

合成プロテアーゼ阻害剤による
ラット副腎褐色細胞腫由来株細胞PC12細胞の
神経細胞への分化誘導およびその機構に関する研究

STUDIES ON NEURONAL DIFFERENTIATION
INDUCED BY A SYNTHETIC PROTEASE INHIBITOR IN PC12 CELLS

斎藤 祐見子

①

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SUMMARY

The rat pheochromocytoma cell line, PC12, is an interesting model applicable to the study of the mechanism of neurite outgrowth. The addition of a leupeptin analogue (Ac-Leu-Leu-Nle-al:ALLNal) to PC12 cells caused neurite initiation and outgrowth. ALLNal also increases the cellular level of both acetylcholinesterase (AChE) and tyrosine hydroxylase (TH). Neuronal differentiation by ALLNal is different in some aspects from that induced by NGF, dbcAMP and basic FGF. Three principal pieces of evidence are presented to support this proposed difference, namely, differences in (a) the relative length and relative number per cell body of neurites (ALLNal, one or two long neurite per cell); (b) the duration of neurite promotion (ALLNal, transitory); and (c) the first intracellular system for neurite initiation (ALLNal does not use A kinase, C kinase or Ca^{2+} flux as a messenger). These findings provide evidence that the protease inhibitor and other known neurotrophic factors elicit neurite initiation by different mechanisms and suggest the existence of a novel molecule(s) which modulates neurite initiation in PC12 cells. Various peptide aldehyde protease inhibitors of the ALLNal analogue were synthesized to examine their effects on neurite outgrowth in PC12 cells. Substitution of the N-terminal acetyl residue by a benzyloxycarbonyl (Z) group and the Nle-3 by Leu-3 (Z-Leu-Leu-Leu-al:ZLLLal) results in 50-fold stronger potency to initiate neurite outgrowth than that of ALLNal. To determine the target molecule, Leu-Leu-Leu-al (LLLal) was immobilized and used as a ligand for affinity chromatography. Proteins of 33K, 35K, and 180K were isolated specifically from the membrane and cytoplasmic fraction of PC12 cells. Z-Leu-Leu-Leu-COOH (ZLLL-COOH) does not induce neurite outgrowth in PC12 cells, and the 33K, 35K and 180K proteins do not bind to a Leu-Leu-Leu-COOH affinity column. Sequences of three peptides obtained from the 33K protein are identical to the partial sequence of the light chain b of clathrin. Two-dimensional gel electrophoresis showed that the mobilities of 33K and 35K proteins are identical to the light chains a and b from authentic clathrin, respectively. On immunoblotting, anti-clathrin heavy chain antibody reacts with the 180K protein. These results suggest that the target molecule involved in neurite outgrowth is clathrin. The

clathrin heavy chain has the ability to bind to an LLLal affinity column directly, while the clathrin light chain does not. Furthermore, ZLLLal enhances the rate of polymerization of clathrin triskelion to the coat structure. On immunocytochemical analysis of PC12 cells with anti-clathrin heavy chain antibody, enhanced staining of the clathrin heavy chain was observed in time with neurite outgrowth induced by ZLLLal.

These results indicate that the specific protease inhibitors ALLNal and ZLLLal penetrate through the cytoplasmic membrane and bind to clathrin via the clathrin heavy chain. This interaction between the protease inhibitor and clathrin induces further reactions leading to neurite initiation. Clathrin is well-known for its role in receptor-mediated endocytosis. This study provides new insights into both the role of the clathrin molecule and the regulatory mechanism of neurite initiation and outgrowth.

ABBREVIATION

NGF: nerve growth factor
PC12 cells: rat pheochromocytoma cell line
ALLNal: Acetyl-leucyl-leucyl-norleucinal
ZLLLal: benzyloxycarbonyl-leucyl-leucyl-leucinal
ZLLL-COOH: benzyloxycarbonyl-leucyl-leucyl-leucine
Dans-LLLal: dansyl-leucyl-leucyl-leucinal
STI: soybean trypsin inhibitor
DFP: diisopropylfluorophosphate
PMSF: phenylmethanesulfonyl fluoride
DMEM: Dulbecco's modified Eagle's medium
PA: plasminogen activator
GDN: glia-derived nexin
CANP: calcium-activated neutral protease
AChE: acetylcholinesterase
TH: tyrosine hydroxylase
ECM: extracellular matrix
bFGF: basic fibroblast growth factor
dBcAMP: dibutyryl cyclic AMP
DRG: dorsal root ganglion
SCG: superior cervical ganglion
LN: laminin
FN: fibronectin
PBS: phosphate-buffered saline
DMSO: dimethyl sulfoxide
SDS: sodium dodecyl sulfate
EDTA: ethylenediaminetetraacetic acid
EGTA: ethyleneglycol-bis (2-aminoethylether)N,N',N'-tetraacetic acid
DTT: dithiothreitol
PVP: polyvinyl pyrrolidone
TFA: trifluoroacetic acid

HPLC: high-performance liquid chromatography

MES: 2-(N-morpholino)ethanesulfonic acid

Buffer A: 0.1M MES/1mM EGTA/0.5mM MgCl₂/ 0.02% NaN₃ (pH6.5)

Buffer B: 0.02M Tris-HCl/1mM EDTA/2mM DTT/ 0.02% NaN₃ (pH7.5)

Buffer C: 0.5M Tris-HCl/1mM EDTA/2mM DTT/0.02% NaN₃/0.1mM PMSF(pH7.5)

Buffer D: 0.02M Tris-HCl/1mM EDTA (pH7.8)

Buffer E: 0.1M MES/1.5mM MgCl₂/1mM EGTA (pH6.2)

GENERAL INTRODUCTION

The development of the vertebrate nervous system is characterized by a series of complex events that cause the apparently homogenous neuroepithelium of the early embryo to form the diverse, highly ordered, and interconnected neural cell types of the adult. Neuronal migration, neurite outgrowth, target recognition, and synaptogenesis are the developmental events in which neurons may interact with as well as modify their local environment. Neurite initiation and outgrowth is one of the first morphological steps leading to the differentiation of the neuronal cells. The timing and rate of neurite outgrowth is of crucial importance, since the first synaptic contacts established appear to have certain advantages in the process of selective synapse stabilization, a key step in the maturation of the nervous system. The elucidation of the mechanisms involved in neurite outgrowth represents a major challenge in developmental neurobiology.

During the past few years, experiments *in vitro* have provided insights into the molecular mechanisms of neuronal process outgrowth. Three distinct classes of proteins that promote axonal outgrowth have been described: diffusible molecules, such as trophic factors and chemotrophic agents, constituents of the extracellular matrix (ECM), and cell adhesion molecules anchored on cell membranes.

Neural cell adhesion molecule (N-CAM)¹⁾ and N-cadherin²⁾ are integral membrane glycoproteins and both molecules promote cell adhesion via a homophilic mechanism. N-CAM and N-cadherin are first expressed on neural ectoderm soon after neural induction and may be important in maintaining the cohesiveness of the neuroepithelium before neuronal differentiation. The axons of differentiated neurons also express high levels of N-cadherin and N-CAM, and antibodies to these glycoproteins reduce the outgrowth of central and peripheral axons on cellular substrates *in vitro*³⁾. These two molecules may therefore permit neurons to adhere to epithelial substrates and to extend axons. The extracellular matrix (ECM) glycoprotein laminin⁴⁾ and fibronectin⁵⁾ are also major components of substrate pathways over which developing axons project and have been shown to promote neurite outgrowth *in vitro*. The functional component of laminin and fibronectin is the tripeptide Arg-Gly-Asp (RGD)⁶⁾. The cellular

receptors that recognize RGD sequences in fibronectin and laminin have been named integrins⁷⁾. Integrins appear to be heterodimeric receptors of ~140kDa. The cytoplasmic domains of some integrins associate with cytoskeletal elements and are substrates for tyrosine kinases. As diffusible factors which induce neurite outgrowth, nerve growth factor (NGF)⁸⁾, brain-derived neurotrophic factor (BDNF)⁹⁾, ciliary neurotrophic factor (CNTF)¹⁰⁾, neurotrophin-3 (NT-3)¹¹⁾, Sl00-b¹²⁾, and acidic/basic fibroblast growth factor (aFGF, bFGF)¹³⁾ have been shown to be identified and characterized. NGF is the first to be discovered and the best characterized of the neurotrophic factors. Several studies have shown that, in a very short time (5-60min), NGF transiently stimulates transcription of a number of genes, such as the proto-oncogene c-fos and c-jun, actin, and others. Some of these early activated genes are also related to cyclic AMP-dependent protein kinase (A kinase)¹⁴⁾ and/or Ca²⁺ and phospholipid-dependent protein kinase (C kinase)¹⁵⁾, two kinases potentially involved in signal transduction. Recent findings^{16,17)} show that NGF actually works through a canonical growth-factor receptor, that is, a protein-tyrosine kinase receptor.

Although neural adhesion molecules, ECM, and soluble neurotrophic factors are essential for neurite regulation, most of the mechanisms for the conveyance of signals to the cell nucleus to induce neuronal differentiation are unknown. Especially, it will be more important to determine the molecular combinations for biologically important responses, that is, neurite outgrowth.

It is known that neurons of the peripheral and central nervous systems secrete proteases during development^{18,19,20)}. These proteases are regulated by their specific inhibitors *in vivo*, so a balance between proteolytic and inhibitory activities may be an important factor in neuronal development. Sympathetic neurons also release plasminogen activator (PA) into the medium²¹⁾, and cardiac myocytes, the target tissue of sympathetic neurons, have been shown to release an inhibitor of PA that induces neurite regeneration from sympathetic neurons *in vitro*²²⁾. Another potent serine protease inhibitor has been purified from C6 glioma conditioned medium, and was shown recently to belong to the protease nexin family²³⁾. This factor induces neurite initiation from neuroblastoma cells and enhances neurite outgrowth from cultured chick sympathetic neurons²⁴⁾. Besides these endogenous protease inhibitors, soybean trypsin inhibitor and

leupeptin induce neurite regeneration from sensory ganglia explants²⁵). Monard et al. showed that hirudin, an inhibitor highly specific for thrombin, initiates neurite outgrowth from mouse neuroblastoma cells²⁶). They suggested that neurite initiation and elongation are controlled by a series of PA or thrombin-like proteases and their inhibitors²⁷).

The PC12 cell line, derived from a rat pheochromocytoma, provides a relatively simple, homogeneous system for studying various aspects of neuronal differentiation²⁸). PC12 cells survive and proliferate in vitro without requiring the presence of neurotrophic factors. The addition of NGF to the culture medium, however, induces a phenotype resembling peripheral neurons. Primary cultured neuronal cells have been previously exposed to NGF or do not require NGF for survival and neurite extension, and already have axons prior to isolation. This is the "primed" condition. On the other hand, the PC12 cell line is derived from the neural crest, is not "primed" with any neurotrophic factor, and is thought to act as a model for the early events in neuronal differentiation²⁹). Despite these characteristics, there have been no reports to show that a specific protease-protease inhibitor system plays a role in neurite initiation or that a specific protease inhibitor can initiate neurite outgrowth in PC12 cells.

The present study was designed to investigate the mechanism of neurite initiation and outgrowth induced by a specific protease inhibitor from PC12 cells. At first, it was found that, among many protease inhibitor species examined, only a leupeptin analogue, Ac-Leu-Leu-Nleu-al (ALLNal), induced neurite outgrowth in PC12 cells. Since neuronal differentiation induced by ALLNal is different from that induced by other known neurotrophic factors, the existence of a specific molecule(s) which regulates neurite formation in PC12 cells was expected^{30,31}). These results are described in Chapter 1. A set of ALLNal analogue peptide protease inhibitors were synthesized and examined for their potency in inducing neurite outgrowth in PC12 cells. It was found that benzyloxycarbonyl-Leu-Leu-Leu-al (ZLLLal) has a stronger potency than ALLNal³²). In an attempt to identify the target molecule of ZLLLal, LLLal was immobilized and used as a ligand for affinity chromatography³³). Unexpectedly, molecules isolated specifically by LLLal affinity chromatography were not proteases but

clathrin, well-known for its role in endocytosis³⁴). These results are described in Chapter 2.

There are no reports of the involvement of clathrin in neurite formation. This study suggests that the role of clathrin should be incorporated into a model describing the pathways for the initiation of neurite outgrowth. At the same time, this study also shows that the protease inhibitor interacts not only with proteases, but also with other molecules.

CHAPTER 1

NEURITE OUTGROWTH AND NEURONAL DIFFERENTIATION INDUCED BY A TRIPEPTIDE ALDEHYDE PROTEASE INHIBITOR (Ac-Leu-Leu-Nle-al) IN PC12 CELLS

INTRODUCTION

As described in the GENERAL INTRODUCTION, protease-protease inhibitor systems are involved in neurite outgrowth in various neuronal cells. Although PC12 cells provide an interesting model for the study from a number of aspects of neuronal development and function, there are no reports on neurite formation using a specific protease inhibitor as a tool.

The influence of exogenously added specific protease inhibitors on neurite outgrowth in PC12 cells have been examined. Of 14 protease inhibitors examined, only a leupeptin analogue, Ac-Leu-Leu-Nle-al (ALLNal) is stimulatory for neurite initiation and outgrowth.

In Chapter 1, experiments to analyse differentiation induced by ALLNal are described, and the neurite-inducing activity of ALLNal as compared to other known trophic molecules is evaluated.

MATERIALS AND METHODS

MATERIALS

Diisopropylfluorophosphate (DFP), soybean trypsin inhibitor (STI), hirudin, and aminocaproic acid were obtained from Sigma. Pepstatin A and leupeptin were from the Peptide Institute (Osaka). Aprotinin was from Boehringer-Mannheim. E64c and E64d were kind gifts from Taisho Pharmaceutical Co. (Omiya). The leupeptin analogues, ALLNal and Ac-Leu-Leu-Met-al (ALLMal), which, unlike leupeptin, do not inhibit trypsin activity, were from Nakalai Tesque (Kyoto). The commercial names of these analogues are calpain inhibitors 1 and 2, respectively, but they are not necessarily specific for calcium-dependent neutral protease (CANP or calpain). Thus, in this study, they are called ALLNal

and ALLMal. Bovine skin collagen type I (0.5% solution in 1mM HCl) and human placenta-derived collagen type IV (0.3% solution in 1mM HCl) were purchased from Koken (Tokyo). EHS sarcoma tumor-derived laminin was from EY Laboratory and bovine plasma fibronectin was from Nitta Gelatin (Tokyo).

Nerve growth factor (NGF), 2.5S, was from Takara (Tokyo). Basic fibroblast growth factor (bFGF) was a kind gift from Dr. Yoshiya Shimada. Dibutyryl cyclic AMP (dbcAMP), A23187, tetradecanoyl phorbol acetate (TPA), D-sphingosine, and 1,2-dioctanoyl-sn-glycerol were from Sigma. H-7 and SC-9 were from Seikagaku-Kogyo (Tokyo).

Anti-tubulin a, b monoclonal antibody, and anti-MAP2 (microtubule-associated protein 2) monoclonal antibody were from Amersham. Anti-GAP 43 (growth cone associated protein) monoclonal antibody and anti-tyrosine hydroxylase antibody were from Oncogene Science and ETI Laboratory, respectively. Anti-MAP 1 polyclonal antibody was kindly supplied by Dr. Taka-aki Kobayashi, Tokyo Jikei University of Medicine.

A specific inhibitor of AChE activity, BW284C51 (Burroughs Wellcome), was kindly provided by Dr. Fumio Mizobe.

CELL CULTURE

PC12 cells, clone h³⁵, were obtained from Dr. Hiroshi Hatanaka and routinely grown in medium containing 90% Dulbecco's modified Eagle's medium (DMEM), 5% newborn calf serum, and 5% heat-inactivated horse serum in plastic tissue flasks (Corning) at 37°C in a humidified 10% CO₂ incubator.

NEURITE OUTGROWTH ASSAY

For the neurite outgrowth assay, the PC12 cells were washed and plated at 2 x 10⁴ cells/cm² in N2 medium (Ham's F12/DMEM=1:1, plus 100 ug/ml transferrin, 5 ug/ml progesterone, 52 ug/ml sodium selenate, and 5 ug/ml insulin)³⁶) onto Lux plates coated with 0.1% collagen type I. When most of cells had attached, the cells were incubated with various protease inhibitors and/or 40 ng/ml NGF, 0.5mM dbcAMP, 10 ng/ml bFGF. At various intervals, cells were fixed with 2% glutaraldehyde in PBS and assayed under a phase-contrast microscope by determining the percentage of cells with neuritic processes longer than the diameter of the

cell body. Only isolated single cells (i.e., those whose cell bodies and neurites do not contact other cells) were counted in order to insure that the observed percentage of neurons with neurites was correct. Usually 400-800 cells were scored each time from randomly chosen fields.

Cells were classified morphologically as to the number of neurites. Cells with one neurite were defined as monopolar, those with two neurites as bipolar, and those with more than two neurites as multipolar type cells. As another classification, neurite length was measured with an eyepiece micrometer, and a distribution pattern of neurite lengths was obtained. At least 100-200 random neurites were counted for each morphological classification. The differences in the percentage of cells with neurite processes were analyzed for significance by Student's t test and the neurite length distribution patterns were analyzed by Wilcoxon's rank-sum test.

REDUCTION OF ALLNal

ALLNal was reduced with NaBH_4 by the method of Kondo et al³⁷⁾. To a solution of 50mM ALLNal in 100 ul of 100% ethanol, 2.5ml of 0.15M NaBH_4 (pH 8.4) was added dropwise in 10min. After 1hr, the reaction mixture was adjusted to pH 3.8-4.0 with acetic acid and lyophilized. The powder was extracted with ethyl acetate, evaporated, and dissolved in H_2O . After reduction, the inhibitory activity of ALLNal against CANP using denatured casein as a substrate was reduced to about 8% of that of the native inhibitor. Reduction was also confirmed by fast atom bombardment mass spectrometry (DX304/DX304: JEOL) with a mass data system.

ACETYLCHOLINESTERASE ACTIVITY ³⁸⁾

The cultures treated with NGF and/or ALLNal were washed with PBS to remove the N2 medium and the cells were scraped from the dishes and homogenized in ice-cold solubilization buffer (10mM Tris-HCl/1M NaCl/50mM MgCl_2 /1% Triton X-100, pH7.2). The extracts were analyzed for total AChE activity by the method of Ellman et al³⁹⁾, using 0.5mM acetylthiocholine as a substrate. Treatment with a specific inhibitor (BW284C51) showed that more than 95% of the enzymatic activity was due to true AChE. Specific activity was calculated on the basis of protein

concentration as determined by the method of Lowry et al⁴⁰). For all experiments, duplicate sister cultures were assayed for each point, and the specific activities are expressed as mean + SEM.

CYTOSKELETON PROTEIN PREPARATION⁴¹

Cells treated with NGF or ALLNal for 2 days were washed three times with PBS at 37°C. Next, an extraction buffer (25mM Na₂HPO₄, 0.4M NaCl, 0.5% SDS, pH 7.2) supplemented with 10 uM benzamidine-HCl, 1mM PMSF, 1 ug/ml o-phenanthroline, 10 ug/ml aprotinin, 10 ug/ml leupeptin, and 10 ug/ml pepstatin A to inhibit proteolysis during preparation was applied to the culture plate. After 3-5min the viscous lysate was drained into a Eppendorf tube and boiled for 3min. The lysate was then centrifuged at 10,000xg for 10min and the DNA-containing pellet was removed.

GEL ELECTROPHORESIS

SDS gel electrophoresis was performed on 6% or 10% polyacrylamide gels⁴²). Molecular weight standards were myosin heavy chain (200,000), b-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

WESTERN BLOTTING

For quantitative immunoblotting, the PC12 cell lysates described above were dissolved in SDS-containing sample buffer and the proteins were separated by SDS gels electrophoresis. Proteins were blotted onto nitrocellulose and immunoperoxidase-stained with antibodies as described by Towbin et al⁴³). Anti-tubulin a and b antibodies, and anti-MAP 2 antibody were used at a 1:1000 dilution, and anti-MAP 1 antibody was used at a 1:100 dilution. Tubulin, MAP 1 and MAP2 levels were quantitated by a TLC scanner (CS-910:Shimazu).

IMMUNOCYTOCHEMISTRY

PC12 cells treated with NGF or ALLNal were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 10min at room temperature, and then treated with

0.5% Triton X-100 in PBS for 5min to permeabilize the cell membrane. The fixed cells were incubated with anti-tyrosine hydroxylase antibody at a 1:200 dilution at 4°C. After incubation for 18hr, the cells were washed with PBS and incubated with biotinylated species specific anti-mouse IgG antibody, and then with streptavidin-biotinylated peroxidase complex, and stained with diaminobenzidine and H₂O₂.

COATING OF DISHES

Poly-L-lysine was dissolved in 0.1M sodium borate buffer (pH 8.5) at a concentration of 10-100 ug/ml, and used to coat the surface area of culture wells by incubation for 18hr at 4°C. Collagen type I or type IV solutions (0.02-0.1% diluted in H₂O) were spread as a film over the culture well and allowed to dry at room temperature under ultraviolet irradiation. Laminin and fibronectin were diluted with PBS at a concentration of 10-100 ug/ml, and applied by air-drying at room temperature for 3hr.

ATTACHMENT ASSAY

PC12 cells were added to each culture well and placed at 37°C in a 10% CO₂ incubator. At various intervals, the medium was aspirated off, and the dishes were rinsed twice with PBS. Attached cells were treated with 0.05% trypsin/0.02% EDTA/PBS at 37°C for 10min. After suspension, cell numbers were counted in a Coulter counter (ZBI type). Data are presented as means of percentage attachment in triplicate wells.

PRIMING OF PC12 CELLS WITH NGF

PC12 cells were primed with 40ng/ml NGF for 5 or 17 days. The cells were washed with N2 medium and mechanically diversified of their neurites by trituration through a narrow pasteur pipet. The cells were replated onto new culture plates at 2x10⁴ cells/cm². After 1hr, the medium was changed to N2 medium containing NGF or ALLNal. The cultures were fixed and assayed by determining the percentage of cells with neurites after 20hr.

RESULTS

Several protease inhibitors including ALLNal, ALLMal, leupeptin, soybean trypsin inhibitor (STI), diisopropylfluorophosphate (DFP), aprotinin, hirudin, 6-aminocaproic acid, pepstatin A, E64c, and E64d were tested at concentrations known to inhibit completely the activity of sensitive proteases in vitro (Table 1). A leupeptin analogue, ALLNal, was found to induce positive neurite initiation at low concentrations during a 48hr bioassay. Leupeptin (Ac-Leu-Leu-Arg-al) and another leupeptin analogue, ALLMal, in which the third amino acid residues are replaced by arginine and methionine, respectively, failed to initiate neurite outgrowth. ALLNal's ability was dose-dependent, with a maximal effect at a concentration of 1.6 μM ; concentrations below 0.6 μM had little effect. This agent had a cytotoxic effect against PC12 cells at concentrations higher than 2 μM .

ALLNal is composed of highly hydrophobic amino acids. To test the effect of this hydrophobicity on neurite outgrowth, the terminal aldehyde group in ALLNal was reduced to an alcohol. Since the aldehyde group is thought to be essential for protease inhibitory activity, the reduced form should have little effect as a protease inhibitor. In fact, the reduced form lacked most of the inhibitory activity against calpain. The reduced form had no effect on neurite outgrowth and also showed no cytotoxicity toward PC12 cells. This means that the effect on neurite initiation is due to the action of a molecule(s) which reacts with the aldehyde group rather than to an interaction between the hydrophobic portions and PC12 cells.

NGF-induced neuronal differentiation in PC12 cells accompanies a wide range of morphological and biochemical changes. The difference between neuronal differentiation induced by ALLNal and that induced by NGF was examined in terms of time course, morphology of neurites, neurotransmitter-synthesizing enzymes, effect of ECM, effect of priming, and transcription-dependent response.

1. Time course

Figure. 1-1 shows the time course for the change in percent of neurite-bearing cells. In the early stages, ALLNal alone showed a potency comparable to that of ALLNal plus NGF and was slightly more effective than NGF alone. More than 10% of cells were found to be neurite-bearing 12hr after treatment with ALLNal. A maximal response was observed 48hr after treatment, with 50% of the cells becoming neurite-bearing (Fig. 1-2B). Cell number was determined by a Coulter counter after an incubation time of 48hr. At ALLNal concentrations of 0.4-1.6 μ M the decrease in cell number did not exceed 8-10% as compared with control cultures (N2 medium only); therefore the neuritogenic activity of ALLNal did not appear to have resulted from its cytotoxic action. However, the morphological change was not permanent and the percentage of cells bearing neurites started to decrease after 48hr. The neurite-bearing cells, of which the processes were seen to retract, became round and finally died (Fig.1-3C). Even if ALLNal was added again, there were no signs of neurite stabilization. When ALLNal and NGF were present together, neurite degeneration and cell death were not apparent (Fig. 1-3D). In contrast, when NGF alone was present, the percentage of neurite-bearing cells increased progressively, and the neurites formed continued to increase greatly in length and exhibited complex branching patterns (compare Fig.1-2B and 1-3B). Thus, in comparison with the effect of NGF, ALLNal-induced morphological differentiation was fast and mostly transitory. ALLNal appears to act as a neurite initiator rather than a survival factor like NGF.

2. Morphological differentiation

Neurites induced by ALLNal had nerve fibers containing growth cones (Fig.1-2B,C). However, with respect to neurite number from a single cell body and neurite length, these neurites were morphologically different from those induced by NGF. After culture for 48hr with ALLNal, the proportions of mono-, bi-, and multipolar cells were 60, 29, and 11%, respectively (Fig.1-4). On the other hand, in cultures with NGF alone, the proportions of mono-, bi-, and multipolar cells were 35,15, and 50%, respectively. Thus, neurites induced by ALLNal had a smaller proportion of the multipolar type and a larger proportion of the mono- and bipolar types than neurites induced by NGF. In culture with both 40ng/ml

NGF and 1.6 μ M ALLNal the distribution of the number of neurites per cell was altered, with about 50% of total cells being of the bipolar type.

As shown in a quantitative assay of neurite outgrowth induced by ALLNal and NGF (Fig.1-5), the length distribution patterns of neurites induced by ALLNal were significantly different from those of neurites induced by NGF ($p < 0.01$) and the average length of the neurites induced by ALLNal was greater than those induced by NGF.

Electron microscopic analysis has shown that neuronal processes are densely packed with parallel arrays of filamentous structures, prominent among which are microtubules. Microtubules are present in all eukaryotic cells, but are especially abundant in neurons where they play essential roles in both the outgrowth and maintenance of neuronal processes. Thus, to understand the biochemical difference between neurites by induced NGF and those induced by ALLNal, it is important to determine how the arrangement of microtubule assembly is targeted within PC12 cells. At first, the level of total tubulin was compared between PC12 cells treated with NGF and those treated with ALLNal for 2 days. Treatment with either NGF or ALLNal resulted in little change in total tubulin levels compared with undifferentiated PC12 cells (10-30%) (Fig.1-6B, Table.1-2B). Next, the levels of two well-characterized microtubule-associated proteins, MAP1 and MAP2, were examined. Black et al. reported that MAP2 levels are elevated in differentiated PC12 cells⁴⁴), but, with the anti-MAP2 antibody employed in this study, MAP2 levels in PC12 cells were very low. Fig.1-6B and Table.1-2B show an immunoblot probed with a polyclonal MAP1 antibody. Two days after either NGF or ALLNal-induced differentiation there was an approximately 2-fold increase in MAP1. This result is in agreement with that of Drubin et al⁴¹).

The growth-associated protein, GAP-43, is believed to be intimately associated with the growth and regeneration of axonal processes⁴⁵). But, anti-GAP-43 stained very faintly on an immunoblot of undifferentiated PC12 cells, and there was little change in PC12 cells treated with either NGF or ALLNal for 2 days.

Thus, as examined with anti-tubulin, -MAP1, -MAP2, and -GAP43, there were no quantitative or qualitative differences between neurites induced by ALLNal and those induced by NGF.

3. NEUROTRANSMITTER-SYNTHESIZING ENZYME

NGF induces an increase in acetylcholinesterase (AChE) activity³⁸). Exposure to ALLNal for 48hr increased AChE activity in PC12 cell cultures to an extent similar to the increase by NGF in a dose-dependent manner (Fig.1-7). However, when tested after 120hr of exposure, this stimulation by ALLNal was no longer observed, in contrast to the case for exposure to NGF. When ALLNal and NGF were added at the same time, no additive effect on AChE activity was observed after either 48 or 120hr of exposure. This means that the pathway for the increase in AChE activity by ALLNal is probably the same as that by NGF.

Tyrosine hydroxylase (TH), a marker enzyme for adrenergic differentiation, catalyzes the first and rate-limiting step in the biosynthesis of catecholamines. The PC12 cell line used in this study is a subclone, named PC12h. The PC12h cells exhibited a significant increase of TH activity in the presence of NGF³⁵). PC12 cells exposed for 2days to ALLNal stained with anti-TH antibody showed strong diffuse cytoplasmic staining compared with undifferentiated cells. This staining in ALLNal-treated cells was stronger than that in NGF-treated PC12 cells (Fig.1-8). The level of TH antibody staining induced by ALLNal, however, returned to control levels when the neurites disappeared after 120hr of exposure, similar to the transient increase in AChE activity induced by ALLNal.

Choline acetyltransferase (CAT), a marker enzyme for cholinergic differentiation, catalyzes the rate-limiting step in the biosynthesis of choline. Green et al. reported that NGF induces an increase in CAT activity in PC12 cells⁴⁶). However, it was reported that CAT activity in PC12 cells is heterogeneous from cell to cell and varies markedly with culture conditions⁴⁶). By subculture, the PC12h cell line readily undergoes a decrease in CAT activity and loses its responsiveness to NGF⁴⁷). In this study, the level of CAT activity in PC12h cells was found to be low (14.8 ± 2.5 pmol/min/mg protein), in comparison with the level reported by Green et al. (100pmol/min/mg protein). The specific activity of CAT was not affected by either NGF or ALLNal (40ng/ml NGF for 2days: 15.2 ± 3.2 pmol/min/mg protein, $1.6 \mu\text{M}$ ALLNal for 2days: 13.5 ± 2.7 pmol/min/mg protein).

4. EFFECT OF EXTRACELLULAR MATRIX (ECM)

Numerous recent studies on ECM factors have established their important role in neurite outgrowth⁴⁸). To determine the effect of ECM on neurite outgrowth, various ECM molecules used to coat dishes were tested for their ability to support neurite outgrowth induced by NGF or ALLNal. ECM molecules did not induce neurite outgrowth in the absence of the agents (NGF or ALLNal), and did not markedly alter the morphology of neurites induced by NGF or ALLNal. However, when collagen types I and IV were used as substrates, ALLNal, but not NGF, caused neurite outgrowth at high percentages (especially collagen type IV, Fig.1-9). Despite the highest efficiency of collagen type IV, the percentage of cells bearing neurites started to decrease after 48hr.

Cell-substratum adhesiveness plays an important role in the initiation of neurite outgrowth in many types of cultured cells. A quantitative assay was used to study the attachment of undifferentiated PC12 cells to substrates coated with each ECM molecule. Using this assay, PC12 cells were shown to attach strongly in the following order: collagen type IV > poly-L-lysine > collagen type I > fibronectin > laminin (Table.1-3). Except for poly-L-lysine, this order is in agreement with the order in which ECM molecules act in neurite outgrowth induced by ALLNal but not by NGF. Cell adhesion to poly-L-lysine is achieved by the electrostatic interactions between the polycation and negative charges on the cell membrane; binding is not through a specific receptor on the cell membrane. These results indicate that effective neurite initiation by ALLNal in PC12 cells depends on components of the ECM. Thus, when stronger physiological adhesiveness between ECMs and a cell surface protein is achieved, more effective neurite initiation by ALLNal occurs.

5. PRIMING AND NEURITE REGENERATION

NGF has a "priming" effect on PC12 cells⁴⁹). That is, NGF-pretreated PC12 cells divested of their processes by mechanical disruption undergo regrowth of long neurites within 24hr of replating (Fig.1-10A). This rapid reaction is termed "priming". Since PC12 cells pretreated with ALLNal for 2 days did not regenerate long neurites rapidly upon addition of ALLNal, ALLNal lacks the ability to prime PC12 cells. However, ALLNal can regenerate neurites in PC12

cells primed with NGF. In PC12 cells primed with NGF for 5 days, 65.9±3.7% of the cells were induced to regenerate neurites by 40ng/ml NGF after 20hr. Treatment with 1.6 μ M ALLNal for 20hr induced neurite regeneration in 62.5±2.8% of the cells, and the neurites were longer than those induced by NGF (Fig.1-10B). PC12 cells pretreated with NGF for a longer time, 17days, also showed regenerated neurite outgrowth upon induction with NGF or ALLNal (NGF: 70.9±8.5%, ALLNal:66.5±6.4%). Neither hirudin, a specific inhibitor for thrombin, nor soybean trypsin inhibitor had any effect on neurite regeneration in primed PC12 cells. These results imply that not only undifferentiated but also differentiated PC12 cells have a target molecule(s) for ALLNal and possess a pathway for neurite outgrowth induced by ALLNal.

6. TRANSCRIPTION-DEPENDENCE

Neurite outgrowth caused by NGF is known to be transcription-dependent²⁹). ALLNal-induced neurite initiation and the NGF-induced neurite initiation show the same sensitivity to an inhibitor of RNA synthesis, actinomycin D (Act.D) (Table.1-4). In the presence of 0.5nM Act.D, the initiation of neurite outgrowth from PC12 cells by NGF was inhibited by about 50% and that by ALLNal was inhibited by about 40%. In the presence of 1.5nM Act.D, the initiation of neurite outgrowth by NGF was inhibited by nearly 90% and that by ALLNal was inhibited by about 80%. Thus, the initiation of neurite outgrowth induced by ALLNal, as well as that induced by NGF, is dependent on transcription.

The neurite-initiating activity of ALLNal was assessed in many aspects and compared with that of NGF. The effect of ALLNal on PC12 cells appears to be different from the action of NGF in terms of neurite morphology, persistence, ECM molecule requirement for effective response, and priming effect. These results provide evidence that ALLNal and NGF elicit neurite initiation by different mechanisms.

Since dibutyryl cyclic AMP (dBcAMP) is known to cause transient neurite outgrowth in PC12 cells^{50,51}), the response to dBcAMP was compared with that to ALLNal. About 40% of PC12 cells displayed neurite outgrowth after 24hr in response to 0.5mM dBcAMP, and the percentage of cells with neurites started to

decrease after 7-8 days. The neurites induced by dBcAMP had a large proportion of the multipolar type after 48hr (Fig.1-11C, 1-12). The larger proportion of multipolar type neurites induced by dBcAMP was in contrast to a smaller proportion of multipolar type neurites induced by ALLNal. The combination of ALLNal and dBcAMP influenced neurite number per cell, and the percentage of the bipolar type increased (Fig.1-11D, 1-12).

Recent experiments have demonstrated that basic fibroblast growth factor (bFGF) can reproduce the entire spectrum of PC12 cell responses previously shown to be elicited by NGF^{52,53}. In the culture system used in this study, neurites induced by 10ng/ml bFGF looked like those induced by NGF with respect to morphology (Fig.1-11A, 1-12). The addition of ALLNal together with bFGF influenced both neurite number per cell and neurite length (Fig.1-11B, 1-12). The percentage of neurite-bearing cells was 65.2±2.3% after 5 days of exposure, and the processes continued to grow to form a network.

These facts suggest that the intracellular mechanisms through which cells initiate neurite outgrowth when induced by ALLNal may be different from those induced by NGF, dBcAMP, and bFGF. Since dBcAMP increases intracellular cyclic AMP, the first intracellular system stimulated by ALLNal does not use cyclic AMP as a messenger.

Since it was reported previously that cytoplasmic Ca²⁺ or C kinase is involved in neurite outgrowth in primary cultured neuronal cells^{54,55}, the effects of the calcium ionophore A23187, the activator of C kinase (TPA, 1,2-dioctanoyl-sn-glycerol, SC-9), and the inhibitor of C kinase (H-7, D-sphingosin) on neurite outgrowth were studied in PC12 cells. Each agent was added to cultures of PC12 cells at various concentrations with or without ALLNal, and the cells with neurites were determined after 2 days (Table.1-5). However, treatment of PC12 cells with these drugs alone had no effect on neurite outgrowth, nor did it alter the effects of ALLNal. These results indicate that the first metabolic system of neurite formation elicited by ALLNal is not stimulated by A kinase, C kinase, nor Ca²⁺ entry.

DISCUSSION

In this study, I showed that a leupeptin analogue, Ac-Leu-Leu-Mle-al (ALLNal) induces morphological and biochemical neuronal differentiation in PC12 cells. Other protease inhibitors or the reduced form of ALLNal failed to induce neurite initiation. Compared with neurites induced by other known molecules (NGF, bFGF, dBcAMP), ALLNal-induced neurites appear to be distinct with respect to morphology and time course of appearance and disappearance. These results suggests the possibility that ALLNal expresses its neurite-initiating effect through an interaction with a specific functional target molecule(s).

As described in the GENERAL INTRODUCTION, neurite initiation from neuroblastoma cells and neurite regeneration from sensory or sympathetic neurons is effected by general protease inhibitors (hirudin=a specific thrombin inhibitor, soybean trypsin inhibitor:STI) or endogenous cell surface-associated serine protease inhibitors (glia-derived nexin:GDN, heart cell-derived plasminogen inhibitor). In PC12 cells, however, hirudin or typical serine protease inhibitors (STI and DFP) have no effect on neurite initiation (Table.1-1), and the endogenous protease inhibitor, GDN, has no ability to initiate neurite outgrowth⁵⁶). These varying responses to serine protease inhibitors might reflect differences in neuronal cell types or in the developmental stage of the neurons. The sensory or sympathetic neuronal cells used in other studies had been previously exposed to NGF or other neurotrophic factors *in vivo*, while the PC12 cell line is derived from neural crest and has never been exposed to any trophic factor. It is easier to cause neurite outgrowth in neuroblastoma cells than in PC12 cells, because the simple withdrawal of serum elaborates neurites in neuroblastoma cells⁵⁷). This means that PC12 cells are a better model for the early stages of neuronal development than neuroblastoma cells. Thus, in the early stages of neuronal development, neither plasminogen activator nor thrombin-like protease might not be expressed and responsible for neurite outgrowth.

Does ALLNal have the ability to regenerate neurite outgrowth from sympathetic or sensory neurons in culture? PC12 cells primed with NGF for a long period (17

days) underwent effective ALLNal-induced neurite outgrowth. Since NGF-primed PC12 cells show a phenotype resembling sympathetic neurons, there is the possibility that ALLNal causes neurite regeneration from sympathetic neurons at an early stage.

Mouse neuroblastoma, NB2a cells, develop neurites upon serum deprivation as mentioned above, and GDN or hirudin initiates neurite outgrowth in the presence of serum^{23,26,57}). Shea et al. reported that ALLNal and leupeptin enhance neurite outgrowth induced by serum deprivation, but do not induce neuritogenesis in the presence of serum⁵⁸). However, in PC12 cells, ALLNal can elicits neurite outgrowth even in the presence of serum⁵⁹), and leupeptin does not enhance the neurite outgrowth induced by NGF. These different responses indicate that the action of ALLNal is mediated by different mechanisms in PC12 cells and NB2a cells.

Numerous reports on ECM factors that regulate neuronal differentiation have established the importance of the role of neuronal adhesion to the ECM. NGF enhances cell-substratum adhesion of PC12 cells⁶⁰), and cellular adhesion is necessary for neurite initiation. The importance of ECM in the neuronal differentiation of PC12 cells induced by ALLNal has also been shown in this study. In early experiments, the culture dishes were coated with acid-soluble collagen from bovine skin (a mixture of the interstitial collagen types I and III), and ALLNal did not have a prominent effect on neurite initiation in the absence of NGF. ALLNal merely accelerated the initiation by NGF³⁰). But, when purified collagen type I was used, ALLNal induced neurite outgrowth efficiently in the absence of NGF³¹). Attachment assays showed that ALLNal itself does not increase the adhesion of PC12 cells to plastic culture dishes or dishes coated with collagen type I at any culture time (10-180min). These facts mean that neurite initiation by ALLNal in PC12 cells depends on a component of ECM, and if the proper ECM is chosen, the initiation is started by protease inhibitor ALLNal alone. PC12 cells were shown to adhere readily to collagen type IV and to undergo more effective neurite formation on substrate coated with collagen type IV (Fig.1-9, Table.1-3). High levels of the integrin for collagen type IV are expressed in PC12 cells⁶¹), so physiological strong adhesiveness via collagen type IV receptor may be required for effective neurite initiation in PC12 cells.

Differentiation induced by ALLNal in PC12 cells was shown to be dependent on transcription, as in the case for differentiation induced by NGF. Differentiation was further characterized by the development of one or two long neurite, by increased cellular levels of AchE activity, and by anti-TH antibody immunoreactivity, and these events are all transient. The transitory neuronal differentiation elicited by ALLNal suggests that ALLNal may play a role as a neurite "initiation" factor, whereas NGF is responsible for the stabilization and long-term maintenance of the differentiated state. When ALLNal and NGF or bFGF were present together, the neurites grew longer and neurite degeneration was not apparent. Thus, ALLNal is not only a neurite "initiation" factor but also an "enhancer" factor of neurite length. Rapid and long neurite outgrowth are advantageous for first synaptic contacts. Analysis of the induction of longer neurites by ALLNal is therefore currently assumed to be an important subject for basic neurobiology.

The induction of one or two long neurites by ALLNal was not due to an increase in the total tubulin or microtubule-associated proteins (MAP) 1 and 2 levels (Fig.1-6, Table.1-2). The majority of the components that have been identified in brain MAPs (MAP1, MAP2, MAP3, MAP5, tau proteins, and chartins) can also be detected in PC12 cells. Recent work has shown that MAP3 and MAP5 are present in PC12 cells and increase by more than 10-fold after NGF treatment⁶²). Chartins are phosphorylated in response to NGF, and the phosphorylation of chartins appears to be required for neurite elongation⁶³). Use of anti-MAP3, anti-MAP5, and anti-phosphorylated chartins antibodies will allow a better understanding of the long neurites induced by ALLNal. Besides the analysis of the regulation of microtubule assembly in neurites, biochemical and morphological examination of the growth cone induced by ALLNal is also necessary. The growth cone is a highly specialized structure equipped with the complex machinery required for neurite elongation⁶⁴). Since the movements of the growth cone apparently play a direct role in the elongation of neurites, a detailed analysis of growth cone motility, structure, and components may help to clarify the causal steps involved in neurite outgrowth induced by ALLNal.

Neither activation nor inhibition of C kinase or the Ca^{2+} flux by A23187 has any obvious effect on neurite outgrowth induced by ALLNal (Table.1-5). dBcAMP caused a rapid extension of multipolar neurites in a transcription-independent manner, and phospholipase A_2 and C promote the rapid extension of multipolar type neurites by stimulating the synthesis of intracellular cyclic AMP⁶⁵). Thus, neurite outgrowth through cyclic AMP is rapid and of a multipolar type. It has also been reported that exogenous potassium, which depolarizes PC12 cells and opens the voltage-sensitive calcium channel, induces neurites^{65,66}). These neurites are spike-like short neurites formed on 8-10% of cells (50mM K^+) after 2 days. ALLNal causes the formation of one or two long neurites in a transcription-dependent manner, suggesting that neither an elevation of cyclic AMP nor Ca^{2+} flux via a voltage-sensitive calcium channel mediates ALLNal-induced neurite outgrowth.

Tsuji et al. reported that the addition of a ganglioside (GQ1b) to neuroblastoma cells induces "one long" neurite, and that some protein kinase systems on the plasma membrane (ecto-type) may play an important role in GQ1b-induced neurite formation⁶⁷). That is, there is a possibility that the ALLNal-activated mechanism somehow involves an ecto-type kinase and consequently makes PC12 cells grow one or two long neurites.

In summary, the findings presented here suggest a new type of neuronal differentiation through specific molecule(s) interacting with a protease inhibitor, ALLNal. As the differentiation of PC12 cells may reflect early events in neuronal development, further studies at the molecular level directed at events specifically associated with ALLNal may provide illumination of the causal steps involved in the "initiating" signal for early neuritogenesis.

TABLE 1-1

Effect of protease inhibitors on neurite outgrowth of PC12 cells

Protease inhibitor	Optimal concentration (μ M)	% cells with neurites
No addition	-	1.67 \pm 0.2
Soybean trypsin inhibitor	1.0	2.8 \pm 0.35
DFP	50	5.4 \pm 0.85
Aprotinin	2.5 ^a	4.1 \pm 0.35
Hirudin	2.5 ^b	2.3 \pm 0.21
6-aminocaproic acid	5000	2.0 \pm 0.35
Pepstatin A	10	2.0 \pm 0.14
E64c	60	5.6 \pm 0.57
E64d	60	1.8 \pm 0.21
Leupeptin (Ac-Leu-Leu-Arg-al)	1.0	2.6 \pm 0.42
Ac-Leu-Leu-Met-al	2.0	2.4 \pm 0.28
Ac-Leu-Leu-Nle-al	1.6	51.1 \pm 1.31
reduced Ac-Leu-Leu-Nle-al	2.0	2.4 \pm 0.42

a μ g/ml b ng/ml

Cells were grown for 2 days with the additive indicated. Figures are mean \pm SEM for 3 experiments, each in duplicate.

TABLE 1-2

Changes in the relative amount of tubulin and MAP1 during PC12 differentiation

A)	N2 only	40 ng/ml NGF	1.6 uM ALLNal
Exp.1	100	115	125
Exp.2	100	110	122
B)	N2 only	40 ng/ml NGF	1.6 uM ALLNal
Exp.1	100	187	210
Exp.2	100	220	200

Equal amounts of PC12 protein exposed to NGF or ALLNal for 2 days exposure to NGF or ALLNal were transferred onto nitrocellulose and stained with the respective antibodies. Changes in the relative amount of tubulin(A) or MAP1(B) were determined by scanning the immunoperoxidase-stained bands with a reflectance densitometer.

TABLE 1-3

PC12 cell attachment to the substrate

substrate	attachment (%)
poly-L-lysine	95±5
col.I	73±8
col.IV	100
FN	45±5
LN	34±5

PC12 cell attachment to substrates coated with poly-L-lysine (20 ug/ml), collagen type I (0.3 %), collagen type IV (0.1 %), fibronectin (20 ug/ml), and laminin (100 ug/ml). Each value represents the mean and SEM for triplicate cultures and is expressed as the percentage of attachment relative to collagen type IV.

TABLE 1-4

Effect of actinomycin D on neurite formation induced by ALLNal and/or NGF

Act.D (nM)		% cells with neurites
0	NGF	45.3 ± 2.3
	ALLNal	48.2 ± 2.9
	NGF + ALLNal	76.2 ± 1.4
0.5	NGF	22.6 ± 1.3
	ALLNal	28.9 ± 2.2
	NGF + ALLNal	46.5 ± 1.2
1.5	NGF	5.1 ± 0.9
	ALLNal	10.5 ± 1.4
	NGF+ ALLNal	17.2 ± 1.0

PC12 cells were treated with the indicated concentrations of actinomycin D in the presence of 40 ng/ml NGF and/or 1.6 μ M ALLNal for 2 days, and the percentage of neurite-bearing cells was scored. Each value represents the mean \pm SEM of 2 experiments, each in duplicate.

TABLE 1-5

Effect of various C kinase modulators and A23187 on neurite outgrowth in PC12 cells

	concentrations examined	optimal concentration	% cells with neurites
ALLNal	-	1.6 μ M	47.3 \pm 2.7
A23187	0.05-0.5 μ M	0.1 μ M	7.8 \pm 1.3
TPA	10-100ng/ml	100ng/ml	5.0 \pm 0.9
SC-9	1-12.5 μ M	12.5 μ M	2.3 \pm 0.1
1,2-dioctanoyl- sn-glycerol	5-40 μ M	40 μ M	2.5 \pm 0.2
H-7	5-20 μ M	10 μ M	3.1 \pm 0.4
D-sphingosine	1-10 μ M	5 μ M	1.7 \pm 0.1
ALLNal 1.6 μ M + A23187	0.05-0.5 μ M	0.1 μ M	48.3 \pm 2.1
ALLNal 1.6 μ M + TPA	10-100ng/ml	100n/ml	48.9 \pm 3.8
ALLNal 1.6 μ M + H-7	5-20 μ M	10 μ M	45.7 \pm 2.7

PC12 cells were treated with the indicated concentrations of drugs in the presence or absence of 1.6 μ M ALLNal for 2 days, and the percentage of neurite-bearing cells was scored. Each value represents the mean \pm SEM of 2 duplicate determinations.

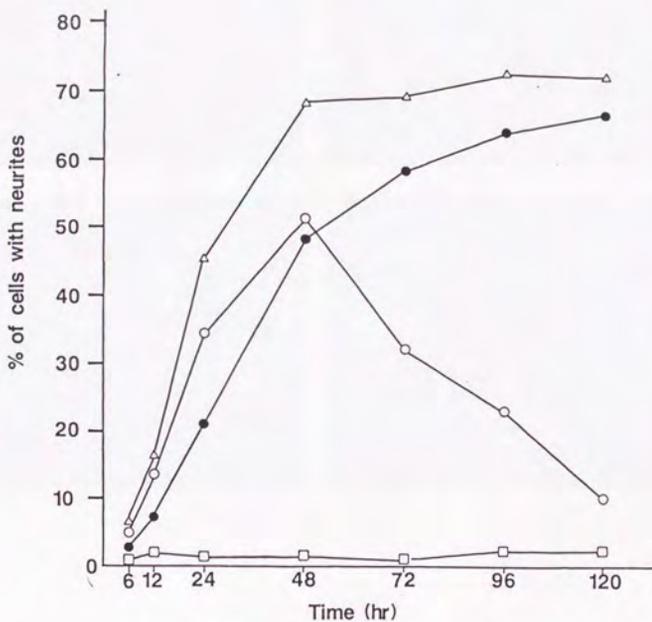


FIGURE.1-1

Time course for the appearance of neurites in PC12 cells following treatment with ALLNal and/or NGF.

Cultures (2×10^4 cells/cm²) were maintained with N2 medium in the presence of 1.6 uM ALLNal (○), 40 ng/ml NGF (●), 1.6 uM ALLNal+40 ng/ml NGF (△), or no addition (□) and the percentage of neurite-bearing cells was scored at intervals. Each value represents the mean of 3 determinations. The SEM did not exceed 10% of the mean.

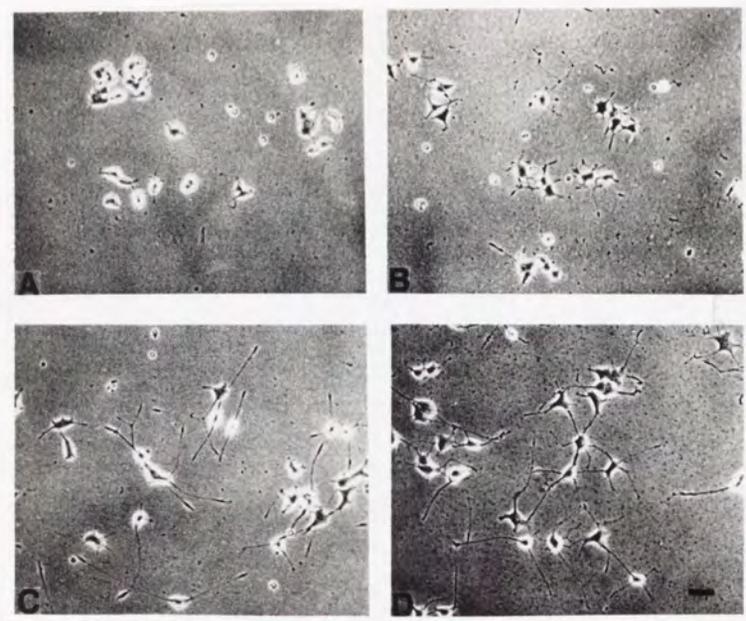


FIGURE.1-2

Phase contrast micrographs of PC12 cells grown for 2 days as described for Fig.1-1.

A, no addition; B, 40 ng/ml NGF; C, 1.6 uM ALLNal;

D, 40 ng/ml NGF + 1.6 uM ALLNal. Bar=35 um.

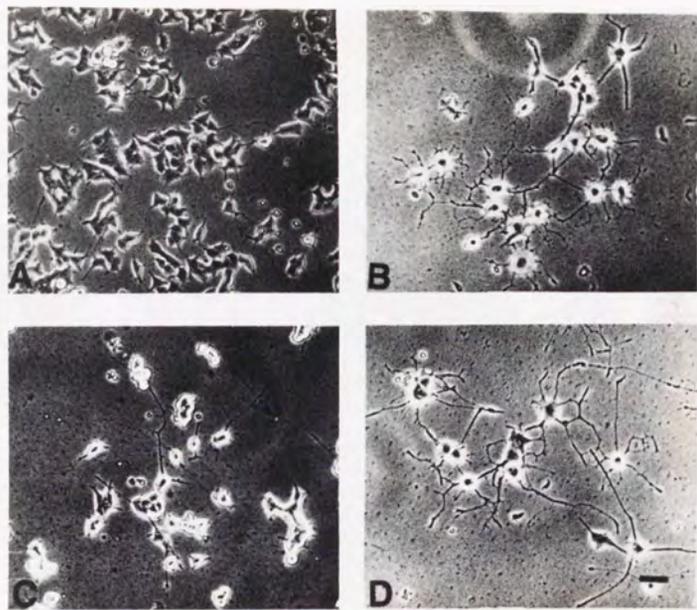


FIGURE.1-3

Phase contrast micrographs of PC12 cells grown for 5 days.

A, no addition; B, 40 ng/ml NGF; C, 1.6 uM ALLNal;

D, 40 ng/ml NGF + 1.6 uM ALLNal. Bar=35 um.

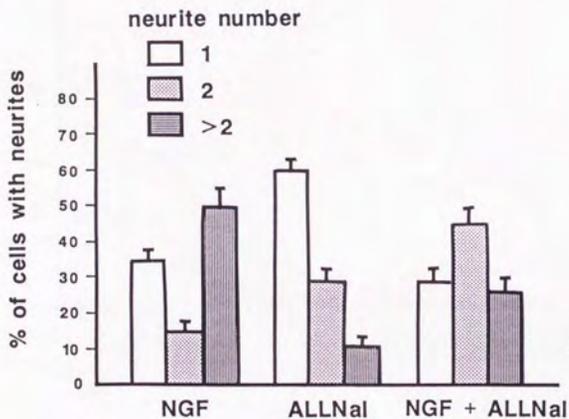


FIGURE.1-4

Distribution of PC12 cells according to neurite number.

PC12 cells were cultured for 2 days with 1.6 μ M ALLNal and/or 40 ng/ml NGF. The number of neurites was counted as indicated in MATERIAL AND METHOD. Each value represents the mean \pm SEM of 3 determinations.

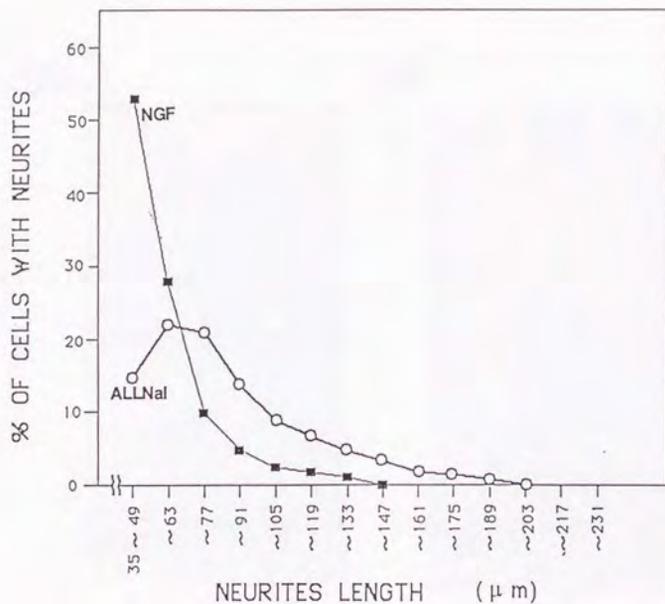


FIGURE.1-5

Neurite length distribution pattern showing the effects of ALLNal or NGF on neurite length.

Bioassays were performed by determining the percentages of neurites of various lengths longer than 35 μm . Cells were scored after 2 days in culture. Each value represents the mean of 3 determinations. The SEM did not exceed 10 % of the mean. The pattern resulting from incubation with 1.6 μM ALLNal (\circ) was significantly different from that resulting from incubation with 40 ng/ml NGF (\blacksquare) ($p < 0.01$) as estimated using Wilcoxon's rank-sum test.

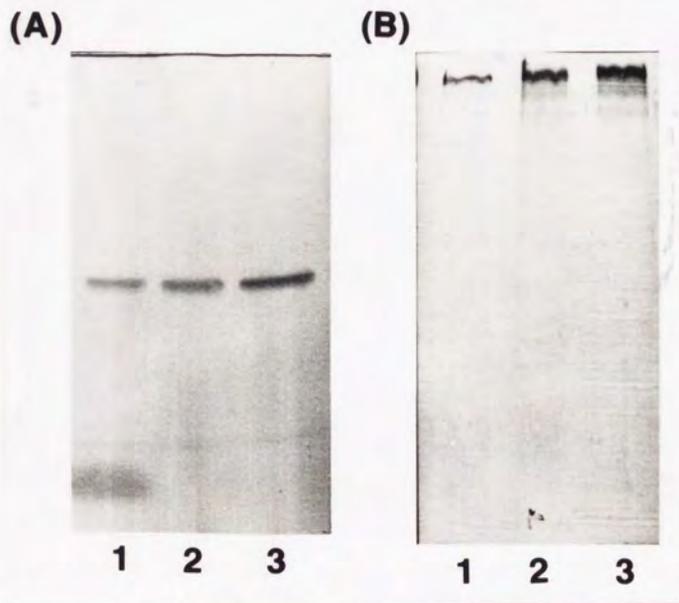


FIGURE.1-6

Changes in the tubulin and MAP1 content of PC12 cells after 2 days of treatment with NGF or ALLNal.

PC12 proteins were loaded onto SDS gel (A:30 ug/slot, B:15 ug/slot) and transferred to nitrocellulose. The nitrocellulose was cut into strips and incubated with antibodies against tubulin a and b(A) and MAP1(B).

lane 1: control (N2 medium only) lane 2: 40 ng/ml NGF

lane 3: 1.6 μ M ALLNal

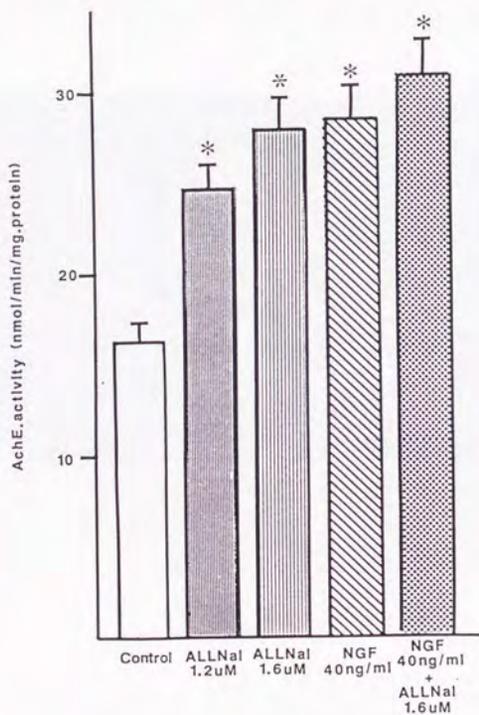


FIGURE.1-7

Effect of ALLNal and NGF on AChE activity in PC12 cells.

Cells were cultured for 2 days with or without ALLNal(1.2 and 1.6 uM) and/or NGF (40 ng/ml). Each value represents the mean \pm SEM of 5 determinations. Significant differences from control at $p < 0.01$ (*) estimated using Student's t test.

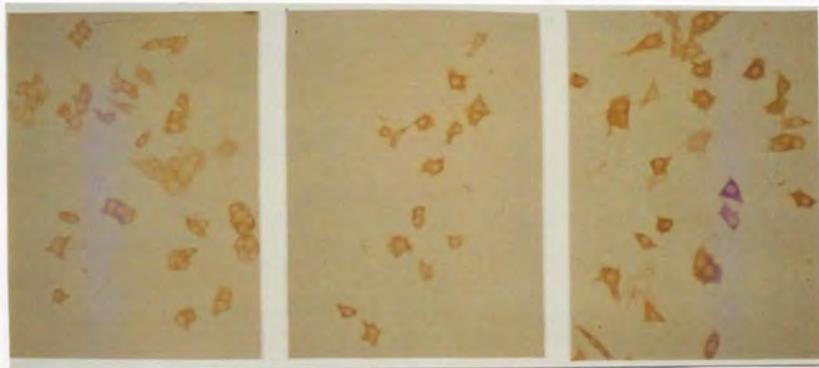


FIGURE.1-8

Immunostaining of PC12 cells for tyrosine hydroxylase.

PC12 cells were treated for 2 days with N2 medium (left), 40 ng/ml NGF (middle), and 1.6 uM ALLNaI (right). Cells were fixed and detergent-treated and immunostained with anti-tyrosine hydroxylase antibody at a dilution of 1:200.

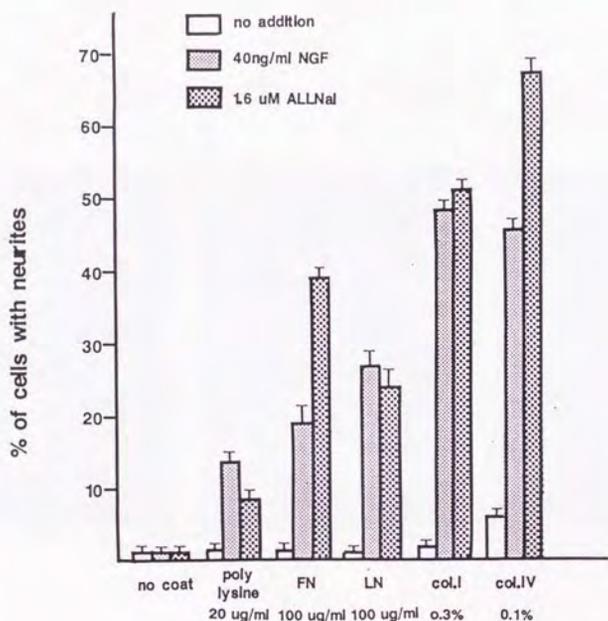


FIGURE.1-9

Influence of ECM molecules on the extent of neurite formation induced by ALLNal or NGF in PC12 cells.

PC12 cells were treated with 40 ng/ml NGF or 1.6 uM ALLNal on culture plates coated with ECM molecules. After 2 days, the percentages of neurite-bearing cells were determined. A maximal response occurred at the indicated concentration of each ECM molecule. Each value represents the mean \pm SEM of 2 duplicate determinations.

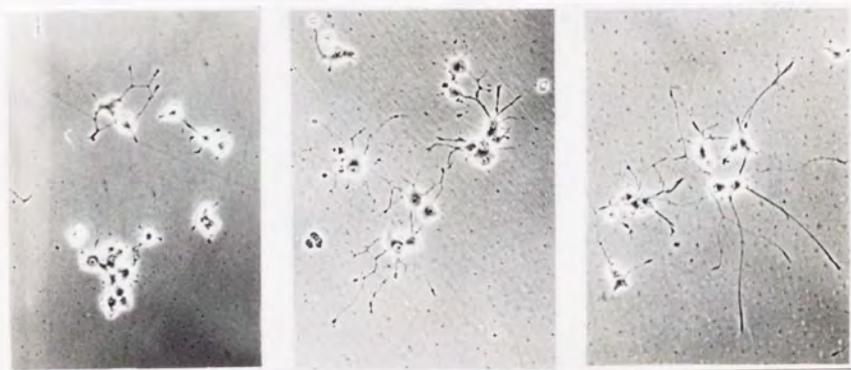


FIGURE.1-10

Phase contrast micrographs of NGF-primed PC12 cells under the following conditions.

PC12 cells were exposed to 40 ng/ml NGF for 5 days. The cells were trituated to shear off neurites, and replated for 20 hr in the presence of N2 medium (left), 40 ng/ml NGF (middle), and 1.6 μ M ALLNal (right).

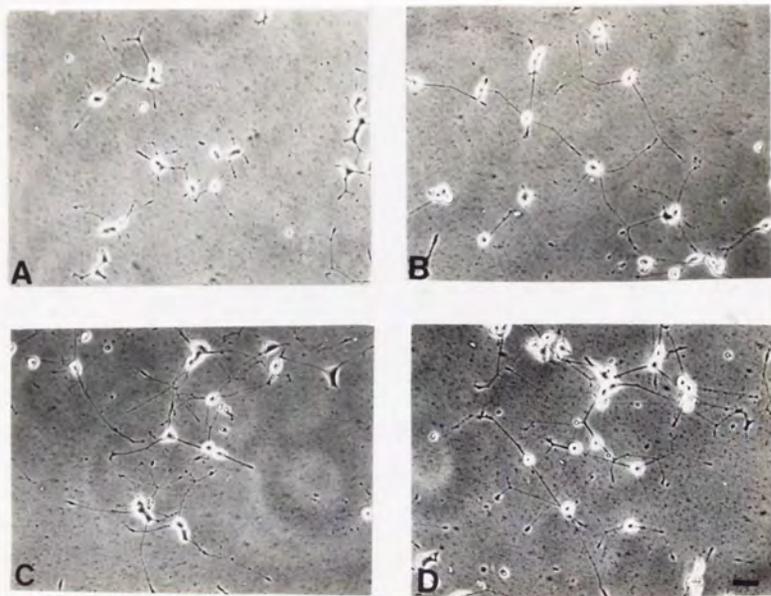


FIGURE.1-11

Phase contrast micrographs of PC12 cells grown for 2 days.

A, 10 ng/ml bFGF; B, 10 ng/ml bFGF + 1.6 uM ALLNal;

C, 0.5mM dBcAMP; D, 0.5mM dBcAMP + 1.6 uM ALLNal. Bar=35 um.

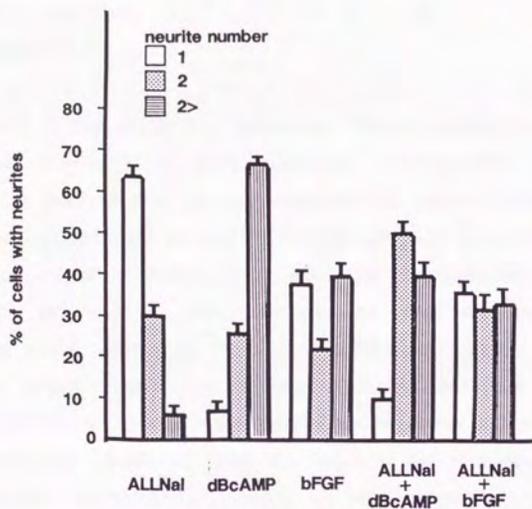


FIGURE.1-12

Distribution of PC12 cells according to neurite number.

PC12 cells were cultured for 2 days with 0.5mM dBcAMP or 10 ng/ml bFGF with or without 1.6 uM ALLNal. Each value represents the mean \pm SEM of 3 determinations.

CHAPTER 2

ANALYSIS OF NEURITE OUTGROWTH INDUCED BY A SPECIFIC PROTEASE INHIBITOR -PURIFICATION AND CHARACTERIZATION OF THE TARGET MOLECULES

INTRODUCTION

In Chapter 1 it was shown that Ac-Leu-Leu-Nle-al (ALLNal) causes the outgrowth of one or two long neurites, MAP1 induction, an increase of AChE activity, and enhancement of anti-TH antibody staining in PC12 cells. Since it seems likely that a specific functional molecule(s) is involved in the initiation of neuronal differentiation in PC12 cells, it is the most important matter to isolate and identify this molecule(s). For this purpose, some analogues of ALLNal were examined for their potency to initiate neurites in PC12 cells, and an attempt was made at affinity purification of the target molecule (s) using a more effective inhibitor (benzyloxycarbonyl-Leu-Leu-Leu-al:ZLLLal) as a ligand. Several lines of evidence indicate that protein isolated specifically from the affinity column was clathrin. Based on the interaction between ZLLLal and clathrin, a role for clathrin in neurite initiation is discussed.

MATERIALS AND METHODS

MATERIALS

Bovine brains were obtained from Shiba-ura slaughterhouse and Wistar rats were from Charles River. The following materials were obtained from the indicated commercial suppliers: Sepharose CL-4B, Pharmacia Co; Ultro gel ACA-34, LKB; Affigel-10, Bio-Rad ; S. aureus V8 protease, Seikagaku-kogyo (Tokyo); TPCK-trypsin, Boehringer-Mannheim ; anti-clathrin heavy chain monoclonal antibody⁶⁸), Progen Co., Ltd.

The series of ALLNal analogues synthesized according to the method of Ito et al.⁶⁹) were kindly provided by members of Prof. Ito's Laboratory at Aoyama-

Gakuin University. The identity of each analogue was confirmed by HPLC, fast atom bombardment mass spectrometry, and the 2,4-dinitrophenyl hydrazine test.

Other chemicals were obtained as listed in Chapter 1.

CELL CULTURE, NEURITE OUTGROWTH ASSAY, GEL ELECTROPHORESIS, and WESTERN BLOTTING were performed as described in Chapter 1.

INHIBITION ASSAY OF mCANP (CALCIUM-ACTIVATED NEUTRAL PROTEASE) ACTIVITY BY ALLNal ANALOGUES

mCANP (active in the presence of calcium ions of mM order) purified from rabbit skeletal muscle was kindly provided by Dr. Seiichi Kawashima⁷⁰). The protease and inhibitory activities were measured using alkali-denatured casein as a substrate. ALLNal analogues in various concentrations were added to a fixed amount of mCANP, and the reaction was started by adding substrate at a final concentration of 0.24% in a mixture containing 28mM 2-mercaptoethanol, 2.5mM CaCl₂, and 0.1M Tris-HCl (pH 7.5). After incubation for 15min at 30°C an equal volume of 10% TCA was added, and the mixture was centrifuged. The absorbance of the supernatants at 280nm was measured. The inhibitory activity of ALLNal analogues was expressed as the concentration required to inhibit 50% of the mCANP activity (IC₅₀).

AFFINITY CHROMATOGRAPHY

For affinity purification of the target molecule, H-Leu-Leu-Leu-semicarbazone was immobilized on Affigel-10 and the C-terminal group was converted to an aldehyde by acid treatment. PC12 cells were washed twice with PBS. The cells were then ruptured in homogenization buffer (0.1M sodium phosphate/0.2M NaCl/1mM EDTA/1mM EGTA/50 uM leupeptin/100 uM DFP, pH 7.5), and centrifuged at 100,000xg for 1hr to separate the cytosolic and membrane fractions. The membrane fraction was subsequently resuspended in 1% Triton X-100/homogenization buffer for 1hr at 4°C, and centrifuged at 100,000xg for 1hr. Each fraction was dialyzed against 0.1M sodium phosphate(pH 7.5)/0.2M NaCl for 18 hr at 4°C to remove EDTA, EGTA, leupeptin, and DFP, and then applied to the affinity column at 4°C. After washing the column with 0.1M sodium phosphate(pH 7.5)/0.2M NaCl/1% Triton X-100

and 0.1M sodium phosphate /1% Triton X-100, the proteins were eluted first with 0.1M citrate buffer (pH 4.0)/1% Triton X-100 at 4°C, and then with 0.1M sodium phosphate(pH 7.5)/6M urea at room temperature. The eluted proteins were precipitated in 10% trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis.

The conditioned media from PC12 cell cultures and rat brain homogenates (embryo 16 days, postnatal 1,3,14 days, and 3 months) were processed similarly. Affinity chromatography was done only for the cytoplasmic fractions of liver, kidney, spleen, heart, and muscle of 3 month old rats.

PEPTIDE MAPPING

Peptide mapping of proteins isolated in gels containing SDS was performed as described by Cleveland et al⁷¹). Proteins in SDS gels were stained with Coomassie Blue, destained briefly, rinsed with cold water, and placed over a light box. The individual bands were cut out, trimmed to 5mm wide, and then soaked 30min in a solution containing 0.125M Tris-HCl (pH 6,8)/0.1% SDS/1mM EDTA. The sample wells in 4% acrylamide were filled with the same buffer and each gel slice was pushed to the bottom of a well. Spaces around the slices were filled by overlaying each slice with 10 ul of the buffer containing 20% glycerol. Finally, 10 ul of the buffer containing 10% glycerol and a given amount of S.aureus V8 protease was overlayed into each slot and electrophoresis was performed in the normal manner except that the current was turned off for 30min when the bromophenol blue dye reached the bottom of the stacking gel. The pattern of the peptide fragments produced was detected using a silver staining kit (Wako).

2-DIMENSIONAL ELECTROPHORESIS

Analysis of proteins by mini-2D-electrophoresis was performed as described by Mikawa⁷²). Isoelectric focusing in a gel containing 1% of pH 3.5-10 ampholines and 0.25% of pH 4-6 ampholines was performed at 100V for 30min and 200V for 3hr. The SDS-polyacrylamide gel for the second dimension contained 10% acrylamide. The gels were stained with Coomassie Blue.

AMINO ACID SEQUENCING

Internal amino acid sequences were analyzed as described⁷³). Proteins were separated by SDS-polyacrylamide gel electrophoresis, and then electrophoretically transferred onto nitrocellulose. The protein-containing regions were detected by reversible staining with 0.1% Ponceau S in 1% acetic acid. The regions were cut out and transferred to Eppendorf tubes and destained by washing with 0.1M Tris-HCl (pH 8.0). Destained bands were incubated for 1hr at 37°C in 1ml of 0.5% PVP-40 dissolved in 0.1M acetic acid in order to prevent adsorption of the protease to the nitrocellulose during digestion. Excess PVP-40 was removed by extensive washing with water, and the nitrocellulose was cut into small pieces. The protein on each nitrocellulose piece was digested as follows. (1) TPCK-trypsin: up to 200 μ l of 0.1M Tris-HCl (pH 8.0)/acetonitrile (90:10, vol/vol), at 37°C for 18hr. (2) lysylendopeptidase: 0.1M Tris-HCl (pH 9.0)/acetonitrile (90:10, vol/vol), at 37°C for 18hr. Purified lysylendopeptidase was a kind gift from Dr. Maho Morishima. Enzyme-to-substrate ratios were kept at 1:25 (mol/mol). After digestion, the whole reaction mixtures were acidified with acetic acid and frozen at -20°C.

Enzymatic cleavage fragments were separated on HPLC system using a C8 column (8x250mm). The following buffer system was used; Buffer A: 0.05% trifluoroacetic acid in water, Buffer B: 0.05% trifluoroacetic acid in acetonitrile/H₂O, (5:95). Peptide-containing fractions were collected manually into Eppendorf tubes based on the UV absorption at 215nm, and sequenced with an ABI 477A Protein Sequencer equipped with an on-line ABI 120A PTH Analyzer.

PREPARATION OF CLATHRIN

Crude coated vesicles were isolated from bovine brains and adult rat livers by a modification of the methods of Blitz et al.⁷⁴) and Lisanti et al.⁷⁵). All operations were performed at 4°C. Four hundred grams of brain or liver was homogenized in an equal volume of buffer A (0.1M MES/1mM EGTA/0.5mM MgCl₂/0.02% NaN₃, pH 6.5) in a mixer employing three 10-s bursts at top speed. The homogenate was centrifuged at 20,000xg for 30min. The supernatant was saved, and the pellet was suspended in buffer A and homogenized as before. The second homogenate was centrifuged as before, and the resulting supernatant was combined

with the first. The combined supernatants were centrifuged at 100,000xg for 1hr. The resulting pellet was resuspended with a loose-fitting Dounce homogenizer in buffer A so as to produce a 30ml suspension. Equal portions of this suspension were layered on each of seven discontinuous sucrose gradients containing 4.5ml each of 5, 10, 20, 30, 40, 50, and 60% sucrose prepared in buffer A. The gradients were centrifuged at 50,000xg for 2hr with an SW28 rotar in the Beckman L8-80M centrifugal separator. All the material was removed from the gradients at the end of the centrifugation, exclusive of a red zone at the top and a dense white pad at the bottom. This material was diluted 1:3 with buffer A and pelleted at 100,000xg for 1hr. The pellets were resuspended in 12ml of buffer A and layered on six discontinuous gradients (5ml each of 20, 30, 40, 50, 55, 60%) and centrifuged at 50,000xg for 16hr. The 50/55% interface was collected, diluted 1:3 with buffer A and pelleted.

Pellets of crude coated vesicles were resuspended in 300ml of buffer B (0.02M Tris-HCl/1mM EDTA/2mM DTT/ 0.02% NaN₃, pH 7.5) and extracted for 18hr at 4°C with stirring. Subsequently, the extract was clarified by centrifugation for 1hr at 100,000xg. The clear supernatant was made 50% in ammonium sulfate saturation, left in the cold for 30min, and centrifuged at 20,000xg for 30min. The resulting pellets were resuspended in 10ml buffer C (0.5M Tris-HCl/1mM EDTA/2mM DTT/0.02% NaN₃/0.1mM PMSF, pH 7.5) and subjected to gel filtration on a Sepharose CL-4B column (2.5x85cm) equilibrated with buffer C. Fractions were screened by SDS gel electrophoresis and pooled (column-purified clathrin).

SEPARATION OF CLATHRIN HEAVY AND LIGHT CHAINS

The clathrin heavy and light chains were separated according to the method of Lisanti et al.⁷⁵⁾ (METHOD-1), and Winkler and Stanley⁷⁶⁾ (METHOD-2).

METHOD-1. Column-purified clathrin was heated in a boiling water bath for 5min and centrifuged at 100,000xg for 60min. Clathrin light chains were obtained in the supernatant and clathrin heavy chains were in the pellet.

METHOD-2. Column-purified clathrin was brought to 50% ammonium sulfate saturation and, after 1hr, centrifuged for 60min at 20,000xg. The precipitate

was resuspended to 4mg/ml in 50mM Tris-HCl/50mM NaCl/2mM EDTA/1mM DTT/0.1mM PMSF, pH 8.0. The concentrated clathrin was brought to 1.3M NaSCN by adding 170 ul of 9M NaSCN/14mM EDTA solution and subjected to gel filtration on a Ultro gel ACA 34 column (1.6x30cm) equilibrated with 50mM Tris-HCl/1.3M NaSCN/2mM EDTA/2mM DTT, pH 8.0. Heavy chains, which eluted close to the void of 0.35 column volumes, and light chains, which eluted at 0.58 column volumes were pooled according to the OD 280 profile and subjected to SDS-polyacrylamide gel electrophoresis. Clathrin heavy and light chains were dialyzed twice against 11 volumes of buffer C, and the aggregates formed were removed by centrifugation for 60min at 100,000xg.

Heavy chains and light chains prepared as described above were dialyzed against 0.1M sodium phosphate (pH 7.5)/0.2M NaCl at 4°C for 18hr, and subjected to LLLal or LLL-COOH affinity chromatography.

ASSEMBLY STUDIES ON CLATHRIN

Clathrin assembly *in vitro* was performed following the procedure of Blank and Brodsky⁷⁷). Column-purified clathrin was dialyzed overnight at 4°C in buffer D (0.02M Tris-HCl/1mM EDTA, pH 7.8). Clathrin was then centrifuged at 50,000xg for 20min and dialyzed against buffer E (0.1M MES/1mM MgCl₂/1mM EDTA, pH6.2) for 1hr at 25°C. Polymerized clathrin was pelleted by centrifugation at 150,000xg for 1hr. The pellet was resuspended, homogenized, and dialyzed against buffer D diluted 1:4 (buffer D') for 90min at 4°C. After centrifugation, clathrin was used for assembly studies.

The effects of ZLLLal and ZLLL-COOH on assembly were examined by mixing the molecules with 120 ul of clathrin (0.4mg protein/ml) for 18hr at 25°C. Assembly was initiated by the addition of 5 ul of 1M MES, pH 6.0. This lowers the pH of the solution to 6.2. Assembly was followed by monitoring the absorbance at 320nm. Disassembly was induced by the addition of 5 ul of 1M Tris-HCl (pH, 9.0).

ELECTRON MICROSCOPY

Clathrin samples (80 ug/ml) were applied to carbon-coated 100-mesh copper

grids, stained with 2% uranyl acetate, and examined using a Hitachi H-600 electron microscope.

IMMUNOCYTOCHEMISTRY

Cells treated with NGF, ALLNal, and ZLLal were fixed for 10min at room temperature with 3% formaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS for 5min at room temperature. Each well was rinsed with PBS and blocked with 20% normal goat serum in PBS for 1hr at room temperature. After blocking, anti-clathrin heavy chain antibody (0.25 ug/ml) was added to each well and the samples were incubated at 37°C for 1hr. The cells were then washed with PBS and incubated first with biotinylated species specific anti-mouse IgG antibody, and then with streptavidin-biotinylated peroxidase complex. Finally, the samples were stained with diaminobenzidine and H₂O₂.

RESULTS

1. Relationship between ALLNal derivatives and the initiation of neurite outgrowth in PC12 cells

Various analogues of ALLNal were synthesized and examined for their effects on neurite formation in PC12 cells. Replacement of the N-terminal acetyl residue with benzyloxycarbonyl (Z) one (Z-Leu-Leu-Nle-al:ZLLNal) resulted in a reduction in the optimal concentration for neurite initiation from 1.6 uM to 0.3 uM. In Chapter 1, it was reported that acetyl-Leu-Leu-Nle-al, but not acetyl-Leu-Leu-Met-al, is an effective neurite initiator, so it is possible that the neurite-initiating effect of the tripeptide aldehyde depends on the amino acid residue at the P3 site. Therefore, P3 site-substituted tripeptide aldehydes with the Z group at the N-terminus were synthesized and used to investigate the subsite specificity involved in neurite initiation (Table 2-1). Analogues with alterations to hydrophobic residues in the P3 site (Ile, Val, Nva, Phe, and Leu) induce neurite outgrowth effectively at various concentrations (0.03-0.3 uM).

The neurites induced by these Z-Leu-Leu-X-al (X=Nle, Ile, Val, Nva, Phe, and Leu) are similar to those induced by ALLNal in morphological terms, namely one or two long neurites produced from a cell body (Fig.2-1C, D, E). Z-Leu-Leu-Leu-al (ZLLLal) is the most potent initiator of neurite outgrowth, while Z-Leu-Leu-Phe-al is the next strongest. ZLLLal induces neurite outgrowth in 50% of the cells at a concentration as low as 30nM, 50-fold lower than the concentration of ALLNal required to produce the same effect. The persistence of the neurites induced by ALLNal analogues was also examined (Table 2-2). In the case of neurites induced by ALLNal, the percentage of cells bearing neurites is maximal after 48hr and then starts to decrease. When ZLLLal was used, the maximal response was also observed 48hr after treatment; this response remained unchanged until 72hr and 30% of the cells still had neurites even after 120hr. The same pattern was found for other Z-Leu-Leu-X-al (X=Nle, Ile, Val, Nva, Phe).

Table 2-3 summarizes the relationships between the hydrophobicity of the 3rd amino acid residue⁷⁸, that of the whole molecule, and the ability to induce neurite outgrowth. The results show that the hydrophobicity of Z-Leu-Leu-X-al is not consistent with the potency for neurite outgrowth. For example, Z-Leu-Leu-Ile-al is the most hydrophobic analogue and the optimal concentration for neurite outgrowth is 0.25 μ M, while less hydrophobic Z-Leu-Leu-Leu-al produces neurite outgrowth at 30nM as mentioned above. However, alteration of P3 to a much less hydrophobic residue (Gly or Ala) results in much weaker induction, and the neurites produced are not so long as those induced by ALLNal (Fig.2-1C,F). This suggests that a moderate hydrophobicity is important for both neurite initiation and elongation.

ALLNal was originally developed as an inhibitor of CANP (calcium-activated neutral protease; see Chapter 1); therefore, the inhibition of mCANP activity by the ALLNal analogues was examined (Table 2-4). All Z-Leu-Leu-X-al have, to a greater or lesser extent, protease inhibitory activity against CANP *in vitro*. The inhibitory activity of Z-Leu-Leu-X-al against CANP is not consistent with the potency for neurite outgrowth. For example, Z-Leu-Leu-Leu-al has the strongest potency for neurite outgrowth, while the strongest inhibitor against CANP is Z-Leu-Leu-Ile-al (IC_{50} =26nM). However, Z-Leu-Leu-Gly-al is the weakest analogue as both an initiator of neurite outgrowth and a CANP inhibitor

(IC₅₀=3700nM).

To confirm the effect of peptide chain length, a Leu residue was either added to or deleted from ZLLLal (Table.1-1). Deletion of a Leu residue increased the effective concentration by about 3300-fold; and in addition, only 20% of cells formed neurites. Addition of a Leu residue had little effect on the effective concentration, but the percentage of neurite-bearing cells was lower than in cells induced by ZLLLal. This implies that a chain length of three amino acid residues is optimal for neurite outgrowth.

Next the subsite specificity of the N- and C-terminal residue involved in neurite outgrowth in PC12 cells was examined (Table 2-1). When the N-terminal blocking group of ZLLLal was replaced by acetyl, butoxycarbonyl (Boc), propyl (Pr), butyl (Bu), or dansyl (Dans), Dans-Leu-Leu-Leu-al was the most effective for neurite outgrowth. Boc, Pr, and Bu, which have aliphatic chains, were not so effective. As for the C-terminal site, semicarbazone (sc), chloromethyl ketone (CH₂Cl), and CF₃ are functional groups that inhibit protease activity as well as aldehyde. Z-Leu-Leu-Leu-CH₂Cl induces neurite outgrowth in 50% of cells at a concentration of 12.5 uM, which is a 400-fold higher concentration than ZLLLal. Z-Leu-Leu-Leu-sc induces neurite outgrowth about in 40% of the cells at 50nM, while Z-Leu-Leu-Leu-CF₃ does not induce neurite outgrowth effectively. Neither ZLLL-COOH nor ZLLL-OH induce neurite outgrowth in PC12 cells.

Besides the P3 residue, analogues with substitutions at P1 or P2 site were synthesized to examine whether these subsites influence neurite outgrowth (Table 2-1). Z-Ala-Leu-Leu-al and Z-Leu-Ala-Leu-al induced neurite outgrowth at 10- and 38-fold lower concentrations than Z-Leu-Leu-Ala-al, respectively. Furthermore, neurites induced by Z-Ala-Leu-Leu-al or Z-Leu-Ala-Leu-al look like those induced by ZLLLal, and are longer than those induced by Z-Leu-Leu-Ala-al. These results show that the amino acid residue at the P3 site is the most critical for neurite outgrowth, that P1 is the next most important subsite, and that P2 is the least important. Substitution at the P1 or P2 site to the hydrophobic residue Phe, results in little change in the concentration required for neurite outgrowth as compared with ZLLLal.

Some details of the structure-function relationship has been revealed for ALLNal analogues. Among ALLNal analogues, the tertiary structures formed by ZLLLal and Dans-LLLal are likely to be the best fitted for eliciting neurite outgrowth in PC12 cells.

2. Purification and identification of the target molecules for ZLLLal by affinity chromatography

Since ZLLLal has a strong potency to induce neurite outgrowth in PC12 cells at low concentrations, an Affigel-10 affinity resin with Leu-Leu-Leu-al(LLLal) immobilized at the amino terminus was prepared.

[1] Purification of the target molecule for ZLLLal

When the cytoplasmic fraction of PC12 cells was applied to the LLLal affinity column and the bound proteins were eluted first with citrate buffer (pH 4.0) and then with 6M urea/sodium phosphate buffer (pH 7.5), 33K, 35K, and 180K proteins were observed on SDS-polyacryamide gel electrophoresis. The proteins eluted readily with citrate buffer (pH 4.0) were found to consist much more of the 33K than 35K species, while the 180K protein was effectively eluted with 6M urea (pH 7.5)(Fig.2-2). Elution with 0.1% SDS after 6M urea (pH 7.5) showed that no proteins remained bound to the affinity column. The 33K, 35K, and 180K proteins did not elute when neither the ionic strength (0-1.5M NaCl) nor the high pH (pH 9.0) were changed. The 33K, 35K, and 180K proteins were also found in the membrane fraction, but the amounts per cell were smaller than in the cytoplasmic fraction (Fig.2-2). Z-Leu-Leu-Leu-COOH had no ability to induce neurite outgrowth in PC12 cells (Table 2-1), and the 33K, 35K, and 180K proteins did not bind to a Leu-Leu-Leu-COOH affinity column (Fig.2-2). Soon after elution from the column, neutralization of the 33K/35K protein fraction or removal of urea from the 180K protein fraction were performed, but the proteins could not bind again to a new LLLal affinity column. This indicates that treatment with low pH or urea rapidly induces an irreversible denaturation of the LLLal-binding site or gross conformational changes.

When conditioned media from the PC12 cell cultures were applied to the LLL-COOH and the LLLal affinity columns, no specific binding of any proteins, including the 33K, 35K and 180K proteins, was detected (Fig.2-3). The concentrated conditioned medium does not block neurite outgrowth induced by ZLLLal in PC12 cells. Moreover, LLLal immobilized to Affigel-10, which does not penetrate the cytoplasmic membrane, has no effect on neurite outgrowth in PC12 cells. These results suggest that the target molecules of ZLLLal are intracellular and not extracellular, and that free ZLLLal can penetrate through the PC12 cell membrane.

Since ZLLLal is an effective inhibitor of CANP (Table 2-4), PC12 cell homogenates were charged to the LLLal affinity column under various buffer conditions with or without Ca^{2+} . However, CANP apparently did not bind to the column under any binding conditions.

The 33K and 35K proteins that elute from the affinity column at low pH were the focus of an attempt to purify and identify the target molecules of ZLLLal. Because PC12 cells were derived from rat, cytosol fractions from the liver, heart, muscle, spleen, kidney, and brain of rats (3 months) were applied to the LLLal affinity column, and the column was eluted with citrate buffer (pH 4.0). Fig.2-4 shows the eluted proteins detected by SDS-polyacrylamide gel electrophoresis. Appreciable amounts of the 33K/35K proteins were identified in heart and muscle.

Proteins eluted from the LLLal affinity column at low pH were investigated for their appearance during brain development in the rat (embryonic 16 days, postnatal 1, 3, 14 days, and 3 months). Fig.2-5 and Table 2-5 summarize the pattern of the eluted proteins detected by SDS-polyacrylamide gel electrophoresis. As a proportion of the total protein applied to the column, the amount of the 33K protein was very low in 16 days embryos, but rapidly increased to maximal levels 1-3 days postnatally, and then decreased from 14 days postnatally. The amount of the 35K protein was greater than that of the 33K protein in 16 days embryos, in contrast to the situation in PC12 cells, but the level decreased from 14 days postnatally. From 1 day postnatally, the 36K/38K proteins began to appear, at which time the proteins were present in the order: 36K > 33K, 35K > 38K. After about 14 days, the amount of the 33K/35K

proteins decreased, as mentioned above, and only small amounts could be detected in adult (3months) rat brain. On the other hand, the amount of the 36K/38K proteins decreased gradually, but were the as only proteins detected in adult rat brain. A similar transition of the 33, 35, 36, 38K proteins was observed in the membrane fraction during brain development. These results indicate that the 33K/35K and 36K/38K proteins are switching proteins in development and are involved in some way brain development.

Fig.2-6 shows the peptide maps of the 33K proteins derived from PC12 cells, 1 day postnatally rat brain, and 3 month rat muscle after limited proteolysis by *S.aureus* V8 protease. Since the same *S.aureus* V8 protease digestion fragments of the 33K protein can be seen despite the tissue source, the 33K proteins isolated from 1 day postnatally rat brain and 3 month rat muscle are thought to be the same as the 33K protein isolated that from PC12 cells. As rat muscle is easily obtained in large amounts, 33K protein isolated from rat muscle was used for internal amino acid sequence analysis.

[2] Identification of the target molecule of ZLLLal

The determination of the partial amino acid sequences of the 33K protein from rat muscle was performed by the micro-sequence analysis method of Aebersold et al.⁷²⁾ using TPCK-trypsin or lysylendopeptidase as the in situ digestion enzyme. Three peptide sequences were obtained which were identical to the clathrin b light chain, a protein that plays a central role in receptor-mediated endocytosis (Table.2-6). The N-terminal residue was found to be blocked in both the 33K protein and the clathrin light chain b^{79,80)}. Clathrin molecules form a triskelion, or "three legged", shape. The projections are often described as arms consisting three heavy and three light chains³⁴⁾ linked noncovalently. Next it was examined whether (1) the 33K protein is the clathrin light chain b itself or a "clathrin-like" protein, and (2) clathrin light chain a and clathrin heavy chain co-exist with the 33K protein.

2D-electrophoresis showed that the 33K/35K proteins derived from 1 day postnatally rat brain are acidic proteins, with mobilities identical to the authentic light chain a (35K) and b (33K) of rat liver clathrin. The molecular weight of the 180K protein eluted from the LLLal affinity column by urea corresponded to that of authentic clathrin heavy chain, and furthermore, reacted with antiserum specific for the clathrin heavy chain on immunoblotting (Fig.2-8).

To verify the functional identity of the proteins derived from the LLLal affinity column and authentic clathrin molecules, purified authentic clathrin from rat liver and bovine brain were applied to the LLLal affinity column. Fig.2-9 A,B shows that authentic clathrins from both rat liver and bovine brain do not bind to the LLL-COOH affinity column but do bind to the LLLal affinity column. The clathrin light chains were eluted by citrate buffer (pH 4.0), while the clathrin heavy chain was eluted by 6M urea (pH 7.5). These elution patterns are the same as for the 33K, 35K, and 180K proteins from PC12 cells (Fig.2-2).

The above results suggest that the target molecule involved in neurite outgrowth in PC12 cells is clathrin. The 33K/35K proteins eluted at low pH are the clathrin light chains b and a, respectively, and the 180K protein eluted with 6M urea is the clathrin heavy chain. A comparison of clathrins from different tissues shows that the higher molecular weight forms of light chain a (38K) and light chain b (36K) are expressed in brain tissue while 33K and 35K are expressed in non-brain tissue^{79,80}). When a homogenate of adult rat brain was applied to the LLLal affinity column, the 36K/38K proteins, but not the 33K/35K proteins, were specifically bound (Fig.2-5). These 36K/38K proteins are regarded as brain type light chains. This result also supports the notion that clathrin itself is able to bind to the LLLal affinity column.

The existence of two intracellular pools of clathrin, assembled and unassembled forms, in various cells has been reported^{81,82}). Therefore, the ratio of clathrin in the assembled and an unassembled forms in PC12 cells was quantitated using Buffer A (0.1M MES/1mM EGTA/0.5mM MgCl₂/0.02% NaN₃, pH 6.5), known to be a stabilizer of clathrin coats *in vitro*⁸²), and immunoblotting with anti-clathrin heavy chain antibody. It was found that about half the clathrin

is in the unassembled form. This unassembled clathrin has the ability to bind to the LLLal affinity column, but not to the LLL-COOH affinity column (data not shown). These results imply that ZLLLal interacts with the unassembled form of clathrin in PC12 cells.

The next principal question is whether it is the heavy chain or light chain that is directly involved in the binding to LLLal affinity column. Two methods were employed to dissociate clathrin into light and heavy chains. The first method is the heat-denaturation⁷⁵⁾, which takes advantage of the fact that the clathrin light chain is heat-resistant and remains soluble, while the clathrin heavy chain, along with other proteins, is highly susceptible to denaturation and can be removed by centrifugation (Fig.2-10A). The other separation method uses the chaotropic ion thiocyanate to destabilize the hydrophobic interactions between the subunits at concentrations that usually do not unfold proteins⁷⁶⁾ (Fig.2-10B). The latter method has the advantage that the heavy chain does not form large irregular aggregates.

Fig.2-11 shows SDS-polyacrylamide gel electrophoretic patterns of heat-treated clathrin heavy or light chains following LLLal affinitychromatography. Even the heat-resistant clathrin light chain is not bound. On the other hand, the clathrin heavy chain isolated by thiocyanate does bind to the LLLal affinity column, but not to the LLL-COOH column (Fig.2-12B). In contrast, the clathrin light chains isolated by thiocyanate do not bind to the LLLal affinity column (Fig.2-12A). As shown in Fig.2-10B, the clathrin light chain fractions from gel filtration in the presence of thiocyanate contain little contamination by clathrin heavy chain. In Fig.2-12A, the contaminating clathrin heavy chain is clearly detected as an eluted protein from the LLLal affinity column. These results indicate that clathrin heavy chain is the component that binds directly to the LLLal affinity column. Since the binding constant between the heavy and light chains is $K_d=10^{-8}-10^{-10} M^{76)}$, clathrin light chain may bind indirectly to the LLLal affinity column via the clathrin heavy chain.

[3] Effect of ZLLLal on clathrin molecules

The results of [1] and [2] strongly suggest that ZLLLal penetrates through the cytoplasmic membrane and binds to clathrin molecules via the clathrin heavy chain. An in vitro clathrin assembly system was used to examine the events the bindings to ZLLLal to clathrin molecules.

Assembly-competent clathrin was prepared from column-purified clathrin using a protocol developed by Blank and Brodsky⁷⁷). This procedure involves one cycle of assembly followed by disassembly and yields clathrin in a low ionic strength buffer (buffer D':4mM Tris-HCl/0.2mM EDTA, pH 7.8). This clathrin assembles into baskets when the pH is lowered to 6.2 by adding 1M MES (pH 6.0). Assembly can be monitored spectrophotometrically at 320nm because of the increased light-scattering properties of assembled clathrin compared with disassembled one. Assembly-competent clathrin preincubated with ZLLLal at various concentrations, undergoes an increase in absorbance at 320nm to near maximal levels within 10min following the addition of MES (Fig.2-13). Although the maximum absorbance in the presence of ZLLLal does not differ from that in the presence of DMSO alone (control), ZLLLal is able to increase the rate of clathrin polymerization in the initial stage. In fact, the rates of polymerization during the first minute in the presence of 8 μ M, 16 μ M, and 33 μ M ZLLLal are about 20, 31, and 73% greater than that of the control(DMSO), respectively. In contrast, 33 μ M ZLLL-COOH does not increase the rate of clathrin polymerization; in fact, the rate decreases slightly. To assess the effects of ZLLLal on the structure of assembled clathrin, the assembly products were examined by electron microscopy (Fig.2-14A,B). Clathrin assembled in the presence of ZLLLal forms normal basket structures which containing various pentagons and hexagons.

ZLLLal was also tested for its effect on assembled clathrin baskets. When ZLLLal was incubated with assembled clathrin baskets for 1hr at 25°C, no effect was seen on the absorbance at 320nm was seen. After 18hr incubation at 25°C with ZLLLal, no difference in the basket structures were observed on electronmicroscopy (Fig.2-14C,D).

The above results indicate that ZLLLal has an effect on the assembly of clathrin into normal basket structures, but not on the assembled clathrin basket

structures. It is possible that ZLLLal binds to free clathrin *in vivo* and that this binding contributes to the increase in the rate of clathrin assembly.

To visualize the distribution of the clathrin heavy chain in the course of neurite formation, PC12 cells treated with NGF or ZLLLal were stained with anti-clathrin heavy chain monoclonal antibody, CHC 5.9⁶⁸). PC12 cells extracted with Triton X-100 and fixed with formaldehyde showed granular structures throughout the cytoplasm with more intense staining in the nuclear regions (Fig.2-15A). After culture for 3-7hr with ZLLLal, no distinct changes in staining were observed. However, after culture for 18-20hr, during which time the PC12 cells began neurite initiation, the distribution of clathrin heavy chain was significantly different from that of untreated cells: the perinuclear staining was more pronounced and evident (Fig.2-15C). In contrast, PC12 cells grown in NGF for 20hr, showed a grainy staining pattern on CHC 5.9 immunocytochemistry which was indistinguishable from untreated cells (Fig.2-15B). Quantitative analysis with immunoblotting indicated that the total content of clathrin heavy chain in PC12 cells does not increase following incubation of the cells with ZLLLal for 20hr. Thus, the enhanced staining of clathrin heavy chain in ZLLLal-treated cells is not due to a change in its level. Since this intensifying of the staining of clathrin heavy chain is induced by ZLLLal but not by NGF, clathrin seems to be involved in the neurite formation initiated by ZLLLal.

DISCUSSION

A set of 24 ALLNal analogues was synthesized and examined for their potency in inducing neurite outgrowth, and the analogues Z-Leu-Leu-Leu-al (ZLLLal), with a has 50-fold stronger potency than ALLNal, was obtained. The neurites induced by ZLLLal are similar to those induced by ALLNal, but persisted longer. To identify the target molecule of ZLLLal, LLLal was immobilized and used as a ligand for affinity chromatography. Proteins of 33K, 35K, and 180K were isolated specifically from PC12 cells, and several lines of evidence suggest that the proteins are components of clathrin. In vitro clathrin assembly studies revealed that the initial rate of clathrin polymerization to normal basket structures is increased by ZLLLal. Moreover, in time with neurite initiation by ZLLLal in PC12 cells, the immunostaining of the clathrin heavy chain is enhanced compared with untreated cells. These results suggest that ZLLLal penetrates through the cytoplasmic membrane and interacts with clathrin molecules. This interaction may result in neurite formation.

The site of action of the neurite-initiating protease inhibitor (namely, the location of the target molecule) in PC12 cells will be intracellular. The reason for this is that (1) neurite outgrowth induced by ZLLLal is not blocked by the concentrated serum-free conditioned medium; (2) LLLal-immobilized Affigel-10, which does not penetrate through the cytoplasmic membrane, has no effect on neurite outgrowth; and (3) all the effective tripeptide aldehydes are hydrophobic in nature (Table.2-3), so they can penetrate through the cytoplasmic membrane. It has been reported that the nervous systems by several species secrete a thrombin-like protease or plasminogen activator, that is, these act all extracellular. These protease-protease inhibitor systems have been implicated in neurite outgrowth stimulated by degradation of the extracellular matrix²⁷). On the other hand, ALLNal and ZLLLal enter the cytoplasm and regulate neurite outgrowth at intracellular sites. Since most protease inhibitors can not penetrate through the cell membrane, these new inhibitors, ALLNal and ZLLLal, are expected to be useful tools for general cell biology.

The 33K, 35K, and 180K proteins that bind to the LLLal affinity column were identified as clathrin light chains (33K, 35K) and clathrin heavy chain (180K). The evidence for this is that 1) the three peptide sequences obtained from the 33K protein are identical to the partial sequence of clathrin light chain b, 2) the electrophoretic mobility of the 33K/35K proteins are identical to those of clathrin light chains b/a derived from rat liver; 3) the 180K protein is recognized by the anti-clathrin heavy chain monoclonal antibody; and 4) authentic clathrin from rat liver and bovine brain bind to the LLLal affinity column, while the 33K, 35K, and 180K proteins and authentic clathrin do not bind to LLL-COOH affinity column.

In addition, there is further evidence that these 33K and 35K proteins are clathrin light chains. Acton and Brodsky reported that the relative levels of expression of clathrin light chain a and b in cells and tissues with and without a regulated secretory pathway⁸³). They found by immunoprecipitation that a higher proportion of clathrin light chain b is present in PC12 cells. This result correlates with the predominance of the 33K protein in the LLLal chromatography of PC12 cells (Fig.2-3). The molecular weight of clathrin light chains depends upon the tissue, that is, the 36K/38K proteins are expressed in brain and the 33K/35K proteins are expressed in non-brain tissues^{79,80}). In this study using the LLLal affinity column, the 33K/35K proteins were obtained from non-brain tissues (adult heart and muscle), while the 36K/38K proteins were obtained from adult brain (Fig.2-4,2-5). This result indicates that the 33K/35K proteins and the 36K/38K proteins that bind to the LLLal affinity column are clathrin light chains.

Clathrin (unassembled form) has been shown to bind not only to the LLLal affinity column but also to free ZLLLal itself, after which the rate of clathrin assembly to the coat structure is increased (Fig.2-13). ZLLL-COOH, which has no effect on neurite outgrowth in PC12 cells, does not enhance the rate of clathrin assembly. Thus, it is likely that the stimulation of the rate of clathrin polymerization following the binding between ZLLLal and free clathrin (unassembled clathrin) is due to the initiation of neurite outgrowth. In PC12 cells, the presence of an intracellular pool of unassembled clathrin with the

ability to bind to the LLLal affinity column may exist. These results imply that ZLLLal in PC12 cells binds and interacts with the unassembled form of clathrin, although the possibility that it also binds to the assembled form of clathrin can not be excluded completely. However, ZLLLal does not induce drastic morphological changes in the assembled clathrin coat structure, even after overnight incubation in vitro, so it seems unlikely that ZLLLal in PC12 cells distorts the structure of assembled clathrin.

The LLLal-affinity chromatography of separated clathrin heavy and light chains suggests that the binding of clathrin to ZLLLal occurs via the clathrin heavy chain (Fig.2-12). Since ZLLLal enhances the rate of clathrin assembly, the binding site of ZLLLal may be important for the regulation of assembly. Clathrin heavy chain is composed of a proximal segment, a distal segment, a link region, and a terminal domain from the triskelion vertex which contains the carboxyl end of the molecule^{34,84}). Among these regions, the proximal region is the region involved in binding with the clathrin light chain⁸⁵), and this binding is very strong ($K_d=10^{-8}-10^{-10}$ M)⁷⁶). Because an exposed site on clathrin is required for the binding to ZLLLal, it is little possible that the proximal region is responsible for the direct binding to the LLLal affinity column. The terminal domain and the link region are protease-sensitive and can be cleaved away from the clathrin heavy chain⁸⁶). By using this characteristic, the region involved in the direct binding to ZLLLal may be identified.

Immunostaining with an antibody against the clathrin heavy chain in PC12 cells is enhanced in parallel with neurite initiation induced by ZLLLal (Fig.2-15). The anti-clathrin heavy chain antibody (CHC.5.9)⁶⁸) employed in this study recognizes a panctate material, that is, clathrin in the assembled state. The enhanced staining induced by ZLLLal in PC12 cells may reflect assembled clathrin formed efficiently by ZLLLal, since ZLLLal stimulates the rate of clathrin assembly. Another explanation for the nature of the enhanced staining is that newly assembled clathrin induced by ZLLLal affects the localization of the pre-existive assembled clathrin. Attempts to confirm these speculations by immunoelectron microscopy are now in progress in an effort to characterize the

strong staining induced by ZLLLal.

Another important point in need of address is what pathway or molecule is responsible for neurite initiation after the interaction between ZLLLal and clathrin.

Coated vesicles or clathrins are reported to interact with cytoskeletal proteins; for example, 1) tubulin α and β are main components of coated vesicles⁸⁷⁾, 2) electron microscopic analysis has shown that actin filaments bind to coated vesicle^{88, 89)}, 3) muscle G-actin, F-actin, and α -actinin are bound by clathrin-coated polystyrene particles *in vitro*⁹⁰⁾. By using the cytoskeleton mechanism, the assembled clathrin cages induced by ZLLLal might play a role as a motive force for neurite membrane formation. Alternatively, clathrin cages induced by ZLLLal could draw the cytoskeletal proteins around and work as cytoskeleton-organizing site for the neurite.

The biochemical analyses of coated vesicle and clathrin also indicate a number of distinct functions. Clathrin light chains bind to calmodulin^{75, 89)} and can bind Ca^{2+} ⁹¹⁾. Coated vesicles contain casein kinase II, which phosphorylates clathrin light chain β ⁹²⁾ and endogenous tubulin⁹²⁾. Coated vesicles also contain a cyclic nucleotide- and Ca^{2+} -independent protein kinase⁹⁴⁾. It is possible that these functions associated with clathrin/coated vesicles are affected by the binding of ZLLLal or the stimulation in the rate of clathrin assembly, which might induce a link up with the proper signal transduction system for neurite initiation.

It is also possible that ZLLLal influences intrinsic functions of clathrin/coated vesicle: receptor-mediated endocytosis, vectorial transport of membranes (from the Golgi apparatus to lysosomes), and exocytosis (from the Golgi apparatus to cytoplasm). Bretscher suggested that the polarized endocytotic cycle may be a part of the cell machinery used in locomotion⁹⁵⁾. On a motile cells, the sites for endocytosis (randomly located on the cell) and membrane return (located at the front of the cell) are not coincident. This causes a bulk flow of lipids, plus receptors in the plasma membrane, away from the front of the cell. Large objects on the cell surface are swept to the rear

of the cell by this flow. As a result, cells use this flow to extend themselves forward during cell locomotion. Thus, the flow of the circulating membrane induced by polarized endocytosis causes the formation of new membrane at the leading edge of cells. In PC12 cells, ZLLal might influence the dynamic equilibrium of the clathrin endocytotic cycle and change the flow of circulating membrane inside the cell. This new flow might produce the addition or formation of membrane for neurite initiation in a restricted region of PC12 cells.

In the nervous system, coated vesicles are thought to be involved in the recycling of synaptic vesicle membranes during transmitter release⁹⁶) and the initiation of fast axonal transport⁹⁷). Recently Wong et al. reported that brain-type clathrin light chain b is restricted in its expression, being found in neuronal cells but not in glial or Schwann cells⁹⁸). Brain type clathrin light chain has a short hydrophobic stretch introduced by an insertion sequence^{79,80}). The authors suggested that a neuron-specific clathrin light chain b is able to bind to some form of axonal-transport protein that also interacts with cytoskeletal elements through the inserted hydrophobic sequence.

Since clathrin binds to the LLLal affinity column, the developmental switching of clathrin light chain in rat brain can be assessed using LLLal affinity chromatography (Fig.2-5, Table.2-5). In 16 days rat embryos (E16), only non-brain type light chain could be detected; in 1 day postnatally rats (P1), both the non-brain type and brain type clathrin light chains are expressed at the same time; beginning subsequently at about 14 days postnatally (P14), the non-brain type light chain begin to diminish. A larger amount of clathrin light chain (non-brain type a and b, brain type b) per applied protein was observed from P1 to P14. The time from P1 to P14 is coincident with the time of the most active neurite outgrowth in rat cerebral brain. This result suggests that clathrin is involved in neuritogenesis of PC12 cells, as well as in in vivo brain neuritogenesis. Triskelion containing brain type light chain b might play a role in the transport of axonal proteins as materials for neurite formation as described by Wong et al., while triskelion containing non-brain type light chains a and b might play another role, as seen in neurite initiation in PC12 cells.

In conclusion, evidence has been presented that clathrin is responsible for neurite initiation induced by ZLLLal in PC12 cells. Clathrin could, therefore, have additional functions in the developing nervous system apart from its role in endocytosis. Immunohistochemistry on brain neurogenesis using anti-clathrin antibody may provide information about the role of clathrin in neuronal differentiation. More specifically, regulation of clathrin expression using specific antibodies, anti-sense mRNA, and gene targetting methods will provide more direct evidence and considerable insight into the processes that control neurite outgrowth.

TABLE 2-1

Effect of Ac-Leu-Leu-Nle-al analogues on neurite outgrowth of PC12 cells

Ac-Leu-Leu-Nle-al analogues	Optimal Concentrations (μ M)	% cells with neurites
Ac-Leu-Leu-Nle-al	1.6	46.8 \pm 1.6
Z-Leu-Leu-Gly-al	40	29.0 \pm 3.0
Z-Leu-Leu-Ala-al	3	30.4 \pm 0.1
Z-Leu-Leu-Nle-al	0.3	48.4 \pm 3.4
Z-Leu-Leu-Ile-al	0.25	50.9 \pm 1.4
Z-Leu-Leu-Val-al	0.25	49.5 \pm 2.3
Z-Leu-Leu-Nva-al	0.1	43.1 \pm 1.6
Z-Leu-Leu-Phe-al	0.05	50.7 \pm 2.9
Z-Leu-Leu-Leu-al	0.03	46.2 \pm 1.0
Z-Leu-Leu-al	100	18.7 \pm 1.2
Z-Leu-Leu-Leu-Leu-al	0.02	34.3 \pm 1.5

Dans-Leu-Leu-Leu-al	0.015	52.2 ± 1.5
Boc-Leu-Leu-Leu-al	0.3	20.0 ± 1.2
Ac-Leu-Leu-Leu-al	0.3	17.5 ± 1.1
Bu-Leu-Leu-Leu-al	1.5	18.5 ± 2.0
Pr-Leu-Leu-Leu-al	5	24.5 ± 1.5
Z-Leu-Leu-Leu-sc	0.05	33.6 ± 2.5
Z-Leu-Leu-Leu-CH ₂ Cl	12.5	48.2 ± 2.1
Z-Leu-Leu-Leu-CF ₃	20	26.7 ± 1.2
Z-Leu-Leu-Leu-COOH	50	1.8 ± 0.2
Z-Leu-Leu-Leu-OH	50	2.5 ± 0.4
Z-Ala-Leu-Leu-al	0.3	45.6 ± 0.6
Z-Leu-Ala-Leu-al	0.08	41.5 ± 1.1
Z-Phe-Leu-Leu-al	0.05	40.0 ± 1.0
Z-Leu-Phe-Leu-al	0.015	31.2 ± 1.1

PC12 cells were grown for 2 days with the additives indicated. Figures are mean ± SEM for 3 duplicates.

TABLE 2-2

Percentage of cells bearing neurites following treatment with ALLNal, ZLLNal, and ZLLLal after 48hr

Optimal concentration (μ M)	Time (hr)			
	48	72	96	120
ALLNal 1.6	46.8 \pm 1.4	39.5 \pm 1.4	21.1 \pm 1.2	13.0 \pm 1.6
ZLLNal 0.25	51.5 \pm 1.6	51.5 \pm 0.9	36.0 \pm 1.8	22.5 \pm 1.7
ZLLLal 0.03	45.0 \pm 1.0	43.5 \pm 1.1	35.5 \pm 1.7	32.5 \pm 1.9

PC12 cells were treated with peptide aldehydes at the indicated concentrations. At various intervals, the percentage of neurite-bearing cells was scored. The medium was changed every other day. Figures are mean \pm SEM for 3 duplicates.

TABLE 2-3

The relationship among the 3rd amino acid residue, hydrophobicity, and ability to induce neurite outgrowth in PC12 cells

	Gly	Ala	Nle	Ile	Val	Nva	Phe	Leu
Optimal conc. (uM)	40	3	0.3	0.25	0.25	0.1	0.05	0.03
Hydropathy index of the 3rd amino acid	0.4	1.8	-	4.5	4.2	-	2.8	3.8
Retention time (min) of ZLLXal on HPLC (C18 column)	3.04	4.17	8.60	9.10	7.14	6.46	7.04	7.62

Optimal concentrations for eliciting neurite outgrowth are from Table 2-1; hydrophobic parameters for the 3rd amino acid are from Kyte-Doolittle's hydrophathy index⁷⁸). Since the C18 column is hydrophobic, the hydrophobicity of ZLLXal is directly proportional to the peak retention time.

TABLE 2-4

Inhibition of mCANP activity by ALLNal analogues

Z-Leu-Leu-X-al	IC ₅₀ (nM)	cf. Optimal conc. (uM)*
Gly	3700	40
Ala	450	3
Nle	200	0.3
Phe	120	0.05
Leu	100	0.03
Nva	55	0.1
Val	36	0.25
Ile	26	0.25

* Optimal concentrations for eliciting neurite outgrowth are from Table 2-1; mCANP purified from rabbit skeletal muscle was incubated at 30°C for 15min with alkali-denatured casein as the substrate in the presence of various ALLNal analogues.

TABLE 2-5

Proteins eluted from the LLLal affinity column at low pH (pH 4.0)

	Rat brain				
	PC12 cell	Embryonic 16days	Postnatal 1day	Postnatal 14days	Postnatal 3months
33K	○	△	◎	△	△
35K	○	○	◎	△	△
36K	×	×	◎	◎	○
38K	×	×	○	○	○

Each cytosol fraction was charged to the LLLal affinity column. The column was washed successively with 0.1M sodium phosphate (pH 7.5)/0.2M NaCl/1% Triton X-100 and 0.1M sodium phosphate (pH 7.5)/1% Triton X-100, and the proteins were eluted with 0.1M sodium citrate buffer (pH 4.0)/1% Triton X-100. Eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Each symbol indicates qualitatively the degree of protein staining by Coomassie Brilliant Blue.

×: undetected △: faint ○: medium ◎: strong

TABLE 2-6

Amino-acid sequences of the 33K protein derived from 3 month rat muscle

33K-1 (K) V T E Q

Lb K V T E Q

33K-2 (K) D L E E X N Q (R)

Lb K D L E E W N Q R

33K-3 (K) V H/A Q L X D/N F N P K

Lb K V A Q L C D F N P K

Partial amino acid sequencing was performed by the method of Aebersold et al⁷³). Sequences of three peptides obtained from the 33K protein by digestion with trypsin (33K-1,2) or lysilendopeptidase (33K-3) were identical to the partial amino acid sequences of the clathrin light chain b (Lb). X:unidentified residue.

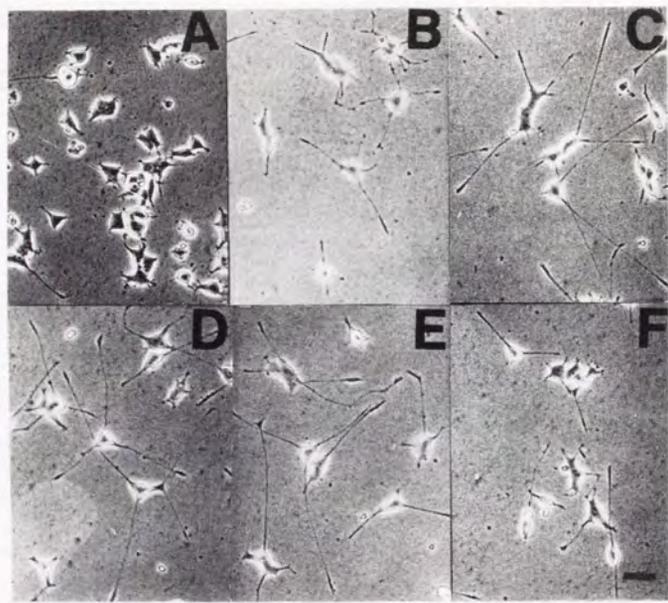


FIG.2-1

Phase contrast micrographs of PC12 cells grown for 2 days in N2 medium on collagen type I-coated dishes.

A, no addition; B, 40ng/ml NGF; C, 1.6 μ M ALLNal; D, 0.3 μ M ZLLNal;
E, 0.03 μ M ZLLLal; F, 40 μ M ZLLGal. Bar=35 μ m

FIG.2-2

LLL-COOH and LLLal affinity chromatographies of PC12 cell homogenates.

-SDS-polyacrylamide gel electrophoresis of the eluted proteins-

The cytosol fraction (2mg protein) or membrane fraction (3 mg protein) of PC12 cells was charged to LLL-COOH or the LLLal Affigel-10 affinity column (4.5 umol of bound LLL-COOH or LLLal/column). The column were washed with 0.1M sodium phosphate (pH 7.5)/0.2M NaCl/1% Triton X-100 and 0.1M sodium phosphate (pH 7.5)/1% Triton X-100, and the proteins were eluted with 0.1M sodium citrate (pH 4.0)/1% Triton X-100 (5 fractions of 1ml each). These operations were performed at 4°C. Subsequently, the affinity columns were washed with 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 and the pH was returned to neutral. The column was then treated with 6M urea/0.1M sodium phosphate (pH 7.5) and 6 fractions of 1ml each were collected. The eluted proteins were precipitated in 10% trichloroacetic acid and analyzed by SDS polyacrylamide gel electrophoresis.

Lane 1: charged proteins

- 2: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluates
- 3-5: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluates
(fractions 1-3, respectively)
- 6-8: 6M urea/0.1M sodium phosphate (pH 7.5) eluate
(fractions 1-3, respectively)

Arrows indicate the 33K, 35K, and 180K proteins that bound specifically to the LLLal affinity column.

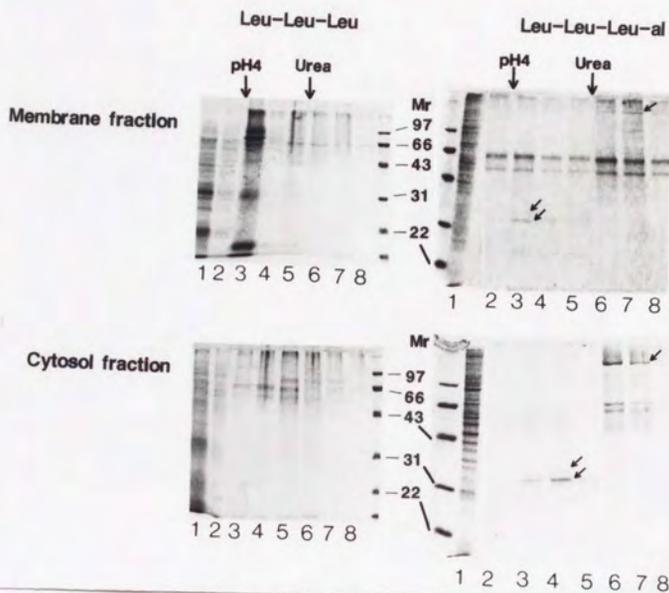




FIG.2-3

The LLL-COOH and LLLal affinity chromatographies of conditioned media from PC12 cells

-SDS-polyacrylamide gel electrophoresis of the eluted proteins-

Forty milliliters of conditioned media from PC12 cell cultures were dialyzed against two changes of 2l volumes of 0.1M sodium phosphate (pH 7.5)/0.2M NaCl, and charged to the LLL-COOH or LLLal Affigel-10 affinity column (9 umol of bound LLL-COOH or LLLal/column). Washing, elution, and electrophoresis were carried out as described for FIG.2-2.

Lane 1: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluate

2-5: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluates
(fractions 1-4, respectively)

6-11: 6M urea/0.1M sodium phosphate (pH 7.5) eluates
(fractions 1-6, respectively)

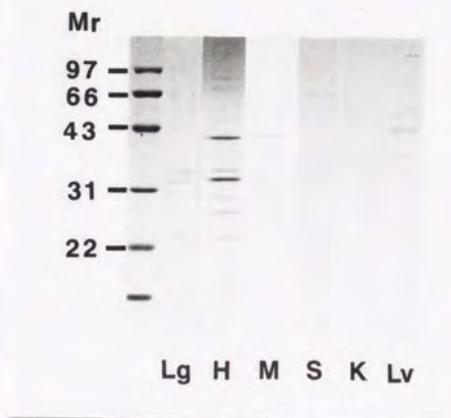


FIG.2-4

The LLLal affinity chromatography of cytosol fractions from the organs of adult (3months) rat

-SDS-polyacrylamide gel electrophoresis of the proteins eluted by low pH treatment-

Cytosol fractions (80mg proteins) from adult rat lung (Lg), heart (H), muscle (M), spleen (S), kidney (K), and liver (L) were charged to the LLLal Affigel-10 column (9 umol of bound LLLal/column). Washing, elution by low pH treatment, and electrophoresis of the combined fractions 1 and 2 of the low pH eluate were performed as described for FIG.2-2.

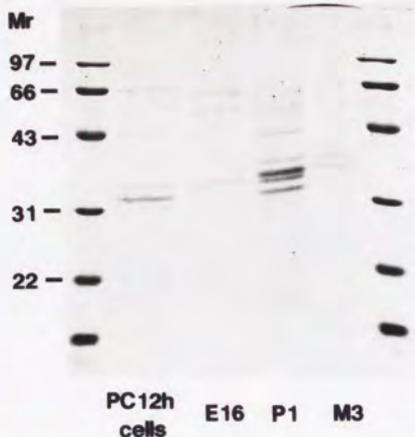


FIG.2-5

The LLLal affinity chromatography of the cytosol fractions from PC12 cells and rat brain

-SDS-polyacrylamide gel electrophoresis of proteins eluted by low pH treatment (pH 4.0)-

Cytosol fractions from PC12 cells (10mg protein), embryonic 16 day brain (E16, 10mg protein), postnatal 1day brain (P1, 10mg protein), and 3month brain (M3, 30mg protein) were charged to the LLLal Affigel-10 affinity column (4.5 μ mol of bound LLLal/column). Washing, elution with 0.1M sodium citrate (pH 4.0)/1% Triton X-100, and electrophoresis of the combined fractions 1 and 2 of low pH eluate were performed as described for FIG.2-2.

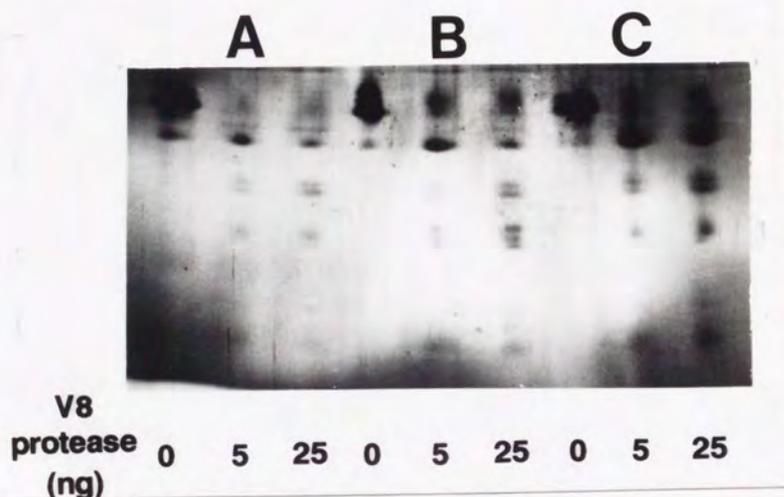


FIG.2-6

Peptide maps of the 33K protein derived from PC12 cells, postnatal 1day rat brain, and adult (3months) rat brain

Peptide mapping was performed as described by Cleveland et al. with the indicated concentrations of *S.aureus* V8 protease . A: the 33K protein deirved from PC12 cells, B: the 33K protein derived from postnatal 1day rat brain, C: the 33K protein from 3month rat brain.

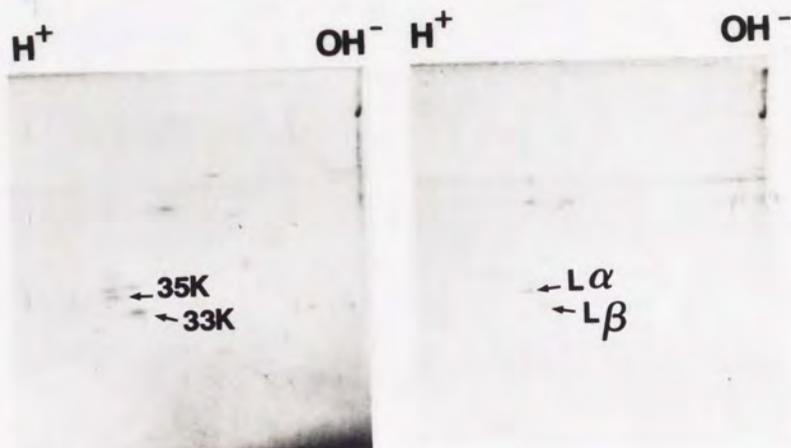


FIG.2-7

Two-dimensional gel electrophoresis profiles of the 33K/35K proteins derived from postnatal 1day rat brain and authentic clathrin light chains purified from rat liver.

Analysis of proteins by two-dimensional electrophoresis was performed as described by Mikawa⁷²). (left) The 33K/35K proteins derived from postnatal 1day rat brain, which were eluted from the LLLal affinity column with 0.1M sodium citrate (pH 4.0)/1% Triton X-100. (right) Authentic clathrin light chains purified from rat liver.

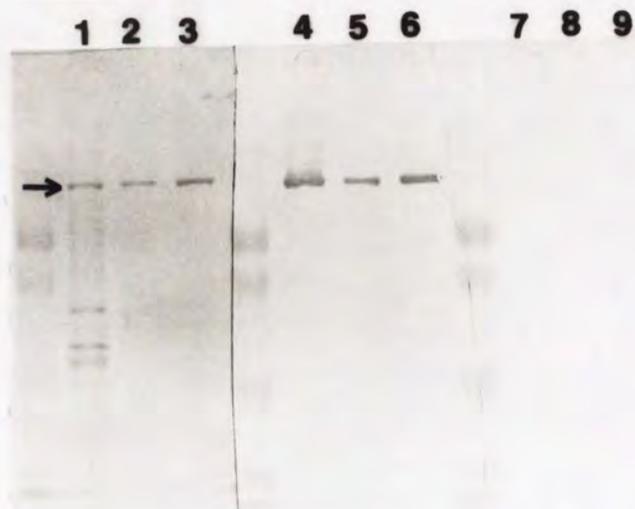


FIG.2-8

Immunoblotting with anti-clathrin heavy chain monoclonal antibody (CHC.5.9). The 180K protein derived from PC12 cells by LLLal affinity chromatography was electrophoresed on a 7.5% polyacrylamide gel and immunostained with anti-clathrin heavy chain antibody. The arrow indicates the position of the 180K protein/clathrin heavy chain.

Lanes 1-3: Amido-black staining

Lanes 4-6: Western blot incubated with 0.25 ug/ml anti-clathrin heavy chain antibody

Lanes 7-9: Western blot incubated with 0.25 ug/ml on non-immune mouse antiserum

Lanes 1,4,7= 180K protein derived from PC12 cells

Lanes 2,5,8= authentic clathrin purified from rat liver

Lanes 3,6,9= doubled amounts clathrin compared to lanes 2,5 and 8, respectively

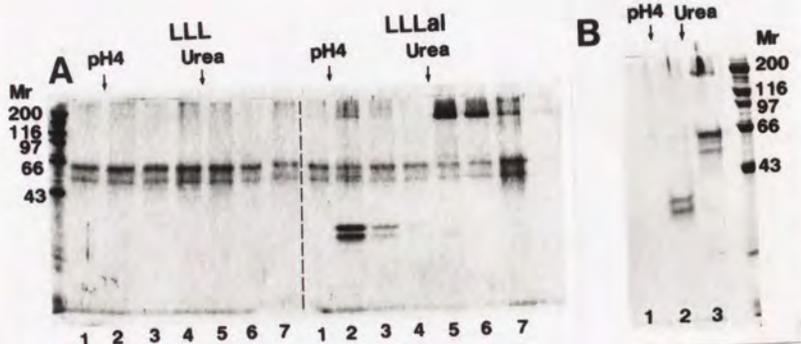


FIG.2-9

The LLL-COOH or LLLal affinity chromatography of authentic clathrin
-SDS-polyacrylamide gel electrophoresis-

Column-purified authentic clathrin (0.6mg) was dialyzed against 2 l of 0.1M sodium phosphate (pH 7.5)/0.2M NaCl, and charged to an LLL-COOH or LLLal Affigel-10 affinity column (4.5 umol of bound LLL-COOH or LLLal/column). Washing, elution, and electrophoresis were carried out as described for FIG.2-2.

A: rat liver clathrin

Lane 1: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluate

2-4: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluates
(fractions 1-3, respectively)

5-7: 6M urea/0.1M sodium phosphate (pH 7.5) eluates
(fractions 1-3, respectively)

B: bovine brain clathrin

Lane 1: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluate

2: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluate (fraction 2)

3: 6M urea/0.1M sodium phosphate (pH 7.5) eluate (fraction 2)

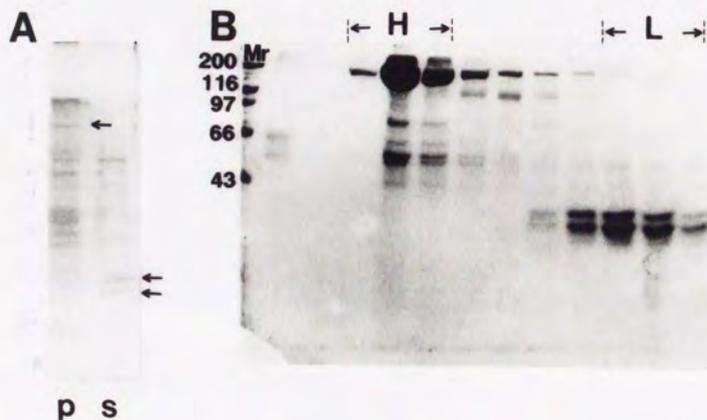


FIG.2-10

SDS-polyacrylamide gel electrophoresis of separated of clathrin heavy chains and light chains

A: Authentic clathrin (0.4mg) purified from rat liver was boiled for 3min at 100°C, and centrifuged at 100,000xg for 1hr. Clathrin heavy chains were sedimented (p), whereas clathrin light chains were non-denaturated and remained in the supernatant (s). The arrows point to clathrin heavy chains and light chains.

B: Authentic clathrin (0.4mg) purified from bovine brain was separated in the presence of a chaotropic agent, thiocyanate. H: heavy chain fractions, L: light chain fractions

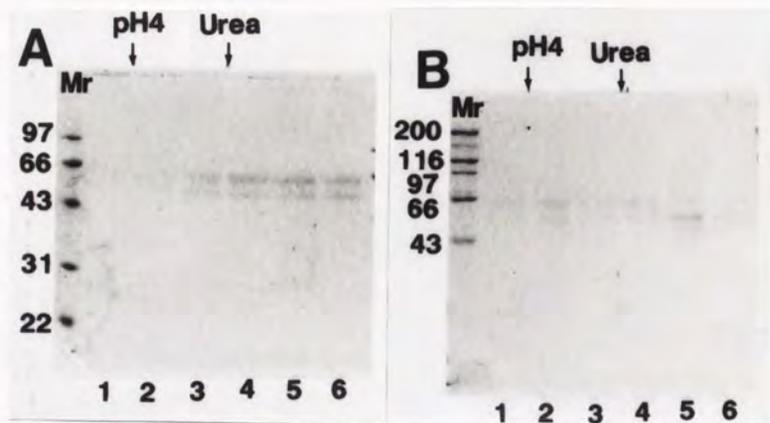


FIG.2-11

The LLLal affinity chromatography of boiled clathrin light chains and heavy chains.

-SDS-polyacrylamide gel electrophoresis-

Clathrin heavy and light chains separated as described for FIG.2-10A were charged to the LLLal Affigel-10 affinity column (4.5 μ mol of bound LLLal/column). Washing, elution, and electrophoresis were performed as described for FIG.2-2.

A:boiled clathrin supernatant (clathrin light chains)

B:boiled clathrin pellet (clathrin heavy chains)

Pellet were homogenized, dialyzed, and charged to the column.

Lane 1: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluate

2-3: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluates
(fractions 1 and 2, respectively)

4-6: 6M urea/0.1M sodium phosphate (pH 7.5) eluates
(fractions 1-3, respectively)

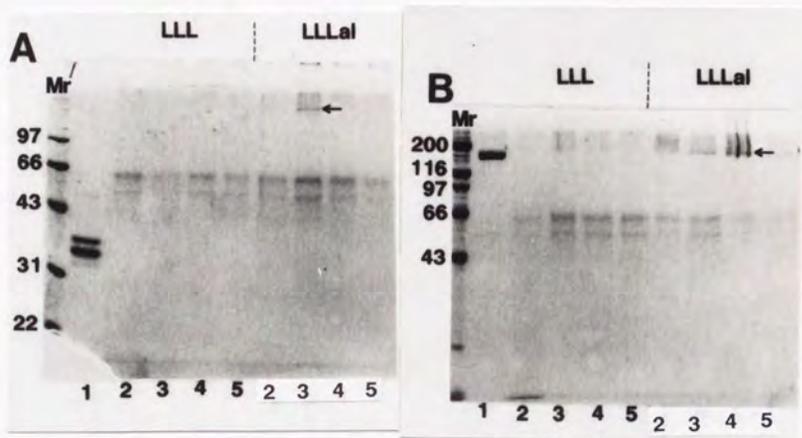


FIG.2-12

The LLLal affinity chromatography of clathrin light chains and heavy chains separated by thiocyanate

-SDS-polyacrylamide gel electrophoresis-

Clathrin heavy chains (0.4mg) and light chains (0.6mg) separated as described for FIG.2-10B were charged to the LLL-COOH or LLLal Affigel-10 affinity column (4.5 μ mol of bound LLL-COOH or LLLal/column). Washing, elution with 6M urea/0.1M sodium phosphate (pH 7.5), and electrophoresis were performed as described for FIG.2-2. The arrows indicate the positions of the clathrin heavy chains.

A: clathrin light chains B: clathrin heavy chains

Lane 1: charged proteins

2: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluate (fraction 2)

3-5: 6M urea/0.1M sodium phosphate (pH 7.5) eluates

(fractions 1-3, respectively)

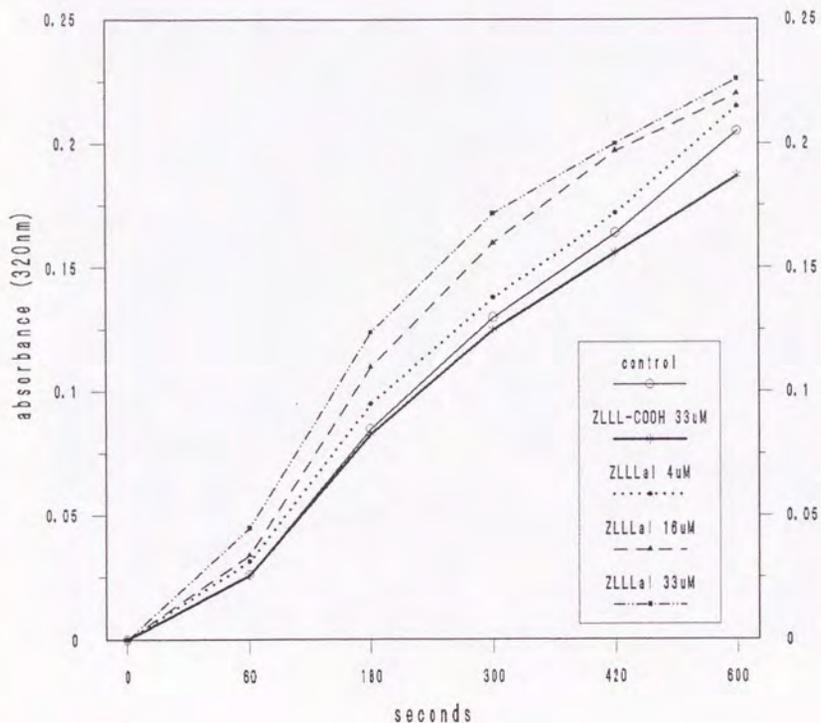


FIG.2-13

Effects of ZLLLal and ZLLL-COOH on the rate of clathrin polymerization

Assembly-competent clathrin was incubated with ZLLLal or ZLLL-COOH at the indicated concentrations for 18hr at 25°C. Then, the clathrin was assembled by the addition of 5 ul of 1M MES (pH 6.0). Assembly was monitored by the change in absorbance at 320nm. Figures are mean \pm SEM for 3 duplicates, and the SEM did not exceed 10% of the mean.

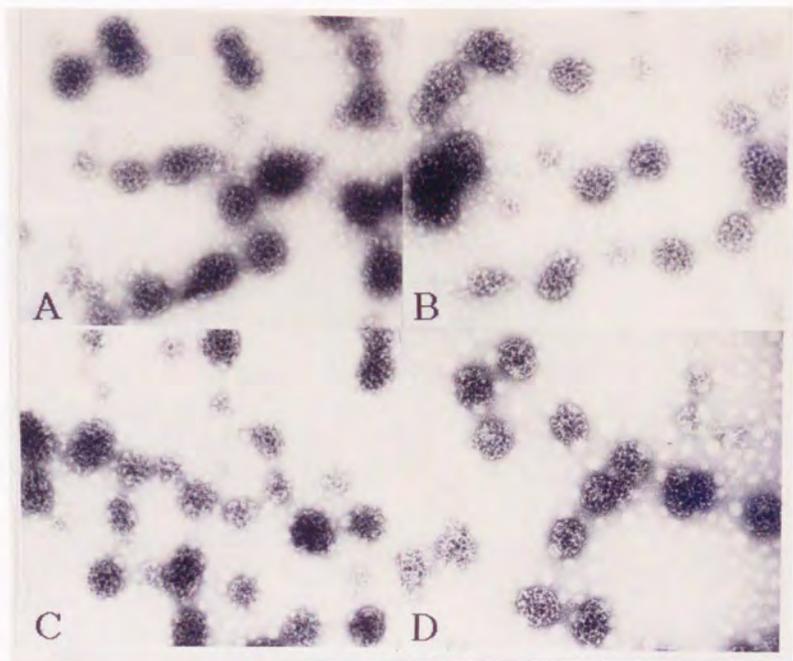


FIG.2-14

Electron micrographs of negatively stained cages reassembled from clathrin.

A: Cage structure assembled from clathrins incubated for 18hr with DMSO (control) at 25°C.

B: Cage structure assembled from clathrins which were incubated for 18hr with 33 uM ZLLLal at 25°C.

C: Cage structure incubated with DMSO (control) at 25°C for 18hr

D: Cage structure incubated with 8 uM ZLLLal at 25°C for 18hr

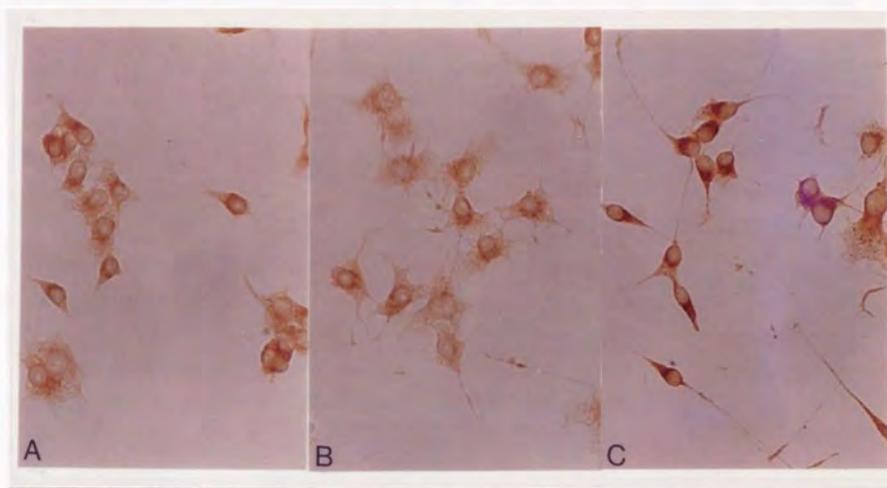


FIG.2-15

Immunostaining of PC12 cells against clathrin heavy chain.

PC12 cells were treated for 18hr with N2 medium (A), 40ng/ml NGF (B), or 1.6 μ M ALLNal (C). Fixed and detergent-treated cells were immunostained with anti-clathrin heavy chain antibody at 0.25 μ g/ml.

GENERAL DISCUSSION

The PC12 cell line has been used in combination with a specific synthetic protease inhibitor to study the mechanism of neuronal differentiation. This approach has revealed that,

(1) a leupeptine analogue (Ac-Leu-Leu-Nle-al:ALLNal) causes morphological and biochemical neuronal differentiation. Compared with neurites induced by other known molecules, ALLNal-induced neurites are unique in terms of their morphology and time course of appearance and disappearance.

(2) Since benzyloxycarbonyl-Leu-Leu-Leu-al (ZLLLal) has a 50-fold stronger potency to initiate neurite outgrowth than ALLNal, Leu-Leu-Leu-al was immobilized and used as a ligand for affinity chromatography. Several lines of evidence indicate that the protein isolated specifically from the affinity column is clathrin, well-known for its role in receptor-mediated endocytosis.

The findings imply a new regulatory mechanism for neuronal differentiation in which the interaction between the specific protease inhibitor and clathrin acts as an intermediary for neurite initiation. The suggestion that clathrin is involved in neurite outgrowth is not limited to tissue culture. Larger amounts of clathrin light chain were found among the proteins applied to the LLLal affinity column in samples taken from rat brains from 1day (P1) to 14days (P14) postnatally than from adult rat brain. Indeed, the time from P1 to P14 is consistent with the time of the most active neurite outgrowth in rat brain.

A computer search using the amino acid sequences of the clathrin heavy chain and clathrin light chain showed no significant homology to other sequenced proteins that listed in the National Biomedical Research Foundation protein data bank (1987)⁸⁴). The amino acid sequences of clathrin heavy and light chains has also show no homology to the catalytic sequence of serine or cysteine proteases. This result shows that the protease inhibitor interacts not only with the actual "protease", but also with other molecules. However, it is possible to predict the presence of a protease that can not be categorized with any protease known previously⁹⁹). Such a novel class of proteases will have catalytic sequence different from those previously known. An example is the multicatalytic high-

molecular-weight proteinase (proteasome). This proteinase is a complex of many subunits, some of which have amino acid sequences deduced from the base sequences of cDNAs; however, none of the sequences show homology with any known proteases¹⁰⁰). Thus, the possibility that clathrin is a "protease" and displays proteolytic activity under the proper conditions can not be excluded.

In this study, the target molecule was identified as clathrin by affinity chromatography using a ligand of the specific protease inhibitor, ZLLLal. It is certain that ZLLLal binds to clathrin and that clathrin plays an important role in neurite outgrowth induced by ZLLLal. However, it is possible that by means of a technique other than LLLal affinity chromatography, another molecule will be found to be involved in the neurite outgrowth induced by ZLLLal.

For example, although ZLLLal is an effective inhibitor of CANP in vitro (Table 2-4), it remains unclear whether CANP is directly involved in the neurite outgrowth induced by ZLLLal in PC12 cells. When crude PC12 cell extracts were charged to the LLLal affinity column under various buffer conditions with or without Ca^{2+} , CANP could not be detected as a bound protein. The inability of CANP to bind to the column may be due to the complex relationship between CANP, calpastatin (an endogenous CANP inhibitor), the synthetic protease inhibitor, and Ca^{2+} ¹⁰¹). CANP can bind to the synthetic protease inhibitor only in the presence of Ca^{2+} . But, in the presence of Ca^{2+} , CANP will bind calpastatin more strongly than the synthetic protease inhibitor. Furthermore the content of calpastatin is generally much greater than that of CANP. Thus, in the in vitro system formed after cell structure is disrupted, calpastatin prevents CANP from binding to the LLLal column in the presence of Ca^{2+} . If calpastatin and CANP co-localize in PC12 cells as in vitro, ZLLLal has no chance to display its ability as a CANP inhibitor. In this case, CANP is not involved in neurite initiation induced by ZLLLal. However, if the localizations of CANP and calpastatin are different, CANP functions upon an increase of intracellular Ca^{2+} and ZLLLal can then interact with CANP. In this case, CANP could be one of the target molecules for ZLLLal.

Of particular interest is the observation that neurite outgrowth induced by ALLNal is different from that induced by NGF. ALLNal induces monopolar/bipolar and longer neurites than those induced by NGF within the first 2 days; but after 2 days, the neurites retract gradually and do not form a network. On the other hand, NGF induces bipolar/multipolar neurites progressively, and the neurites formed exhibit a complex network after 5 days. In the course of PC12 cell differentiation by NGF, a number of early and late genes are activated¹⁰²). Identification and characterization of the mRNAs regulated by ALLNal, and a comparison of the mRNAs induced by ALLNal with those induced by NGF are of considerable importance to basic neuroscience. The mRNAs induced in common by NGF and ALLNal may represent a general gene for neuronal differentiation, spite the differences in morphology and duration of the neurites. mRNAs induced only by NGF may be responsible for the maintenance of neurites and network formation. These questions must be addressed in order to study the mechanism of neuronal differentiation and network formation.

Long-term potentiation (LTP), a very persistent enhancement of synaptic efficacy following high-frequency stimulation, has attracted considerable interest as a potential memory mechanism. Recently, Lynch's group reported that ALLNal reduces the degree of LTP induced by theta burst stimulation¹⁰³). They used ALLNal as a CANP inhibitor, and suggested that CANP produces effects like those associated with LTP. They also reported that ALLNal improves the recovery from hypoxia of synaptic transmission in hippocampal slices, and CANP activation may be triggered by even relatively brief periods of hypoxia¹⁰⁴). Since ZLLLal can inhibit CANP activity at lower concentrations than ALLNal, ZLLLal can be used as a more potent calpain inhibitor for the elucidation of mechanism of LTP or hypoxia.

ALLNal can regenerate long neurites in PC12 cells. Therefore, an in vivo experiment to study neurite regeneration by the protease inhibitor is important matter for the near future. Since the direct in vivo administration of a highly hydrophobic drug may produce an ill effect, a less hydrophobic drug with a strong potency for neurite outgrowth is required. In fact, Z-Leu-Ala-Leu-al is not hydrophobic compared with ZLLLal, but has the ability to induce neurite

outgrowth effectively. A better understanding of the relationship between structure and ability to initiate neurite outgrowth might suggest attractive possibilities for direct administration in the treatment of traumatic and degenerative diseases of the peripheral and central nervous systems.

In conclusion, this study has shown the following.

1. The development of a synthetic protease inhibitor of a new type that penetrates through the cell membrane.
2. Neuronal differentiation induced by a specific protease inhibitor in PC12 cells.
3. The possible involvement of clathrin in neurite initiation and outgrowth.

These results will yield useful insights into the cellular mechanism involved in neuronal differentiation.

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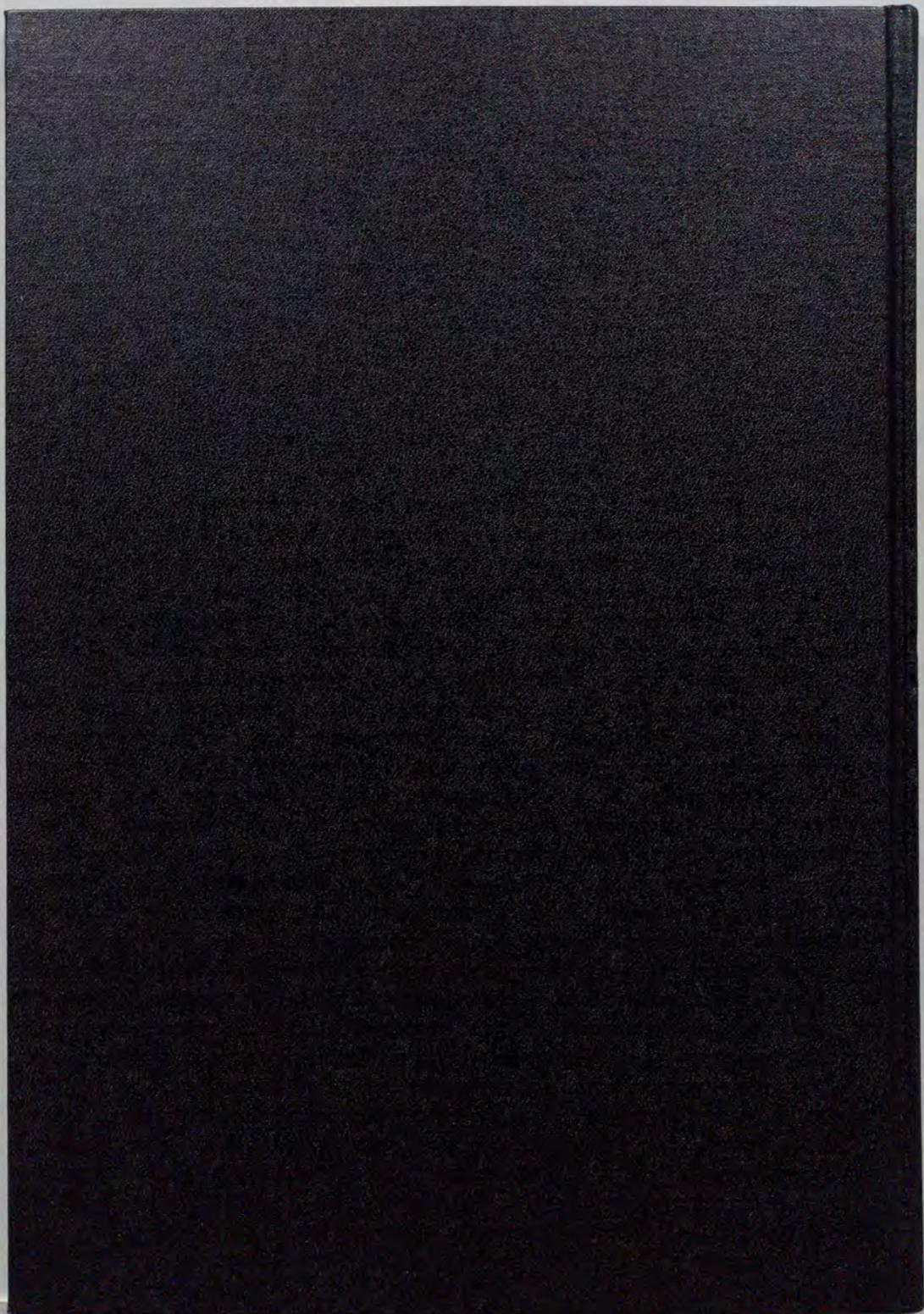
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