STUDY ON THE BIOLOGICAL ACTIONS OF β-AMYLOID PEPTIDES

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CONTENTS

Abbreviations

Abbreviations used are those reccomended by IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) : *Eur. J. Biochem.*, 138, 9 (1984)

Ala (A):	L-alanine	Arg (R):	L-arginine
Asn (N):	L-asparagine	Asp (D):	L-aspartic acid
Gln (Q):	L-glutamine	Glu (E):	L-glutamic acid
Gly (G):	glycine	His (H):	L-histidine
Ile (I):	L-isoleucine	Leu (L):	L-leucine
Lys (K):	L-lysine	Met (M):	L-methionine
Phe (F):	L-phenylalanine	Pro (P):	L-proline
Ser (S):	L-serine	Thr (T):	L-threonine
Trp (W):	L-tryptophan	Tyr (Y):	L-tyrosine
Val (V):	L-valine		

AD:	Alzheimer's disease
APP:	amyloid protein precursor
βAP:	β-amyloid protein
βDPN:	β-diphosphopyridine nucleotide
Boc:	t-butyloxycarbonyl
BOP:	benzotriazolyl N-oxytrisdimethylamino phosphonium
Bt_2cAMP:	dibutyryl cyclic AMP
DCC:	N,N'-dicyclohexylcarbodiimide
DMSO :	dimethylsulfoxide
Et_3N:	triethylamine
FCS:	fetal calf serum
Fmoc:	9-fluorenylmethyloxycarbonyl
HF:	hydrogen fluoride

HMP resin:	p-hydroxymethylphenoxymethyl-polystyrene resin
HOBt :	1-hydroxybenzotriazole
HPLC:	high performance liquid chromatography
HS:	horse serum
KPI:	Kunitz-type serine protease inhibitors
LDH:	lactate dehydrogenase
MBHA resin:	4-methylbenzhydrylamine resin
MeOH:	methanol
MTT:	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium NGF: nerve growth factor
NKA:	neurokinin A
NKB:	neurokinin B
NMP:	N-methyl-2-pyrrolidinone
n-PrOH:	n-propanol
Pac:	phenacyl ester
PAM resin:	phenylacetamidomethyl resin
PMA:	phorbol 12-myristate 13-acetate bromide
PNCS:	precolostrum newborn calf serum
PTX:	pertussis toxin
SAL resin:	super acid labile resin, 4-(2',4'-dimethoxyphenyl-fluorenyl-aminomethyl)-phenoxy resin
SP:	substance P
TFA:	trifluoroacetic acid
WST-1:	water soluble tetrazolium

Chapter I GENERAL INTRODUCTION

General introduction

β-Amyloid protein (βAP) is a peptide containing 39-43 amino acid residues and it is one of the major constituent of senile plaques in Alzheimer's disease (AD). Glenner et al. first reported the primary structure of amyloid filaments isolated from meningeal vessels of AD patients (1). Also, βAP in the senile plaques of AD brain was confirmed to be almost indistinguishable from that of meningeal vessels (2). The molecular cloning of a cDNA encoding the βAP revealed that it is synthesized as a large precursor molecule, amyloid protein precursor (APP) encoded by a gene on human chromosome 21 (3). APP is a receptor-like transmembrane protein which may be coupled to GTP-binding protein (G_O) (4). βAP is located on the region from the C-terminus of the extracellular domain to the N-terminus of the transmembrane domain of APP and it seems to be generated through proteolytic processing of the molecule.

Further molecular studies subsequently revealed several different isoforms of APP that are produced by alternative splicing (Fig. I-1). The major and ubiquitous primary translation products 695, 751 and 770 amino acid residues (APP695, APP751, and APP770, respectively) (3, 5-7). Both larger APP751 and 770 contain a 56-amino acid insert with high sequence homology to the Kunitz-type serine protease inhibitors (KPI) which is missing from APP695, and they have protease inhibitory activity (7, 8). There are also other isoforms such as APP714, which is APP695 with a 19 amino acids-insert (9), and APP563, which is a secreted form lacking the transmembrane region (10). APP is expressed in almost every tissue and in the brain, and it is found in neuronal and non neuronal cells.

Under normal conditions, the processing enzyme (α-secretase) cleavage may represent the major metabolic pathway that cleaves the APP molecule within the βAP-region between 16 and 17, suggesting that mature βAP can not be generated (Fig. I-2) (11). On the production of βAP from APP, it is

postulated that the β-secretase cleaves APP at the N-terminal first amino acid of the βAP sequence and that the βAP-region is further processed by γ-secretase at the C-terminal leading to the formation and subsequent secretion of the intact βAP peptide (Fig. I-2) (12). The α-secretase pathway can be activated, whereas βAP production is inhibited by compounds that stimulate protein kinase-C (13-17).

βAP is mainly found in the brain and cerebral blood vessels (1, 18), but βAP deposits have been detected in non-neural tissues or blood vessels of AD patients (19). Presumably, the proteolytic cleavage producing the mature βAP occurs during normal metabolism and this protein is widespread in the circulatory systems. It appears that βAP is generated by some normal cultured cells *in vitro* (20), and observed in the biological fluids as a soluble peptide (21). These findings also indicate that soluble βAP may have a regulatory role in a physiological function.

Since insoluble βAP deposits are associated with the presence of dystrophic neurites and since neuronal loss is evident in severely affected AD brain regions, it can be assumed that βAP has direct neurotoxic effects upon the central nervous system. To understand βAP toxicity, various studies have been undertaken, mainly using primary neuronal cultured cells or clonal cell lines. Yankner et al. indicated that rat pheochromocytoma PC12 cells transfected with the APP gene containing the βAP-region gradually degenerated when they were differentiated into neuronal cells by nerve growth factor (NGF), and its cultured medium was toxic to neurons in primary hippocampal cultures (22). They further reported that synthetic βAP had a neurotrophic effect upon undifferentiated neurons at lower concentrations and a neurotoxic effect upon mature hippocampal neurons at a higher concentration range (23). In addition, they showed that a short peptide fragment of βAP (βA25-35) was a bioactive region on both effects and that tachykinin peptide reverses the both activities of βAP (Fig. II-1) (23).

However, the direct neurotoxicity induced by β-amyloid peptides is not always reproducible. On the reason of the problem, Pike et al. have found that β-amyloid peptides in an aggregated state were directly toxic to cultured neurons, whereas the soluble peptides lack direct toxicity, suggesting that the biological properties of βAP are influenced by changes in its physical state (24). Thus, the aggregation of β-amyloid peptides is thought to be one of the critical properties involved in the induction of the neurotoxic effect (25, 26).

The tendency of βAP to form peptide aggregates appears to require a portion of the C-terminal hydrophobic domain (27). The C-terminal longer βAPs, such as 42 and 43 residues, are easily aggregated compared with the shorter peptide (39 or 40 residues). βAP aggregation to form insoluble amyloid may be initiated by a soluble, monomeric hydrophobic cluster (Fig. I-3) (28). Since amyloid fibril formation follows a nucleation-dependent mechanism, a small change in the concentration of the aggregating species can have a dramatic effect on the rate of aggregation. Likewise, the active peptide fragment βA25-35, located near the transmembrane region of βAP, exhibits a concentration-dependent aggregation which is accompanied by a reversible random coil ↔ β-sheet transition (29).

Extensive immunohistochemical studies indicate that other proteins are co-deposited with βAP in senile plaque. Amyloid P-component, α_1-antichymotrypsin (ACT), apolipoprotein E (apoE), apolipoprotein J (apoJ), complement components, vitronectin, glycosaminoglycans (GAG), and extracellular matrix proteins are among the amyloid-associated proteins (30, 31). Within these molecules, the protease inhibitor ACT and the lipid transport protein apoE may have a strong stimulatory role in the polymerization of βAP into amyloid firaments, and progress its neurotoxicity (32). Inheritance of the E4 allele of the gene encoding apoE is particularly considered to be as one of the main risk factors for late-onset AD (33, 34).

Conversely, the administration of polysulphated GAGs and dyes, such as Congo Red and thioflavin S which are used to specifically stain amyloid deposits, attenuate the neurotoxic effects of β-amyloid peptides (35, 36). The association of these compounds with βAP may prevent the formation of intermolecularly stacked β-pleated sheet aggregates characteristic of the toxic form of βAP. In addition, rifampicin, which is an antibiotic widely used in the treatment of tuberculosis and leprosy, inhibits the aggregation and fibril formation of βAP, and protects against βAP-induced neurotoxicity on PC12 cells (37).

Metal ions reportedly accelerate the aggregated formation of βAP and may be lethal in AD brains. βAP can bind Zn^{2+} in a saturable and specific manner (38, 39). The structure of βAP possesses 3 histidines and several negatively charged residues, and these are structural features that support Zn^{2+} binding. Physiological concentrations of Zn^{2+} increase the resistance of the peptide to proteolytic catabolism and promote βAP precipitation. Also, Al^{3+} can be seen in senile plaques with βAP and progress βAP aggregation, suggesting that Al^{3+} should be a risk factor in AD brain (40).

As one well documented change in the central nervous system associated with aging is oxidative damage caused by free radicals, it is possible that βAP neurotoxicity is mediated by oxidative free radicals. Behl et al. have demonstrated that β-amyloid peptides are cytotoxic for cultured cortical nerve and PC12 cells, and that the free radical scavenger vitamine E inhibits the cell death (41). Several antioxidants protect cells from βAP toxicity, suggesting that at least one pathway to βAP cytotoxicity results in free radical damage (42). βAP could cause increased levels of H_2O_2 and lipid peroxides to accumulate in cells. Since the H_2O_2-degrading enzyme catalase protected the cells from βAP toxicity, hydroxyl radicals derived from H_2O_2 may mediate the oxidative damage caused by βAP (42). Furthermore, it was reported that a synergistic effect between βAP and interferon-γ (IFN-γ) triggers the production of reactive

nitrogen intermediates and tumor-necrosis factor-α (TNFα) from microglia, the activation of which, leads to neuronal cell injury *in vitro* (Fig. I-4) (43). These findings suggested that oxidative free radicals have an important role on βAP neurotoxicity, and the pathogenesis of neuronal degeneration observed in AD.

Several lines of evidence suggest that cell death occurs by one of two general pathways, necrosis or apoptosis. Necrosis is associated with nonphysiological conditions that disrupt cellular homeostasis (e. g., hypoxia, ischemia, and excitotoxicity). By contrast, apoptosis is a type of regulated cellular self-destruction that functions in the normal control of development and tissue homeostasis and may be regulated by various factors throughout life. Whether the neuronal cell death caused by βAP is necrosis or apoptosis has been examined. In PC12 cells stably transfected with the *bcl-2* gene, βA25-35-induced cell death could not be prevented by the gene product protein Bcl-2 which reportedly blocks apoptotic cell death induced by a wide variety of stimuli, suggesting that the toxicity of the peptide was not apoptotic (44). It is also concluded that βA25-35 induces necrosis rather than apoptosis in PC12 cells and rat cortical neurons according to analyses of DNA degradation and ultrastructural studies using a transmission electron microscope (45). On the other hand, several studies have indicated that the cell death caused by βAP should be apoptosis (46-48).

Recent molecular genetic findings support the notion of the prominent involvement of βAP in AD pathology, because several mutations in the APP gene have been linked with familial AD in terms of the overall production and increasing the levels of the longer, more amyloidgenic form of βAP (49-54). Understanding the pathological events in AD will likely require knowledge of the biological effects induced by β-amyloid peptides. Additionally, soluble βAP may have a physiological role, because it is also found under conditions of normal metabolism. For this thesis, the biological actions of β-amyloid peptides were investigated in cultured cell lines to estimate or assess the

pathophysiological or physiological function of βAP. These peptides were mainly examined in relation to the actions of the tachykinin neuropeptide substance P (SP), since biological actions of βAP might be associated with SP (23). In the following chapter, several pharmacological properties of β-amyloid peptides that are similar to those of SP in cultured cell lines are described and discussed.

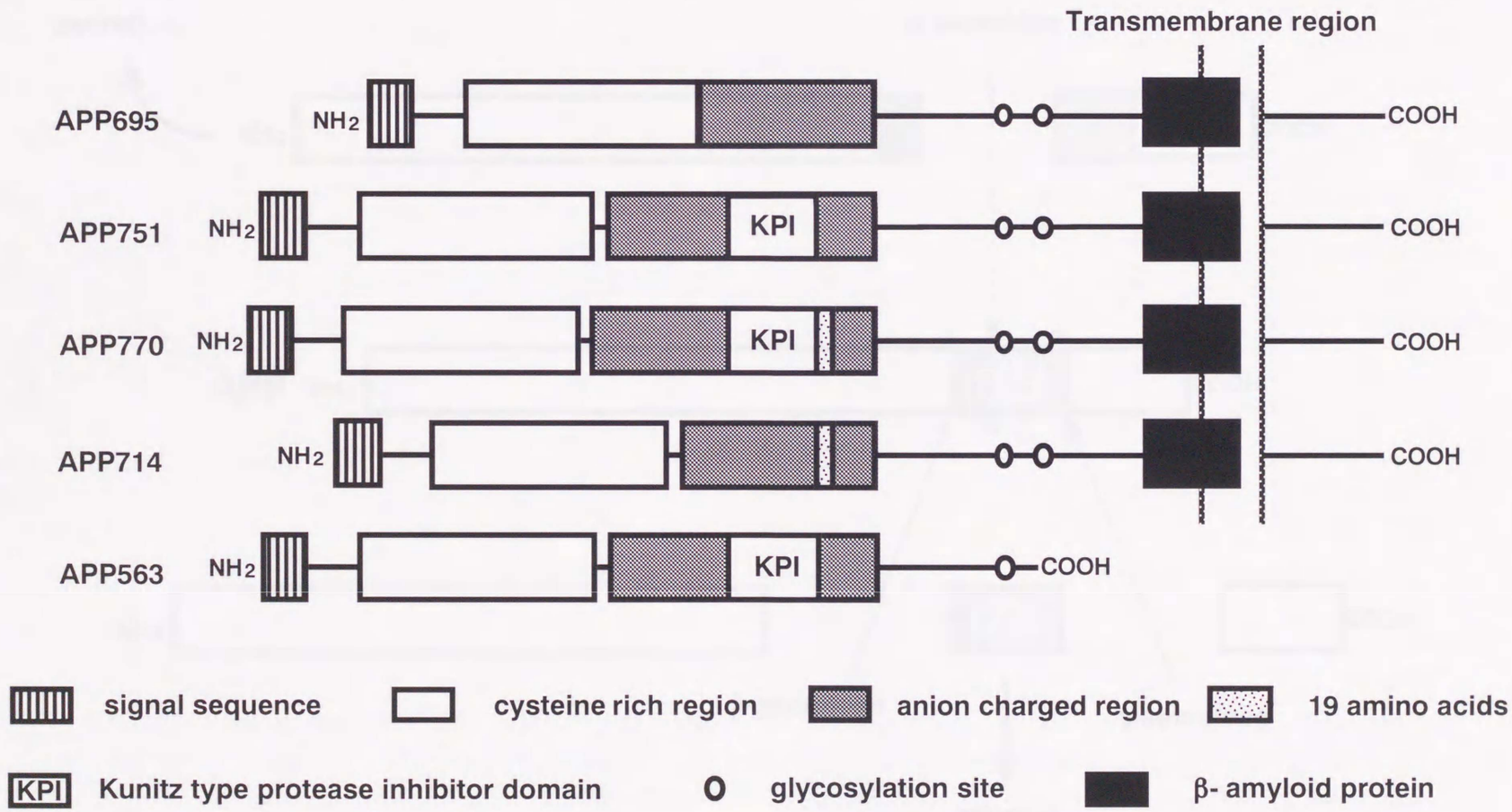

Fig. I-1. Schematic structures of the isoforms of APP.

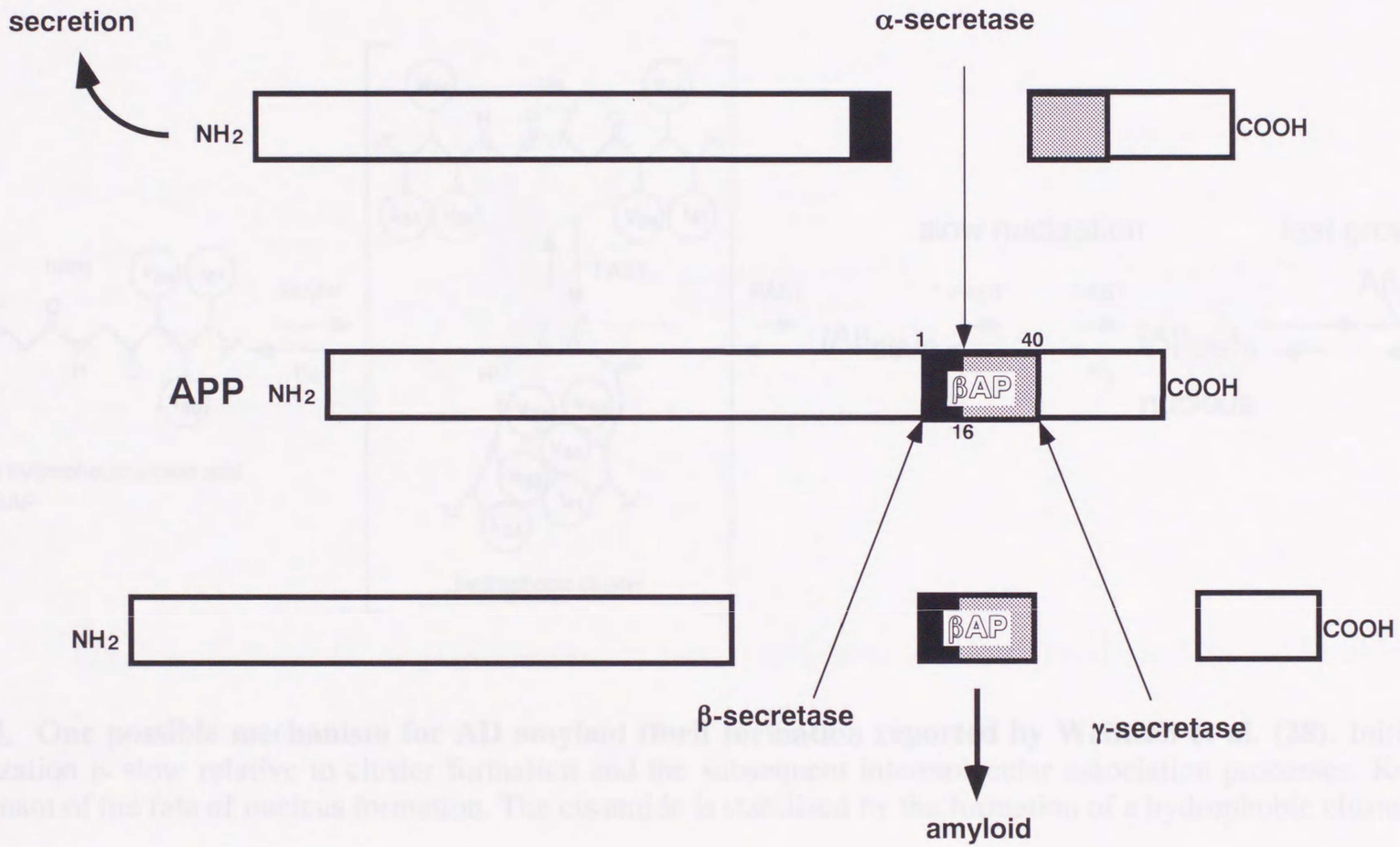

Fig. I-2. Schematic overview of the metabolic fate of APP.

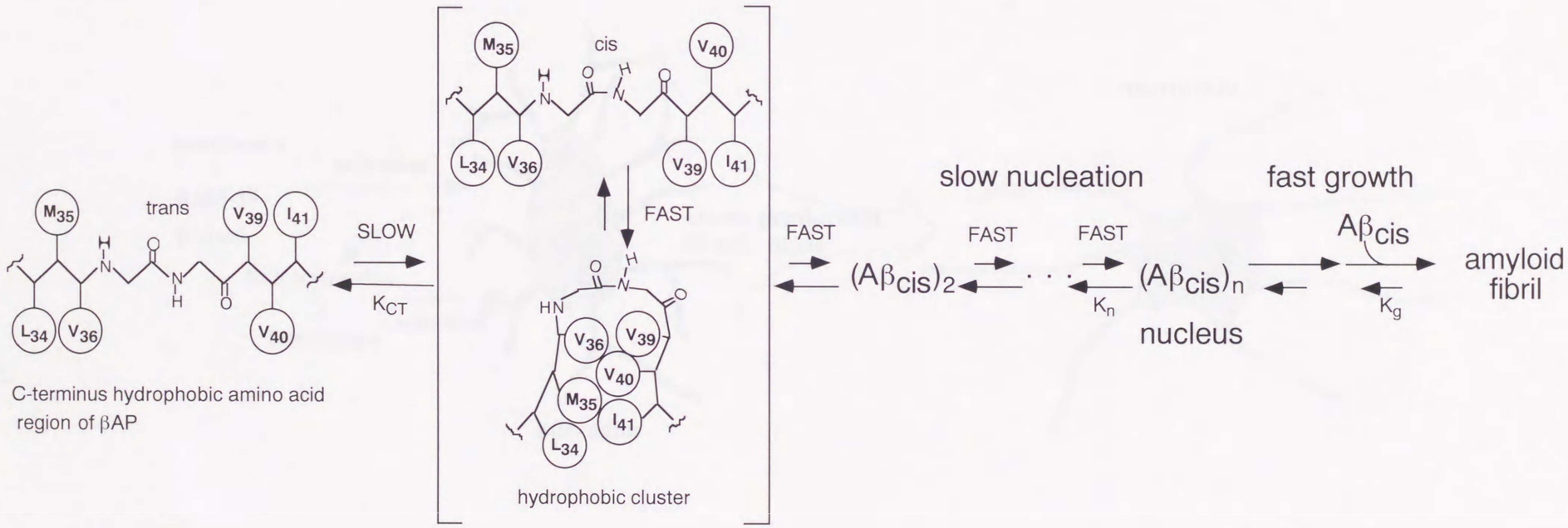

Fig. I-3. One possible mechanism for AD amyloid fibril formation reported by Weinreb et al. (28). Initial trans to cis isomerization is slow relative to cluster formation and the subsequent intermolecular association processes. Kct is a critical determinant of the rate of nucleus formation. The cis amide is stabilized by the formation of a hydrophobic cluster.

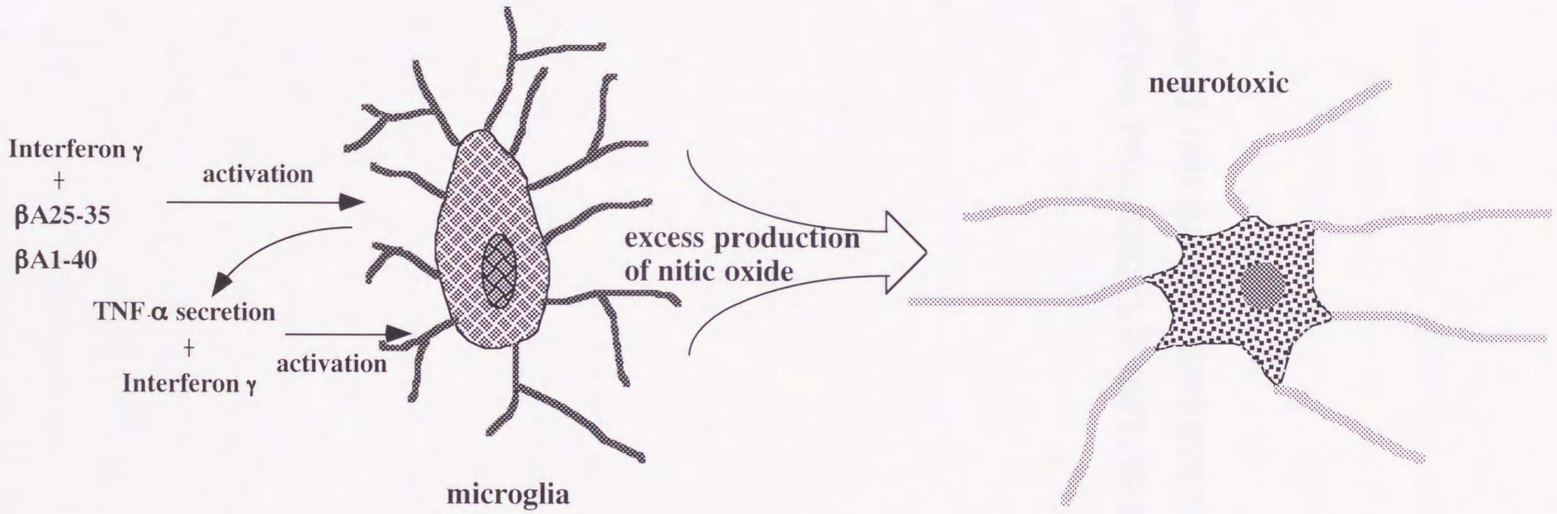

Fig. I-4. Schematic representation of β-amyloid neurotoxicity mediated by nitric oxide (43).

Chapter II INHIBITORY EFFECTS OF β-AMYLOID PEPTIDES ON NICOTINE-INDUCED Ca^{2+} INFLUX IN PC12h CELLS IN CULTURE

II-1 ABSTRACT

The ability of synthetic β-amyloid peptides and the neuropeptide substance P (SP) to modulate the nicotinic response in PC12h cells (a subclone of PC12 cells) was examined. SP, βA1-40 and its peptide fragment βA25-35-NH_2 significantly inhibited the increase in cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) induced by nicotine in a dose-dependent manner. Furthermore, βA1-40 inhibited the $[Ca^{2+}]_i$ increase induced by depolarization with a high concentration of potassium. These findings indicated that both βA1-40 and βA25-35-NH_2 mimic the function of SP on inhibition of nicotinic response through different mechanisms.

II-2 INTRODUCTION

βAP has neurotrophic or neurotoxic effects on cultured neuronal cells. Yankner et al. first reported that the undecapeptide fragment of βAP (amino acid residue 25 to 35, βA25-35) had activities similar to the full length βAP in undifferentiated and differentiated neurons (23). Since βA25-35 shares the C-terminal structure, Gly-Leu-Met, with the tachykinin family of peptides, the effects of tachykinins on the trophic and toxic actions of βAP were tested. In particular, SP which is a representative tachykinin peptide and regarded as a neurotransmitter of primary sensory neurons, reverses the effect of βAP on hippocampal neurons in culture *in vitro* (23) and prevents the neuronal death caused by βAP in the adult rat brain *in vivo* (55). These findings have prompted studies of the relationship between SP and βAP on neuronal cells.

Initially, βAP was assumed to exhibit these activities through the tachykinin receptors on the surface of the neurons. However, βAP and the minimal active sequence βA25-35, do not significantly interact with the NK-1 receptor (also known as the SP receptor) and other tachykinin receptors, such as

NK-2 and NK-3, according to some studies of tissue contraction and radioligand binding assays (56), or cultured cell lines responding to tachykinin (57, 58). Hence, there must be unknown pathways to account for these βAP effects, which are distinguishable from conventional tachykinin receptor mediation.

Although SP has several pharmacological properties, such as contracting activity on various smooth muscles, hypotensive effects and sialogogic effects (59), all of these activities can not be explained by the mediation of only the NK-1 receptor. One of the functions of SP that is thought to be independent of the NK-1 receptor, is the modulation of the nicotinic response on adrenal chromaffin cells (60) or on PC12 cell lines, a transplantable rat adrenal pheocromocytoma (61), because antagonists of SP on the NK-1 receptor, such as [D-Pro^{2}, D-Trp^{7,9}]-SP and [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]-SP, did not display antagonistic activities against this action (62, 63).

The modulation of the nicotinic response is that SP inhibits nicotine-induced catecholamine secretion (62, 63), accompanying an inhibition of Na^{+} or Ca^{2+} influx through the cation channels at lower nicotine concentrations (64), and protects nicotinic acetylcholine receptors from desensitization under high concentrations of nicotine (65). The modulatory effect of SP on acetylcholine receptors is shown to be specific for nicotinic receptors, because SP fails to change excitation responses with glutamate, aspartate, or muscarinic agonists (66), and also fails to block secretions induced by depolarization with a high concentration of potassium (67).

SP inhibition of $^{22}Na^{+}$ influx is reportedly noncompetitive with respect to carbamylcholine (68), and SP inhibits carbamylcholine-induced $^{45}Ca^{2+}$ uptake despite the Na^{+} concentration (64), suggesting that SP does not directly interact with Na^{+} entry into the nicotinic acetycholine receptor. It also has been thought that these effects of SP occurred through the novel SP binding site. Geraghty et al. suggested that another SP binding site exists that is distinguished from the NK-1 receptor in the bovine adrenal medulla (69).

In this chapter, it was examined whether a peptide corresponding to the first 40 amino acids of βAP (βA1-40), βA25-35 peptides, the C-terminal carboxyl free form βA25-35-OH and the amidated derivative βA25-35-NH_2 (Fig. II-1) can modulate the nicotinic response as well as SP in PC12h cells, (in this PC12 subclone, choline acetyltransferase activity is restrained at a lower level and there is an increase of tyrosine hydroxylase activity in the presence of NGF) (70), as a model system for studying SP function. To clarify the actions, we examined the inhibitory activity of these peptides against the intracellular increase in free Ca^{2+} concentrations ($[Ca^{2+}]_i$) induced by nicotine using the calcium sensitive indicator dye, fura-2 (71).

II-3 MATERIALS AND METHODS

II-3-1 Cell culture

PC12h cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 Nutrient Mixture (Ham) containing 5% horse serum (HS) and 5% precolostrum newborn calf serum (PNCS), and incubated in a humidified 90% air / 10 % CO_2 incubator at 37°C.

II-3-2 Peptide synthesis

βA25-35-OH, βA25-35-NH_2 and βA1-40 were manually synthesized in a conventional glass vessel by the solid-phase methodology using 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids (Table II-1). Fmoc amino acids were coupled stepwise to SAL-resin which was used to form the C-terminal amide of βA25-35, and to HMP resin which was used to form the C-terminal carboxyl free peptides. Coupling reactions in chain elongations were performed by means of the BOP-HOBt method (72). SP and Neurokinin B

(NKB) were synthesized by the Boc solid-phase strategy using DCC-HOBt coupling (Table II-1) (73). Boc amino acids were coupled stepwise to Boc-methionine-4-methylbenzhydrylamine resin which was used to form the C-terminal amide of the peptide. In both methods, each coupling step was monitored by Kaiser's ninhydrin test (74). Peptides were cleaved from the resin by trifluoroacetic acid (for Fmoc method) or anhydrous hydrogen fluoride (for Boc method), and purified by reversed phase high performance liquid chromatography (RP-HPLC). The purity of the peptides was confirmed by the analytical RP-HPLC and amino acid analysis. The molecular weight of βA1-40 was also checked by fast atom bombardment mass spectrometry (FAB-MS) (JEOL JMS-HX110 double focusing mass spectrometer) (Fig. II-2), and amino acid sequencing.

II-3-3 Intracellular calcium measurements

The PC12h cell suspension was centrifuged, then the cells were resuspended and washed in buffer A (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM NaH_2PO_4, 11 mM glucose, 0.2 % bovine serum albumin, pH 7.4). Fura 2-AM was added to the cell suspension (5 μM final concentration) and loaded for 1 hr at 20°C. After two washes with buffer A, the cell suspension (about 1×10^6 cells / ml) was transferred to a quartz cuvette and stirred continuously in a CAF-100 spectrofluorometer (Japan Spectroscopy Inc. Tokyo, Japan) for intracellular Ca^{2+} measurements. Fluorescence was excited at 340 nm and 380 nm, and the intensity of the fluorescence (F340 and F380) at 500 nm was measured at 30 °C. The Ca^{2+} concentrations were calculated from the ratio of the fluorescence intensity (R = F340 / F380) and a K_d value of 224, dissociation constant for fura-2, using the equation:

$$[Ca^{2+}]_i = K_d [(R - R_{min}) / (R_{max} - R)](F_{min} / F_{max}).$$

F_{max} and F_{min} were values of F380 after treatment with Triton X-100 (1 % final concentration) and excess EGTA (4 mM final concentration), respectively. The R_{max} and R_{min} were also obtained after exposure to Triton X-100 and EGTA, respectively. βA1-40 peptide and NKB were dissolved in dimethylsulfoxide (DMSO) since these peptides had poor solubility in aqueous solution.

II-3-4 Materials

PC12h cells were a gift from Dr H. Hatanaka (Osaka University). PNCS and HS were purchased from Mitsubishi Chemical Industries (Tokyo, Japan), Neurokinin A (NKA) from the Peptide Institute (Osaka, Japan), 1 mM Fura 2-AM DMSO solution from DOJINDO. For peptide synthesis, all Boc-amino acids, DCC and HOBt were purchased from the Peptide Institute, BOP from Kokusan Chemical Works, Ltd. (Tokyo, Japan), HMP resin from Applied Biosystems, all Fmoc-amino acids and SAL resin from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan).

II-4 RESULTS

II-4-1 Effects on nicotinic response

To evaluate the precise effect of the peptides on the $[Ca^{2+}]_i$ increase stimulated by nicotine in PC12h cells, 32 μM of nicotine was used because the nicotinic acetylcholine receptor was rapidly desensitized by nicotine itself, which caused a decrease of $[Ca^{2+}]_i$ at concentrations higher than 32 μM. These peptides were dissolved and immediately used for the experiments after lyophilization, meaning that the peptides were not aggregated. The addition of 32 μM nicotine evoked a rapid $[Ca^{2+}]_i$ increase, followed by a gradual decrease in the Ca^{2+} concentration and came to a sustained phase that was a higher Ca^{2+}

concentration than the basal level. The nicotine-induced $[Ca^{2+}]_i$ increase disappeared in the presence of 4 mM extracellular EGTA, suggesting that it is due to an extracellular Ca^{2+} influx (data not shown). When the cells were first exposed to 10 μM SP, this nicotinic response was changed, accompanied by rapid desensitization (Fig. II-3). The 10 μM βA25-35-NH_2 inhibition of the nicotinic response was very similar to that of SP (Fig. II-3). Although βA1-40 significantly inhibited the nicotinic response (Fig. II-4), the profile was quite different from that of SP in the rapid desensitization state and the sustained phase. βA1-40 seemed to directly block the nicotine-induced extracellular Ca^{2+} flux into cells rather than enhance desensitization of the nicotinic acetylcholine receptor. βA25-35-OH did not cause a measurable inhibitory response in the $[Ca^{2+}]_i$ increase stimulated by 32 μM nicotine at a peptide concentration of 10 μM (Fig. II-4). Other mammalian tachykinins, neurokinin A (NKA) and neurokinin B (NKB), could not significantly inhibit the $[Ca^{2+}]_i$ increase induced by 32 μM nicotine at 10 μM (Fig. II-5).

Fig. II-6 shows the dose-dependent inhibitory actions of SP and β-amyloid peptides on the nicotinic response. SP inhibited the nicotine-induced $[Ca^{2+}]_i$ increase from a concentration of about 1 μM, and showed nearly 70% inhibition at 10 μM. βA25-35-NH_2 dose-dependently inhibited to the nicotine-induced $[Ca^{2+}]_i$ increase. βA1-40 also had similar inhibitory activity. The dose-dependent inhibition curves of βA25-35-NH_2 and βA1-40 were shifted slightly to the right compared with those of SP.

II-4-2 Effects on muscarinic response

In PC12h cells, the $[Ca^{2+}]_i$ increase was stimulated by 100 μM muscarine (Fig. II-7). Muscarinic acetylcholine receptors transduce their signals by interacting with guanine nucleotide regulatory proteins (G-proteins), such as rhodopsin, β-adrenergic receptors, and other members of the family of seven

transmembrane-spanning domain receptors. Stimulation of the muscarinic receptors includes Ca^{2+} mobilization from the intracellular stores in the adrenal chromaffin cells, which is independent of extracellular Ca^{2+} (75). SP reportedly does not inhibit the muscarinic response in adrenal chromaffin and PC12 cells. In this study, the inhibitory effect of 10 μM SP on muscarine-stimulated change in $[Ca^{2+}]_i$ was undetectable (Fig. II-7). Likewise, βA25-35-NH_2, βA25-35-OH and βA1-40 did not significantly affect the 100 μM muscarine-induced $[Ca^{2+}]_i$ increase at a peptide concentration of 10 μM (Fig. II-7).

II-4-3 Effects on 60mM-K^+ stimulation

The difference in the inhibition profiles between βA1-40 and SP prompted us to investigate the effect of βA1-40 on the $[Ca^{2+}]_i$ increase induced by depolarization with a high concentration of potassium. The rapid $[Ca^{2+}]_i$ increase stimulated by 60 mM-K^+ was due to an extracellular Ca^{2+} influx through the voltage-dependent calcium channels on the surface of PC12h cells, since this $[Ca^{2+}]_i$ increase disappeared upon the addition of 4 mM EGTA and it was blocked by 100 nM nifedipine, which is an L-type voltage-dependent calcium channel blocker (Fig. II-8). SP and βA25-35-NH_2 could not significantly inhibit the $[Ca^{2+}]_i$ increase induced by 60 mM-K^+ at 10 μM (Fig. II-8). It is known that SP does not directly inhibit voltage-dependent calcium channels in chromaffin cells. βA1-40 inhibited the 60 mM-K^+ induced $[Ca^{2+}]_i$ increase in a dose-dependent manner (Fig. II-8, 9), suggesting that it blocks the Ca^{2+} influx from extracellular in PC12h cells, probably caused by direct interaction with the cell membranes because of its hydrophobicity.

II-5 DISCUSSION

This study shows that the β-amyloid peptides, βA1-40 and βA25-35-NH_2 inhibited the nicotine-induced $[Ca^{2+}]_i$ increase in a dose-dependent manner. The inhibition profile of βA25-35-NH_2 is very similar to that of SP with respect to accompanying the rapid desensitization of the nicotinic acetylcholine receptor. The finding raises the possibility that βA25-35-NH_2 acts via the same mechanism as SP, which does not involve competition with nicotine for the receptor recognition site on the PC12h cells. Since βA25-35-OH did not inhibit the nicotinic response, the structure of the C-terminal is important to conserve the inhibitory potency of the βA25-35 peptide to say nothing of SP (76).

The inhibition profile of βA1-40 is distinct from that of SP in its sustained phase. βA1-40 seems to directly depress the nicotine-induced $[Ca^{2+}]_i$ increase rather than enhance desensitization of the nicotinic acetylcholine receptor. The high-K^+ study indicated that βA1-40 may block the dihydropyridine-sensitive calcium channels of PC12h cells stimulated by high-K^+. It also suggests a possibility that βA1-40 directly depresses the cation (Ca^{2+} or Na^+) flux into the cells through voltage-dependent calcium channels or the nicotinic acetycholine receptor, which is a cation channel activated by the depolarization.

Two actions of SP have been identified in the adrenal medulla and in PC12 cells: [1] inhibition of nicotine stimulation and [2] potentiation of nicotine stimulation through protection of nicotinic desensitization. The mammalian tachykinins are more effective in protecting against desensitization than in inhibiting the nicotinic response (77). NKA and NKB did not significantly inhibit the nicotine-induced $[Ca^{2+}]_i$ increase at 10 μM in PC12h cells (Fig. III-6). Thus, the inhibitory effects were relatively specific for SP and β-amyloid peptides within the tachykinin-like peptides.

Cheung et al. have reported that βA25-35-NH_2 inhibits the 10 μM nicotine-induced catecholamine secretion in a manner analogous to SP in

cultured bovine adrenal chromaffin cells (78). Concerning these inhibitory actions of βA25-35-NH_2 on the nicotinic response, our findings were in agreement with theirs because the catecholamine release was necessary to increase the $[Ca^{2+}]_i$ in these cells (79). Although they could not test the effect of βA1-40 due to its low solubility in aqueous media, we found that βA1-40 inhibited the nicotinic response in the presence of 1.0 % DMSO in the buffer; the low concentration of DMSO had no influence on the $[Ca^{2+}]_i$ induced by nicotine.

One common feature of some neurotoxins is the unusual activation of $[Ca^{2+}]_i$ (80, 81). β-Amyloid peptides has been reported to elevate resting levels of calcium and enhance calcium responses to excitatory amino acids and calcium ionophore in human cortical neurons (82). This increases the vulnerability of the neurons to excitotoxic damage (83). Also, cultured hippocampal neurons exhibiting the calcium binding protein calretinin are relatively resistant to the degeneration resulting from exposure β-amyloid peptides, suggesting that the intrinsically buffering characteristics of calretinin against the enhanced $[Ca^{2+}]_i$ are effective (84). Though it is difficult to consider that this inhibitory effects of β-amyloid peptides are directly connected with its neurotoxicity, our findings indicate that βA25-35-NH_2 may interact with an unidentified SP binding site on PC12h cells that is distinguished from the NK-1 receptor and thus provide a means with which characterize the inhibitory activity of SP.

In conclusion, this study showed that βA1-40 and βA25-35-NH_2 inhibited the $[Ca^{2+}]_i$ increase caused by nicotine in PC12h cells as well as SP, but that the actions of both peptides might be mediated by different mechanisms. βA1-40 might also directly block the voltage-dependent channels against the cation influx in these cells, since the increase in $[Ca^{2+}]_i$ induced by high-K^+ was depressed by the peptide. This finding also raises the possibility that high

concentrations of β-amyloid peptides prevent normal neuronal transmission in the sympathoadrenal system.

β-amyloid peptides

βA1-40

1 10 20 30 40

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

βA25-35 (active site) GSNKGAIIGLM

mammalian tachykinin peptides

Substance P (SP) RPKPQQFFGLM-NH_2

Neurokinin A (NKA) HKTDSFVGLM-NH2

Neurokinin B (NKB) DMHDFFVGLM-NH2

Fig. II-1. Primary structures of β-amyloid and mammalian tachykinin peptides.

Table 1. Program of Boc and Fmoc solid-phase peptide synthesis

Boc method	Fmoc method
(1) CH_2Cl_2 (2 min wash × 2)	(1) CH_2Cl_2 (2 min wash × 2)
(2) 50% TFA in CH_2Cl_2 deprotection of Boc-group (30 min)	(2) 20% piperidine in NMP deprotection of Fmoc-group (30 min)
(3) n-PrOH (2 min)	(3) MeOH (2 min)
(4) 10% Et_3N in CH_2Cl_2 neutralization (2 min)	(4) CH_2Cl_2 (2 min)
(5) n-PrOH (2 min)	(5) Fmoc-AA-OH, BOP, and HOBt coupling in NMP
(6) CH_2Cl_2 (2 min)	(6) MeOH (2 min)
(7) Boc-AA-OH, DCC, and HOBt coupling in CH_2Cl_2 or DMF	(7) CH_2Cl_2 (2 min)
(8) MeOH (2 min)	(8) MeOH (2 min)
(9) CH_2Cl_2 (2 min)	
(10) MeOH (2 min)	

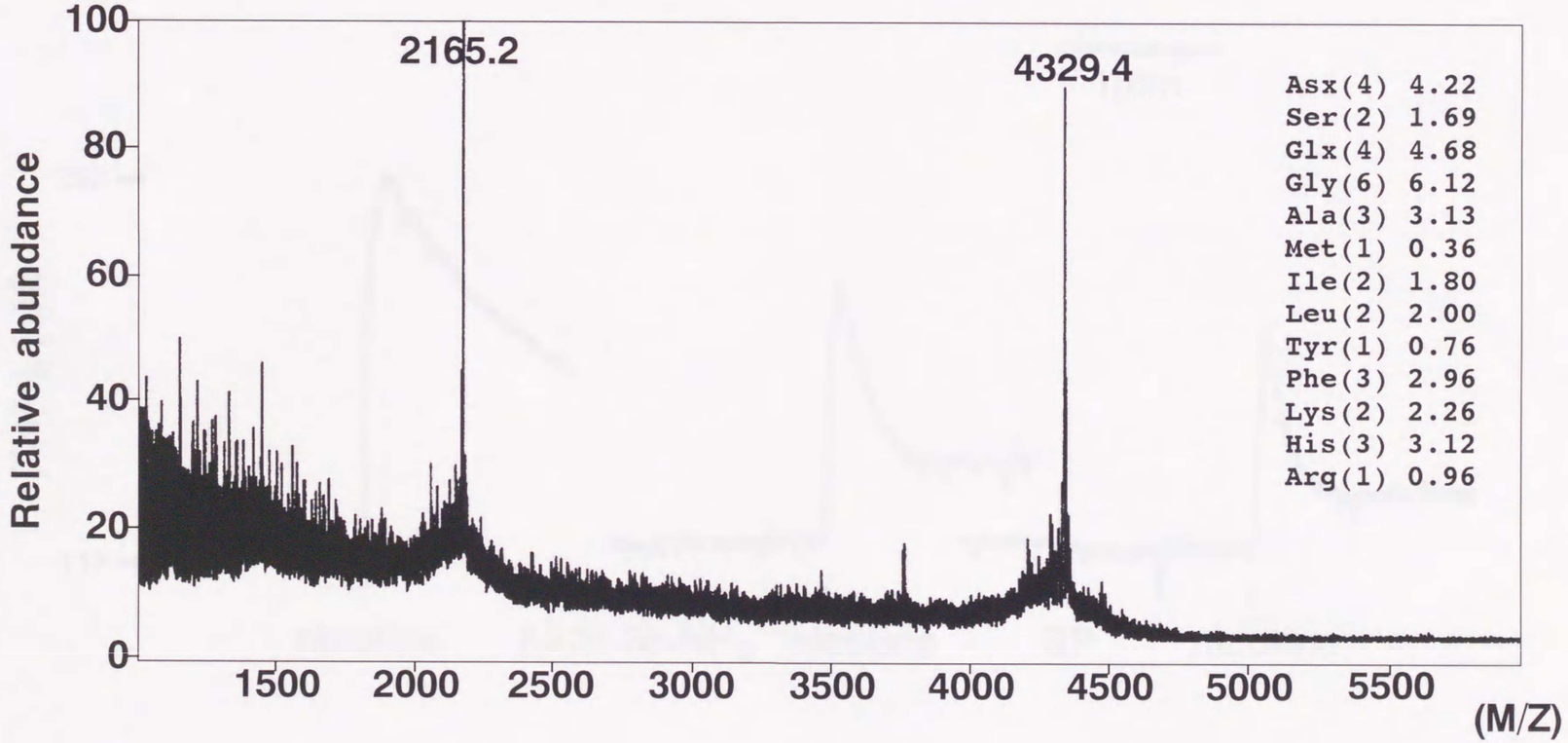

Fig. II-2. Positive ion FAB mass spectra and amino acid composition of βA1-40. The calculated value is M/Z = 4330.

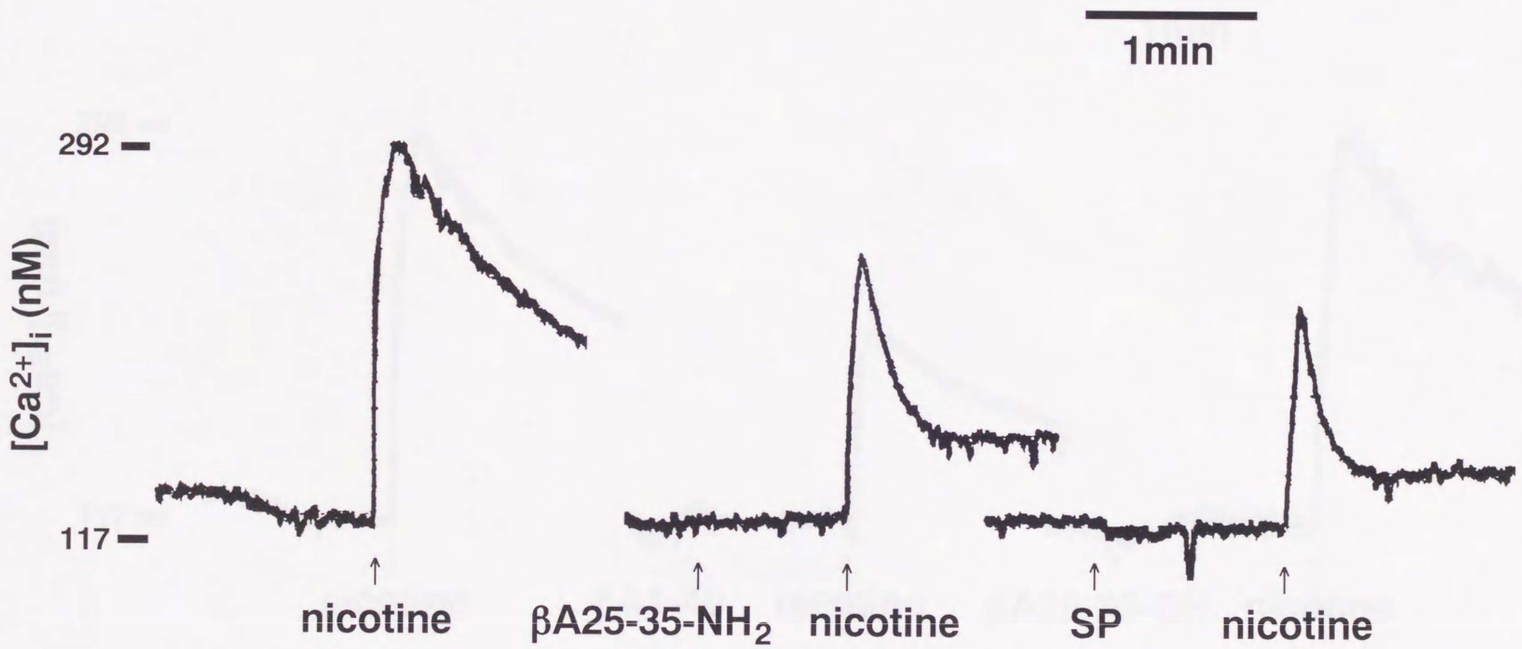

Fig. II-3. Inhibitory effects of βA25-35-NH_2 and SP on $[Ca^{2+}]_i$ influx induced by nicotine in PC12h cells. After adding 10 μM βA25-35-NH_2 and SP, the increase in $[Ca^{2+}]_i$ induced by 32 μM nicotine was remarkably inhibited as compared with no addition.

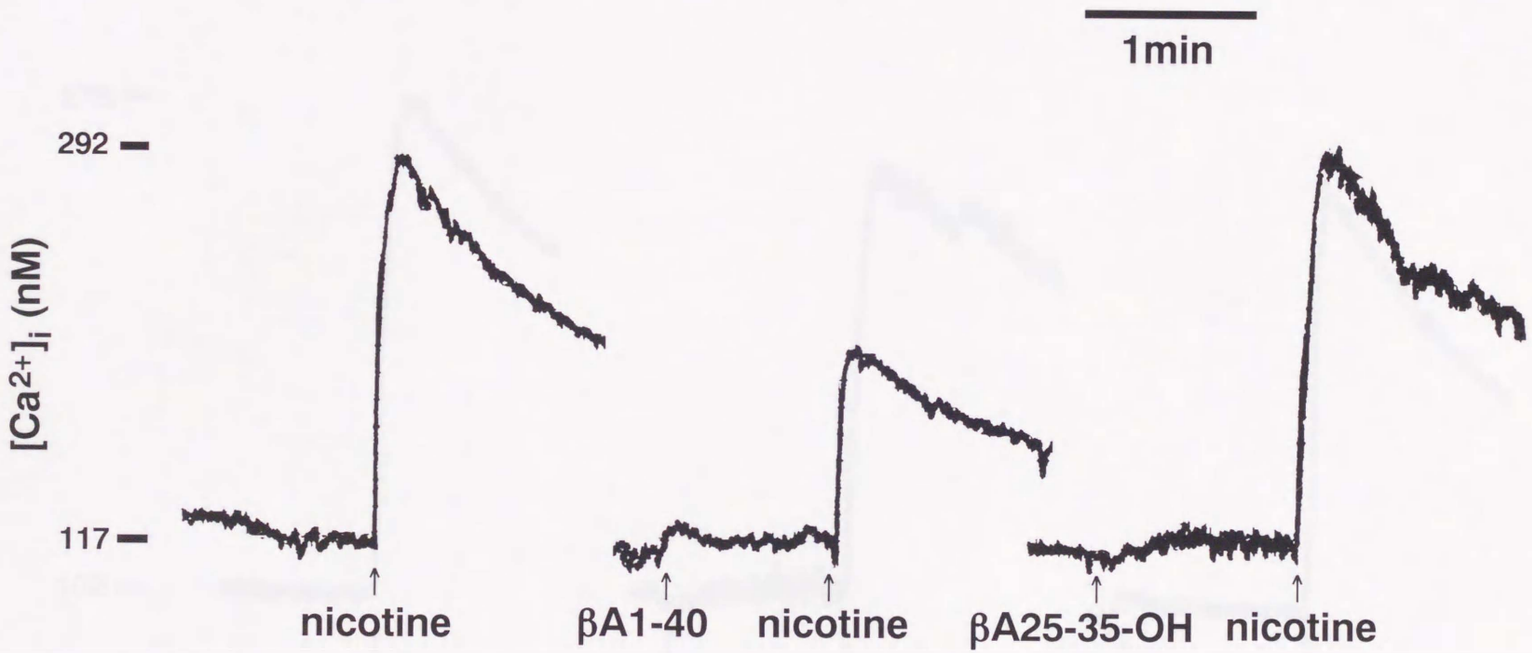

Fig. II-4. Inhibitory effects of βA1-40 and βA25-35-OH on $[Ca^{2+}]_i$ influx induced by nicotine in PC12h cells. After adding 10 μM βA1-40, the increase in $[Ca^{2+}]i$ induced by 32 μM nicotine was remarkably inhibited as compared with no addition. However, the inhibition by 10 μM βA25-35-OH was not significant.

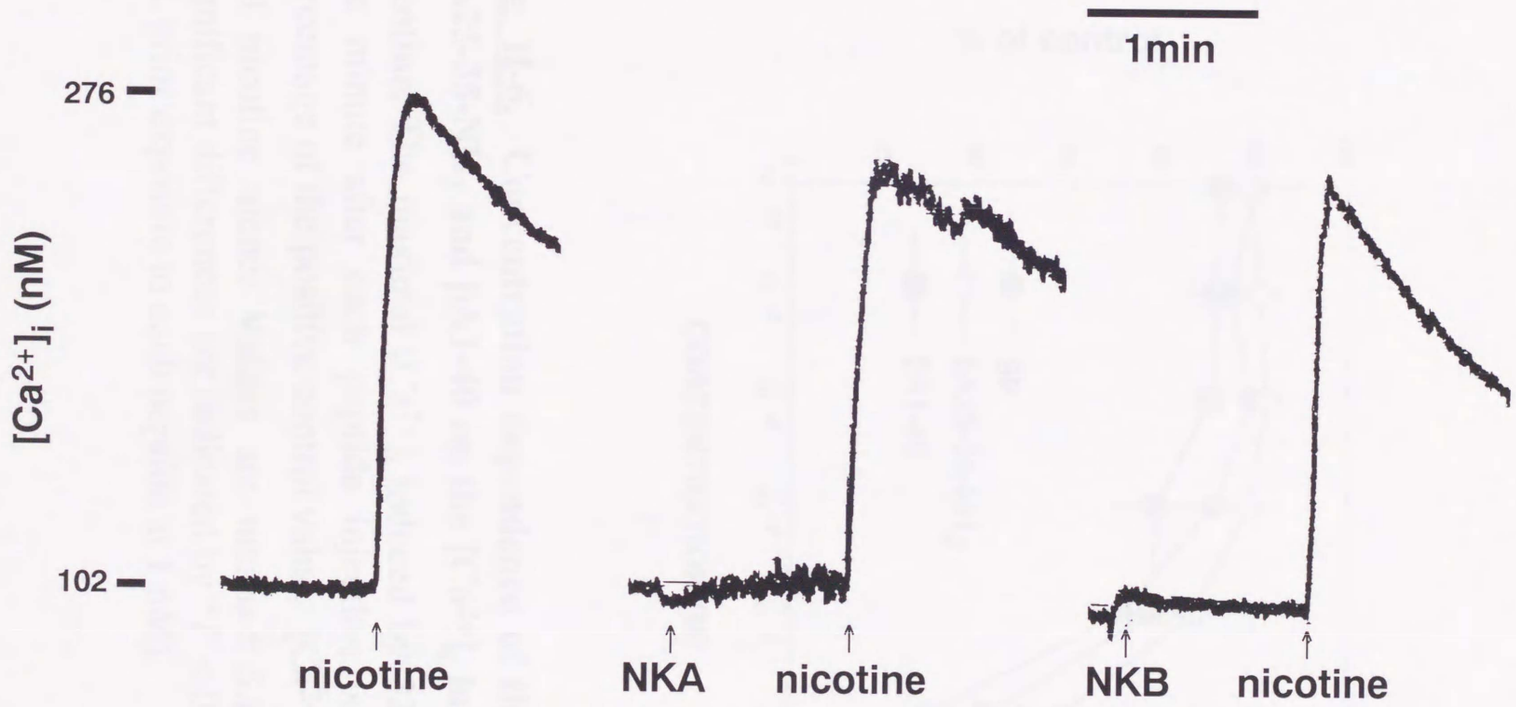

Fig. II-5. The effects of NKA and NKB on $[Ca^{2+}]_i$ influx induced by nicotine in PC12h cells. The increase in $[Ca^{2+}]_i$ induced by 32 μM nicotine was not significantly inhibited by 10 μM NKA and NKB as compared with no addition.

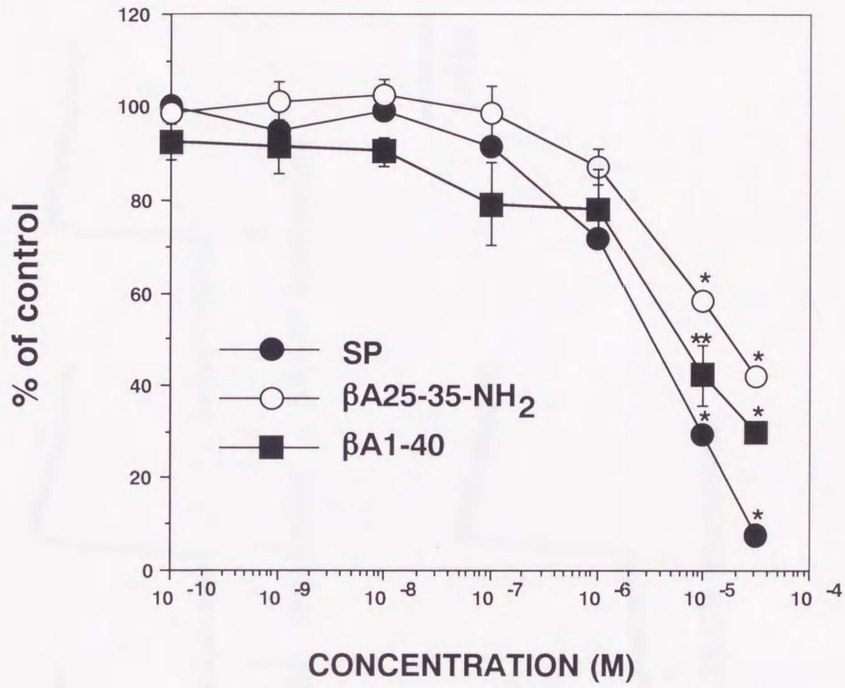

Fig. II-6. Concentration dependence of the effects of SP, βA25-35-NH_2 and βA1-40 on the $[Ca^{2+}]_i$ influx induced by nicotine. The maximal $[Ca^{2+}]_i$ induced by 32 μM nicotine at one minute after each peptide injection is indicated as a percentage of the positive control value, $[Ca^{2+}]_i$ induced by 32 μM nicotine alone. Values are means ± S.E.M. (n = 3-5). Significant differences are indicated by $*P < 0.001$, $**P < 0.01$ (vs. prior exposure to each peptide at 1 nM).

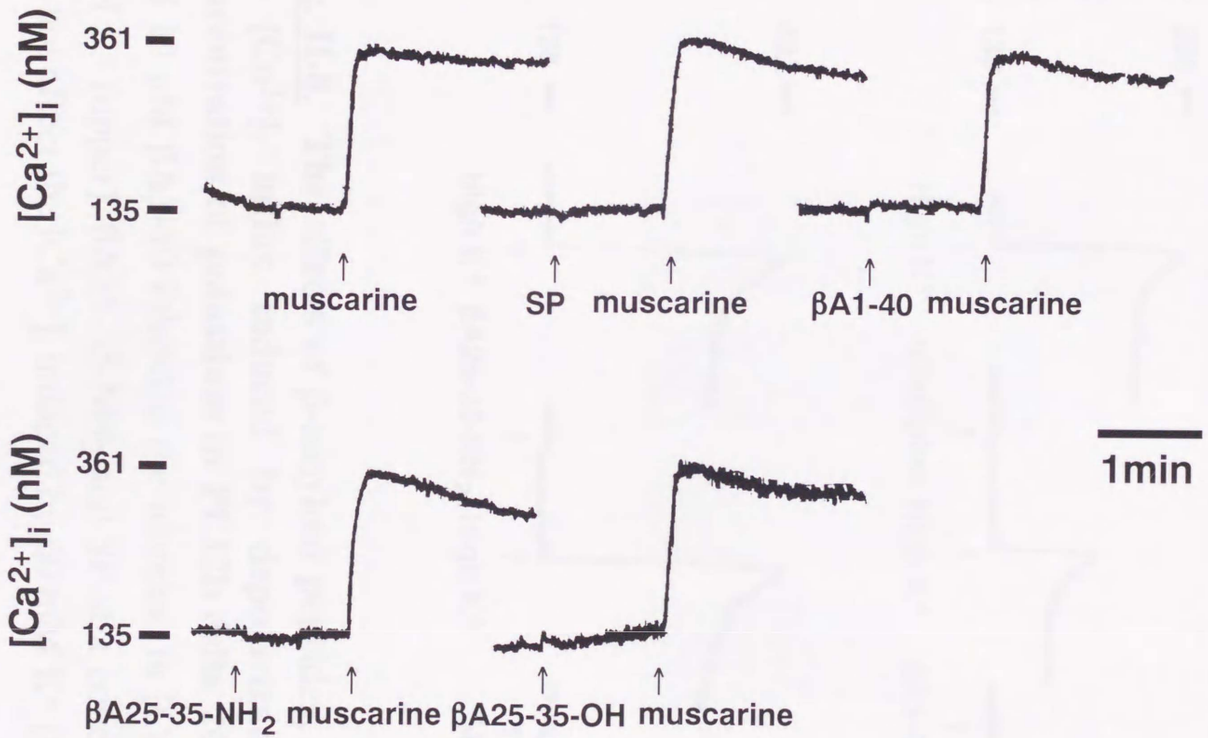

Fig. II-7. The effects of SP and β-amyloid peptides on the $[Ca^{2+}]_i$ influx induced by muscarine in PC12h cells. SP, βA1-40 (upper), βA25-35-NH_2 and βA25-35-OH (lower) had no effect on the increase in $[Ca^{2+}]_i$ induced by 100 μM muscarine at a peptide concentration of 10 μM.

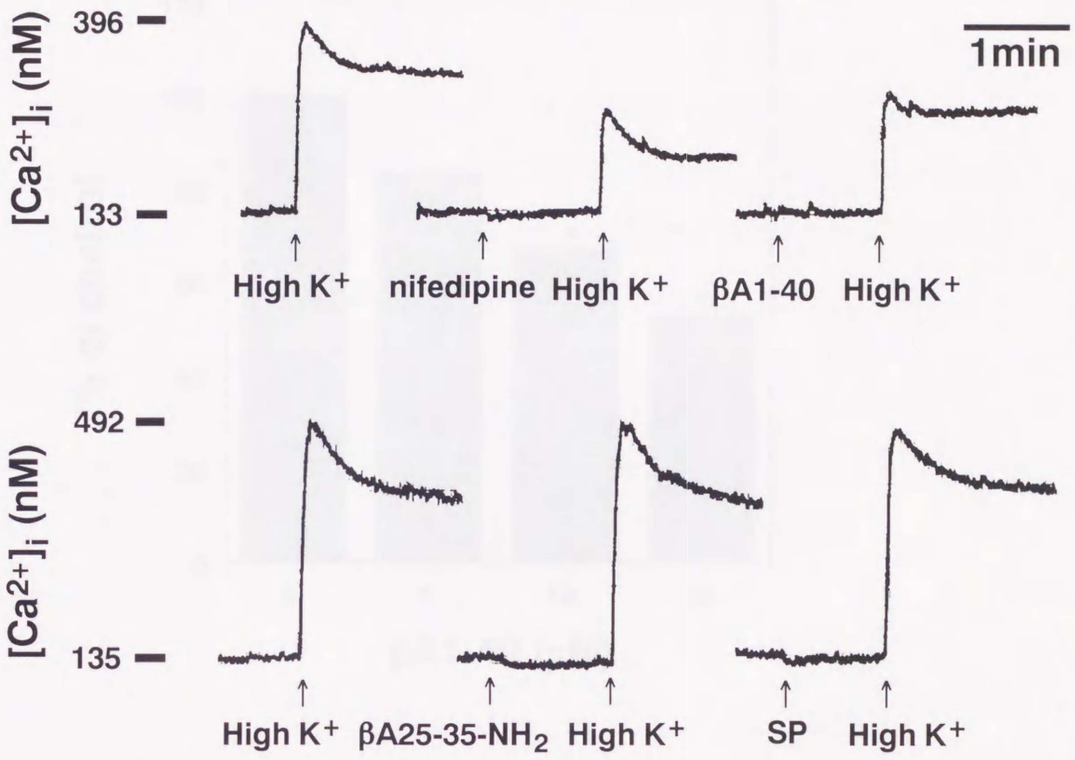

Fig. II-8. The effects of β-amyloid peptides, SP and nifedipine on $[Ca^{2+}]_i$ influx induced by depolarization with a high concentration of potassium in PC12h cells. Nifedipine (100 nM) and 10 μM βA1-40 inhibited the increase in $[Ca^{2+}]_i$ induced by 60 mM K^+ (upper). βA25-35-NH_2 and SP at a concentration of 10 μM did not affect the $[Ca^{2+}]_i$ induced by 60 mM K^+ (lower).

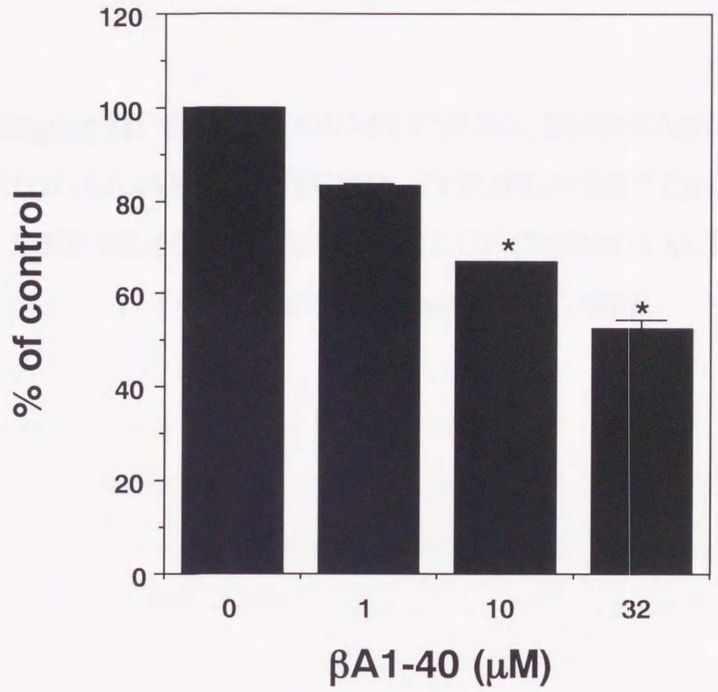

Fig. II-9. Concentration dependence of the effects of βA1-40 on the $[Ca^{2+}]_i$ influx induced by 60 mM K^+. The maximal $[Ca^{2+}]_i$ induced by 60 mM K^+ at one minute after βA1-40 injection is indicated as a percentage of the positive control value, $[Ca^{2+}]_i$ induced by 60 mM K^+ alone. Values are means ± S.E.M. (n = 3). Significant differences are indicated by $*P < 0.001$ (vs. prior exposure to βA1-40 at 1 μM).

Chapter III β-AMYLOID PEPTIDES, SUBSTANCE P AND SEC-RECEPTOR LIGAND ACTIVATE CYTOPLASMIC Ca^{2+} IN NEUTROPHIL-LIKE HL-60 CELLS: EFFECT OF CHEMOTACTIC PEPTIDE ANTAGONIST BOC-MLF

III-1 ABSTRACT

A discrete peptide fragment of βAP, βA25-35, and substance P (SP) possess sequence homology and bind the serine protease inhibitor (Serpin) Enzyme Complex (SEC) receptor. Thus, it has been thought that these peptides and SEC-receptor ligand have close relationships in terms of their biological activities. In this study, we found that C-terminal amidated βA25-35-NH_2, SP and SEC-receptor ligand, Phe-Val-Phe-Leu-Met (FVFLM), potently induced an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neutrophil-like differentiated human leukemic (HL-60) cells. A prior exposure to pertussis toxin (PTX) potently inhibited the $[Ca^{2+}]_i$ increase stimulated by these peptides, suggesting that these responses are mediated by PTX-sensitive G-proteins. Furthermore, the effect of *t*-butyloxycarbonyl-methionyl-leucyl-phenylalanine (BocMLF), which is a competitive antagonist of chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) upon its receptor was examined. BocMLF scarcely inhibited the $[Ca^{2+}]_i$ increase stimulated by βA25-35-NH_2. However, the FVFLM-induced $[Ca^{2+}]_i$ increase was potently inhibited by BocMLF. The results suggest that the $[Ca^{2+}]_i$ activation of βA25-35-NH_2 may have a different mechanism from that of FVFLM in neutrophil-like HL-60 cells, which is not mediated by the SEC-receptor.

III-2 INTRODUCTION

The undecapeptide fragment of βAP (βA25-35) mimics the biological effects of full length βAP and shares a sequence similar to that of SP (23). β-amyloid peptides reportedly possess close relationships with SP in terms of their biological actions (chapter II, 23, 55, 85). The inflammatory response surrounding the senile plaques in the AD brains has been studied in detail (18, 86), and it is thought that βAP is included in the inflammation. β-amyloid

peptides directly activate the cell types involved in the inflammatory and immune processes, such as human neutrophils (87) and rat peritoneal macrophages (88). Also, SP is well-known as one of the mediators of neurogenic inflammation.

The chemotactic activity stimulated by βA25-35 may be mediated by the serine protease inhibitors (Serpins) Enzyme Complex (SEC) receptor in human neutrophils (87). The SEC-receptor recognizes complexes of serpins with their cognate enzymes, and mediates increases in the de novo synthesis of serpins or degradation of the complexes by endocytosis (Fig. III-1) (89, 90). It is expressed on the surface of human neutrophils (87), human hepatoma HepG2 cells and human monocytes (91). The pentapeptide fragment of α_1-antitrypsin, Phe-Val-Phe-Leu-Met (FVFLM), is the minimum sequence which is sufficient for binding to the SEC-receptor (92). Furthermore, it has been reported that β-amyloid peptides and SP directly bind the SEC-receptor (93). These findings indicate that the β-amyloid peptides, SP and FVFLM have interrelated biological activities through interactions with the same receptor, such as the SEC-receptor (94).

In this chapter, we examined the effects of β-amyloid peptides, SP and FVFLM on cytoplasmic Ca^{2+} change in human leukemic (HL-60) cells differentiated by dibutyryl cyclic AMP (Bt_2cAMP). HL-60 cells differentiated by Bt_2cAMP express a large number of chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptors and are functionally similar to neutrophils (95). Cytoplasmic Ca^{2+} change was monitored by using the calcium sensitive indicator dye, fura-2 (71). The results showed that the C-terminal amidated βA25-35-NH_2, SP and FVFLM potently stimulated an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells. The $[Ca^{2+}]_i$ increase was induced by these peptides in the presence of an extracellular excess EGTA and inhibited by a prior exposure to pertussis toxin (PTX). Also, *t*-butyloxycarbonyl-methionyl-leucyl-phenylalanine (BocMLF) which is a

competitive antagonist of fMLP on its receptor (96) potently inhibited the FVFLM-induced $[Ca^{2+}]_i$ increase, although it inhibited βA25-35-NH_2 stimulation only slightly . The results suggest that βA25-35-NH_2 may activate the Bt_2cAMP-differentiation of HL-60 cells through a different mechanism from that of FVFLM .

III-3 MATERIALS AND METHODS

III-3-1 Cell culture

HL-60 cells were routinely cultured in RPMI 1640 containing 10 % heat inactivated fetal calf serum (FCS) supplemented with 100 μg / ml streptomycin and 100 U / ml penicillin, and incubated in a humidified 95 % air / 5 % CO_2 incubator at 37 °C. Cells were induced to differentiate into neutrophil-like cells in this growth medium with 500 μM Bt_2cAMP for 3 days at a concentration of about 10^6 cells per ml. Furthermore, cells were incubated with 200 ng / ml PTX for 4 hr to examine its effects.

III-3-2 Peptide synthesis

FVFLM and SP were manually synthesized in a conventional glass vessel by means of the Boc solid-phase methodology as described in Chapter II. BocMLF was synthesized by the Boc liquid-phase methodology. The C-terminal of Boc-Phe-OH was protected by phenacyl ester (Pac). The Boc group was deprotected by TFA and Boc-Leu-OH was coupled to NH_2-Phe-OPac by using water-soluble carbodiimide (WSCD) - HOBt coupling reagents. After Boc-Met-OH coupling by the same method, the product was exposed to an excess of zinc powder to deprotect the phenacyl group at the C-terminus. The

BocMLF was purified by preparative RP-HPLC and confirmed by amino acid analysis.

III-3-3 Intracellular calcium measurement

HL-60 cell suspensions were centrifuged, then the cells were resuspended and washed in buffer A. Fura 2-AM, a sensitive luminescence calcium chelator, was added to the cell suspension (5 μM final concentration) and loaded at 37 °C for 45 min. Intracellular free calcium was measured as described in Chapter II.

III-3-4 Inositol phosphate studies

Differentiated HL-60 cells were resuspended in 1% FCS / 199 medium with a low inositol concentration. Thereafter, *myo*-[^{3}H] inositol (10 μCi / ml) was added and the cells were incubated for 24 hr at 37 °C in a humidified 95 % air / 5 % CO_2 incubator. The cells were then washed twice in buffer A containing 5 mM unlabelled inositol and resuspended in inositol-free buffer A with 10 mM LiCl at a density of 10^6 cells / ml. The optimal reaction time (10 min) of the peptides was determined in preliminary experiments. After an incubation for 15 min at 37 °C, various concentrations of βA25-35-NH_2 and 10 μM fMLP were added to a 1 ml cell suspension and incubated for 10 min at 37 °C. At a fixed time, 200 μl of 20 % perchloric acid was added and placed on ice for 20 min. The precipitate was removed by centrifugation for 10 min at 4 °C. A 1 ml portion of the supernatant was neutralized with 1.5 M KOH containing 60 mM Hepes. After remaining on ice for 1 hr, the precipitate was removed by centrifugation and radiolabelled inositol phosphates in the supernatant were extracted by anion exchange chromatography as described by Berridge (97). All supernatants were applied to columns containing Dowex 1 × 8 resin, 100-200 mesh, formate form. The columns were washed with 10 ml water and 6 ml of 5

mM disodium tetraborate / 60 mM sodium formate to elute free inositol and glycerophosphoinositol. The fractions of inositol 1-phosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol triphosphate (IP_3) were sequentially eluted with 6 ml each of 0.1 M formic acid / 0.2 M ammonium formate, 0.1 M formic acid / 0.4 M ammonium formate and 0.1 M formic acid / 1.0 M ammonium formate. The 6 ml eluates containing ^{3}H-labeled inositol phosphates were quantified by scintillation counting.

III-3-4 Materials

All Boc protected amino acids, HOBt, DCC and fMLP chemotactic peptide were from the Peptide Institute (Osaka, Japan), WSCD from EIWEISS (Yokohama, Japan). HL-60 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). FCS was purchased from Biotech International, RPMI 1640 and 199 medium from GIBCO, phorbol 12-myristate 13-acetate (PMA) and Bt_2cAMP from SIGMA, Pertussis toxin was from List Biological Laboratories, Inc. (California, U.S.A.). Radiolabelled *myo*-[^{3}H] inositol was purchased from Amersham.

III-4 RESULTS

III-4-1 Effects of SP and β-amyloid peptides on $[Ca^{2+}]_i$

βA1-40 was dissolved in DMSO since it is poorly soluble in water. A low concentration of DMSO (1.0 % of final concentration in buffer) had no influence on the basal $[Ca^{2+}]_i$ in the experimental conditions. SP and βA25-35-NH_2 were dissolved in buffer. Figure III-2 shows that adding 10 μM β-amyloid peptides and SP into the Bt_2cAMP-differentiated HL-60 cell suspension resulted in a significantly increase in $[Ca^{2+}]_i$. βA1-40 and βA25-35-OH showed weakly

activated $[Ca^{2+}]_i$ (Fig. III-2). βA25-35-NH_2 potently increased the $[Ca^{2+}]_i$ in a similar manner to SP, which then gradually decreased nearly to the basal level (Fig. III-2). The $[Ca^{2+}]_i$ increase was induced by βA25-35-NH_2 and SP in the presence of extracellular 4 mM EGTA (Fig. III-2, lower). Also, the $[Ca^{2+}]_i$ activations stimulated by the both peptides were dose related over a concentration range of 1-100 μM (Fig. III-3). From these findings, βA25-35-NH_2 was used to examine the function of β-amyloid peptide in the following experiments, since it was more effective on the $[Ca^{2+}]_i$ response.

III-4-2 Effects of PMA on βA25-35-NH_2-induced $[Ca^{2+}]_i$ mobilization

Figure III-4 shows the effect of PMA, a direct protein kinase C activator, on the $[Ca^{2+}]_i$ change elicited by 10 μM βA25-35-NH_2. PMA was also dissolved in DMSO. The low concentration of DMSO had no influence on the $[Ca^{2+}]_i$ increase induced by βA25-35-NH_2. These concentrations of PMA did not affect the basal $[Ca^{2+}]_i$ under the present experimental conditions. The maximal $[Ca^{2+}]_i$ obtained by stimulation with 10 μM βA25-35-NH_2 was inhibited by PMA dose-dependently over a concentration range of 1-100 nM (Fig. III-4).

III-4-3 Inositol polyphosphates accumulation induced by βA25-35-NH_2

The inositol phosphate formation induced by βA25-35-NH_2 was investigated. At a concentration of 10 μM, fMLP activated phosphoinositide hydrolysis as a positive control in this experimental conditions (Fig. III-5). The accumulation of inositol polyphosphates, IP, IP_2 and IP_3, was also stimulated by βA25-35-NH_2 in a dose dependent manner (Fig. III-5), indicating that the βA25-35-NH_2 may produce inositol phosphates as an intracellular second messenger for calcium mobilization from the intracellular store.

III-4-4 Effects of FVFLM on $[Ca^{2+}]_i$

FVFLM was dissolved in DMSO for the same reason described above. FVFLM at a concentration of 10 μM induced a prompt increase in $[Ca^{2+}]_i$, suggesting a possibility that this response is associated with SEC-receptor mediation (Fig. III-6). The $[Ca^{2+}]_i$ was increased in the presence of 4 mM extracellular EGTA (Fig. III-6), and it was dose related over a concentration range of 0.1-100 μM (Fig. III-6).

III-4-5 Effects of PTX on peptide-induced $[Ca^{2+}]_i$ mobilization

To characterize the $[Ca^{2+}]_i$ changes induced by these peptides, Bt_2cAMP-differentiated HL-60 cells were incubated with PTX to examine whether or not the actions are mediated by a PTX-sensitive guanine nucleotide regulated protein (G-protein). The ADP ribosylation of the α-subunit of specific G-protein by PTX completely uncouples the fMLF receptor from phospholipase C and regulates cell responses such as secretion, inositol phosphate formation and $[Ca^{2+}]_i$ mobilization in neutrophils and in differentiated HL-60 cells (98-100). PTX also partially inhibits the functions mediated by P_2-purinergic receptors for extracellular ATP in Bt_2cAMP-differentiated HL-60 cells (101). Figure III-7 shows that the $[Ca^{2+}]_i$ increase induced by 10 μM βA25-35-NH_2, SP and FVFLM was potently inhibited by PTX. The response of differentiated cells to 10 nM fMLP stimulation was used to assess the effect of PTX. The $[Ca^{2+}]_i$ mobilization induced by 10 nM fMLP was completely abolished after exposure to PTX (Fig. III-8).

III-4-6 Effects of BocMLF on peptide-induced $[Ca^{2+}]_i$ mobilization

We further examined the effects of BocMLF on the increase in $[Ca^{2+}]_i$ induced by peptides. BocMLF binds to the fMLP receptor as a competitive antagonist of the chemotactic peptide fMLP. Since Marasco et al. have reported that SP binds to the fMLP receptor on rabbit neutrophils (102), these peptides may interact with the fMLP receptors which are expressed on the surface of Bt_2cAMP-differentiated HL-60 cells. BocMLF was also dissolved in DMSO for the reasons described above. BocMLF barely affected the basal $[Ca^{2+}]_i$ under the present experimental conditions. BocMLF at 100 μM completely inhibited the $[Ca^{2+}]_i$ increase stimulated by 10 nM fMLP (Fig. III-8). BocMLF (100 μM) potently inhibited the $[Ca^{2+}]_i$ increase stimulated by 10 μM FVFLM, but hardly affected that stimulated by 10 μM βA25-35-NH_2 (Fig. III-9). The increase in $[Ca^{2+}]_i$ induced by 10 μM SP was also significantly inhibited by BocMLF at 100 μM (Fig. III-9).

III-5 DISCUSSION

A disordered cytoplasmic Ca^{2+} homeostasis caused by β-amyloid peptides on neuronal cells has been shown by several investigations (82, 103, 104), because one common feature of some neurotoxins is the activation of cytoplasmic Ca^{2+} (80, 81). These findings suggested that the disrupted cytoplasmic Ca^{2+} homeostasis was caused by extracellular Ca^{2+} mobilization and that these effects were absent when the extracellular Ca^{2+} was chelated with excess EGTA. Since β-amyloid peptides are thought to directly interact with cell surface membrane and form ion channels (105-107), these Ca^{2+} responses in neuronal cells have been considered not to be mediated and regulated by a physiological pathway. This study revealed that βA25-35-NH_2 activates $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells in the presence of extracellular 4 mM EGTA, suggesting that the increase in $[Ca^{2+}]_i$ contains a mobilization from the intracellular calcium store. It is also suggested that the response may be

regulated by second messengers which can activate the intracellular calcium store. These results are different from those showing the mechanism of $[Ca^{2+}]_i$ mobilization induced by β-amyloid peptides on neuronal cells.

Protein kinase C (PKC) activation reportedly modulates the subsequent metabolism of inositol polyphosphate products as well as the phospholipase C (PLC) catalyzed breakdown of polyphosphoinositide substrates. PMA, a direct PKC activator, apparently inhibits the ATP and fMLP-induced $[Ca^{2+}]_i$ increase through inhibiting the inositol polyphosphate products at 100 nM [18]. As the $[Ca^{2+}]_i$ increase induced by 10 μM βA25-35-NH_2 was inhibited by PMA at concentrations of 1-100 nM, the $[Ca^{2+}]_i$ from the intracellular store may be associated with the activation of phosphoinositide turnover.

One mechanism of the $[Ca^{2+}]_i$ influx from the intracellular store is that mediated by the receptor recognized by IP_3 as a second messenger, which is the product of phosphoinositide hydrolysis catalyzed by PLC (108). In fact, βA25-35-NH_2 stimulated the production of inositol polyphosphates in a dose dependent manner. These results suggest that βA25-35-NH_2 follows a functional pathway to activate the Bt_2cAMP-differentiated HL-60 cells and that it is connected with phosphoinositide turnover.

PTX significantly attenuated the $[Ca^{2+}]_i$ increase stimulated by βA25-35-NH_2, SP and FVFLM. PTX-sensitive G-proteins couple the Ca^{2+} mobilizing receptors to phospholipase C activation in Bt_2cAMP-differentiated HL-60 cells (98, 100). Thus, this PTX inhibition suggests that the responses of peptides are regulated by one of these toxin sensitive G-proteins.

FVFLM, a ligand for the SEC-receptor, potently increased $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells. Hence, it has been assumed that FVFLM and βA25-35-NH_2 might stimulate $[Ca^{2+}]_i$ activation via the same mechanism such as SEC-receptor mediation. In this study, the effect of the chemotactic peptide antagonist BocMLF was investigated upon the $[Ca^{2+}]_i$ increase induced by FVFLM and βA25-35-NH_2. BocMLF at 100 μM did not prevent the βA25-

35-NH_2-induced $[Ca^{2+}]_i$ increase, suggesting that this response is not mediated by the fMLP receptor. However, BocMLF potently inhibited the $[Ca^{2+}]_i$ increase induced by 10 μM FVFLM. Since BocMLF can block the calcitonin gene-related peptide (CGRP)-mediated effects in human neutrophils (96), we can not conclude from only this finding that FVFLM interacts with the fMLP receptor. In addition, SEC-receptor is reportedly distinct from the fMLP receptor (91). As a result, it is suggested that βA25-35-NH_2 and FVFLM follow a distinct pathway to increase $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells.

SP is a mediator of neurogenic inflammation and it activates various cell types involved in inflammatory and immune processes. It has been reported that SP can directly activate human neutrophils at a micromolar concentration range (109). Likewise, we showed here that SP directly stimulated the $[Ca^{2+}]_i$ increase in neutrophil-like HL-60 cells at concentrations above 1 μM. The action of SP was considered not to be mediated by the NK-1 tachykinin receptor because the expression of this response required a higher concentration range of SP than other conventional function of SP, such as contractile activity on smooth muscles (110) and $[Ca^{2+}]_i$ activation on the rat pancreatic acinar cell line AR 4-2J cells (111). It was demonstrated that βA25-35-NH_2 mimics the function of SP in inhibiting the nicotinic response in PC12h cells that is thought not to be mediated by the NK-1 receptor (Chapter II). βA25-35-NH_2 and SP also similarly activated $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells. Thus, we suppose that βA25-35-NH_2 mimics the effect of SP in these cells via the same mechanism that is thought not to be mediated by the NK-1 receptor, although BocMLF inhibited nearly 35 % of the SP-induced $[Ca^{2+}]_i$ increase.

To summarize, βA25-35-NH_2, FVFLM and SP induced $[Ca^{2+}]_i$ activation in neutrophil-like differentiated HL-60 cells, and it was regulated by a PTX-sensitive G-protein. Although these peptides are known as SEC-receptor ligands and are assumed to activate $[Ca^{2+}]_i$ via the same mechanism as SEC-receptor mediation, BocMLF, a chemotactic peptide antagonist, showed different effects

on the $[Ca^{2+}]_i$ increase induced by each peptide. The results raise the notion that βA25-35-NH_2 action is mediated by a receptor which is not the SEC-receptor in Bt_2cAMP-differentiated HL-60 cells. It would also be of interest to determine the interaction site of the active β-amyloid peptide fragment.

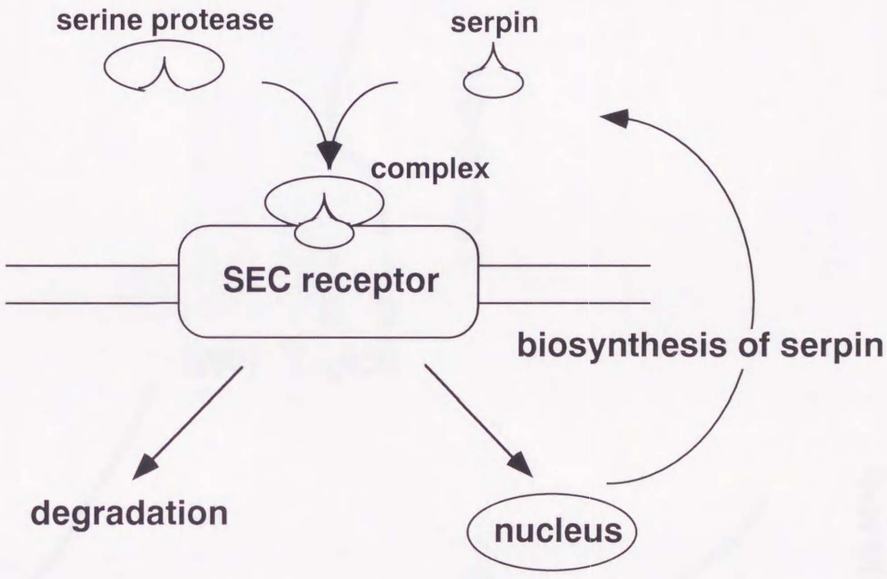

Fig. III-1. **Schematic representation of the function mediated by the SEC receptor.**

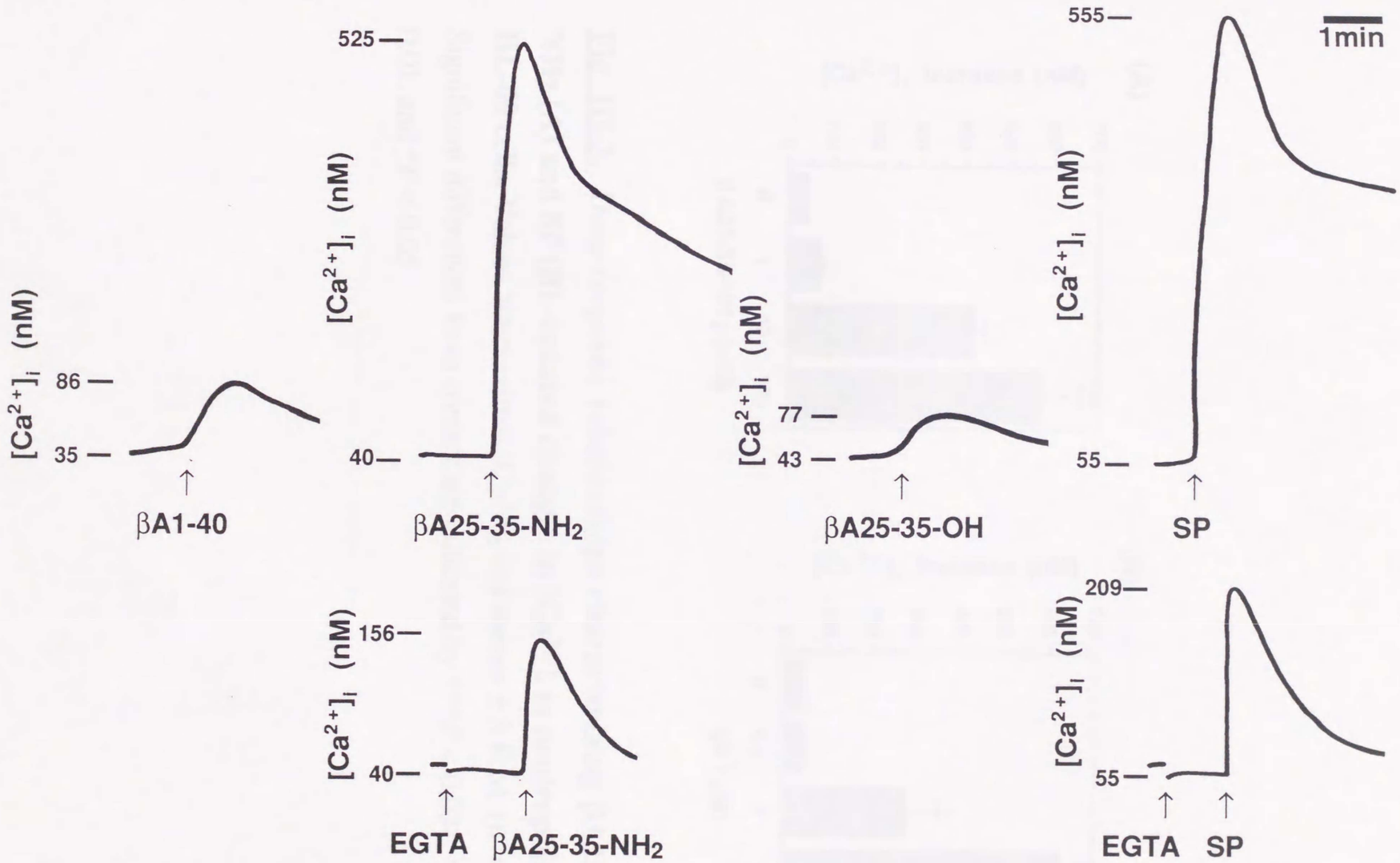

Fig. III-2. The effects of β-amyloid peptides and SP on $[Ca^{2+}]_i$ in neutrophil-like HL-60 cells. 10 μM βA25-35-NH_2 and SP increased the $[Ca^{2+}]_i$ in the absence (upper) and presence (lower) of extracellular 4 mM EGTA. 10 μM βA1-40 and βA25-35-OH weakly increased the $[Ca^{2+}]_i$ in these cells.

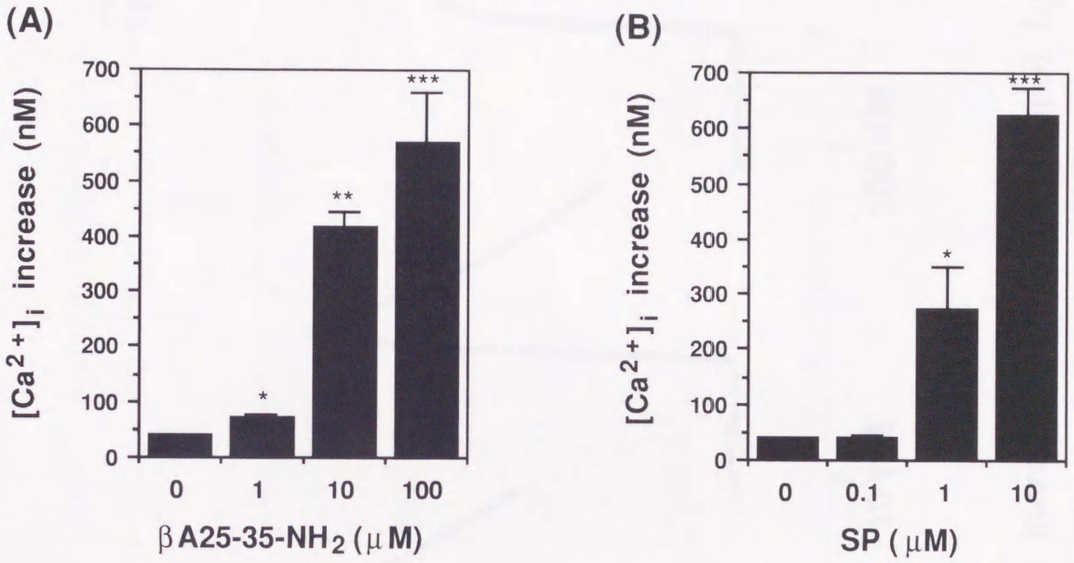

Fig. III-3. Dose-response relationships characterizing βA25-35-NH_2 (A) and SP (B) -induced changes in $[Ca^{2+}]_i$ in neutrophil-like HL-60 cells. Values are maximal $[Ca^{2+}]_i$ and means ± S.E.M. (n = 3-5). Significant differences from control are indicated by ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

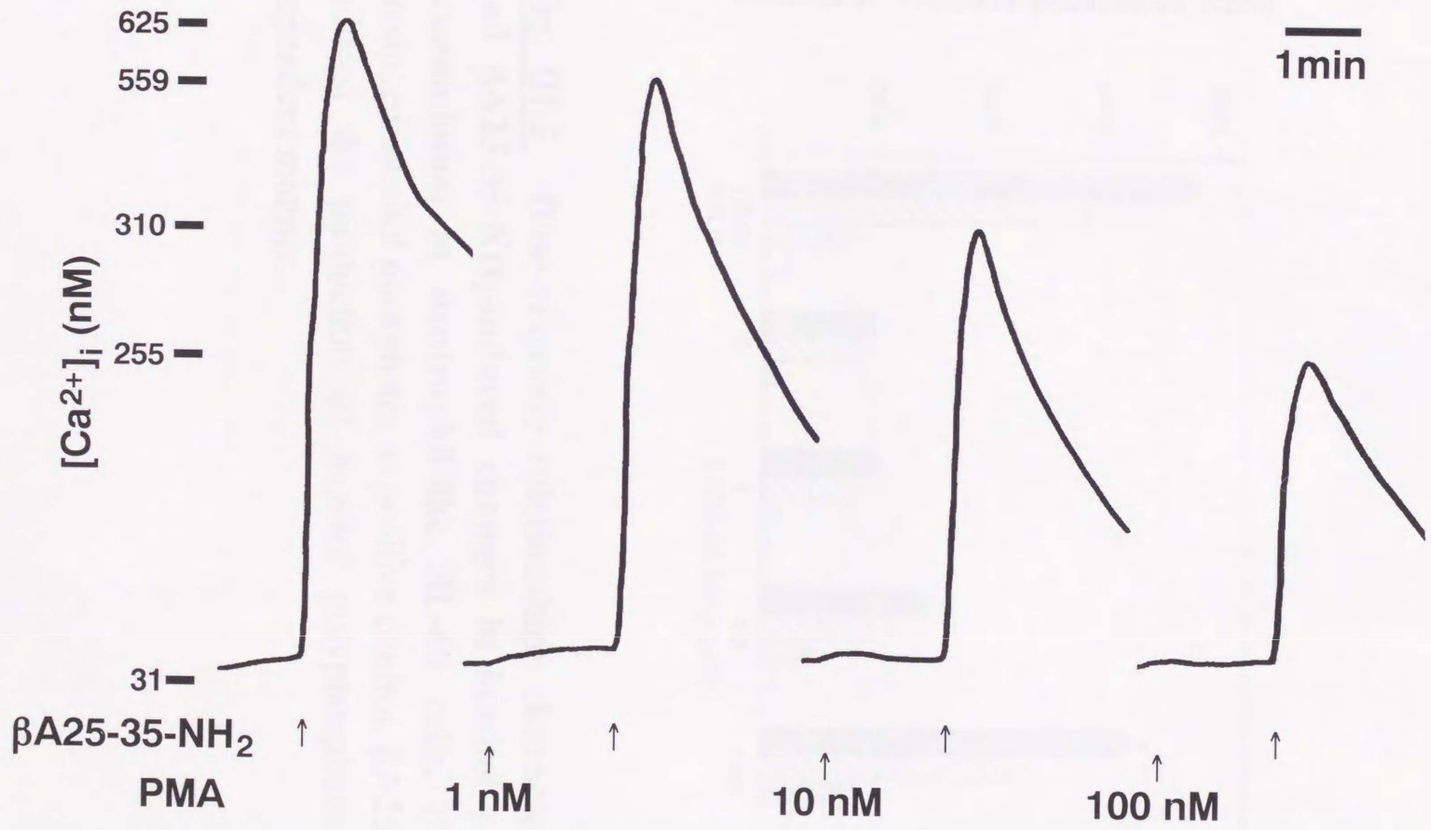

Fig. III-4. Inhibitory effects of PMA on the increase in $[Ca^{2+}]_i$ induced by βA25-35-NH_2 in neutrophil-like HL-60 cells. PMA inhibited the 10 μM βA25-35-NH_2-induced $[Ca^{2+}]_i$ increase in a dose-dependent manner.

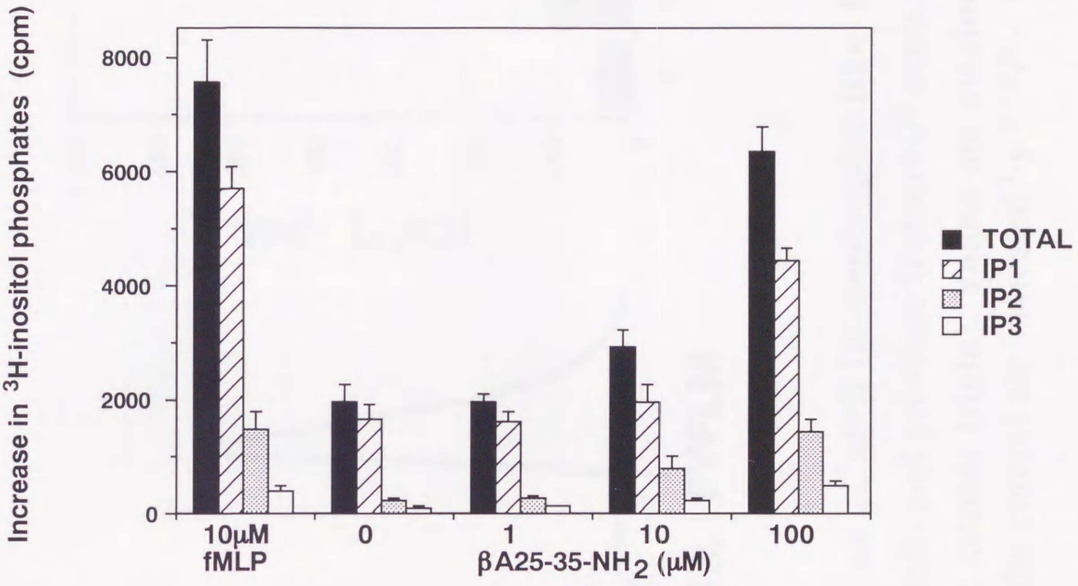

Fig. III-5. Dose-response relationships characterizing fMLP and βA25-35-NH$_2$-induced changes in inositol polyphosphate accumulation in neutrophil-like HL-60 cells. 10 μM fMLP produced inositol phosphates as positive control. βA25-35-NH$_2$ also induced the production of inositol polyphosphates in a dose-dependent manner.

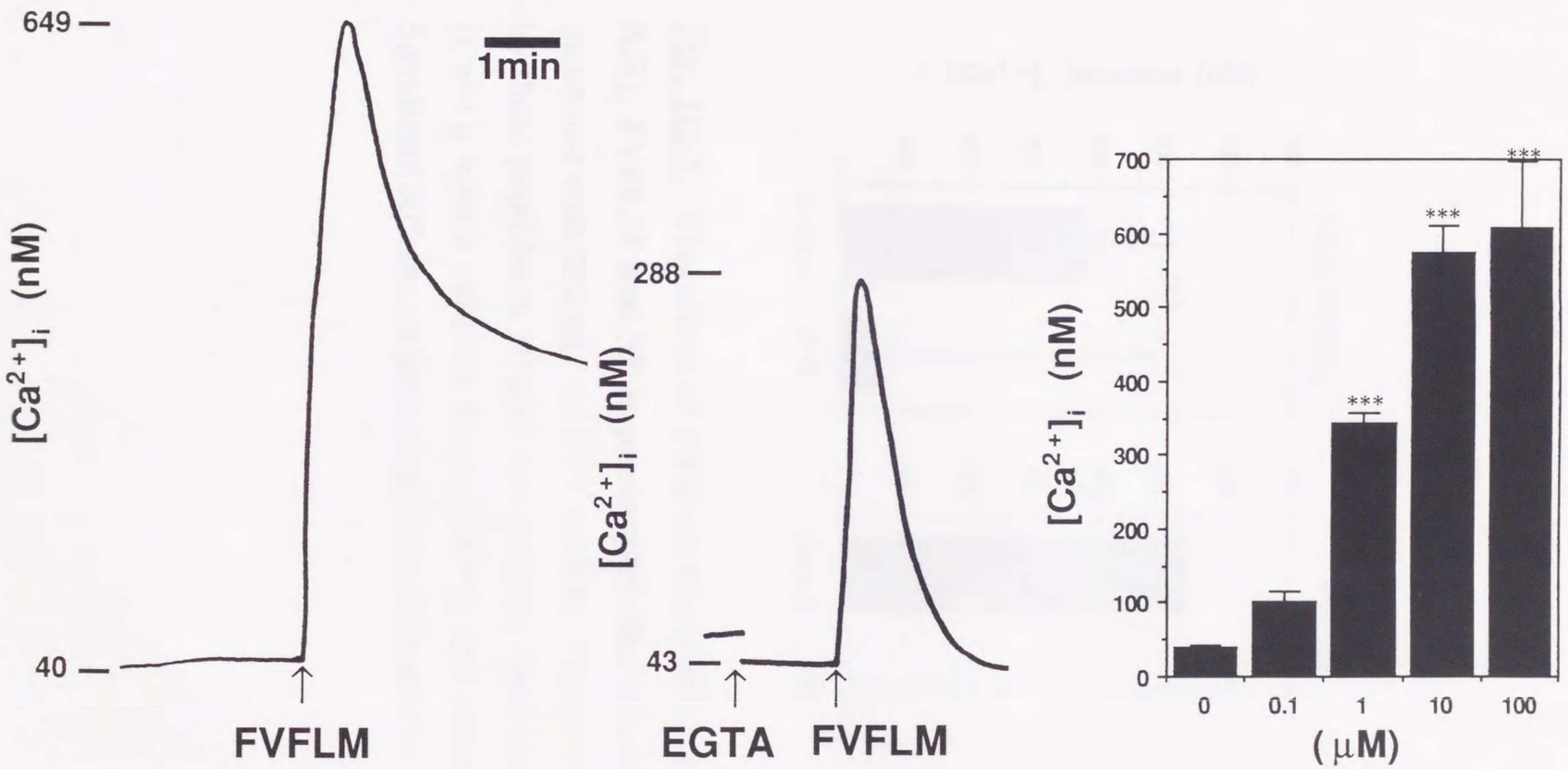

Fig. III-6. The effect of FVFLM peptide on $[Ca^{2+}]_i$ in neutrophil-like HL-60 cells. 10 μM FVFLM increased the $[Ca^{2+}]_i$ in the absence (left) and presence (center) of extracellular 4 mM EGTA. The $[Ca^{2+}]_i$ was increased in a dose-dependent manner (right). Values are maximal $[Ca^{2+}]_i$ and means ±S.E.M. (n = 3-5). Significant differences from the control are indicated by ***$P < 0.001$.

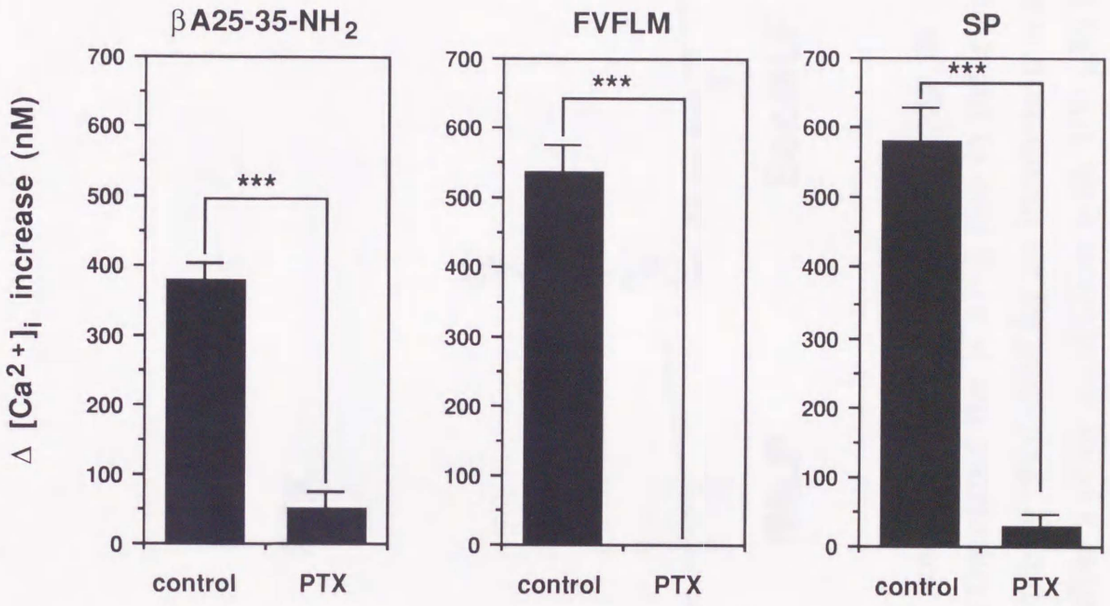

Fig. III-7. The effect of PTX on the $[Ca^{2+}]_i$ response to βA25-35-NH_2, FVFLM and SP in neutrophil-like HL-60 cells. The cells were incubated with 200 ng / ml PTX for 4 hr. The increase in $[Ca^{2+}]_i$ induced by these peptides at 10 μM was potently inhibited. Values are maximal $[Ca^{2+}]_i$ which subtract basal $[Ca^{2+}]_i$ and means ±S.E.M. (n = 3-5). Significant differences from control are indicated by $^{***}P < 0.001$.

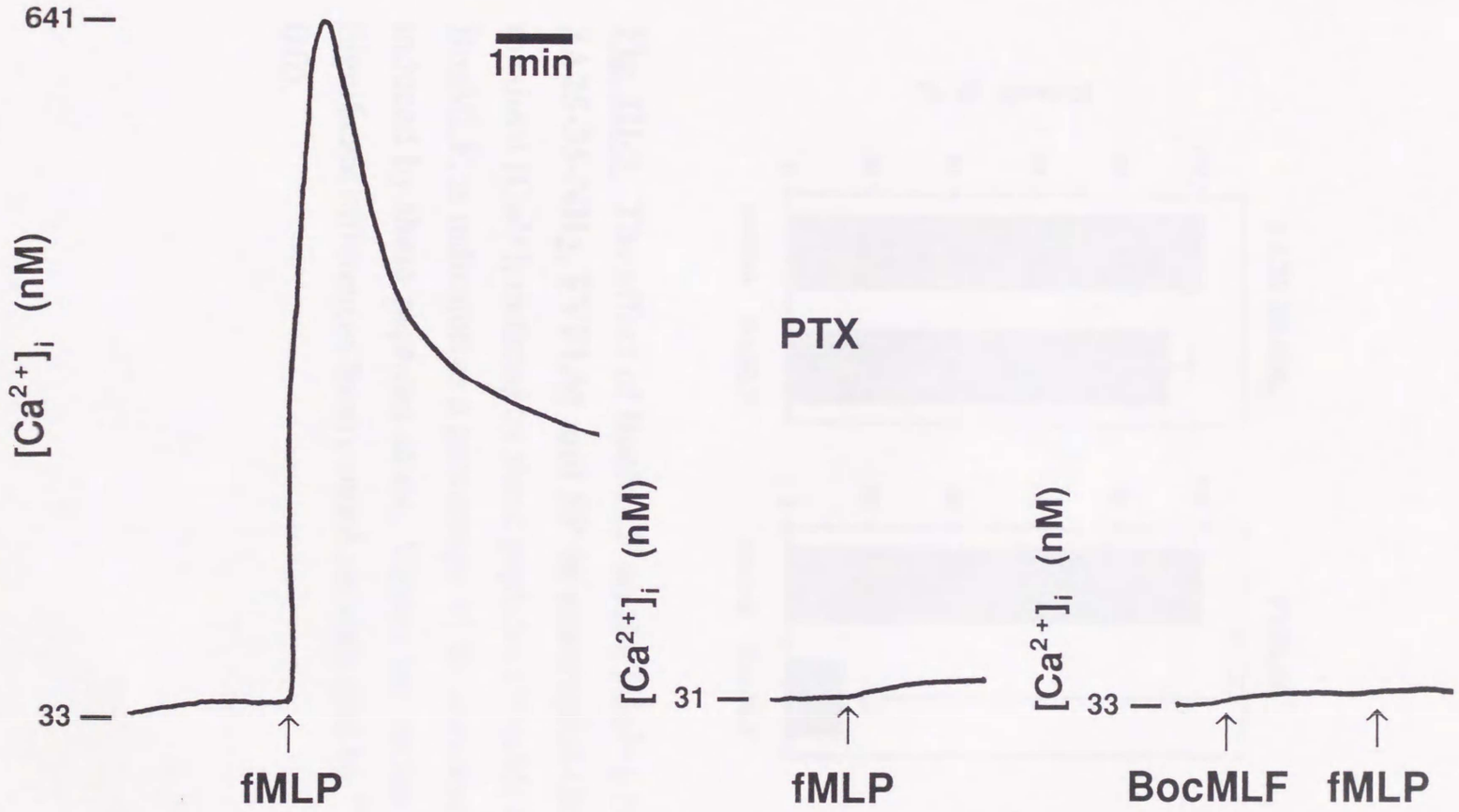

Fig. III-8. The effect of a prior exposure to PTX and BocMLF on the change in $[Ca^{2+}]_i$ caused by fMLP in neutrophil-like HL-60 cells. fMLP potently stimulated the $[Ca^{2+}]_i$ rise in these cells at 10 nM (left). The increase in $[Ca^{2+}]_i$ caused by 10 nM fMLP was completly abolished by an incubation with 200 ng / ml PTX for 4 hr (center). Also, the $[Ca^{2+}]_i$ response disappeared after a prior incubation with 100 μM BocMLF (right).

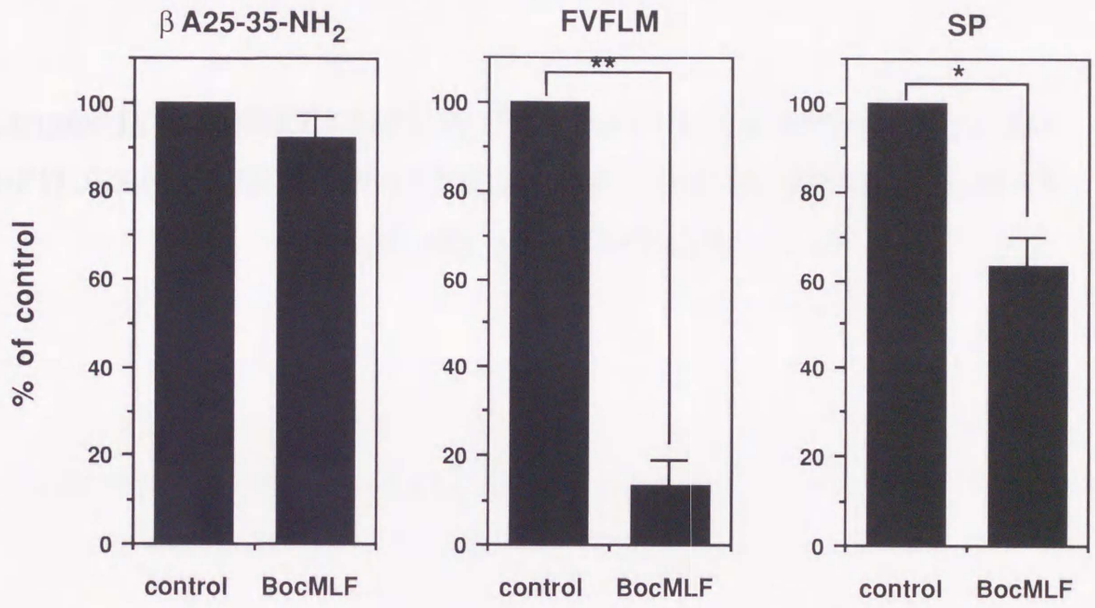

Fig. III-9. The effect of BocMLF on the $[Ca^{2+}]_i$ increase induced by βA25-35-NH_2, FVFLM and SP in neutrophil-like HL-60 cells. The maximal $[Ca^{2+}]_i$ induced by these peptides (10 μM) after adding 100 μM BocMLF, is indicated as a percentage of the maximal increase in $[Ca^{2+}]_i$ induced by these peptides alone. Values are means ±S.E.M. (n = 3-5). Significant differences from control are indicated by $^{**}P < 0.001$ and $^{*}P < 0.05$.

Chapter IV TROPHIC EFFECTS OF SUBSTANCE P AND β-AMYLOID PEPTIDES ON DIBUTYRYL CYCLIC AMP-DIFFERENTIATED HUMAN LEUKEMIC (HL-60) CELLS

IV-1 ABSTRACT

The neuropeptide SP is thought to be a mediator of neurogenic inflammation. Also, β-amyloid peptides can directly activate the cell types involved in inflammatory processes, such as neutrophils and macrophages. The relationship between SP and β-amyloid peptides in terms of their biological actions has been thoroughly studied. SP is trophic in neuronal cells and protects them against the death induced by βAP *in vivo*. In this study, we examined the effects of SP and β-amyloid peptides on cell viability in neutrophil-like differentiated HL-60 cells, by means of the WST-1 tetrazolium and lactate dehydrogenase (LDH) release assays. The results showed that SP promoted the cell survival on neutrophil-like HL-60 cells under serum-free conditions. Also, β-amyloid peptide showed trophic, rather than toxic effects in these cells in WST-1 assay, although it is reportedly toxic in neuronal cells.

IV-2 INTRODUCTION

SP is widely distributed throughout the central and peripheral nervous systems, and it is regarded as a neurotransmitter of primary sensory neurons. In addition, SP is a mediator of neurogenic inflammation and it activates various cell types associated with inflammatory and immune processes. SP can directly activate human neutrophils at a micromolar concentration range (109), and potentiate the stimulation induced by the chemotactic peptide fMLP and the complement fraction C5a at nanomolar concentrations (112).

The neuropeptide SP may have putative roles not only as a neurotransmitter or neuromodulator but also as a neurotrophic factor in cultured neuronal cells. SP reportedly induces neurite outgrowth in cultured neuroblastoma cells (113) and promotes neuronal survival on hippocampal neurons *in vitro* (114). Furthermore, the administration of SP protects against

neuronal cell death caused by βAP, which is one of the main components of senile plaques in AD, in the adult rat brain *in vivo* (55). Although SP is thought to be one of the mediators in inflammation, little is known about the effect of SP on the viability of the cells involved in the inflammatory response.

βAP also directly activates cell types such as human neutrophils (87) and rat peritoneal macrophages (88). As SP and βAP closely interact to effect on their biological activities, the biological actions of SP and β-amyloid peptides should be compared.

In this chapter, we describe the effects of SP and β-amyloid peptides upon the viability of human leukemic (HL-60) cells differentiated by dibutyryl cyclic AMP (Bt_2cAMP) under serum-free conditions, determined by means of WST-1 tetrazolium (115) and lactate dehydrogenase (LDH) release assays. The results showed that SP promoted differentiated HL-60 cells survival under our experimental conditions. Also, β-amyloid peptides showed trophic, rather than toxic effects in these cells in the WST-1 assay.

IV-3 MATERIALS AND METHODS

IV-3-1 Cell culture

HL-60 cells were routinely cultured as described in Chapter III.

IV-3-2 Peptide synthesis

SP and β-amyloid peptides were manually synthesized in a conventional glass vessel using the solid-phase methodology described in Chapter II.

IV-3-3 Survival assay

The differentiated cells were washed with serum and Bt_2cAMP-free RPMI 1640 medium, and plated on 96 well plates (Nunc) at a density of 1×10^5 cells / well in the same medium in all except peripheral wells. Thereafter, 10 μl of sample peptides or Bt_2cAMP was added. After a 24 hr incubation at 37 °C, the surviving cells were assessed by means of the WST-1 assay. Ten microliters of WST-1 solution (1 mM WST-1 and 0.2 mM 1-methoxy-5-methylphenazinium methylsufate), a new tetrazolium compound, was added and incubated for a further 4 hr. The absorbance was then measured with ELISA plate reader (Corona electric) at a wavelength of 415 nm. Likewise, after a 24 hr incubation in serum-free medium in the presence of samples, LDH activity in the supernatant of the cultured medium was evaluated using lithium lactate as a substrate and oxidized β-diphosphopyridine nucleotide (βDPN). Briefly, 10 μl of cultured supernatant was added to 500 μl of substrate-βDPN buffer (114 mM lithium lactate, 667 mM 2-amino-2-methyl-1-propanol, 6.25 mM βDPN, pH 9.0) and incubated for 30 min at 37 °C. After placing on ice, the absorbance was measured at a wavelength of 340 nm (JASCO spectrophotometer). Moreover, viable cells were directly counted by Trypan blue exclusion using a Haemacytometer after the 24 hr incubation.

IV-3-4 Superoxide measurement

Generation of superoxide anion (O_2^-) was assessed as the superoxide disumutase (SOD)-inhibitable reduction of ferricytochrome *c*. Differentiated HL-60 cells (3.0×10^6 / 80 μl) were suspended in buffer A (BSA free). After an incubation for 5 min at 37 °C, 10 μl of ferricytochrome *c* (42 mg / ml) and peptides were added and the cells were incubated for a further 10 min. The reaction was stopped with SOD (10 μl of 10 units / ml) and the cells were placed on ice. After centrifugation at 4 °C, the absorbance in 90 μl of

supernatants was measured with an ELISA plate reader (Colona electric) at a wavelength of 550 nm.

IV-3-5 Materials

WST-1 and 1-methoxy-5-methylphenazinium methylsufate were purchased from DOJINDO (Kumamoto, Japan). Lithium lactate and βDPN were purchased from Tokyo Kasei, Ltd. (Tokyo, Japan). SOD and ferricytochrome *c* were from Wako (Osaka, Japan).

IV-4 RESULTS

IV-4-1 WST-1 tetrazolium survival assay

In the WST-1 assay, the viable cell number is reflected by the absorbance because this tetrazolium compound is thought to be mainly cleaved by various dehydrogenase enzymes in mitochondria of living cells and it produces a water-soluble formazan that is detected by absorption at a wavelength of 415 nm (115). A high percentage of the Bt_2cAMP-differentiated HL-60 cells died after a 24 hr incubation in serum and Bt_2cAMP-free medium (absorbance of the control was 0.210 ± 0.017), since the initial absorbance was 0.844±0.049 when the cells were seeded. Figure IV-1 shows the effects of fMLP, SP, β-amyloid peptides and Bt_2cAMP on the cell death caused by serum-starvation using the WST-1 tetrazolium assay. In the presence of 500 μM Bt_2cAMP, the number of viable cells potently increased. The absorbance was significantly increased in the presence of 10 μM SP and βA1-40 as compared with the control, raising the notion that these peptides promote the cell survival. The effects of βA25-35-NH_2 and βA25-35-OH were weaker than βA1-40 (Fig. IV-1). The trophic effects of SP and βA1-40 in WST-1 assay were dose-dependent (Fig. IV-2). The

effect was not seen upon adding 10 μM fMLP, a potent activating peptide of differentiated HL-60 cells (Fig. IV-1). These effects were confirmed to be absent on undifferentiated HL-60 cells (Fig. IV-3).

IV-4-2 LDH assay and direct cell counting

We further examined the trophic effects of the peptides by means of the LDH-release assay and direct cell counting. LDH, a cytosolic marker enzyme, accumulates in culture medium as the cells die. Bt_2cAMP (500 μM) potently decreased LDH activity in the supernatant of cultured medium (Fig. IV-4 A) and over 2 fold more cells survived as compared with the control (Fig. IV-4 B). LDH activity was significantly decreased by 10 μM SP (Fig. IV-4 A), indicating that SP prevented differentiated HL-60 cell death. Also, the number of viable cells significantly increased in the presence of 10 μM SP (Fig. IV-4 B). These findings were in agreement with the results of the WST-1 assay. However, the differences were not significant between the cells cultured with or without 10 μM βA1-40 in both experiments (Fig. IV-4 A, B).

IV-4-3 The effects of peptides on cell functions

We next examined the change in superoxide anion (O_2^-) production to understand the function of SP and β-amyloid peptides in differentiated HL-60 cells. SP transiently increased $[Ca^{2+}]_i$ at 10 μM, but the $[Ca^{2+}]_i$ activation induced by βA1-40 was much weaker (Fig. III-2). fMLP has been reported to induce O_2^- production in differentiated HL-60 cells (116). Although 10 μM fMLP potently stimulated O_2^- production under our experimental conditions, SP and βA1-40 had no effect on O_2^- production (Fig. IV-5).

IV-5 DISCUSSION

In human neutrophils, inflammatory mediators such as endotoxic lipopolysaccharide, human recombinant C5a and human recombinant granulocyte-macrophage colony-stimulating factor markedly inhibit the rate of neutrophil apoptosis and prolong the functional life span of neutrophils (117). SP is also a mediator of neurogenic inflammation. Although we did not determine whether or not the differentiated HL-60 cell death induced by the serum-starvation is due to apoptosis, our results raise the possibility that SP as well as other mediators can prolong the life span of neutrophil-like cells to maintain their functions.

The cytotoxic effects of β-amyloid peptides are characterized by the reduction of redox activity as measured by the MTT tetrazolium assay on PC12 or neuronal cells (118). However, β-amyloid peptides showed a trophic effect on differentiated HL-60 cells in the WST-1 assay. As significant differences were not evident in the LDH assay and direct cell counting after exposure to βA1-40, we suppose that βA1-40 promotes or maintains the differentiation state of HL-60 cells, since the absorbance in the WST-1 assay increased along with the HL-60 cell differentiation.

SP increases $[Ca^{2+}]_i$ and activates phosphoinositide turnover as well as the secretion in human neutrophils (109). In Chapter III, we confirmed that SP markedly increases the $[Ca^{2+}]_i$ at 10 μM in Bt_2cAMP-differentiated HL-60 cells. It is suggested that the transient increase in $[Ca^{2+}]_i$ induced by SP may have an important role in preventing the differentiated HL-60 cell death caused by serum-starvation. Although the chemotactic peptide fMLP potently increased the $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells (Fig. III-8), we could not identify measurable cell survival activity induced by fMLP at 10 μM in the WST-1 assay. Since 10 μM fMLP potently induced O_2^- production, the free radical might be toxic to the cells under our experimental conditions.

In summary, SP promoted the survival of neutrophil-like differentiated HL-60 cells under serum-free conditions. The results suggested that SP may have functional roles not only as a mediator of inflammation but also as a trophic factor in the cell types involved in inflammatory processes. Furthermore, β-amyloid peptides exerted trophic, rather than toxic effects in these cells in the WST-1 assay, though it is reportedly toxic to neuronal cells.

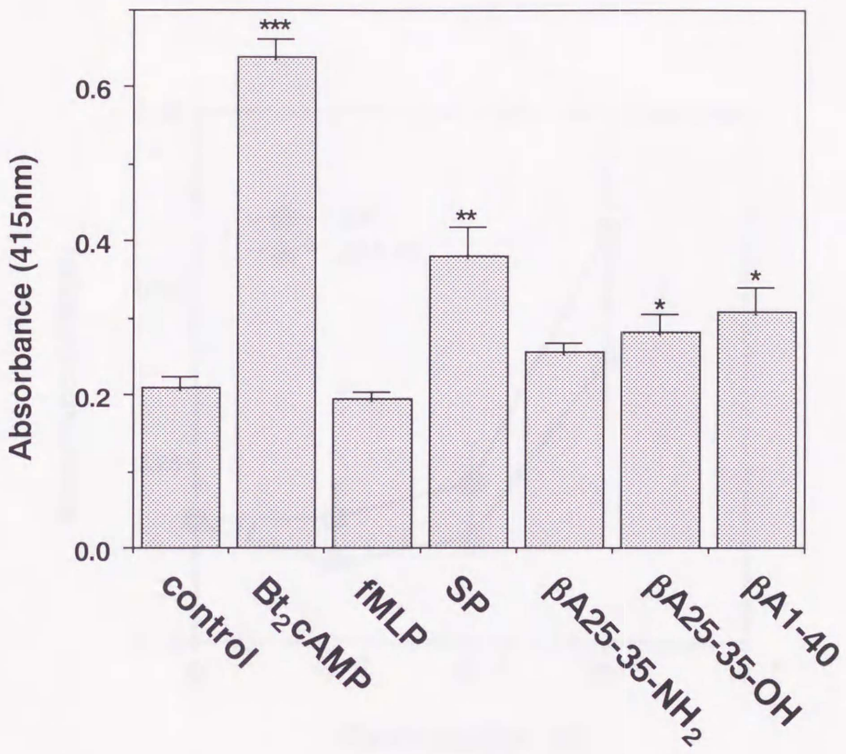

Fig. IV-1. The effects of Bt_2cAMP, fMLP, SP and β-amyloid peptides on neutrophil-like HL-60 cell viability, measured by the WST-1 assay. The tetrazolium assay was performed after a 24 hr incubation with 500 μM Bt_2cAMP and 10 μM peptides under serum-free conditions. Values are means ±S.E.M. (n = 4-5). Significant differences from control are indicated by $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$.

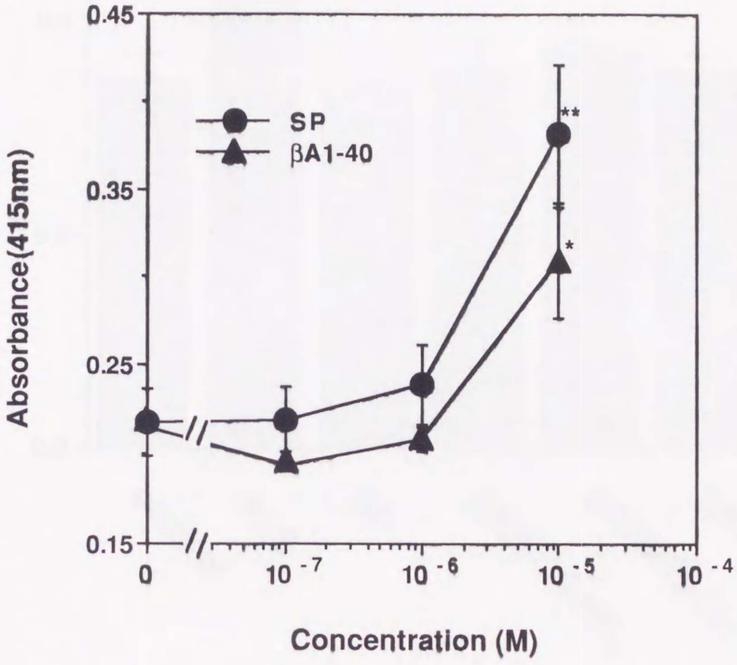

Fig. IV-2. Concentration dependence of the trophic effects of SP and βA1-40 in neutrophil-like HL-60 cells measured by the WST-1 assay. The tetrazolium assay was performed after a 24 hr incubation with these peptides under serum-free condition. Values are means ±S.E.M. (n = 3-5). Significant differences are indicated by $^{**}P < 0.01$ and $^{*}P < 0.05$.

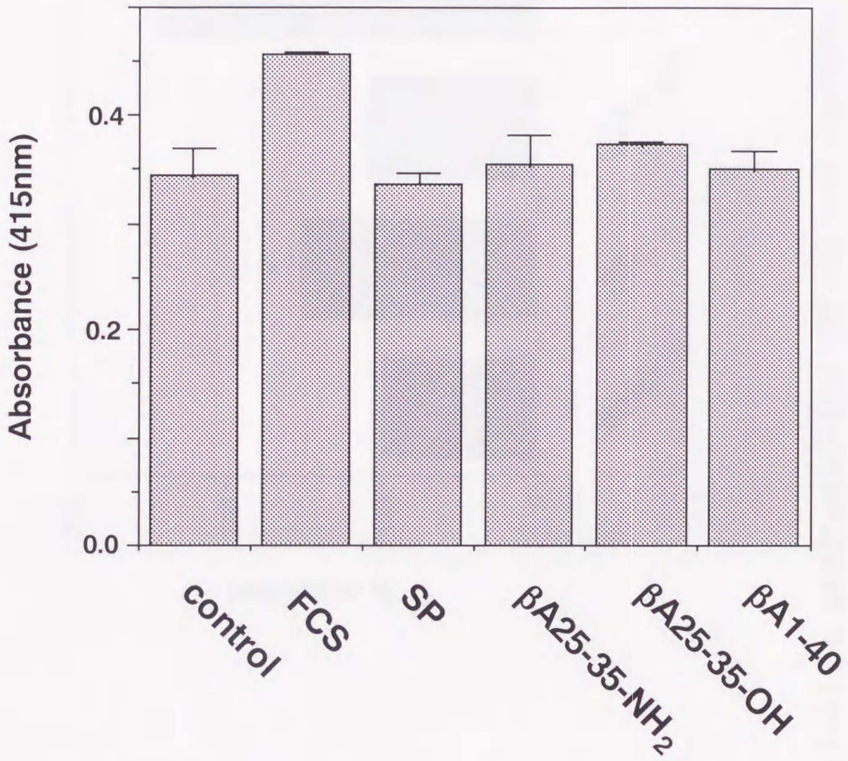

Fig. IV-3. The effects of FCS, SP and β-amyloid peptides on undefferentiated HL-60 cell viability, measured by the WST-1 assay. The tetrazolium assay was performed after a 24 hr incubation with 10 % FCS and 10 μM peptides under serum-free conditions. Values are means±S.E.M. (n = 3).

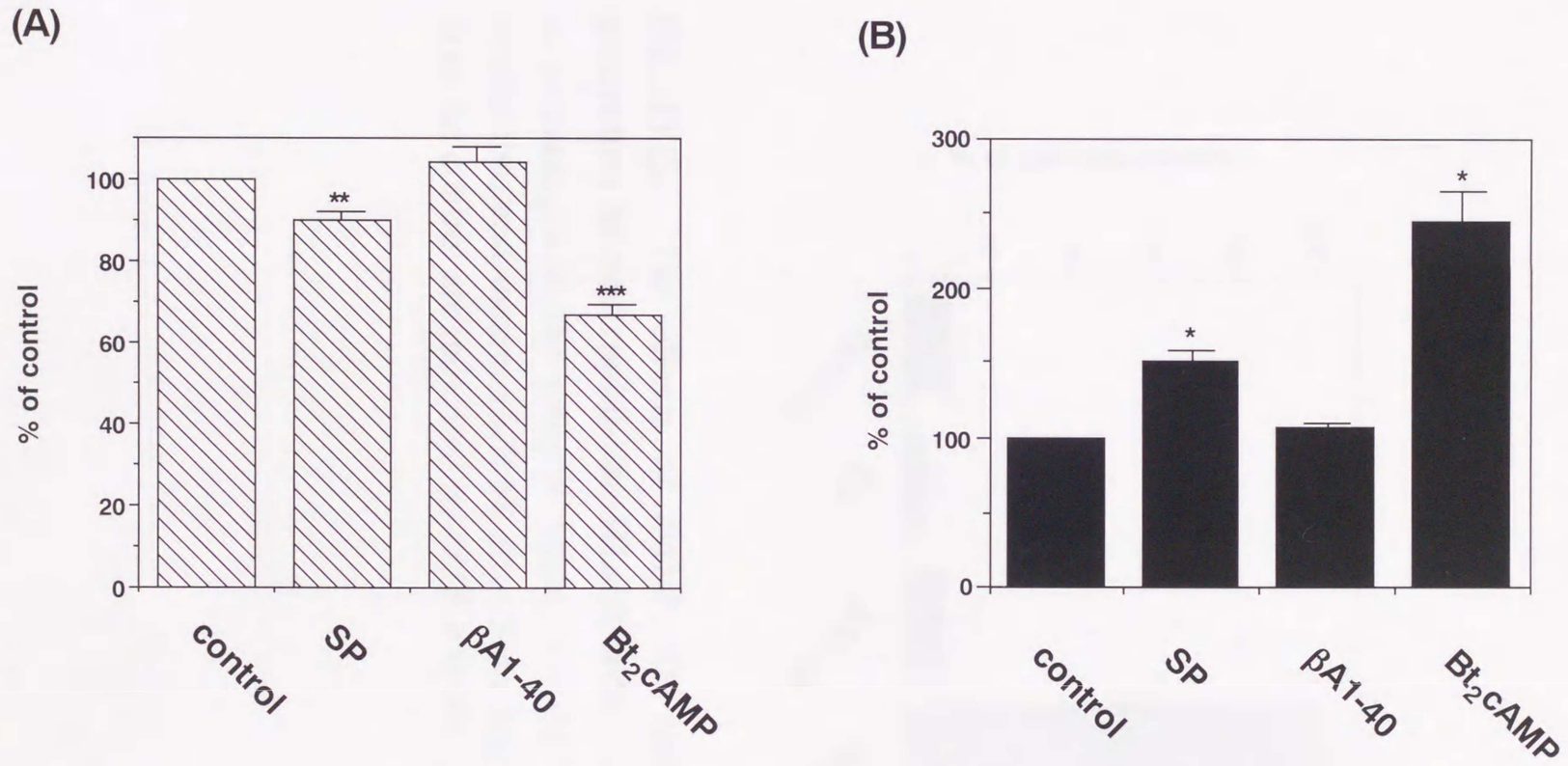

Fig. IV-4. The effects of Bt_2cAMP, SP and βA1-40 on neutrophil-like HL-60 cell viability, evaluated by the LDH release assay (A) and direct cell counting (B). Both assays were performed after a 24 hr incubation with 500 μM Bt_2cAMP and 10 μM peptides under serum-free condition. Values are indicated as a percentage of the control and means ±S.E.M. (n = 5-7 for LDH assay, and n = 3 for cell counting). Significant differences from the control are indicated by $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$.

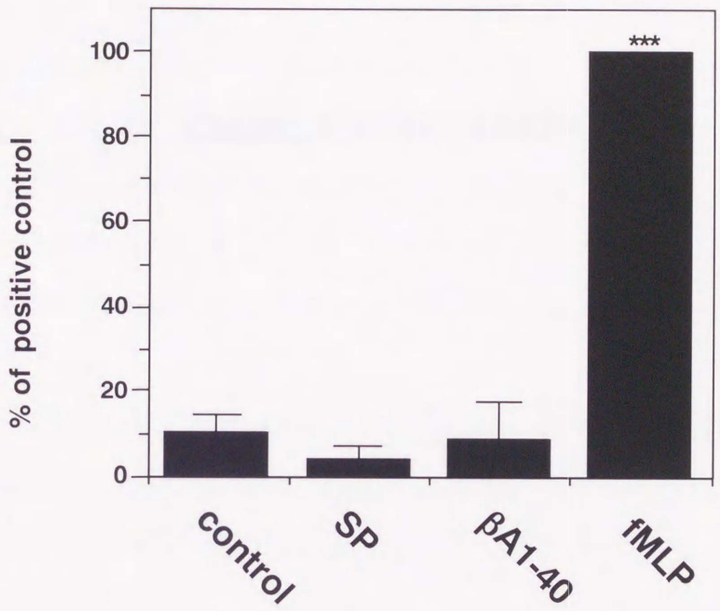

Fig. IV-5. The effects of fMLP, SP and βA1-40 on O_2^- generation in neutrophil-like HL-60 cells. Values are indicated as percentages of the positive value, 10 μM fMLP-induced O_2^- production, and means ±S.E.M. (n = 3-4). Significant differences from the control are indicated by $^{***}P < 0.001$.

Chapter V CONCLUSION

Conclusion

It was thought after the study of Yankner et al. (23) that SP would be important in understanding the physiological or pathological features of βAP. Including the present thesis, several findings have shown that β-amyloid peptides might exert their effects through the same mechanism as SP. Some reports suggest that the conventional SP receptor (NK-1 receptor) mediates the biological actions of β-amyloid peptides (119, 120). However, we identified other effects of SP which were not exerted via the NK-1 receptor, because β-amyloid peptides initially have been thought not to directly interact with the receptor.

At first, we examined the modulatory action of β-amyloid peptides on the nicotinic response , since the effect of SP is reportedly not mediated by the NK-1 receptor. βA1-40 and βA25-35-NH_2 mimicked the inhibitory activity of SP on the nicotine-induced calcium influx in PC12h cells with a similar potency. The other mammalian tachykinins, NKA and NKB, did not show the effect at the same concentration range, indicating that the biological activity is comparatively specific for the SP and β-amyloid peptides. Cholinergic neurons are remarkably degenerated in the AD brain, and the binding ability of acetylcholine with the nicotinic receptor is reduced. On the basis of the results in Chapter II, we suppose that βAP directly affects the nicotinic cholinergic system in terms of pathophygiological function.

SP is a mediator of neurogenic inflammation and it directly activates the various cell types that are seen at sites of inflammation / tissue injury. Since senile plaques in the AD brain accompany the inflammatory response and βAP reportedly activates the neutrophils, macrophages and microglia which is resident macrophages in the central nervous system, understanding the activation mechanism may help unravel the pathogenesis of AD. As described in Chapter III, it was confirmed that β-amyloid peptides activated $[Ca^{2+}]_i$ in

Bt_2cAMP-differentiated HL-60 cells as a neutrophils model. Further investigation indicated that the β-amyloid peptides are associated with physiological pathways in differentiated HL-60 cells, and so these cells may be useful to analyze the putative inflammatory function of β-amyloid peptides.

The toxic effect of β-amyloid peptides against neuronal cells is most noteworthy feature to directly related to the induction of neurodegeneration in AD brain. The MTT tetrazolium survival assay has been frequently used to assess the toxicity because β-amyloid neurotoxicity particularly disorders redox activity against MTT cleavage. With respect to βA25-35 toxicity, we confirmed that βA25-35-OH was remarkably toxic to PC12h cells as measured by the MTT assay, but the C-terminal amide analoge was not, because βA25-35-OH was tightly aggregated, as opposed to the soluble βA25-35-NH_2 (Fig. V-1). However, in Chapter IV, we described that β-amyloid peptides were trophic rather than toxic in differentiated HL-60 cells. βAP may help prolong the life span of cells involved in the inflammatory response as distinguished from the effects of neurons.

Various biological actions of β-amyloid peptides have been reported and there is interest in their association with AD pathology. βA1-40 and βA1-42 have been examined as full length βAP. Also, several fragment peptides are found to be bioactive regions of βAP. βA25-35 peptide is the most thoroughly examined as a functional region of βAP. βA1-28 appeared to have neurotrophic activity because it enhanced neuronal survival in vitro (121), and βA22-35 is described as a neurotoxic region (122). Although the biological functions induced by these peptides did not always directly connect with AD, a detailed investigation of β-amyloid peptides should provide essential information in understanding the pathogenesis of AD.

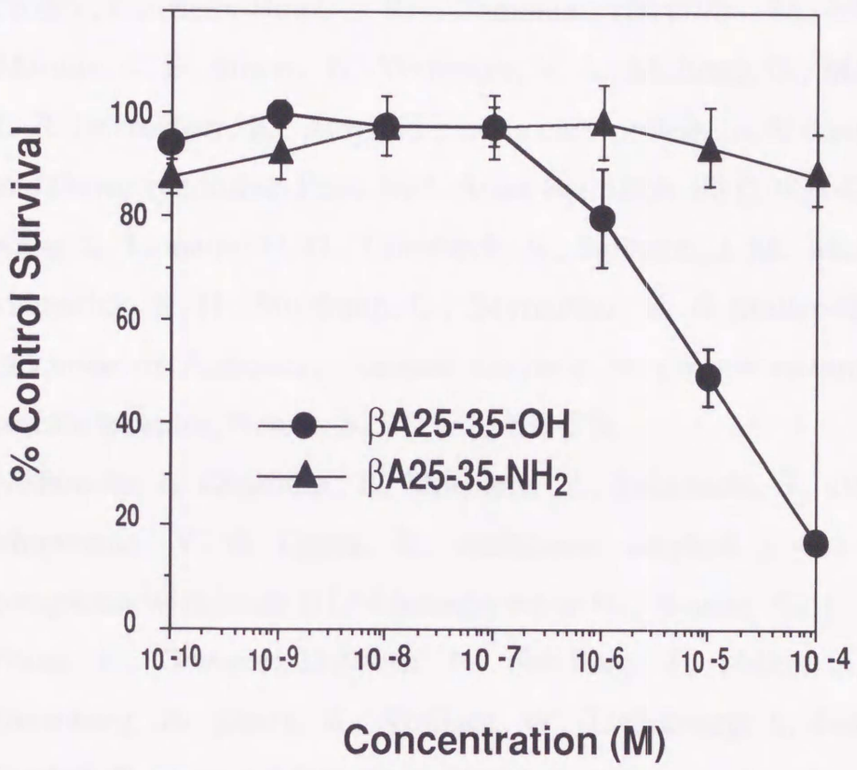

Fig. V-1. Concentration dependence of the effects of βA25-35-NH_2 and βA25-35-OH in PC12h cells assessed by the MTT assay. The tetrazolium assay was performed after a 48 hr incubation with these peptides under serum-free conditions. Values are means ±S.E.M. (n = 4-5). The C-terminal amide analogue was toxic, but the C-terminal carboxyl free analogue was not.

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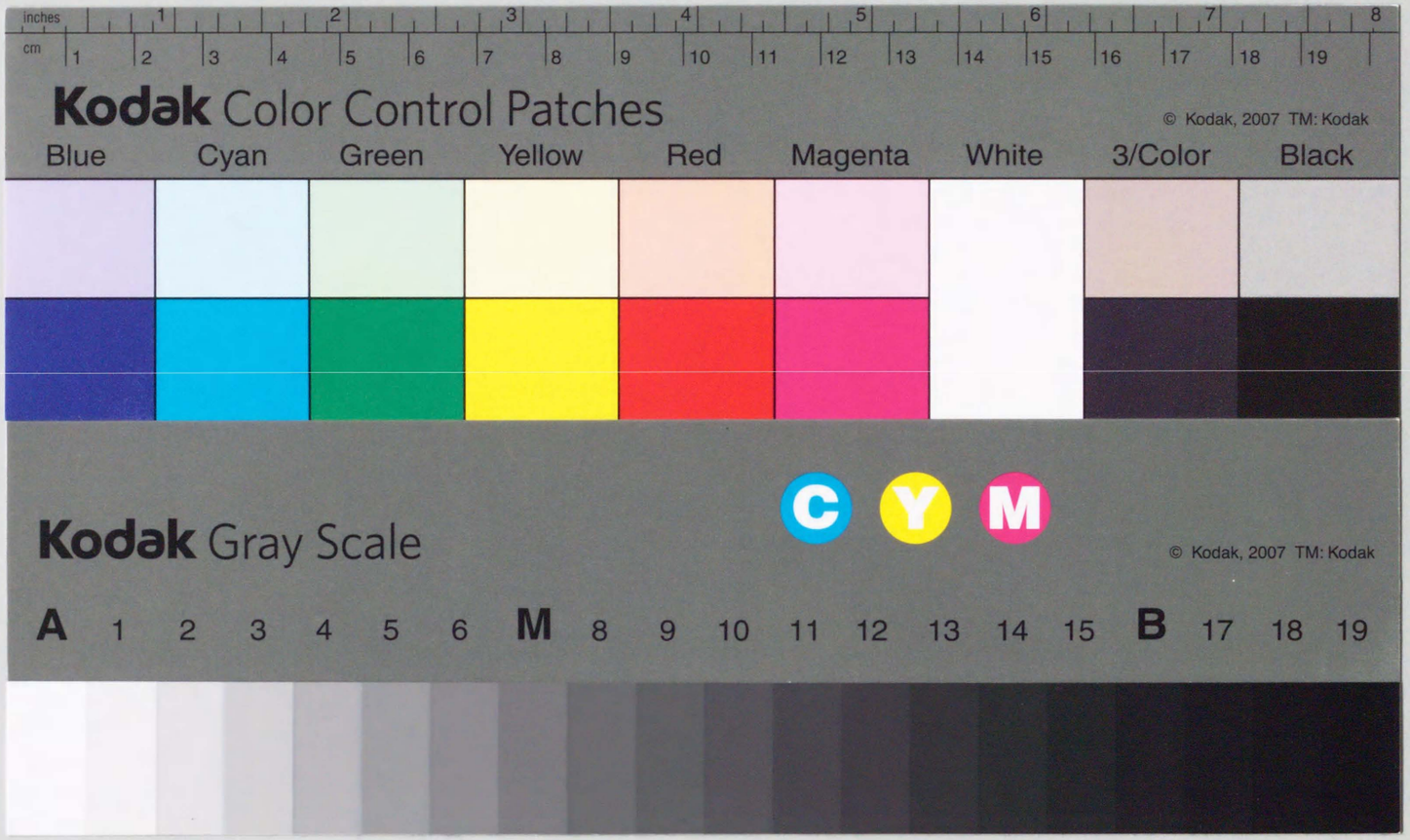
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