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# Cholinergic agonists and interleukin 1 regulate processing and secretion of the Alzheimer β/A4 amyloid protein precursor

(dementia/proteolysis/protein kinase/protein phosphorylation)

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**ABSTRACT** **Activation of protein kinase C by phorbol esters is known to accelerate the processing and secretion of the β/A4 amyloid protein precursor. We have now examined various first messengers that increase protein kinase C activity of target cells for their ability to affect β/A4 amyloid protein precursor metabolism. Acetylcholine and interleukin 1, which are altered in Alzheimer disease, were shown to increase processing of the β/A4 amyloid protein precursor via the secretory cleavage pathway. Cholinergic agonists stimulated secretion in human glioma and neuroblastoma cells as well as in PC12 cells transfected with the $M_1$ receptor, while interleukin 1 stimulated secretion in human endothelial and glioma cells.**

Alzheimer disease is characterized by distinct neuropathological lesions, including intracellular neurofibrillary tangles, extracellular parenchymal and cerebrovascular amyloid deposits, and selective cell death that particularly affects cholinergic neurons in the basal forebrain (1). The death of cholinergic neurons is associated with decreased levels of acetylcholine in the cortex and other projection areas. Other biochemical changes that occur in Alzheimer disease include increases in the levels of interleukin 1 (IL-1) in the brain and cerebrospinal fluid (2, 3).

The principal component of parenchymal amyloid plaque cores and cerebrovascular amyloid is the β/A4 amyloid protein (4–6), which is derived from cleavage of a large transmembrane protein, the β/A4 amyloid protein precursor (APP) (7–13). APP exists in three major isoforms ($APP_{695}$, $APP_{751}$, and $APP_{770}$), which arise from alternative splicing of a single primary transcript. APP undergoes secretory processing, which leads to release of the extracellular domain of APP into the extracellular space (14). Normal secretory processing involves cleavage of APP within the β/A4 amyloid domain and thus precludes amyloidogenesis (15, 16). Some of us have previously demonstrated that agents that lead to an increased state of phosphorylation of APP (17, 18), including phorbol esters (which activate protein kinase C) and okadaic acid (which inhibits protein phosphatases 1 and 2a), accelerate processing and secretion of this protein (19, 20). These same compounds may also increase alternative, potentially amyloidogenic, processing of APP (19).

The demonstration that processing of APP is regulated by agents that alter phosphorylation and dephosphorylation raises the question as to the nature of the physiological stimuli (i.e., intercellular signals or first messengers) involved in regulation of APP processing. In the present report we describe the regulation of APP secretion in various cultured cells by muscarinic agonists and antagonists, IL-1, the naturally occurring IL-1 receptor antagonist (IL-1ra), and thrombin.

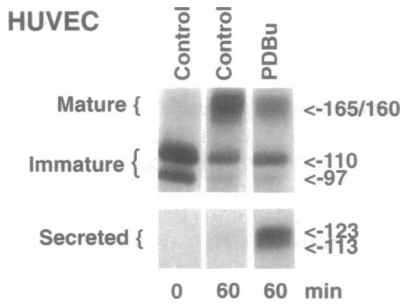


FIG. 1. APP maturation and secretion. Metabolically labeled HUVEC were incubated for either 0 or 60 min in the absence or presence of 1 μM phorbol dibutyrate (PDBu). Immature and mature (*Upper*) and secreted (*Lower*) APP were analyzed as described in text. The results shown are representative of six experiments. Apparent molecular masses (kDa) are indicated.

## MATERIALS AND METHODS

Acetylcholine chloride, carbachol, bethanechol chloride, and atropine sulfate were purchased from Research Biochemicals (Natick, MA); recombinant human IL-1β (>$10^7$ units/mg) and thrombin (from human plasma), from Boehringer Mannheim; IL-1ra, from R & D Systems (Minneapolis); phorbol 12,13-dibutyrate, from LC Services (Woburn, MA); and chloroquine, from Sigma. All media and animal sera for culturing cells were purchased from GIBCO/BRL.

Human umbilical vein endothelial cells (HUVEC) were prepared and maintained as described (21) and used at the second or third passage. The Hs 683 cell line was purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum (FBS); SH-SY5Y cells (22) were the gift of J. Biedler (Memorial Sloan-Kettering Cancer Center, New York) and were maintained in 1:1 (vol/vol) minimal essential medium/Ham's F-12 medium containing 15% FBS. PC12 cells, stably transfected with the $M_1$ receptor (23), were maintained in RPMI 1640 containing 10% FBS and 5% horse serum.

Pulse-chase labeling for $PC12M_1$ and SH-SY5Y cells was carried out as described (19), with the addition of 1% fetal bovine serum to the medium; for HUVEC and for Hs 683 cells, pulse-chase labeling was performed identically except that labeling was performed on cell monolayers in 12-well

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Abbreviations: APP, β/A4 amyloid protein precursor; HUVEC, human umbilical vein endothelial cells; IL-1, interleukin 1; IL-1ra, interleukin 1 receptor antagonist.

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Table 1. Regulation of APP secretion by phorbol dibutyrate

| Cell type | Control | PDBu |
|---|---|---|
| SH-SY5Y | 1.00 ± 0.09 | 2.82 ± 0.45* |
| $PC12M_1$ | 1.00 ± 0.19 | 4.30 ± 0.40* |
| HUVEC | 1.00 ± 0.05 | 6.79 ± 0.51* |
| Hs 683 | 1.00 ± 0.10 | 7.08 ± 1.55* |

The indicated cell types were incubated for 60 min in the absence or presence of 1 $\mu$M of phorbol dibutyrate (PDBu). Secreted APP was then analyzed as described in text. Within each experiment, incubations were carried out in triplicate, and data were normalized to the average amount of APP recovered under control conditions. The results shown represent the means ± SEM of two or three experiments. *, $P < 0.003$.

culture dishes (Corning) with 200 $\mu$l of medium per well. Metabolic labeling was carried out for 20 min, followed by a chase period of 0–240 min. The chase was initiated by the addition of an equal volume of medium containing excess unlabeled methionine. Two minutes later, test compounds dissolved in medium were added. Thus, the effects observed were attributable to changes in APP metabolism rather than APP transcription. Following incubation, APP was purified from cell lysates by immunoprecipitation with an anti-APP COOH-terminal antibody (369) (19, 24) and from conditioned medium with an anti-APP $NH_2$-terminal antibody (22C11) (14, 24). Purified APP was subjected to PAGE and quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). ANOVA followed by Bonferroni *t* test was used to determine the significance of observed differences.

## RESULTS

**APP Turnover in Cultured Cells.** To identify physiological regulators of APP metabolism, we have undertaken an analysis of the regulation of APP processing and secretion by different first messengers in various cell types. In the present study, the following cells were used: (*i*) HUVEC; (*ii*) human glioma cells (Hs 683); (*iii*) human neuroblastoma cells (SH-SY5Y); and (*iv*) PC12 cells transfected with the $M_1$ receptor ($PC12M_1$). In metabolically labeled HUVEC (Fig. 1) and in labeled Hs 683, SH-SY5Y, and $PC12M_1$ cells (not shown), APP was first detected as lower molecular weight proteins which then matured (with a concomitant increase in apparent molecular weight), the mature molecules in turn serving as substrates for cleavage and secretion. As we previously found for PC12 cells (19, 24), mature APP reached maximal levels at between 45 and 60 min, while secreted proteins reached maximal levels after 3 hr (not shown). Secreted APP could not be immunoprecipitated by an antibody against the cytoplasmic domain of APP (19, 24), indicating that secreted APP had been cleaved normally (14–16).

**Regulation of APP Metabolism by Phorbol Dibutyrate and Chloroquine.** In PC12 cells, APP can either undergo protein kinase C-regulated secretion (19, 20) or be degraded in a chloroquine-sensitive intracellular compartment (24). To confirm that both pathways were present in the four cell types used in these studies, we incubated metabolically labeled cells for 60 min in the absence or presence of either 1 $\mu$M phorbol dibutyrate or 50 $\mu$M chloroquine. In all cell types under control conditions, a small but detectable amount of APP was secreted in this time period. Phorbol dibutyrate enhanced the levels of secreted APP by 3- to 7-fold in all cell types (Figs. 1–3; Table 1). The increased secretion of APP was accompanied by a decrease in the levels of mature, cell-associated APP (e.g., Fig. 1).

Incubation with chloroquine resulted in significantly higher (approximately 2-fold) amounts of mature APP recovered in the cell lysate at 60 min of chase, compared with control cells (not shown). Chloroquine had no significant effect on secretion by these cells (not shown). These data indicate that, in these cells, two pathways exist for APP metabolism and that first messengers that activate protein kinase C can regulate APP processing in these cells.

Table 2. Regulation of APP secretion by cholinergic agonists and antagonists

| Cell type | Treatment | Relative secretion |
|---|---|---|
| $PC12M_1$ | Control | 1.00 ± 0.10 |
| | Acetylcholine (1 mM) | 3.59 ± 0.61* |
| | Carbachol (1 mM) | 5.73 ± 0.83* |
| | Bethanechol (1 mM) | 4.29 ± 0.67* |
| | Bethanechol (1 mM) + atropine (100 $\mu$M) | 1.30 ± 0.28$^{NS}$ |
| SH-SY5Y | Control | 1.00 ± 0.08 |
| | Carbachol (1 mM) | 1.61 ± 0.10* |
| Hs 683 cells | Control | 1.00 ± 0.10 |
| | Bethanechol (1 mM) | 2.07 ± 0.12* |

Cells were incubated for 60 min in the absence or presence of the indicated test compounds. Secreted APP was then analyzed as described in text. Within each experiment, incubations were carried out in triplicate, and data were normalized to the average amount of APP recovered under control conditions. The results shown represent the means ± SEM of two to four experiments. *, $P < 0.003$; NS, not significant, $P = 0.21$.

**Cholinergic Agonists Regulate APP Processing and Secretion.** The effects of the cholinergic agonists acetylcholine, carbachol, and bethanechol on APP metabolism were studied. These compounds activate protein kinase C in some cells. When PC12 cells that had been transfected with the $M_1$ receptor ($PC12M_1$) were incubated with the cholinergic agonist acetylcholine (1 mM), there was a more than 3-fold increase in the levels of secreted APP during 60 min of chase (Table 2) compared with control cells. Carbachol (1 mM), and the muscarinic agonist bethanechol (1 mM), stimulated secretion by 4- to 6-fold (Fig. 2; Table 2). The effects of 1 mM bethanechol on APP secretion were blocked by the muscarinic antagonist atropine (100 $\mu$M) (Fig. 2; Table 2).

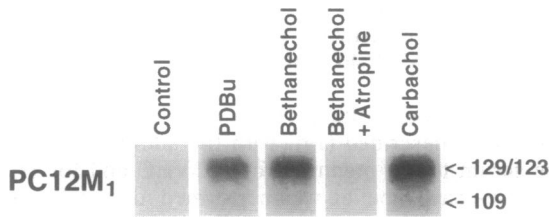


FIG. 2. Regulation of APP secretion by cholinergic agonists. $PC12M_1$ cells were incubated for 60 min in the absence or presence of 1 $\mu$M phorbol dibutyrate (PDBu), 1 mM bethanechol, 100 $\mu$M atropine, or 1 mM carbachol. Secreted APP was then analyzed as described in text. Apparent molecular masses (kDa) are indicated.

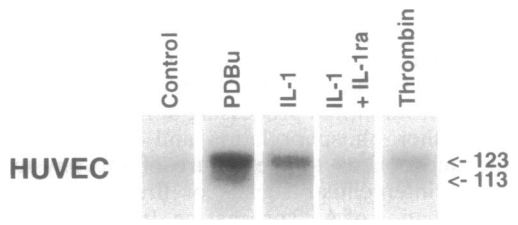


FIG. 3. Regulation of APP secretion by interleukin 1 and thrombin. HUVEC were incubated for 60 min in the absence or presence of 1 $\mu$M phorbol butyrate (PDBu), 20 units of IL-1 per ml, 2 $\mu$g of IL-1ra per ml, or 2 units of thrombin per ml. Secreted APP was then analyzed as described in text. Apparent molecular masses (kDa) are indicated.

Table 3. Regulation of APP secretion by interleukin 1 and thrombin

| Cell type | Treatment | Relative secretion |
|---|---|---|
| HUVEC | Control | 1.00 ± 0.05 |
| | IL-1 (20 U/ml) | 3.66 ± 0.26* |
| | Thrombin (2 U/ml) | 1.91 ± 0.19* |
| Hs 683 | Control | 1.00 ± 0.10 |
| | IL-1 (20 U/ml) | 2.57 ± 0.26* |
| | Thrombin (2 U/ml) | 2.21 ± 0.19* |

Cells were incubated for 60 min in the absence or presence of the indicated test compounds. Secreted APP was then analyzed as described in text. Within each experiment, incubations were carried out in triplicate, and data were normalized to the average amount of APP recovered under control conditions. The results shown represent the means ± SEM of three to five experiments. *, $P < 0.003$; U, units.

Carbachol and bethanechol stimulated APP secretion in human neuroblastoma cells (SH-SY5Y) and human glioma cells (Hs 683) by 60–100% (Table 2), supporting the idea that the effects of muscarinic agonists on APP metabolism are of physiological relevance. In HUVEC no significant effect of bethanechol on APP secretion was observed (not shown).

**IL-1 Regulates APP Processing and Secretion.** IL-1 can increase APP expression in HUVEC (25) and other cells (26, 27) by what is probably a protein kinase C-mediated mechanism (25). When metabolically labeled HUVEC were incubated for 60 min in the presence of IL-1 (20 units/ml), APP secretion increased more than 3-fold (Fig. 3; Table 3) compared with control cells. The effect of various concentrations of IL-1 on APP secretion is shown in Fig. 4. A half-maximal effect ($ED_{50}$) was observed at about 3.2 units/ml. The effect of IL-1 was antagonized by IL-1ra: IL-1ra blocked the effect of 20 units of IL-1 per ml with an $ED_{50}$ value of approximately 30 ng/ml (Fig. 5).

Glial cells may be involved in the brain's response to injury and can respond to IL-1 (see ref. 2); as a model for this phenomenon, we also studied the effects of IL-1 on human glioma (Hs 683) cells. IL-1 (20 units/ml) increased APP secretion in these cells by more than 2-fold (Table 3). In SH-SY5Y and $PC12M_1$ cells, IL-1 did not lead to significant increases in APP secretion (not shown).

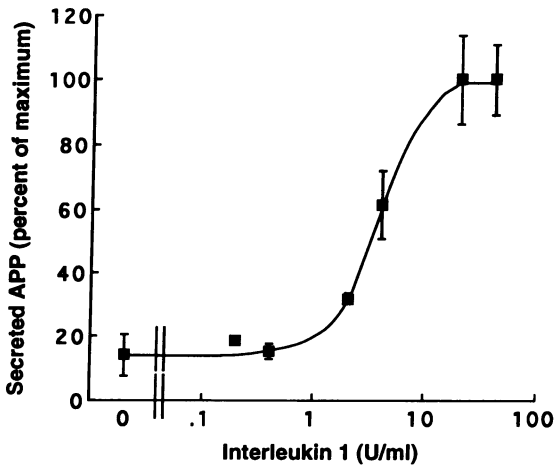


FIG. 4. Regulation of APP secretion by various concentrations of IL-1. HUVEC were incubated for 60 min in the presence of the indicated concentrations of IL-1. Secreted APP was then analyzed as described in text. Within each experiment, incubations were carried out in duplicate or triplicate, and data were normalized to the maximal levels of secreted APP. The results shown represent the means ± SEM of three experiments. U, units.

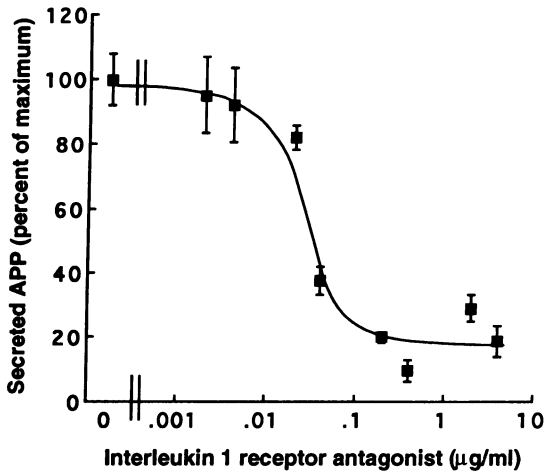


FIG. 5. Regulation of APP secretion by various concentrations of IL-1ra. HUVEC were incubated for 60 min with 20 units of IL-1 per ml and the indicated concentrations of IL-1ra. Secreted APP was then analyzed as described in text. Within each experiment, incubations were carried out in duplicate or triplicate, and data were normalized to the levels of APP secreted in the absence of IL-1ra. The results shown represent the means ± SEM of three experiments.

Thrombin, like IL-1, is involved in the acute-phase response and has been shown to stimulate APP secretion in platelets (28, 29). Because other cells involved in the acute-phase response (including HUVEC) are known to respond to thrombin, we characterized the effects of 2 units of thrombin per ml on APP secretion. In both HUVEC and Hs 683 cells, this compound increased APP secretion by about 2-fold (Fig. 3; Table 3). In SH-SYSY and $PC12M_1$ cells, thrombin did not lead to significant increases in APP secretion (not shown).

## DISCUSSION

It is now well established that activation of protein kinase C can increase the processing and secretion of APP (19, 20). In the present study we have examined several first messengers known to activate protein kinase C for their ability to affect APP metabolism. Some of these first messengers, specifically acetylcholine and IL-1 (1–3), are reported to be altered in Alzheimer disease. We found that cholinergic agonists and antagonists, IL-1, IL-1ra, and thrombin can regulate secretory processing of APP. For each of the classes of compounds we tested, some cell types responded while others did not. This is probably attributable to the different complement of receptors expressed in the various cells.

It has been reported that secretion of APP is regulated by incubation of cells for 1–12 days in the presence of nerve growth factor (30, 31), epidermal growth factor (31), or fibroblast growth factor (30). In contrast to the work presented here, those earlier studies did not distinguish effects of those compounds on APP expression from their effects on APP processing and secretion. Other compounds that have been identified as stimulating APP secretion include thrombin (28, 29) and phytohemagglutinin (32). Further characterization of signals regulating APP metabolism both *in vitro* and *in vivo* should contribute to the understanding of the physiological role of this molecule and may facilitate the development of effective therapies for Alzheimer disease (33).

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