Inhibition of Protein Phosphatase 1 Stimulates Secretion of Alzheimer Amyloid Precursor Protein

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ABSTRACT

Background: Aberrant metabolism of the Alzheimer amyloid precursor protein (APP) or its amyloidogenic Aβ fragment is thought to be centrally involved in Alzheimer's disease. Nonamyloidogenic processing of APP involves its cleavage within the Aβ domain by a protease, termed α-secretase, and release of the large extracellular domain, termed APP^S. Secretion of APP^S can be stimulated by phorbol esters, activators of protein kinase C, with concurrent inhibition of Aβ production. While the role of protein kinases on APP metabolism has been investigated, considerably less effort has been devoted to elucidating the role played by protein phosphatases. Okadaic acid, a protein phosphatase inhibitor, has been shown to stimulate secretion of APP^S, but the identity of the phosphatase involved has not been investigated.
Materials and Methods: The secretion of APP^S from COS-1 cells was measured in the absence or presence of various doses of serine/threonine-specific phosphatase inhibitors. Quantitation of the derived IC_{50} values was used to determine the identity of the phosphatase involved in the control of APP secretion.
Results: The availability of protein phosphatase inhibitors with different relative potencies against the different types of serine/threonine-specific protein phosphatase allowed us to examine which of the four known types of protein phosphatase might be involved in the regulation of APP secretion. Both okadaic acid and calyculin A stimulated the secretion of APP from COS-1 cells in a dose-dependent manner. The half-maximal dose for stimulation of APP secretion was approximately 100-fold higher with okadaic acid than with calyculin A.
Conclusions: The nearly 100-fold difference in the observed IC_{50} values for okadaic acid and calyculin A implicates a type 1 protein phosphatase in the control of APP^S production. Protein phosphatase 1 (PP1) is known to be highly expressed in adult mammalian brain, both in neurons and glia. The identification of a specific phosphatase type in the control of APP secretion opens new avenues to the development of rational therapeutic intervention strategies aimed at the prevention and/or treatment of Alzheimer's Disease.

INTRODUCTION

Alzheimer's disease (AD) is the most common of the neurodegenerative diseases, probably affecting more than 50% of individuals over the age of 80. Central to the pathology of the disease is the formation of senile plaques in the brains of affected individuals. The amyloid core of these plaques is comprised largely of a small 39–43 amino acid peptide (1), termed Aβ, derived from the Alzheimer amyloid precursor protein (APP). APP can also be processed via a nonamyloidogenic pathway by a protease (α-secretase) that cleaves APP within the Aβ domain and releases the large extracellular domain (APP^S).

Previous work has shown that treatment of a variety of cells with phorbol esters stimulates the release of APP^S, presumably via protein kinase C-mediated phosphorylation of a target protein (2–5). A similar effect can be obtained using okadaic acid (an inhibitor of serine/threonine-specific protein phosphatases), possibly via increased phosphorylation of the same target protein due to phosphatase inhibition. In addition, protein kinase C (PKC) was shown to phosphorylate the cytoplasmic domain of APP (6,7) at a

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site that is also phosphorylated in vivo (M. Oishi and P. Greengard, unpublished observations). However, APP^S secretion can still be stimulated by phorbol esters or okadaic acid in the absence of the cytoplasmic domain of APP (4,5). Thus, although the identity of the target protein has not been elucidated, it is unlikely to be the APP protein itself. Phorbol ester stimulation of APP^S secretion implicates PKC in APP processing. Likewise, the stimulation of APP^S secretion by okadaic acid implicates a phosphatase. However, the question of which type of serine/threonine-specific protein phosphatase is involved in APP secretion has not been addressed previously.

Four major types of serine/threonine-specific protein phosphatase have been identified in eukaryotic cells (termed 1, 2A, 2B, and 2C), which can be distinguished by a variety of biochemical and enzymatic properties (reviewed in Ref. 8). Of the four types of protein phosphatases, PP1 and PP2A exhibit broad and overlapping substrate specificities in vitro, accounting for almost all measurable activity towards a variety of phosphoproteins regulating cellular processes. PP2B (also known as calcineurin) is active on a much more restricted range of substrates, and PP2C is structurally unrelated to the other types. Recent recombinant DNA approaches identified the existence of several isoforms for each phosphatase type and revealed that types 1, 2A, and 2B belong to a gene family distinct from that of phosphatase 2C. A number of "novel" phosphatases have also been identified in a variety of organisms, the first of which was termed protein phosphatase X (9).

A variety of compounds is now available capable of specifically inhibiting serine/threonine-specific protein phosphatases. Two of the best characterized are okadaic acid and calyculin A (10). Since they readily enter intact cells, they can be used to determine which phosphatase is active in a particular process by virtue of their different relative potencies against the various phosphatase types. In this study we have used them to identify the protein phosphatase involved in the control of APP^S production. The data presented here were the subject of a preliminary report (11).

MATERIALS AND METHODS

Materials

Calyculin A, okadaic acid, PDBu (phorbol 12,13-dibutyrate), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were obtained from Life Technologies Inc. (Gaithersburg, MD, U.S.A.). Cantharidin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Murine monoclonal antibody 22C11, prepared against the amino terminus of APP (12), was purchased from Boehringer Mannheim Corporation (Indianapolis, IN, U.S.A.). Murine monoclonal antibody 6E10, whose epitope is located within residues 1–17 of Aβ, was obtained from Drs. H. M. Wisniewski and K. S. Kim (New York State Institute for Basic Research in Developmental Disabilities).

Cell Culture, Drug Treatment, and Detection of Secreted APP

COS-1 cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. The cells were subcultured in triplicate into six-well plates and grown to confluency. After two washes with phosphate buffered saline, the cells were incubated in the absence or presence of varying concentrations of either PDBu (for 60 min) or the indicated phosphatase inhibitors (for 90 min). When a combination of drugs was used, the time of exposure was kept to 60 min. All drugs were prepared by diluting the stock solutions into serum-free DMEM. Following incubation as described above, the conditioned medium was collected into tubes containing SDS (1% final concentration), and boiled for 5 min. The samples, normalized for protein content (13), were subjected to SDS-polyacrylamide gel electrophoresis on 7.5% gels (14). The separated proteins were then electrophoretically transferred onto nitrocellulose and probed with antibodies 22C11 or 6E10, as described in the figure legends. The immunoblots were washed and then incubated successively with rabbit anti-mouse IgG (Cappel, Durham, NC) and ^{125}I-protein A (Amersham, Arlington Heights, IL, U.S.A.). Immunoreactive bands were detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Several previous studies showed that APP secretion can be stimulated in a variety of cells using phorbol esters. Indeed, in a previous report we demonstrated that PDBu (phorbol 12,13-dibutyrate) could stimulate the secretion of both en-

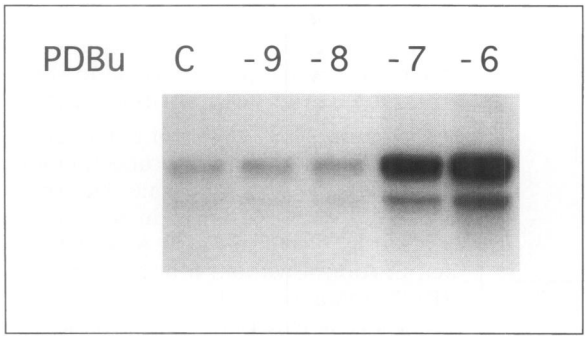

FIG. 1. Stimulation of APP^S production from COS-1 cells in response to PDBu treatment

Cells were treated with the PDBu concentrations indicated (10^{-9}–10^{-6} M), and the APP^S released into the medium was quantitated by immunoblotting with antibody 22C11. C, control without PDBu.

TABLE 1. Concentration of test substance required to achieve half-maximal secretion of APP^s

Test Substance	EC_{50} (nM)	
	22C11	6E10
Okadaic Acid	80	110
Calyculin A	1	1
Cantharidin	500	ND
PDBu	54	ND

The range of concentrations used for each test substance was as follows: 10^{-10}–10^{-6} M okadaic acid, 10^{-11}–10^{-7} M calyculin A, 10^{-9}–10^{-5} M cantharidin, and 10^{-10}–10^{-6} M PDBu. The amount of APP secreted into the medium was expressed as a percentage of the maximal amount, as exemplified in Fig. 2B. Immunoreactive bands were quantitated by immunoblotting using a PhosphorImager. The drug concentration required to achieve half-maximal secretion of APP from COS-1 cells (EC_{50}) was calculated from the average results obtained in several experiments, each performed in triplicate, and are tabulated above. ND, not determined.

dogenous APP and transfected wild-type and mutant APP expressed in COS-1 cells (4). In the present study, we have further characterized the control of secretion of endogenous APP from COS-1 cells in response to agents capable of modulating the state of phosphorylation of intracellular proteins. PDBu treatment of COS-1 cells was shown to stimulate secretion of endogenous APP in a dose-dependent manner (Fig. 1). The concentration of PDBu required for half-maximal effect (EC_{50}) was approximately 50 nM (Table 1). We have previously reported the EC_{50} value for PDBu-stimulated processing of APP to be 17 nM in PC12 cells, as measured by following the disappearance of mature intracellular APP using an antibody whose epitope lies in the C-terminal intracellular domain of APP (15). Thus, there is reasonable agreement between the two experimental systems, even though different parameters were measured and different cell types were used.

APP secretion can be stimulated not only by activators of PKC, such as PDBu, but also by inhibitors of serine/threonine-specific protein phosphatases. Thus, okadaic acid was shown to stimulate APP secretion in PC12 cells (2) and in COS-1 cells (4). However, the identity of the phosphatase targeted by such inhibitors was not elucidated. The availability of other naturally occurring compounds that are cell permeable and act as specific inhibitors of certain of the serine/threonine-specific protein phosphatases has allowed us to investigate the nature of the phosphatase involved in the control of APP secretion. Both okadaic acid and calyculin A stimulated the production of APP^S from COS-1 cells in a dose-dependent manner (Fig. 2A). Quantitation of the results from several dose-response experiments using antibody 22C11 yielded average EC_{50} values of 80 and 1 nM for okadaic acid and calyculin A, respectively (Fig. 2B, Table 1). Since antibody 22C11 has been reported to cross-react with APP-related proteins (16,17) and does not differentiate between α-secretase and β-secretase cleaved APP^S, we sought to confirm and extend our results using a more specific antibody. Thus, using antibody 6E10, which specifically recognizes APP^S produced by the α-secretase pathway, we obtained EC_{50} values of 110 and 1 nM for okadaic acid and calyculin A, respectively (Fig. 3, Table 1). Given the approximately 100-fold difference in the calculated EC_{50} values for okadaic acid and calyculin A, these results identify protein phosphatase 1 (PP1) as being the target of the phosphatase inhibitors used (see Discussion below), and therefore implicate it in the control of APP cleavage and secretion.

Cantharidin, isolated from blister beetles, is a recently described phosphatase inhibitor (18,19) thought to be the active ingredient in the purported aphrodisiac, "Spanish fly". Cantharidin, like okadaic acid and calyculin A, was also capable of stimulating APP^S production from COS-1

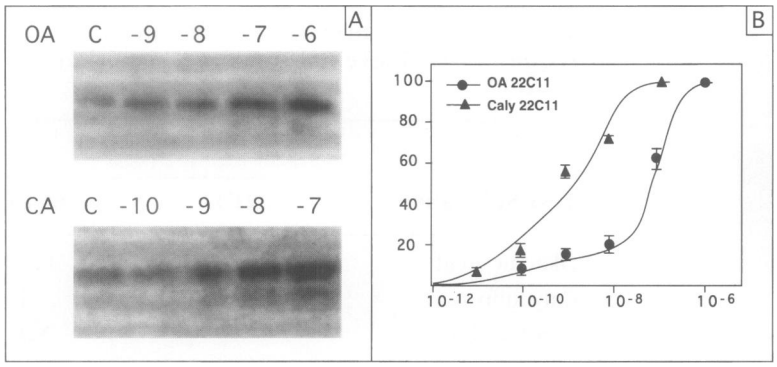

FIG. 2. Stimulation of APP^S production from COS-1 cells, in response to treatment with phosphatase inhibitors, measured with antibody 22C11

(A) Immunoblot analysis of COS-1 cells treated with the indicated concentrations of OA (10^{-9}–10^{-6} M okadaic acid) or CA (10^{-10}–10^{-7} M calyculin A; higher concentrations of calyculin A were toxic). C, control without drug treatment. (B) Quantitation of the dose response from several experiments. The values plotted are the mean ± SEM.

cells in a dose-dependent manner (Fig. 4A). In this case, the calculated EC_{50} was approximately 500 nM (Table 1). The higher EC_{50} value obtained for cantharidin in comparison with okadaic acid and calyculin A is in agreement with their relative in vitro phosphatase inhibitory potencies (Ref. 19 and E. F. da Cruz e Silva et al., manuscript in preparation). Using submaximal doses of PDBu (10^{-8} M) and cantharidin (10^{-7}), an additive effect was measured (Fig. 4B); the use of maximally effective doses of both did not result in further stimulation of APP^S production beyond the levels achieved with either drug alone (Fig. 4B and data not shown). These results are consistent with phorbol esters and phosphatase inhibitors affecting the state of phosphorylation of the same target protein via the activation of PKC or the inhibition of PP1.

The evidence presented above indicating that PP1 regulates APP^S production in COS-1

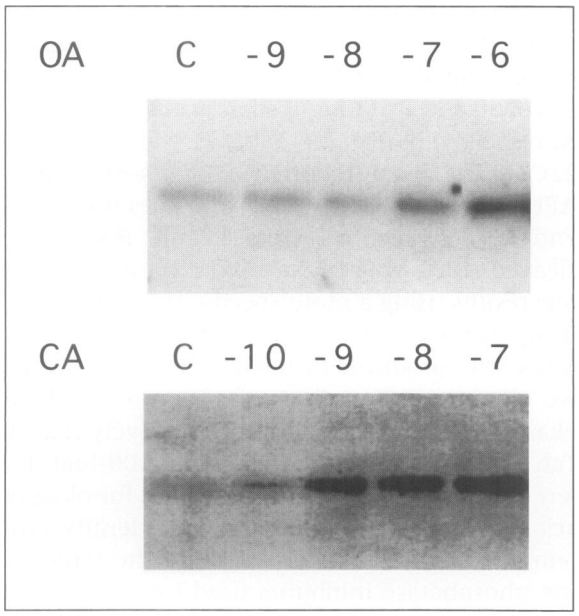

FIG. 3. Stimulation of APP^S production from COS-1 cells, in response to treatment with phosphatase inhibitors, measured with antibody 6E10

Cells were treated with the indicated concentrations of OA (10^{-9}–10^{-6} M okadaic acid) or CA (10^{-10}–10^{-7} M calyculin A). C, control without drug treatment.

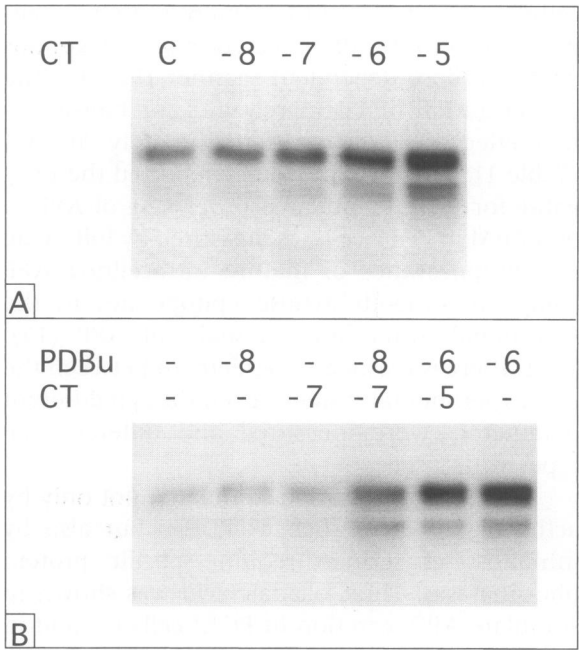

FIG. 4. Cantharidin stimulation of APP^S production from COS-1 cells as measured with antibody 22C11

(A) Immunoblot analysis of the dose response using the indicated concentrations of cantharidin (10^{-8}–10^{-5} M). C, control without cantharidin treatment. (B) Immunoblot analysis of APP^S production in response to the indicated concentrations of PDBu and cantharidin (CT). −, absence of drug.

cells led us to examine these cells for the expression of PP1. The mammalian genome is known to contain at least three different genes encoding PP1 catalytic subunits, namely, PP1α, PP1β, and PP1γ (20,21). PP1γ is known to undergo tissue-specific alternative splicing to generate two proteins differing solely at their extreme carboxyl termini. One of these, PP1γ_1, is ubiquitously expressed in most tissues, whereas the other isoform, PP1γ_2, is thought to be testis specific (20,22). Previously described isoform-specific antibodies (22,23) detected both PP1α and PP1γ_1 in COS-1 cells (data not shown).

DISCUSSION

The availability of several phosphatase inhibitors with different potencies against the various types of phosphatase allowed us to investigate the identity of the phosphatase involved in the control of APP secretion. Both okadaic acid and calyculin A are potent inhibitors of protein phosphatase types 1 and 2A, whereas PP2B is only inhibited at much higher concentrations and PP2C is essentially resistant to these compounds. However, while calyculin A is equally potent in vitro against purified PP1 and PP2A (IC_{50} = 1–2 nM), okadaic acid inhibits PP1 with an IC_{50} of 10–100 nM and PP2A with an IC_{50} of 0.5–1 nM (10,24,25). Thus, okadaic acid and calyculin A can be used to characterize which type of phosphatase is involved in a particular physiological process (e.g., Refs. 26 and 27). In our experiments, calyculin A was approximately 100-fold more potent than okadaic acid at stimulating APP^S production (Figs. 2 and 3, Table 1). Thus, our data implicate a type 1 phosphatase in the control of APP^S production. Furthermore, we have shown by immunoblotting that COS-1 cells express at least two of the known isoforms of PP1 (namely, PP1α and PP1γ_1), which exhibit very similar sensitivity profiles to okadaic acid, calyculin A and cantharidin (E. F. da Cruz e Silva et al., manuscript in preparation). Given the dynamic nature of the protein phosphorylation process, and given that PP1 has been detected in all eukaryotic cells examined to date (including neurons and glia), it is likely that PP1 also regulates APP^S production in other cell types. PP1 has been shown to be particularly enriched in brain compared with peripheral tissues, and the occurrence and distribution of known PP1 isoforms has been described in mammalian brain both by immunoblotting, immunocytochemistry, and in situ hybridization (22,23).

Several neurotransmitters affect the physiological properties of neurons by regulating the phosphorylation state of PP1 inhibitor proteins (28–30), hence affecting PP1 activity. For example, dopamine, by increasing intracellular cAMP levels and activating protein kinase A, causes the phosphorylation and activation of the PP1 inhibitor DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein, M_r 32,000 daltons) in the medium-sized spiny neurons of the neostriatum (28,31). Conversely, glutamate, by increasing intracellular calcium levels and activating PP2B, causes the dephosphorylation and inactivation of DARPP-32 (32). The convergence of major neurotransmitter pathways (e.g., dopaminergic, glutamatergic, and GABAergic) on PP1 suggests a critical role for this phosphatase in mediating the actions of these neurotransmitters.

Defects in signal transduction-dependent regulation of APP cleavage may play a critical role in the pathogenesis of AD. For instance, deficits in both neurotransmission (33,34) and PKC activity (35–37) have been reported to be associated with AD. Consistent with this notion, the stimulation of APP^S release in response to neurotransmitters and other first messengers known to act via PKC has been reported in several studies (e.g., Refs. 38 and 39). The possibility now arises that regulation of APP metabolism, including APP^S release and Aβ formation, may also be modulated by neurotransmitters or first messengers whose receptors are linked to modulation of PP1 activity. The characterization of the protein kinases and phosphatases involved in the control of APP metabolism should facilitate the development of novel rational therapeutic intervention strategies for AD.

ACKNOWLEDGMENTS

We are grateful to Drs. J. D. Buxbaum, S. E. Gandy, and A. C. Nairn for critical reading of the manuscript. This work was supported by U. S. Public Health Service Grant AG-09464. CTBVZ was supported by a postdoctoral fellowship from CNP_q and by PICD from Universidade Estadual de Londrina (Brazil).

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Contributed by P. Greengard on April 21, 1995.