Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4489–4493, May 1994
Neurobiology

Calcium regulates processing of the Alzheimer amyloid protein precursor in a protein kinase C-independent manner

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ABSTRACT **Various first messengers linked to phospholipase C, including acetylcholine and interleukin 1, regulate the production both of the secreted form of the amyloid protein precursor (APP) and of amyloid β-protein. We have now identified intracellular signals which are responsible for mediating these effects. We show that activation of phospholipase C may affect APP processing by either of two pathways, one involving an increase in protein kinase C and the other an increase in cytoplasmic calcium levels. The effects of calcium on APP processing appear to be independent of protein kinase C activation. The observed effects of calcium on APP processing may be of therapeutic utility.**

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 principal component of parenchymal amyloid plaque cores and cerebrovascular amyloid is the amyloid β-protein (Aβ) (2–4). It has been shown that this ≈4-kDa protein is produced by various cultured cells (5–7), including transfected cells stably expressing the amyloid protein precursor (APP), from which Aβ is derived (8–14).

During the past few years, a variety of evidence has emerged indicating that the processing of APP is regulated by signal transduction pathways. Thus, phorbol esters (activators of protein kinase C) and okadaic acid (an inhibitor of protein phosphatases 1 and 2A) increase APP metabolism and secretion (15–18). More recently, it has been shown that first messengers known to activate the phospholipase C/protein kinase C cascade increase the secretion of APP (17, 19). It was also shown that the formation of a peptide with properties similar to those of Aβ was decreased by phorbol esters, by okadaic acid, by direct activators of phospholipase C, and by first messengers that activate phospholipase C (20–22). However, activation of phospholipase C not only activates protein kinase C (through the formation of diacylglycerol) but also increases cytoplasmic calcium (through the action of inositol 1,4,5-trisphosphate, IP_3). For this reason, we undertook an investigation to determine whether the IP_3/calcium limb of this pathway might, like the diacylglycerol/protein kinase C limb, affect APP processing.

MATERIALS AND METHODS

Cell culture conditions and the sources of analytical reagents have been described (17, 20). HTB 148 (H4 neuroglioma) cells and CHO cells stably transfected with DNA encoding M_1 or M_3 muscarinic acetylcholine receptors were purchased from the American Type Culture Collection. CHO cells stably transfected with cDNA encoding the 751-aa isoform of APP (APP_{751}) were the gift of E. H. Koo (Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA). Transient transfection of cells with cDNA coding for APP_{751} (the gift of E. H. Koo) was carried out with Lipofectin (GIBCO/BRL) according to the manufacturer's guidelines. Antibodies 4G8 and 6E10 were supplied by H. Wisniewski and K. S. Kim (Institute for Basic Research in Developing Disabilities, Staten Island, NY).

Pulse–chase labeling of cells was carried out on confluent cell monolayers in six-well culture dishes (Corning) with 1 ml of methionine-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mCi (37 MBq) of [^{35}S]methionine/cysteine (EXPRE^{35}S^{35}S; NEN). Metabolic labeling was carried out for 2 hr, followed by a chase period of 2 hr. The chase was initiated by replacing the labeling medium with DMEM containing 0.2 mM unlabeled methionine. Two minutes after the start of the chase, the indicated test compounds were added to maximize the probability that any observed effects were attributable to changes in APP metabolism rather than APP transcription. Following incubation, conditioned medium was collected and centrifuged for 5 min at 10,000 × *g*. Secreted APP (APP_s) and Aβ were immunoprecipitated from the conditioned medium with a mixture of two monoclonal antibodies (4G8 and 6E10). Immunoprecipitated APP fragments were subjected to SDS/10–20% PAGE with Tris/tricine buffer, autoradiographed, and quantified with a PhosphorImager (Molecular Dynamics). Results presented are means ± SEM of three or more experiments performed in triplicate. Analysis of variance followed by Fisher's post-hoc analysis was used to determine the significance of observed differences.

RESULTS

CHO cells stably transfected with cDNA encoding either the M_1 (CHO-M_1) or the M_3 (CHO-M_3) muscarinic acetylcholine receptor were transiently transfected with cDNA encoding APP_{751}. When these cells were metabolically labeled and the medium was subjected to immunoprecipitation with a mixture of two antibodies (4G8 and 6E10) raised against a synthetic peptide corresponding to the first 24 residues of Aβ [Aβ-(1–24)], three major bands were observed (Fig. 1). In the higher molecular weight region of the gel, a major band with an apparent molecular mass of 130 kDa was observed, while in the lower molecular weight region of the gel, two small peptides with apparent molecular masses of 3 and 2 kDa were observed. The monoclonal antibody 6E10, which recognizes an epitope around amino acid 11 of Aβ (24), immunoprecipitated the 130-kDa protein and the 3-kDa peptide but not the 2-kDa peptide (data not shown). The monoclonal antibody 4G8, which recognizes an epitope within amino acids 17–24 of Aβ (25), immunoprecipitated the 3- and 2-kDa peptides but not the 130-kDa protein (data not shown). None of these three

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Abbreviations: Aβ, amyloid β-protein; APP, amyloid protein precursor; APP_s, secreted APP; APP_{751}, 751-aa isoform of APP; IP_3, inositol 1,4,5-trisphosphate; PBt_2, phorbol 12,13-dibutyrate.

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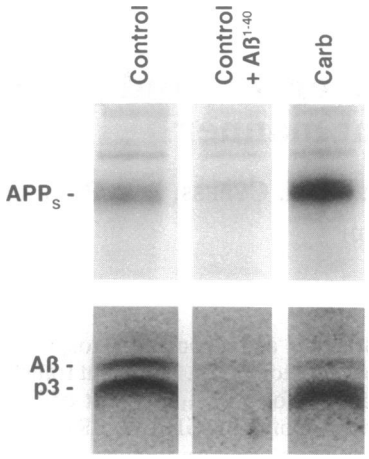

FIG. 1. Carbachol regulates formation of APP_s and Aβ. APP_s, Aβ, and p3 were immunoprecipitated from the medium of metabolically labeled CHO cells stably expressing the M_3 muscarinic acetylcholine receptor. (*Upper*) Higher molecular weight region of a 10–20% polyacrylamide Tris/tricine gel, showing APP_s. (*Lower*) Lower molecular weight region of a 10–20% polyacrylamide Tris/tricine gel, showing Aβ and p3, an Aβ fragment deleted of the first 16–17 aa (23). Control, no additions; $Aβ^{1-40}$, immunoprecipitation was carried out in the presence of synthetic Aβ-(1–40)-peptide at 5 μg/ml; Carb, 1 mM carbachol.

proteins were recovered when immunoprecipitation was carried out in the presence of synthetic Aβ-(1–40) (Fig. 1). The immunochemical properties of these three proteins, their apparent molecular weights, and the fact that they were preferentially observed in cells transiently transfected with cDNA encoding APP_{751} (data not shown) allow tentative assignment as follows: the 130-kDa protein corresponds to the portion of APP which is secreted (APP_s), the 3-kDa peptide represents an Aβ-like peptide, and the 2-kDa peptide represents a p3-like peptide (23). Aβ-like peptides are heterogeneous in many cells; therefore the identity of the two low molecular weight bands awaits amino acid sequencing to determine their precise composition.

The levels of APP_s increased significantly when cells expressing either the M_1 or the M_3 receptor were incubated with the cholinergic agonist carbachol (Fig. 1; Table 1; refs. 17 and 19). At the same time, production of Aβ decreased dramatically (Fig. 1; Table 1; ref. 21). Similarly, incubation of CHO-M_3 cells with the muscarinic agonist bethanechol stimulated APP_s production and decreased Aβ production (Fig. 2).

Activation of M_1 or M_3 receptors stimulates phospholipase C activity in various cells, leading to the activation of protein kinase C as well as the release of calcium from intracellular stores. The effects of activators of phospholipase C on APP_s and Aβ levels might therefore arise from either the activation of protein kinase C or the activation of other calcium-dependent processes. Consistent with previous results (16–18, 20–22), incubation of CHO-M_1 cells or CHO-M_3 cells with PBt_2, a compound which activates protein kinase C, resulted in increased production of APP_s and decreased production of Aβ (e.g., Fig. 2). This suggests that activation of protein kinase C following phospholipase C activation is sufficient to mediate the effects of muscarinic agonists on APP processing. To test whether activation of protein kinase C was necessary for muscarinic regulation of APP processing, we incubated CHO-M_3 cells, transiently expressing APP_{751}, for 17 hr in 1 μM PBt_2, a treatment that leads to the down-regulation of protein kinase C. Under these conditions, bethanechol still increased the formation of APP_s and decreased Aβ formation even though PBt_2 was no longer effective (Fig. 2). These results support the possibility that bethanechol and other phospholipase C activators may affect the formation of APP_s and Aβ through an action on the IP_3/calcium pathway.

Table 1. Phospholipase C-linked first messengers regulate formation of APP_s and Aβ in CHO cells

Condition	APP_s, relative units	Aβ, relative units
CHO-M_1 cells		
Control	1.00 ± 0.07	1.00 ± 0.10
Carbachol (1 mM)	2.10 ± 0.17*	0.55 ± 0.12*
CHO-M_3 cells		
Control	1.00 ± 0.05	1.00 ± 0.07
Carbachol (1 mM)	1.97 ± 0.30*	0.49 ± 0.07*

APP_s and Aβ were immunoprecipitated from the medium of metabolically labeled CHO cells which had been transiently transfected with cDNA encoding APP_{751}. *, Different from control ($P < 0.005$).

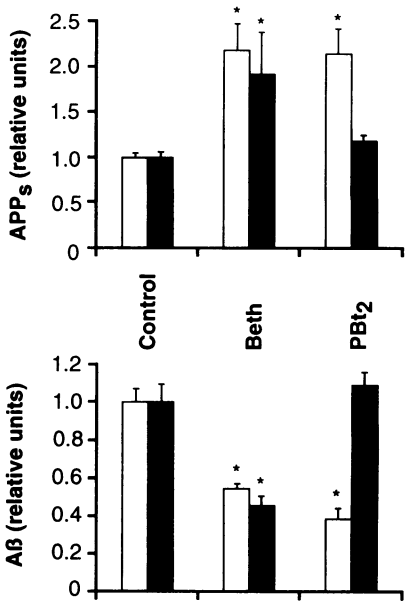

FIG. 2. Bethanechol regulates formation of APP_s and Aβ in cells lacking functional protein kinase C. CHO-M_3 cells transiently expressing APP_{751} were either untreated (open bars) or treated for 17 hr with 1 μM phorbol 12,13-dibutyrate (PBt_2) to down-regulate protein kinase C (filled bars) before the start of the metabolic labeling. After metabolic labeling, APP_s and Aβ were immunoprecipitated from the medium. Control, no additions; Beth, 1 mM bethanechol; PBt_2, 1 μM PBt_2. *, Different from control ($P < 0.003$).

Incubation of CHO cells stably transfected with cDNA encoding APP_{751} (CHO-APP_{751}) with the phospholipase C-linked first messenger ATP stimulated APP_s formation. This effect was also observed after down-regulation of protein kinase C (Fig. 3). ATP had no effect on Aβ production under conditions in which it stimulated APP_s secretion, suggesting that APP was not limiting for the formation of Aβ under these conditions (see ref. 20).

To evaluate further the possibility that changes in intracellular calcium levels were sufficient to mediate the effects of phospholipase C on APP processing, we incubated CHO-APP_{751} cells with thapsigargin, a compound which increases cytoplasmic calcium by irreversibly inhibiting the uptake of calcium into the endoplasmic reticulum. Incubation of cells with 20 nM thapsigargin led to a dramatic increase in APP_s production (Fig. 4). A study of the effects of various concentrations of thapsigargin on APP_s production demonstrated an EC_{50} value of ≈8 nM (data not shown). To ascertain whether the effect of thapsigargin on APP_s formation was due to activation of protein kinase C, we down-regulated protein

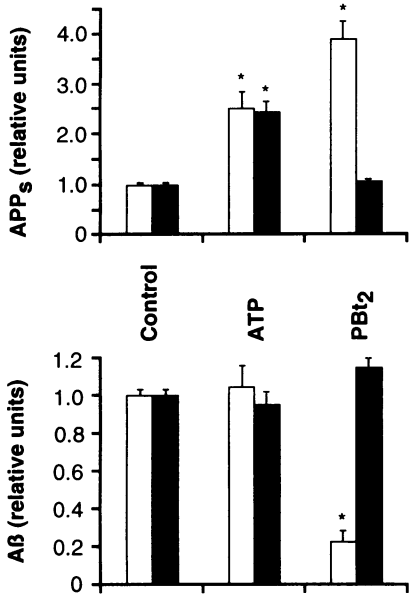

FIG. 3. ATP regulates formation of APP_s and Aβ in cells lacking functional protein kinase C. CHO-APP_{751} cells were either untreated (open bars) or treated for 17 hr with 1 μM PBt_2 to down-regulate protein kinase C (filled bars) before the start of the metabolic labeling. After metabolic labeling, APP_s and Aβ were immunoprecipitated from the medium. Control, no additions; ATP, 1 mM ATP; PBt_2, 1 μM PBt_2. *, Different from control ($P < 0.0001$).

kinase C by incubating CHO-APP_{751} cells with 1 μM PBt_2 for 17 hr before carrying out metabolic labeling. Incubating such cells with thapsigargin led to increased APP_s production which was indistinguishable from that observed in naive cells, whereas PBt_2 no longer had an effect on the treated cells (Fig. 4).

Experiments similar to those using thapsigargin were carried out using cyclopiazonic acid, which elevates cytoplasmic

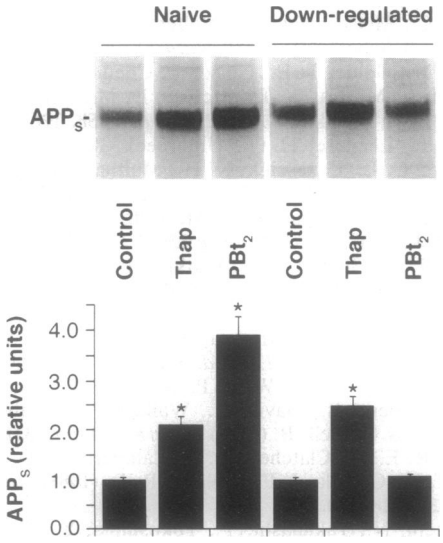

FIG. 4. Thapsigargin, a compound which raises cytoplasmic calcium levels, regulates formation of APP_s in cells lacking functional protein kinase C. CHO-APP_{751} cells were either untreated (Naive) or treated for 17 hr with 1 μM PBt_2 to down-regulate protein kinase C (Down-regulated) before the start of the metabolic labeling. After metabolic labeling, APP_s was immunoprecipitated from the medium. (*Upper*) Higher molecular weight region of a 10–20% polyacrylamide Tris/tricine gel. (*Lower*) Quantitation of APP_s. Control, no additions; Thap, 20 nM thapsigargin; PBt_2, 1 μM PBt_2. *, Different from control ($P < 0.0001$).

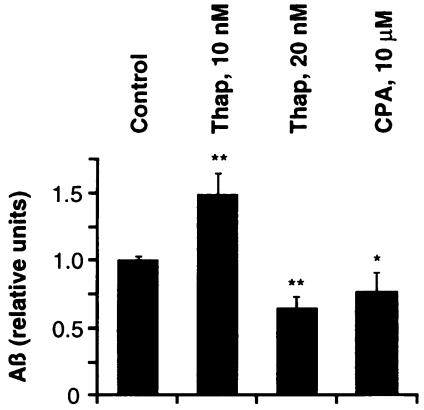

FIG. 5. Agents which raise cytoplasmic calcium levels regulate formation of Aβ. Aβ was immunoprecipitated from the medium of metabolically labeled CHO-APP_{751} cells. Control, no additions; Thap, thapsigargin; CPA, cyclopiazonic acid. *, Different from control ($P < 0.05$); **, different from control ($P < 0.0005$).

calcium levels by reversibly inhibiting the uptake of calcium into the endoplasmic reticulum. Incubation of naive CHO-APP_{751} cells with 10 μM cyclopiazonic acid stimulated APP_s production, resulting in a 3.9 ± 0.74-fold increase in APP_s recovered in the medium. Incubation of protein kinase C-down-regulated CHO-APP_{751} cells with 10 μM cyclopiazonic acid resulted in a 3.1 ± 0.45-fold increase in APP_s production.

In contrast to the effect of thapsigargin on APP_s production, which was stimulatory at all concentrations tested (5 nM and above), its effect on Aβ production depended qualitatively on the concentration used. Thus, Aβ production was increased in the presence of 10 nM thapsigargin and decreased in the presence of 20 nM thapsigargin (Fig. 5). Cyclopiazonic acid at 10 μM decreased Aβ production (Fig. 5).

We also tested the effects of phospholipase C activators, of thapsigargin, and of PBt_2 on APP processing in a human neuroglioma cell line (HTB 148) (Fig. 6; Table 2). In this cell line, these agents increased APP_s formation and decreased Aβ formation. As can be seen from the data in Table 2, there was a direct correlation between the ability of various test substances to increase APP_s formation and their ability to decrease Aβ formation. These data support the notion that the amount of APP substrate available for metabolism is rate limiting under these experimental conditions (20). The effects we observed with PBt_2 disagree with those of a previous report in which PBt_2 had no effect on APP_s formation but decreased Aβ formation (22).

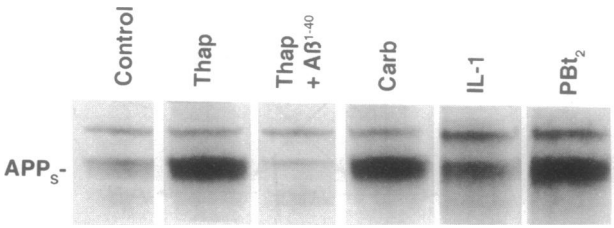

FIG. 6. Regulation of formation of APP_s in human neuroglioma cells. APP_s was immunoprecipitated from the medium of metabolically labeled HTB 148 cells. The higher molecular weight region of a 10–20% polyacrylamide Tris/tricine gel is shown. Control, no additions; Thap, 20 nM thapsigargin; $A\beta^{1-40}$, immunoprecipitation was carried out in the presence of synthetic Aβ-(1–40)-peptide at 5 μg/ml; Carb, 1 mM carbachol; IL-1, interleukin 1 at 100 units/ml; PBt_2, 1 μM PBt_2.

Table 2. Regulation of formation of APP_s and Aβ in human neuroglioma cells by various substances

Treatment	APP_s, relative units	Aβ, relative units
Control	1.00 ± 0.07	1.00 ± 0.06
Thapsigargin (20 nM)	3.74 ± 0.61**	0.64 ± 0.18**
Carbachol (1 mM)	4.53 ± 1.17**	0.75 ± 0.09*
Interleukin 1 (100 units/ml)	3.01 ± 0.23*	0.92 ± 0.14
PBt_2 (1 μM)	8.82 ± 1.67**	0.20 ± 0.05**

APP_s and Aβ were immunoprecipitated from the medium of metabolically labeled HTB 148 cells. *, Different from control ($P < 0.008$); **, different from control ($P < 0.01$).

DISCUSSION

Our results suggest that phospholipase C activation can affect APP processing via either of two pathways (Fig. 7). Diacylglycerol-mediated activation of protein kinase C stimulates APP_s production and decreases Aβ production. IP_3-mediated increases in cytoplasmic calcium also stimulate APP_s production and, under most circumstances (see below), inhibit Aβ production. The data presented here indicate that calcium can regulate APP processing in a protein kinase C-independent fashion. The parallel and independent effects of diacylglycerol and IP_3 on APP processing suggest that cells lacking one of these two pathways could still respond to phospholipase C-linked first messengers with altered APP processing.

The results further suggest that various ligand-operated ion channels and voltage-operated ion channels that control intracellular calcium levels may also regulate APP processing. This idea is supported by recent studies of the effects of a calcium ionophore on APP_s production in cultured cells (27). Both APP_s and Aβ have been demonstrated to modulate intracellular calcium levels (28–31), raising the possibility, when taken together with the data presented here, that these compounds may regulate their own formation.

Thapsigargin at higher concentrations decreased the formation of Aβ in CHO cells as well as in HTB 148 cells (Fig. 5; Table 2). Similarly, cyclopiazonic acid decreased the formation of Aβ in CHO cells (Fig. 5). These data support the idea that the ability of bethanechol to decrease Aβ formation in CHO-M_3 cells without functional protein kinase C is mediated by calcium. In contrast, thapsigargin, at lower concentrations, caused a small increase in the formation of Aβ in CHO cells. The effect of low levels of thapsigargin on the production of Aβ suggests that, under some conditions in some cells, first messengers may stimulate Aβ production. However, as mentioned above, it will be important to confirm by sequencing the identity of the Aβ-like peptide we have observed.

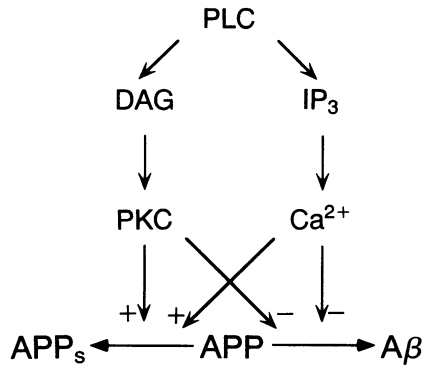

FIG. 7. Activation of phospholipase C may regulate formation of APP_s and Aβ via two distinct mechanisms. Phospholipase C (PLC) hydrolyzes phosphatidylinositol bisphosphate, yielding two intracellular signaling molecules, diacylglycerol (DAG) and IP_3. DAG activates protein kinase C (PKC), which enhances APP_s production and diminishes Aβ production. IP_3 stimulates the release of calcium from intracellular stores, which enhances APP_s production and, at least under certain conditions, diminishes Aβ production. For further discussion, see text.

The regulation of the formation of Aβ is clearly of potential clinical relevance. As such, the apparent role of calcium as a key regulator in this process may be of importance. Calcium has been implicated in aging and in Alzheimer disease (e.g., 32–37). Targeting calcium for potential therapies in Aβ amyloidosis is therefore worth consideration. Such an approach could prove advantageous over attempts to regulate protein kinase C activity because, unlike protein kinase C, intracellular calcium levels do not seem to affect APP transcription (26). The possible physiological relevance of the calcium regulation of APP processing is supported by the studies on neuroglioma cells (HTB 148), which indicate that calcium and phospholipase C-linked first messengers can regulate APP processing in this human cell line.

We thank Dr. E. H. Koo for providing CHO cells transfected with APP_{751} and for cDNA constructs for expression of APP_{751}. We thank Dr. S. E. Gandy for critical review of this manuscript. This work was supported by United States Public Health Service Grants AG09464 and AG10491 (to P.G.). J.D.B. is the recipient of an Alzheimer's Association Faculty Scholar Award.

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