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PHOSPHOLIPASE A_2 ACTIVATION INFLUENCES THE PROCESSING AND SECRETION OF THE AMYLOID PRECURSOR PROTEIN

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Summary: The phospholipase A_2 (PLA_2) inhibitors quinacrine, manoalide and scalaradial inhibit the carbachol-stimulated secretion of the amyloid precursor protein (APP) from cells transfected with the human m1 muscarinic receptor. Conversely, activation of PLA_2 by melittin increases secretion of an apparently immature species of APP from these cells. These results implicate PLA_2 in regulating APP processing and secretion, which may have important implications for understanding the pathogenesis of Alzheimer's Disease.

The amyloid precursor protein (APP) carries within its sequence a stretch of amino acids (APP 597 to APP 639) that, when cleaved from the parent molecule, gives rise to the ß-amyloid peptide (ß/A4) (1). ß/A4 is the major component of the amyloid plaque in Alzheimer's Disease (AD) and is thought to be a major contributor to the pathogenesis of the disease. The pathway by which newly synthesized APP is processed determines if it will contribute to amyloid plaque formation. APP is processed primarily for secretion by proteolytic cleavage of membrane-associated holo-APP in the middle of its ß-amyloid region, eliminating production of ß/A4 (2). Alternatively, ß/A4 is produced when holo-APP is shuttled to an acidic intracellular compartment in which proteolysis creates amyloid-containing peptides, which are then secreted from the cells (3-5). This latter event is suspected of being critical to the development of AD.

Recent evidence suggests that amyloidogenic and non-amyloidogenic processing of APP are influenced by second messenger systems. Activation of protein kinase C (PKC) by phorbol esters increases the secretion of APP by cells in culture (6) and concomitantly reduces the production of amyloidogenic peptides (7, 8). This implies that the amyloidogenic and non-amyloidogenic pathways for APP processing are interrelated. Activation of G-protein linked receptors that secondarily increase phosphatidyl inositol (PI) turnover and PKC activity (e.g., m1 and m3 muscarinic receptors) appear to mimic the effects of phorbol esters on APP processing (9-12). This apparent reciprocal relation between APP secretion and ß/A4 production upon PKC activation raises the question of whether activation of other second messenger systems promote the production of ß/A4.

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The levels of both APP and ß/A4 in brain tissue increase upon trauma (13-16), suggesting that nerve cell injury promotes the production of amyloid and non-amyloid containing fragments of APP. One consequence of brain trauma is the activation of the enzyme phospholipase A_2 (PLA_2) (17-19). PLA_2 (E.C. 3.1.1.4) is responsible for the hydrolysis of membrane phospholipids at their sn-2 position to produce arachidonic acid (20). This lipid can be further metabolized into leukotrienes and prostaglandins, mediators of inflammation. PLA_2 exists in two forms, the low molecular weight (14-18 kDa), secretory PLA_2 and the high molecular weight (31-110 kDa), cytoplasmic PLA_2. The cytoplasmic PLA_2 can be activated by G-protein linked receptors. Because the activation of cytoplasmic PLA_2 influences secretion of several substances from cells (21-25), we sought to determine if PLA_2 activation affects APP processing and secretion.

MATERIALS AND METHODS

Tissue culture: Stable chinese hamster ovary cells (CHO-K1) transfected with human m1 muscarinic receptor subtype (26) were grown to near confluence in 75 cm filter cap tissue culture flasks (Costar) in DMEM containing 10% fetal bovine serum, 1% non-essential amino acids and additional 150 µg/mL L-proline (all media and sera for culturing cells were purchased from Gibco/BRL, Gaithersberg, MD). Cells were dislodged from the flask using trypsin-EDTA, plated onto 100 mm tissue culture dishes (Falcon) at 2 x 10^{-6} cells per plate in 10 mL complete media and incubated for 1-2 days until 80% confluence was reached. Upon reaching the desired confluence, media was aspirated from plates and the cells were washed twice with 5 mL of Opti-mem (Gibco/BRL). Opti-mem containing the agent to be tested was added to the plates of cells for a 1.0 hr pretreatment. Afterward the media was replaced with fresh media containing the agent and carbachol or melittin for a 1.0 hr incubation period. After 1.0 hr, the conditioned media was removed from the plate to a centrifuge tube containing protease inhibitor cocktail (PMSF 10 mg/ml, EDTA 5 mM and the following at 2 mg/ml: aprotinin, leupeptin, pepstatin A, antipain). The conditioned media was centrifuged for 30 min at 2000 x g and concentrated to 40-80 µl using Amicon Centriprep and Centricon concentrators with a 30 kDa molecular weight cut-off filter. Protein concentrations were determined by the Pierce Bicinchoninic acid microtiter assay (27) on the supernatant. The supernatant was then aliquoted 4:1 with 5x sample buffer (glycerol, SDS, 2-mercaptoethanol and Bromophenol Blue), mixed and frozen at -20° C.

Manoalide and scalaradial were purchased from BIOMOL (Plymouthmeeting, PA). Quinacrine, melittin and other compounds were purchased from Sigma Chemical Company (St. Louis, MO).

Protein separation by gel electrophoresis and Western blotting: Samples of supernatant were thawed and heated to 95° C for 2 min. Tris/glycine precast gels (4-20% from ISS/Daiichi, Natick, MA) were loaded with equal amounts of total protein. Electrophoresis was performed at 45 mA/gel until the dyefront arrived at 0.5 cm from the bottom of the gel. The gels were soaked for 30 min in transfer buffer containing tris, glycine and 20% methanol (v/v) and stacked in BioRad blotting cartridges. Proteins were transferred to nitrocellulose (45µm), at 100 volts for 105 min. Following blotting, a monoclonal antibody (5 µg/ mL, MAb 22C11, Boehringer-Mannheim, Indianapolis, IN) to the N-terminus of the APP was applied to the blots in TBS containing 0.05 % Tween 20 (TTBS) and 1% gelatin for at least 2 hr. Excess MAb was removed and then a second antibody goat anti-mouse IgG, conjugated to alkaline phosphatase (Biorad, Riverside, CA) was applied for 2 hr. The blots were then rinsed and the nitrocellulose stained for alkaline phosphatase activity. All manipulations were performed at room temperature. The blots were then scanned using optical reflectance and the integrated optical density (IOD) of each band was calculated. All gels/blots contained an internal no-treatment control, to which treatment lanes were compared. Molecular weights of the bands were estimated using prestained molecular weight markers.

RESULTS

The activation of PI-linked muscarinic receptors in our m1 CHO cells affects APP secretion (10, 11). Treatment of the m1 CHO cells with 1 mM carbachol increases APP secretion from 3 to

6-fold over basal secretion (see Fig. 1). The effects are inhibited by 1.0 μM of the protein kinase inhibitor staurosporine (data not shown) showing that carbachol-stimulated release of APP is a protein kinase-dependent event.

The effects of various inhibitors of PLA_2 were tested on carbachol-stimulated secretion of APP. Our results indicate that PLA_2 also plays a role in APP secretion from m1 CHO cells. The reversible PLA_2 inhibitor quinacrine (20) at 50 μM diminishes the carbachol-stimulated release by about 40% (Fig. 1A). Likewise, the irreversible PLA_2 inhibitors, manoalide and scalaradial (20), have effects on APP secretion similar to quinacrine (Figs. 1B and 1C). Both inhibitors reduce carbachol-stimulated secretion of APP by 50% to 65%, at a concentration (3.2 μg/mL) known to inhibit PLA_2 mediated Golgi transport (28). However, manoalide and scalaradial have little effect on the basal secretion of APP (Fig. 1B and 1C) unlike quinacrine (Fig. 1A). Thus, quinacrine-mediated reduction of basal APP secretion may be due to effects other than inhibition of PLA_2. Whether or not this is a factor in quinacrine's effects on carbachol-stimulated secretion of APP remains to be determined.

Direct activation of PLA_2 using melittin, a 26 amino acid peptide isolated from bee venom, also implicates PLA_2 as having a role in APP secretion. Melittin treatment (5 μg/mL) increases secretion of APP from the m1 CHO cells (Fig. 2) to a level similar to that produced by 1 mM carbachol. However, the form of APP released by melittin and carbachol are different. The molecular weight of APP secreted into the culture media after melittin treatment is lower (about 97 kDa) (Fig. 3, lane c) than that found after basal and carbachol-stimulated secretion (117 kDa and 113 kDa) (Fig. 3, lanes a and b). The 97 kDa APP has the same molecular weight as the cell-associated APP found in m1 CHO cells (Fig. 3, lane d). Manoalide (3.2 μM) inhibits the melittin-induced increase in secretion of APP by more than 60% (Fig. 2). However, staurosporine at 1.0 μM fails to inhibit the melittin effect (data not shown). These results indicate that activation of PLA_2 by melittin is required to increase APP secretion and is not dependent upon activation of protein kinases.

DISCUSSION

Our results suggest that carbachol-stimulation of m1 CHO cells in addition to activating a protein kinase dependent pathway also activates PLA_2. Studies by Namenoff and his colleagues

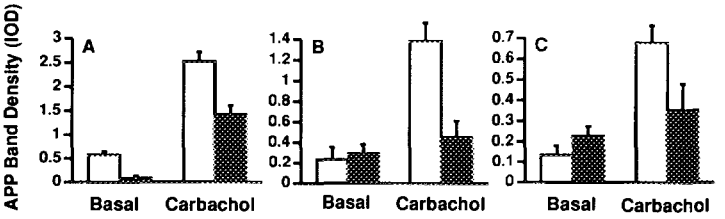
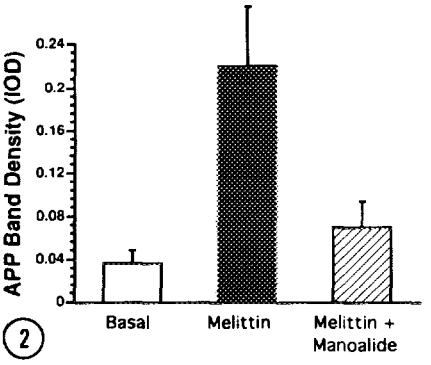


Figure 1. The effects of PLA_2 inhibitors on basal and 1 mM carbachol-stimulated secretion of APP. The open bars indicate no inhibitor and the closed bars indicate the presence of inhibitor. (A) 50 μM quinacrine, (B) 3.2 μM manoalide, and (C) 3.2 μM scalaradial. The values in each graph are the mean and standard error for each experimental condition (n= 3).



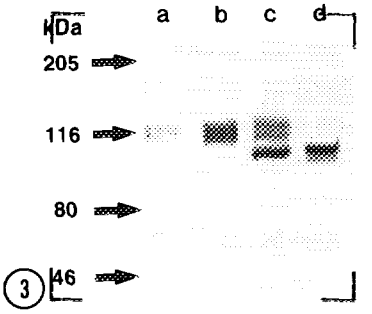


Figure 2. The effects of 5 μg/ mL of melittin on APP secretion. Manoalide (3.2 μM) was used to inhibit the melittin effect. The values represent the mean and standard error for each experimental condition (n= 3).

Figure 3. Immunoblot of secreted and cell-associated APP from m1 CHO cells. the sample order of the lanes is as follows: (a) basally secreted APP, (b) 1 mM carbachol-stimulated APP, (c) 5 μg/mL melittin-stimulated APP, and (d) m1 CHO cell-associated APP. The numbers and bars to the right side of the figure represent the values and position of the molecular weight markers used on the gels.

support this conclusion (29). They showed that carbachol treatment of CHO cells, transfected with the m1 muscarinic receptor, increased cytoplasmic PLA_2 activity as measured by increased arachidonic acid release. Furthermore, down-regulation of PKC by chronic exposure to phorbol ester inhibits the effect of carbachol on arachidonic acid release, indicating that PLA_2 activation is downstream from the carbachol activation of PKC. The activation of PKC followed by PLA_2 activation explains how APP secretion increased by carbachol is inhibited by staurosporine, while that produced by melittin, which activates PLA_2 directly, is not . Thus, our data suggest that activation of PLA_2 may provide a common pathway mediating APP secretion stimulated by carbachol and melittin. Whether PLA_2 exclusively mediates APP secretion, or other second messenger systems that can function independently of PLA_2 are also involved, remains to be determined.

Activation of PLA_2 alone is not sufficient to produce secretion of mature APP. Melittin stimulation causes a lower molecular weight form of APP (about 97 kDa) to be secreted than is normally released under basal or carbachol-stimulated conditions. This form of APP may correspond to the cell-associated 97 kDa form of APP found in the m1 CHO cells, representing an immature nonglycosylated form (30). This implies that the activation of PKC may play an important role in controlling the processing of APP other than stimulating its secretion. These data also may indicate that the normal maturation and secretion of APP is dependent upon a coordinated series of processing events orchestrated by several different signal transduction systems, not just one. This has important implications for understanding the pathogenesis of AD, since evidence for abnormality in second messenger systems involved in protein phosphorylation in AD brains already exists (31). Alterations in the orchestration of signal transduction systems may indirectly affect APP maturation. This may affect, in turn, the normal function of APP and also influence the production of ß/A4.

To our knowledge, this is the first report implicating PLA_2 activity in the secretion of APP from cells. The involvement of PLA_2 in secretory processes is not unusual. PLA_2 is implicated in the secretion of neurotransmitters (21,22), amylase (23), insulin (24) and parathyroid hormone (25). The effects of PLA_2 are thought to be mediated by increased arachidonic acid production which potentiates the fusion of secretory vesicles with other intracellular vesicles and with the plasma membrane (32). This relationship may explain how APP increases in nervous tissue at sites of trauma, since trauma and ischemia lead to activation of PLA_2 (17-19). In turn, activation of PLA_2 may increase APP release. Brain trauma also increases the accumulation of ß/A4 containing peptides (13, 16). At present, it is not known what role PLA_2 activation plays in this process. However, given the increasing evidence for an ongoing inflammatory process in AD brain (33) and the likelihood that PLA_2 is activated under these conditions, it is important to investigate the possible linkage between these two events.

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