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Phospholipase A_2 is involved in muscarinic receptor-mediated sAPPα release independently of cyclooxygenase or lypoxygenase activity in SH-SY5Y cells

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Abstract

The release of soluble amyloid precursor protein α (sAPPα), produced during α-secretase processing by cleavage within the β amyloid peptide domain of APP, is highly regulated by several external and internal signals. Because evidence suggests the involvement of inflammatory processes in the pathology of Alzheimer's disease and APP formation, we examined the involvement of the phospholipase A_2 (PLA_2) pathway and of its downstream cyclooxygenase (COX) and lipoxygenase (LOX) pathways in the regulation of sAPPα, release induced by muscarinic receptor activation in SH-SY5Y cells. The amount of sAPP released into the culture medium was analyzed using a monoclonal 6E10 antibody detecting sAPPα. Treatment with the PLA_2 inhibitor, manoalide, blocked the release of oxoM (muscarinic receptor agonist)-stimulated sAPPα, and the muscarinic receptor-mediated sAPPα release was increased by the non-selective PLA_2 activator mellitin. COX and LOX inhibitors inhibited exogenous AA-induced sAPPα release, but upregulated basal constitutive sAPPα release. However, treatment with COX or LOX inhibitors failed to significantly change oxoM-stimulated sAPPα release, and furthermore, muscarinic receptor activation inhibited AA-stimulated COX activity. Our results suggest that sAPPα release induced by muscarinic receptor activation is regulated by AA generation via PLA_2 activation independently of COX and LOX activities, but that the COX and LOX pathways are possibly involved in the constitutive release of sAPPα.

Keywords: Muscarinic receptors; sAPPα release; PLA_2; COX; LOX; Arachidonic acid

Chronic inflammatory processes are known to be associated with the pathology of Alzheimer's disease (AD). The expressions of cyclooxygenase-2 (COX-2) [21] and phospholipase A_2 (PLA_2) [27] appear to be strongly activated in AD indicating the importance of inflammatory gene pathways in response to brain injury. Amyloid precursor protein (APP) is a transmembrane protein that produces β amyloid (Aβ) peptide, a characteristic of the AD brain, by proteolytic cleavage. APP normally undergoes proteolytic cleavage within its Aβ sequence by an unidentified enzyme designated α-secretase, thus liberating a soluble N-terminal fragment (sAPPα). The biological activities of sAPPα include the promotion of neuronal cell survival, adhesive interactions, neurite outgrowth, synaptogenesis, and synaptic plasticity [28]. Moreover, it has been reported that muscarinic (M1 and M3) receptor activations increase sAPPα release [20,26]. However, the signaling mechanism underlying the regulation of sAPPα release mediated by muscarinic receptor activation is not understood.

PLA_2 is a heterogeneous group of enzymes that specifically hydrolyze fatty acid from the sn-2 position of cell membrane phospholipids. The products of PLA_2 catalyzed phospholipid hydrolysis include arachidonic acid (AA) and lyso-platelet activating factor (PAF), which are precursors of a wide spectrum of pro-inflammatory mediators like prostaglandins, thromboxanes, leukotriene, and PAF [16]. Moreover, the stimulation of G-protein coupled receptors (GPCRs) activates cytosolic PLA_2, and the receptor-mediated activation of PLA_2 generates free fatty acids (e.g., AA) and lysophosphatidylcholine from membrane phospholipids [7,6]. An initial study suggested that PLA_2 could partially mediate the muscarinic receptor stimulation of sAPP formation [5], and subsequent studies have extended these results to include both the serotonergic [17] and glutamatergic

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[18] regulation of APP processing. Likewise, the inhibition of COX, an enzyme that metabolizes AA, increases sAPP release in human glioma cells [18]. Moreover, it has been reported that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence and progression of AD [2,23,29]. Weggen et al. [29] demonstrated that a subset of NSAIDs preferentially downregulate highly amyloidogenic Aβ42 peptide, which is produced in a variety of cultured cells, independently of COX activity, and recently, NSAIDs were reported to markedly reduce the levels of the cellular APP holoprotein, thus further accelerating non-amyloidogenic processes like sAPPα release [1]. The purpose of the present study was to investigate the involvement of a signaling mechanism, especially PLA_2-related pathways, in the regulation of sAPPα release mediated by muscarinic receptor activation in a human neuronal cell line, SH-SY5Y, which endogenously expresses both muscarinic receptors (M3 and M1) and APP.

To determine the effects of PLA_2 inhibitors, COX or LOX, on muscarinic receptor-mediated sAPPα release, the equal numbers of SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were cultured in multiwell dishes (6-well format) to confluence in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37 °C under humidified conditions in a 5% CO_2 atmosphere. After pretreating cells with various agents in serum-free media for 30 min, cells were treated with oxotremorine M (oxoM; Research Biochemicals International, Natick, MA, USA) in serum-free media for 1 h. Conditioned media from each well were centrifuged to remove debris by micro-centrifugation for 5 min at 4 °C, desalted using PD-10 desalting columns (Amersham Pharmacia Biotech), dried, and reconstituted in a SDS loading buffer (125 mM Tris, 2% w/v SDS, 10% glycerol, 1% bromophenol blue and 10% β-mercaptoethanol; pH 6.8). Total protein in the cell lysates per well was measured with the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were fractionated by sodium dodesyl-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Normalization of protein loading on each blot was obtained by loading a sample of concentrated conditioned medium, standardized to the protein concentration in the cell lysates. Membranes were incubated in a blocking buffer containing 5% bovine serum albumin in Tween-containing Tris-buffered saline (TTBS; 137 mM NaCl, 20 mM Tris–Cl, 0.1% Tween 20, pH 7.6) for 2 h and then incubated for 2 h at room temperature with a monoclonal antibody to sAPPα (clone 6E10, 1:200), which recognizes amino acid residues 1–17 of APP (Chemicon International, CA, USA). Blots were washed four times with TTBS before adding secondary antibody (horse peroxidase conjugated goat anti-mouse at a dilution of 1:3000) in TTBS containing 3% BSA. After 1 h incubation, blots were washed four times with TTBS. The sAPPα band signals were detected by enhanced chemiluminescence by following the manufacturer's recommendations (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Data collection and processing were performed using a luminescent image analyzer LAS-1000 and IMAGE GAUSE software (Fujifilm, Japan). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from the control sample assayed under the sample conditions. Controls were processed in parallel with stimulated samples and always included in the same blot.

To measure prostaglandin E_2 (PGE_2) release, SH-SY5Y cells were cultured in 12-wells dishes, pretreated with indomethacin and/or AA in serum-free media for 30 min, and then treated with vehicle or oxoM in serum-free media for 1 h. The PGE_2 tracer were added to PGE_2-antibody 96-well plates and incubated at room temperature for 18 h. After incubation, plates were washed to remove any unbound reagents and then treated with Ellman's Reagent containing acetylthiocholine and 5,5′-dithiobis-(2-nitrobenoic acid). After incubating plates for 7 h, optical densities were measured at 405 nm. Color intensities were proportional to the amount of PGE_2 tracer bound to the wells, which is inversely proportional to the amount of free PGE_2 present in wells during incubation.

Statistical analysis was done by one-way ANOVA followed by two-tailed Student's *t*-test; a value of $p < 0.05$ was considered significant.

AA, PGE_2 and melittin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Indomethacin, esculetin, manoalide were purchased from Biomol (PA, USA). Stock solutions were prepared by dissolving compounds in dimethyl sulfoxide (DMSO), and then further diluted in serum-free medium. Final DMSO concentrations did not exceed 0.2% (v/v).

We found that in SH-SY5Y cells, sAPPα secretion to culture medium was significantly increased by treating with the muscarinic agonist oxoM, in a time-dependent manner, and that oxoM-induced sAPPα release was blocked by pretreating with the muscarinic receptor antagonist atropine (Fig. 1A). This observation correlates well with previous observations, whereby the activations of muscarinic receptors (M1 and M3) increased sAPPα release [20,26]. To assess the stimulation of muscarinic receptor-mediated sAPPα release by PLA_2 activation in SH-SY5Y cells, we first examined that the effect of a PLA_2 inhibitor on sAPPα release. Muscarinic receptor-mediated increased sAPPα secretion was effectively blocked by the non-selective PLA_2 inhibitor, manoalide (3.2 μM), indicating that PLA_2 may play a role in the cellular signaling cascade coupled to muscarinic receptor-mediated sAPPα secretion in SH-SY5Y cells. Moreover, treatment with melittin (a non-selective PLA_2 activator, 5 μg/ml) stimulated basal sAPPα release and induced an additive increase in muscarinic agonist-induced sAPPα release (Fig. 1B). Treatment with agents used in this study had no effect on the full-length cellular APP leves (data not shown). Our data suggests that PLA_2 pathway is involved in the muscarinic receptor stimulation of sAPPα formation in SH-SY5Y cells.

We also investigated whether the COX and LOX pathways (downstream of PLA_2) are involved in sAPPα secretion. To determine the effect of COX activity on sAPPα secretion, we treated SH-SY5Y cells with COX inhibitors, indomethacin and nimesulide, to observe their effects on sAPPα release. Treatment of SH-SY5Y cells with indomethacin increased basal sAPPα release into medium. However, muscarinic receptor-mediated sAPPα release was unchanged by indomethacin treatment. Another COX inhibitor, nimesulide, showed same effect