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The Role of Phospholipase D in Amyloid β Precursor Protein Processing

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Abstract: The generation of a secreted N-terminal fragment of the amyloid precursor protein (AβPPs) can be stimulated by a variety of signaling pathways many of which are also known to modulate the activity of the phospholipase D (PLD) enzyme. This study used primary rat neuronal cerebellar granule (CG) cultures and SH-SY5Y human neuroblastoma cell lines to determine the potential role of PLD in the protein kinase C (PKC)-associated generation of AβPPs. Protein release was markedly enhanced by direct PKC stimulation following treatment of both cell type with either phorbol ester or indirectly by the muscarinic agonist carbachol and these effects were greatly attenuated by co-incubation with the PKC inhibitor GF109203X. A partial inhibition of PKC- and carbachol-stimulated AβPPs secretion was also achieved by pre-treatment of the cells with toxin B, a PLD inhibitor. This suggested that PLD may play a role downstream of PKC in the control of AβPPs secretion.

Key words: Alzheimer, muscarinic, protein kinase C, toxin

INTRODUCTION

The amyloid precursor protein (AβPP) is widely expressed in eukaryotic cells[1]. Following transcription, the newly synthesised proteins are transported in lipid vessicles via the ER/Golgi to the neuronal plasma membrane where they have a rapid turnover rate due to the actions of a variety of proteolytic processing pathways[2]. Cleavage of the protein proximal to the plasma membrane by the α-secretase protease enzyme[3] generates a soluble N-terminal polypeptide, AβPPs, which exhibits both neurotrophic and neuroprotective actions. Alternatively, the Aβ peptide, which forms the basis of the neuritic plaques characteristic of Alzheimer's disease (AD) pathology, can be generated from AβPP by the β and γ-secretase cleavage pathway[4]. Therefore, there is a delicate balance between the secretory and amyloidogenic processing pathways, with the two considered as being mutually exclusive.

A diverse number of factors can influence the processing of AβPP following the activation of specific signal transduction pathways[2]. These include the heterotrimeric G protein-coupled metabotropic glutamatergic and adrenergic receptors, the EGF- and PDGF-activated phosphotyrosine receptor kinase[5] and steroid receptors[6,7]. The stimulation of AβPPs release can also occur by receptor-independent activators such as phorbol ester-activation of protein kinase C or elevated intracellular calcium concentrations that may activate a variety of cell signaling pathways which have been identified by the use of specific inhibitory agents[8]. This strategy has identified a phospholipase C (PLC)-mediated signaling pathway as a likely candidate for the coupling between muscarinic receptor activation and AβPPs secretion since there is a correlation between protein secretion and the generation of the PLC product DAG, which subsequently activates PKC[9,10]. The receptor-associated elevation in DAG is transient and returns to basal levels within minutes whilst AβPPs generation can be maintained for much longer time periods suggesting that other downstream mechanisms may also be involved in the process. Another potential mechanism for the prolonged phase of AβPPs secretion is the activation of the enzyme phospholipase D (PLD)[11]. This enzyme can also be stimulated by a variety of many activators that are common to PLC although it has a more sustained period of activation[12].

PLD catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline with the latter serving as a precursor for acetylcholine biosynthesis[12]. In AD, a deficit in the cholinergic neurotransmitter system contributes significantly to the cognitive decline that is

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characteristic of the disease[13]. This investigation was carried out to determine the specific role of PLD in the secretion of the soluble AβPPs fragment.

MATERIALS AND METHODS

Cell culture: Primary cerebellar granule (CG) cells were isolated and cultured as described previously[14]. Briefly, cerebella from 7-day old Wistar rats (Harlan, U.K.) were mechanically and enzymatically triturated with insoluble tissue clumps being removed by centrifugation. The cell pellet was resuspended at a density of $3x10^6$ cells/mL in minimal essential medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS), 30 mM glucose, 2 mM glutamine, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were cultured in 6 well tissue culture plates (30 mm diameter, Nunc) pre-coated with poly-D-lysine at a density of $3-4x10^6$ cells cm^{-2} and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Fresh culture medium containing 20 mM cytosine arabinose was added after 24 h to inhibit astroglial cell proliferation. The cells were routinely used for experimentation at days 6-7 after plating. The procedures used here were designed to minimize animal suffering and were carried out in accordance with the recommendation of the EEC (86/609/EEC).

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and containing penicillin 2 U mL^{-1} and streptomycin (0.25 mg mL^{-1}) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultures in 6 well plates at an initial density of $2.5x10^5$ and the experiments were conducted on the cells when they reached approximately 70% confluency.

Measurement of AβPPs secretion: The FCS-containing culture medium was removed from the cells that were washed once with serum free media (SFM) prior to the addition of 1 mL of SFM for 2 h to each well in order to remove any FCS-associated AβPPs that may have become loosely attached to the plastic surface. The medium was then removed and replaced with fresh SFM containing either carbachol (1 mM) or PDBu (100 nM). Where inhibitors were tested, GF109203X (2-5 μM) was added to cells in SFM 5 min prior to the addition of stimulus while Toxin B from *Clostridium difficile* (a generous gift from Prof. K.H. Jakobs, University of Essen) was present for 18 h in the serum-containing media, throughout washes and the subsequent incubation of the cells in SFM. This represents the optimal time course for PLD inhibition by the toxin[15]. At the end of the 1 h incubation, the medium was collected, the protein content precipitated with 10% (w/v) trichloroacetic acid and the AβPPs content determined by western blot analysis as previously described using the 22C11 antibody which recognises an N-terminal epitope on AβP [8]. The protein bands were visualized by enhanced chemiluminescence (ECL, Amersham, U.K.) and quantified using the Scion Image software (Scion Corporation).

Determination of cytoplasmic calcium responses in CG cells: The measurement of cellular calcium flux has been previously described[16]. Briefly, CG neuronal cells cultured on cover slips were pre-loaded with the fluorescent fura-2/AM dye (1-2 μM) for 30 min at 37°C. Calcium measurements were performed at 36-37°C in a thermostatically controlled chamber on the stage of a Nikon diaphot fluorescence microscope. Changes in intracellular calcium levels were estimated from the ratio of fura-2 emission at 510 nm with alternate excitation at 340 and 380 nm. Fura-2 images for digital analysis were generated by an 8-bit camera and analysed with Miracal software[16].

Statistical analysis: Statistical significance of differences between values was assessed by conducting analysis of variance (ANOVA) followed by Bonferonni's post hoc comparison test using Graphpad Prism software (GraphPad Software Inc.) with $P<0.05$ being considered as statistically significant.

RESULTS

Previous reports have characterised both the constitutive and PKC-stimulated release of AβPPs from various cell lines and tissue preparations[8,17]. The initial experiments in the present study confirmed the constitutive release of AβPPs from SH-SY5Y human neuroblastoma cells and primary rat neuronal CG neuronal cells and their relative responsiveness to the muscarinic agonist carbachol in addition to direct PKC activation by PDBu in order to verify the functionality of the experimental system. The 22C11 antibody recognises an epitope that is present on both AβPP and the homologue APLP2, although the protein detected will be referred to as AβPP in this study. There was a robust and reliable basal level of protein secretion from both cell preparations that was detectable after 1 h. In general, the SH-SY5Y cells were more responsive to the stimulation of muscarinic receptors or the activation of PKC with an average 7-8 fold increase over basal (Fig. 1A) when compared with a 3-4 fold increase in the CG cells (Fig. 1B).

Pre-treatment of both cell lines with the PKC inhibitor GF109203X resulted in a significant attenuation of both carbachol- and PDBu-stimulated AβPPs secretion (Fig. 1). This provides robust evidence that PKC plays a key role

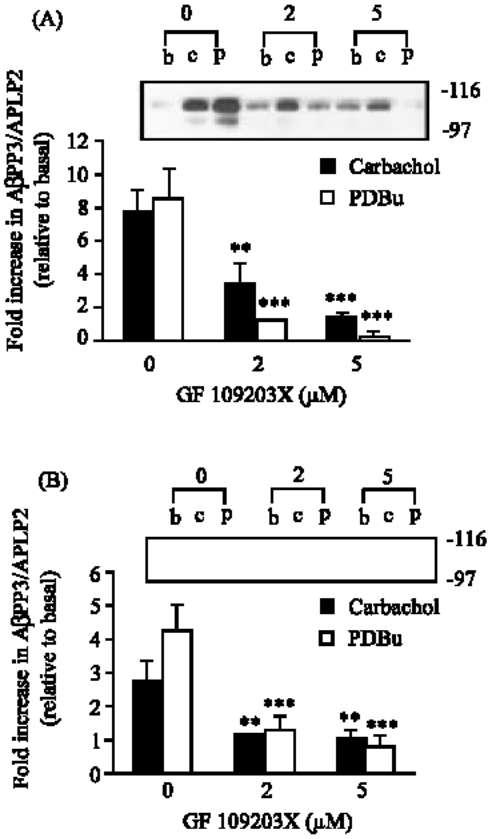

Fig. 1: The effect of 10 min pre-treatment with the protein kinase C inhibitor G F109203X on carbachol (c)- and PDBu (p)-stimulated AβPPs secretion from (A) SH-SY5Y and (B) cerebellar granule cells as determined by densitometric analysis of the protein bands. The values indicated the fold increase over basal (b) (unstimulated) after treatment with agonist for 1 h and are represented as mean±SEM (n=3). * indicated $p<0.05$ and ** indicated $p<0.01$ versus control (no drug) (ANOVA followed by Bonferonni's post-hoc comparison test). A representative blot of AβPPs expression is also displayed

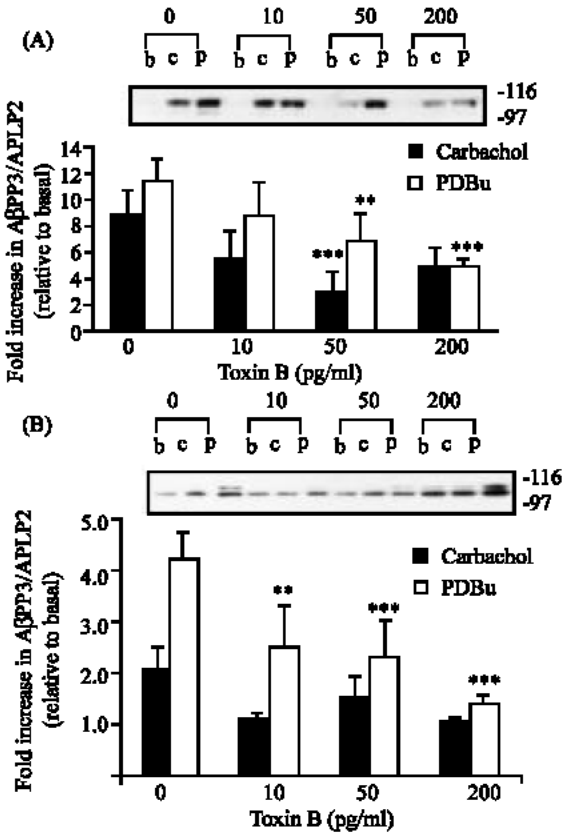

Fig. 2: The effect of 18 h pre-treatment of the phospholipase D inhibitor Toxin B on carbachol- and phorbol dibutyrate-stimulated AβPP secretion from (A) SH-SY5Y and (B) cerebellar granule cells as determined by densitometric analysis of the protein bands. The values indicated the fold increase over basal (unstimulated) after treatment with agonist for 1 h and are represented as mean±SEM (n=3). * indicated $p<0.05$, ** indicated $p<0.01$ and *** indicated $p<0.001$ versus control (no drug) (ANOVA followed by Bonferonni's post-hoc comparison test). A representative blot of AβPPs expression is also displayed

in carbachol stimulation of protein secretion from both primary neuronal and neuroblastoma cells. However, carbachol-stimulated AβPPs secretion from SH-SY5Y cells was only partially inhibited at a concentration of 2 μM while CG cells were more sensitive to the inhibitor with both PDBu- and carbachol-stimulated AβPPs secretion being totally abolished at this concentration of drug.

A partial inhibitor of PLD, toxin B, was used to investigate the potential its role in the regulated component of AβPPs secretion[15]. The toxin partially blocked both the carbachol- and PDBu-associated protein release from SH-SY5Y cells at 50 pg mL^{-1} and PDBu stimulated protein secretion from CG cells at 10 pg mL^{-1}, in parallel with the effects previously observed for GF109203X (Fig. 2). The reduction in carbachol-induced secretion from CG cells failed to achieve statistical significance and may in part be due to the relatively modest response of this cell type to the actions of carbachol.

As the higher doses of Toxin B also elevated slightly the basal levels of AβPPs detected in the medium derived from the CG cells, it was important to determine whether the toxin might be mediating its effects via a

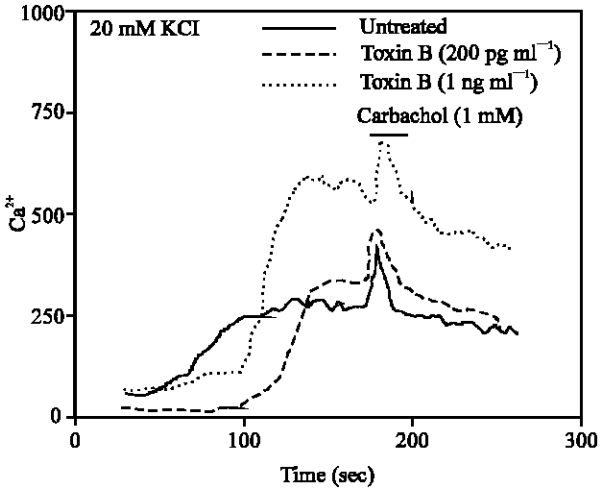

Fig. 3: The effect of Toxin B on the depolarisation-induced calcium influx and the carbachol-induced release of calcium from internal cellular stores in rat CG neuronal cells. Changes in intracellular calcium levels were estimated from the ratio of fura-2 emission at 510 nm with alternate excitation at 340 and 380 nm.

non-specific influence on cell integrity. A disruption of calcium homeostasis is a clear indication of such an effect. We therefore tested the influence of the toxin on the depolarisation-induced calcium influx and the carbachol-induced release of calcium from internal cellular stores using doses of the toxin equivalent to, or higher than, those employed in (Fig. 2). An increase in the depolarisation-associated Ca^{2+} influx that was likely to be associated with a loss of cell integrity was only observed at the highest dose of the drug (1 ng mL^{-1}) (Fig. 3). This suggests that the results obtained (Fig. 2) at the doses of drug used were not due to changes in cellular integrity but were rather directly associated with the direct inhibition of PLD by the toxin.

DISCUSSION

The processing of AβPP is a key biochemical component of CNS function, with both the membrane-bound and secreted forms of the proteins playing distinct but complementary roles in key processes such as synaptic plasticity[18]. Changes in the balance between the particulate and secreted pools of the protein would be expected to have significant functional consequences and there is a significant body of evidence that receptor activation plays an important role in controlling this balance[2,8]. It is therefore of particular importance to have a clear understanding of the role of neurotransmitter receptors and the associated second messenger systems in AβPP processing as alterations in the processing pathways of these proteins exert an influence on the onset and development of AD.

A key role for PKC in AβPPs release from the plasma membrane using a synaptosomal model system was previously demonstrated[8]. The present study has investigated some of the downstream signaling pathways associated with AβPPs release. The activation of AβPPs release by muscarinic receptor stimulation has been well characterised and these studies invariably conclude that the activation of PKC is associated, at least in part, to PLC stimulation[9,19]. The present study used two cell lines to investigate the role of PLD in AβPP processing. The initial characterisation of AβPPs secretion compared the sensitivity of both cell types to a receptor-dependant and -independent activation of the PKC-mediated release of the proteins. The SH-SY5Y human neuroblastoma cells were more sensitive to the actions of PDBu and carbachol that the CG cells. This line has been used extensively to study AβPP processing, although the tumorigenic nature of these cells makes their sensitivity to various growth factors and receptor ligands distinct from those of non-mitotic neurons.

The inhibition of muscarinic receptor-stimulated release of AβPPs by the PKC inhibitor GF109203X confirmed that carbachol acts primarily via at a PKC-associated second messenger system. However in the SH-SY5Y cells, the partial inhibition of carbachol-induced release at the lower dose of the drug (2 mM) when compared with a complete abolition of direct PKC activation by PDBu, highlights potential differences between these mechanisms. GF109203X acts by the inhibition of ATP binding to PKC, thus preventing its phosphorylation and the associated catalytic activity[20]. Therefore, it is possible that components of the carbachol pathway may operate in an ATP-independent manner.

To consolidate a role for PLD in both carbachol and PKC-associated AβPP processing, we employed the partial PLD inhibitor toxin B[15]. There was a moderate decrease in carbachol-stimulated AβPPs secretion in the presence of toxin B suggesting that at least a part of the induction pathway is mediated via a PLD downstream component. However, it was interesting to note that PDBu-associated AβPPs secretion was also partially inhibited by toxin B. Again, there was a difference in the response of the SH-SY5Y and CG cells with the latter being much more susceptible to the actions of toxin B[21]. While previous studies have reported that toxin B inhibits muscarinic receptor-stimulated PLD, it has not been shown to modify phorbol ester induced enzyme

activation[15]. Therefore, the inhibition of PKC-mediated AβPPs generation by toxin B may occur via a PLD-independent pathway that may involve other signaling mechanisms that are also be blocked by the toxin. These findings underline the complexity of second messenger component interactions.

Treatment of the CG cells with toxin B resulted in a slight increase in the basal level of AβPPs generation, suggesting that it may be exerting a deleterious effect on the cell. This was investigated using cellular calcium imaging techniques. However, at the highest doses of toxin B used, there was a minimal effect on cell well-being. Therefore, the effect of the concentrations of toxin used in the study on basal AβPPs generation could not be accounted for by a general toxic effect but rather is likely to be due to a specific action on the cellular second messenger systems.

PLD may also play a role in modulating the fusion of synaptic vesicles with the plasma membrane, thus providing an additional pathway by which the enzyme may modify AβPPs generation[22]. It was previously demonstrated that second messenger modification of AβPPs occurs primarily at the level of the plasma membrane[8] and any agents that modify membrane functioning may act to influence the pattern of AβPPs generation. Following PKC activation, however, it is likely that there will be extensive "cross-talk" between PLD and other second messenger pathways that may also influence its role in AβPPs processing.

The roles of numerous second messenger systems in the control of AβPP processing have been investigated with a particular interest in the potential design of therapeutic agents that could decrease the amyloid burden in the early stages of AD and thus attenuate the rate of disease progression. However, such systems are extremely complex with a significant level of interaction between the individual pathways. While the direct or receptor-mediated stimulation of AβPPs generation has been well characterised, this study demonstrated that PLD appears to serve as a downstream component of the effect of PKC-associated events. The results from this study highlight the complexity of the mechanisms underlying the control of AβPP processing and the myriad of factors that may act to influence the biochemical events associated with the amyloidoigenic component of AD pathology.

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