Amyloid beta protein (25–35) stimulation of phospholipases A, C and D activities of LA-N-2 cells

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Abstract [^{3}H]Myristic acid prelabeled LA-N-2 cells were exposed to varying concentrations of amyloid beta protein (25–35), from 20 to 250 μg/ml, and the activation of phospholipases A and D estimated. A progressive increase in phosphatidylethanol formation, a measure of phospholipase D activity, and of free fatty acid release, a measure of phospholipase A activity, was observed over a time–course of 60 min. [^{3}H]Inositol prelabeled LA-N-2 cells were exposed to varying concentrations of AβP, from 20 to 125 μg/ml, and phospholipase C activation was measured. There was an increased release of inositol phosphates in the presence of amyloid beta protein as a function of incubation time. The effects of adrenergic, metabotropic amino acid and bombesin antagonists on the AβP mediated stimulation of phospholipase C activity was investigated. Propranolol, a β adrenergic antagonist, 7-chlorokynurenic acid, a metabotropic amino acid antagonist, and [Tyr^4-D-Phe^{12}]bombesin, a bombesin antagonist, blunted the AβP stimulation of phospholipase C activity in [^{3}H]inositol prelabeled LA-N-2 cells. This suggests that amyloid beta protein activation of phospholipase C may be receptor mediated. The phospholipase C inhibitor U 71322 prevented the activation of phospholipase C by AβP. However, this activation was not effected by tocopherol, propylgallate, or vitamin C.

Key words: Amyloid beta protein; Phospholipase A; Phospholipase D; Phospholipase C; Receptor

1. Introduction

The neuropatholigical hallmark of Alzheimer's disease (AD) is the abundance and pattern of distribution of senile plaques and neurofibrillary tangels [1]. The predominant protein present in these structures is amyloid beta protein (AβP) having a 40–42 amino acid chain length [2]. The possible contribution of AβP to the neuronal degeneration characteristic of AD is largely based upon experimental evidence obtained both from in vitro and in vivo observations. The presence of AβP at the time of hippocampal cell seeding increased the numbers of cells during the first 2 days, suggesting a neurotropic effect, but decreased the numbers of cells at longer times of exposure [3]. The neurotoxicity of AβP or its truncated form containing amino acids 25–35 has been observed in vivo [4] and in vitro [5] and there are reported neurotoxic effects by intracerebral injections of AβP1 → 40 [6, 7]. A possible explanation for this duality of neurotropic and neurotoxic properties may be related to the state of AβP aggregation. Freshly prepared solutions of AβP do not possess neurotoxic properties but 'aged' preparations which contain aggregates are neurotoxic to cell cultures [5, 8]. AβP-generated free radicals and/or lipid peroxides may contribute to this neurotoxicity [9–11]: this was prevented by several antioxidants [11, 12].

The AβP neurotoxicity seen with cultured human fetal cortical cells was not prevented by the classical excitatory amino antagonists kynurenate or 20-amino-5-phosphonovalerate [13]. This suggests that excitatory amino acids or their receptors are not involved in AβP-provoked neurotoxicity. We wish to report that AβP (25–35) stimulates phospholipase A, C and D activities of the human neuroblastoma LA-N-2 cells.

2. Materials and methods

2.1. Materials

[9,10-^{3}H]Myristic acid (39.3 Ci/mmol) and *myo*-[2-^{3}H]inositol (17.6 Ci/mmol) were purchased from Dupont-NEN, Boston, MA. AβP (25–35), propylgallate, tocopherol and L-ascorbic acid were purchased from Sigma Chemicals, St. Louis, MO, and Bachem California, Torrance, CA. L-Norepinephrine bitartrate, *trans*-1-aminocyclopentyl-1,2-dicarboxylic acid (ACPD), propranolol-HCL, 7-chlorokynurenic acid and U 73122 were obtained from Research Biochemicals International, Natick, MA, USA. Bombesin and [Tyr^4-D-Phe^{12}]bombesin were obtained from Sigma Chemicals, St. Louis, MO, USA. Liebovitzs' L-15 medium and heat inactivated fetal calf serum were from Flow Laboratories, Mississauga, Ont. Silica gel G60 TLC plates were from Merck Darmstadt, Germany. Phosphatidylethanol standard was prepared as previously described [14]. The human neuroblastoma cell line, LA-N-2 (passage 81) was obtained from Dr. R. Seeger, UCLA, and maintained as previously described [15].

2.2. Cell prelabeling and analytical procedures

The cell labeling with [^{3}H]myristic acid, lipid extraction and the thin layer chromatographic procedures employed for measurement of phospholipase A (PLA) and D (PLD) activities were as previously described [16]. Confluent monolayer cultures of LA-N-2 cells were incubated with 2 μCi/ml *myo*-[^{3}H]inositol for 48 h in L-15 medium containing 15% fetal bovine serum to label the phosphatidylinositols. The cells were harvested, washed twice with L-15 medium containing 10 mM lithium chloride (LiCl), and these [^{3}H]inositol prelabeled cells were incubated in 1.0 ml L-15 medium containing 10 mM LiCl with the various agonists and antagonists as described, and the reactions were terminated by adding 200 μl of ice-cold 50% trichloroacetic acid (TCA). Samples were transferred into Eppendorf centrifuge tubes, kept on ice for 15 min, were centrifuged and the TCA extracts transferred into 13 × 100 mm glass tubes. The TCA extracts were washed five times with diethylether (1:1 ratio) and the pH of these samples adjusted to neutral with 1 M NaOH. The extracts were applied to Dowex-1 formate columns, and the labeled inositol phosphates were eluted as described [17].

3. Results

3.1. AβP dose–reponses

[^{3}H]Myristic acid or [^{3}H]inositol prelabeled LA-N-2 cells were exposed to varying concentrations of AβP. There was a progressive increase in free fatty acid release, a measure of PLA

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Abbreviations: AβP, amyloid beta protein; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; AD, Alzheimer's disease; PtdETOH, phosphatidylethanol; FFA, free fatty acid.

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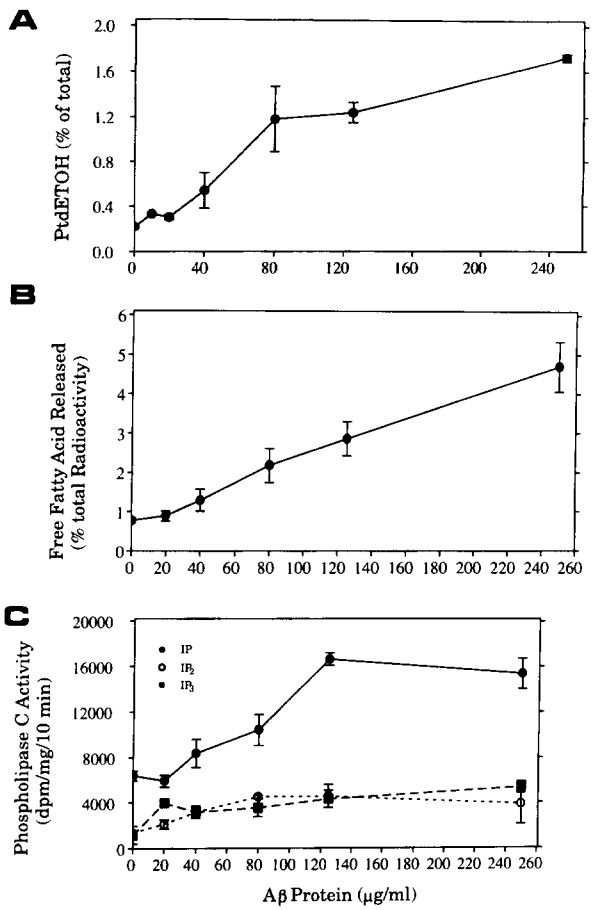

Fig. 1. Activation of phospholipases A, C and D of cultured LA-N-2 cells by increasing amounts of AβP. The final concentrations of freshly prepared AβP are as indicated. The activations of PLD (panel A) and PLA (panel B) were determined with [^{3}H]myristic acid-prelabeled cultures. The appearance of the radioactive inositol phosphates from [^{3}H]inositol-prelabeled cells was regarded as a measure of PLC activation (Panel C).

activity (Fig. 1B) with increasing quantities of AβP present. The amount of PtdETOH, a measure of PLD activity, increased linearly with 20–80 μg AβP/ml and appeared to plateau (Fig. 1A). Inositol monophosphate was the major product of PLC activation and the quantity increased with 20–125 μg AβP/ml to reach a plateau (Fig. 1C).

3.2. Time–course

There was an increase in the appearance of the total inositol phosphates with the duration of exposure to AβP and a plateau was reached by 5 min (Fig. 2B). There was a progressive increase in PtdETOH formation over the complete time–course of 60 min (Fig. 2A). Free fatty acid release reached a plateau by 15 min of exposure to AβP (Fig. 2A).

3.3. 'Aged' AβP

'Aged' AβP was produced by incubation of sterile aquaous solutions at 37°C for 7 days [5] and the ability to activate the phospholipases determined. 'Aged' AβP (25–35) at 125 μg/ml was capable of activating PLA 2.2 fold, PLC 1.8 fold and PLD 3.3 fold.

3.4. PLC activity and receptors

We have previously reported that LA-N-2 cellls have both metabotropic and adrenergic receptors coupled to PLC [18] but not to PLA or PLD. Exposure of the inositol prelabeled cells to bombesin results in an increased release if inositol phosphates. Maximum PLC activation occured with 100 nM bombesin and this was blunted by 10 μM [Tyr^4-D-Phe^{12}]bombesin, its antagonist [19] (Table 1). We investigated the possibility that metabotropic, adrenergic and bombesin receptor occupancy may be involved in the stimulation of PLC activity by 'fresh' and 'aged' AβP. Preincubation of [^{3}H]inositol labeled LA-N-2 cells with either 50 μM propranolol or 50 μM 7-chlorokynurenic acid or 25 μM [Tyr^4-D-Phe^{12}]bombesin for 15 min blunted the formation of inositol phosphates provoked by subsequent exposure in these cells to 'fresh' AβP (Table 2).

3.5. Antioxidants and a PLC inhibitor

U 73122 is a PLC inhibitor and prevents the ligand-stimulated inositol phosphate increase in human neuroblastoma cells [20] pancreatic acinal cells [21] and NG108-15 cells [22]. The presence of 2 μM U 73122 completely suppressed the AβP stimulation of PLC activity. In contrast the presence of 250 μM tocopherol, 25 μM propylgallate or 300 μM–L-ascorbic acid did not effect this AβP stimulation. (data not shown).

4. Discussion

AβP (25–35) activates the phospholipases A, C and D of LA-N-2 cells in a dose- and time-dependent manner (Figs.1 and 2). Previous attempts to relate AβP effects to a particular receptor type have been either unsuccessful or inconclusive. Indeed there is no consensus currently available supporting the possibility that the cellular responses to AβP are dependent upon receptor occupancy. A possible mechanism contributing to the neurotoxic effects of AβP focused upon calcium homeostasis [23]. Exposure of hippocampal cultures to aggregated AβP caused elevations in intracellular calcium [24]. The neurodegeneration of primary cortical neuronal cultures exposed to AβP was prevented by the presence of nimodipine, a calcium channel blocker, supporting a possible role for calcium in the AβP neurotoxicity [25]. The neurotoxicity of AβP toward hippocampal cultures was not prevented by several voltage- or ligand-gated blockers [26] employing different culture conditions than by others [25]. Elevated internal calcium levels could be responsible for the phospholipase A and D activations since these are calcium-dependent enzymes [27].

The pretreatment of inositol-prelabeled LA-N-2 cells with propranolol or 7-chlorokynurenic acid or [Tyr^4-D-Phe^{12}]-

Table 1
Activation of phospholipase C (PLC) by bombesin and prevention by [Tyr^4-D-Phe^{12}]bombesin[a]

	Total inositol phosphates[b]
Control	388,399 ± 87,902
Bombesin (100 nM)	1,173,770 ± 420,599[c]
[Tyr^4-D-Phe^{12}]bombesin (10 μM)	229,343 ± 23,563
Bombesin (100 nM) + [Tyr^4-D-Phe^{12}]bombesin (10 μM)	301,166 ± 49,160

[a] Representative of 3 separate experiments performed in triplicate.
[b] Expressed as DPM's/mg protein/10 min.
[c] $P < 0.01$ vs. controls.

bombesin blunted the PLC activation provoked by AβP (Table 2). Thus, these studies suggest that AβP stimulation of PLC activity may be mediated through adrenergic, metabotropic and bombesin-type receptors. The prevention of AβP activation of PLC by U 73122 was anticipated since this compound inhibits PLC activation by a number of agonists in a number of cell types. The inability of the antioxidants, tocopherol, propylgallate and vitamin C, to blunt the AβP activation of PLC indicates that this activation is not mediated by free radical formation.

Increased quantities of free *myo*-inositol have been detected by ^{1}H NMRS in vivo [28] and in perchloric acid extracts of autopsy [29] tissues from Alzheimer's disease patients. It is possible that this elevation of inositol might be a reflection of increased PLC activation in Alzheimer's disease. There are no detectable quantitative differences in PLC activity of membranes derived from Alzheimer's patients as compared to controls [30,31], however, responsiveness in vitro to agonists or the preponderance of a particular PLC isoform are altered. Perhaps the increased *myo*-inositol observed in the brain tissue of Alzheimer's disease individuals is a consequence of the AβP activation of PLC. The excessive quantities of AβP in proximity to the cell surface could activate phospholipases and, therefore, trigger second messenger cascades which would provoke an unnecessary cellular response. Continuous aberrant activation could be deleterious to neurons and ultimately lead to the neuronal loss seen in Alzheimer's disease.

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Table 2
The effect of β adrenergic, metabotropic and bombesin antagonists on PLC stimulation by 'fresh' and 'aged' AβP (25–35)

Treatment	Total inositol phosphates[a]	
A. Fresh		
Control	311,149 ± 39,067	
AβP (125 μg/ml)	764,194 ± 116,873[b]	(146%[c])
AβP (125 μg/ml) + 50 μM propranolol	296,465 ± 53,415	
AβP (125 μg/ml) + 50 μM 7-chlorokynurenic acid	272,293 ± 64,842	
AβP (125 μg/ml) + 25 μM [Tyr^4-D-Phe^{12}] bombesin	272,533 ± 9,975	
B. Aged		
Control	165,129 ± 21,252	
AβP (250 μg/ml)	317,407 ± 78,821[d]	(92%)
AβP (250 μg/ml) + 500 μM propranolol-treated cells	212,206 ± 62,173[e]	(29%)
AβP (250 μg/ml) + 500 μM 7-chlorokynurenic acid-treated cells	180,018 ± 41,964[f]	(10%)
AβP (250 μg/ml) + 250 μM [Tyr^4-D-Phe^{12}]bombesin-treated cells	212,992 ± 28,192[e]	(28%)

All values are the average ± S.D. for independent experiments, each conducted in triplicates.
[a] Expressed as DPM's/mg protein/10 min.
[b] $P < 0.001$ vs. control cells.
[c] Values in parenthesis are percentage increases compared to controls.
[d] $P < 0.005$ vs. control cells.
[e] $P < 0.025$ vs. control cells.
[f] Not significantly different.

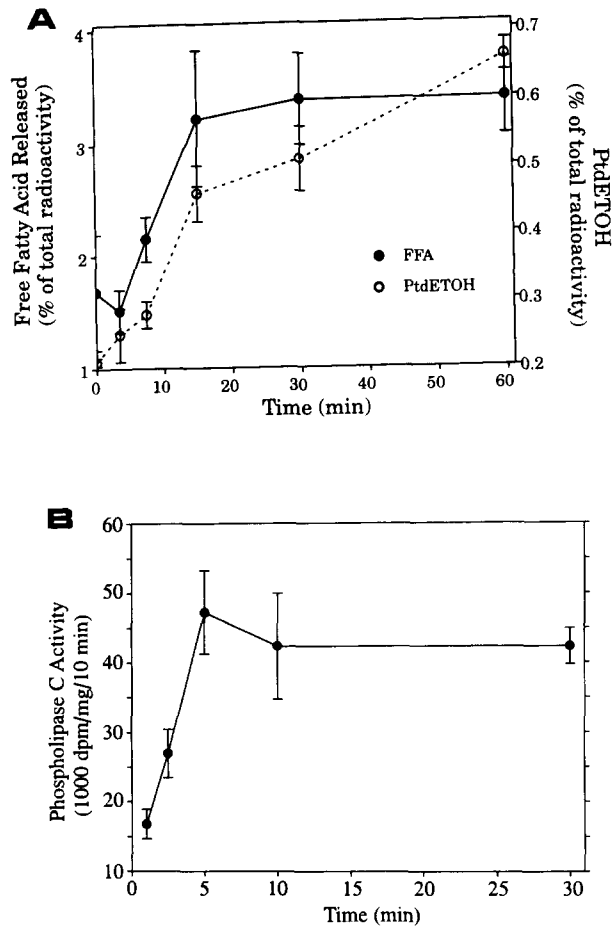

Fig. 2. Time-course of the activation of PLC (panel B) PLA (panel A) and PLD (panel A) by AβP. The concentration of AβP was 125 μg/ml for both the [^{3}H]inositol- and the [^{3}H]myristate-prelabeled cells.

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