Activation of LA-N-2 cell phospholipases by single alanine substitution analogs of amyloid β peptide (25–35)

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Abstract A series of single alanine substituted analogs of amyloid β peptide (25–35) were tested for their ability to activate the phospholipases of cultured LA-N-2 cells. Substitution of alanine for the amino acids 29–34 prevented the activation of phospholipases A2 and D. In addition substitution of alanine at 28 prevented phospholipase D but not phospholipase A2 activation. All the alanine substitutions, except for positions 33 and 35, blunted phospholipase C activations. There were no activations by scrambled amyloid β peptide.

Key words: Alzheimer's disease; Amyloid β peptide; Phospholipase A2; Phospholipase C; Phospholipase D; Analogue

1. Introduction

It has been suggested that amyloid β peptide (AβP), a major component of the senile plaques characteristic of Alzheimer's disease (AD) [1], contributes to the pathophysiology of this disease [2,3]. This suggestion is supported by the observation that AβP can be neurotoxic for neuronal cells [4]. The region of the full length AβP (1–42) associated with its neurotoxic properties is present in AβP (25–35) [5–7]. Systematic amino acid substitutions localized the neurotoxic region from 27 to 33 of AβP (25–35) [7,8].

We have observed that AβP (25–35) was capable of stimulating the phospholipase A, C and D activities of LA-N-2 cells in a dose dependent manner [9] and have characterized the activation of phospholipase A2 in detail [10]. A series of single alanine substituted AβP (25–35) analogs [8] were tested for their ability to stimulate the phospholipase A2, C and D activities of LA-N-2 cells. Because alanine is the simplest L-amino acid, this substitution is useful for identifying functional residues in biologically active peptides.

2. Materials and methods

2.1. Materials

[5,8,9,11,13,14,15-^{3}H]Arachidonic acid, 209 Ci/mmol, [2-^{3}H]myo-inositol, 17.6 Ci/mmol, and [9,10-^{3}H]myristic acid, 51 Ci/mmol, were purchased from Amersham Life Sciences, Oakville, Ont., Canada. Leibovitz L-15 medium and heat-inactivated fetal calf serum were obtained from ICN Pharmaceuticals, Costa Mesa, CA, USA. Dowex AG-1X8 formate (200–400) mesh was obtained from Bio-Rad Labs, Hercules, CA, USA. Silica gel G60 thin layer chromatography (TLC) plates were from Merck, Darmstadt, Germany. Arachidonic acid, diglycerides and phosphatidic acid (PtdA) were from Serdary Research Labs, Englewood Cliffs, NJ, USA. Phosphatidylethanol (PtdEtOH) was from Avanti Polar-Lipids, Inc., Alabaster, AL, USA. AβP (1–40) was purchased from Bachem, Torrance, CA, USA. The single alanine substituted AβP (25–35) analogs were synthesized as described [8] and the sequences are shown in Fig. 1. AβP (28–34) and AβP (1–42) were synthesized in a similar manner. The peptides were dissolved in water to provide stock solutions of 10 mg/ml. The human neuroblastoma cell line LA-N-2 was obtained from Dr. Robert Seeger of the University of California, Los Angeles, CA, USA, and maintained as previously described [11].

2.2. Measurement of phospholipase D (PLD) activation

The LA-N-2 cells were prelabeled for 48 h with [^{3}H]myristic acid (5 μCi/ml) in L-15 medium, containing 15% fetal calf serum to provide a pool of radioactive lipids. The radioactive medium was removed and the cells rinsed three times with fresh L-15 medium containing 1 mg/ml BSA. The cells received L-15 medium containing 0.5% ethanol (85 mM), the various analogs at a final concentration of 100 μM, and the flasks were returned to the incubator for 1 h. The PtdEtOH produced was quantitated as previously described [12]. The lipid extracts of the cell pellets were subjected to TLC, after the addition of PtdA and PtdETOH carrier, with the upper organic phase from ethylacetate/isooctane/acetic acid/water (130:20:30:100) as solvent. The quantity of radioactivity present in PtdETOH is expressed as percentage of total radioactivity recovered from the TLC plate for each sample.

2.3. Measurement of phosphoinositide (PtdIns) specific phospholipase C (PLC) activation

Confluent monolayer cultures of LA-N-2 cells were incubated with myo-[^{3}H]inositol (2 μCi/ml) for 48 h in L-15 medium containing 15% fetal calf serum in order to label the cellular PtdIns. The cells were harvested, washed twice with L-15 medium containing 10 mM LiCl, and these [^{3}H]inositol prelabeled cells were incubated in 1.0 ml L-15 medium containing 10 mM LiCl at 37°C for 10 min with the various analogs at a final concentration of 100 μM. The neutralized trichloroacetic acid extracts of the cell pellets were applied to Dowex-1-8-formate columns and the [^{3}H]inositol phosphates eluted. The inositol phosphates produced were quantitated as previously described [13] and results expressed as total inositol phosphates which is the sum of inositol monophosphate+inositol diphosphate+inositol triphosphate. Protein was quantitated by a standard procedure [14].

2.4. Measurement of phospholipase A2 (PLA2) activation

Confluent monolayer cultures of LA-N-2 cells were incubated with [^{3}H]arachidonic acid (1 μCi/ml) for 48 h in L-15 medium containing 15% fetal calf serum to provide a pool of radioactive lipids. The cells were rinsed 5 times with serum-free L-15 medium containing 1 mg/ml of fatty acid-free BSA. Treatment with the various analogs at a final concentration of 100 μM was carried out in serum-free L-15 medium. The [^{3}H]arachidonic acid produced was quantitated as previously described [10]. The lipid extracts of the cell pellets were subjected to TLC, after addition of arachidonic acid and diglyceride carrier, with petroleum ether/diethyl ether/glacial acetic acid (70:3:2) as solvent. The quantity of radioactivity present in arachidonic acid is expressed as a percentage of the total radioactivity recovered from the TLC plate for each sample.

All experiments were carried out in triplicate and each experiment was repeated on three separate occasions. Data are presented as mean ± S.D. and the Student's *t*-test for *P* value estimation.

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25					30					35	
Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	Met	AβP(25-35)
Ala											G25A
	Ala										S26A
		Ala									N27A
			Ala								K28A
				Ala							G29A
					Ala						I31A
						Ala					I32A
							Ala				G33A
								Ala			L34A
										Ala	M35A
Ile	Met	Leu	Lys	Gly	Asn	Gly	Ala	Ser	Ile	Gly	Scrambled

Fig. 1. Amino acid sequences of AβP (25–35) and its analogs.

3. Results

3.1. AβP (25–35) analogs

The results of incubating these 12 peptides on the activation of phospholipases A2, C and D are shown in Fig. 2.

3.1.1. Phospholipase A2. The information contained in Fig. 2A shows that there is little detectable PLA2 activity in untreated LA-N-2 cells and that the activity is increased by AβP (25–35). Comparable activations were obtained with S26A, N27A, K28A and M35A. In contrast the activations by G29A, I31A, I32A, G33A, L34A and scrambled were comparable to the control values. Activation by G25A was approximately 56% that of AβP (25–35) ($P < 0.025$).

3.1.2. Phospholipase C. The information contained in Fig. 2B shows that there is little detectable PLC activity in untreated LA-N-2 cells and that the activity is increased by AβP (25–35). Comparable activations were obtained with

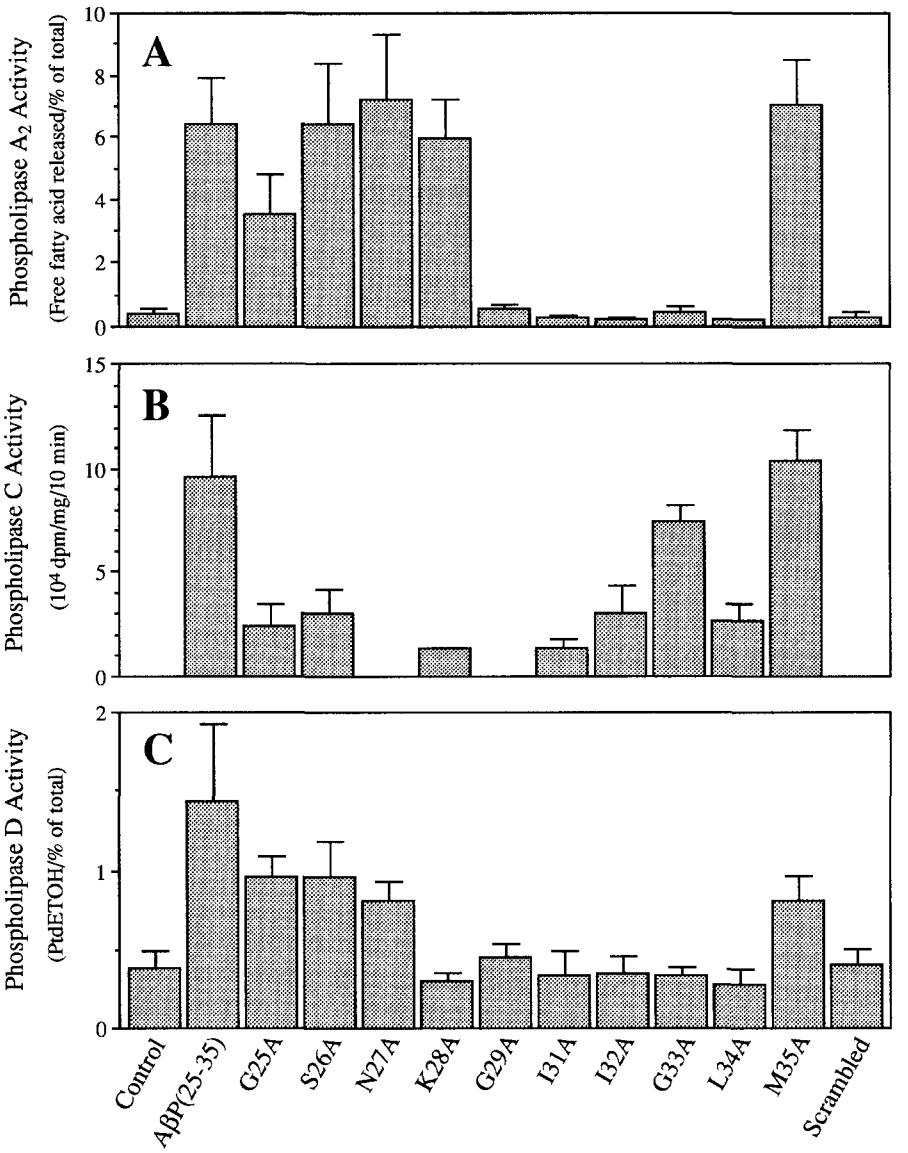

Fig. 2. The activation of phospholipases by AβP (25–35) and its analogs. A: The appearance of radioactive arachidonic acid from [^{3}H]arachidonate prelabeled LA-N-2 cells is the measure of phospholipase A2 activity. B: The appearance of radioactive inositol phosphates from [^{3}H]inositol prelabeled LA-N-2 cells is the measure of phospholipase C activity. The control value for the appearance of [^{3}H]inositol phosphates was subtracted from all the experimental results. C: The appearance of radioactive phosphatidylethanol from [^{3}H]myristic acid prelabeled LA-N-2 cells is the measure of phospholipase D activity.

M35A and perhaps G33A. The activations by G25A, S26A, N27A, K28A, G29A, I31A, I32A, L34A and scrambled were either greatly reduced or comparable to the control values.

3.1.3. Phospholipase D. The information contained in Fig. 2C shows that there is low PLC activity in untreated LA-N-2 cells and the activity is increased by AβP (25–35). Similar activations were obtained with G25A, S26A, N27A and M35A. The activations by K28A, G29A, I31A, I32A, G33A, L34A and scrambled were similar to the control values.

3.2. Comparisons of activations by AβP (1–42), (1–40) and (25–35)

It was relevant to compare the activation of phospholipase A2, C and D by AβP (1–42), AβP (1–40) and AβP (25–35). There was a 2.2-fold, 2.0-fold, and 1.9-fold increase of PLD activity in the presence of 50 μM AβP (25–35), 50 μM AβP (1–42) and 30 μM AβP (1–40) respectively. There was a 3.5-fold, 3.2-fold and 3-fold increase of PLA2 activity in the presence of 50 μM AβP (25–35), 50 μM AβP (1–42) and 30 μM AβP (1–40) respectively. There was a 2.04-fold, 1.95-fold and 1.9-fold increase of PLC activity in the presence of 50 μM AβP (25–35), 50 μM AβP (1–42) and 30 μM AβP (1–40) respectively.

4. Discussion

These studies show that structural requirements exist for the phospholipase activations by AβP (25–35). There is no activation by the scrambled AβP. The structural requirements for phospholipase A2 and D are similar but not identical. The peptides in which the amino acids present at 29–34 were replaced by alanine are inactive for both phospholipases. The lysine at 28 is necessary for PLD but not PLA2 activation. The requirements for PLC activation differ from those for the other two phospholipases. Except for substitution at glycine-33 and methionine-35, replacement of the other amino acid residues by alanine results in a decrease or loss of stimulatory activity. The neurotoxicity for hippocampal neuronal cultures of G25A, S26A, K28A, G29A and M35A was equivalent to that of AβP (25–35) [8]. This suggests some correlations between the previously reported neurotoxicity in hippocampal cultures [8] and LA-N-2 phospholipase activation. AβP (28–34) did not activate the phospholipases. There were similar activations of phospholipases A2, C and D by 50 μM AβP (25–35), 50 μM AβP (1–42) and 30 μM AβP (1–40). These results indicate that AβP (25–35) is as effective an activator of these phospholipases as AβP (1–42).

The four amino acids from the N terminus are hydrophilic and the seven at the C terminus are hydrophobic. The three-dimensional structure of AβP (25–35) was deduced from 2D ^{1}H-NMR spectroscopy. It was concluded that the C terminal region would be buried in the membrane with an α helical conformation and the flexible N terminal would be exposed to the environment [15]. The interaction of AβP (25–35) with 1-palmitoyl-2-oleoyl phosphatidylcholine was examined by X-ray diffraction analysis. The authors suggested that lysine-28 may be at the hydrocarbon-water interface and the seven hydrophobic amino acids positioned in the hydrocarbon core as an α helix [16]. The intercalation of AβP (25–35) may provoke a restricted or generalized membrane perturbation. This might account for the stimulation of phospholipase A2 and D activations (Fig. 2A,C). However, the stimulation of PLC must have an additional factor. The hydrophilic amino acids at the N terminal position of AβP (25–35) are thought to be unrestricted [15] but may interact with phosphate headgroups of the membrane phospholipids [16]. This additional interaction seems necessary for the observed PLC activation in addition to the hydrophobic interactions with the membrane core.

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