Essential and non-essential phosphatidylinositol residues in acetylcholinesterase and arylacylamidase of sheep basal ganglia

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1. INTRODUCTION

Acetylcholinesterase (AChE) is a tightly membrane-bound enzyme in the mammalian system and the most commonly used agent for solubilizing this enzyme is Triton X-100. We have indicated that a serotonin-sensitive arylacylamidase (AAA) is identical with AChE in different mammalian sources [1–3].

Phosphatidylinositol (PI)-specific phospholipase C has found widespread use in the solubilization of membrane-bound enzymes [4–6]. Moreover, sodium deoxycholate (DOC) activated the endogenous PI-specific phospholipase C in the brain and platelets [7–9]. Here we show that PI-specific phospholipase C of *S. aureus* or DOC-activated endogenous phospholipase C solubilizes AChE and AAA of sheep brain basal ganglia and that beyond a critical [DOC], a loss in activity of these enzymes occurs which can be restored by externally added PI.

2. MATERIALS AND METHODS

2.1. *Materials*

PI (soybean), phosphatidylcholine dimyristoyl and dipalmitoyl (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phospholipase A_2 *(Naja naja)*, phospholipase C (*Bacillus cereus*) and DOC were obtained from Sigma Chemicals (St Louis MO). Concanavalin A (con A)–Sepharose was prepared as in [10]. Basal ganglia isolated from sheep brain was kept frozen at −20°C until used. PI-specific phospholipase C from *Staphylococcus aureus* (Cowan strain) was cultured and purified by Amberlite CG-50 column chromatography as in [11]. The peak fraction of phospholipase C eluted from the column was dialyzed against 50 vol. distilled water (6 h) before use. Phospholipids were used as sonicated suspensions in water.

2.2. *Solubilization experiments*

A 10% homogenate of basal ganglia in 50 mM Hepes (*N*-2 hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid)/NaOH buffer (pH 7.4) was prepared (9 mg protein/ml). This homogenate was subjected to solubilization by two methods:

(i) By adding 0.3 ml *S. aureus* phospholipase C (20 units); or

(ii) By adding DOC at different concentrations, to 0.2 ml homogenate in final 0.6 ml vol.in 30 mM Hepes/NaOH buffer (pH 7.4).

The above mixture was incubated for 30–40 min at 37°C with shaking. After cooling the mixture in ice, it was centrifuged at 105 000 × *g* for 60 min and the supernatant was considered as the solubilized portion.

2.3. *ConA–Sepharose column chromatography*

This was done on a 4 × 1 cm column using 2–3 ml solubilized enzyme. The column was washed with 100 ml 0.5 M NaCl/20 mM Tris–HCl buffer (pH 7.4) and eluted with 1 M α-methylglucoside/20 mM Tris–HCl buffer (pH 7.4). The eluted enzyme was dialyzed against 20 mM Tris–HCl buffer (pH 7.4).

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2.4. *Enzyme assays*

AChE, AAA and alkaline phosphatase were assayed as in [3,12,13]. Phospholipase C activity was assayed as in [11] using 1 mg PI in the incubation mixture and one unit of activity was defined as 0.01 μmol water-soluble organic phosphorus released in 90 min at 37°C.

3. RESULTS

3.1. *Solubilization by* S. aureus *phospholipase C*

Under the conditions of solubilization described, PI-specific phospholipase C solubilized ~60% of both AChE and AAA from basal ganglia. Use of higher units of phospholipase C (up to 26 units) or longer incubation periods (up to 60 min) did not result in anything >65% solubilization. There was no loss of enzyme activities as the insolubilized activity was fully recovered in the 105 000 × *g* sedi-

Table 1

Solubilization of AChE and AAA from sheep basal ganglia by *S. aureus* phospholipase C

Addition	% Activity released into 105 000 × *g* supernatant	
	Ache	AAA
No addition	9	10
Phospholipase C	61	60
+ 2 mM $CaCl_2$	61	60
+ 10 mM $CaCl_2$	36	39
+ 0.2 M KCl	32	32
+ 100 μM PMSF	60	60
+ 1 mg PI	48	47
+ 2 mg PI	25	26
+ 4 mg PI	15	16
+ 1 mg PC	57	56
+ 4 mg PC	49	48

Sheep basal ganglia homogenate in 50 mM Hepes/NaOH buffer (pH 7.4) was incubated as in section 2 with the additions shown, for 40 min at 37°C then centrifuged at 105 000 × *g* for 60 min. Phospholipase C used was 20 units. The supernatant was assayed for AChE and AAA. Values are expressed as % of the activity of an unincubated control homogenate. (There was no detectable loss of enzyme activity when the homogenate was incubated at 37°C for 40 min.) $CaCl_2$, KCl, PMSF, PI or PC alone at the concentrations used did not affect AChE or AAA activity or their solubilization as observed in appropriate control tubes

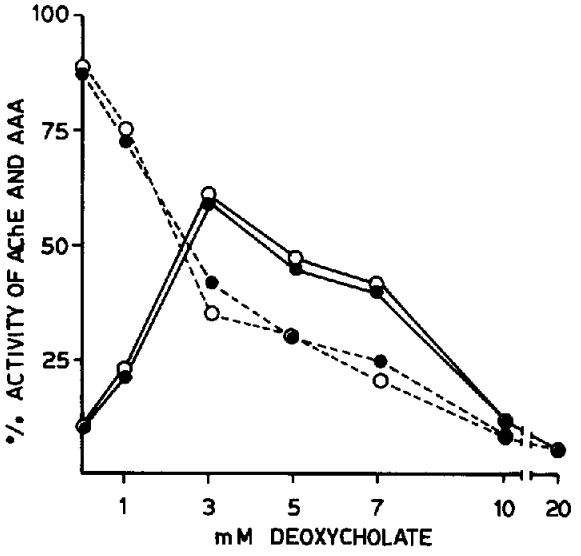

Fig.1. % activity of AChE (-•-) and AAA (-○-) in the 105 000 × *g* supernatant and 105 000 × *g* residue of basal ganglia homogenate after DOC treatment. The homogenate was incubated with DOC at various concentrations as in section 2. After incubation at 37°C for 30 min, it was centrifuged at 105 000 × *g* for 60 min. AChE and AAA activity in the supernatant and residue (suspended in 50 mM Hepes/NaOH) buffer (pH 7.4) was estimated. Activity is expressed as % of incubated control sample of homogenate: (---) activity in the residue; (——) activity in the supernatant.

ment. Only those fractions from the Amberlite CG-50 column which had phospholipase C activity were able to solubilize the enzymes. Several lines of evidence indicated that the solubilization was brought about by a PI-specific phospholipase C (table 1). Thus 0.2 M KCl a known inhibitor of bacterial phospholipase C(5) reduced the solubilization. Although 2 mM $CaCl_2$ did not affect, 10 mM $CaCl_2$ suppressed solubilization. Addition of 100 μM phenyl-methyl-sulphonyl fluoride (PMSF) an inhibitor of serine protease did not inhibit solubilization. More convincing evidence came from the fact that PI at different concentrations (used as competitive inhibitor) was able to prevent solubilization to a different extent while PC was inadequate to do so. Use of *B. cereus* PC-specific phospholipase C or phospholipase A_2 (up to 100 units) resulted in ≤10% solubilization over the controls. There was no inactivation of AChE and AAA by these phospholipases.

3.2. *Use of DOC for solubilization*

The use of different concentrations of DOC for solubilizing AChE and AAA is shown in fig.1. While the maximum solubilization (60%) in the 105 000 × *g* supernatant was observed at 3 mM DOC, at higher concentrations there was a loss in activity of both AChE and AAA reaching ~80% loss at 10 mM DOC. The loss in activity in the supernatant was not due to decreased solubilization as may be seen from the activity values given for the 105 000×*g* residue in fig.1.

Numerous lines of evidence suggested that the solubilization at 3 mM DOC was due to an endogenous PI-specific phospholipase C. Thus the phospholipase C activity in the basal ganglia homogenate with no DOC, with 3 mM and 10 mM DOC were 2, 24 and 10 units/0.3 ml, respectively. There was only 12–15% solubilization with 3 mM DOC if the pH of the medium was 5.0 or 9.2, values unfavourable for phospholipase C action [7]. Inclusion of 1.2 and 4 mg PI (as competitive inhibitor) with 3 mM DOC at pH 7.4 resulted in 45–50%, 65–67% and 80% inhibition of solubilization of AChE and AAA. Use of 4 mg PC instead of PI resulted in 40% inhibition. This relatively high inhibition by PC may be due to its ability to alter the PC to endogenous PI ratio which is known to affect the phospholipase C activity [14,15].

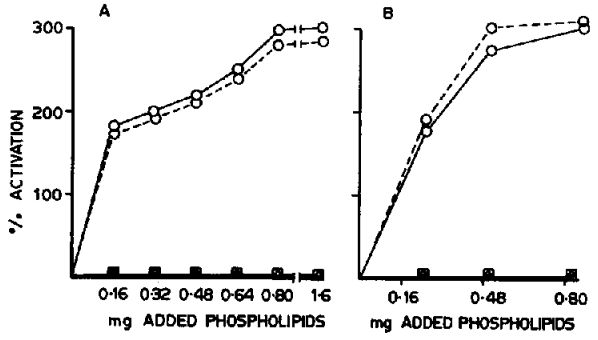

Fig.2. Activation by PI of 10 mM DOC-inactivated AChE and AAA. (A) Basal ganglia homogenate was incubated with 10 mM DOC at 37°C for 30 min and centrifuged at 105 000×*g* for 60 min. The supernatant was purified by passage through con A–Sepharose as in section 2. (B) AChE and AAA solubilized by *S. aureus* phospholipase C from basal ganglia homogenate was treated with 10 mM DOC and purified by con A–Sepharose chromatography as in section 2. PI (○), PC (□), PE (△) and PS (●) were added at the given amounts to 0.15 ml of the purified enzymes, kept for 10 h at 4°C with shaking and then assayed for AChE and AAA activity: (---) AAA activity; (——) indicates AChE activity.

The endogenous phospholipase C activatable by DOC appeared to be a soluble enzyme which needed Ca^{2+} for activity. If the homogenate was first centrifuged at 105 000×*g* and the residue after washing with 50 mM Hepes/NaOH buffer (pH 7.4) subjected to 3 mM DOC treatment, either in presence or absence of 1 mM Ca^{2+}, there was no significant solubilization of AChE and AAA from the residue. Furthermore, the phospholipase C activity was detectable in the 105 000×*g* supernatant which upon dialysis failed to exhibit the activity unless 1 mM Ca^{2+} was included in assay. Mixing the 105 000×*g* supernatant with the 105 000×*g* residue in the presence of 3 mM DOC resulted in 60% solubilization of both AChE and AAA.

3.3. *Inactivation of AChE and AAA by 10 mM DOC and reactivation by PI*

The solubilized enzymes obtained by bacterial phospholipase C or 3 mM DOC treatment after purification by con A–Sepharose chromatography did not exhibit any activation by different phospholipids including PI.

When the bacterial phospholipase C-solubilized enzyme was incubated with 10 mM DOC there was 80% inactivation of both AChE and AAA similar to the observation made when 10 mM DOC alone was used for solubilization. There was no such inactivation when 3 mM DOC was used. This indicated that DOC at 10 mM caused the inactivation of AChE and AAA irrespective of whether the enzyme was solubilized by bacterial or endogenous phospholipase C.

The inactivated, solubilized enzyme obtained either by treatment with 10 mM DOC alone or by incubation of the bacterial phospholipase C solubilized enzyme with 10 mM DOC, were purified by con A–Sepharose chromatography and the effect of various phospholipids on the enzyme activities tested (fig.2A,B). It was found that PI alone but not PC, PE or PS was able to reactivate AChE and AAA up to a maximum of 300% at the highest concentration of PI used. This reactivation resulted in ~60–75% restoration of the activity present in the active solubilized enzyme (either with 3 mM DOC or by bacterial phospholipase C).

3.4. *Alkaline phosphatase*

This membrane-bound enzyme was also solubilized from the basal ganglia to ~65% by the *S. aureus* phospholipase C under these conditions. However, there was no significant inactivation of the alkaline phosphatase by 10 mM or even 20 mM DOC.

4. DISCUSSION

PI-specific phospholipase C has found use as a powerful tool for the release of several insoluble enzymes such as alkaline phosphatase, AChE and 5′-nucleotidase from mammalian membranes [5,6,13]. These studies clearly indicated that either the bacterial or DOC-activated endogenous PI-specific phospholipase C solubilized the basal ganglia AChE and AAA. We also observed that 10 mM DOC inactivated the released AChE and AAA and reactivation could be achieved selectively by PI but not by other phospholipids. This inactivation–reactivation phenomenon was observed specifically for AChE and AAA but not for alkaline phosphatase. It is not yet clear whether the inactivation by 10 mM DOC is due to its detergent action or not, but the specific reactivation by PI suggested that this inactivation involved certain PI residues. The most plausible explanation for our observations is the following:

There are two species of PI involved in the anchoring of AChE and AAA to basal ganglia membrane, one essential for catalytic activity and the other non-essential. While 3 mM DOC or *S. aureus* phospholipase C treatment affect only the non-essential PI residues resulting in solubilization of enzyme, 10 mM DOC affects the essential PI residues needed for catalytic activity. It appears that the disposition of PI in the membrane-bound enzyme is an essential factor for determining its solubilization as well as for protection of its catalytic activity. The identical behaviour of AChE and AAA in the solubilization procedure and reactivation by PI also provide additional evidence for the identity in the lipid environment of these two enzymes.

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