Control of the Activity of Brain Synaptosome-Associated Acetylcholinesterase by Acidic Phospholipids

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Incubation of synaptosomal plasma membranes (SPM) with liposomes of phosphatidylserine (PS), phosphatidylinositol (PIN) or phosphatidylglycerol (PGL), led to an increase of acetylcholinesterase (AchE) activity at concentrations of 0.1—1 μmol phospholipids per mg SPM protein. The use of higher concentrations (1—7 μmol/mg protein), however, led to a progressive inhibition of the activity with respect to the maximal percentage of enzyme stimulation. To explain the enzyme stimulation by the acidic phospholipids, AchE was solubilized with the detergent Lubrol-PX and showed no change in the enzyme activity at any PS, PIN or PGL concentration used, indicating that these compounds do not act on the protein molecule directly. Arrhenius plots of AchE activities in untreated SPM (control), exhibited a break point at 23 °C, which was decreased to 16—17 °C in PS-treated SPM. Moreover, the Arrhenius activation energy (Ea) value in PS-treated SPM was increased related to the Ea below the break point in the control. These results indicate that acidic phospholipids do not act on AchE directly, but indirectly, affecting the membrane fluidity probably. Such modifications of interactions between lipid and AchE may control physiological processes in the central nervous system.

Introduction

Even though the negatively charged phospholipids PS and PIN occur in minute amounts in SPM [1], they cause important physiological processes in the central nervous system. PIN lipids are well known to be involved in synaptic transmission and their catabolism plays an important and perhaps primary central role in the biochemical events associated with the physiological activity [2, 3]. Intravenous injection of a sonicated dispersion of brain phospholipids results in a significant increase both the dopamine-sensitive adenylate cyclase activity, and the cyclic AMP content of mouse brain [4]. Under these experimental conditions, PS liposomes increase in the metabolism of catecholamines in the brain of animals [5], stimulate the release of acetylcholine from rat cerebral cortex and the release of histamine from mast cells [6, 7]. Moreover, PS vesicles (liposomes) interact with biological membranes causing changes of the membrane physicochemical properties [8]. Such alterations of the state in the bilayer can modify the activities of membrane-bound enzymes, e.g. Na⁺, K⁺-ATPase, acetylcholinesterase, Ca²⁺-stimulated ATPase and adenylate cyclase [9—11]. Changes in the viscosity of the membrane can markedly enhance or depress the activities of the enzymes by affecting the conformation, rotation, diffusion and association of their subunits. The fluid properties of the lipid matrix, also, has been suggested to play an important role in the transport of different ligands by membrane-bound proteins [12] and other functions including receptor-neurotransmitter binding and receptor stimulation [13].

This study was carried out in order to investigate: a) whether PS only or other acidic phospholipids as well, can induce changes in the activity of AchE, b) which is the mechanism of action of these compounds.

Material and Methods

Synaptosomal plasma membranes (SPM) from dog brain were prepared and qualitatively assessed as previously described [14]. Phosphatidylserine (PS), phosphatidylinositol (PIN) or phosphatidylglycerol (PGL) liposomes were prepared essentially as previously described [15]. Briefly, 1 ml of above phospholipids in solution (20 mg/ml) in chloroform (Sigma) was pipetted into 20 ml test tubes. The solvent was evaporated under nitrogen. Each of the dried phospholipids was then dispersed in 2 ml of 5 mM Tris-HCl, pH 7.4, by sonication for 20 min at 0 °C under...
nitrogen in a Branson LS-75 sonicator. The lipid dispersions were then centrifuged at 30000 × g for 40 min. The sediment containing large liposomes was discarded. The supernatant was applied to a column (1 cm × 40 cm) of Sepharose-4B (Pharmacia, Uppsala) and eluted with 5 mM Tris-HCl, pH 7.4. Fractions (approx. 1 ml) were collected and analyzed for PS, PIN or PGL respectively. Fractions of PS, PIN or PGL were pooled and kept at 4 °C. They were used within 2 days.

To determine binding of phospholipids-liposomes to SPM, preincubations of SPM protein with different concentrations of PS, PIN or PGL were performed for 3 h at 25 °C in an incubation mixture of 1.15% (w/v) KCl, 5 mM Tris-HCl, pH 7.4, in a final volume of 5 ml, with continuous magnetic stirring. Samples (4 ml) were withdrawn and layered over 30 ml of 20% (w/v) sucrose, 5 mM Tris-HCl, pH 7.4, and centrifuged for 60 min at 95000 × g in a SW-27 rotor of a Spinco L5-75 Ultracentrifuge, so that the bound and free phospholipids were separated. The protein content was determined by the Lowry method as described by Miller [16] using bovine serum albumin (Sigma) as standard. Then, the enzyme activity was determined. The bound and free phospholipids fractions were pooled separately and extracted according to the method of Folch et al. [17]. The pooled chloroform extracts were dried under a stream of nitrogen and the residues were dissolved in 1.0 ml chloroform. Samples of these solutions were directly spotted on a silica-gel G TLC plate (Merck). Samples of chloroform extracts of a mixture of unincubated SPM with each phospholipid and the phospholipid alone were also chromatographed similarly. Chromatography was developed as described by Breckenridge et al. [1].

The migration extracts of membrane-bound and free phospholipids was identical with those of unincubated SPM with each phospholipid and the phospholipid alone, suggesting that no significant metabolic conversion of membrane-bound and free PS, PIN or PGL occurred under the present incubation conditions.

AchE activity was determined by measuring the hydrolysis of acetylthiocholine by the method of Ellman et al. [18] at temperatures varying from 4° to 42°C at 2–3 degree intervals. The assay mixture (3 ml) contained 0.5 mM acetylthiocholine iodide, 0.125 mM 5,5'-dithionitrobenzoic acid, 120 mM NaCl, 0.24 M Sucrose and 0.1 M Tris-HCl, pH 8.0. Protein concentration was 0.1 mg/3 ml incubation mixture. The reaction was followed spectrophotometrically by the increase in absorbance at 412 nm by using a Beckman Acta MVI Spectrophotometer.

Solubilization of the membranes was performed using 0.5% (w/v) Lubrol-PX at 4 °C for 4 h under magnetic stirring, in a medium 0.24 M Tris-HCl, pH 7.4, containing 8.4% (w/v) Sucrose and 1.5 mg of SPM protein per ml. After the solubilization, the sample was centrifuged at 150000 × g for 1 h. The supernate contained 1.2 mg of protein per ml and solubilized AchE [19]. In the assay mixture of the enzyme, the detergent was in a final concentration less than 0.008%, which was not able to influence the measurement of enzyme activity.

Lines were fitted to the data points in Arrhenius plots by regression analysis. Statistical comparisons were made by regression analysis and Student's t-tests.

Results

Previous experiments have shown that of the negatively charged phospholipids incorporated into SPM, only PS can stimulate the endoenzyme N⁺, K⁺-ATPase [9, 10]. Moreover, it can stimulate the ectoenzyme AchE [10]. Further study was carried out in order to investigate: a) whether PS only or other acidic phospholipids as well, can induce changes in AchE activity, b) which is the mechanism of action of these compounds. Fig. 1 illustrates the changes of AchE activity at different concentrations of incubated phospholipids. A significant increase in the enzyme activity (40%) was induced up to the concentration of 1 μmol PS or PIN or PGL per mg SPM protein. Incubation at higher concentrations led to a progressive inhibition of enzyme activity, which eventually was reduced to levels lower than that of the control. To explain the enzyme stimulation by the acidic phospholipids, AchE was solubilized with the non-ionic detergent Lubrol-PX and showed no change in the enzyme activity at any PS, PIN or PGL concentration used, indicating that these compounds do not act on the protein molecule directly, but indirectly, affecting the membrane fluidity probably. Moreover, a slight increase in the activity (≥ 25%) was succeeded by the solubilization.
Fig. 1. Effect of different concentrations of acidic phospholipids liposomes on the activities of SPM-bound AchE and the solubilized one. A) Effect of phosphatidylserine (PS) liposomes on the enzyme activity. B) Effect of phosphatidylinositol (PIN) liposomes on the enzyme activity. C) Effect of phosphatidylglycerol (PGL) liposomes on the enzyme activity. The value of the activity measured at 20 °C in untreated SPM (control), was 0.68 ± 0.02 A OD/mg prot./min. Points and bars represent means ± coefficient of variation respectively from three different experiments. The average value of each experiment came from three determinations.

Because all acidic phospholipids used displayed similar biphasic changes of AchE activity in relation to their concentration, the investigation was continued further using only PS. Arrhenius plots of AchE activities exhibited a break point approx. 23 °C to non-treated SPM, but there was a lower break point approx. 16.5 °C to those treated with 1 µmol PS per mg SPM protein at 25 °C for 3 h (Fig. 2, Table I). Moreover, Table I shows that the Arrhenius activation energy (Ea) value in PS-treated SPM was increased (48.9 ± 4.8 KJ/mol) related to the Ea below the break point in the untreated SPM (35.5 ± 3.0 KJ/mol). This difference of Ea and the decrease of the break point consist with an increase in bilayer fluidity by PS. This lipid-mediated effect is presumably located to the external half of the bilayer, because AchE is an ectoenzyme of SPM and its activity is apparently insensitive to perturbations of the inner half of the bilayer [20]. Similar effect appeared on the activity of ectoenzyme 5'-nucleotidase in liver plasma membranes [21].

Discussion

The present study indicated that AchE is stimulated by acidic phospholipids incorporated into SPM. This effect seems to be unspecific. However, as regards to Na⁺, K⁺-ATPase the stimulating effect
Table I. Effect of phosphatidylserine on the break point and activation energies derived from Arrhenius plots of AchE activity.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Break point</th>
<th>Activation energy</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Above break</td>
<td>Below break</td>
</tr>
<tr>
<td>Non-treated SPM (Control)</td>
<td>23.0 ± 0.6</td>
<td>24.4 ± 2.2</td>
</tr>
<tr>
<td>+ Phosphatidylserine (1 µmol/mg prot.)</td>
<td>16.5 ± 1.1</td>
<td>26.1 ± 1.5</td>
</tr>
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Values are means ± S.D. of three independent experiments. The average value of each experiment came from duplicate determinations.

of PS seems to be specific [9, 10]. The solubilization of AchE revealed that these compounds do not act on the enzyme directly, but indirectly, through lipid-protein interactions probably. Because lower phospholipid concentration (approx. 1 µmol PS/mg protein) used for maximal stimulation for AchE (Fig. 1) and Na⁺, K⁺-ATPase [10] than adenylate cyclase (approx. 2 µmol PS/mg protein, [11], we may suggest that AchE and Na⁺, K⁺-ATPase are more sensitive than adenylate cyclase in these lipid-protein interactions. For example, Mg⁺-ATPase is relatively insensitive under such interactions [10].

AchE activity was increased (± 25%) after the solubilization of SPM by the detergent Lubrol-PX (see Fig. 1). After the solubilization with Lubrol-PX, some (annular) phospholipids remain associated with the solubilized enzyme [22]. Therefore, the observed increase in AchE activity after solubilization, may be achieved by a relief of a physical constraint, which was imposed by the bilayer upon the enzyme before the solubilization.

Discontinuities in Arrhenius plots of membrane-bound enzymes have widely been considered to reflect a lipid-phase transition and therefore to indicate a lipid-dependence of the enzymes [23]. Abrupt changes in slope at a particular temperature have been taken to represent a phase transition in the lipid environment of endoenzymes and ectoenzymes [24, 25, 10, 11, 20]. In the present study the AchE showed a break point at 23 °C, which was decreased to 16–17 °C in PS-treated SPM (Fig. 2, Table I). The values of the activation energies below \( (E_{aL}) \) and above \( (E_{aH}) \) the break point and the observed break point \( (T_0) \), put in an Arrhenius equation \( \Delta S_{L}^+ - \Delta S_{H}^+ = \frac{E_{aL} - E_{aH}}{T_0} \) [26], where \( \Delta S \) the entropy of activation. The calculation gave difference in the entropy \( \Delta S_{L}^+ - \Delta S_{H}^+ \) 0.48 KJ per mole per degree for the control, but 1.38 KJ per mole per degree in PS-treated SPM. These values suggest that the increase of AchE activity at all temperatures by PS, is achieved by an increase of entropy of activation, so that the enzyme conformation may be fitted better by its substrate (Koshland's theory). This lipid-mediated effect is presumably located to the external half of the bilayer, because AchE is an ectoenzyme of SPM and its activity is apparently insensitive to perturbations of the inner half of the bilayer [20].

The increased activities of the AchE achieved by low concentrations of phospholipids, presumably due to a slight fluidization of the membranes resulting to an increase of the conformational flexibility of the enzyme achieved by a relief of a physical constraint imposed by the bilayer upon the protein molecules. Further increases of the membrane fluidity induced by high concentrations of phospholipids, inhibit the enzyme activity, probably by displacing of annular lipids from around the protein molecules, as it was suggested by Houslay et al. [27].

In addition, changes in AchE activity can influence the postsynaptic binding of acetylcholine to its receptor, whose sensitivity may be controlled by membrane fluidity, as well [28]. In conclusion, modulation of SPM architecture caused by changes in lipid fluidity by acidic phospholipids, could modify the activities of other membrane-bound enzymes, binding of neurotransmitters, conformation of receptors and processes of ion channels, events which could control the synaptic transmission.
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