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IDENTIFICATION OF COVALENTLY BOUND INOSITOL IN THE HYDROPHOBIC MEMBRANE-ANCHORING DOMAIN OF TORPEDO ACETYLCHOLINESTERASE

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SUMMARY: The hydrophobic, membrane-bound form of Torpedo acetylcholinesterase is specifically solubilized by a phosphatidylinositol-specific phospholipase C, suggesting that acetylcholinesterase is bound to the membrane via a direct and specific interaction with phosphatidylinositol (Futerman et al., Biochem. J. (1985) 226, 369-377). Here we demonstrate the presence of covalently bound inositol in the membrane-anchoring domain of purified Torpedo acetylcholinesterase. Upon removal of this domain, levels of inositol are reduced to only 15-20% of those found in the intact enzyme. The results presented strongly support our suggestion that phosphatidylinositol is indeed involved in anchoring acetylcholinesterase to the plasma membrane.

We have recently suggested that the hydrophobic, dimeric form of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) in Torpedo electric organ is anchored to the plasma membrane via a direct and specific interaction involving phosphatidylinositol (PI) (1). This suggestion is based on the observation that AChE from Torpedo is released from the membrane by the action of a phosphatidylinositol-specific phospholipase C (PIPLC) from Staphylococcus aureus (2,3). Furthermore, PIPLC can modify the hydrodynamic behavior of the detergent-solubilized, affinity-purified enzyme by removal of

ABBREVIATIONS: AChE, Acetylcholinesterase; PIPLC, Phosphatidylinositol-specific phospholipase C; PI, Phosphatidylinositol.

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a hydrophobic, detergent-binding site (1). Our hypothesis predicts that purified AChE should contain stoichiometric amounts of PI tightly attached to the membrane-anchoring domain of the AChE molecule. In this communication, we substantiate this hypothesis by direct determination of the inositol content of purified AChE, solubilized by various treatments, using a GC/MS technique (4).

MATERIAL AND METHODS

Materials

Torpedo californica electric organ was obtained frozen from Pacific Biomarine (Venice, CA) and stored at -80°C. PIPLC was purified from Staphylococcus aureus (5). Cholic acid (Merck) was recrystallized from hot ethanol. Proteinase K (proteinase type XI from Tritirachium album) was from Sigma.

Purification of AChE

Torpedo AChE, solubilized with either sodium cholate or proteinase K, was purified by affinity chromatography as previously described (1). AChE was purified after solubilization with PIPLC by a modification of the above procedures. After removal of the low-salt-soluble fraction (see Ref. 1), the resulting homogenate was incubated with PIPLC (1 µg/ml) for 18h at 22°C in 40 mM $MgCl_2$-100mM NaCl-10mM Tris, pH 8.0. Essentially quantitative solubilization of the dimeric form of AChE was obtained by this treatment. After centrifugation in a 35Ti rotor using a Beckman L8-70 ultracentrifuge (78,000g, 1h, 4°C), the supernatant was passed over an affinity-chromatography column consisting of the affinity ligand (m-aminophenyl)trimethylammonium coupled via a dicaproyl spacer to Sepharose 2B (1). The column was extensively washed and the AChE was eluted with 2mM decamethonium bromide. The eluted enzyme was dialysed to remove decamethonium and then lyophilized before GC/MS analysis of inositol content. All three enzyme forms thus purified appeared as one major band on SDS-polyacrylamide gel electrophoresis and all had a specific activity greater than 2500 units/mg with 3mM [^{3}H]-acetylcholine as substrate at pH 7.4 and 25°C.

GC/MS analysis of AChE

Samples of AChE plus 200ng of a scyllo-inositol (Calbiochem-Behring) internal standard were hydrolysed in sealed glass ampoules with 6N HCl at 110°C for 24 h. The scyllo-inositol was used to correct for losses of the inositols during hydrolysis. In separate experiments the recoveries of each inositol, subjected to the same hydrolytic conditions, were: myo-inositol, 63%; scyllo-inositol, 62%; chiro-inositol, 60%. Following hydrolysis, aliquots of the samples were taken to dryness at 40°C in a stream of nitrogen, briefly placed under a rotary-pump vacuum so as to ensure thorough drying, and then derivatized with 50 µl of a 1:1 mixture of dry pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide containing 10% trimethylsilyl chloride (Regis, Morton Grove, IL). Aliquots of each sample were subjected to GC/MS using electron ionization on a Finnigan Model 3200 system employing a 5'x¼" glass column packed with 1% SE-30 on Gas-Chrom Q (Applied Science Laboratories, State College, PA). Dilutions of trimethylsilyl myo-, scyllo- and (-)-chiro-inositol (the latter a gift from L. Anderson, Univ. of

Wisconsin, Madison, WI) were measured so as to determine that the analysis was linear and that the standard curve passed through x,y = 0. Samples were then compared with individual standards which were run several times each day so as to assess drift in sensitivity. The mass-spectrometric fragment ions of the trimethylsilyl inositols at m/z 305 and 318 were used in these measurements (6). The ratio of these ions was measured in each analysis and compared with the ratios of the standard myo-, chiro- and scyllo- inositols in each case. The ratio of these ions for the AChE-derived inositols were at all times identical with the standards within experimental error, as were the gas chromatographic retention times, confirming the identity of the inositols in each analysis.

RESULTS AND DISCUSSION

Torpedo AChE, affinity-purified after solubilization in the detergent cholate, contains substantial amounts of myo-inositol, i.e. approximately 0.7 moles per mole of catalytic subunit (Table 1). We have previously shown that this form of AChE retains intact the PIPLC-sensitive hydrophobic domain which anchors the enzyme to the membrane, and which should, therefore, contain the putative PI moiety responsible for this anchoring (1). AChE similarly purified, but after solubilization with PIPLC, contains approximately one mole of myo-inositol per mole enzyme (Table 1). This is also consistent with our model since PIPLC treatment should leave inositol phosphate attached to the enzyme after removal of the hydrophobic region of the molecule, 1,2-diacylglycerol (1). Furthermore, AChE purified after solubilization with proteinase K, which is believed to cleave off the hydrophobic moiety by digestion of a peptide to which PI is attached (1), contains less than 0.15 moles myo-inositol per mole of enzyme, or less than 20% and 15% of the myo-inositol content of the cholate- and PIPLC- solubilized enzymes respectively (Table 1).

In all three cases above, interpretation of the data is complicated by the unanticipated presence of chiro-inositol in addition to myo-inositol in the hydrolysed AChE samples. The predominant isomer detected in our analyses, myo-inositol, is the isomer normally present in PI; chiro-inositol is a rare isomer which has been previously reported in seagrass and other plant sources(7,8). In most of the AChE samples analysed, however, chiro-inositol was present in significant amounts. In one of our initial analyses (n=3),

TABLE 1: INOSITOL CONTENT OF PURIFIED AChE FROM TORPEDO ELECTRIC ORGAN

Method of AChE solubilization	Inositol content, moles per mole AChE subunit	
	myo-isomer	chiro-isomer
Cholate	1.06 ± 0.03 (3)	0.50 ± 0.11 (3)
	0.62 ± 0.07 (8)	0.26 ± 0.03 (8)
	0.57 ± 0.03 (8)*	0.31 ± 0.02 (8)*
	0.58 ± 0.01 (4)*	0.17 ± 0.02 (4)*
PIPLC	0.96 ± 0.03 (6)	0.09 ± 0.01 (6)
	1.12 ± 0.10 (5)	<0.01 (5)
Proteinase K	0.10 ± 0.02 (6)*	0.04 ± 0.02 (6)*
	0.27 ± 0.05 (5)*	0.05 ± 0.01 (5)*
	0.21 ± 0.02 (3)	0.04 ± 0.01 (3)
	0.06 ± 0.01 (4)	<0.01 (4)

Inositol content was determined on several different purified preparations for each type of solubilization method. Values are given as moles inositol per mole of AChE catalytic subunit ± S.D. Figures in parentheses denote the number of separate replicate analyses done on each preparation. Samples with n>5 were analysed in separate groups several weeks apart. The low S.D. values show the precision of the replicate analyses. The amount of AChE was determined from the amino acid content of each sample, the amino acid composition obtained being indistinguishable from that published for the AChE dimer (14), and assuming a subunit molecular weight of 64,000 (1). In some cases*, AChE content was determined from the absorbance at 280 nm, assuming $\varepsilon^{1\%}_{280}$ = 18 (15) and the values were corrected by comparison with the UV absorption of samples of known amino acid content.

AChE purified after proteinase K solubilization contained an unusually high amount of chiro-inositol (0.38 ± 0.06 moles inositol per mole AChE subunit). The myo-inositol level in this initial analysis was much lower (0.09 ± 0.01 moles inositol per mole AChE subunit), similar to levels subsequently found for this form of the enzyme (Table 1). Levels of myo-inositol and chiro-inositol in non-hydrolysed samples were less than 5% and 10% respectively of the levels detected in the hydrolysed samples. Furthermore, less than 10% of the inositol in cholate-purified AChE was extracted in chloroform-methanol.

These data preclude the possibility that the inositols found in AChE are present as PI or as free inositols non-specifically adsorbed to the enzyme and strongly suggest that they are covalently bound to the AChE molecule.

The source of chiro-inositol in Torpedo AChE is not clear. Its apparent association with the hydrophobic domain of Torpedo AChE raises the possibility that a chiro-inositol containing PI is found in Torpedo electric organ. However, in separate experiments, chiro-inositol was not reproducibly detected in hydrolysed lipid extracts of Torpedo electric organ, nor is chiro-inositol formed from myo- or scyllo-inositol or from PI by acid hydrolysis. Thus, the variable ratio of myo- to chiro-inositol in AChE samples purified after different solubilization procedures, and even within each procedure from experiment to experiment, does not allow us to draw a conclusion regarding the relationship between or the origin of chiro-inositol and AChE. However, it should be noted that whereas in the PIPLC-solubilized AChE preparations myo-inositol is, as predicted, almost stoichiometric with respect to the catalytic subunit, and chiro-inositol values are low, in cholate-solubilized samples myo-inositol is generally substoichiometric, and chiro-inositol levels are substantially higher.

The results presented clearly demonstrate the presence of covalently bound inositol in the hydrophobic domain of the Torpedo AChE molecule. Previous studies had shown that the hydrophobic membrane-anchoring domain of Torpedo AChE is removed by PIPLC and we have proposed that this domain is in fact PI or some closely related molecule. The observations reported here strongly support this proposal. It is relevant that purified preparations of alkaline phosphatase, another enzyme whose hydrophobic domain is removed by PIPLC (9,10), contain a similar molar ratio of myo-inositol to enzyme as in AChE (Low and Sherman, unpublished observations). Our previous work also led us to propose that the interaction between PI and both AChE and alkaline phosphatase is covalent (1,10) and the data presented here are consistent with such a model. Several instances of covalent modification of membrane proteins by lipids have been reported (reviewed in Ref. 11, see also literature quoted

in Ref. 1), but AChE and alkaline phosphatase appear to be the first to involve a phospholipid, namely PI. However, recent work on the variant surface glycoprotein of Trypanosoma brucei (Refs. 12,13, and Ferguson, Low and Cross, in preparation) and the Thy-1 antigen (Low and Kincade, in preparation) suggest that these proteins may also be anchored to the membrane via a covalent linkage involving PI.

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