constraints required for the elucidation of the solution structure of NMT.

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Conformation Change of the Intestinal Calcium-Binding Protein Induced by Phospholipids in the Presence and Absence of Ca^{2+}

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ABSTRACT: Effects of phospholipids (PL's) and lyso-PL's on the conformation of the porcine intestinal calcium-binding protein (CaBP) were studied fluorometrically with 1-(dimethylamino)naphthalene-5-sulfonyl- (DNS-) labeled CaBP. The fluorescence intensity of DNS-labeled CaBP was much higher in the presence of excess EGTA than in its Ca^{2+}-bound state. In the absence of free Ca^{2+} (with 1 mM EGTA) the fluorescence of the labeled CaBP was greatly enhanced by addition of lysophosphatidylcholine (lyso-PC), lysophosphatidylserine (lyso-PS), or lysophosphatidylinositol (lyso-PI). With addition of 25 μM Ca^{2+} the enhancement of the fluorescence by these lyso-PL's was depressed; especially that due to lyso-PC became small. Lysophosphatidylethanolamine (lyso-PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and mono- and dipalmitoylglycerols had no or much less effect on the fluorescence in the presence and absence of Ca^{2+}. Lyso-PC attenuated in a concentration-dependent manner the quenching of the fluorescence of the DNS–CaBP by high temperatures and increase of ionic strength in the presence of EGTA. Lyso-PL's generally protected the CaBP from digestion with proteases in the presence of EGTA. These experimental results suggest that particular lyso-PL's have Ca^{2+}-sensitive interaction with the porcine CaBP and induce conformation change of the CaBP molecules.

The porcine intestinal CaBP[1] was first reported by Hitchman et al. (1972). We previously demonstrated by a quantitative determination of Ca^{2+} binding and measurement of Ca^{2+}-induced changes in the intrinsic fluorescence, CD, and UV absorption that the CaBP molecule has two noncooperative Ca^{2+}-binding sites with essentially the same affinity for Ca^{2+} (Chiba et al., 1983a,b) and increases α-helix content of it by about 5% on maximal binding of Ca^{2+} (Chiba et al., 1983b).

Wasserman (1970) has reported that lyso-PC interacts with the chick CaBP and releases bound Ca^{2+} from the protein. He also suggested that interaction of the CaBP with the intestinal membrane components affects its Ca^{2+} binding and plays an important role in the process of Ca^{2+} absorption. We previously suggested that the porcine CaBP changes its conformation on binding Ca^{2+} so as to increase hydrophobicity in

[1] Abbreviations: CaBP, calcium-binding protein; DNS, 1-(dimethylamino)naphthalene-5-sulfonate; ANS, 1-anilinonaphthalene-8-sulfonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; CD, circular dichroism; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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the microenvironment of the 1-anilinonaphthalene-8-sulfonate-(ANS-) binding site in the protein (Chiba & Mohri, 1987).

In this study we have prepared such a fluorescent conjugate of the porcine intestinal CaBP with DNS whose fluorescence is highly sensitive to both calcium and lyso-PL's and investigated the interaction of CaBP with PL's or Lyso-PL's in the presence and absence of Ca^{2+}.

Materials and Methods

Chemicals. Lyso-PC (egg), PC (egg), synthetic lyso-PC (palmitoyl), and synthetic mono- and dipalmitoylglycerols were obtained from Sigma Chemical Co. (St. Louis, MO), lyso-PS (bovine brain) and lyso-PI (bovine liver) were from Avanti Polar Lipids Inc. (Birmingham, AL), and lyso-PE (egg), PS (bovine brain), and PE (egg) were from Serdary Research Laboratory (London, Ontario, Canada). Trypsin (type I), papain (type IV), DNS chloride, and N^{α}-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma Chemical Co. (St. Louis, MO), soybean trypsin inhibitor was from P-L Biochemicals Inc. (Milwaukee, WI), and 1-anilinonaphthalene-8-sulfonate (ANS) was from Nakarai Tesque Co. (Kyoto, Japan), as the magnesium salt. The stock solutions of insoluble lipids were prepared as follows: each phospholipid solution in organic solvent was evaporated, and the dried lipids were dispersed with sonication in appropriate buffer prior to use. All other chemicals used were of the purest grade commercially available.

Preparation of the Porcine CaBP. The porcine CaBP was prepared from the fresh small intestine as previously described (Chiba et al., 1983a). It was not passed through Chelex-100 column (Chiba et al., 1983b) and not checked on Ca^{2+} content.

Dye Labeling of the CaBP. DNS labeling of the CaBP was carried out as follows. Twenty microliters of stock solution of 100 mM DNS chloride in acetone was added to 2 mL of 0.1 M bicarbonate buffer (pH 9.5) containing 100–150 μM CaBP in the presence of 1 mM EGTA at room temperature. After 30–60 min of reaction time, unreacted dye was removed by centrifugation (3000 rpm, 10 min) and the supernatant was applied on a Sephadex G-25 column (1 × 30 cm). The DNS-labeled CaBP (DNS–CaBP) containing fraction was pooled, concentrated, and dialyzed against 10 mM Tris-HCl buffer (pH 7.4) at 4 °C for 48 h. The molar ratio of bound DNS to protein in the DNS–CaBP used in the present experiments was about 1, unless otherwise specified.

Fluorescence Measurements. Fluorescence of each CaBP sample was measured at 25 °C in 2.5 mL of 50 mM Tris-HCl buffer (pH 7.4) with a Hitachi MPF-4 fluorescence spectrophotometer. The excitation and emission wavelengths used to determine dye fluorescence were 330 and 520 nm for the DNS–CaBP and 380 and 500 nm for ANS, respectively, unless otherwise specified. Intrinsic fluorescence of the CaBP was measured with 275 and 305 nm as excitation and emission wavelengths, respectively. It took several minutes for the intensity of the fluorescence of the labeled CaBP to become stable after addition of PL's, lyso-PL's, $CaCl_2$, or EGTA.

Treatment of DNS–CaBP with Various Temperatures or Ionic Strengths. To examine the effect of temperature on the fluorescence intensity of the DNS–CaBP, the labeled protein was incubated at various temperatures for 10 min with 20 μM Ca^{2+} or 1.5 mM EGTA and concentrations of lyso-PC (palmitoyl) in 50 mM Tris-HCl (pH 7.4) as specified under Results and Discussion.

The effect of ionic strength was examined by addition of 74 μM lyso-PC (palmitoyl) to the DNS–CaBP in the presence of various concentrations (~98 mM) of KCl and 1 mM EGTA.

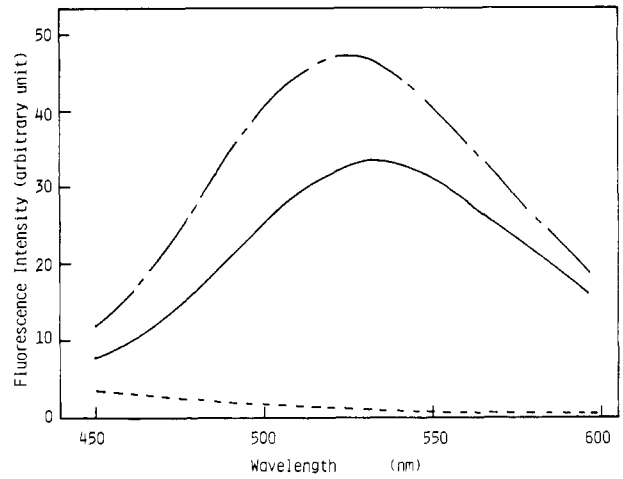

FIGURE 1: Emission spectra of the fluorescence of the DNS–CaBP with addition of Ca^{2+} or EGTA. The DNS–CaBP concentration used was 0.6 μM. (—) 20 μM $CaCl_2$; (- - -) 1 mM EGTA; (- - -) buffer.

Digestion of CaBP with Trypsin and Papain. Tryptic or papain digestion was carried out in cuvettes by incubating the DNS–CaBP (about 1 μM) with trypsin (6.2 or 13.4 μg/mL) or papain (10 μg/mL) at 37 °C in 50 mM Tris-HCl, pH 7.4, under various conditions as specified under Results and Discussion, and fluorescence of the samples was periodically determined.

Results and Discussion

Effect of Ca^{2+} on the Fluorescence of DNS–CaBP. Figure 1 shows the fluorescence spectra of the DNS–CaBP after addition of Ca^{2+} (20 μM $CaCl_2$) and EGTA. The fluorescence of the DNS–CaBP on excitation at 330 nm with addition of Ca^{2+} has an emission peak at 530 nm. The intensity of the fluorescence was greatly enhanced by addition of 1 mM EGTA, and the emission maximum showed blue shifting by about 10 nm. These fluorescence changes in intensity and emission maximum were generally observed in the samples different in DNS/CaBP molar ratio varied from 1.00 to 2.53.

As has been previously reported (Chiba et al., 1983b), intrinsic fluorescence of the porcine CaBP increased with increasing bound Ca^{2+}. Figure 2 shows that increase of the intrinsic fluorescence of the CaBP and decrease of the fluorescence of the DNS–CaBP with increase of bound Ca^{2+} are in good correlation. This result suggests that the changes of intrinsic and DNS fluorescences induced by Ca^{2+} addition are related to a common conformation change of the CaBP induced by Ca^{2+} binding.

Effects of PL's on the Fluorescence of DNS–CaBP. The fluorescence of the DNS–CaBP without addition of PL's was not dependent on the concentration of added Ca^{2+} (Figure 3). Lyso-PS and lyso-PI enhanced the fluorescence intensity of the DNS–CaBP with addition of 5–25 μM Ca^{2+}, but much less enhancement of it was observed with lyso-PC (egg) in the same range of added Ca^{2+} (Figure 3). With addition of 1 mM EGTA these lyso-PL's further enhanced the intensity; much greater enhancement was observed with lyso-PC (egg) than with the other two lyso-PL's. Thus it seems that lyso-PC has a high-affinity interaction preferentially with the apo form of the CaBP. PC (24 μg/mL) had no effect on the fluorescence of the DNS–CaBP with addition of 5–25 μM Ca^{2+} (Figure 3). Other PL's (PS, PI, and PE) and mono- and dipalmitoylglycerols had no effect on the fluorescence in the same condition, either (data not shown). The mechanism for lyso-PS and lyso-PI to increase the fluorescence depending on the Ca^{2+} concentration is not elucidated yet, but it is suspected

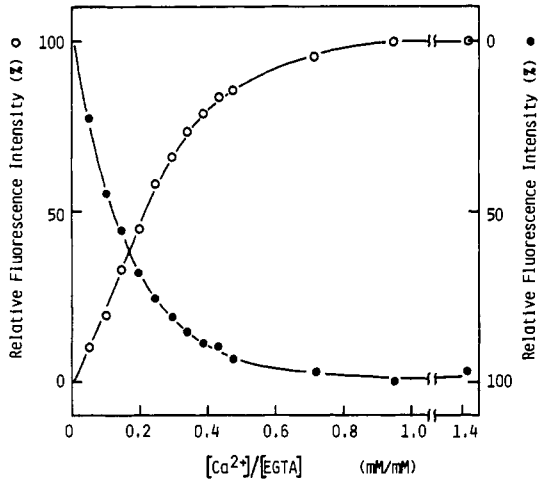

FIGURE 2: Correlation between intrinsic and DNS fluorescence changes of the CaBP with increasing added Ca^{2+}/EGTA ratio. The CaBP and DNS–CaBP concentrations were 12.0 and 0.5 μM, respectively. Ca^{2+} concentrations used were varied from 0 to 1.4 mM in the presence of 1 mM EGTA. (○) Intrinsic fluorescence; (●) DNS fluorescence. Changes of fluorescence intensities were expressed relative to the maximal changes. Other assay conditions are described under Materials and Methods.

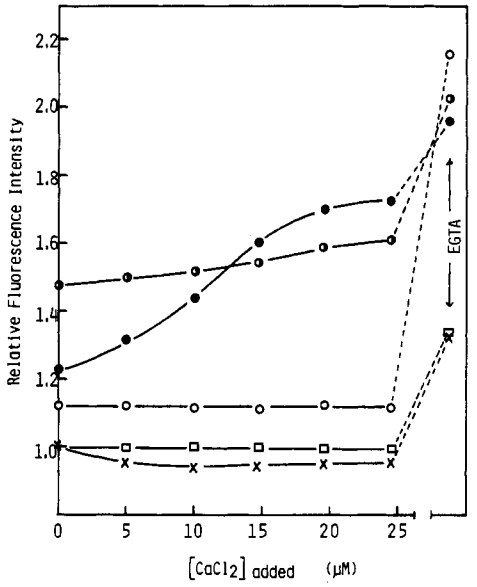

FIGURE 3: Ca^{2+} titration of the DNS–CaBP in the presence of various lyso-PL's and PC. The DNS–CaBP and PL concentrations used were 0.6 μM and 24 μg/mL, respectively. (○) Lyso-PC (egg); (◑) lyso-PI; (●) lyso-PS; (□) PC; (×) no PL. Fluorescence intensity is expressed relative to the value with addition of neither Ca^{2+} nor PL. EGTA (1 mM) was added to each sample after titration with $CaCl_2$ up to 25 μM. Other assay conditions are described under Materials and Methods.

that the former binds Ca^{2+} and the resultant complex could interact with the CaBP to affect the fluorescence of bound DNS. Anyway the specific binding of Ca^{2+} to the protein is considered to be saturated in the condition (0–25 μM $CaCl_2$ added without addition of EGTA) shown in Figure 3 [cf. Figure 2 and Chiba et al. (1983b)]. Fluorometric titration profiles of the DNS–CaBP with the lyso-PL's in the absence of free Ca^{2+} showed saturation curves (Figure 4). Apparent dissociation constants for lyso-PC (egg), lyso-PI, and lyso-PS calculated from the fluorometric titrations were 28.1, 53.5, and 32.5 μg/mL, respectively. In the presence of saturable Ca^{2+} lyso-PC (egg) dependence of the fluorescence of the DNS–CaBP was not observed. The fluorescence enhancement by lyso-PL addition was extremely diminished in the presence

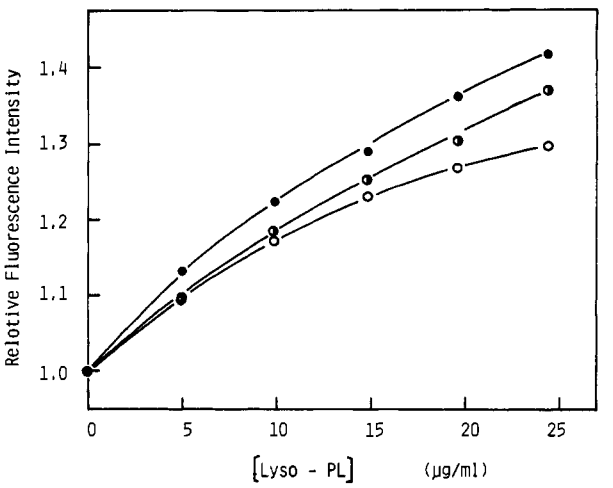

FIGURE 4: Lyso-PL titration of the DNS–CaBP in the absence of Ca^{2+}. The DNS–CaBP and EGTA concentrations used were 0.6 μM and 1 mM, respectively. (○) Lyso-PC (egg); (◑) lyso-PI; (●) lyso-PS. Fluorescence intensity is expressed relative to the value with no addition of lyso-PL. Other assay conditions are described under Materials and Methods.

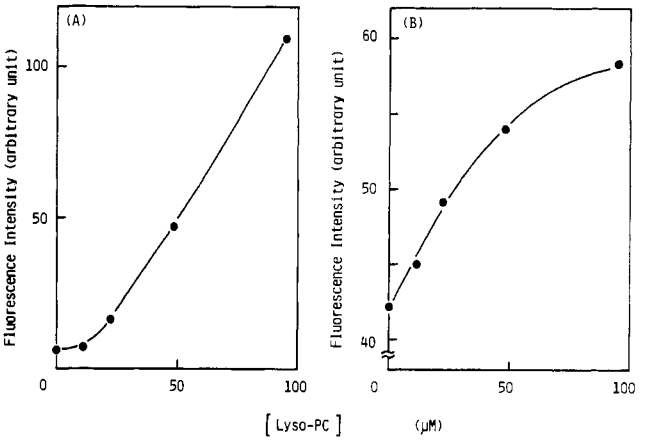

FIGURE 5: Lyso-PC-dependent enhancement of ANS and the DNS–CaBP fluorescences. (A) ANS (20 μM). (B) DNS–CaBP (0.8 μM). Ex: 335 nm; Em: 525 nm. Fluorescence intensities of ANS and the DNS–CaBP were measured 24 h (at 4 °C) after preparation of the mixtures containing various concentrations of lyso-PC (palmitoyl) and ANS or the labeled protein with addition of 1 mM EGTA.

of 24 μg/mL sodium deoxycholate or taurocholate (data not shown). The good correlation between changes of intrinsic fluorescence of the CaBP and the fluorescence intensity of the DNS–CaBP with increasing binding of Ca^{2+} and a high Ca^{2+} sensitivity of the fluorescence enhanced by addition of lyso-PC (egg) (Figures 3 and 4) suggest that the specific Ca^{2+}-binding sites of the CaBP interact with lyso-PC and conformation change of the protein is induced as a consequence of this interaction so as to alter the nature of the microenvironment around bound DNS residue.

The critical micelle concentration (cmc) of lyso-PC (palmitoyl) is about 10 μM (Harerland & Reynold, 1975). Schneider and Edelhoch (1972) have suggested that the rate of micelle dissociation of lyso-PC (egg) below its cmc is rather slow with a half-time of about 2 h at 37.6 μM and that the micelles which are derived from the stock solution would remain associated during fluorescence measurement. As shown in Figure 5A, the critical concentration of lyso-PC (palmitoyl) for enhancement of ANS fluorescence is near the cmc for it. On the contrary, such a critical concentration was not observed for the increase of DNS–CaBP fluorescence with increasing lyso-PC (palmitoyl) (Figure 5B). All the solutions containing

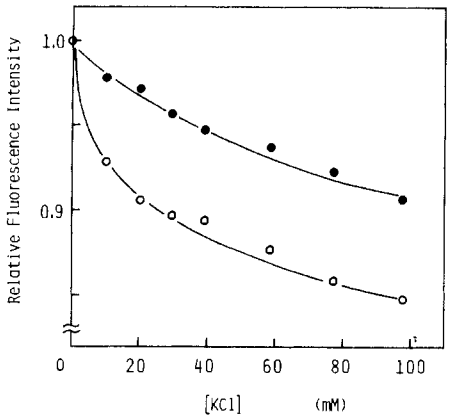

FIGURE 6: Effect of lyso-PC on the quenching of the DNS–CaBP fluorescence by high ionic strengths in the absence of Ca^{2+}. The CaBP concentration used was 0.8 μM. (○) 1 mM EGTA; (●) 1 mM EGTA plus 74 μM lyso-PC (palmitoyl). Fluorescence intensity is expressed as relative to the value at no addition of KCl. Other assay conditions are described under Materials and Methods.

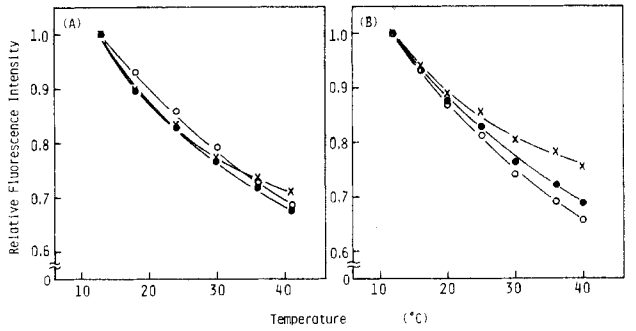

FIGURE 7: Effect of lyso-PC on temperature-induced quenching of the DNS–CaBP fluorescence with (A) or without (1.5 mM EGTA present) (B) addition of $CaCl_2$ (20 μM). The DNS–CaBP concentration used was 1.3 μM. (○) No addition; (●) 30 μM lyso-PC (palmitoyl); (×) 60 μM lyso-PC (palmitoyl). Fluorescence intensity is expressed as relative to the value at 12 °C in each case. Other assay conditions are described under Materials and Methods.

various concentrations of lyso-PC (palmitoyl) used in this experiment were prepared 24 h before measurement to equilibrate the dissociation of micelles. It seems from the above result that micelle formation is not essentially required for the interaction between lyso-PC and the CaBP.

Effect of Lyso-PC on Quenching of the Fluorescence of DNS–CaBP by Increasing Ionic Strength and Temperature. The fluorescence of the DNS–CaBP was quenched progressively with increasing concentrations of KCl in the presence of excess EGTA (Figure 6). Addition of lyso-PC (palmitoyl) partially prevented the quenching induced by elevation of ionic strength. In the presence of Ca^{2+} the KCl-induced quenching of the fluorescence was not observed (data not shown). Lyso-PC (palmitoyl) moderated the quenching of the fluorescence of the DNS–CaBP induced by increase of temperature depending on the concentration of the lipid in the absence of Ca^{2+} (Figure 7B). However, with addition of 20 μM Ca^{2+} (Figure 7A) lyso-PC (palmitoyl) did not affect the quenching.

These findings suggest that lyso-PC induces conformation change of the CaBP molecule to stabilize the structure in the vicinity of the DNS-binding site, preferentially in the apo form of it.

Effect of Phospholipids on Tryptic and Papain Digestion of DNS–CaBP. It has been reported that tryptic digestion of the porcine (Bryant & Crith, 1986) and bovine (Fullmer et al., 1975) CaBP's is suppressed progressively with increasing

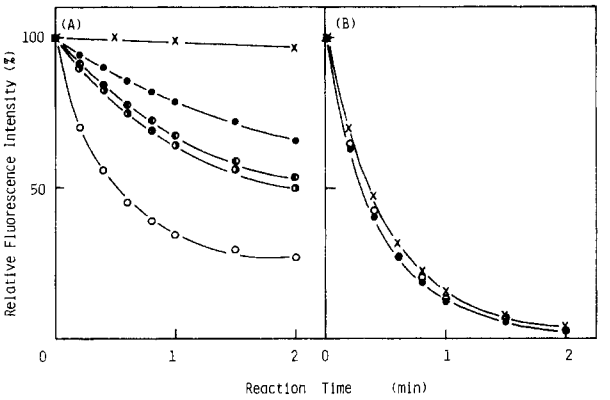

FIGURE 8: Effect of PL's on the quenching of the DNS–CaBP fluorescence by trypsin in the presence of EGTA (1 mM). (A) Effect of lyso-PC, lyso-PI, and lyso-PS. Concentration of each lyso-PL used was 24 μg/mL. (○) EGTA only; (◑) lyso-PC (egg); (◐) lyso-PI; (●) lyso-PS; (×) 1 mM $CaCl_2$ (no EGTA). (B) Effect of PC and lyso-PE. PC and lyso-PE concentrations used were 50 and 30 μg/mL, respectively. (○) EGTA only; (●) PC; (×) lyso-PE. Reaction was started by the addition of trypsin (13.4 μg/mL) at time 0. Fluorescence intensity was expressed as relative to the value determined just before addition of trypsin. Other assay conditions are described under Materials and Methods.

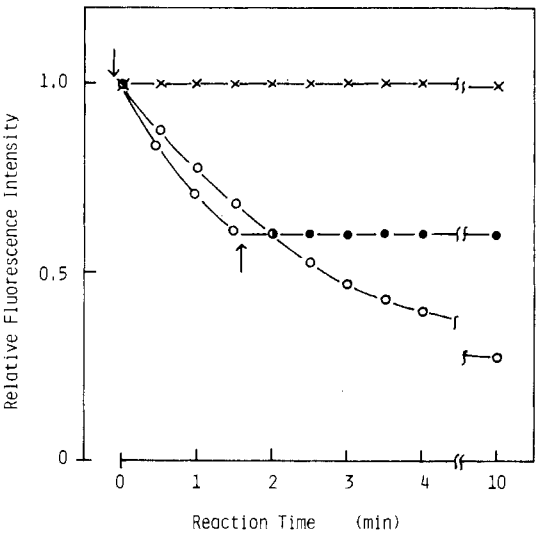

FIGURE 9: Blocking effect of the soybean trypsin inhibitor on fluorescence quenching by tryptic digestion of the DNS–CaBP in the absence of Ca^{2+}. EGTA concentration used was 1 mM. (○) EGTA only; (● and ×) EGTA plus the soybean trypsin inhibitor (10 μg/mL). The inhibitor was added at arrows in the figure. The reaction was started by the addition of trypsin (6.2 μg/mL) at time 0. Fluorescence intensity was expressed relative to the value determined just before addition of trypsin in each case. Other assay conditions are described under Materials and Methods.

Ca^{2+} concentration. As shown in Figure 8, the fluorescence intensity of the DNS–CaBP was decreased with time of the treatment with trypsin up to 2 min in the presence of EGTA, whereas no such a quenching occurred with addition of Ca^{2+} (1 mM). Addition of the soybean trypsin inhibitor (10 μg/mL) to the reaction mixture completely suppressed the quenching of the fluorescence (Figure 9). Lyso-PC (egg), lyso-PI, and lyso-PS, all of which can induce the enhancement of the fluorescence of the DNS–CaBP in the absence of Ca^{2+}, showed an inhibitory effect on the quenching of the fluorescence by tryptic digestion (Figure 8A). Both lyso-PE and PC, which cannot induce the enhancement of the fluorescence, could not affect the quenching (Figure 8B). It has been demonstrated by an electrophoretic analysis on SDS–polyacrylamide gel (20%) for the time course of digestion of the CaBP and DNS–CaBP with trypsin that addition of lyso-PC

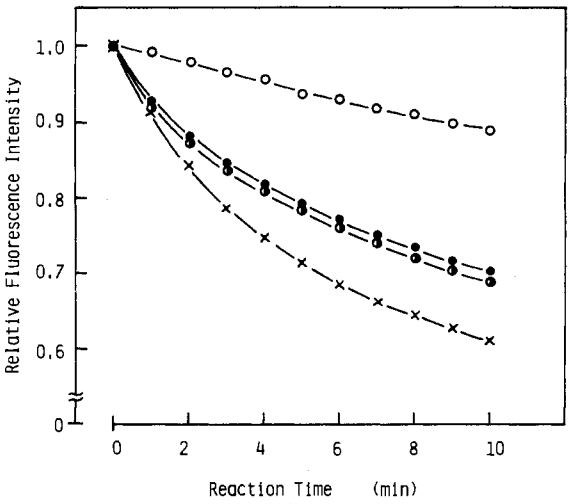

FIGURE 10: Effect of lyso-PL's on digestion of the DNS–CaBP by papain. Concentration of each lyso-PL used was 24 μg/mL. EGTA was added to all samples at 1 mM. (○) Lyso-PC (egg); (◑) lyso-PI; (●) lyso-PS; (×) EGTA only. The reaction was started by the addition of papain (10 μg/mL) at time 0. Fluorescence intensity was expressed as relative to each value before addition of papain. Other assay conditions are described under Materials and Methods.

to the reaction mixture in the absence of Ca^{2+} obviously delays the disappearance of the spot of intact molecules and appearance of smaller peptides on the test gel columns (data not shown). Since hydrolysis of BAEE, a model substrate for trypsin, by the enzyme is not affected by the presence of lyso-PL's, the inhibitory effect of lyso-PL's on the tryptic digestion is not considered to be due to direct interaction of them with trypsin.

All three of the lyso-PL's shown inhibitory on trypsin digestion above also protected to various extents the CaBP from papain digestion in the absence of Ca^{2+} (Figure 10).

All these results suggest that certain lyso-PL's can bind to the porcine CaBP preferentially in the absence of Ca^{2+} and induce conformation change of the molecule, particularly lyso-PC. Chapelle and Gilles-Baillien (1983) have reported that lyso-PC content of the rat intestinal brush border membranes is more than twice that of the basolateral membranes (about 8.7 and 3.6% of total phospholipids, respectively). It is suggested that lyso-PL's, lyso-PC most prominently, have specific affinity for the CaBP and cause a conformation change in it in the specific region including DNS-binding site(s) located near the Ca^{2+}-binding sites.

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