Calcium-dependent association of 33 kDa protein in polymorphonuclear leukocytes with phospholipid liposomes containing phosphatidylserine or cardiolipin

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The effects of lipids and Ca^{2+} on the association of cytoplasmic proteins of polymorphonuclear leukocytes (PMN) with dipalmitoylphosphatidylcholine liposomes containing phosphatidylserine (PS) or cardiolipin (CL) were examined. In the presence of Ca^{2+}, a cytoplasmic protein (33 kDa) of PMN was found to be a major component that bound to these liposome membranes. The maximum association with the liposomes was obtained at the molecular concentration of 25% CL and 50% PS; the required concentrations of Ca^{2+} were in the nanomolar and micromolar ranges for CL and PS, respectively. The liposome-associated 33 kDa protein was released from liposomes by an addition of EGTA. These results suggest that the 33 kDa protein reversibly associates to the cytoplasmic surface of PMN plasma membranes where PS localizes depending on a small change in intracellular concentration of free Ca^{2+}.

Ca^{2+}-binding protein *Protein-lipid interaction* *Liposome* *Polymorphonuclear leukocyte* *Phosphatidylserine* *Cardiolipin*

1. INTRODUCTION

It is generally agreed that a wide variety of hormones and neurotransmitters exert their effects via mobilization of intracellular Ca^{2+}. The calcium-dependent regulation of cellular processes is mediated mainly by specific intracellular calcium-binding proteins, such as troponin C, protein kinase C and calpain [1–3]. With the binding of Ca^{2+} to these proteins, conformational changes are induced by which hydrophobic domains are exposed to an aqueous phase [4–6]. Thus, Ca^{2+}-induced exposure of hydrophobic domains of these proteins might increase their interactions with the cytoplasmic surface of plasma membranes, where certain phospholipids, such as phosphatidylserine (PS), are enriched. Binding to the inner surface may also enhance interaction of these cytoplasmic proteins with some transmembranous proteins, such as membrane-bound enzymes and receptors [7–10].

Based on these ideas, we tested the effect of Ca^{2+} on the interaction of the 33 kDa protein derived from cytoplasmic proteins of polymorphonuclear leukocytes (PMN) with liposomes containing different lipid components. This work demonstrates that the protein associates selectively and reversibly to liposome membranes containing cardiolipin (CL) or PS in a Ca^{2+}-dependent manner.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), cholesterol (C), cetylamine (CA), dicetyl phosphate (DCP), PS, CL, phosphatidic acid (PA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). All reagents used were analytical grade.

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2.2. *Preparation of PMN*

Guinea pig PMN were obtained after intraperitoneal injection of 2% casein and washed with calcium-free Krebs-Ringer-phosphate solution (KRP) (pH 7.4) as described [11]. KRP contained 154 mM NaCl, 6 mM KCl, 10 mM phosphate buffer (pH 7.4) and 1 mM $MgCl_2$.

2.3. *Preparation of cytoplasmic proteins of PMN*

Cells were suspended in hypotonic solution (5×10^8 cells/ml) containing 10 mM NaCl or KCl, 10 mM Hepes-Na or -K buffer (pH 7.4), 1 mM EGTA, and 1 mM PMSF, a serine-protease inhibitor, and homogenized with a Teflon-glass homogenizer at 4°C for 10 min with 20 strokes. The homogenate was centrifuged for 60 min at 100000 × *g* at 4°C and the supernatant fraction was kept at 4°C until use.

2.4. *Preparation of liposomes*

Liposomes were prepared by sonication under N_2 gas with DMPC, DPPC, C, CA, DCP, CL, PS, PA and total lipids of rat liver mitochondria or microsomes and/or human erythrocyte ghosts in a medium of 0.1 M KCl or NaCl, 10 mM Hepes-K or -Na buffer (pH 7.4) as described [12,13]. Both uni- and multilamellar vesicles were used for the experiments.

2.5. *Interaction of liposomes and proteins*

The supernatant of PMN (10 mg protein/ml) was incubated with 15 mM phospholipid liposomes at room temperature for 10 min in a medium containing 0.1 M KCl or NaCl, 10 mM Hepes-K or -Na buffer (pH 7.4) and various concentrations of $CaCl_2$ or EGTA. The liposome-associated proteins were separated from non-associated proteins by discontinuous Ficoll density gradient centrifugation technique (flotation method) as described in [12,13]. After separation of the protein associated-liposomes, the liposome-associated proteins were analyzed by SDS-polyacrylamide disk gel electrophoresis (SDS-PAGE) according to the method of Laemmli [14]. Proteins were detected by Coomassie brilliant blue R250 or silver staining methods [15].

2.6. *Protein and phospholipid content*

Protein concentration was determined by the Biuret method using bovine serum albumin as a standard [16]. The lipids from rat liver mitochondria or microsomes and/or human erythrocyte ghosts were extracted by the method of Folch et al. [17] using $CHCl_3/CH_3OH$ (2:1, v/v). The extracted lipids were analysed by thin-layer chromatography using basic silica plates and developed with $CHCl_3/CH_3OH$/acetic acid/H_2O (50:25:7:3, v/v) [18]. Lipid phosphorus was determined by using the method of Lowry et al. [19].

3. RESULTS AND DISCUSSION

3.1. *Membrane-associated proteins in supernatant of the homogenate*

By using the flotation method with Ficoll density gradient centrifugation, several cytoplasmic proteins of PMN have been identified as membrane-associated proteins [12,13]. Many proteins including actin, tubulin, α-actinin and the 33 kDa protein were found to associate with DMPC or DPPC liposomes [12,13]. In the case of DMPC liposomes, the degree of association of these proteins was greatly reduced by C, and the reduced association by C was overcome by adding CA. However, the association to liposomes was limited to certain proteins, such as actin, tubulin and α-actinin. Association of the 33 kDa protein with the DMPC/C/CA liposomes did not occur even in the presence of Ca^{2+}, as described [13]. In contrast, actin, tubulin and α-actinin always associated with DPPC liposomes which contained various lipid species either in the presence of 1 mM Ca^{2+} or 1 mM EGTA. In the presence of Ca^{2+}, however, the 33 kDa and small amounts of other proteins firmly associated with the DPPC liposomes which contained CL or PS (table 1 and fig.1A). It should be noted that the association of the 33 kDa protein to DPPC/C/PS liposomes required K^+ in the solution; the association was reduced by substituting K^+ with Na^+.

3.2. *Calcium-dependent association of the 33 kDa protein*

Most cytoplasmic proteins that associated to various liposomes did not require Ca^{2+} for this association. However, the association of the 33 kDa and some of other proteins with DPPC/C/PS or DPPC/CL liposomes was Ca^{2+} dependent; the association with DPPC/CL was

Table 1

Association of the 33 kDa protein to the various liposomes in the medium of K^+ or Na^+ and its dependency on Ca^{2+}

Liposome	Ca^{2+} (M)	EGTA (M)	Association of the 33 kDa protein in	
			Na^+	K^+
Microsomal lipid	10^{-4}	–	+	+ + +
	–	10^{-3}	–	–
Ghost lipid	10^{-4}	–	+	+ +
	–	10^{-3}	–	–
Mitochondrial lipid	10^{-6}	–	+ + +	+ + +
	–	10^{-3}	–	–
DPPC	10^{-4}	–	–	–
	–	10^{-3}	–	–
DPPC/C	10^{-4}	–	–	–
(1:0.5)	–	10^{-3}	–	–
DPPC/C/PS	10^{-6}	–	+	+ + +
(1:0.5:1)	–	10^{-3}	–	–
DPPC/PS	10^{-4}	–	–	+ +
(1:0.25)	–	10^{-3}	–	–
DPPC/CL	10^{-6}	–	+ + +	+ + +
(1:0.25)	–	10^{-3}	–	–

Supernatant of PMN was incubated with various liposomes in 0.1 M KCl, 10 mM Hepes-K buffer (pH 7.4) (K^+) or 0.1 M NaCl, 10 mM Hepes-Na buffer (pH 7.4) (Na^+) and protein associated liposomes were isolated by the flotation method as described in section 2. Molar ratios are quoted in parentheses. –, less than 10% association; +, 11–25% association; + +, 26–50% association; + + +, greater than 50% association

more sensitive to Ca^{2+} than that with DPPC/C/PS (fig.2). The half-maximum concentrations of Ca^{2+} required for the association of the 33 kDa protein with DPPC/C/PS and DPPC/CL were 10^{-5} and 10^{-8} M, respectively. Consequently, association of the 33 kDa protein with these liposomes did not occur in the presence of EGTA; the liposome-associated 33 kDa protein was reversibly dissociated by adding an excess amount of EGTA (fig.1C).

3.3. *Effect of CL and PS on the association of the 33 kDa protein to DPPC liposomes*

The association of the 33 kDa protein with the DPPC liposomes also depended on the concentrations of PS and CL in liposomes (fig.3). The half-maximum associations to DPPC/C/PS and DPPC/CL occurred at PS/PC and CL/PC molar ratios of 0.5 and 0.25, respectively. Similar associations of the 33 kDa protein also occurred with liposomes consisting of the lipids extracted either from mitochondria, microsome or erythrocyte ghosts (table 1). In this case, the association of the 33 kDa protein with liposomes did not solely depend on the electrostatic force of the liposome surface; no association was observed when the liposomes consisted of charged components, such as DPPC/C/DCP, DPPC/DCP, DPPC/CA, DPPC/C/CA, DPPC/PA or DPPC/C/PA. Accordingly, PS and CL were the most effective lipids for the association of the 33 kDa protein with DPPC liposomes. Although the exact mechanisms for the interaction of protein with liposomes containing PS or CL are still unknown, the Ca^{2+}-binding capacity of these two lipids may enhance the association of the 33 kDa

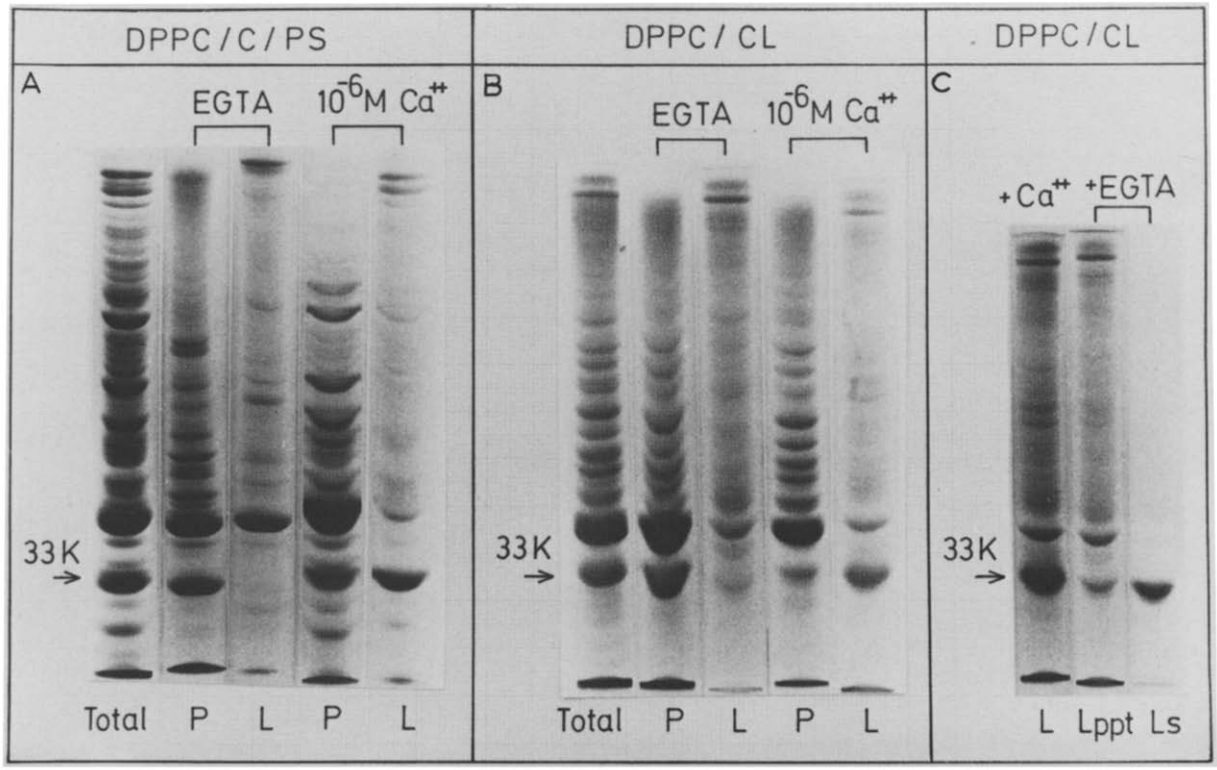

Fig.1. Electrophoretic analysis of the 33 kDa protein associated with DPPC liposomes containing PS or CL. Effect of Ca^{2+}. The supernatant fraction of PMN homogenate (10 mg protein) was incubated with liposomes (15 μmol DPPC) containing DPPC/C/PS (1:0.5:1, M/M) or DPPC/CL (1:0.25, M/M) at room temperature for 10 min in the potassium medium containing Ca^{2+} or EGTA, and protein associated-liposomes were isolated by Ficoll density gradient centrifugation. (A) Association with DPPC/C/PS liposomes. (B) Association with DPPC/CL liposomes. (C) Dissociation of the 33 kDa protein from DPPC/CL liposomes by 5 mM EGTA. Total, total proteins of supernatant fraction; P, non-associated proteins; L, liposome-associated proteins; EGTA, association was carried out in the presence of 1 mM EGTA; Ca, association was carried out in the presence of 1 μM Ca^{2+}; Lppt, undissociated protein from DPPC/CL liposomes by EGTA; Ls, dissociated proteins from DPPC/CL liposomes by EGTA.

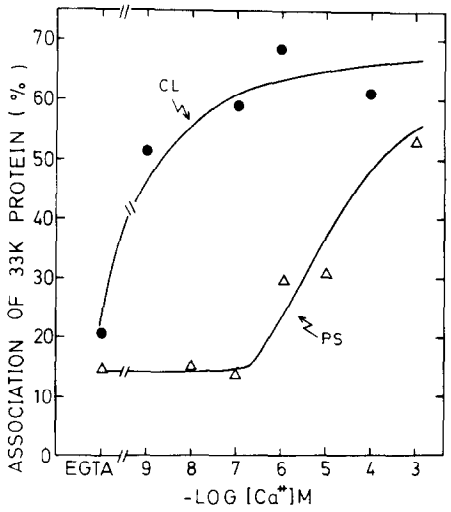

Fig.2. Ca^{2+} requirement for the association of 33 kDa protein to DPPC/C/PS or DPPC/CL liposomes. Experimental conditions were as described in fig.1 except that the Ca^{2+} concentration was varied in the incubation mixtures and the DPPC/C/PS was 1:0.5:0.5 (M/M). CL, association of the 33 kDa protein with DPPC/CL liposomes; PS, association of the 33 kDa protein with DPPC/C/PS liposomes. Data expressed as the % association of the 33 kDa protein.

protein with the liposome membranes.

These results suggested that the protein whose hydrophobic domains are exposed by calcium has a capacity to associate some lipid moiety, such as PS or CL of the liposomes. There are several proteins, such as calmodulin [5], calcimedins [20],

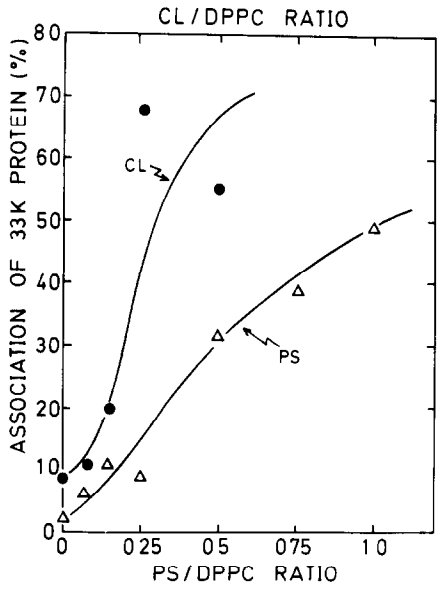

Fig.3. Requirement of PS or CL for the association of the 33 kDa protein with DPPC liposomes. Experimental conditions were as described in fig.2 except that the concentrations of PS or CL in DPPC liposomes were varied and the concentration of Ca^{2+} was 10^{-6} M.

protein kinase C [2,10], and calpain [3], whose hydrophobic binding activities were increased by Ca^{2+}. A recent report [21] revealed that protein kinase C was activated by liposomal vesicles consisting of a 1:4 molar ratio of PS/PC and constituted a physiological bilayer structure. These findings suggest that protein kinase C, which exists in cytoplasm in its inactive state, might bind to the internal surface of plasma membranes where PS localizes, thereby enhancing the catalytic activity of the enzyme. Thus, hydrophobic interaction of proteins and lipid membrane surface might play an important role in the regulation of calcium-related cellular metabolism. At present, the physiological importance of the 33 kDa protein remains obscure. However, Ca^{2+}-dependent reversible interaction of this protein with the liposome membrane suggests that the biological action of this protein may be affected significantly by changes in the intracellular Ca^{2+} levels of PMN.

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REFERENCES

[1] Moore, P.B., Kraus-Friedmann, N. and Dedman, J.R. (1984) J. Cell Sci. 72, 121–133.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Murachi, T. (1983) in: Calcium and Cell Function (Cheung, W.Y. ed.) vol.4, pp.377–410, Academic Press, New York.
[4] Tanaka, T. and Hidaka, H. (1981) Biochem. Int. 2, 71–75.
[5] LaPorte, D.C., Wierman, B.M. and Storm, D.R. (1980) Biochemistry 19, 3814–3819.
[6] Tanaka, T. and Hidaka, H. (1980) J. Biol. Chem. 255, 11078–11080.
[7] Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) J. Biol. Chem. 257, 13193–13196.
[8] Wolf, M., LeVine, H., May, W.S. jr, Cuatrecasas, P. and Sahyoun, N. (1985) Nature 317, 546–549.
[9] Moskowitz, N., Andres, A., Silva, W., Shapiro, L., Schook, W. and Puszkin, S. (1985) Arch. Biochem. Biophys. 241, 413–417.
[10] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. and Horecker, B.L. (1985) Proc. Natl. Acad. Sci. USA 82, 6435–6439.
[11] Utsumi, K., Sugiyama, K., Miyahara, M., Naito, M., Awai, M. and Inoue, M. (1977) Cell Struct. Funct. 2, 203–209.
[12] Utsumi, K., Okimasu, E., Takehara, Y., Watanabe, S., Miyahara, M. and Moromizato, Y. (1981) FEBS Lett. 124, 257–260.
[13] Utsumi, K., Okimasu, E., Morimoto, Y.M., Nishihara, Y. and Miyahara, M. (1982) FEBS Lett. 141, 176–180.
[14] Laemmli, U.K. (1970) Nature 227, 180–185.
[15] Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980) Anal. Biochem. 105, 361–363.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Forch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 469–509.
[18] Miyahara, M., Nishihara, Y., Moromizato, Y. and Utsumi, K. (1981) Biochim. Biophys. Acta 641, 232–241.
[19] Lowry, O.H., Roberts, N.R., Leiner, K.Y., Mei-Ling, W.V. and Farr, A.L. (1954) J. Biol. Chem. 207, 1–17.
[20] Boni, L.T. and Rando, R.R. (1985) J. Biol. Chem. 260, 10819–10825.
[21] Walsh, M.P., Valentine, K.A., Ngai, P.K., Carruthers, C.A. and Hollenberg, M.D. (1984) Biochem. J. 224, 117–127.