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Calcium binding protein from porcine intestine binds to phosphatidylserine vesicles in the presence of calcium

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Protein II, a 32K cytoskeleton-associated protein isolated from porcine intestinal epithelium, binds to vesicles composed of phosphatidylserine in the presence, but not the absence, of 10 μM Ca^{2+}. Binding was saturable and was specifically inhibited by chelation of free Ca^{2+} with EGTA. Binding was also inhibited by trifluophenothiazine. Vesicles composed of dimyristoylphosphatidylcholine did not bind protein II, suggesting that interaction with phosphatidylserine was selective. These properties are consistent with a possible role for protein II in Ca-regulated cytoskeleton-cell membrane events.

Introduction

Changes in free calcium concentration play an important role in regulating cytoskeletal changes that underlie such cell behaviors as secretion, movement, and adhesion [2–4]. We have described three calcium binding proteins isolated from porcine intestine and liver that appear to be associated with the cytoskeleton when cells are solubilized in the presence of Ca^{2+} [1,4,5]. Similar or identical proteins have been described in bovine liver, chromaffin granules, and in electric organ of *Torpedo* [6–8], and Geisow and others speculated that they may mediate exocytosis [9]. Our data indicated [4] that the three proteins were unrelated immunologically, had different affinities for Ca^{2+}, and had different tissue distributions. Protein I is a tetramer composed of 10K and 36K subunits, while proteins II and III are monomers of 32K and 68K, respectively [1,4]. All three proteins undergo Ca-dependent conformational changes that can be detected by ultraviolet spectroscopy [1,4].

We now show that protein II is able to bind to phosphatidylserine vesicles in the presence, but not the absence, of Ca^{2+}.

Methods

Purification of porcine intestinal protein II

Protein II was purified from porcine intestinal epithelia [1,4]. Briefly, membrane vesicles derived from epithelial cells were demembranated in extraction buffer (0.1 M KCl/0.5 mM DTT/1 mM NaN_3/10 mM imidazole-HCl (pH 7.4)) containing 1% Triton X-100 and 1 mM $CaCl_2$. The $10\,000 \times g$ pellet was washed twice with Ca^{2+} extraction buffer without Triton and then re-extracted with extraction buffer containing 5 mM EGTA. After centrifugation at $100\,000 \times g$ the EGTA extract was precipitated with 75% ammonium sulfate, dialyzed, and applied to DEAE-cellulose in 10 mM imidazole-HCl (pH 7.4). Pro-

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Abbreviations: PS, phosphatidylserine.

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tein II was eluted with a 0–150 mM gradient in NaCl and final purification achieved using gel filtration on Sephadex G-100.

Radioiodination of purified protein II was performed by the Bolton-Hunter method [10]. Specific activity achieved was 10^6 cpm/μg protein.

Multilamellar vesicles of phosphatidylserine (PS) (Sigma) were prepared as follows [10]. PS was dried by rotary evaporation at 37°C and resuspended at 2 mg/ml in 10 mM phosphate buffer (pH 7.0). Vesicles were formed by rotating samples at 37°C for 30 min, and washed by centrifugation at 8000 × g for 3 min. Vesicles were multilamellar and uniform in size (about 10 μm diameter) as assessed by phase microscopy. Vesicles composed of dimyristoylphosphatidylcholine, prepared by the same method, were the kind gift of A. Corin and E. Blatt (Max Planck Institute for Biophysical Chemistry, Göttingen).

Binding assays

Labelled protein II was diluted with unlabelled protein II to give a final stock solution that contained 0.05 mg/ml protein II and about 1000 cpm/μl. Assay buffer consisted of 10 mM imidazole-HCl (pH 7.4)/150 mM NaCl/2 mM $MgCl_2$/0.1 mM $CaCl_2$/0.1 mg/ml bovine serum albumin (Sigma). Vesicles were preequilibrated in assay buffer for 30 min before the assay to ensure that free calcium concentrations would not change during the assay. Protein II, diluted in assay buffer, was added to 10 μl of lipid vesicle suspension in volume of 100 μl, and samples incubated 30 min at 20°C. Samples were diluted to 300 μl with assay buffer and lipid-bound material was collected by centrifugation at 10000 × g for 2 min. The amount of ^{125}I-labeled protein II in the lipid vesicle pellet was determined by counting ^{125}I in a gamma counter.

Calcium concentration in the binding assay was varied with balanced Ca/EGTA buffers [12]. Inhibition studies with trifluophenothiazine (Chemical Dynamics) were done by adding trifluophenothiazine (15 mg/ml in DMSO) to a final concentration of 200 μM. Control samples received an equal volume of DMSO, which did not affect the assay.

Porcine liver plasma membranes were prepared by the method of Therien et al. [13]. The final preparation was sonicated for 20 s with a microprobe tip to form vesicles, which were used in the assay as described above.

Results

Radioiodinated protein II bound to PS lipid vesicles in the presence of 0.1 mM free calcium (Fig. 1). Binding was saturable and was competed by unlabelled protein II (Fig. 1B). In the presence of 1 mM EGTA, binding was not detectable (Fig.

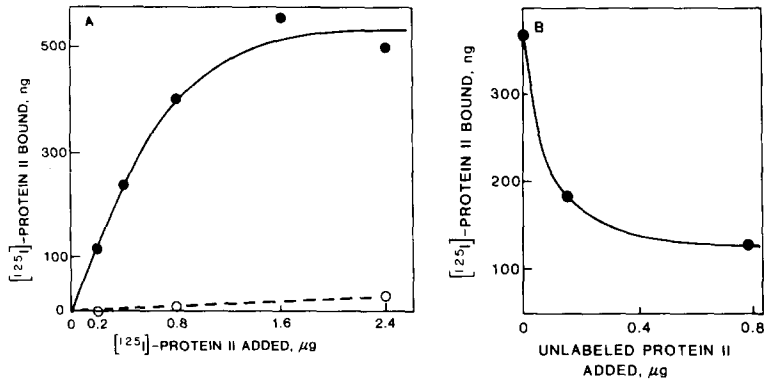

Fig. 1. (A) Protein II binds to phosphatidylserine vesicles in the presence, but not the absence, of calcium. Solid line, the presence of 0.1 mM free Ca^{2+}; dashed line, in the presence of 1 mM EGTA. Protein II was purified from porcine intestine and radioiodinated as described in Methods. Incubations were performed at 20°C in assay buffer. Lipid vesicles (10 μg), prepared as described, were incubated with ^{125}I-labelled protein II in a final volume of 100 μl. (B) Binding of labelled protein II to PS vesicles is competed by addition of unlabelled protein II. 10 μg of PS vesicles were incubated with 780 ng labelled protein II and increasing amounts of unlabelled protein II in the presence of 0.1 mM free calcium. Binding was determined after a 30 min incubation.

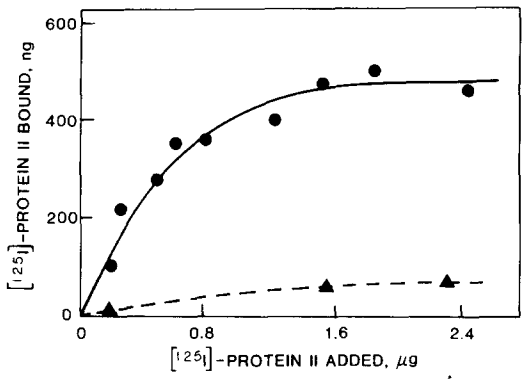

Fig. 2. Trifluophenothiazine inhibits binding of ^{125}I-labelled protein II to phosphatidylserine vesicles. Trifluophenothiazine (1 μl) was added to assay tubes containing lipid vesicles (Final concentration 200 μM). After 20 min, ^{125}I-labelled protein II was added and the assay was performed. Control samples received an addition of an equal volume of DMSO. Closed circles, control samples. Triangles, trifluophenothiazine added.

1A). These results were obtained in the presence or absence of 2 mM $MgCl_2$. Binding depended on the type of lipid used as vesicles of dimyristoylphosphatidylcholine did not bind measurable amounts of ^{125}I-labelled protein II.

To investigate the role of Ca^{2+}, trifluophenothiazine was added to the assay. This drug binds selectively to calmodulin and other calcium binding proteins. It is thought to bind to hydrophobic regions of the proteins exposed in the presence of Ca^{2+} [14]. At 200 μM, the drug blocked protein II binding to PS vesicles over 90% (Fig. 2), suggesting that Ca^{2+} binding sites of protein II are required for interaction with lipid vesicles.

To verify that the ^{125}I bound to vesicles represented binding of labelled protein II, the PS vesicle fraction was collected by centrifugation and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Radioactivity was found in the 32K protein II band (Fig. 3a). A short incubation of the pelleted vesicle fraction in assay buffer containing 1 mM EGTA released 82% of the lipid-associated protein II into the supernatant (Fig. 3, lanes b and c). In a parallel incubation, in which EGTA was present throughout the first incubation, less than 4% of the ^{125}I-labelled protein II added bound to PS vesicles.

The Ca^{2+} concentration dependence of ^{125}I-labelled protein II binding to PS vesicles was

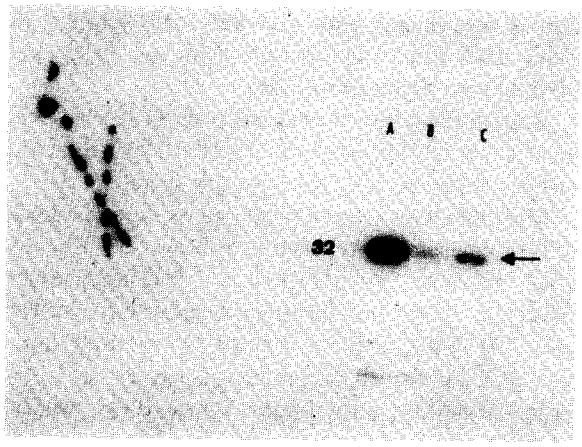

Fig. 3. Lipid-associated ^{125}I-labelled protein II can be released by short incubation in EGTA-containing buffer. Autoradiography of a 10% SDS gel of lipid vesicle bound protein II. Binding assay was performed as described, and the final pelleted lipid vesicle fraction was analyzed by SDS-PAGE and autoradiographed. Arrow (32K) indicates position of a protein II standard run in parallel and stained with Coomassie blue. Lane a, PS vesicle pellet after incubation with ^{125}I-labelled protein II in assay buffer and centrifugation. Lane b, ^{125}I-labelled Protein II bound to lipid vesicles was incubated for 5 min in a fresh aliquot of assay buffer that was made 1 mM in EGTA, centrifuged, and the lipid-associated pellet applied to the gel. Lance c, material solubilized from (b) during 5-min EGTA incubation.

determined by performing the assay at varying concentrations of Ca^{2+} [12]. Assay buffer was modified by calculated additions of EGTA and

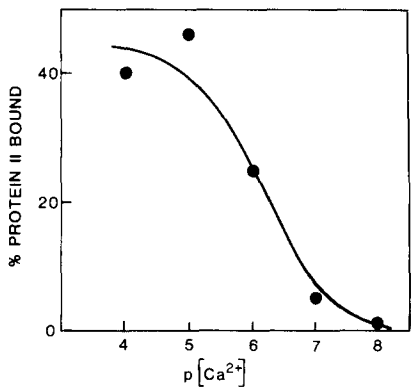

Fig. 4. Binding of ^{125}I-labelled protein II to PS vesicles is dependent upon free Ca^{2+} concentration. Binding assay was performed upon 10 μl of lipid suspension and 10 μl (780 ng) protein II stock solution in a final volume of 0.2 ml modified assay buffer. Calcium concentration was varied as described. Binding was determined in duplicate.

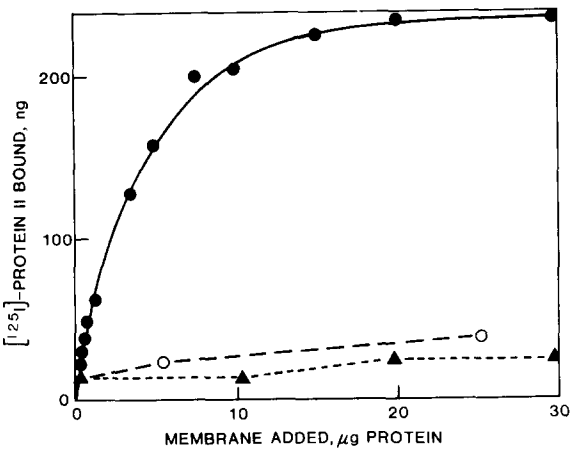

Fig. 5. ^{125}I-labelled protein II binds to liver plasma membrane preparation in the presence of Ca^{2+}. Liver plasma membranes were prepared as described and protein II binding determined as in Methods. Solid circles, in standard assay buffer (contains 0.1 mM Ca^{2+}). Open circles, addition of trifluophenothiazine (200 μM final concentration) to assay buffer. Triangles, assay buffer modified by the addition of EGTA to 1 mM.

Ca^{2+} to produce desired Ca^{2+} concentrations, and protein II binding was determined as described, under conditions of slight excess of protein I. Fig. 4 shows that protein II binding depended on Ca^{2+} concentration and correlated well with the estimated K_d ($2.5 \cdot 10^{-5}$ M) of protein II for Ca^{2+} [4]. When protein II concentration was varied, the maximal nanomoles of protein bound also depended upon Ca^{2+} concentration, and ws not affected by increasing the incubation time, in agreement with this conclusion (data not shown). The same result was obtained when the assay was performed at 2°C, a temperature at which the lipid is below its phase transition (data not shown).

Plasma membrane vesicles were prepared from liver cells by differential centrifugation and sonication [13] and bound ^{125}I-labelled protein II with properties that were very similar to those described here for pure PS vesicles. Binding was optimal at 10 μM Ca^{2+}, was inhibited by either trifluophenothiazine or EgtA, and was not affected by pretreatment of membrane vesicles with trypsin.

Discussion

We have shown that protein II, a calcium binding protein of porcine intestine [1,4], binds to phosphatidylserine vesicles specifically and reversibly in the presence of 10^{-5} M Ca^{2+}. Unlabelled protein II inhibited the interaction and chelation of Ca^{2+} resulted in the release of lipid-bound protein II. In contrast, lipid vesicles composed of dimyristoylphosphatidylcholine failed to bind ^{125}I-labelled protein II under identical conditions.

The mechanism by which protein II binds to lipid bilayers is not known. Binding may be indirect via Ca^{2+} ions that are electrostatically bound to the lipid bilayer. Alternatively, since Ca^{2+} induces conformational changes in protein II that increase the exposure of hydrophobic residues [4], it is possible that direct interaction between protein II and the phospholipid occurs.

After our results were completed Geisow et al. [8] noted that 'endonexin', a 32.5K calcium binding protein isolated from bovine liver, bound to vesicles composed of phosphatidylethanolamine and phosphatidylinositol in the presence of Ca^{2+}, but did not interact with phosphatidylcholine or phosphatidylserine vesicles. Endonexin and protein II are very similar in their purification properties, Ca^{2+} binding affinities and ability to associate with membranes. We have purified protein II from porcine liver [4], and it is very similar to intestinal protein II in isoelectric point, molecular weight and reactivity with antibodies raised against intestinal protein II. The difference in our results might be due to the different assays used to detect lipid binding. Geisow et al. [8] relied on SDS-PAGE detection of bound proteins, a procedure less sensitive than the ^{125}I-binding assay we used. Alternatively, it is possible that liver endonexin differs from intestinal protein II in its lipid binding properties. Regardless of this discrepancy lipid selectivity is indicated for endonexin and protein II since phosphatidylcholine is not bound. This together with recent results on protein I [15,16] supports the notion that a group of related Ca^{2+} binding proteins show selectivity in lipid binding. Ca^{2+} dependent binding to lipids involves so far only lipids thought to be present at the cytoplasmic side of membranes.

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