Calcium-dependent Association of a Protein Complex with the Lymphocyte Plasma Membrane: Probable Identity with Calmodulin–Calcineurin

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ABSTRACT A protein complex is shown to participate in a calcium-dependent association with plasma membranes purified either from pig mesenteric lymph node lymphocytes or from human lymphoblastoid cell lines. Plasma membranes prepared in the presence of calcium possess this complex; those prepared in the absence of calcium (5 mM EGTA) do not. The complex associates itself with the inner cytoplasmic surface of the plasma membrane. This complex is referred to as the "acidic protein band" because of its location during migration upon alkaline–urea gel electrophoresis. The complex dissociates from the plasma membrane during electrophoresis on 8-M urea gels, irrespective of calcium levels during electrophoresis; at intermediate urea concentrations (4–6 M), the complex is not dissociated in the presence of calcium. Upon purification of the acidic protein band, SDS acrylamide gel electrophoresis, immunoblotting, and radioimmunoassay techniques suggest that the acidic protein band is composed of at least four peptides (designated 68K, 59K, 20K, 20K): two of these (68K, 20K) are immunopositive for calcineurin and one (20K) is immunopositive for calmodulin. Immunoblots of urea gels also indicate that the calcineurin heavy chain (68K) can also appear at three different locations on the urea gel. Patches and caps induced in human peripheral blood lymphocytes by fluorescein-conjugated goat anti-human IgG are not coincident with the location of calcineurin, which remains distributed throughout the cell.

The lymphocyte plasma membrane is of considerable significance with regard to lymphocyte function. It possesses appropriate external receptors for antigen and/or mitogen binding which, when occupied, send a transmembrane signal leading to differentiation and blastogenesis (1). In addition the plasma membrane has recognition sites for cell–cell interactions as well as the machinery necessary for transmembrane coupled phenomena such as patching and capping (2). It has been averred that phosphorylation of certain lymphocyte plasma membrane proteins might be the initial response to transmembrane signaling which accompanies the above phenomena (3–5). Evidence for both endogenous cAMP-dependent and -independent protein kinases bound to the lymphocyte plasma membrane has been presented (4, 6). Tyrosine-specific protein kinases have been found in association with the lymphocyte plasma membrane (7, 8) and elevated levels of such activity have been found in at least one lymphoma cell line (9). However, to date, there has been no evidence for the presence of a plasma membrane–bound phosphatase, which presumably would have a key role to play in the regulation of membrane-bound protein phosphorylation reactions.

Brain calcineurin is composed of two peptides, calcineurin A (60–65 kD) and calcineurin B (15–20 kD) (10, 11); the active site is located on the high molecular weight subunit (12, 13). In the presence of calcium and calmodulin, calcineurin acts as a protein phosphatase (14), a property confirmed in several laboratories (15, 16, 18). Calcineurin has also been shown to be capable of cleaving phosphotyrosine (16, 18), even in the absence of calmodulin. Although calcineurin was originally thought to be confined to nervous tissue (10, 11), it has since been found in rabbit skeletal muscle (14, 19).

This paper provides evidence for a protein complex which is associated, in a calcium-dependent manner, with plasma membranes from human lymphoblastoid cells and pig lymphocytes. The complex is immunopositive to antisera for calmodulin and calcineurin and displays activities and a

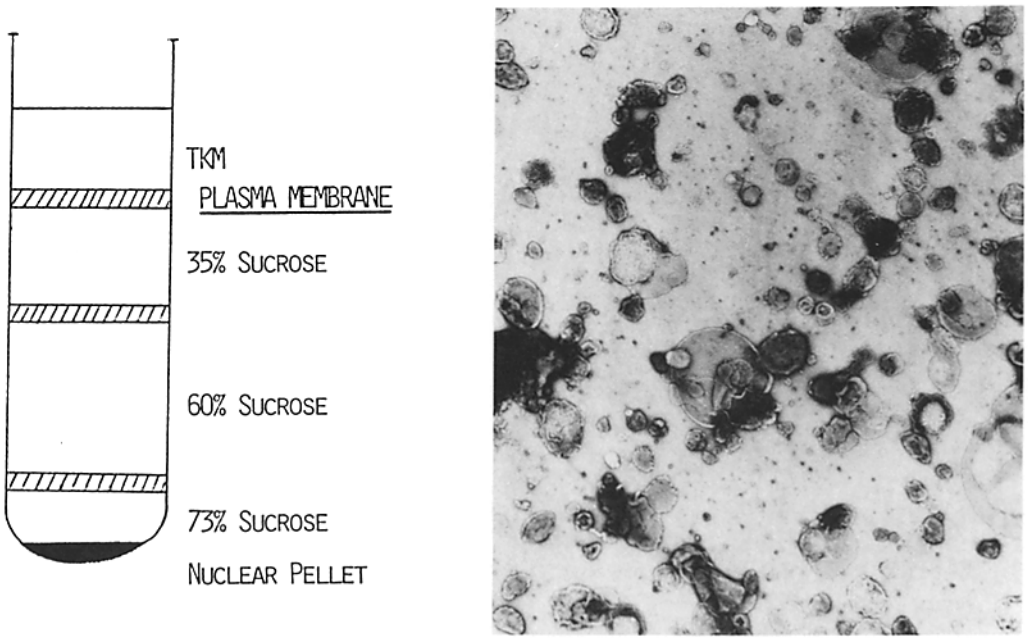

FIGURE 1 (a) Diagram of stepwise sucrose gradient used in the lymphocyte plasma membrane preparations described in this study. *TKM*, 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4. (*b*) Negatively stained electron micrograph images of typical plasma membrane vesicles prepared during this study. × 17,000.

peptide composition consistent with this identification. Using fluorescently labeled antibodies, it is demonstrated that calcineurin is distributed throughout the lymphocyte cytoplasm and does not co-cap or co-patch with anti-surface immunoglobulin (sIg)–induced[1] caps and patches. Antibodies to calcineurin do not react with the external cell surface of viable lymphocytes indicating that the calcium-dependent attachment seen in vitro is most probably to the inner surface of the plasma membrane. The ability of a calmodulin–calcineurin complex to attach itself to the lymphocyte plasma membrane places a versatile phosphatase at a key location, consistent with it having an important role in the termination of transmembrane signaling.

A preliminary account of this work has been presented earlier (20).

MATERIALS AND METHODS

Lymphocyte Plasma Membrane Preparation: In earlier experiments, using human lymphoblastoid cells, plasma membranes were prepared by use of a cell disrupter (21). Most experiments, however, were performed on pig lymphocyte plasma membranes, prepared from pig mesenteric lymph node lymphocytes by a modification of a procedure described by Monneron & d'Alayer (22). Individual nodes were pressed through 20-mesh stainless steel cloth into Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum and 1% penicillin, 1% streptomycin. Material from 20–30 nodes was then passed through eight layers of cheesecloth before washing in phosphate-buffered saline (PBS) (170 mM NaCl, 3 mM KCl, 12 mM phosphate, 1 mM $CaCl_2$, pH 7.2). The cellular suspension in PBS was loaded onto Ficoll-Isopaque (Pharmacia Fine Chemicals, Piscataway, NJ) and spun at 800 *g* for 30 min at 20°C. The lymphocyte layer was resuspended in PBS and the viability of the cell population ascertained by trypan blue exclusion. Viability was routinely better than 90% at this stage. Lymphocytes were spun down and resuspended (0.5–5.0 × 10^7 cells per ml) in 70% sucrose dissolved in buffer A (50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4) which was subsequently adjusted to 60% sucrose before homogenization in a glass/teflon motorized Potter homogenizer (Sorvall, DuPont Co., Newtown, CT). Viability subsequent to this step was routinely <5%. This broken cellular suspension was then loaded onto a stepwise sucrose gradient (22) and spun overnight at 25,000 *g* in a Beckman SW 27.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). The top plasma membrane layer (see Figure 1*a*) was collected, diluted with buffer A, then spun for 30 min at 50,000 *g* in a Beckman Ti50 rotor (Beckman Instruments, Inc.). The smooth, vesicular nature of a typical preparation is seen in Figure 1*b*. Plasma membrane pellets were resuspended in 10 mM Tris, 3 mM NaN_3 plus 8% sucrose, pH 7.5, and stored at −20°C.

Preparation of Human Lymphocytes and Human Lymphoblastoid Cells: Human peripheral blood lymphocytes were isolated from Source Leukocytes, a white cell–enriched fraction obtained from the American Red Cross. The Source Leukocytes were loaded onto Ficoll-Isopaque and spun at 800 *g* for 30 min at 20°C; the buffy coat was selectively removed, washed in DME supplemented with 2% fetal calf serum, 1% penicillin, and 1% streptomycin, and then resuspended in the same. Cell viability was checked at this point by trypan blue exclusion and was routinely better than 90%.

The human lymphoblastoid cell line, Bri-8, was cultured in Roswell Park Memorial Institute 1640 supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin.

Antibodies: Rabbit polyclonal antibodies raised to both calcineurin (A + B) and calmodulin were generous gifts from Dr. Claude Klee (National Cancer Institute, Bethesda, MD). The calmodulin radioimmunoassay kit was obtained from Amersham Corp., Arlington Heights, IL. Peroxidase-conjugated swine anti-rabbit IgG was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). Fluorescein-conjugated goat anti-human IgG was obtained from Gibco Laboratories (Grand Island, NY). Rhodamine-conjugated goat anti-rabbit IgG was obtained from Miles Laboratories, Inc., Elkhart, IN.

Electrophoresis and Transfer Techniques: Urea–polyacrylamide gel electrophoresis was performed in the presence and absence of calcium, under standard conditions (23, 24). SDS polyacrylamide gel electrophoresis was performed using procedures attributed to Laemmli (25). Gels were routinely stained using Coomassie Blue; silver staining (26) was performed when applicable.

Western transfer of material from gels to nitrocellulose paper followed the protocol of Towbin et al. (27). After overnight transfer at 8 V/cm, a single lane was cut from the nitrocellulose paper and checked for successful transfer by Amido black staining (5 min in 0.1% Amido black, 45% methanol, 10% acetic acid, then destained by 5 min in 90% methanol, 2% acetic acid). The remainder of the nitrocellulose paper was divided up as necessary, fixed in 25% isopropanol, 10% acetic acid for 30 min, and then washed once in PBS. Next, the

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; PNPP, *p*-nitrophenyl phosphate; sIg, surface immunoglobulin.

nitrocellulose paper was gently shaken in blocking solution (10% horse serum, 3% BSA) for 5 h before overnight incubation with primary antibody. Excess primary antibody was then removed by copious washes with PBS before a second blocking step (2 h) and incubation with horseradish peroxidase–conjugated second antibody (2–10 h). After removal of excess secondary antibody by copious washes, the reaction was developed using 0.01% hydrogen peroxide and 0.5 mg/ml 4-chloro-1-naphthol as substrate (28). Color was allowed to develop over a 10-minute to overnight period. Densitometry of the developed color was performed on a Leitz MPV photometer using incident light optics.

Electroelution of proteins from gel slices (150 V for 1 h) was facilitated by the use of Isco sample cups (Isco, Lincoln, NE); the buffer used was the standard urea gel running buffer (24). Molecular mass standards used were myosin heavy chain, β-galactosidase, BSA, ovalbumin, carbonic anhydrase, and cytochrome *c* (200, 135, 68, 45, 30, and 12 kD, respectively).

Activity Assays: Activity was measured spectrophotometrically by monitoring the cleavage of *p*-nitrophenyl phosphate (PNPP) at 410 nm using an extinction coefficient of $1.75 \times 10^4\ M^{-1}\ cm^{-1}$ (29). All samples contained 0.1 ml of purified plasma membrane and 20 mM PNPP in a total sample volume of 1.0 ml. In addition, samples either contained 50 mM Tris, pH 8.6, 20 mM $MgCl_2$, 10 μM $CaCl_2$ (29), or 50 mM Tris, pH 7.2, 2 mM Ni^{2+} (18, 29). Blanks were identical except that they lacked plasma membrane. Assays for the acidic protein band were carried out in a similar manner.

Protocol for Patching and Capping Experiments: Human peripheral blood lymphocytes (3×10^6 cells per 0.5 ml medium) were incubated with fluorescein-labeled goat anti-human IgG (1:20 dilution) for 20 min at 0°C in order to label and cross-link the sIg on the cell surface of B lymphocytes. The cells were washed three times in DME supplemented with 2% fetal calf serum, 1% penicillin, and 1% streptomycin, and then resuspended in 0.5 ml of the same and incubated at 37°C for 0-, 10-, 20-, and 30-min intervals. The cells were then fixed by addition of an equal volume of 2% paraformaldehyde and were incubated at 0°C for 20 min. After three washes in DME (supplemented as above), the cells were resuspended in 0.5 ml of the same. In double-labeling experiments, the cells were next incubated with rabbit anti-calcineurin (A + B) (1:1,000 dilution) for 30 min at room temperature. The cells were then washed as above before a 20-min incubation with the second antibody: rhodamine-labeled goat anti-rabbit IgG (1:20 dilution). After three final washes, the cells were resuspended in 0.5 ml of DME (supplemented as above). One drop of solution was placed on a microscope slide together with one drop of 0.1% *p*-phenylenediamine in 90% glycerol to prevent fluorescence quenching (30). Cells were examined with a Leitz Dialux fluorescence microscope set up with phase-contrast optics and a Leitz Orthomat-W camera. Leitz filter cubes I2 and N2 were used for fluorescein and rhodamine fluorescence, respectively. Photographs were taken using Kodak Plus-X film.

Protocol To Determine if Antigens Are Located on the Outer Cell Surface: Human peripheral blood lymphocytes (3×10^6 cells per 0.5 ml medium), 95% viable as determined by trypan blue exclusion, were incubated for 20 min with either fluorescein-labeled goat anti-human IgG (1:20 dilution), rabbit anti-calcineurin (A + B) (1:1,000 dilution), or rhodamine-labeled goat anti-rabbit IgG (1:20 dilution). The cells were then fixed by addition of an equal volume of 2% paraformaldehyde and were incubated at 0°C for 20 min. Cells that had initially been incubated with fluorescein-labeled goat anti-human IgG or with rabbit anti-calcineurin (A + B) were washed before incubation with rhodamine-labeled goat anti-rabbit IgG for 20 min at 0°C. The purpose of the batch of cells incubated with rhodamine-labeled goat anti-rabbit IgG alone was to determine the degree of cross-reaction, if any, of this antibody with human lymphocyte sIg. After three final washes, the cells were prepared for fluorescence microscopy as described above.

RESULTS

Urea–Acrylamide Gel Electrophoresis of Plasma Membrane Preparations from Human Lymphoblastoid Cell Lines and Pig Lymphocytes

SDS acrylamide gel electrophoretic patterns of typical plasma membrane preparations from pig lymphocytes (e.g., see Figs. 5 and 7*b*) show that major peptides of 200, 83, 72, 58, and 45 kD were routinely obtained in a calcium-independent manner, together with minor bands at 125, 56, 43, 37, and 34 kD (seen in eight preparations). This detailed pattern is simplified significantly when preparations are run on alkaline urea–acrylamide gels (Fig. 2). At pH 8.6, most components of the plasma membrane remain close to the cathode upon urea–acrylamide gel electrophoresis. A major band, which we refer to as the principal band, is seen about one quarter of the way into the gel. Another band, which we refer to as the acidic protein band, migrates close behind the dye front at the anodal end of the gel. In 8 M urea, the acidic protein band is observed irrespective of the presence or absence of calcium (Fig. 2). However, in 4–6 M urea, the appearance of the acidic protein band is dependent upon the calcium level. In 4–6 M urea, the band is only observed in the absence of calcium (5 mM EGTA); in the presence of calcium (1 mM $CaCl_2$) the band cannot be seen. The principal band is observed over the whole range of calcium and urea concentrations studied. Results shown in Fig. 2 are from plasma membranes isolated from the human lymphoblastoid B-cell line, Bri-8. Similar results were obtained from other human lymphoblastoid B-cell lines (MST, JY, and Maja), and from the human Burkitt lymphoma cell line Daudi (data not shown), as well as from pig mesenteric lymph node lymphocytes (Fig. 3*a*). These results are only obtained if the membrane preparation is performed in the presence of calcium (see below).

SDS Gel Electrophoresis of the Acidic Protein Band Followed by Immunoblotting

The acidic protein band arising from the loading of pig lymphocyte plasma membrane (≅1 mg) onto a preparative urea slab gel (5 M urea; 5 mM EGTA) was excised from the gel, electroeluted, dialyzed, and then rerun on an SDS polyacrylamide gel (Fig. 3). The acidic protein band from the preparative urea gel gave rise to three separate peptides on the SDS gel with molecular masses of 68, 59, and 20 kD (see Figs. 3*b* and 5). Western transfer of the material from the SDS gel to nitrocellulose paper, followed by development of the transferred material using anti-calcineurin (A + B) as primary antibody, yielded the pattern seen in Fig. 3*c*. The anti-calcineurin antibody reacts with both the 20-kD and the 68-kD bands. The anti-calcineurin antibody has been fully

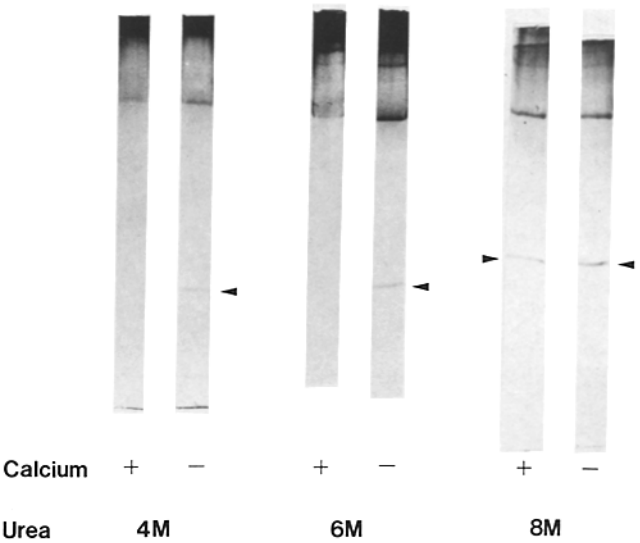

FIGURE 2 Urea–acrylamide electrophoresis of a plasma membrane preparation from Bri-8 lymphoblastoid cells, run as a function of urea concentration (4 M, 6 M, 8 M) in the presence (1 mM $CaCl_2$) or absence (5 mM EGTA) of calcium. 10% acrylamide gels, pH 8.6. ≅400 μg loading per lane. Gels stained with Coomassie Blue. Arrowheads mark the position of the acidic protein band.

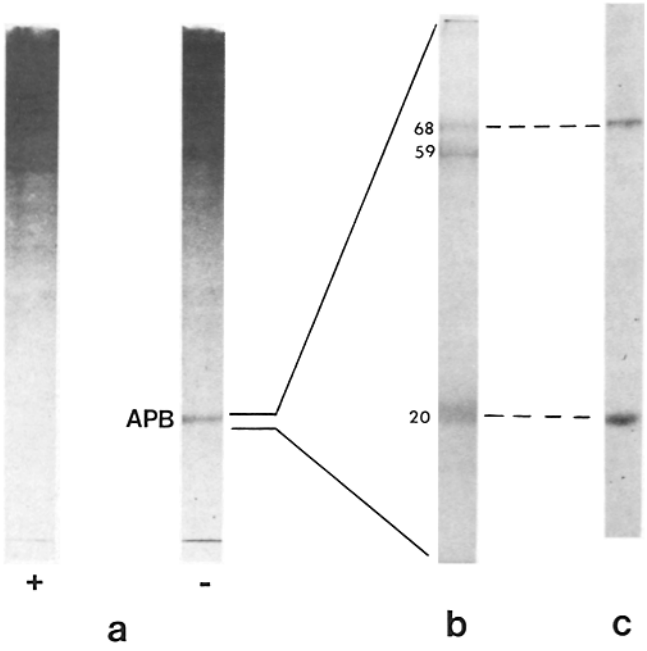

FIGURE 3 (a) Urea-acrylamide electrophoresis of a plasma membrane preparation from pig mesenteric lymph node lymphocytes, run either in the presence (1 mM $CaCl_2$) or absence (5 mM EGTA) of calcium. 10% acrylamide gels in 5 M urea, pH 8.6. ≅400 μg loading per lane. Gels stained with Coomassie Blue. (*b*) The acidic protein band obtained from preparative urea slabs, in the absence of calcium, was excised from the gel, electroeluted, dialyzed, lyophilized, and then rerun on a 12.5% acrylamide SDS slab. ≅6 μg loading per lane. Gels stained with Coomassie Blue. (c) Western blot of a sister lane of *b* developed using anti-calcineurin (A + B) as primary antibody and horseradish peroxidase-conjugated swine anti-rabbit IgG as second antibody; color developed using 0.01% H_2O_2 and 0.5 mg/ml 4-chloro-1-naphthol. For details see Materials and Methods. *APB*, acidic protein band.

characterized (Klee, C. B., manuscript in preparation). In our hands the antibody did not cross-react with myosin, β-galactosidase, BSA, ovalbumin, actin, carbonic anhydrase, cytochrome *c*, or calmodulin. Appropriate negative controls, with non-immune serum in place of the primary antibody, were run with each experiment; these showed no signs of staining even when the peroxidase reaction was allowed to continue for several hours.

Immunoblotting of the Urea–Acrylamide Gel with Anti-calcineurin and Anti-calmodulin

Pig lymphocyte plasma membranes, run on 8 M urea–acrylamide gels, were subjected to Western transfer onto nitrocellulose paper followed by development of the transferred material using anti-calcineurin as primary antibody (see Figs. 4 and 6). Anti-calcineurin stained the replica lane at three different locations, one of which coincided with the position of the acidic protein band. Material close to the origin of the gel also stained with anti-calcineurin, but by far the majority of the anti-calcineurin staining was located about half way along the gel and was not coincident with any band visualized by Coomassie Blue staining of the urea gel (Fig. 4*b*). When the appropriate region of the preparative sister urea gel was excised, electroeluted, dialyzed, lyophylized, and then rerun on an SDS polyacrylamide gel, two bands of 68 and 59 kD were obtained (Fig. 4*c*). These bands were in exactly the same position as the two bands of higher molecular weight seen in the acidic protein band (Fig. 5). No 20-kD band was seen here. Western transfer from the SDS gel onto nitrocellulose paper and development with anti-calcineurin (A + B) as primary antibody showed that only the 68-kD band was immunopositive for calcineurin (Fig. 4*d*).

When pig lymphocyte plasma membrane preparations were run on an 8-M urea gel and the gel was immunoblotted onto nitrocellulose paper followed by development of sister lanes with both anti-calcineurin (A + B) and anti-calmodulin as primary antibodies, the results seen in Fig. 6 were obtained. The three bands corresponding to anti-calcineurin staining were obtained as in Fig. 4; in contrast, the anti-calmodulin activity resided in a region close to the cathode as well as with the acidic protein band. No anti-calmodulin staining was observed in the region of maximal anti-calcineurin staining.

This result was also confirmed using a radioimmunoassay for calmodulin. Various zones, isolated from preparative urea gels of lymphocyte plasma membranes, were analyzed by calmodulin radioimmunoassay; whole plasma membranes from both pig lymphocytes and human Bri-8 lymphoblastoid cells were also assayed for calmodulin. Results are seen in Table I. Calmodulin is found in the acidic protein band but not in the region of the major calcineurin–immunopositive band.

The concentration of calmodulin in the pig lymphocyte

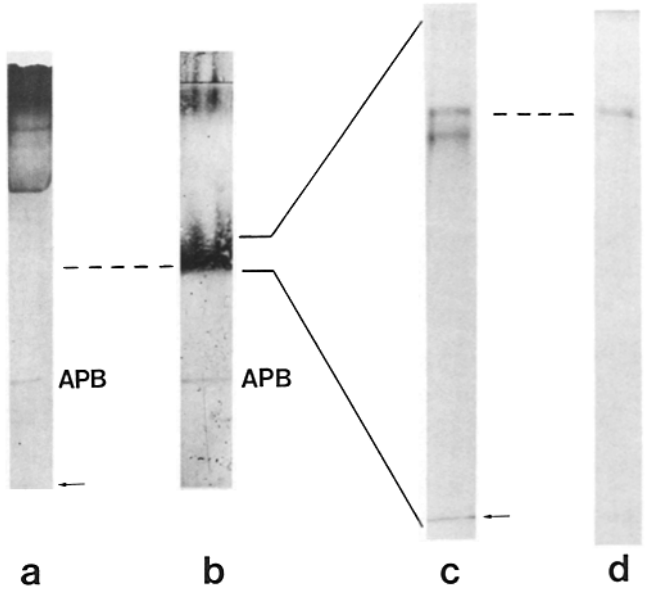

FIGURE 4 (a) Urea–acrylamide electrophoresis of a plasma membrane preparation from pig mesenteric lymph node lymphocytes. 5 mM EGTA. 10% acrylamide gels in 8 M urea, pH 8.6. ≅400 μg loading. Gel stained with Coomassie Blue. (*b*) Western blot of a sister lane of *a* developed using anti-calcineurin (A + B) as primary antibody, horseradish peroxidase–conjugated swine anti-rabbit IgG as second antibody; color developed using 0.01% H_2O_2 and 0.5 mg/ml 4-chloro-1-naphthol. For details see Materials and Methods. (c) The region of major anti-calcineurin activity was excised from a preparative urea slab, electroeluted, dialyzed, lyophilized, and then rerun on a 12.5% acrylamide SDS slab. ≅6 μg loading. Gels stained with Coomassie Blue. (*d*) Western blot of a sister lane of c developed using anti-calcineurin (A + B) as primary antibody, horseradish peroxidase–conjugated swine anti-rabbit IgG as second antibody; color developed using 0.01% H_2O_2 and 0.5 mg/ml 4-chloro-1-naphthol. For details see Materials and Methods. Arrows point to the dye fronts of the gels. *APB*, acidic protein band.

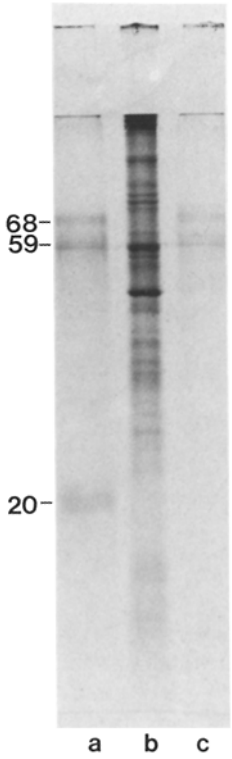

FIGURE 5 12.5% acrylamide SDS slab. Gels stained with Coomassie Blue. (*a*) Acidic protein band. ≅6 μg loading. (*b*) Plasma membrane preparation from which a and c were derived. ≅15 μg loading. (*c*) Major anti-calcineurin-positive band. ≅6 μg loading. Molecular mass assignations obtained from protein standards (see Materials and Methods) run on adjacent lanes.

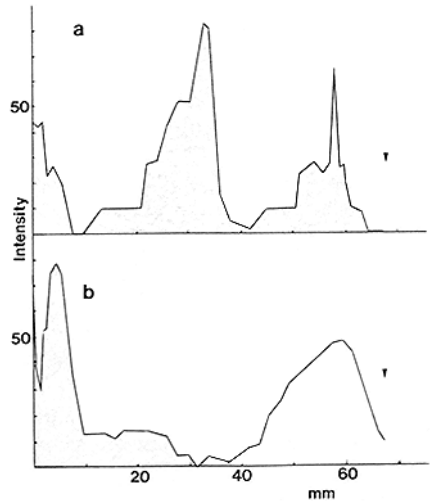

FIGURE 6 Densitometry traces of Western blots from alkaline 10% acrylamide gels in 8 M urea, 5 mM EGTA, on which pig lymphocyte plasma membranes (≅125 μg per lane) had been run. The blots were developed using anti-calcineurin (A + B) (*a*) and anti-calmodulin (*b*) as primary antibody. See Materials and Methods for details. Arrowheads mark the dye-front of the gels, found at the anodal end of each gel. Intensity is measured in arbitrary units (different for *a* and *b*).

plasma membrane was similar to that found in the human lymphoblastoid cell plasma membrane (Table I) and averaged 4.3 ± 1.0 μg/mg membrane protein.

Calcium-dependent Appearance of the Acidic Protein Band

The presence (1 mM $CaCl_2$) or absence (5 mM EGTA) of calcium during cell breakage and throughout all subsequent steps of the plasma membrane preparation determined whether or not one saw an acidic protein band by urea–acrylamide gel electrophoresis. If calcium was present during the preparation, subjection of the plasma membrane to 8-M urea gel electrophoresis yielded an acidic protein band; if calcium was absent, the acidic protein band either did not appear or was considerably reduced in amount (Fig. 7*a*). Preparations ± Ca^{++}, where traces of the acidic protein band can still be seen in the $-Ca^{++}$ lanes by silver staining (e.g., Fig. 7), show that the acidic protein band does not exhibit a calcium-dependent mobility on urea gels and therefore does not complicate this type of analysis.

Appropriate excision of the urea gel at the acidic protein band location, followed by electroelution and calmodulin radioimmunoassay, confirmed these results by showing a 65-fold enhancement of calmodulin in the acidic protein band prepared from plasma membranes in the presence of calcium, as opposed to its absence. Material prepared in the presence of calcium yielded 39 ng calmodulin/μg acidic protein; material prepared in the absence of calcium (5 mM EGTA) yielded 0.6 ng calmodulin/μg acidic protein.

SDS gel electrophoresis of the plasma membrane preparations ± calcium indicated that a 68-kD peptide was present when the preparation was performed in the presence of 1 mM calcium, whereas the appearance of a 96-kD peptide was intensified when the preparation was performed in the absence of calcium (5 mM EDTA) (Fig. 7*b*). Furthermore, the relative intensities of two bands in a doublet centered at 37 kD appeared to be reversed dependent upon whether the preparation was performed in the presence or absence of calcium (Fig. 7*b*).

Activity Assay

The phosphatase activity of pig lymphocyte plasma membrane preparations was measured using the PNPP assay procedure (see Materials and Methods). Activities were of the order of 6.3 ± 0.6 nmol P_i/mg plasma membrane/min for the assay in 20 mM $MgCl_2$, 10 μM $CaCl_2$, pH 8.6, and 2.8 ± 0.7 nmol P_i/mg plasma membrane/min for the assay in 2 mM Ni^{2+}, pH 7.2. Assays of the isolated acidic protein band or the purified calcineurin heavy chains yielded zero activity; possible reasons for this are considered in the Discussion.

TABLE I. *Various Parameters of the Plasma Membrane and Acidic Protein Preparations*

Proportion of plasma membrane protein as acidic protein band	3.7% (4)
Concentration of calmodulin in acidic protein band	133 ng/μg protein (3)
Concentration of calmodulin in main calcineurin-staining band from urea gel	0.0 ng/μg protein (3)
Concentration of calmodulin in pig lymphocyte plasma membrane	5.3 μg/mg protein (4)
Concentration of calmodulin in Bri-8 lymphoblastoid cell plasma membrane	3.6 μg/mg protein (6)
Proportion of plasma membrane calmodulin as % of total cell calmodulin	1.8% (4)
Proportion of lymphocyte calmodulin as % of total cell protein	0.15% (4)

Calmodulin quantitation was performed by radioimmunoassay (see Materials and Methods).

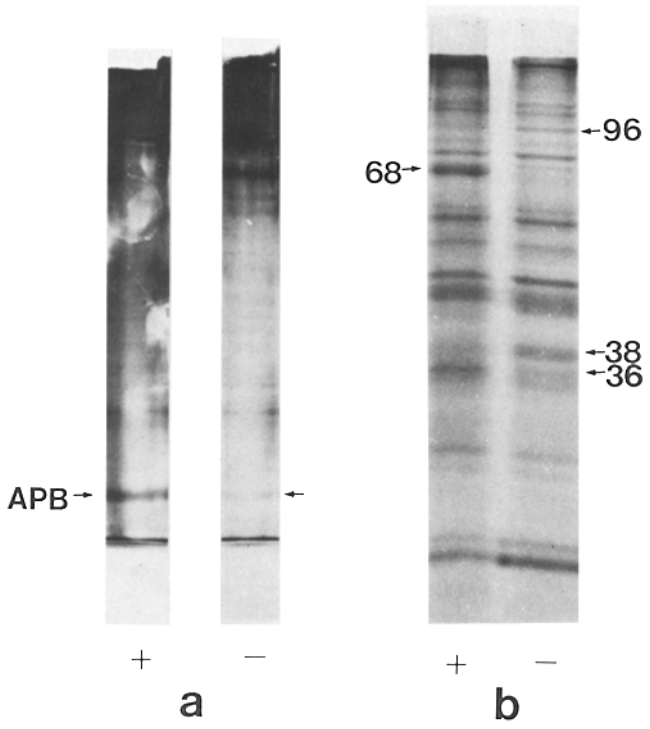

FIGURE 7 Electrophoresis of lymphocyte plasma membranes prepared in the presence (1 mM $CaCl_2$) and absence (5 mM EGTA) of calcium. (*a*) 8 M urea–acrylamide gel electrophoresis of lymphocyte plasma membrane prepared in the presence (+) and absence (−) of calcium. The gels were silver stained. Note marked reduction in the presence of the acidic protein band (APB) when the plasma membrane is prepared in the absence of calcium. ≅400 μg loadings. (*b*) SDS acrylamide gel electrophoresis (12.5%) of same lymphocyte plasma membrane preparation as in *a*, prepared in the presence (+) and absence (−) of calcium. Note the calcium-dependent appearance and disappearance of the 68- and 96-kD bands, respectively. Note also the intensity shift in the 36- and 38-kD bands. ≅100 μg loadings. Gels stained with Coomassie Blue.

Does Anti-sIg–induced Capping Result in Calcineurin Co-capping?

Incubation of human peripheral blood lymphocytes with fluorescein-labeled goat anti-human IgG at 0°C results in sIg surface labeling of the B lymphocytes in the cell population (Fig. 8, *a* and *b*). Under these conditions, anti-calcineurin (A + B) immunoreactivity is distributed throughout the cell in a diffuse manner (Fig. 8*c*). When the temperature is raised to 37°C, sIg patches form, as viewed by fluorescein-labeled goat anti-human IgG staining, prior to their coalescence into a cap (Fig. 8, *d*, *e*, *g*, and *h*). This occurs during a 10–30-min period; up to 30% of the cell population forms caps. Under all these conditions the majority of anti-calcineurin (A + B) immunoreactivity appears to remain distributed about the cell in a diffuse pattern (Fig. 8, *c*, *f*, and *i*). Less than 3% of all capped cells exhibit sIg/calcineurin co-capping.

Is Lymphocyte Calcineurin Located on the External Cell Surface?

Because calcineurin-immunopositive material associates with the lymphocyte plasma membrane in a calcium-dependent manner and because occasional examples of sIg/calcineurin co-capping are seen, it was necessary to determine whether lymphocyte calcineurin might be located on the internal or external cell surface. This was performed by incubating human peripheral blood lymphocytes (>95% viability) with either anti-calcineurin (A + B) or fluorescein-labeled goat anti-human IgG (as a positive control) at 0°C or 37°C before fixation. Fixed cells were subsequently incubated at 0°C with rhodamine-labeled goat anti-rabbit IgG (see Materials and Methods). At 0°C, sIg-positive B lymphocytes exhibited surface staining with fluorescein-labeled goat anti-human IgG (Fig. 9, *a* and *e*); no staining was observed using intact lymphocytes treated with anti-calcineurin (A + B) and rhodamine-labeled goat anti-rabbit IgG (Fig. 9, *b* and *f*). At 37°C, cells staining with fluorecein-labeled goat anti-human IgG exhibited patching and capping (Fig. 9, *c* and *g*); no staining was observed with intact lymphocytes treated with anti-calcineurin (A + B) and rhodamine-labeled goat anti-rabbit IgG (Fig. 9, *d* and *h*). Calcineurin-immunopositive material therefore does not exist on the external cell surface of intact lymphocytes.

DISCUSSION

The plasma membrane preparation of Monneron and d'Alayer was chosen because of its good yield, purity, and extensive characterization of products compared to other methods (22, 31). The procedure was modified slightly so as to combine Fractions 1 and 2, indistinguishable in the original protocol (22), at a single step in the sucrose gradient; this yielded a population of plasma membrane vesicles of average diameter 0.1–0.7 μm (Fig. 1). Fraction 3 of the original protocol was discarded due to slight contamination with rough endoplasmic reticulum vesicles and mitochondria (22).

SDS gel electrophoresis of the lymphocyte plasma membrane preparations reproducibly revealed major peptides of 200-, 83-, 72-, 58-, and 45-kD together with minor components of 125-, 56-, 43-, 37-, and 34-kD, in a calcium-independent manner. Due to discrepancies in molecular mass assignments between different laboratories, it is difficult to compare these results exactly with others, but the overall pattern is very similar to that seen by a number of laboratories (21, 31, 32). The peptides at 200 and 45 kD are almost certainly assignable to myosin heavy chain and actin, respectively (31, 33). We did not run low percentage polyacrylamide gels and so we are unable to comment on the major band at 280 kD seen by Monneron and d'Alayer (31).

Plasma membranes, prepared in the presence of calcium from the cell rupture stage onwards, yield an acidic protein band when they are run on alkaline 8-M urea-acrylamide gels (Figs. 2 and 3). This acidic protein band was observed with plasma membranes prepared from a variety of sources including pig mesenteric lymph node lymphocytes, the human lymphoblastoid B cell lines Bri-8, MST, JY, and Maja, and the human Burkitt lymphoma cell line Daudi. The appearance of the acidic protein band on 8-M urea gels was independent of the presence or absence of calcium; however, if lower concentrations of urea were used for both sample preparation and gel formation, the acidic protein band appeared only in the absence of calcium (i.e., 5 mM EGTA; 4–6 M urea, pH 8.6). In the presence of calcium, the acidic protein band is not seen at intermediate urea concentrations (4–6 M urea). This is presumably because of a calcium-dependent association of the acidic protein band with other material which remains close to the origin of the gel; this

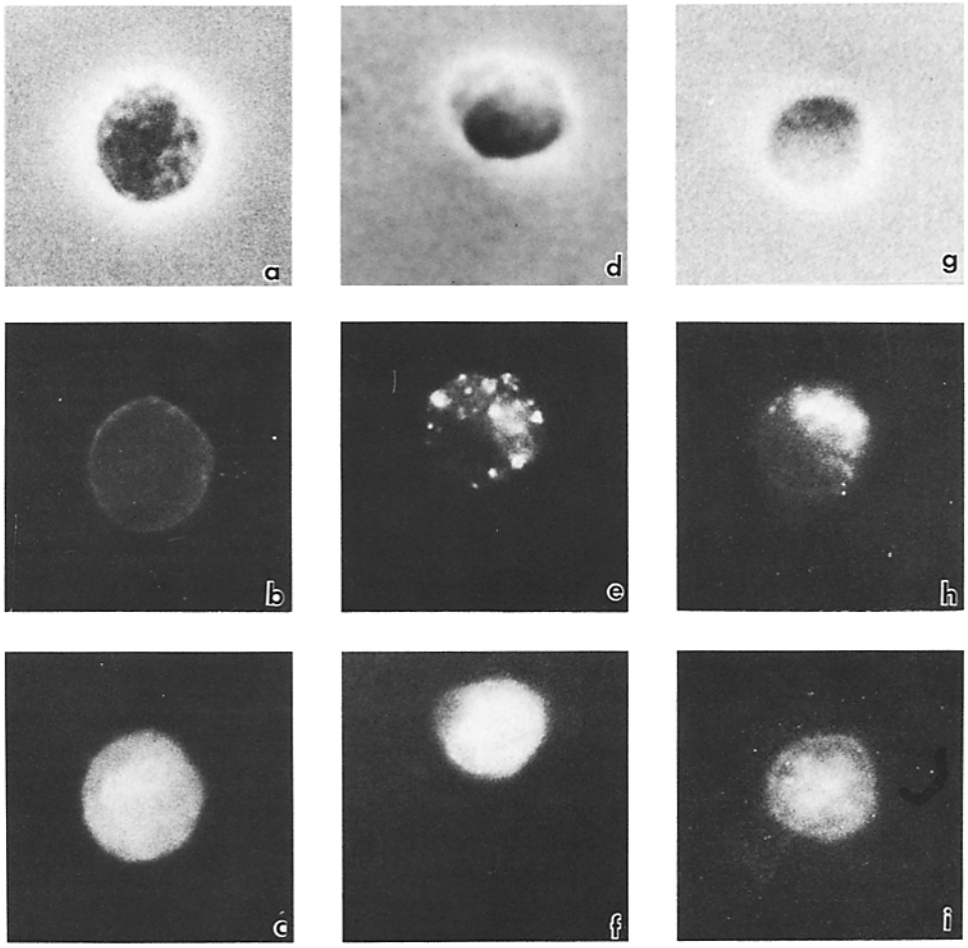

FIGURE 8 Patching and capping of human peripheral blood lymphocytes using a double-labeling technique so as to observe the location of sIg and calcineurin simultaneously. Full details given in Materials and Methods. (*Top row*) Phase images; (*middle row*) fluorescence images using fluorescein-conjugated anti-sIgG; (*bottom row*) fluorescence images using rhodamine-conjugated goat anti-rabbit IgG and rabbit anti-calcineurin (A + B). (*a–c*) Lymphocytes surface labeled with anti-sIg at 0°C. Images of the same cell show peripheral surface labeling of sIg (*b*) whereas calcineurin is oriented throughout the cell (*c*). (*d–f*) Lymphocytes surface labeled with anti-sIg allowed to patch at 37°C for 10 min. Images of the same cell show patches corresponding to sIg (*e*) whereas calcineurin remains distributed throughout the cell (*f*). (*g–i*) Lymphocytes surface labeled with anti-sIg allowed to cap at 37°C for 20 min. Images of the same cell show that the sIg cap (*h*) does not correspond to the location of calcineurin which remains distributed throughout the cell (*i*). × 4,000.

association is overcome when the urea concentration is increased to 8 M. It is of interest to note, however, that even 8 M urea is not capable of breaking the acidic protein band into its constituent peptides, for upon SDS acrylamide gel electrophoresis (Figs. 3 and 5), it became apparent that the acidic protein band is composed of at least three peptides with molecular masses of 68, 59, and 20 kD.

Radioimmunoassay of the isolated acidic protein band (Table I) as well as immunoblotting of plasma membrane preparations run on 8-M urea gels (Fig. 6) indicate that calmodulin is a component of the acidic protein band. Western transfer of plasma membranes run on 8-M urea gels (Fig. 6) or of the isolated acidic protein band run on SDS gels (Fig. 3), followed by immunoassay using anti-calcineurin (A + B), indicate that calcineurin is also a component of the acidic protein band. Only the 68- and 20-kD peptides were immunopositive for calcineurin. Although calf brain calcineurin consists of only one heavy chain (subunit A) and one light chain (subunit B) (11), a more complex peptide pattern for rabbit skeletal muscle calcineurin has been noted (14); in this latter case, heavy chain peptides with molecular masses of 61 and 58 kD were seen.

It would appear, therefore, that the acidic protein band is a complex of calcineurin and calmodulin. Further work, such as peptide mapping and partial sequencing, is required to absolutely prove this contention. Whether or not the 59-kD peptide is also related to the calcineurin heavy chain but is not cross-reactive to the anti-calcineurin (A + B) antibody used, is not known at this time. Certainly the 59-kD peptide always appears to be present in stoichiometric ratio to the 68-kD peptide. Because anti-calcineurin (A + B) does not cross-react with calmodulin and because anti-calmodulin does not cross-react with calcineurin, at least two peptides must be present within the 20-kD band. Hence, the acidic protein band possesses a minimum of four polypeptide chains. From the quantitative radioimmunoassay data for calmodulin, obtained from the isolated acidic protein (Table I), a stoichiometry of calcineurin:calmodulin of 1:0.61 can be calculated. We are unable to distinguish between two plausible alternatives at this time: either both the 59- and 68-kD peptides bind to one molecule of calmodulin, or each of these two peptides separately bind to one molecule of calmodulin. In the former

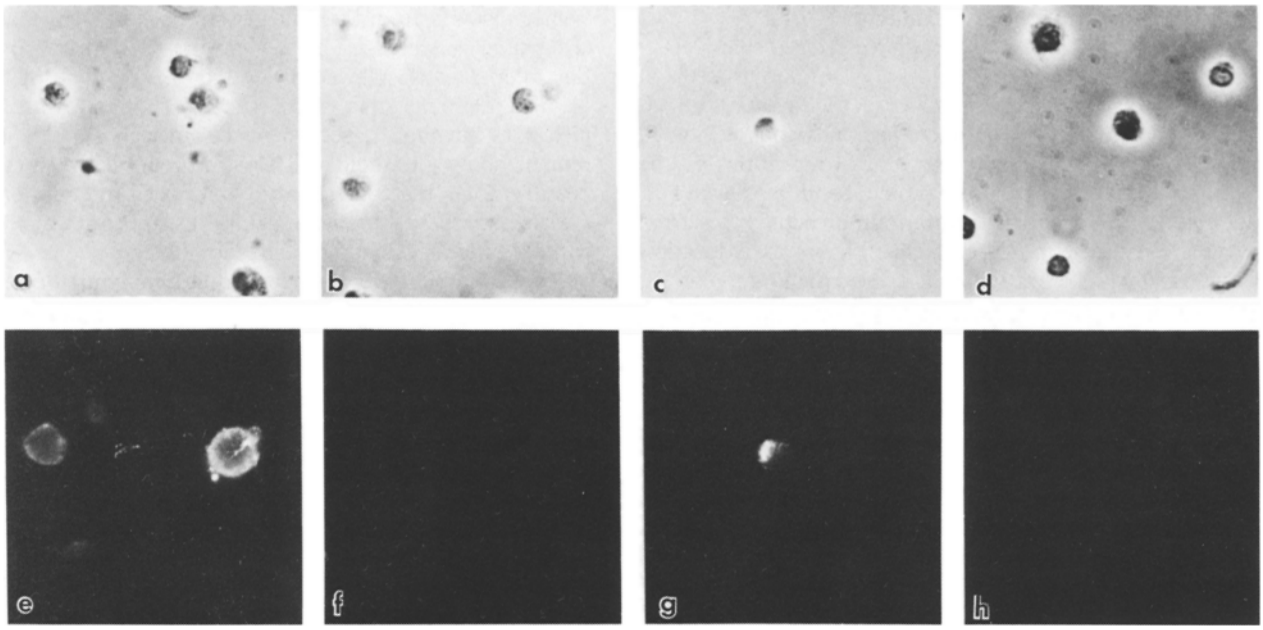

FIGURE 9 Detection of surface labeling of human peripheral blood lymphocytes by either anti-sIg or anti-calcineurin. See Materials and Methods for details. (*Top row*) Phase photography. (*Bottom row*) Fluorescence photography. (*a* and e) Same cells, surface labeled at 0°C with fluorescein-conjugated anti-sIg. (*b* and *f*) Same cells after incubation at 0°C with rabbit anti-calcineurin (A + B) before fixation and treatment with rhodamine-conjugated goat anti-rabbit IgG. (c and *g*) Same cell, surface labeled at 0°C with fluorescein-conjugated anti-sIg before capping at 37°C for 20 min. (*d* and *h*) Same cells, treated as in c and *g* but with rabbit anti-calcineurin instead of anti-sIg. Cells were then fixed and treated with rhodamine-conjugated goat anti-rabbit IgG. (a–d) × 1,600; (*e–h*) 2,000.

case our experimental result underestimates the amount of calcineurin; in the latter case we underestimate the amount of calmodulin.

The ability to observe the dissociation of the acidic protein band from lymphocyte plasma membranes on an 8-M urea gel depends critically on whether calcium was present or absent during the plasma membrane preparation—both during cell rupture and throughout the sucrose gradient. If calcium is absent during the plasma membrane preparation (5 mM EGTA), the acidic protein band is either not seen or its presence is considerably reduced (Fig. 7 and Results).

SDS gel electrophoresis of plasma membrane preparations obtained in the presence and absence of calcium indicate the additional presence of either a 68-kD peptide (in the presence of calcium) or a 96-kD peptide (in the absence of calcium) (Fig. 7*b*). It is unlikely that the 68-kD peptide seen in the SDS gel (Fig. 7*b*) corresponds to the 68-kD peptide of the acidic protein band (Fig. 5), for it is present in much greater quantity than the acidic protein band. Furthermore, there is no indication of a corresponding calcium-dependent appearance of a 59-kD component. More likely, this 68-kD component may be related to a 68-kD peptide described by others (34) as a component of the lymphocyte cytoskeleton. Detergent-extracted cytoskeletons, prepared from plasma membranes in the presence of calcium, possessed a 68-kD peptide that was absent if calcium was omitted from the cytoskeleton preparation (these authors prepared lymphocyte plasma membranes in the presence of calcium). Their 68-kD peptide shared an antigenic determinant in common with all classes of intermediate filament protein (34).

Calcium-dependent association of the acidic protein band with the plasma membrane suggests a possible functional attachment of calmodulin–calcineurin to the plasma membrane which may be controlled by physiological calcium levels. Furthermore, this labile attachment suggests that the acidic protein complex is not an intrinsic membrane protein. In addition, anti-calcineurin (A + B) immunoreactivity is not directed to the external surface of intact lymphocytes (Fig. 9). Taken together, these data suggest a calcium-dependent interaction of the calmodulin–calcineurin complex with a component attached to the inner cytoplasmic surface of the lymphocyte plasma membrane. From this, one might also expect the complex to be found distributed throughout the cytoplasm as well as attached to the plasma membrane. This is in fact seen. Anti-calcineurin (A + B) (Fig. 8) and anti-calmodulin (unpublished results and reference 35) immunoreactivity is distributed throughout the cytoplasm in a diffuse manner. Furthermore, lymphocyte cytosol fractions prepared free of nuclei, mitochondria, endoplasmic reticulum, and plasma membranes, possess a component which co-migrates with the acidic protein band on 8-M urea gels (data not shown). There would appear to be a large intracellular reserve of this protein complex.

It is unlikely that the acidic protein band simply represents a contaminant of the plasma membrane preparation, trapped within its vesicles. As discussed above, the preparation of Monneron and d'Alayer gives a pure product which has been well characterized (22, 31). The presence of the acidic protein band is seen only when plasma membranes are prepared in the presence of calcium. Under these conditions its appearance is a reproducible phenomenon and it makes up a constant proportion of the plasma membrane fraction even after extensive washings after recoupment from the sucrose density gradient. The data of Figs. 2 and 3, together with the stoichiometric relationship of the subunits, would be difficult to explain simply on the basis of either entrapment or co-

migration of polypeptides with similar pI's.

Direct electrophoretic transfer of pig lymphocyte plasma membrane proteins from 8-M urea–acrylamide gels to nitrocellulose paper, followed by development with anti-calcineurin (A + B) and the peroxidase-conjugated second antibody, revealed the presence of calcineurin at two locations on the urea gel in addition to the acidic protein band (Figs. 4 and 6). The majority of anti-calcineurin immunoreactivity occurred about half-way down the gel; excision of the gel at this location in a preparative sister gel followed by electroelution, dialysis, and SDS gel electrophoresis, revealed this component to be a doublet of 59 and 68 kD, which co-migrated with peptides of the same molecular mass from the acidic protein band (Fig. 5). Only the 68-kD peptide was immunopositive for calcineurin (Fig. 4). It would appear that the associated membrane complex of calcineurin with calmodulin, together with a putative membrane component, is incompletely dissociated by 8 M urea under the conditions used. The various associated peptides migrate along the alkaline urea gel according to their individual charges on each complex in 8 M urea, pH 8.6: thus calmodulin confers an acidic charge to the acidic protein complex, whereas the two heavy chains together run as a complex which forms the major calcineurin immunopositive band about halfway down the urea gel.

The lack of phosphatase activity exhibited by the isolated lymphocyte calcineurin complexes, using PNPP as substrate, was not surprising. It has been reported that brain calcineurin loses its phosphatase activity irreversibly by exposure to 6 M urea (13) and all lymphocyte calcineurin complexes reported here were prepared using 8 M urea preparative gels. By contrast, the purified lymphocyte plasma membrane does exhibit PNPP cleavage activity although probably not all of this activity can be attributed to calcineurin.

Measurement of the yields of acidic protein suggest that 1 mg plasma membrane gives rise to ~37 μg acidic protein (Table I). Radioimmunoassay of the acidic protein band suggests that 13% of this material is calmodulin (Table I), i.e., 4.8 μg calmodulin/mg plasma membrane; this value compares well with an average value of 4.3 μg calmodulin/mg plasma membrane obtained by direct radioimmunoassay for calmodulin using purified plasma membranes (Table I). These results, in turn, suggest a range of ~15–30 μg calcineurin/mg plasma membrane, the limits depending on whether one views the 59-kD component as being part of calcineurin or not.

In the majority of lymphocytes, calcineurin does not appear to co-cap with capped sIg (Fig. 8). Throughout both patching and capping, anti-calcineurin immunoreactivity is distributed in a diffuse manner throughout the cell, sometimes forming small punctate regions which are not coincident with anti-Ig immunoreactivity. The force-generating proteins, actin (36, 37) and myosin (38), as well as diverse components of the cytoskeleton including α-actinin (39, 40), tubulin (36, 41), vimentin (42), and fodrin (43) co-cap on the cytoplasmic side of the plasma membrane during anti-IgG–induced sIg co-capping; some of these components may be actively involved in the capping process themselves. It would appear, as judged by this criterion, that lymphocyte calcineurin is not a component of the lymphocyte cytoskeleton and may represent a class of membrane-bound molecules, on the cytoplasmic surface, normally refractory to capping phenomena. Occasional examples of sIg/calcineurin co-capping are found in every experiment (<3% of all capped cells). As capping has been shown, phenomenologically, to be associated with mobilization of calcium from internal sources, together with its subsequent release into the external medium (44), it could be that internal calcium levels drop so low during sIg capping so as to cause dissociation of the calmodulin–calcineurin complex. Only in those cases where the internal calcium levels remain above threshold will sIg–calcineurin co-capping be possible. The exact mechanism of capping is still poorly understood; some investigators suggest that it can even occur without internal calcium mobilization (45).

The sequence of events whereby quiescent lymphocytes are stimulated to proliferate and differentiate, in response to specific antigens or less-specific mitogens, are still unknown. Transmembrane signaling, via transient phosphorylation of membrane-bound component(s) is a particularly attractive hypothesis (3–5). Such changes would have to occur within time intervals of less than 5 min from their initiation by receptor binding, in order to account for the fact that changes in the plasma membrane phosphorylation pattern are not seen subsequent to this time interval (6). An increase in intracellular calcium exchange (46, 47) is thought to be an immediate consequence of this transmembrane signal; calcium as second messenger leads, in turn, to a cascade of events resulting in an increase in nuclear ADP-ribosyltransferase activity and the subsequent repair of single-strand breaks in DNA (48), a prerequisite to differentiation and blastogenesis. Evidence for both endogenous cAMP-dependent and independent protein kinases, bound to the lymphocyte plasma membrane, has been obtained (4, 6) but no evidence for a plasma membrane–bound phosphatase, necessary for a transient phosphorylation signal, has been forthcoming. The evidence presented in this paper, describing a calcium-dependent attachment of a calcineurin-containing complex to the cytoplasmic surface of the lymphocyte plasma membrane, places a well-known phosphatase at a key location where it could control transmembrane signaling. Future experiments will attempt to test this hypothetical role for lymphocyte calcineurin.

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