

PERIPHERAL HYALINE BLEBS (PODOSOMES) OF MACROPHAGES

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ABSTRACT

The plasmalemma and hyaline ectoplasm together constitute the sensory and motor organ of macrophages. The purpose of this study was to isolate this cell fraction in order to analyze it biochemically and functionally. Brief sonication of warmed rabbit lung macrophages caused release of heterodisperse hyaline blebs and filopodia, which were easily collected by differential centrifugation. Viewed in the electron microscope, these structures consisted of membrane-bounded sacs principally containing actin filaments. Some contained secondary lysosomes. They were enriched threefold over whole cell homogenates in specific adenylate cyclase activity and in trichloroacetic acid-precipitable ^{125}I when derived from cells labeled with ^{125}I by means of a lactoperoxidase-catalyzed reaction. These markers were found to have identical isopycnic densities when macrophage homogenates were subjected to sedimentation in a focusing sucrose density gradient system, and these markers had densities distinct from those of other cytoplasmic organelles. These markers were therefore assumed to be associated with macrophage plasma membranes. The specific β -glucuronidase activity of the bleb fraction was similar to that of homogenates, but the blebs had considerably lower specific succinic dehydrogenase activity and RNA content, and DNA was undetectable.

Electrophoresis of blebs solubilized in sodium dodecyl sulfate on polyacrylamide gels revealed polypeptides co-migrating with macrophage actin-binding protein, myosin, and actin; blebs also had EDTA-activated adenosine triphosphatase activity characteristic of myosin. The concentrations of actin-binding protein and myosin were higher in blebs than in cells or cytoplasmic extracts, whereas actin concentrations were similar (relative to extracts) or only slightly greater (than in cells). Blebs and intact cells had high lactate dehydrogenase activities in the presence but not the absence of Triton X-100. Blebs and cells oxidized 1- ^{14}C glucose, and the rate of glucose oxidation was increased substantially in the presence of latex beads.

We conclude that intact sacs of plasmalemma encasing contractile proteins and cytoplasmic enzymes can be isolated from macrophages. They are enriched in myosin and actin-binding protein, indicating that the contractile apparatus is regulated in the cell periphery. These structures have the capacity to respond to

environmental signals. We suggest the name "podosomes" for them because of their resemblance to macrophage pseudopodia. We propose that podosome formation results from rapid dissolution of the cortical gel when the membrane is in an actively extended configuration.

KEY WORDS podosomes · contractile proteins · membranes · macrophages · cytoplasmic gels

Locomotion, spreading, and phagocytosis by macrophages are activities which involve the plasma membrane and a cortical layer of organelle-excluding peripheral cytoplasm, now known to contain contractile proteins. The components of this unit, plasmalemma and hyaline ectoplasm, somehow cooperate to produce pseudopodial veils, ruffles, filopodia, blebs, and microvilli in response to stimuli from the environment. A variety of treatments induce bleb formation, including: mechanical stress applied to cells (5), placement of cells in hypotonic medium (33), addition of heavy metals or sulfhydryl alkylating agents (2), cytochalasins (3, 19), aldehydes (26), heating of cells to 46°C (16), and viral transformation (23). In some cases, these blebs spontaneously bud off from the cell, becoming freely suspended in the medium. Potentially, this budding is a very useful phenomenon which can be exploited to isolate a specific subfraction of the whole cell.

Our interest lies in determining how pseudopodia surround and engulf particles during phagocytosis. This determination requires, in addition to the characterization of contractile proteins and their interactions, an understanding of the relationship between them and the plasma membrane. In this report, we describe the isolation and some properties of metabolically viable peripheral hyaline blebs from rabbit lung macrophages which we show to be sacs of plasmalemma encasing cytoplasmic contractile proteins. Because of the resemblance between these structures and pseudopodia, we propose to name them "podosomes." A preliminary report of this work has been published (9).

MATERIALS AND METHODS

Rabbit Lung Macrophages

Rabbit lung macrophages were isolated by tracheal lavage, 10–15 days after priming animals with 1 ml of Freund's complete adjuvant, injected into the marginal ear vein (20, 21). Cells, collected with 360 ml of 0.15 M NaCl solution at room temperature, were washed

twice with modified Krebs, Ringer phosphate medium, pH 7.4 (KRP) (27) at 4°C by centrifugation (260 g, 10 min), and finally resuspended in KRP at the desired concentration. Yields were typically 3–6 ml of packed cells per rabbit, of which >90% were macrophages.

Preparation of Podosomes

Several different techniques were tested and these are described in the Results section. The method here is the one which was finally adopted for routine use. Washed macrophages were suspended in KRP at a concentration of 5% (vol/vol) and were divided into lots of 10 ml in conical glass centrifuge tubes. These tubes were warmed to 37°C in a water bath and then individually placed into a sonicator bath (model F, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) for 5 s. Immediately thereafter, the tubes were briefly agitated on a vortex mixer and immersed in ice. Cell bodies were pelleted by centrifugation (260 g, 10 min), leaving a suspension of blebs in the supernatant fluid which were isolated by sedimentation at 12,000 g for 10 min at 4°C, and subsequently resuspended in 0.2 ml of KRP.

Homogenization of Cells

Washed macrophages were washed again in calcium-free KRP, once in ice-cold sodium phosphate buffer solution containing 2 mM EDTA, pH 7.4, and finally resuspended in 3 vol of homogenizing medium (sucrose 0.34 M, EDTA 1 mM, dithiothreitol [DTT] 10 mM, adenosine triphosphate [ATP] 0.5 mM, tris-maleate buffer 10 mM, pH 7.4). Cells were broken with 15–30 strokes in a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.); cell rupture was monitored in the phase-contrast microscope.

Cytoplasmic Extracts

Extracts were prepared from the whole homogenate by centrifuging it at 10⁵ g for 1 h at 2°C (27). The resultant supernates are referred to as "extracts."

Gradient Pouring

5 ml of homogenates were diluted to 25 ml with homogenizing medium and split into two 12.5-ml lots. These were converted into 40 ml each of "heavy" and "light" component, containing 20% (wt/vol) and 55% (wt/vol) sucrose, respectively, by the addition of the appropriate volumes of concentrated sucrose solution (sucrose 75% (wt/vol) in tris-maleate buffer, 10 mM, EDTA 1 mM, pH 7.4). Two identical gradients were poured into 40-ml cellulose nitrate centrifuge tubes,

using a Buchler Instruments gradient maker (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.). These were spun at 25,000 rpm, using a Beckman SW-27 rotor (Beckman Instruments, Inc., Los Angeles) in a Sorvall OTD-2 ultracentrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) for 4 h and fractionated into 20 × 2-ml fractions by aspiration from the bottom of the tube.

Iodination

Lactoperoxidase-catalyzed iodination of intact macrophages was done according to established principles (15). Each milliliter of reaction mixture contained 2.5×10^7 macrophages in KRP without added Mg^{2+} or Ca^{2+} , containing 2 mM EDTA, pH 7.4, (EDTA buffer), lactoperoxidase 10 μ g, glucose 5 μ mol, and 50 μ Ci carrier-free $Na^{125}I$ (New England Nuclear, Boston, Mass.). The reaction, initiated by the addition of glucose oxidase (1.4 orthodiansidine units of type V, Sigma Chemical Co., St. Louis, Mo.), proceeded for 30 min at room temperature, after which time unincorporated label was removed from cells by two washes in EDTA buffer (260 g, 10 min). Lactoperoxidase (LPO) was omitted from control incubations. Covalently bound ^{125}I was separated from residual free forms of the label by precipitation at 4°C with 5 vol of 5% TCA, containing 5 mM KI. Precipitates were centrifuged at 1,000 g for 10 min. The pellets were counted in a Packard Auto Gamma Counter (Packard Instrument Co., Downers Grove, Ill.), and washed with 5% TCA 5 mM KI solution, until successive counts fell by <10%.

Incorporation of [3H]Uridine

A cell suspension (5% in KRP medium) was incubated at 37°C for 2 h in the presence of 5 μ Ci/ml of [3H]uridine (24.2 Ci/mmol, New England Nuclear). Cells were washed twice with ice-cold KRP to remove excess label, and podosomes were made from the cells in the usual way. Cells and podosomes were precipitated by the addition of ice-cold 10% TCA and precipitates were collected on 0.4 μ m pore-size Millipore filters (Millipore Corp., Bedford, Mass.). The filters were washed with ~5 ml of ice-cold 10% TCA, transferred to scintillation vials, and counted for 3H -radioactivity in a Packard Tricarb scintillation counter (model 3255, Packard Instruments Co.) using "Instagel" scintillant (Packard Instruments Co.).

1-[^{14}C]Glucose Oxidation

Analysis of basal and particle-stimulated oxidation of 1-[^{14}C]glucose by intact macrophages was performed as previously described (29). Each reaction vial contained 100 μ l of macrophages, 5% suspension, or 0.2 mg protein of podosomes in KRP, containing 8.2 μ M glucose and 50 nCi of 1-[^{14}C]glucose (30.7 mCi/mmol, New England Nuclear) in the presence and absence of 3

mg of 1 μ m-diameter polystyrene latex spheres (Difco Laboratories, Detroit, Mich.). Incubations were carried out with gentle agitation in sealed plastic vials whose caps were fitted with cups containing 2 M NaOH-saturated paper wicks. After 10 min of incubation at 37°C, dissolved CO_2 was liberated from solution by the addition of 1 ml of 5 M H_2SO_4 . The wicks, containing adsorbed labeled CO_2 , were transferred to scintillation vials for counting.

Chemical Analyses

Protein concentration was determined by the method of Lowry et al. (18). As a standard, a solution of bovine serum albumin was dialyzed against water, and calibrated by drying an aliquot to constant weight. When known amounts of DTT were present in samples, a standard curve containing the same levels of DTT was used. Deoxyribonucleic acid was determined by using the diphenylamine reagent and herring sperm DNA as a standard, a method recommended by Schneider et al. (25).

Dodecyl Sulfate Polyacrylamide

Gel Electrophoresis

Electrophoresis was performed using 5% polyacrylamide gels containing 0.1% dodecyl sulfate, according to the method of Fairbanks et al (11). Various tissue samples were solubilized and reduced in a solution of 1% dodecyl sulfate containing 2% mercaptoethanol before being applied to the gels. Coomassie Blue stained gels were scanned, and a densitometric trace was recorded at 550 nm, using the Gilford Linear Transport (Gilford Instrument Laboratories Inc., Oberlin, Ohio) and a Linear Instruments integrating chart recorder (Linear Instruments Corp., Irvine, Calif.).

Enzyme Analysis

(a) β -glucuronidase activity was assessed as the initial rate of hydrolysis of *p*-nitrophenyl- β -D-glucuronide at pH 5.0, in the presence of 0.1% Triton X-100, as previously described (28). (b) Uridine diphosphogalactose: *N*-acetylglucosamine glycosyl transferase activity was determined according to the method of Leelavathi et al. (17). The name of the enzyme is abbreviated to "transferase." (c) Lactate dehydrogenase activity was assayed, using the method of Wacker et al. (30). Each test contained 0.1 mM nicotinamide adenine dinucleotide (NADH) and 0.5 mM sodium pyruvate at pH 7.4. Rates of change of optical density at 340 nm were recorded continuously (Gilford Spectrophotometer, Gilford Instrument Laboratories) both in the presence and absence of pyruvate at 37°C. A value of 6.2×10^9 was used for the molar extinction coefficient of fully reduced NADH. (d) Succinate dehydrogenase activity was assayed according to the principles of Cooperstein et al. (4). Into a 3-ml cuvette at 25°C were placed 2.3 ml of

sodium phosphate buffer 0.1 M, pH 7.4, 50 μ l of cytochrome C (Sigma grade VI Sigma Chemical Co.) 1 mM, 25 μ l of NaCN 50 mM, and 50 μ l of enzyme material. Rates of change of optical density at 550 nm were recorded continuously both in the presence and absence of 1 ml of sodium succinate 0.1 M, pH 7.4. Activity of the enzyme was calculated, using a value of 1.96×10^4 for the molar extinction coefficient of fully reduced cytochrome C with respect to its oxidized form. (e) Fluoride Stimulated Adenylate Cyclase Activity. Enzyme material, 20 μ l, was incubated with 50 μ l of substrate to give a mixture of the following composition: ATP, 1 mM, containing 100,000 CPM of 14 C-labeled ATP (45 Ci/mol, New England Nuclear); $MgCl_2$, 5 mM; cyclic 3'-5' adenosine monophosphate (cAMP), 2 mM; theophylline, 10 mM; bovine serum albumin, 0.1%; NaF, 10 mM, tris-HCl, 50 mM; pH 7.4. Incubations were carried out for 1 h at 37°C and the reaction was terminated by the addition of 20 μ l of EDTA, 0.1 M, containing 3,000 cpm of 3H cAMP (37.7 Ci/mmol, New England Nuclear), which served as a means of calculating recovery of cAMP in subsequent steps. Assay tubes were placed into a boiling water bath for 2 min and coagulated protein sedimented at 1,000 g for 10 min. Separation of cAMP from its precursor and other labeled nucleotides was effected using columns of neutral alumina and 50 mM tris-Cl buffer, pH 7.4, according to the method of White and Zenser (31).

In some experiments, adenylate cyclase activity was determined in material previously labeled with ^{125}I . Contamination of the final alumina column effluent by free radioactive iodine was removed by a single extraction with 1 ml of 5 mM I_2 in $CHCl_3$. Scintillation fluid was added to the resultant aqueous layer and counting was performed as previously described. Rates of conversion of ATP to cAMP were found to be constant with time over 2 h and with amounts of enzyme material over the ranges routinely employed. (f) Myosine EDTA-Activated ATPase Activity. The EDTA-activated activity unique to muscle and cytoplasmic myosins was assayed in 0.6 M KCl solution as previously described (27).

Electron Microscopy

Podosomes were pelleted at 12,000 g for 10 min at 4°C. Particulate material in focusing gradient fractions was sedimented at 10^8 g for 60 min at 4°C. Pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 50 min at room temperature. Fixed pellets were washed three times in Sabatini's solution (0.18 M sucrose, 0.1 M sodium cacodylate buffer, pH 7.2), post-fixed in Dalton's chrome osmium (8), and processed through uranyl acetate and graded acetones into 1:1 and 1:3 propylene oxide/Epon Araldite mixtures before being embedded in full-strength Epon. Thin sections were cut and examined in a Phillips 300 electron microscope.

Disrupted podosomes were also examined by negative

staining. To make them more adherent, Formvar-coated grids were coated with protamine sulfate by soaking them in a solution (1 mg/ml) for 1 min. Podosome suspension, diluted 1/10 with 0.1 M KCl, 0.01 M tris-HCl, 1 mM Mg^{2+} , pH 7.5 was contacted to the grids. The adherent podosomes were lysed by treatment with various concentrations of Triton X-100 and for various times of exposure. Specimens were finally negatively stained with 1% of uranyl acetate solution and examined in a Philips 200 electron microscope.

RESULTS

Preparation of Podosomes

The first experiments employed a sonicator probe immersed in a beaker of water. Cells contained in a glass test tube were placed in this beaker and sonicated in this indirect manner for various times. When viewed under phase-contrast microscopy, it was apparent that this procedure did not break open many cells but instead caused them to project blebs. These were spherically shaped protuberances (Fig. 1 inset), some of which remained attached to the cell, while others floated freely. Because blebs were usually much smaller than the cell, they were easily separated by differential centrifugation. Later, the sonicator probe was replaced by a sonicator bath. This bath transmitted energy more efficiently than the previous setup to such an extent that after long exposure, extensive lysis of cells occurred. After 20 s in the bath, a significant drop in cell numbers occurred, and in 3 min nearly all the cells were lysed (Fig. 1). For podosome preparations, cells were immersed for periods no longer than 5 s. By the standard procedure, podosomes were obtained in a yield representing ~3% of the total cell protein. If the sonication was done at 0°C instead of at 37°C, no podosomes were obtained.

Morphology of Podosomes

Under phase-contrast microscopy (Fig. 2), podosomes appeared as almost clear spheres ranging in size from barely visible to ~10 μ m in diameter. Some were apparently spherical, and others were "threads" and other intermediate shapes. The smaller podosomes gave evidence of being agitated by Brownian motion and did not settle and remain still on the slide. Larger podosomes settled more easily but never appeared to adhere strongly to the glass slide and did not spread on it like intact cells.

Examined as thin sections in the electron micro-

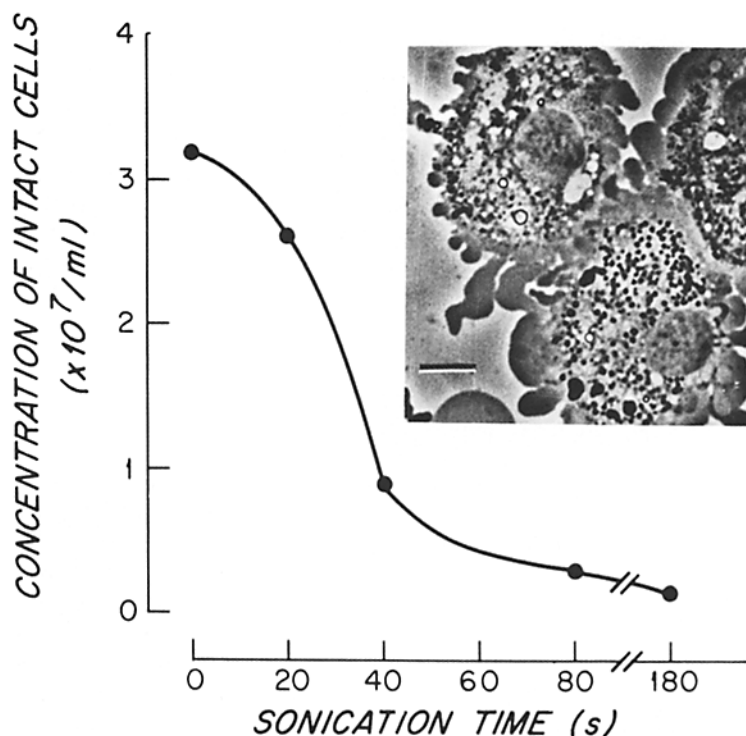


FIGURE 1 Survival of macrophages during indirect sonication. Macrophages suspended in 5 ml of KRP medium at 37°C in a conical centrifuge tube were immersed in a sonicator bath for a total of 3 min. At various times, samples were removed, diluted, and numbers of intact cells determined on a Coulter counter. Inset, appearance of macrophages after 5 s sonication; phase-contrast optics. Bar, 10 μ m.

scope (Fig. 3), podosomes appeared either as round vesicles bounded by bilaminar membranes containing randomly oriented filaments, or else as round-to-elongated structures filled with thin filaments apparently organized into bundles parallel with the long axis of the podosomes. Very few organelles other than glycogen granules and occasional secondary lysosomes were seen. Podosomes were treated with 0.03% Triton X-100 in an attempt to reveal the internal network of filaments. They were then negatively stained with uranyl acetate and examined in the electron microscope (Fig. 4). Small spheres were the predominant structures seen, but extensively elongated structures were also present which, on closer inspection, contained 4–6-nm diameter filaments arrayed in parallel.

Validation of Macrophage Plasma Membrane Markers

The distribution of TCA-precipitable 125 I, after

fractionation of cells subjected to lactoperoxidase-catalyzed iodination, and the distribution of adenylate cyclase activity, were chosen as possible markers for the macrophage plasma membrane. Results were evaluated on focusing sucrose density gradients. Whole cells labeled with 125 I were homogenized and fractionated on the focusing sucrose density gradient, and adenylate cyclase activities and TCA-precipitable radioactivities were determined on each fraction (Fig. 5a). Peak activity for both markers occurred in the same fraction or position in the gradient, representing a buoyant density of 1.16–1.17, and the profiles of the two activities were similar. This fraction contained membrane vesicles (Fig. 6).

Marker enzymes for other organelles of the cell were also determined in similar focusing gradients to ensure that both of the above markers belonged uniquely to membranes of a distinct isopycnic density. Therefore, glucuronidase (lysosomes), succinate dehydrogenase (mitochondria), and “transferase” (golgi) activities were also measured

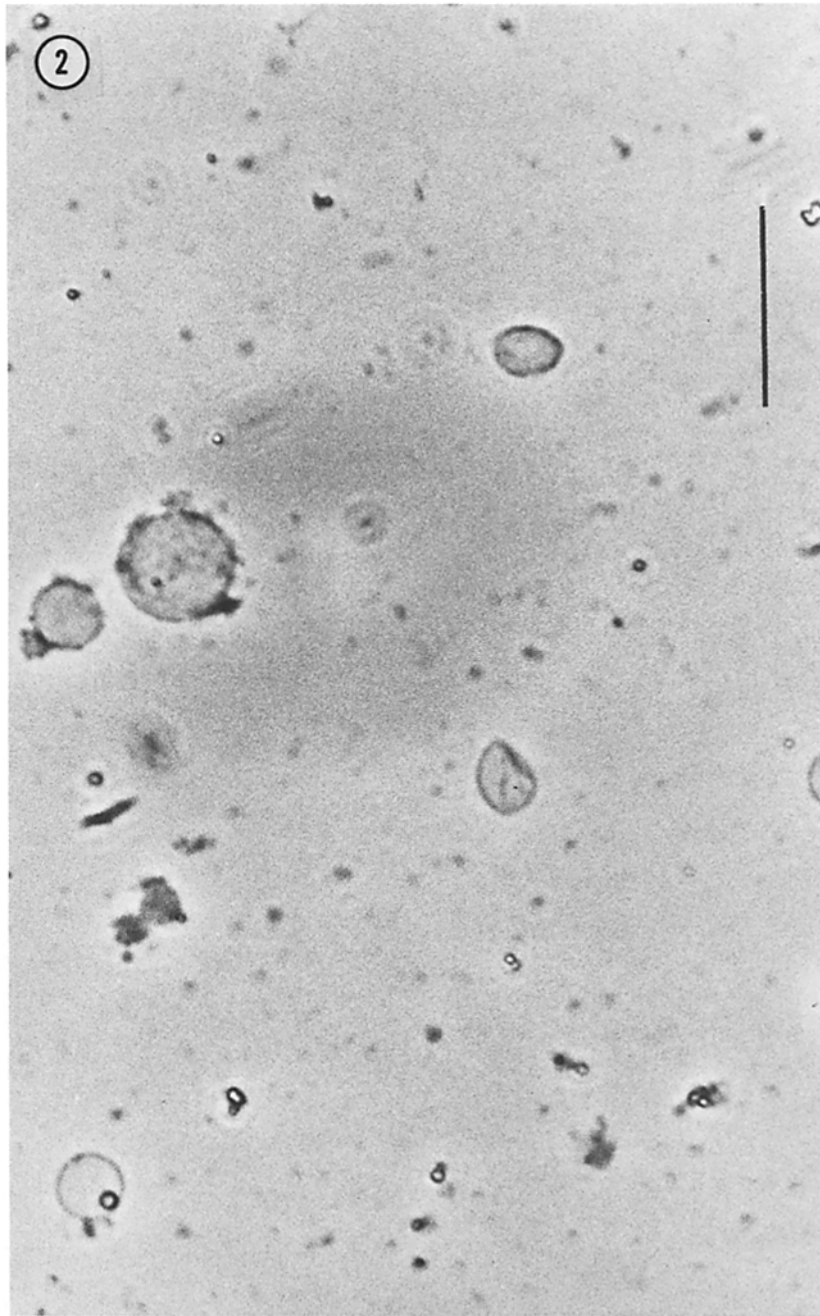


FIGURE 2 Appearance of podosomes. Phase-contrast optics. Bar, 20 μm .

across the gradient. Fig. 5*b* and *c* show that peaks of activity of these last enzymes occurred at positions in the gradient distinct from the peaks in Fig. 5*a* and the peak of adenylate cyclase activity in Fig. 5*b*. We concluded that TCA-

precipitable ^{125}I and adenylate cyclase activity are markers for the macrophage plasma membrane.

The total protein content of a typical fractionated focusing sucrose density gradient is shown in Fig. 5*d*. The protein content remained largely



FIGURE 3 Appearance of podosomes. Electron micrograph of a thin section. Bar, 1 μm .

constant from the most dense fraction (fraction 1) until fraction 14, where the amount of protein in each fraction progressively decreased with increasing sucrose concentration.

Presence of Membrane and Organelle Markers in Podosomes

Podosomes were prepared from cells previously

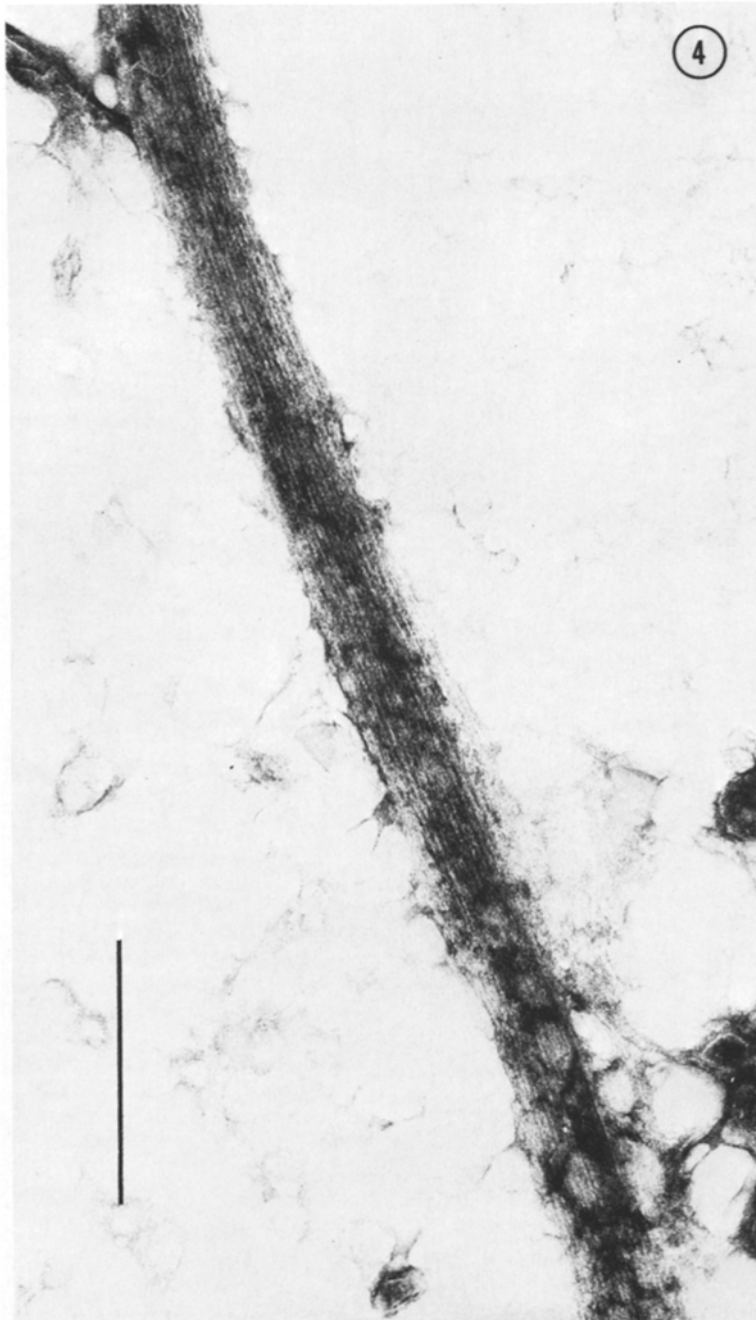


FIGURE 4 Appearance of podosomes. Electron micrograph of podosomes treated with 0.03% Triton X-100 and negatively stained with uranyl acetate. Bar, 0.5 μm .

labeled with ^{125}I and assessed for TCA-precipitable radioactivity. Podosomes from unlabeled cells were assessed for adenylate cyclase activity. Specific activities for both markers (Table I) show

that there was an approximately threefold increase in the podosomes compared to the homogenate. Activities of markers taken to represent the presence of other organelles of the cell were also

assessed. Levels of DNA (nucleus) and succinate dehydrogenase (mitochondria) were all considerably lower than those of the whole cell homogenate, whereas levels of β -glucuronidase (lysosomes) were slightly raised. The presence of soluble cytoplasm was determined by assessing levels of lactate dehydrogenase activity in the presence of Triton X-100. This surfactant effectively removed the membrane barrier between enzyme and substrates, as amplified below. The specific activity of lactate dehydrogenase (LDH) in the presence of Triton X-100 is ~60% of that of whole cells. Therefore, it is concluded that podosomes are essentially organelle-free (apart from lysosomes) packets of cytoplasm bounded by plasma membrane.

Intactness of Podosomes

To evaluate the integrity of the podosome membrane relative to that of the whole cell in an isotonic buffered salt solution, the activity of the cytoplasmic "soluble" enzyme, lactate dehydrogenase was measured. One of the substrates of the LDH reaction, NADH, cannot easily permeate the plasma membrane of intact cells. The degree of intactness of podosome membranes can therefore be determined by measuring LDH specific activities and comparing these values to the specific activity of the enzyme when the membrane barrier is removed with a suitable surfactant like Triton X-100. In whole cells, values of LDH activity were greatly increased by the addition of Triton X-100 (Table I), indicating that their membranes had limited permeability to NADH. In podosomes, also, Triton X-100 produced an increase in specific LDH activity.

Contractile Proteins of Podosomes Compared with Macrophage Extracts

The polypeptide composition of podosomes was determined by dodecyl sulfate polyacrylamide gel electrophoresis and compared to that of whole macrophages and of sucrose extracts of whole macrophages (Fig. 7). Podosomes and extracts exhibited different staining patterns, with extracts displaying a greater number of major bands. Positions of actin-binding protein (ABP), myosin heavy chain, and actin were identified by interpolation from a standard gel containing proteins of known molecular weight and by comparison with previous data (27). The levels of these contractile proteins were quantified, using densitometric

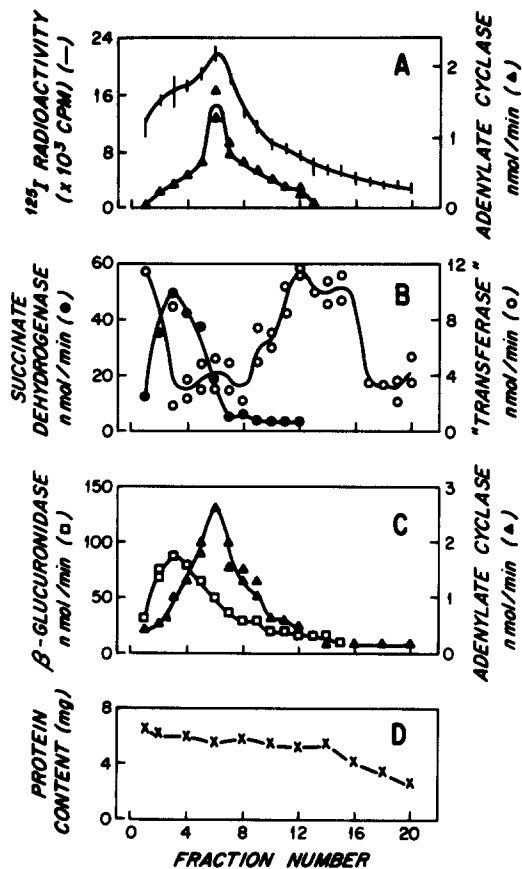


FIGURE 5 Focusing sucrose density gradients; profiles of marker activities. (A) Adenylate cyclase activity and TCA-precipitable ^{125}I -radioactivity in the same gradient. (Recoveries were 98 and 110%, respectively.) (B) Succinate dehydrogenase and "transferase" activities. (C) β -glucuronidase and adenylate cyclase activities in the same gradient. (D) Protein content of a typical gradient. Results in duplicate are represented by two points or a line. One point only signifies that duplicates were identical. Marker activities are given as total activity in each 2-ml fraction. Fraction 1 represents the bottom, most dense, fraction.

scans (Table II). The concentrations of actin-binding protein and myosin increased in the podosomes relative to the extracts, whereas actin levels remained about the same. Relative to cells, the content of all three contractile proteins was increased in podosomes, but actin was increased the least. The concentrations of actin-binding protein and myosin in podosomes were therefore enriched relative to extracts and cells, both with respect to the actin concentration and with respect to the total podosome protein content.

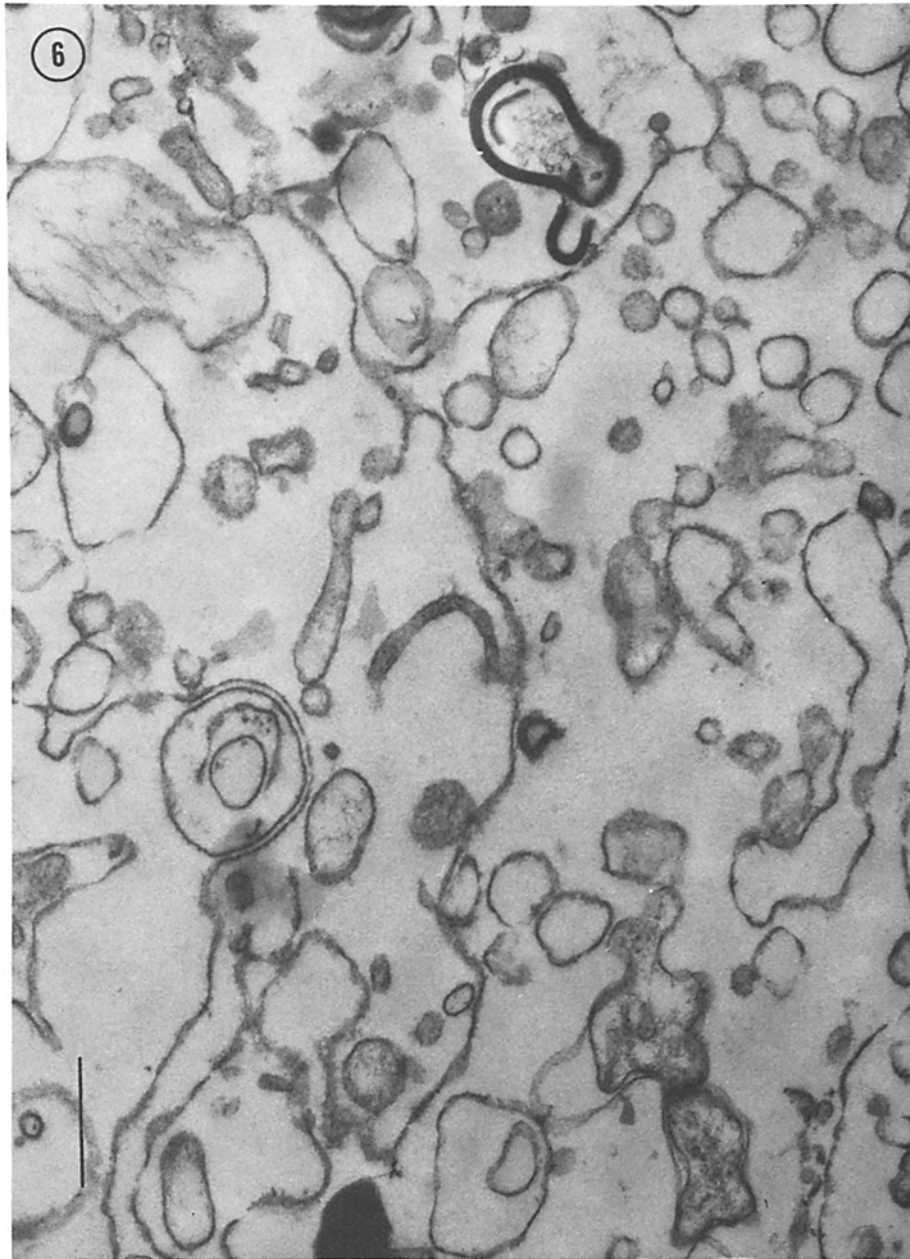


FIGURE 6 Appearance of the focusing gradient fraction with peak adenylated cyclase activity and TCA-precipitable ^{125}I . Electron micrograph of a thin section. Bar, $0.5\ \mu\text{m}$.

The specific EDTA-activated ATPase activity of podosomes was $\sim 80\%$ greater than that of cytoplasmic extracts (Table II). This value is in good agreement with the estimate of myosin content by electrophoretic analysis. Presumably, the presence of EDTA and the high salt concentration

of the assay rendered myosin accessible to the substrate ATP.

Metabolic Responsiveness of Podosomes

The ability of podosomes to oxidize 1- ^{14}C]glucose to $^{14}\text{CO}_2$ was determined in the pres-

TABLE I
Biochemical Properties of Podosomes

	Homogenate or whole cells*	Podosomes	Ratio of: podosomes:homogenate
Protein yield			0.03 ± 0.003
DNA content	0.017 ± 0.004 (2)	N.D.	—
TCA-precipitable [³ H]uridine*	147 ± 19 (6)	59 ± 6 (6)	0.41 ± 0
TCA-precipitable ¹²⁵ I*	13.2 ± 0.4 (5)	40 ± 6.6 (6)	3.0 ± 0
Marker enzymes			
Adenylate cyclase	118 ± 24 (7)	379 ± 67 (8)	3.2 ± 0.9
β-glucuronidase	3.4 ± 0.5 (4)	4.0 ± 0.5 (12)	1.2 ± 0.2
Succinate dehydrogenase	129 ± 15 (4)	14.8 ± 0.7 (4)	0.11 ± 0.01
Lactate dehydrogenase*			
+ Triton X-100	1,400 ± 150 (2)	800 ± 110 (4)	0.57 ± 0.1
- Triton X-100	170 ± 20 (2)	290 ± 20 (4)	1.7 ± 0.2

Podosomes were assayed for the indicated enzyme or marker. Values above represent specific activities based on unit protein of the enzyme or marker, together with standard errors and the number of determination in parentheses. Units of activity are as follows: DNA, mg/mg; incorporation of TCA-precipitable [³H]uridine, fmol/mg/2 h; incorporation of TCA-precipitable ¹²⁵I, cpm/μg; adenylate cyclase, pmol/min/mg; β-glucuronidase, nmol/min/mg; succinate dehydrogenase, pmol/min/mg; lactate dehydrogenase, nmol/min/mg. N.D., not detected.

* Whole cells rather than homogenate were used.

ence and absence of latex particles (Table III). Both whole macrophages and podosomes liberated ¹⁴CO₂ in the absence of latex, and both were stimulated in this respect by the presence of latex. The resting specific glucose-oxidation rate of cells was greater than that of podosomes. However, the specific oxidation rate of podosomes in the presence of latex was greater than that of cells.

DISCUSSION

The evidence presented supports the notion that podosomes isolated from macrophages are virtually intact sacs of organelle-free cytoplasm surrounded by plasma membrane. Proof of this idea relies partly on reliable markers for plasma membrane. We utilized two independent marker techniques, LPO-catalyzed iodination and the presence of adenylate cyclase activity, both of which are believed to be markers for the plasmalemma in diverse types of cells. Plasma membrane's accessibility to the iodination reaction suggests that this organelle should be labeled preferentially, and there is convincing evidence that the iodination technique primarily labeled one population of membranes of macrophages. TCA-precipitable ¹²⁵I-radioactivity appeared as a distinct peak on the focusing sucrose density gradient, implying that the label was covalently attached to organelles of a common isopycnic density. The shoulder to the left of the peak, however, argues for another population of organelles being labeled, possibly

lysosomes or mitochondria which were shown to cofocus in this region. Pinocytosis of iodinated membrane is known to occur (12), and because the macrophage is active in pinocytosis, it is possible that pinocytic vesicles, pinolysosomes, are labeled. However, divalent cations, known to enhance pinocytosis by rabbit lung macrophages (10), were removed from the iodination reaction. The small degree of labeling in the least dense part of the gradient, fractions 12–20, indicates that there is a low general background of activity across the gradient, and that labeling of soluble protein could have occurred also. This background is not likely to be caused by trapping or adsorption of ¹²⁵I to TCA precipitates, as carrier iodide ion was present in the TCA wash solution, in order to permit exchange of noncovalently attached label for carrier. Additionally, the recovery of total TCA-precipitable radioactivity from the fractions of the sucrose density gradient is in good agreement with the radioactivity applied, suggesting that discrepancies due to variable iodide adsorption were absent. The most probable cause of internal labeling, as evidenced by the background and shoulder in Fig. 5a, is breakdown of the LPO-1 complex to iodide radical, which is capable of diffusing readily through the plasma membrane (6).

Adenylate cyclase was chosen as another marker because it is thought to be localized exclusively on the plasma membrane of many cells. In

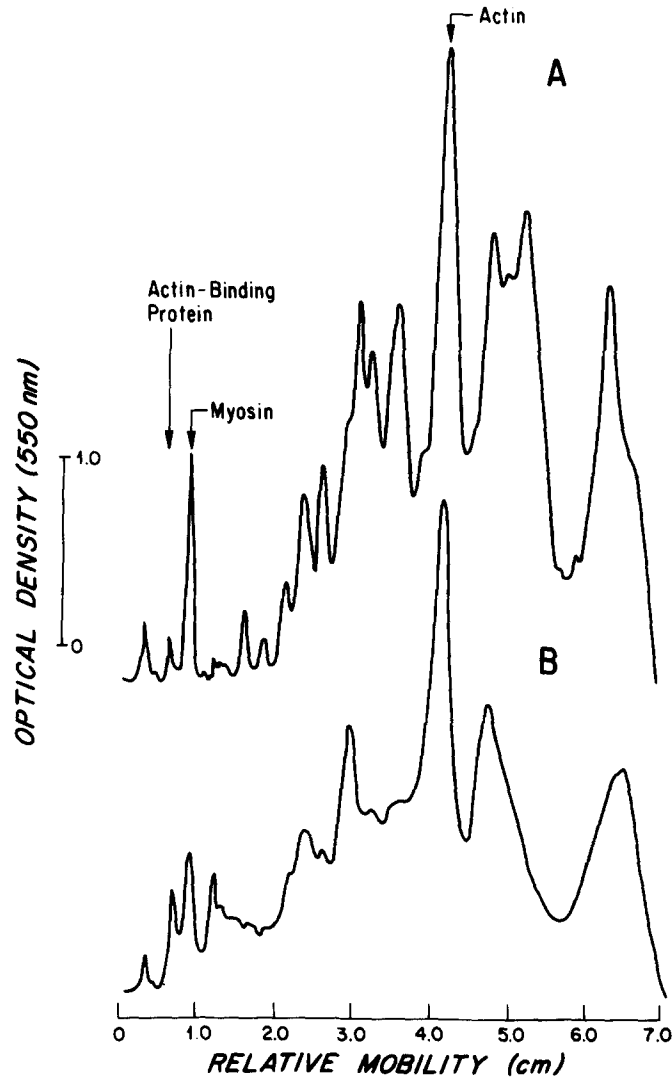


FIGURE 7 Densitometric scans of dodecyl sulfate polyacrylamide gels. Top, organelle-free sucrose extract of whole macrophages. Bottom, podosomes.

the presence of fluoride, the activity of this enzyme is stimulated maximally and the stability is enhanced (24). Adenylate cyclase activity appeared as a symmetrical peak with almost zero background activity in the focusing sucrose density gradient. This finding suggests that the enzyme is located exclusively on one class of organelles which are distinct from those represented by the markers for mitochondria, lysosomes, or golgi (Fig. 5*b* and *c*). Because adenylate cyclase activity and TCA-precipitable ^{125}I -radioactivity focused into the identical fraction and in the same sucrose

gradient, their profiles of activity are similar, and their buoyant density is of an order reported for plasma membranes (32), both labels are concluded to be plasma membrane markers. Some evidence exists that the golgi membrane, as well as the plasma membrane of rat liver cells, contain adenylate cyclase activity (7). Because in the present study the adenylate cyclase activity exhibited a peak which was far removed from the peak of "transferase" activity which was taken to indicate the presence of golgi, a significant contribution of golgi to the total activity and to the

TABLE II
Content of Contractile Proteins in Podosomes, Extracts, and Cells

	Extract	Cell	Podosomes	Ratio of:	
				Podosomes: Ex-tract	Podosomes: Cell
Densitometry					
Actin-binding protein (3)	0.6 ± 0.1	0.6 ± 0.2	1.5 ± 0.3	2.5	2.5
Myosin (heavy chain) (3)	1.5 ± 0.06	1.3 ± 0.1	2.5 ± 0.2	1.7	1.9
Actin (3)	17 ± 0.5	12.2 ± 2.0	18 ± 1.3	1.1	1.5
EDTA-activated ATPase (3)	2.9 ± 0.1		5.1 ± 0.8	1.8	

Percentage content of proteins (with standard errors) were obtained by integrating densitometric scans of 5% polyacrylamide gels. Units of EDTA-activated ATPase are nmol Pi/min/mg protein. Number of determinations is shown in parentheses.

TABLE III
Oxidation of [^{14}C]Glucose

	Whole cells	Podosomes
+ Latex particles	8.1 ± 1.6 (2)	9.9 ± 2.9 (4)
- Latex particles	6.0 ± 1 (2)	4.8 ± 0.6 (4)

Podosomes or whole macrophages were incubated for 10 min at 37°C in the presence of [^{14}C]glucose, either with or without latex particles. ^{14}C -labeled CO_2 was collected and determined as described. The units are pmol of CO_2 collected/min/mg protein. The number of determinations is shown in parentheses.

peak of the focusing sucrose density gradient can be ruled out.

The use of the focusing sucrose density gradient (1) in the present study was a necessary departure from the more usual practice of using "loading zone" gradients. The present method was adopted after it was found that macrophage homogenate, applied atop a sucrose density gradient, often would not enter the gradient even after prolonged centrifugation. Presumably interfacial tension was responsible, but in some cases concentrated homogenates gelled, preventing organelle penetration. This gelation is a result of the interaction of actin and actin-binding protein (27). No such difficulties were encountered with the focusing method, as homogenate was distributed evenly within the gradient from the start, and the dilution thus achieved prevented gelation.

The two plasma membrane markers showed increases in specific activity in podosomes of about threefold with respect to whole cell homogenates. This similarity between two independent markers implies that the podosome membrane is a representative sample of that bounding the whole cell.

If a significant difference between the two had arisen instead, the inference would be that components of the original cell were not represented proportionately on the podosome membrane. The increase in specific activities can therefore be interpreted as due to the greater surface-to-volume ratio of the smaller podosomes compared to that of the larger cells, and not to preferential concentration of one marker on the podosome's membrane.

Podosome membranes are comparably intact to those of the whole macrophage and maintain responsiveness to particle stimulation. Large increases in LDH activity in podosomes after treatment with Triton X-100 indicate that this enzyme is made much more accessible to its substrate when the membrane is removed, and therefore the enzyme, normally contained within the podosome, cannot leak out. Increases in glucose oxidation in the presence of latex particles (Table III) indicate that the podosome membrane contains the necessary receptor mechanisms, transducers, and subsequent biochemical pathways to respond in a manner similar to that of the whole cell, and therefore their membranes are functionally viable.

Thin-section electron microscopy and biochemical markers show that podosomes contain few of the intact cell's organelles. These techniques indicate the virtual absence of nuclei, mitochondria, and golgi membranes. The presence of lysosomal activity was consistent with the occasional findings of dense bodies, presumably secondary lysosomes, in some of the podosomes viewed in electron micrographs of thin sections. As shown in the accompanying paper (13), the specific activity of a lysosomal enzyme in podosomes can be altered when podosomes

are prepared under different circumstances.

The presence of considerable LDH activity in podosomes and the ability of the structures to oxidize glucose both imply that podosomes have an intact complement of cytoplasmic "soluble" enzymes. The capacity for glucose oxidation also suggests that the podosomes have the ability to generate ATP. On the other hand, podosomes contain fewer major polypeptide bands than organelle-free macrophage extracts, although, by electrophoretic and biochemical analysis, podosomes are enriched in the contractile proteins, actin-binding protein, and myosin. These proteins respectively gel and aggregate actin of rabbit lung macrophages, and therefore the enrichment of these proteins in podosomes fits the idea that the contractile apparatus of macrophages is regulated in the hyaline peripheral cytoplasm.

The formation of podosomes from whole cells can be tentatively explained. Cytoplasmic extracts prepared from whole macrophages change state from a free-flowing sol to a firm gel when warmed from 0°C to higher temperatures (27). These gels are disrupted rapidly by mechanical agitation or by the addition of cytochalasin B, are slowly dissolved by cooling to 0°C, and are actively contracted in the presence of myosin and ATP (14, 27). We feel justified in assuming that the physical state of the extract is indicative of the state of cytoplasm within the cell. Apparently, the formation of blebs is dependent on the dissolution of the gelled cytoplasm in the cell, because sonication of macrophages in the cold does not produce them. Furthermore, the disruption must be rapid, because cooling the cells to 0°, which slowly dissolves the gel in vitro, does not produce blebs, but agents which rapidly dissolve the gel, cytochalasin B (3), or mechanical stress (5), do produce them. Sonication provides mechanical stress which, by virtue of its short wavelength, is effective in the dimensions of the cell, and is sufficiently effective not only to produce blebs but to separate them from the cell body. Active contraction of filaments in the dissolved gel could also participate in podosome formation. Concentrations of cytochalasins that produce blebs (3, 19) and dissolve actin gels (14) do not inhibit the interaction between actin filaments and myosin (14). Furthermore, metabolic poisons inhibit bleb formation by the cytochalasins (19), possibly by preventing ATP production required for actomyosin contraction.

The implication of this theory is that a cytoplasmic gel acts as a cytoskeleton to the plasmalemma. If this cytoskeleton is disrupted, the membrane is subsequently left unsupported, whereupon it automatically adopts a shape of minimum free energy, becoming spherical. This event occurs when cells "round up" in the cold. However, if focal disruption of the cortical gel happens rapidly when the membrane is in an extended configuration, it is conceivable that surface forces could produce many small spheres of membrane-bound cytoplasm, or podosomes, before the whole membrane can retract itself into one large sphere. As shown in the accompanying paper (13), macrophages with pseudopods extended on nylon fibers easily yield podosomes in response to shear stress.

This work was supported by grants from the U. S. Public Health Service (HL 19429) and the American Heart Association (77 979). Dr. Davies is a recipient of an Australian-American Educational Foundation Travelling Fellowship Grant. Dr. Stossel was an Established Investigator of the American Heart Association.

Received for publication 14 April 1977, and in revised form 24 August 1977.

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