INTERACTION OF PHOSPHOLIPID VESICLES WITH CELLS

Endocytosis and Fusion as Alternate Mechanisms for the

Uptake of Lipid-Soluble and Water-Soluble Molecules

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ABSTRACT

Depending on their phospholipid composition, liposomes are endocytosed by, or fuse with, the plasma membrane, of *Acanthamoeba castellanii*. Unilamellar egg lecithin vesicles are endocytosed by amoebae at 28°C with equal uptake of the phospholipid bilayer and the contents of the internal aqueous space of the vesicles. Uptake is inhibited almost completely by incubation at 4°C or in the presence of dinitrophenol. After uptake at 28°C, the vesicle phospholipid can be visualized by electron microscope autoradiography within cytoplasmic vacuoles. In contrast, uptake of unilamellar dipalmitoyl lecithin vesicles and multilamellar dipalmitoyl lecithin liposomes is only partially inhibited at 4°C, by dinitrophenol and by prior fixation of the amoebae with glutaraldehyde, each of which inhibits pinocytosis. Vesicle contents are taken up only about 40% as well as the phospholipid bilayer. Electron micrographs are compatible with the interpretation that dipalmitoyl lecithin vesicles fuse with the amoeba plasma membrane, adding their phospholipid to the cell surface, while their contents enter the cell cytoplasm. Dimyristoyl lecithin vesicles behave like egg lecithin vesicles while distearoyl lecithin vesicles behave like dipalmitoyl lecithin vesicles.

Phospholipid vesicles (unilamellar liposomes) consist of a single, closed spherical bilayer of phospholipid, of outside diameter approximately 30 nm, which defines an internal aqueous space about 20 nm in diameter (4, 16, 17). Water-soluble molecules can readily be incorporated into the vesicle's interior space, and lipophilic molecules can be incorporated into the phospholipid bilayer. The interaction of phospholipid vesicles with cells is of interest for several reasons. On the one hand, endocytosis of phospholipid vesicles provides a mechanism for the introduction of water-soluble molecules, contained within the interior aqueous space of the vesicle, or lipophilic molecules, introduced into the vesicle's bilayer, directly into the lysosomes of endocytic cells. In contrast, if phospholipid vesicles were to fuse with the plasma membrane of cells, the contents of the vesicles would be introduced directly into the cell's cytoplasm while lipophilic molecules in the vesicle bilayer would be incorporated into the cell's plasma membrane. The potential experimental and therapeutic applications of these modes of entry of soluble and insoluble molecules into cells are obvious. Finally, phospholipid vesicles and cells provide a potentially useful model system for investigating the biochemical and ultrastructural events of membrane fusion.

Acanthamoeba castellanii, a small soil amoeba that is nutritionally dependent on endocytosis (8),

is a convenient organism for studying pinocytosis (8) and phagocytosis (20, 35). Both processes are energy dependent being inhibited by anaerobiosis, azide, cyanide, and 2,4-dinitrophenol, and by incubation below 12°C. Pinocytosis of solutes is a continuous process in growing cells (8, 18) and requires the "turnover" of the cell surface about 5-50 times per h. The cells have a generation time of about 10-12 h. The quantity of nutrient ingested by pinocytosis is much less than would be needed to replace the plasma membrane internalized during the endocytic process, even if it were all to be used for that purpose. Therefore, almost certainly, the membrane internalized by pinocytosis must recirculate to the cell surface by exocytic processes. Phagocytosis of latex beads by Acanthamoeba is a saturable process (20, 35), perhaps because the plasma membrane used to form the phagocytic vesicle cannot readily return to the cell surface. Despite the fact that isolated amoebae plasma membranes contain phospholipases, acyl-CoA transferase and acyl-CoA synthetase (18, 34), no evidence has been found for increased turnover of membrane phospholipids associated with endocytosis.

In the experiments reported in this paper, we present evidence that vesicles prepared from dipalmitoyl lecithin or distearoyl lecithin can fuse with the plasma membrane of A. castellanii whereas vesicles prepared from egg lecithin or dimyristoyl lecithin are taken up by the amoebae only by endocytosis. After this research was completed, other investigators have published similar but not identical conclusions derived from their experiments on the interaction of phospholipid vesicles with other cells (23, 25-27). Phagocytosis of multilamellar liposomes* has also been reported (12-15, 22).

MATERIALS AND METHODS

Egg lecithin was purchased from Lipid Products (South Nutfield, England) and stored at −20°C under a nitrogen atmosphere. The fatty acid composition of egg lecithin is palmitic 30%, palmitoleic 3%, stearic 15%, oleic 29%, linoleic 16%, arachidonic 4%, and other 3% (2). Dipalmitoyl lecithin and distearoyl lecithin were purchased from Calbiochem, San Diego, Calif. and dimyristoyl lecithin from Sigma Chemical Co., St. Louis, Mo. The purity of the lecithins was checked by thin-layer chromatography, and they were used without further purification. L-[α-¹⁴C]dipalmitoyl lecithin (50 mCi/nmol) was purchased from Applied Science Labs. Inc., State College, Pa. Its purity was determined by thin-layer chroma-

tography, and when used immediately, further purification was unnecessary. Degradation occurred upon storage at −20°C, requiring purification by silicic acid column and thin-layer chromatography before use (32). Radioactive lecithin (5.2 × 10⁷ cpm/μmol) was also prepared from Acanthamoeba grown in the presence of 25 mCi of neutralized [9,10-³H]oleic acid purchased from Amersham/Searle Corp., Arlington Heights, Ill., and used without purification. When 90-95% of the radioactive fatty acid was removed from the medium, the cells were harvested, and lecithin was isolated by column and thin-layer silicic acid chromatography (32) and stored as a chloroform solution at −20°C under nitrogen. The purity of the lecithin was monitored by thin-layer chromatography before use. The fatty acid composition of Acanthamoeba lecithin has been previously determined (32, 33): 16% saturated, 41% monounsaturated, 43% polyunsaturated fatty acids.

Stearylamine was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisc., dicetyl phosphate from K & K Laboratories, Inc., Plainview, N. Y., and bacterial α-amylase (type II-A) from Sigma Chemical Co., St. Louis, Mo.). L-[¹⁴C]glucose (566 mCi/nmol), D-[1-³H]glucose (5.5 Ci/nmol) and [³H]inulin (300 mCi/nmol) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. Inulin was purified by mixing 1 mCi with 1 g of washed (O-diethylaminoethyl)cellulose (DEAE-cellulose) (DE-52, Whatman Biochemicals Ltd., Maidstone, England) in 20 ml of 0.01 M phosphate buffer, pH 6.8, for 1 h and removing the DEAE-cellulose by centrifugation. The supernatant solution contained 90-95% of the radioactivity. This procedure minimized the nonspecific adsorption of radioactivity when cells were incubated with inulin.

Single bilayer phospholipid vesicles were prepared according to the procedure of Batzri and Korn (4). Lecithin (120 μmol including 0.1-0.2% radioactive lecithin and 12 μmol of stearylamine or dicetyl phosphate for positively or negatively charged vesicles) was evaporated from organic solvent under reduced pressure. The lipid was dissolved in ethanol, taken to dryness, and dissolved in 3 ml of ethanol. The ethanolic solution of lipid was rapidly injected through a Hamilton syringe (Hamilton Co., Reno, Nev.) into 60 ml of 0.16 M KCl maintained at a temperature above the transition temperature of the phospholipid (room temp, 40°C, 55°C, and 65°C for egg lecithin, dimyristoyl lecithin, dipalmitoyl lecithin, and distearoyl lecithin, respectively). The vesicles were concentrated to 3.5 ml at the same temperature by filtration under nitrogen pressure on an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, Mass.) using an XM-100A membrane. The concentrated vesicles were then rapidly cooled below their transition temperatures (except for egg lecithin when the entire procedure was carried out at room temperature). Slow cooling resulted in a larger percentage of multilamellar liposomes. The suspension was centrifuged for 20 min at 48,000 g to remove large aggregates, and the supernate was analyzed

for radioactivity and phospholipid phosphorus. The vesicles were stable under a nitrogen atmosphere for at least several days at room temperature.

When it was desired to trap solute molecules within the internal aqueous space of the phospholipid vesicles, D-[1-³H]glucose (15 μmol/ml), [³H]inulin (0.05 μmol/ml), and/or α-amylase (50 mg/ml) were dissolved in the 0.16 M KCl in which the vesicles were formed. After concentration of the vesicles by ultrafiltration, the vesicles were separated from external solutes by chromatography on a 1.5 × 25-cm column of Sephadex G-50 (for glucose and inulin) or 2.5 × 55-cm column of Sephadex G-200 (for amylase). The vesicles appeared in the void volume, and the external solute was in the included volume of the columns. By comparing the radioactivity or enzyme activity of the vesicle fraction to their concentrations in the original solution, the internal volume of the vesicles can be calculated, assuming an absence of adsorption to the bilayer surface. Internal volumes were found to be 0.39, 0.5, and 0.33 μl/μmol of phospholipid for vesicles labeled with glucose, inulin, and amylase (each vesicle will contain about two molecules of amylase), respectively. These values are in good agreement with the calculated volume of 0.3–0.5 μl/μmol of phospholipid (4), supporting the assumption that most, if not all, of the marker molecules were within the vesicles. As determined by enzyme assays in the presence and absence of 0.1% sodium dodecyl sulfate, at least 85% of the amylase was latent (in several preparations) and, therefore, presumably inside the phospholipid vesicles. The 15% of the amylase activity measurable in the absence of detergent may represent either enzyme adsorbed to the surface of the vesicles or partial activity of internal amylase. Only negligible inactivation of amylase occurred during the preparation of the vesicles.

Multilamellar liposomes were prepared from dipalmitoyl lecithin in 0.16 M KCl at 55–60°C essentially according to the method of Bangham et al. (3). The liposomes were sonicated for 10 s in a bath sonicator before use.

A. castellanii (Neff strain) was cultured axenically at 28°C in medium containing 1.5% glucose and 1.5% proteose peptone as described previously (29). After 3–5 days of growth, the cells were harvested by low-speed centrifugation, suspended in a solution containing 0.01 M phosphate, pH 6.8, 0.02 M NaCl, and 0.1 M glucose, counted in a cell counter (Particle Data, Inc., Elmhurst, Ill.), centrifuged, and resuspended at a concentration of 2.5–3 × 10⁶ cells/ml. The cells were equilibrated at the desired temperature for 20 min while agitated in a reciprocal shaker at 120 cycles/min. In experiments with glutaraldehyde-fixed cells, 50% glutaraldehyde was added to the washed cells at a final concentration of 3% glutaraldehyde, and the cells were left at room temperature for 1 h. The fixed cells were washed three times with the above buffer, suspended, and equilibrated as above.

Unless otherwise noted, all incubations were carried out with phospholipid vesicles at a concentration of 3

μmol of phospholipid/ml and amoebae at 2 × 10⁶ cells/ml in the above buffer with 15–35 mM KCl derived from the vesicle suspension. When present, 2,4-dinitrophenol was added at a final concentration of 1 mM 10–20 min before the addition of the phospholipid vesicles. Aliquots of 2 ml were removed from the incubation flasks at timed intervals and were washed three times with 5 vol of ice-cold 0.9% NaCl by centrifugation at 500 g and suspended in 0.9% NaCl for analysis. Since, as will be seen, the uptake of some phospholipid vesicles is not entirely inhibited at 0°C, a true "zero time" value is not always obtained, and the apparent "uptake" at zero time has been subtracted from the reported values. Cells that were incubated with multilamellar liposomes were washed with 10% sucrose, the increased density being necessary to separate the cells from the liposomes.

Radioactivity was measured using 10 ml of Aquasol (New England Nuclear) in a Beckman LS-250 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). Quench corrections were made for single and double isotope counting by the channels ratio method, using external standardization. For single isotope counting the efficiency was 40% for ³H and 85% for ¹⁴C.

Phospholipid was extracted from 1 × 10⁷ cells by the procedure of Bligh and Dyer (6) and determined as lipid-soluble phosphorus by the method of Ames and Dubin (1). Thin-layer chromatography of phospholipids was carried out on silica plates (Supelco, Inc., Bellefonte, Pa.) previously dehydrated for 1–2 h at 110°C, prerun in chromatographic solvent (chloroform:methanol:acetic acid:water, 25:15:4:2), and dried again. Samples were applied in chloroform. Phospholipids were detected by iodine vapor and scraped into scintillation vials for determination of radioactivity. When necessary, recovery of radioactivity was corrected on the basis of the recovery of known amounts of radioactive lecithin treated similarly.

Amylase was assayed according to Bernfeld (5) at 30°C, with maltose as standard, sometimes in the presence of 0.1% sodium dodecyl sulfate. The buffer solution contained bovine serum albumin (1 mg/ml) to stabilize the amylase.

Phagocytosis of polystyrene latex beads (1.1 μm in diameter) was measured as described by Weisman and Korn (35).

To determine cell volume, 2 × 10⁷ cells (pellet volume about 0.2 ml) were mixed with 0.2 ml of D-[1-³H]glucose (13 μCi), and an aliquot of 25 μl was taken for cell count and aliquots of 5 μl for determination of radioactivity. The cells were centrifuged, and aliquots of 5 μl were taken from the cell-free supernate for measurement of radioactivity. *Acanthamoeba* are impermeable to glucose, and pinocytosis is negligible over the period of the experiment. The average cell volume (V) was calculated as $V = [(T/A) - (T/B)]/C$, where T = total counts per minute added to the sample, A = counts per minute/milliliter of cell suspension, B = counts per minute/milliliter of cell-free supernate, and C = number of cells in sample.

For electron microscopy, washed cells were fixed with 3% glutaraldehyde in 0.1 M phosphate, pH 6.8. Cells incubated with multilamellar liposomes were fixed without washing. Postfixation in 1% OsO$_4$, dehydration in ethanol, and embedding in Epon 812 (Shell Chemical Co., N. Y.) were carried out as described previously [7]. Thin sections (pale gold) were picked up on 300-mesh copper grids and were stained with uranyl acetate and lead citrate before examination in a Siemens model 101 electron microscope at 80 kV.

For electron microscope autoradiography, phospholipid vesicles were prepared from 82 μmol of egg lecithin, 8 μmol (1 mCi) of amoeba [^{3}H]lecithin and 10 μmol of stearylamine. The phospholipid vesicles were incubated with cells in the usual way, and aliquots of cells were washed, fixed, dehydrated, embedded, and sectioned as usual. About 10-15% of the radioactivity was lost from the cells during the dehydration and embedding procedures as determined by monitoring the waste solutions. The sections were picked up on parlodion-coated, 200-mesh copper grids and were then coated with a thin layer of carbon. The grids were placed on a clean glass slide (2-3 per slide) and were coated with Ilford L-4 emulsion (Ilford Ltd., Ilford, England) by the loop method of Caro and Van Tubergen [9]. The grids were exposed for 3-7 wk in the cold room and were then developed with Microdol X developer (Eastman Kodak Company, Rochester, N.Y.), fixed, and washed. Before electron microscope examination, the sections were stained with uranyl acetate and lead citrate in the usual manner.

RESULTS

Phospholipid vesicles are rapidly taken up by the amoebae at 28°C, the optimal growth temperature (Figs. 1 A and C). That the radioactive assay used in Fig. 1 is a valid measure of net uptake of phospholipid is demonstrated in Table I where the radioactive assay is compared to the increase in cell phospholipid. Unless otherwise specified, the data in this paper were calculated from the radioactive assay. Positively charged vesicles are taken up more actively than negatively charged vesicles (Fig. 1 A and C), although the effect of charge is greater with vesicles made from egg lecithin than with vesicles made from dipalmitoyl lecithin. The major differences between egg lecithin vesicles and dipalmitoyl lecithin vesicles, however, are the effects of incubation at 4°C or in the presence of dinitrophenol, both of which inhibit pinocytosis of solute molecules and phagocytosis of latex particles below reliably measureable levels (8, 35, and Table II).

The uptake of phospholipid vesicles prepared from egg lecithin is inhibited almost completely by dinitrophenol and at 4°C while the uptake of

vesicles prepared from dipalmitoyl lecithin is inhibited only about 40% by dinitrophenol and 20% at 4°C (Figs. 1 B and D). Even when cells are fixed with glutaraldehyde, the uptake of dipalmitoyl lecithin vesicles is only slightly or not at all impaired (Fig. 1 and Table II): fixation, of course, inhibits pinocytosis (Table II) and phagocytosis (data not shown). At 4°C, dimyristoyl lecithin vesicles behave like those prepared from egg
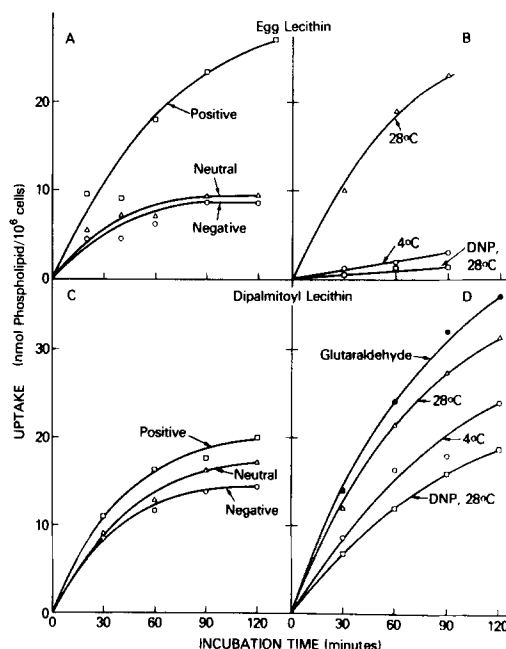

FIGURE 1 Uptake of unilamellar phospholipid vesicles by *A. castellanii*: effect of phospholipid composition, charge, temperature, and metabolic inhibitors. (A) Neutral and positively and negatively charged egg lecithin vesicles (3 μmol/ml) labeled with [^{3}H]lecithin were incubated with amoebae (2 × 10^6/ml) at 28°C, and the uptake of phospholipid was calculated from the uptake of radioactivity as described in Materials and Methods. (B) The uptake of positively charged egg lecithin vesicles was compared at 28°C, 4°C, and at 28°C in the presence of 1 mM dinitrophenol (DNP). (C) Same experimental conditions as in A except that the vesicles consisted of dipalmitoyl lecithin. (D) Same as B, except for the use of positively charged dipalmitoyl lecithin vesicles. In a separate experiment the cells were fixed for 1 h with glutaraldehyde before incubation at 28°C with the dipalmitoyl lecithin vesicles; the data were normalized for the uptake at 4°C and in the presence of dinitrophenol, in that experiment, in order to plot them in this figure. The differences in uptake between C and D reflect the variations between different cultures of amoebae and are not experimental error.

TABLE I

Uptake of Phospholipid Vesicles by A. castellanii Measured by Uptake of Radioactivity and Phospholipid Phosphorus

	Vesicle uptake	
Incubation	Phospho-lipid	Radio-activity
	nmol/10⁶ cells	
Egg lecithin, 28°C	52	45
Dipalmitoyl lecithin, 4°C	18	21
Distearoyl lecithin, 4°C	18	20
Dimyristoyl lecithin, 4°C		1.6
Egg lecithin, 4°C		2.3

Amoebae (2 × 10⁶/ml) were incubated with unilamellar phospholipid vesicles (3 μmol/ml) for 2 hr, at which time aliquots containing 3 × 10⁶ cells and 1 × 10⁷ cells were analyzed for uptake of radioactivity and phospholipid phosphorus, respectively, as described in Materials and Methods. Egg lecithin vesicles contained 0.1% [³H]lecithin (from *Acanthamoeba*); all other phospholipid vesicles contained [¹⁴C]dipalmitoyl lecithin. The data are the average of duplicate analyses. The experiment with egg lecithin vesicles at 28°C was carried out at a different time from the other incubations, all of which were with the same batch of amoebae.

lecithin (little or no uptake) while distearoyl lecithin vesicles are taken up as well as those prepared from dipalmitoyl lecithin (Table I). The relative rates of uptake of positive, neutral, and negative dipalmitoyl lecithin vesicles are the same at 4°C or in the presence of dinitrophenol as at 28°C (data not shown).

As mentioned in Materials and Methods, all the data for uptake have been corrected for the radioactivity (or vesicle phospholipid phosphorus) associated with the cells at "zero time." For cells incubated at 4°C or in the presence of dinitrophenol the apparent uptake at zero time is about 10% of the value at 2 h, and for glutaraldehyde-fixed cells the value at zero time is about 25% of the uptake at 2 h. The corrections are less under all other conditions. This background "uptake" may represent adsorption of vesicles to cells, or it may be, at least partly, the result of uptake during the period it takes to separate the amoebae from the vesicles.

Although there is some variation among experiments, the uptake of phospholipid by the amoebae is frequently sufficient to increase the phospholipid content of the cells (normally about 40 nmol/10⁶ cells) by about 50-75% in 2 h. Expressed as

percentage of the material in the incubation medium, the uptake of phospholipid vesicles at 20°C is about 15-30 times the rate of pinocytosis of solute molecules (glucose, inulin, or amylase) (Table II). In contrast, the rate of uptake of dipalmitoyl lecithin vesicles at 4°C, in the presence of dinitrophenol or by glutaraldehyde-fixed cells, is about 200 times the rate of pinocytosis (Table II). At 28°C, latex particles are also taken up more rapidly than solute molecules are pinocytosed (35), but, under the inhibitory conditions, there is no detectable phagocytosis of latex particles.

The rates of uptake of the phospholipid bilayer and of the aqueous space within the vesicle are compared in Table II using radioactive lecithin as tracer for the bilayer and either radioactive inulin, radioactive glucose, or amylase, alone or in combination, as marker of the internal space. It can be seen that vesicles prepared from egg lecithin are taken up by the amoebae with no loss of internal contents. The contents of vesicles prepared from dipalmitoyl lecithin, however, are taken up by the amoebae only about 40% as well as the phospholipid bilayer of the vesicles, irrespective of the size of the solute molecule.

Electron microscopy (Fig. 2) of cells incubated at 28°C with egg lecithin vesicles reveals an increase in cytoplasmic vacuoles containing numerous membranous profiles which, as demonstrated by autoradiography, are derived from the radioactive phospholipid vesicles. The cells are otherwise normal in appearance despite the large uptake of phospholipid (45 nmol/10⁶ cells in this experiment), suggesting that all of the excess phospholipid is within the cytoplasmic vacuoles (digestive vacuoles).

Electron microscopy of amoebae incubated at 4°C with dipalmitoyl lecithin vesicles reveals no increase in cytoplasmic vacuoles and none of the digestive vacuoles that are present contain membranous profiles (Fig. 3 *b* and *c*) despite the significant uptake of phospholipid (24 nmol/10⁶ cells in this experiment). The electron micrographs of cells incubated with dipalmitoyl lecithin vesicles do show an increase in the infoldings of the cell surface (Fig. 3 *b*) that also seem to be revealed in cross section within the section (Fig. 3 *c*). In this experiment the uptake of dipalmitoyl lecithin was equal to about 50% of the phospholipid content of the amoebae or about 5-10 times greater than the phospholipid content of the amoeba plasma membrane (10, 33). There was little or no increase in cell volume (3.5 vs. 3.67

TABLE II

Comparison of the Uptake of Bilayer and Internal Contents of Phospholipid Vesicles and Pinocytosis of Solute Molecules by A. castellanii

Vesicles	Label	Incubation conditions	Uptake	
			%	Content/Bilayer
Egg lecithin*	[³H]lecithin	28°C	3.1	
	[³H]inulin (inside)		3.1	1
	[³H]inulin (outside)		0.1	
Dipalmitoyl lecithin‡	[¹⁴C]lecithin	4°C	1.05	
	Amylase (inside)		0.41	0.39
	[³H]glucose (inside)		0.5	0.48
	Amylase (outside)		0	
	[¹⁴C]glucose (outside)		0.004	
Dipalmitoyl lecithin§	[³H]lecithin	Glutaraldehyde-fixed, 28°C	0.77	
	[³H]glucose (inside)		0.32	0.42
	[¹⁴C]glucose (outside)		0.003	
Dipalmitoyl lecithin§	[³H]lecithin	Dinitrophenol, 28°C	1.0	
	Amylase (outside)		0.003	
	[³H]inulin (outside)		0.005	

Unilamellar phospholipid vesicles were prepared with and without radioactive phospholipid (0.1%) in the bilayer and radioactive solute markers or amylase in their internal aqueous space. Incubations were carried out for 1 h as described in Table I, and aliquots of 4×10^6 cells (inulin and glucose experiments) or 2×10^7 cells (amylase experiments) were analyzed for uptake of radioactivity and amylase as described in Materials and Methods. Radioacitve glucose or inulin, or amylase, were added to the medium (20 times more than was present inside the vesicles) to measure the rate of pinocytosis.

* Three similar preparations of vesicles (one labeled with [³H]lecithin, one labeled with internal [³H]inulin, and one unlabeled but with external [³H]inulin added) were incubated in parallel flasks with amoebae from the same culture flask.

‡ One preparation of phospholipid vesicles contained three labels, [¹⁴C]lecithin in the bilayer, and amylase and [³H]glucose in the internal aqueous space. Another preparation of unlabeled dipalmitoyl lecithin vesicles was incubated in a parallel flask containing external amylase and [¹⁴C]glucose to measure pinocytosis of solute molecules.

§ In these two experiments, [³H]phospholipid vesicles were incubated with amoebae alone or in the presence of external amylase or [¹⁴C]glucose. In parallel flasks, amoebae were incubated with phospholipid vesicles labeled only with internal [³H]glucose or with unlabeled vesicles and external [³H]inulin.

μl/10⁶ cells). Although incubation of amoebae at 4°C in the absence of phospholipid vesicles may cause some invagination of the cell surface, the difference between the experimental and control cells (Fig. 3 a) is striking. Examination of 35 random sections of experimental and control cells showed that 18 of the sections of cells incubated with dipalmitoyl lecithin and only two sections of the control cells had both invaginations at the cell periphery and apparent cross sections of invaginated cell surface within the cell, while 13 experimental sections and 20 control sections showed no manifestation of increased surface membrane.

Similar results were obtained upon incubation of amoebae at 4°C with multilamellar dipalmitoyl lecithin liposomes. Liposomes have the practical advantage, compared to unilamellar vesicles, of a morphologically detectable content (internal bilayers). In the experiment from which the micrographs in Fig. 4 were obtained, 3.2% of the liposomes were taken up by the cells under conditions in which there was no detectable pinocytosis of solute radioactive glucose (in the same incuba-
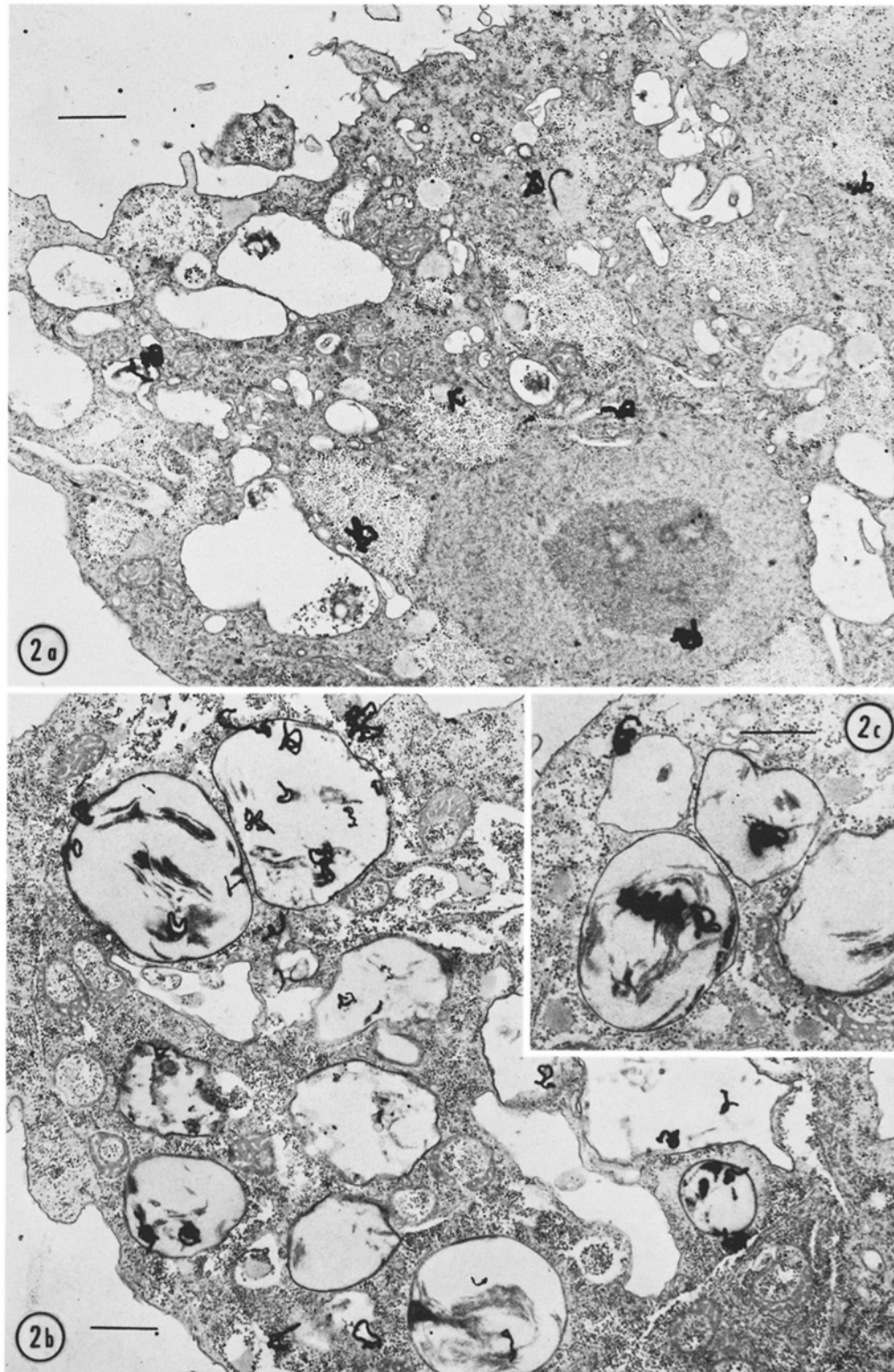
FIGURE 2 Electron microscope autoradiography of *A. castellanii* after incubation with positively charged egg [³H]lecithin vesicles. (*a*) Incubated with vesicles for 10 min, fixed, embedded, sectioned, coated with emulsion, and exposed for 10 wk before examination in the electron microscope. Uptake was about 4 nmol of phospholipid/1 × 10⁶ cells. (*b*) Cells were fixed after incubation with vesicles for 2 h, and the coated sections were exposed for 4 wk. Uptake was 45 nmol/1 × 10⁶ cells. (*c*) Same as *b*. Bars are 1 μm.
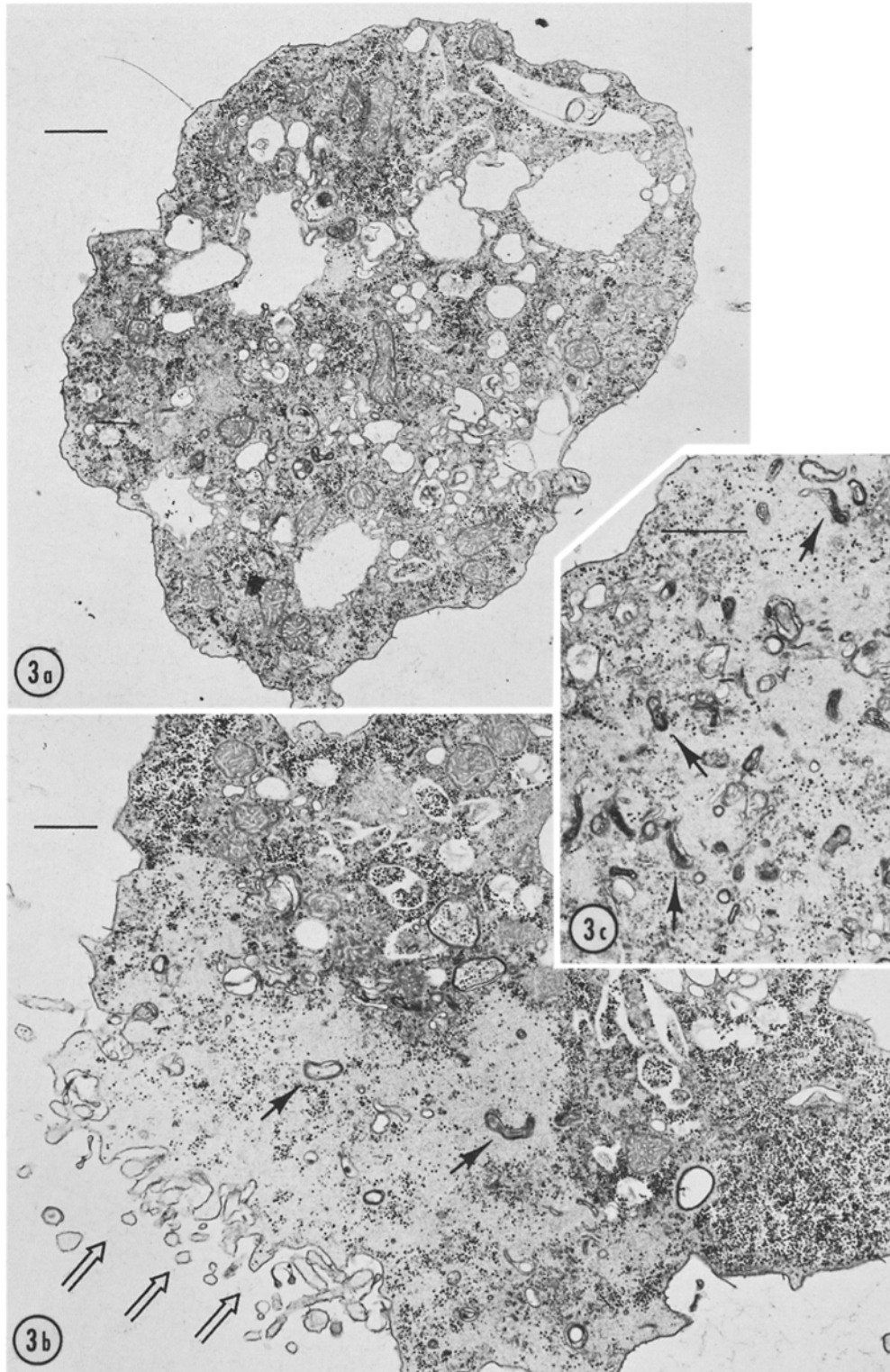
FIGURE 3 Electron microscopy of *A. castellanii* after incubation with neutral unilamellar dipalmitoyl lecithin vesicles. (*a*) Control cells incubated at 4°C without vesicles. (*b*) Amoebae after incubation with dipalmitoyl lecithin vesicles for 2 h at 4°C. (*c*) Same as (*b*) Double arrows (*b*) indicate a region with an unusually high number of surface projections and invaginations. Single arrows (*b* and *c*) indicate surface invaginations in cross section. Bars are 1 μm.
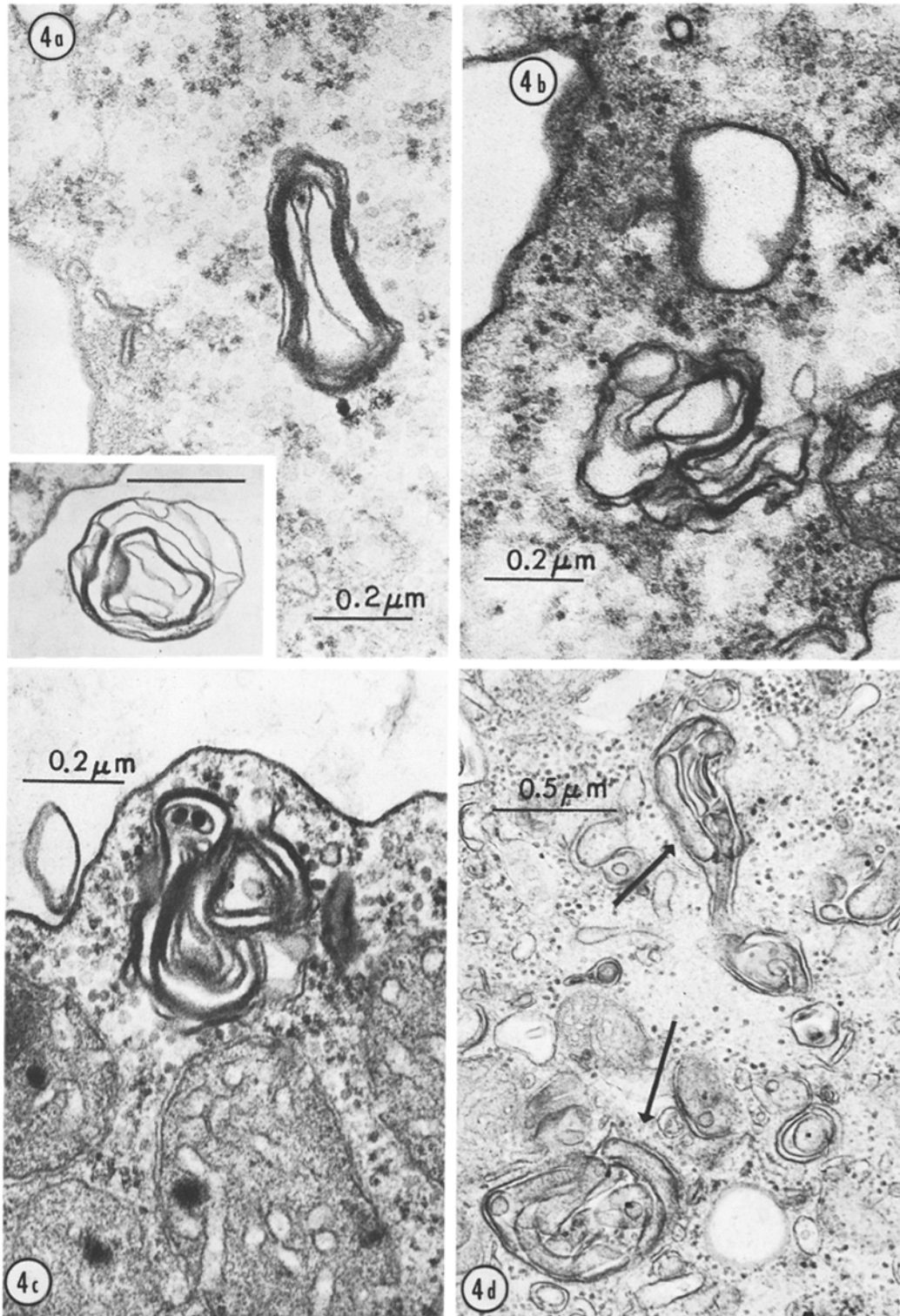
FIGURE 4 Electron microscopy of *A. castellanii* after incubation with multilamellar neutral dipalmitoyl lecithin liposomes at 4°C for 2 h. Uptake was 3.2% of the added liposomes. Images *a*, *b*, and *c* show multilamellar liposomes within cells but not within a cytoplasmic vacuole. The inset in *a* shows a liposome near the surface of an amoeba (the bar is 1 μm). Image *d*, shows cross sections of membrane (arrows) interpreted as cytoplasmic invaginations of the plasma as a consequence of the increased surface derived from fusion with liposomes. Invaginations such as these are not seen in control sections.

tion flask) or phagocytosis of latex particles (in a parallel flask). The electron micrographs show occasional images of multilamellar liposomes lying near cells (Fig. 4 a inset). Multilamellar vesicles within the cell, but not within a cytoplasmic vacuole, are frequently observed (Fig. 4 a–c), as are cross sections of membranes most readily interpreted as cytoplasmic invaginations of the cell surface (Fig. 4 d).

DISCUSSION

The results of the experiments with egg lecithin vesicles can be interpreted unambiguously as demonstrating endocytosis because (a) the uptake of the vesicles is inhibited by incubation at 4°C and in the presence of dinitrophenol, (b) contents of the internal vesicle space are taken up at the same rate as the phospholipid bilayer, and (c) electron microscope autoradiography shows numerous membranous profiles within digestive vacuoles as if egg lecithin vesicles had fused with each other subsequent to their endocytosis. The possibility that radioactive phospholipids might be incorporated into the amoeba plasma membranes by exchange with the vesicles is eliminated by the fact that the increase in cell phospholipid agrees with the uptake calculated from the radioactive data. The possibility of significant adsorption of egg lecithin vesicles to the cell surface is incompatible with the electron microscope observations and the lack of uptake at 4°C or in the presence of dinitrophenol. Endocytosis of phospholipid vesicles, schematically illustrated in Fig. 5 A, would introduce lipid- or water-soluble molecules directly into the digestive vacuoles (lysosomes in mammalian cells) if the additive were present in the phospholipid bilayer or the internal aqueous space of the phospholipid vesicle, respectively. Preparation of unilamellar vesicles by the procedure of Batzri and Korn (4), rather than the more commonly used procedure of high energy sonication, may facilitate the use of this procedure with unstable molecules such as proteins and nucleic acids. Multilamellar liposomes can be, and have been (12–15, 22), used to introduce enzymes into lysosomes, but unilamellar phospholipid vesicles may have some advantages because of their small size. It may also be possible to direct vesicles to specific target organs and cells in higher animals by incorporation of receptor molecules into the phospholipid bilayer, by controlling the composition or charge of the bilayer (13), or by varying the size of the vesicles (24).

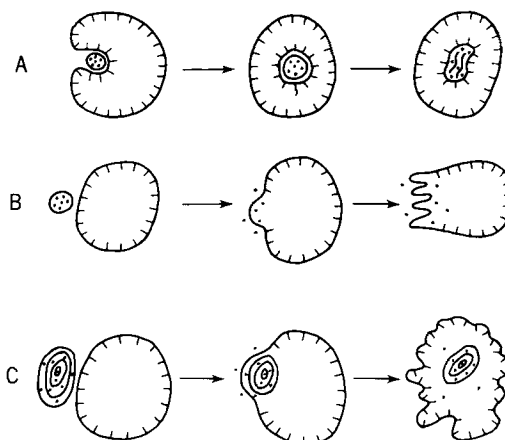The interaction of dipalmitoyl lecithin vesicles

FIGURE 5 Schematic representation of the possible interactions of phospholipid vesicles with A. castellanii. (A) Pinocytosis of unilamellar phospholipid vesicles and their contents. One or more vesicles is ingested within an endosome; the endosomes fuse with each other and preexisting digestive vacuoles. The phospholipid vesicles fuse with each other, possibly undergoing some hydrolysis, to form membranous profiles within cytoplasmic vacuoles. This is the fate of egg lecithin vesicles (and probably some dipalmitoyl lecithin vesicles) incubated with amoebae at 28°C (Fig. 2). (B) Fusion of unilamellar phospholipid vesicles with the amoeba plasma membrane. The vesicle phospholipid will add to the plasma membrane, increasing the surface area of the cell and causing invaginations of plasma membrane. The fusion is partially "leaky" so that some of the contents of the vesicle enter the cell cytoplasm while some of the contents are lost to the medium. This is the suggested fate of dipalmitoyl lecithin vesicles incubated with amoebae at 4°C (Fig. 3) in the presence of dinitrophenol or with glutaraldehyde-fixed cells. (C) Fusion of multilamellar phospholipid liposomes. The outermost bilayer of the liposomes fuses with the amoeba plasma membrane, causing an increase in surface area and invagination of the excess plasma membrane. The remaining liposome (minus one bilayer) together with its contents will enter the cytoplasm of the cell. This is the suggested fate of multilamellar dipalmitoyl liposomes (Fig. 4).

with the amoebae is more complicated. Endocytosis is probably responsible for the uptake that is inhibited by dinitrophenol or at 4°C, but fusion of phospholipid vesicles with the cell plasma membrane is the best explanation of the uptake that occurs in the presence of dinitrophenol, at 4°C or with glutaraldehyde-fixed cells. Adsorption of the vesicles to the amoebae can be eliminated as a possible explanation for the nonendocytic uptake, for several reasons: (a) dipalmitoyl lecithin vesicles are taken up irrespective of their charge although

at somewhat different rates; (b) the surface of the vesicles prepared from dipalmitoyl lecithin should be the same as the surface of the egg lecithin vesicles, since the two phospholipids differ only in the composition of their fatty acid chains, yet only the dipalmitoyl lecithin vesicles are taken up by cells under conditions that inhibit endocytosis; and (c) the contents of the dipalmitoyl lecithin vesicles are taken up only about 40% as well as their phospholipid bilayer. This last observation is compatible with fusion as the mechanism of uptake of dipalmitoyl lecithin vesicles, as schematically illustrated in Fig. 5 B, since known fusion processes have been shown to be totally or partially "leaky" in other experimental systems (11, 30, 31, 36). The inequality of uptake of vesicle content and vesicle bilayer also argues against adsorption of vesicles to the amoeba cell surface. Demonstration of an increase in cell phospholipid equal to that calculated from the radioactivity data argues against exchange of phospholipid between vesicles and cells as contributing significantly to the observations.

The electron micrographs of cells after incubation with unilamellar dipalmitoyl lecithin vesicles and multilamellar dipalmitoyl lecithin liposomes also support the interpretation of fusion as the mechanism of their uptake. In both cases there is morphological evidence for an increase in the surface area of the cells, in neither case is there morphological evidence for endocytosis, and in the latter case there is morphological evidence for multilamellar liposomes within the cytoplasm, not surrounded by a cell membrane, as would be expected if the outermost bilayer of a multilamellar liposome had fused with the plasma membrane of the cell. This image, in fact, may be the visualization with multilamellar liposomes of what one would anticipate (Fig. 5 C) as the consequence of fusion of unilamellar vesicles with cells; the contents of the vesicles should enter directly into the cytoplasm. It is interesting, but of unknown significance, that the infoldings of the cell surface that occur as the apparent consequence of fusion of cells with dipalmitoyl lecithin vesicles are consistently localized adjacent to cytoplasmic areas that are relatively free of organelles and glycogen and enriched in cytoplasmic actin filaments.

If this apparent fusion of vesicles and cells can be confirmed and generalized, a mechanism will be available for introduction of lipophilic molecules into plasma membranes (by fusion with vesicles containing the molecule in their phospholipid bilayer) or water-soluble molecules (contained within the vesicle's interior) into the cell's cytoplasm. In our experiments, one cell with a volume of about 4,000 μm^3 fuses in 1 h with about 2×10^6 unilamellar vesicles with a total internal volume of about 8 μm^3. If a solute were contained within the vesicles at a concentration of 0.1 M, its intracellular concentration would be 0.2 mM, assuming uniform distribution, after fusion. We have made stable impermeable vesicles containing 0.5 M potassium ferricyanide and, as in experiments reported in this paper, 5% amylase, so this is a feasible protocol.

Alternatively, one could use multilamellar liposomes to place lipophilic molecules into the cytoplasm and into the plasma membrane, or water-soluble molecules, enclosed within phospholipid bilayers, into the cytoplasm. The several aqueous compartments separating the liposomal bilayers provide a greater internal volume than is available with the unilamellar phospholipid vesicles. Suitable modification of the composition of the phospholipid bilayer may allow selectivity for target organs.

Definitive proof of fusion of phospholipid vesicles with cells requires demonstrating that the vesicle phospholipid is incorporated into the cell plasma membrane and that solute markers known to be within the vesicles' internal aqueous space appear free within the cell cytoplasm, not enclosed within membrane vesicles. In our experiments we have shown that dipalmitoyl lecithin is taken up by the Acanthamoeba, and the morphological evidence for an increase in the surface area of the cell suggests that the vesicle phospholipid has been incorporated into the amoeba plasma membrane. The experiments with multilamellar dipalmitoyl lecithin provide morphological evidence that the "contents" of liposomes (a liposome with presumably one less bilayer than the original substrate) are introduced into the cytoplasm as expected if the outermost bilayer of the liposome fused with the cell plasma membrane.

Grant and McConnell (11) have previously demonstrated the incorporation of the phospholipid of dipalmitoyl lecithin vesicles into the plasma membrane of Acholeplasma laidlawii, fulfilling the first of the above two criteria for fusion, but in their experiment the soluble contents of the vesicles were lost completely to the external medium. Since the completion of the experiments described in this paper, several papers have been published that suggest, but also do not prove, that

unilamellar vesicles may fuse with mammalian cells. Martin and MacDonald (23) have observed that phospholipid vesicles containing 20% lysolecithin induce cell fusion in monolayers of KB cells and in suspensions of Ehrlich ascites tumor cells (similar results were reported by others previously [28]). Such results are most readily explained by the fusion of a single phospholipid vesicle with two cells, thus forming a bridge between the cells. Martin and MacDonald (23) have also suggested, on the sole basis of an electron micrograph of negatively stained preparations, that multilamellar liposomes can fuse with the surface of human erythrocytes.

Pagano et al. (25) have studied the interaction of unilamellar and multilamellar dioleoyl lecithin and egg lecithin vesicles with Chinese hamster V79 cells. All preparations of phospholipids were taken up by the cells at 37°C by a process that is insensitive to inhibitors of energy metabolism and is only partially affected by incubation at 2°C or by fixing the cells with glutaraldehyde. These controls argue against endocytosis as the mechanism of uptake. With unilamellar vesicles at 37°C, the uptake of vesicle content (inulin) was about 50% of the uptake of vesicle phospholipid. However, at 2°C, vesicle content was taken up only 8% as well as the phospholipid bilayer, and with multilamellar liposomes, at 37°C and 2°C, the uptake of content was only 10% and 1% as great as the uptake of phospholipid. These results were interpreted as demonstrating a lack of fusion of multilamellar vesicles with cells regardless of temperature while unilamellar vesicles appear to fuse with cells only at 37°C. Electron micrographs of thin sections of cells showing unilamellar vesicles possibly in the process of fusion with cells could be obtained only when the cells were treated with cationized ferritin before incubation with the phospholipid vesicles.

Papahadjopoulos et al. (26) have published a convincing freeze-cleavage electron micrograph of the product of fusion of multilamellar liposomes with a human erythrocyte ghost. Their evidence for fusion of unilamellar phospholipid vesicles with cultured 3T3 cells is less strong (26, 27). It depends essentially on the observation that cell growth was inhibited by cyclic AMP, presumably trapped within "fluid" phospholipid vesicles, but not by cyclic AMP trapped within "solid" phospholipid vesicles despite the equal uptake of both vesicles by the cells. It was postulated that "fluid" vesicles

may fuse with the cell plasma membrane thus allowing the cyclic AMP to enter the cytoplasm and inhibit growth while "solid" vesicles are pinocytosed, the cyclic AMP being ineffective within the lysosomes. However, one can calculate from their data that 10–20 times more cyclic AMP was associated with the phospholipid vesicles than could be contained within their internal aqueous space. Most of the cyclic AMP was probably bound to the surface of the positively charged vesicles from where it might have affected cell growth by processes not involving vesicle-cell fusion.

It is not clear why, in our experiments, the amoeba plasma membrane fuses with dipalmitoyl lecithin and distearoyl lecithin vesicles but not with egg lecithin and dimyristoyl lecithin vesicles. The plasma membranes of A. castellanii consist of approximately one-third each of protein, phospholipid plus sterol, and a novel polymeric lipophosphonoglycan (10, 19, 21). The plasma membrane phospholipids consist mostly of mono- and polyunsaturated fatty acids (33), but the lipophosphonoglycan contains about 15% by weight of predominantly very long chain and 2-hydroxy fatty acids (21) and 13% by weight of long chain phytosphingosine bases.[1] Any interpretation of the molecular and physical events of fusion between phospholipid vesicles and Acanthamoeba plasma membranes must take these compositional data into account. It should be noted, however, that in the experiments of Grant and McConnell (11) it was dipalmitoyl lecithin vesicles that fused with the Acholeplasma membrane, and that Pagano et al. (25) observed that Chinese hamster cells took up phospholipid vesicles better, by a process suggested to be fusion, at a temperature below the transition temperature of the vesicle phospholipid. Fusion may be favored when the vesicles consist of phospholipids below their transition temperatures.

Our data suggest that phospholipid metabolism is not necessary for the fusion of two membranes. Within experimental limits, all of the lecithin of the vesicles remains unaltered after uptake by the amoebae. Furthermore, the fact that dipalmitoyl lecithin vesicles undergo fusion but egg lecithin vesicles do not also argues against the involvement of the phospholipases and acyltransferase of the amoeba plasma membrane since these enzymes are

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[1] Korn, E. D., and D. G. Dearborn. Manuscript in preparation.

equally active with unsaturated and saturated lipids as substrate (34). Moreover, one would expect appreciable inhibition of uptake of dipalmitoyl lecithin vesicles at 4°C and by glutaraldehyde fixation were enzymatic activity required for their fusion with the cell plasma membrane.

Finally, nonenzymatic fusion between phospholipid vesicles and cells suggests that, in the regions where fusion occurs, the plasma membrane phospholipids are exposed at the cell surface.

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