Phorbol esters stimulate macropinocytosis and solute flow through macrophages

JOEL A. SWANSON

Department of Anatomy and Cellular Biology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115, USA

Summary

The morphology and kinetics of pinocytosis by bone marrow-derived macrophages were studied to determine how stimulation by phorbol esters increases net solute accumulation. Application of phorbol myristate acetate (PMA) increased both the abundance of macropinosomes and the rate of solute flow through the endocytic compartment. The large pinosomes originated as ruffles at the cell margins that folded back on themselves, internalizing extracellular medium and solutes. I examined how stimulation affects the kinetics of pinocytic influx, accumulation, and subsequent efflux of the fluorescent dye Lucifer Yellow (LY) in macrophages. Both the accumulation of LY and its subsequent efflux were temperature-dependent and directly proportional to the concentration of LY in the extracellular medium. Macrophages incubated in PMA and LY for 2 h accumulated four to six times more LY than did macrophages in LY alone. If after pinocytosis the macrophages were washed and reincubated in unlabeled medium for a 1 h chase period, some of the internalized LY was regurgitated from the cells. Inclusion of PMA in the chase medium increased efflux of LY. In contrast, a smaller percentage of LY was regurgitated from macrophages which were both loaded and chased in the presence of PMA. This indicates that although efflux is increased by PMA, influx increases more, and therefore more of the LY entering by pinocytosis is retained within the cell. I suggest that macropinocytosis increases the size difference between pinosomes and efflux vesicles, and that that difference increases greatly both solute accumulation and membrane flow through the endocytic compartment.

Key words: pinocytosis, macrophage, phorbol ester, Lucifer Yellow.

Introduction

Macrophages are capable of continuous internalization of plasma membrane by pinocytosis. Stereological measurements indicate that they internalize the equivalent of their cell surface area once every 33 min (Steinman *et al.* 1976), yet they maintain a flattened morphology with a high cell surface to volume ratio. After endocytosis, membrane receptors and some fluid solute probes of pinocytosis return to the cell surface, indicating that internalized membrane is recycled. Despite such partial recycling, however, solute probes accumulate inside macrophages linearly for many hours, indicating that much of the fluid volume entering the cell does not recycle. How does the macrophage maintain steady state rates of solute accumulation without internalizing more membrane than it recycles?

Treatment of macrophages with the tumor-promoting phorbol ester phorbol myristate acetate (PMA) stimulates pinocytosis. Within several minutes of addition of PMA, macrophage accumulation of the fluid phase solute probes horseradish peroxidase or Lucifer Yellow (LY) increases several fold (Phaire-Washington *et al.* 1980; Swanson *et al.* 1985). Increased accumulation is continuous for many hours, and occurs without any noticeable loss of cell surface area. Indeed, surface area appears to increase during the first hour of stimulation (Phaire-Washington *et al.* 1980). Examination of the kinetics of stimulation in thioglycollate-elicited murine peritoneal macrophages revealed that PMA stimulated both the rate of influx and the net intracellular retention of LY (Swanson *et al.* 1985). It was proposed that PMA increased the efficiency of pinosome–lysosome fusion, possibly *via* the extension of lysosomes into more peripheral regions of cytoplasm (Swanson *et al.* 1987).

The present work considers constitutive and stimulated pinocytosis in light of their effect, or lack of effect, on cell shape. It was prompted first by the observation that in bone marrow-derived macrophages PMA increases LY accumulation without noticeable redistribution of lysosomes. Using time-lapse video microscopy and measurement of the kinetics of LY pinocytosis, I report that PMA stimulates macropinocytosis and increases the net rate of flow through the endocytic

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compartment. Accumulation is increased not by inhibiting efflux, but by a differential increase of both influx and efflux. I propose a model to explain how rates of constitutive and stimulated pinocytosis could be sustained without net redistribution of plasma membrane into the cell.

Materials and methods

Cells

Bone marrow-derived macrophages were obtained by the method of Celada *et al.* (1984), with some modifications. Female mice (ICR, Trudeau Inst., Saranac Lake, NY, USA or C3H/HeJ, Jackson Lab., Bar Harbor, ME, USA) were killed by cervical dislocation and their femurs removed. The ends of the bones were clipped off, then the bone marrow was extruded using a 26 g needle and a syringe containing cold Dulbecco's Modified Eagle's Medium plus 10 % heat-inactivated fetal bovine serum (DME–10F). The reticular matrix was agitated gently to loosen cells, then removed by low speed centrifugation (500 revs min^{-1} for 1 min). The suspension of cells was then washed by centrifugation and resuspended in complete bone marrow medium (DME plus 30 % L-cell-conditioned medium +20 % HiFBS). 5×10^6 bone-marrow cells in 25 ml of bone marrow medium were plated into 100 mm diameter Lab-Tek petri dishes, then incubated for six days at 37°C in a 5 % CO_2 incubator. Three days after plating, each dish received 10 ml of bone marrow medium. On day six, adherent macrophages were harvested by first washing the plates with ice-cold divalent cation-free phosphate-buffered saline (PD: 137 mM-NaCl, 3 mM-KCl, 7 mM-phosphate buffer, pH 7·4), leaving dishes 15 min on ice, then washing the macrophages off the dishes with a gentle stream of cold PD. These suspended bone marrow-derived macrophages (BMM) were plated either onto coverslips for microscopic observation, or into 16 mm diameter wells of a 24-well culture dish (Costar). For most experiments cells were plated at 3×10^5 cells per well. After allowing 30 min at 37°C for the suspended cells to adhere to the dish, PD was replaced with 0·5 ml of DME-10F, and the cultures were left overnight at 37°C and 5 % CO_2. Experiments were performed with these cells within the next two days. Greater than 95 % of these cells were macrophages, as judged by their ability to phagocytose opsonized sheep red blood cells. Nearly all the data shown here was obtained using the ICR mice. C3H/HeJ mice, which are insensitive to bacterial lipopolysaccharide (Vogel *et al.* 1981), were used to confirm the essential observations reported here.

Microscopy

Suspended, day six BMM were plated at low density onto 25 mm diameter circular no. 1 coverslips placed into 35 mm tissue culture dishes. They were studied within the next two days. For time-lapse microscopy, coverslips were put into Sykes-Moore chambers, which were then mounted into a temperature-controlled stage (Extech Instr.) on a Zeiss inverted microscope and held at 36°C. The Sykes-Moore chamber was perfused with PBS containing 10 % heat-inactivated fetal bovine serum (PBS–10F), with or without phorbol myristate acetate (PMA, Sigma Chem. Co., St Louis, USA) at 60 ng ml^{-1}. Phase microscope images were collected *via* a ×100 objective lens (NA 1.25) and a Dage NC-66X video camera mounted onto the microscope. A Panasonic optical disc recorder (TQ 2025F) provided a time-lapse record of events, speeded up ×36.

For fluorescence microscopic observation of pinosomes, BMM on coverslips were preincubated for 60 min in PBS–10F ± PMA, then incubated for 60 s in 1·5 mg ml^{-1} Lucifer Yellow CH (LY; potassium salt, Molecular Probes, Eugene, OR, USA), rinsed in PBS, and fixed for 30 min in a paraformaldehyde fixative (McLean and Nakane, 1974). Specimens were mounted in glycerol and observed in a Zeiss Photomicroscope III equipped for epi-illumination of fluorescent specimens (Lucifer Yellow filter set) and for 35 mm photography (Tmax 400 film).

To quantify pinocytic vesicles, macrophages on coverslips were preincubated 90 min in PBS–10F ± PMA (37°C) then were placed into fixative containing 2·5 % glutaraldehyde in PBS, plus 4·5 % sucrose, and fixed for 45 min at room temperature (21°C). Fixed preparations were washed with several changes of PBS, then mounted in glycerol for microscopic study. Photomicrographs were taken using Pan X film, and were enlarged to a final magnification of ×1800. For every macrophage whose entire profile was visible in a print, all of its phase-bright vesicles were measured and recorded.

Quantitation of LY pinocytosis

Pinocytosis of LY was quantified by allowing BMM in 24-well dishes to incubate in PBS–10F containing LY, then washing away extracellular dye, lysing the cells, and measuring the fluorescence of the lysate. Typically, 3×10^5 BMM per well were incubated in PBS–10F on ice for 30–45 minutes. Cold PBS–10F was then replaced by 0·35 ml of a cold solution of LY dissolved in PBS–10F at 0·5 mg ml^{-1}, ±30 ng ml^{-1} PMA (48 nM). The culture dish was then either maintained on ice, or warmed to 37°C for 2 h. For time course experiments, dishes were not chilled before adding the drinks, but were instead maintained at 37°C throughout. Drinks were added such that all incubations ended at the same instant.

To terminate LY pinocytosis, dishes were drained, then immersed in 1 l of ice-cold PD with 1 mg ml^{-1} bovine serum albumin. Dishes were washed by repeated immersion and drainage, then were transferred to a second 1 l beaker of ice-cold PD. Dishes were rinsed for 5 min in this second beaker before passing through a third and final liter of cold PD. They were drained and aspirated dry, then were either reincubated in PBS–10F (chase period, see below), or were lysed in 0·50 ml of 0·1 % Triton X-100. Wells with Triton were incubated 60 min at 37°C before analyzing LY fluorescence or protein content. In the pulse–chase experiments, washed dishes of LY-loaded cells were reincubated for 60 min in 0·45 ml PBS–10F. Here, too, solutions were added cold, then dishes were either warmed to 37°C or maintained on ice for the chase period. Following this chase, cells were washed in 2×1 l of ice-cold PD, drained and lysed in Triton as described above.

Pinocytosed LY was measured by combining 0·40 ml of lysate with 0·75 ml of 0·1 % Triton X-100 and 100 μg ml^{-1} bovine serum albumin, then reading the fluorescence in an SLM-Aminco 500C spectrofluorometer. Specimens were excited at 430 nm (4 nm bandpass) and measured at 540 nm (4 nm bandpass). Protein was measured using a modified version of the bicinchoninic acid (Pierce Chem. Co., Rockford, IL, USA) method (Smith *et al.* 1985): for each sample 50 μl of lysate was combined with 1 ml of working reagent. The protein standard curve was made using bovine serum albumin. By this method, I determined that there are 180 μg protein per 10^6 BMM.

To determine whether pinocytosed LY was degraded to a non-fluorescent form, a pulse–chase experiment was performed in which both cells and chase medium were analyzed for fluorescence. Adherent BMM were incubated in 0·5 mg ml^{-1} LY for 2 h at 37°C, then were washed and reincubated in PBS (37°C) for 0, 1, 2, or 3 h. At the end of each chase period, PBS was removed and saved as 'medium', and 0·5 ml Triton X-100 (0·1 %) was added to each well. Fluorescence of the lysate

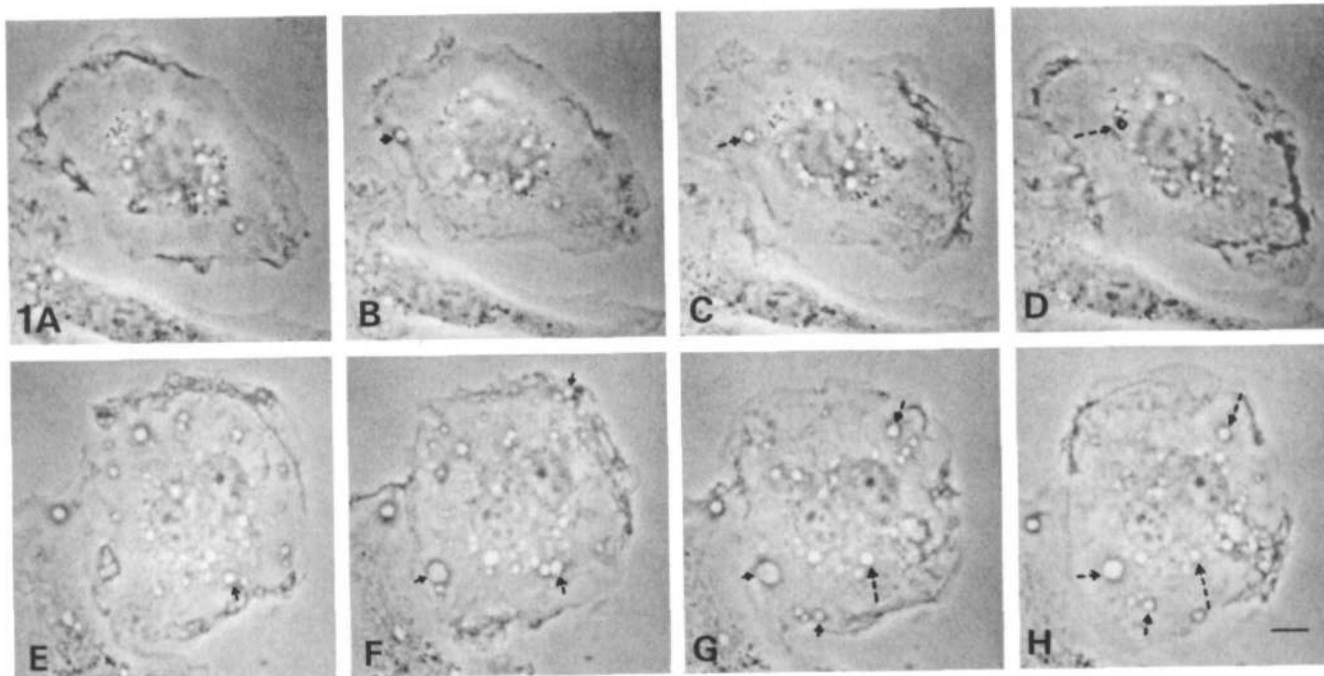

Fig. 1. Macropinocytosis in macrophages. Micrographs taken at 2 min intervals from a time-lapse video sequence. A–D. Unstimulated macrophages display relatively few phase-bright pinosomes (arrow), but do exhibit ruffling activity at the cell margins. E–H. Macrophages incubated in $60\,\mathrm{ng\,ml^{-1}}$ PMA for 60 min prior to observation. Phase-bright pinosomes are generated at the ruffling margin, then shrink or fuse with other pinosomes as they move toward the nucleus. Broken lines indicate the course of the pinosomes through the photographic series. Bar, 5 μm.

(termed 'dish-bound') and medium were measured, and the ng of LY per well calculated. The total fluorescence per well, medium plus dish-bound, provided a measure of how much LY fluorescence was lost from the system due to degradation.

Results

Morphology

When observed by time-lapse video microscopy, BMM exhibited ruffling along their upper and marginal surfaces (Fig. 1, A–D). These ruffles were relatively small, however, and seldom formed the large, phase-bright macropinosomes prominent in early films of pinocytosis (Lewis, 1931). After addition of $60\,\mathrm{ng\,ml^{-1}}$ PMA, there was a 5 to 10 min delay, then activity increased. The ruffles appeared as broad sheets of membrane, or lamellipodia, which sometimes flattened out against the coverglass in a spreading response, and at other times folded back toward the nucleus. These folding lamellipodia often resealed with the cell surface, enclosing extracellular medium in phase-bright vesicles. Typically they moved from the ruffling margin of the cell toward the nucleus, shrinking in size and sometimes fusing with other newly formed vesicles (Fig. 1, E–H). Piranhalysis, the nibbling of macropinosomes by small lysosomes (Willingham and Yamada, 1978), was not observed. Macropinocytosis continued for several hours of observation. Because macropinosomes sometimes formed out of the plane of focus, it was difficult to measure precisely by video microscopy the fluid volume entering *via* macropinocytosis. Rates of influx varied considerably from cell to cell, and the stimulation was greater at higher cell densities, a less photogenic condition. Nonetheless, it was clear from the time-lapse recordings that macropinosomes ranged from 0·5 to 5 μm in diameter, and formed at an approximate rate of one or two per minute.

To compare pinosome dimensions in the presence or absence of PMA, 70 fixed cells from each condition were photographed by phase microscopy, then the diameter of each phase-bright vesicle was measured and its volume calculated assuming its dimensions were spherical. As shown in Table 1, vesicles larger than 1 μm diameter occurred more frequently in PMA-stimulated BMM. The small increase in vesicle diameter distribution represents a large shift in the volume distribution, as the volume of a spherical vesicle increases with the cube of its radius. The phase-bright vesicles included newly formed

Table 1. *Macropinosomes in macrophages**

	No. of vesicles		Per cent of total		Volume per cent†	
Vesicle diameter	−	+	−	+	−	+
0·5–1 μm	534	910	85	70	22	7
1–2 μm	93	314	15	24	39	36
>2 μm	5	73	1	6	38	57
Total	632	1297				

* BMM were incubated 60 min with or without PMA, then were fixed and photographed by phase optics. Micrographs were enlarged to a final magnification of ×1800, and all phase-bright vesicles were counted and their diameters measured. −, control; +, PMA-treated.

† The volume of each vesicle was calculated assuming it was spherical.

macropinosomes and those which had shrunken since their formation. The images therefore represented the steady state dimensions of macropinosomes and their derivatives. From the data used to generate Table 1, I determined that control macrophages contained $1 \cdot 50 \pm 2 \cdot 50$ vesicles larger than $1\,\mu m$ diameter per cell (n = 72), and that PMA-stimulated macrophages contained $5 \cdot 68 \pm 5 \cdot 77$ large vesicles per cell (n = 69). This difference was significant ($P < 0 \cdot 001$, two-sampled Student's t-test for independent samples with unequal variance).

To detect micropinosomes, cells were preincubated for 60 min in the presence or absence of PMA, then were exposed to $1 \cdot 5\,mg\,ml^{-1}$ LY in PBS–10F ± PMA, for 60 s. After a rapid wash and fixation, the coverslip preparations were observed by fluorescence microscopy. In both PMA-treated and untreated BMM, the cell periphery was labeled with many punctate, fluorescent spots (Fig. 2). These micropinosomes were too small for resolution by phase optics (see Osborn *et al.* 1978). Labeled macropinosomes could be identified in both conditions, but were more abundant among those cells stimulated with PMA (Fig. 2 C,D). Not every cell contained a labeled macropinosome, indicating either that only a subpopulation of cells exhibited macropinocytosis, or that the process was slower than micropinocytosis, and therefore during a 60 s pulse would not have labeled macropinosomes in all of the cells. The time-lapse video microscopy indicated the latter.

Quantitation of LY pinocytosis

The rate of LY influx and accumulation were measured in time-course experiments. During the first 5 min of exposure to LY, BMM accumulated 87 ng LY per mg protein, which, if taken as an estimate of the fluid influx rate, translates into $6 \cdot 3$ fl per cell per min (Fig. 3, inset; $0 \cdot 5\,mg\,ml^{-1}$ LY, $0 \cdot 18$ mg protein per 10^6 BMM). Within

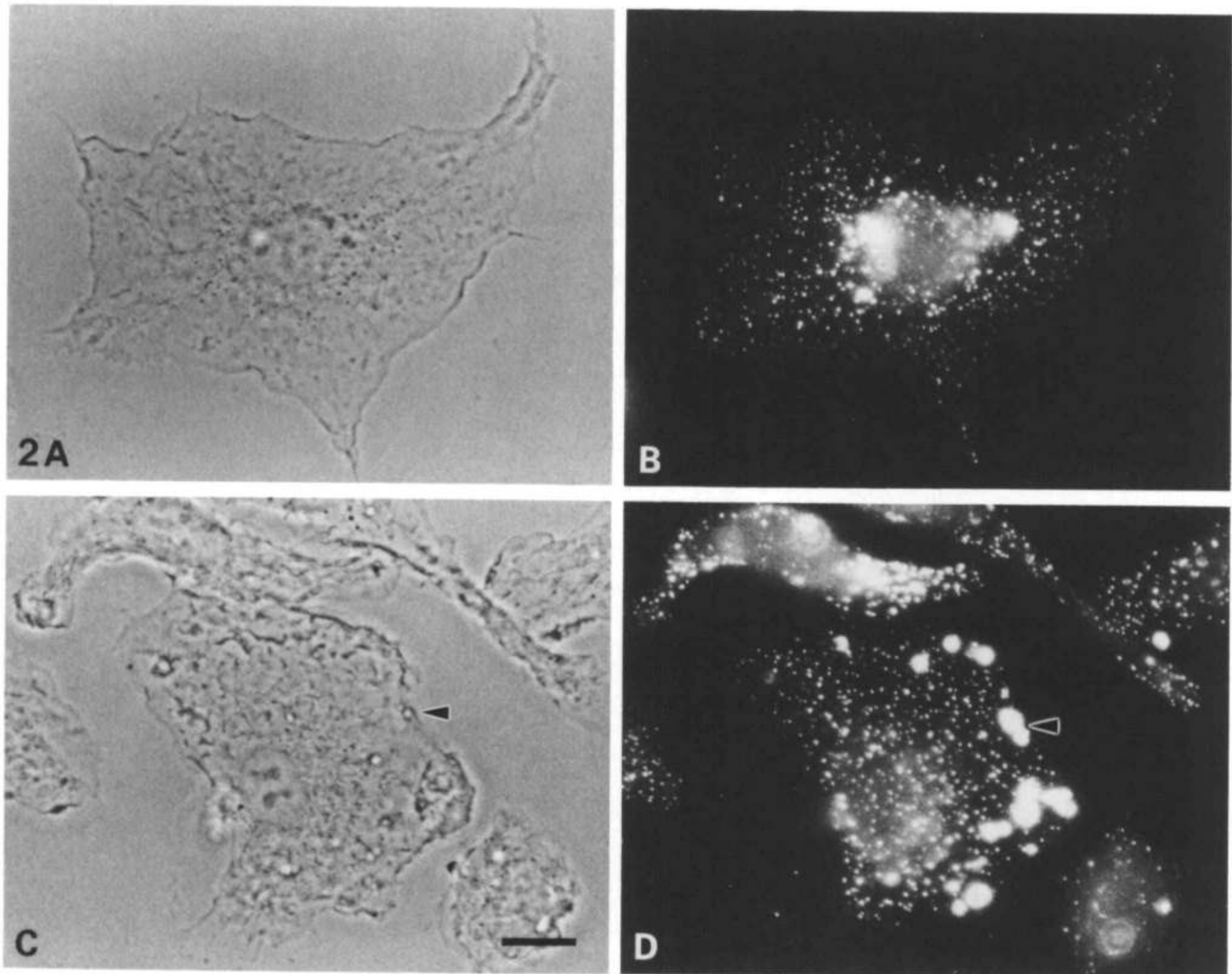

Fig. 2. LY-labeled pinosomes in stimulated and unstimulated macrophages. Macrophages were incubated in $1 \cdot 5\,mg\,ml^{-1}$ LY in PBS–10F for 1 min, following a 1 h preincubation in PBS–10F with (C,D) or without (A,B) $60\,ng\,ml^{-1}$ PMA. Cells were fixed before microscopic observation. Both treated and untreated cells display punctate fluorescence which does not correspond to any vesicular structures visible by phase optics. The PMA-treated cell contains phase bright macropinosomes labeled during the brief pulse with LY (arrows). Unlabeled phase-bright vesicles presumably represent pinosomes formed prior to the LY pulse. A and C are phase micrographs, and B and D are fluorescence micrographs of the corresponding cells. Bar, $5\,\mu m$.

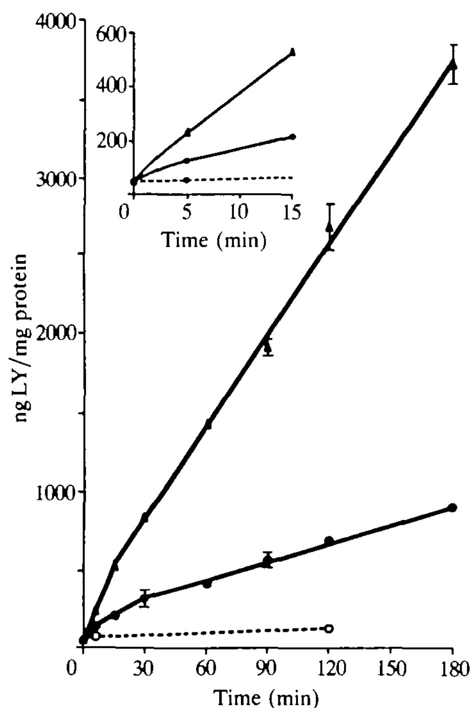

Fig. 3. Accumulation of LY by unstimulated macrophages (●——●); control and by macrophages in 30 ng ml^{-1} PMA (▲——▲) both at 37°C. 3×10^5 cells per sample were preincubated 60 min in PBS–10F ± 30 ng ml^{-1} PMA, then for the times indicated in 0·5 mg ml^{-1} LY ± PMA. At the end of the incubation, cells were washed in cold PD, lysed in Triton X-100 and the fluorescence and protein content of that lysate measured. Macrophages incubated at 4°C showed little accumulation of LY (-----). The inset shows the early time points with the scale expanded. Points represent mean ± S.D. (n = 3). Similar results were obtained on three occasions.

the first 30 min of exposure to LY, the accumulation rate slowed to 3·8 ng LY per mg protein per min, which was only 22 % of the influx rate. This lower rate was maintained for several hours (Fig. 3, and data not shown). A high initial rate of accumulation slowing to a lower sustained rate was similar to what was observed in thioglycolate-elicited mouse peritoneal macrophages (thio-macrophages), in which it was found that the slower rate reflected the net accumulation due to influx and efflux of LY (Swanson *et al.* 1985). Cells maintained at 4°C exhibited little accumulation of LY.

PMA stimulated both the influx and the net accumulation of LY. Taking the 5 min accumulation rate as an estimate of influx rate, the stimulation of fluid influx by PMA was 2·1 times greater than the control rate (184 ng LY per mg protein in 5 min, or 36·8 ng LY per mg protein per min or 13·2 fl per cell per min) representing an increased influx of 6·9 fl per cell per min (Fig. 3). This corresponds to one vesicle of 2·36 μm diameter formed per cell per min, a value which is consistent with the stimulation observed by time-lapse microscopy. The initially high rate of accumulation slowed to a linear rate of accumulation, which was 53 % of the influx rate (19·5 ng LY per mg protein per min). This finding was in agreement with earlier studies (Swanson *et al.* 1985), in which the long-term linear rate of accumulation in PMA-

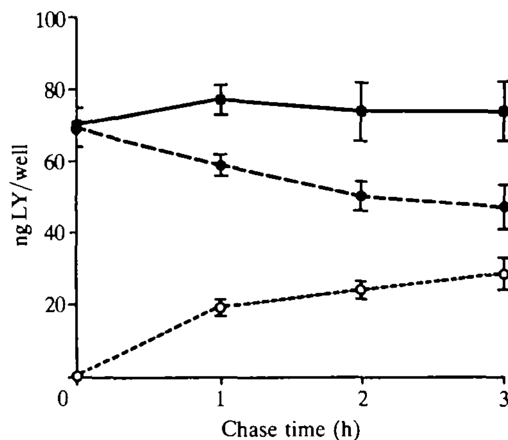

Fig. 4. LY pinocytosed by macrophages is not degraded to some non-fluorescent form. BMM were incubated for 2 h in 0·5 mg ml^{-1} LY, then were washed and reincubated in PBS for the indicated times. At the end of this chase period, PBS was removed for measurement of fluorescence, then cells were lysed in Triton X-100. Fluorescence of the lysate (---) and fluorescence of the medium (-----) when added yield the total fluorescence of the system (■——■). Points represent the mean and S.D. (n = 6), pooling data from two separate experiments.

stimulated cells was a higher percentage of the influx rate relative to unstimulated cells, indicating that more of the LY entering by pinocytosis was being retained within the macrophages.

To assure that the measurements were not altered by LY degradation within lysosomes, the fluorescence of LY loaded into cells by pinocytosis was followed through a chase period. BMM loaded with LY by pinocytosis were washed free of dye and incubated for up to 3 h in warm PBS. That PBS was collected, then the cells were lysed in Triton X-100. Figure 4 shows that LY fluorescence accumulated in the medium and was lost from the cells (dish-bound), but the total amount of fluorescence in each well remained unchanged. Thus, during the 3 h chase period, LY was not degraded to some non-fluorescent form.

To report the pinocytosis of fluid solutes, a probe molecule should accumulate in cells at a rate proportional to its concentration in the extracellular medium. If there is efflux of fluid solutes from the cell *via* vesicular transport, then loss of the reporter molecule pinocytosed by cells should be inhibited at low temperatures and should at 37°C be proportional to the concentration of LY loaded into the cell. These conditions held for LY, as shown in Fig. 5A. Accumulation of LY was temperature-dependent, and was proportional to the concentration of LY in the medium. If, after pinocytosis of LY, cells were washed and reincubated in LY-free PBS–10F, there was a temperature-dependent egress of LY from the cells. Moreover, after 60 min of chase, the amount of LY remaining inside the cells was proportional to the concentration of LY initially provided during pinocytosis. Thus, the loss of LY from BMM appeared to represent bulk flow out of the cell, and was most likely a vesicle-mediated efflux. Figure 5B shows that PMA-stimulated

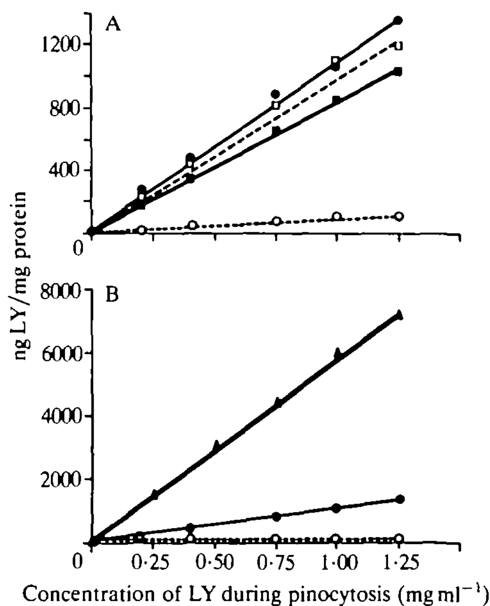

Fig. 5. Accumulation and efflux of LY are proportional to the concentration of LY in the extracellular medium. (A) Macrophages were incubated in the indicated concentrations of LY for 2 h at 37 °C or 4 °C. They were then either washed and lysed, or washed and reincubated in unlabeled medium for 1 h. ●——●, 37 °C. □——□, first incubation 37 °C, second incubation 4 °C. ■——■, first and second incubations, 37 °C. ○——○, 4 °C. (B) Stimulated accumulation of LY by PMA is also proportional to LY concentration outside the cell. Fluorescence values represent the cell-associated LY fluorescence. Values are the mean of three points; in all but two of the points shown the standard deviation was smaller than the symbol used to mark the point. Similar results were obtained on three occasions. ▲——▲, with PMA at 37 °C. ●——●, control at 37 °C. ○——○, 4 °C.

accumulation was also proportional to the concentration of LY provided in the medium.

In earlier work using thio-macrophages, we found that in PMA less of the LY pinocytosed by the cells returned *via* efflux to the extracellular medium (Swanson *et al.* 1985). Loss of LY from cells exhibited an exponential decline, represented best as the emptying of two intracellular compartments (fast and slow; see Besterman *et al.* 1981). Although rates of efflux could be estimated, they were difficult to measure precisely because (1) the initial rate of efflux was high, (2) the cells were changing temperature, warming from 4 °C to 37 °C during the earliest and most critical minutes of efflux, and (3) the size of the compartments that were emptying was indeterminable due to concentrative properties of the lysosomes. I therefore chose not to attempt measurement of efflux rates from BMM.

The kinetics of accumulation already indicated that PMA was increasing the proportion of internalized LY remaining inside the cell: steady state accumulation was 53 % of the influx rate in PMA-treated cells and 22 % in control cells (Fig. 3). To measure exocytosis of LY from BMM, cells were allowed to pinocytose LY for 1 h at 37 °C (or 4 °C), with or without PMA. These LY-loaded

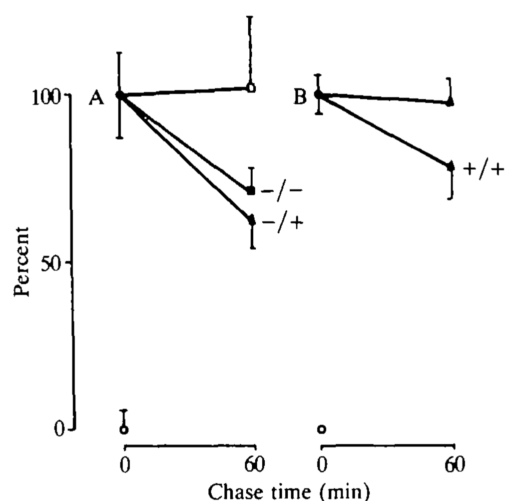

Fig. 6. PMA stimulates efflux yet increases retention of pinocytosed LY in macrophages. A. Macrophages were incubated in 0·5 mg ml^{-1} LY without PMA for 1 h at 4 °C (○) or at 37 °C (●, □, ■, ▲). They were then either washed and lysed (○, ●), or washed and reincubated in unlabeled medium for 1 h at 4 °C (□) or 37 °C with (▲, −/+) or without (■, −/−) 60 ng ml^{-1} PMA, before final washing and lysis. B. Macrophages were incubated in 0·5 mg ml^{-1} LY plus 60 ng ml^{-1} PMA for 1 h at 4 °C (○) or 37 °C (●, △, ▲). They were then washed and lysed (○, ●) or washed and reincubated at 4 °C (△) or at 37 °C with PMA (▲, +/+). The amount remaining in the cells is shown as the percent of that accumulated at 37 °C without subsequent chase. Shown are the mean and S.D. from the pooled data of four experiments ($n = 23$ or 24).

cells were then washed and reincubated for 1 h in LY-free medium, at 37 °C or 4 °C, with or without PMA. Temperature-dependent accumulation and loss were then calculated for both stimulated and unstimulated cells. Figure 6 shows the percentage of internalized LY that returned to the medium during a one-hour chase. Cells loaded in the absence of PMA lost 30 % of their LY during chase (Fig. 6A, −/−). Addition of PMA during the chase period increased LY efflux to 38 % (Fig. 6A, −/+; $P < 0.01$). If PMA was included during the pinocytosis of LY (Fig. 6B), cells accumulated four to six times more LY, which is not evident in the figure, but returned a smaller percentage of that LY during the 1 h chase in PMA (+/+, 22 %; $P < 0.01$ comparing −/− with +/+). This indicates that although efflux was increased by PMA, influx increased more, and consequently more internalized LY was retained within the macrophage. As discussed below, this may be due to differences in the sizes of vesicles entering and exiting the endocytic compartment.

Discussion

These experiments demonstrate that PMA elicits sustained macropinocytosis in BMM. Microscopic inspection provided the clearest evidence of this. The fact that the pinocytic response to PMA continued for several hours permitted quantitative analysis of solute move-

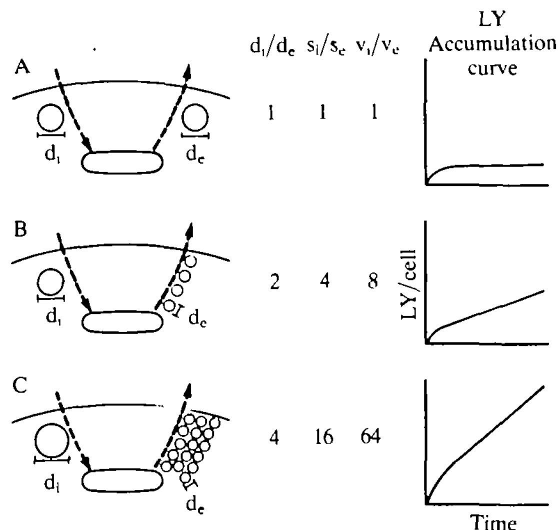

Fig. 7. The size ratio of incoming and outgoing vesicles may regulate solute accumulation and membrane flow through the endocytic compartment. d_i indicates the diameter of incoming vesicles (pinosomes); d_e indicates the diameter of recycling vesicles. Increasing d_i/d_e increases differentially s_i/s_e and v_i/v_e. See accompanying text for details.

ments into and out of the cells. Analysis of pinocytosis using LY as a probe for fluid solute movement showed that PMA increased net solute movement through the endocytic compartment.

Increased solute retention in stimulated macrophages could result by increasing pinosome size or the rate of pinosome formation, by decreasing efflux, or by some combination of these. The kinetic measurements reported here indicate that PMA increases both influx and efflux, with influx increasing more. This could occur transiently by simply increasing the rate of pinosome formation, with a lesser increase in the rate of efflux. However, such a change could not be maintained for long without net redistribution of plasma membrane into the cell and some consequent change in cell shape.

Enlarged pinosomes could increase solute retention

The observations that macropinocytosis and efflux are increased during stimulation suggest a mechanism that would permit a sustained change in the dynamics of solute flow. I suggest that pinosomes, the influx vesicles, are in general larger than recycling vesicles. This idea has been presented previously (van Deurs and Christensen, 1984), and there is some evidence to support it: coated vesicles are generally 100 nm in diameter (Steinman *et al.* 1976), macropinosomes are larger, and the reported dimension of recycling vesicles is less than 100 nm (van Deurs and Nilausen, 1982). If a macrophage is to pinocytose constitutively without rounding up, it must return to its surface as much membrane as it has internalized.

Figure 7 shows how the relative diameters of pinosomes and recycling vesicles could control the rate of solute accumulation. The average pinosome diameter is termed d_i, and the average recycling vesicle diameter d_e. Assuming the vesicles are spherical, their membrane areas, s_i and s_e, can be represented as the surface areas of spheres, which increase with the square of the diameter. Similarly, their volumes and their fluid contents are given as v_i and v_e, and will increase with the cube of the vesicle diameter. If one considers first a cell with equally sized influx and efflux vesicles (Fig. 7A), the ratios d_i/d_e, s_i/s_e, and v_i/v_e all equal unity. The rate of LY accumulation by such a cell would initially be high, as the endocytic compartment filled with LY from outside the cell. Eventually, however, a condition of zero net flux would be attained, with efflux balancing influx, and the LY accumulation rate equal to zero. Such accumulation kinetics have been observed in *Entamoeba histolytica* (Aley *et al.* 1984) and in lymphoid cells (Goldmacher *et al.* 1986).

Increasing the ratio d_i/d_e to 2 (Fig. 7B) would increase s_i/s_e to 4 and v_i/v_e to 8. To maintain a constant distribution of membrane between the cell surface and the endocytic compartment, four recycling vesicles must return for each incoming vesicle. However, these four vesicles will return only half of the incoming volume. The remaining 50% must be compensated for by water and solute flux across the membrane into the cytosol or by solute concentration (e.g. LY accumulation) within the compartment. If this differential, $d_i > d_e$, were maintained constitutively, the rate of water and solute flux across endocytic membranes could be held constant. This could be achieved by restricting pinosomes to a given size range using, perhaps, clathrin-coated vesicles, and similarly holding efflux vesicles to some constant, smaller size.

Increasing d_i/d_e to 4, as might occur by stimulating macropinocytosis, would increase s_i/s_e to 16 and v_i/v_e to 64 (Fig. 7C). Balancing the distribution of membrane between the endocytic compartment and the cell surface would now require 16 recycling vesicles per incoming pinosome, and would increase greatly the difference in volume flow. If 16 vesicles were recycling per pinosome, the volume difference would be 48 (64 minus 16), which implies that more water must flow across endosomal membranes into cytoplasm, with greater retention of LY in the endocytic compartment. The increase in the number of efflux vesicles would explain why PMA stimulates LY exocytosis from cells loaded in the absence of PMA (Fig. 6A): increased recycling resulting from larger pinosomes 'flushes out' more LY from the previously loaded endocytic compartment. However, the increased exocytosis is not sufficient to recycle the even greater increase in volume of fluid and solute entering *via* macropinosomes, and cells loaded with LY in the presence of PMA will have a higher *rate* of efflux, but will return a smaller percentage of what they internalize. Thus, increased pinosome diameter could increase solute influx, efflux and retention simultaneously.

Although PMA has been shown to elicit a net increase in cell surface area (Phaire-Washington *et al.* 1980), it is still reasonable to propose that, at steady state, membrane influx equals membrane efflux. Most of the shape changes and membrane redistributions occur within the first hour of PMA stimulation, yet the enhanced rates of LY accumulation can be observed for many hours after

addition of PMA. Therefore, after the first hour in PMA, steady state accumulation of LY should occur without gross redistribution of membrane.

One puzzling aspect of the kinetics remains, and that is why the LY accumulation curves become linear during the first hour and remain so for many hours. In theory, concentrating LY in endocytic compartments should continually raise its concentration in the efflux vesicles, and should consequently increase its rate of efflux indefinitely; that is, accumulation should always be curvilinear. Linear accumulation kinetics indicate that at some point, independent of LY concentration outside the cell, both influx and efflux are constant terms. One possible explanation is that solutes such as LY are sequestered into a compartment that does not recycle its contents. Such a compartment was proposed by Besterman *et al.* (1981). How does one fit a non-participating organelle into a scheme of balanced membrane flow into and out of the cell? Here again morphology offers a clue. Cohn and Benson (1965) observed, and I confirm that observation here, that after their formation, macropinosomes gradually shrink, ultimately either vanishing or condensing into a dense, lysosomal granule. Pinosomes, and the endosomes and lysosomes they fuse with, may recycle membrane and solutes at a progressively decreasing rate. LY is trapped in these maturing pinosomes, and more is trapped in those that start out larger (macropinosomes). Repeated cycles of this could allow the non-degradable probe to accumulate indefinitely.

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