# Phorbol Myristate Acetate Stimulates Pinocytosis and Membrane Spreading in Mouse Peritoneal Macrophages

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ABSTRACT Phorbol myristate acetate (PMA) at a concentration of 0.01 μg/ml causes an approximately threefold increase in surface area of resident, proteose-peptone-elicited, and thioglycolate-broth-elicited mouse peritoneal macrophages. Resident and proteose-peptone-elicited macrophages, cultured for 24 h in the presence of PMA, increase their pinocytic rate twofold in response to addition of PMA (0.01 μg/ml) to the medium. Thioglycolate-broth-elicited macrophages, cultured for 24 h in the absence of PMA, immediately increase their pinocytic rate 2- to 3.5-fold in response to a single challenge with PMA (0.01 μg/ml). Cytochalasin B, colchicine, and podophyllotoxin have only modest inhibitory effects on the basal rate of pinocytosis and on PMA-induced cellular spreading, but completely block the stimulatory effects of PMA on pinocytosis in thioglycolate-broth-elicited macrophages. Cytochalasin D markedly inhibits both basal and PMA-stimulated pinocytosis in these cells. Thus, PMA is a useful tool for studying mechanisms of macrophage spreading and for enhancing the overall rate of pinosome formation.

Pinocytosis is a ubiquitous cellular process that plays an important role in the uptake and transport of macromolecules into and through cells (1), and in the turnover and recirculation of plasma membrane constituents (2–4). We recognize two types of pinocytosis: fluid-phase pinocytosis, the bulk uptake from the medium of water and dissolved solutes; and adsorptive pinocytosis, the uptake of molecules that are bound to the cell surface. Sucrose, inulin, and horseradish peroxidase (5) are examples of molecules interiorized by fluid-phase pinocytosis. Concanavalin A (6), antigen-antibody complexes (7), protein hormones (8), low density lipoproteins (9), and some animal viruses (10) are endocytosed by adsorptive pinocytosis, and consequently are removed from the medium at rates several orders of magnitude faster than are fluid-phase markers.

Both fluid-phase and adsorptive pinocytosis are temperature sensitive processes (11) that are fueled by cellular metabolism (1, 12) and require membrane movement and fusion. Cytochalasin B slows pinocytosis in Chang liver cells (13), but has no inhibitory effect on this process in mouse macrophages (14, 15).

Several investigators have studied the factors that regulate fluid phase pinocytosis, using horseradish peroxidase to quantitate the rate of solvent uptake. Edelson et al., (16) reported that macrophages elicited by the intraperitoneal injection of proteose-peptone or thioglycolate broth have higher rates of fluid phase pinocytosis than resident macrophages, and Steinman et al. (12) showed that mouse fibroblasts pinocytose more rapidly under confluent than under sparse culture conditions. These studies indicate that a cell's pinocytic rate is determined both by its developmental history and by its culture conditions. However, there is little information about factors that stimulate fluid-phase pinocytosis, with the exceptions of the reports of Edelson and Cohn (6) that concanavalin A stimulates pinocytosis in resident mouse macrophages, and of Haigler et al. (17) that epidermal growth factor promotes pinocytosis in carcinoma A-431 cells. Compounds that enhance membrane internalization would be useful to study membrane recycling and turnover and to examine the cellular factors that regulate pinocytosis. The studies reported here were begun when we noted that the tumor promotor, phorbol myristate acetate



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(PMA), stimulates fluid-phase pinocytosis in mouse peritoneal macrophages. A preliminary account of these experiments has been published as an abstract (18).

## MATERIALS AND METHODS

### Cell Culture Medium

Macrophages were cultured in Eagle's minimal essential medium with Earle's salt solution containing 5.5 mM glucose (MEM) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 20% heat-decomplemented (56°C, 30 min) fetal bovine serum (FBS). The complete medium is referred to as MEM-FBS. Phosphate-buffered saline (PBS) was prepared as described (19). PD is PBS without $Ca^{++}$ and $Mg^{++}$.

### Macrophage Cultures

Mouse macrophages were harvested and cultured as described by Michl et al. (19). Thioglycoate-medium-elicited or proteose-peptone-elicited macrophages were harvested from mice injected intraperitoneally 4 d previously with 1.5 ml of 4% Brewer's thioglycolate medium (Difco Laboratories, Detroit, Mich.) or 10% proteose-peptone (Difco).

For studies involving the determination of pinocytic rates, 1-ml aliquots of peritoneal cells, at a concentration of $3 \times 10^6$/ml in MEM-FBS, were plated in each 16-mm well of Costar culture plates (Costar, Data Packaging, Cambridge, Mass.). Cells were allowed to adhere to the wells for 3–4 h at 37°C in a 5% $CO_2$/95% air atmosphere. The wells were then washed vigorously two to three times with 1 ml of MEM and further incubated at 37°C in 1 ml of MEM-FBS for 24–48 h. For phase-contrast microscopy studies, $1.5–2 \times 10^6$ peritoneal cells were plated in 35-mm tissue culture petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing .5 inch diameter glass coverslips (Gold Seal coverglass, Scientific Supply Co., Linden, N. J.). After 3-4 h, cells were washed two times with MEM to remove nonadherent cells and incubated at 37°C in 2 ml of MEM-FBS for 24–48 h.

### Determination of Pinocytic Rates

The rates of pinocytosis of macrophages in monolayer cultures were determined by measuring the uptake of horseradish peroxidase from the culture medium by a slight modification of the method of Steinman and Cohn (5).

Horseradish peroxidase (HRP) Type II (Sigma Chemical Co., St. Louis, Mo.) was dissolved in MEM at a concentration of 10 mg/ml. 0.1 ml of this HRP stock solution was added to macrophages in 0.9 ml of MEM-FBS in Costar tissue culture plates. PMA was added as indicated to some of the cultures 2 h before the addition of HRP, and pinocytosis of HRP was allowed to proceed for 90 min at 37°C in a 5% $CO_2$/95% air humidified atmosphere. The cells were washed six times with 1 ml of MEM containing 0.1% BSA (MEM-BSA), and the entire Costar plate was then immersed five times in 800 ml of phosphate-buffered saline, pH 7.4 (PBS). The cells were lysed by adding 1 ml of an 0.05% solution of Triton X-100 to each well and incubating the plates for 15 min at room temperature. Cell lysates were diluted 1:10 in PD containing 1% heat-decomplemented FBS. The quantity of HRP in cell lysates was assayed spectrophotometrically at 460 nm, using *o*-dianisidine (Sigma) and 0.3% $H_2O_2$ as substrates.

To be certain that extensively spread cells (PMA treated) and less spread cells (untreated controls) were not lost from the dish surface at different rates during the washing procedure, the amount of cell protein remaining in the wells of the Costar culture dishes was measured by use of two different methods. In the first method, the cells were incubated for 2–4 h in MEM-FBS. Plates were washed as described in the pinocytosis assay and PMA (0.01 μg/ml) was added to some of the cultures. 1 ml of 0.1 N NaOH was added to each Costar well to dissolve the cells. The protein content of this solution was measured by the method of Lowry et al. (20). In the second method, the cells were labeled for ~12 h with [$^3$H]leucine (5 μCi/ml) in MEM-FBS, washed, and further incubated for 12 h in MEM-FBS. As before, some of the cultures were treated with PMA for 2 h and washed, and the cells were released from the Costar wells by scraping with a rubber policeman into 1 ml of 10% TCA. 0.1 ml of a 1 mg/ml solution of BSA was added as carrier and the precipitated protein was collected on Millipore filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filters were dried and counted in Liquefluor toluene in a Nuclear Chicago Scintillation spectrometer. Using both methods, an equivalent amount of cell protein or TCA-precipitated radioactivity was recovered in control and PMA-treated cultures (data not shown). Consequently, all results in this report reflect nanograms of peroxidase pinocytosed in each 16-mm culture well containing $1–2 \times 10^6$ cells. The values reported represent the average peroxidase uptake of three separately processed samples.

### Phagocytosis Assay

Phagocytosis of IgG or IgM and complement-coated sheep erythrocytes was assayed as described by Griffin et al. (21). In studies using cytochalasin D, macrophages were incubated with this drug at a concentration of $10^{-5}$ M in MEM for 30 min at 37°C. 0.1 ml of a 1% suspension of IgG or IgM and complement-coated erythrocytes was then added and the macrophages were further incubated at 37°C for an additional 60 min.

### Determination of Surface Area Covered by Spread Cells

To obtain quantitative data on the extent of spreading of cells treated with various drugs, representative photographs of macrophages on coverslips were enlarged, cell outlines were traced, and the tracings were cut out and weighed. A standard graph relating paper weight to surface area was used to calculate the surface area covered by spread cells. At least 20 cell profiles were measured for each sample.

### Chemicals

All chemicals used were reagent grade or the best grade available. Vinblastine sulfate and Vincristine sulfate were obtained from Lilly Chemical Co., Indian-

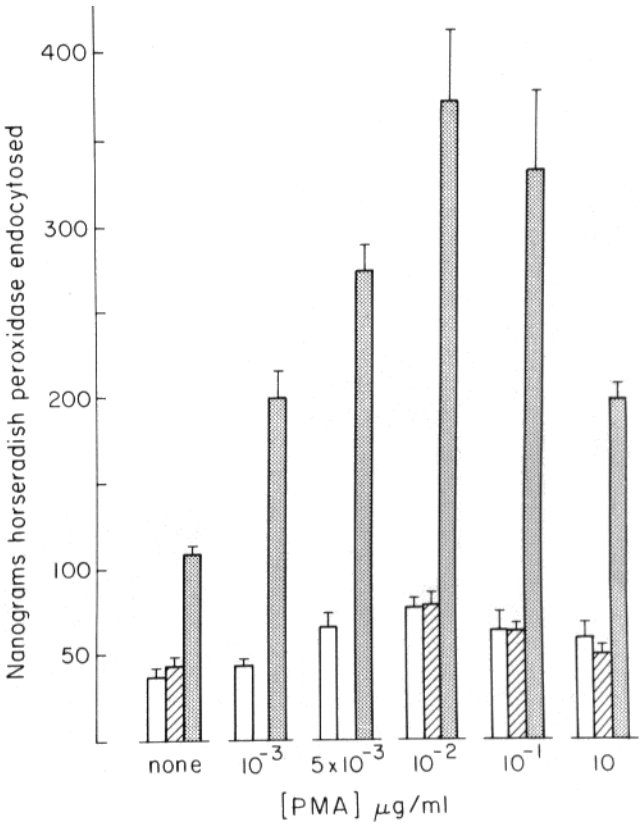


FIGURE 1 Effects of PMA on pinocytosis by resident, proteose-peptone and thioglycolate broth-elicited macrophages. Macrophages were plated in Costar dishes as described in Materials and Methods. 48-h Cultures of resident macrophages (open bars) or proteose-peptone macrophages (striped bars) were incubated with PMA in MEM 20% FBS as indicated for 24 h at 37°C. The cultures were washed, and then incubated with PMA at the same concentration in fresh medium for 2 h at 37°C. Horeseradish peroxidase (final concentration, 1 mg/ml) was then added, and the cells were incubated for an additional 90 min at 37°C and then washed and assayed for peroxidase as described in Materials and Methods. 24-h Cultures of thioglycolate macrophages were treated with PMA at the concentrations indicated for 2 h at 37°C and incubated with HRP for 90 min at 37°C to measure pinocytic rates as described above.

apolis, Ind., and cytochalasin B and cytochalasin D, from Sigma. Podophyllotoxin and VP-16-213 were gifts from Dr. John Loike of The Rockefeller University. PMA (Consolidated Midland Corp., Brewster, N. Y.) (10 mg/ml in dimethylsulfoxide [DMSO]) was stored at −70°C in the dark. To prevent repeated thawing, the PMA stock solution was stored in 25-μl aliquots that were thawed and diluted immediately before use in MEM-BSA.

## RESULTS

### PMA Stimulates Pinocytosis

PMA stimulates pinocytosis of HRP by resident, proteose-peptone-elicited, and thioglycolate-elicited macrophages in a dose-dependent fashion. The concentration of PMA required to promote maximal stimulation of HRP uptake was 0.01 μg/ml for all three macrophage types. Thioglycolate macrophages have the highest basal pinocytic rate of the three macrophage populations examined (reference 16 and Fig. 1), and exhibit the greatest stimulation of HRP uptake (3.7-fold) after PMA treatment. PMA induces a twofold stimulation of pinocytosis in resident and proteose-peptone macrophages (Fig. 1). Resident macrophages, however, require a 26-h incubation in PMA-containing medium before a significant stimulation in their pinocytic rate was evident. In contrast, pinocytosis in thioglycolate macrophages was stimulated within 30 min of addition of PMA to the medium. Consequently, thioglycolate macrophages were used for most of the experiments reported here.

Control experiments showed that PMA did not induce binding of HRP to the macrophage surface; thioglycolate macrophages treated with PMA in MEM-FBS for 2 h showed the same background binding after incubation with HRP (1 mg/ml, 30 min, 4°C) as parallel cultures without PMA treatment. Moreover, neither phorbol alcohol nor DMSO stimulated pinocytosis in thioglycolate macrophages (data not shown).

Mouse macrophages contain no detectable endogenous peroxidase. In the absence of added HRP, PMA did not induce peroxidatic activity in resident or thioglycolate macrophages.

It was possible that the increased HRP found in PMA-treated cells reflected decreased HRP degradation and not increased uptake. To examine this possibility, thioglycolate macrophages were incubated with HRP in the presence or absence of PMA (0.01 μg/ml) for 90 min. The cultures were then washed and incubated in MEM-FBS for an additional 24 h. As expected, the PMA-treated cells pinocytosed 3.3-fold as much HRP as the control cells. Nevertheless, the half-life of HRP activity in both cultures was 7–8 h (Fig. 2, open symbols), a value consistent with that reported by Steinman and Cohn (5). Thus the enhancement of HRP activity in PMA treated macrophages is not an artifact of slowed intracellular degradation of this enzyme.

### PMA Does Not Promote Secretion of Endocytosed HRP

PMA stimulates plasminogen activator secretion by polymorphonuclear leukocytes (22), macrophages (23), and HeLa cells (24), and the release of specific granules by polymorphonuclear leukocytes (25). The experiment described above suggests that PMA does not promote secretion or enhance the rate of degradation of pinocytosed HRP. To examine this question under conditions where both control and PMA-treated cells contained equivalent amounts of HRP, thioglycolate macrophages were allowed to pinocytose HRP for 2 h at 37°C, washed, and further incubated in MEM-FBS at 37°C. PMA was added to one set of cultures after the period of HRP uptake. At the end of the 2-h HRP pulse, all cells contained the same amount of peroxidase. Cell-associated peroxidase activity at 4 and 8 h decreased at equivalent rates in control and PMA-treated macrophages (Fig. 2, solid symbols).

Steinman et al (26) showed that HRP is concentrated in secondary lysosomes within 5 min after its uptake from the medium. Thus, virtually all of the HRP pinocytosed in a 2-h period is contained in this cellular compartment. Failure of PMA to reduce cellular HRP content indicates that this compound does not markedly stimulate secretion of HRP from the lysosomal compartment during the period examined. However, we cannot exclude the possibility that PMA causes a small (<10% of total cellular HRP content) increase in HRP release from the macrophages.

### Effects of Colchicine, Podophyllotoxin, and Cytochalasins B and D on PMA-stimulated Pinocytosis

Colchicine at a concentration of $10^{-5}$ M inhibited basal HRP uptake by ~30% (Fig. 3), whereas $10^{-5}$ M colchicine had no inhibitory effect on this process (data not shown). However, PMA-stimulated HRP uptake was inhibited by colchicine at both these concentrations. In the presence of PMA and colchicine, pinocytosis continued at basal rate. The inhibition of the PMA effect was not reversed by removal of colchicine from

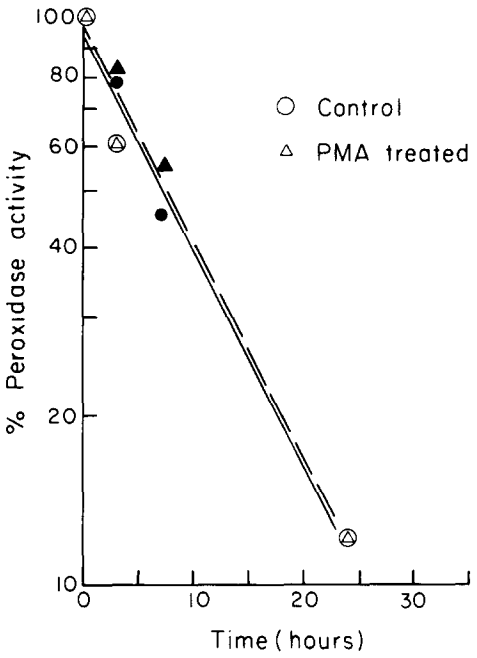


FIGURE 2 PMA does not alter the rate of degradation of horseradish peroxidase by thioglycolate macrophages. Macrophages plated 24 h previously in Costar dishes were incubated in HRP-containing medium as described in the text. The cultures were washed, incubated at 37°C in fresh medium for the indicated time periods, and assayed for their content of HRP as described in Materials and Methods. Results are plotted as percent of macrophage HRP content at the end of the HRP-uptake period. Open symbols: cells incubated in HRP-containing medium for 90 min in the presence (△) or absence (○) of PMA (0.01 μg/ml) before washing. Closed symbols: cells incubated in HRP-containing medium for 2 h, then washed and further incubated in medium in the presence (▲) or absence (●) of PMA (0.01 μg/ml). Dotted line: PMA. Solid line: control.

the medium (data not shown). The effects of vinblastine ($10^{-5}$ M) and vincristine ($10^{-5}$ M) on basal level and PMA-stimulated pinocytosis were similar to those described for colchicine (data not given).

Colchicine inhibits nucleoside transport in mammalian cells (27). To be certain that the inhibitory effect of colchicine on PMA-stimulated pinocytosis was not related to inhibition of PMA transport, macrophages were first incubated with PMA (0.01 μg/ml) and then treated with colchicine ($10^{-5}$ M). PMA stimulation of HRP uptake was abolished under these conditions as well (data not shown).

To confirm that the inhibitory effect of colchicine on PMA-stimulated pinocytosis was related to the effect of colchicine on microtubule function, we used podophyllotoxin and its congener VP-16-213. VP-16-213 shares the nucleoside transport inhibitory properties of podophyllotoxin but differs from the parent compound in that it lacks tubulin binding activity (28). Cells were treated sequentially with PMA, podophyllotoxin or VP-16-213, and HRP, and the amount of HRP pinocytosed was quantitated. Podophyllotoxin ($10^{-5}$ M) was as effective as colchicine in inhibiting PMA-stimulated HRP uptake. However, VP-16-213 had no inhibitory effect on basal or PMA-stimulated pinocytosis (Table I). Thus the effects of colchicine and podophyllotoxin on nucleoside transport appear unrelated to their effects on PMA-stimulated pinocytosis.

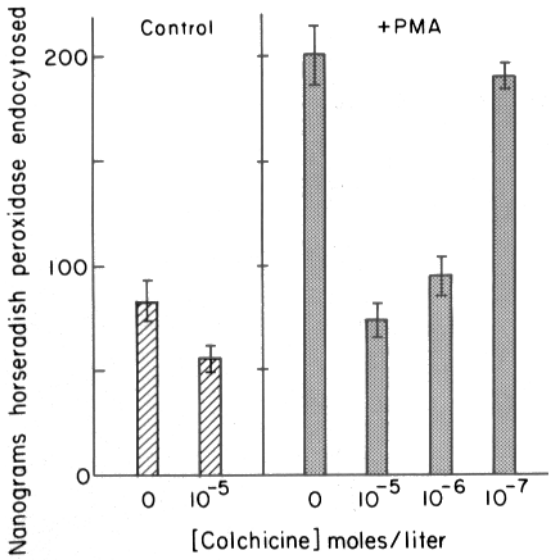


FIGURE 3 Colchicine inhibits PMA-stimulated pinocytosis. Thioglycolate macrophages, cultured for 24 h in Costar wells, were treated with PMA (0.01 μg/ml), and/or colchicine in HRP-containing medium for 90 min at 37°C as indicated, washed, and assayed for HRP content.

TABLE I

*Effects of Podophyllotoxin and VP-16-213 on PMA-stimulated Pinocytosis* *

| Macrophage treatment | Peroxidase pinocytosed | % Control |
|---|---|---|
| | *ng per culture* | |
| None | 80 ± 10 | 100 |
| Podophyllotoxin ($10^{-5}$ M) | 63 ± 33 | 79 |
| VP-16-213 ($10^{-5}$ M) | 82 ± 10 | 98 |
| PMA | 222 ± 4 | 278 |
| PMA + podophyllotoxin ($10^{-5}$ M) | 82 ± 4 | 98 |
| PMA ± VP-16-213 ($10^{-5}$ M) | 210 ± 5 | 263 |

* Thioglycolate-elicited macrophages that had been maintained in culture for 24 h were treated with 0.01 μg/ml PMA in MEM-FBS for 2 h at 37°C. Control cultures received MEM-FBS alone. Macrophages were then treated with podophyllotoxin or VP-16-213 in MEM-FBS for 1 h at 37°C in PMA-containing medium, and HRP (1 mg/ml final concentration) was added for an additional 90 min. Cells were washed and assayed for their content of HRP as described in Materials and Methods. Each determination is the mean of three independently processed cultures.

Cytochalasins B and D ($10^{-5}$ M) inhibited the basal rate of pinocytosis by thioglycolate macrophages by 14 and 55%, respectively (Fig. 4). The PMA-stimulated uptake of HRP was completely abolished by cytochalasin B at $10^{-5}$ M. Cytochalasin B was slightly less effective than cytochalasin D in inhibiting PMA-stimulated pinocytosis at $10^{-6}$ M (cf. Fig. 4 *a* and *b*). In these experiments the macrophages were treated with PMA before the addition of the cytochalasins. Thus the inhibitory effect of the cytochalasins cannot be attributed to reduced entry of PMA into cytochalasin-treated macrophages. Other experiments (data not shown) confirmed that $10^{-5}$ M cytochalasin D completely inhibited phagocytosis of IgG and of complement-coated erythrocytes in thioglycolate macrophages, and that this inhibition could not be overcome by PMA (0.01 μg/ml).

## PMA-stimulated Macrophage Spreading

PMA induced a variety of morphological effects within the first 10–20 min after its addition to macrophage monolayers. The peripheral cytoplasm became thin and the mitochondria aligned radially. Increased numbers of phase-lucent vacuoles appeared in both the perinuclear region and the cell's periphery. The nucleus became very distinct, presumably as a result of cellular flattening. However, cellular spreading is the most

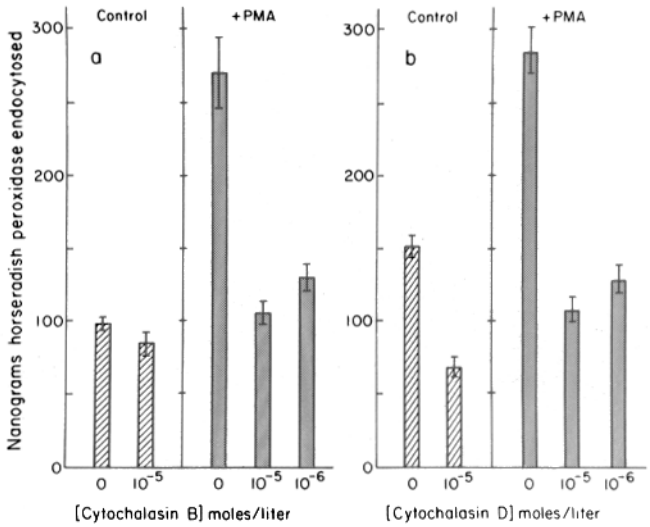


FIGURE 4 Cytochalasins B and D inhibit PMA-stimulated pinocytosis. Thioglycolate macrophages maintained in culture for 24 h in Costar dishes were incubated in MEM-BSA for 2 h at 37°C. PMA (0.01 μg/ml) was added where indicated. (*a*) The cells were then incubated with cytochalasin B at the concentrations indicated, in medium containing HRP (1 mg/ml) and 0.01 μg/ml PMA for 90 min at 37°C and assayed for HRP as described in Materials and Methods. (*b*) The cells were then treated with cytochalasin D at the concentrations indicated, in the presence of PMA (0.01 μg/ml) for 1 h at 37°C, washed three times with MEM, further incubated in MEM containing PMA (0.01 μg/ml), cytochalasin D at the concentrations indicated, and HRP (1 mg/ml) for 90 min at 37°C, washed, and assayed for HRP as described in Materials and Methods.

striking of these changes. As shown in Fig. 5 *a* and *b* and in Table II, resident and thioglycolate macrophages treated with 0.001–10 μg/ml PMA occupied up to threefold more surface area than untreated cells. The extent of spreading was nearly equal at all concentrations of PMA tested. This distinguishes PMA-induced spreading from PMA-stimulated pinocytosis; the latter effect is strongly dependent upon the concentration of PMA used (Fig. 1).

### PMA Induces Spreading of Macrophages Treated with Colchicine or Cytochalasins

After 15–30 min treatment with colchicine ($10^{-5}$–$10^{-6}$ M) resident, proteose-peptone-elicited, and thioglycolate-elicited macrophages exhibited extensive changes in shape and organization. Spikes and clublike processes protruded from the cells. There was an uneven distribution of cytoplasm, and the nucleus was frequently juxtaposed against the plasma membrane. Despite these changes the cells remained attached to the substrate and viable as evidenced by their ability to exclude trypan blue and to pinocytose peroxidase.

Colchicine-treated macrophages covered 22% less surface area than did untreated controls. PMA (0.01 μg/ml) caused rapid respreading of colchicine-treated ($10^{-5}$ M) macrophages (Table III), and colchicine-induced cell rounding was markedly inhibited in PMA-stimulated macrophages. In the presence of colchicine, however, PMA-treated cells spread unevenly. Portions of the cell surface resembled fans and contained parallel arrays of phase-dense cytoplasm.

Macrophages incubated with $10^{-6}$ M colchicine and PMA (0.01 μg/ml) exhibited a more normal appearance than cells treated with $10^{-5}$ M colchicine and PMA. The former exhibited a well-defined centrosphere region, and spreading occurred evenly around the cell's circumference. Macrophages treated with $10^{-5}$ or $10^{-6}$ M colchicine and PMA spread over a 2- to 2.8-fold greater area than macrophages incubated in the absence of either drug (Table III). PMA also induced spreading of podophyllotoxin-treated macrophages (data not shown).

Cytochalasins B and D ($10^{-5}$ M) induced macrophage rounding and retraction. The cells remained attached to the substrate by numerous filapodia. Additional of PMA to the medium of cells treated with $10^{-5}$ M cytochalasin B promoted cell spreading; these respread cells covered a much greater surface area than control macrophages (Table III). In contrast, PMA (0.01 μg/ml) was relatively ineffective in inducing spreading in macrophages treated with $10^{-5}$ M cytochalasin D (data not shown).

Thioglycolate macrophages incubated in medium alone or in medium containing PMA together with colchicine or cytochalasin B exhibited similar rates of pinocytosis (Figs. 3 and 4) but showed 2- to 2.6-fold differences in extent of spreading over the surface of the culture vessel (Table III). These findings further support the conclusion that PMA-induced membrane spreading does not by itself account for the stimulatory effect of this compound on pinocytosis.

## DISCUSSION

The tumor promotor PMA exerts a variety of apparently unrelated effects on animal cells (29). Prominent among these are its effects on phagocytic leukocytes. PMA stimulates release of azurophilic granules (25) and of plasminogen activator (22) by polymorphonuclear leukocytes, and the secretion of $H_2O_2$ (30), $O^-_2$ (31), prostaglandins (32), and plasminogen activator (23) by macrophages. The experiments reported here show that

TABLE II

*PMA Stimulates Macrophage Spreading* *

| PMA | Surface area | |
|---|---|---|
| | Thioglycolate macrophages | Resident macrophages |
| μg/ml | μm² | |
| None | 460 ± 80 | 380 ± 50 |
| 0.001 | 1,460 ± 410 | 980 ± 250 |
| 0.01 | 1,560 ± 370 | 1,180 ± 260 |
| 0.1 | 1,540 ± 460 | 1,180 ± 280 |
| 1.0 | 1,600 ± 440 | 1,200 ± 180 |
| 10.0 | 1,520 ± 420 | 1,180 ± 220 |

* Thioglycolate-elicited or resident macrophages maintained in culture for 24 h were incubated with PMA in MEM-FBS for 1 h at 37°C. Afterward, cells were washed and fixed, and the surface area covered by spread cells was determined as described in Materials and Methods.

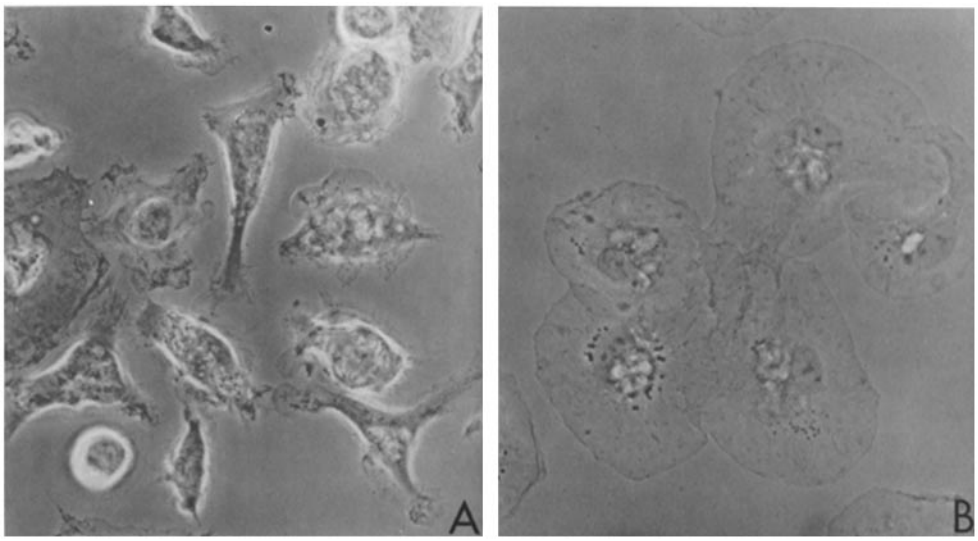


FIGURE 5 Phase-contrast micrographs of thioglycolate-macrophages, explanted 24 h previously, incubated for 1 h at 37°C in MEM-FBS in the absence (*a*) or presence (*b*) of PMA (0.01 μg/ml), and fixed in glutaraldehyde.

TABLE III

*PMA-induced Spreading of Colchicine- or Cytochalasin-Treated Macrophages* *

| Macrophage Treatment | Thioglycolate macrophages | | Resident macrophages | |
|---|---|---|---|---|
| | Surface area covered by cell | % Control | Surface area covered by cell | % Control |
| | $\mu m^2$ | | $\mu m^2$ | |
| None | 460 ± 80 | 100 | 380 ± 50 | 100 |
| $10^{-5}$ M cytochalasin B | 360 ± 70 | 78 | ND | ND |
| $10^{-5}$ M colchicine | 320 ± 70 | 70 | ND | ND |
| $10^{-6}$ M colchicine | 320 ± 50 | 70 | ND | ND |
| PMA | 1,560 ± 370 | 340 | 1,180 ± 260 | 311 |
| PMA + $10^{-5}$ M cytochalasin B | 1,180 ± 200 | 260 | 880 ± 260 | 232 |
| PMA + $10^{-5}$ M colchicine | 930 ± 320 | 200 | 980 ± 270 | 258 |
| PMA + $10^{-6}$ M colchicine | 1,300 ± 380 | 280 | 980 ± 220 | 258 |

* Resident or thioglycolate macrophages explanted for 24 h were treated with cytochalasin or colchicine, as indicated, for 30–60 min at 37°C in MEM-FBS. PMA (0.01 μg/ml) was added to some of the cultures without removing the cytochalasin or colchicine, and they were further incubated at 37°C for 1–2 h. The surface area covered by the cells was measured as described in Materials and Methods. *ND*, not determined.

PMA is a potent inducer of cell spreading and fluid phase pinocytosis in mouse macrophages.

In suspension, macrophages have highly irregular surfaces with numerous lamellopodia and ruffles (33). Upon attachment to a substrate, these cells spread out and lose many of their surface irregularities (34). For this reason there is good agreement between the value obtained by stereological methods for the surface area of cultured resident macrophages by Steinman et al. (26) and our measurements by planimetric methods of the surface area of similar cells. Steinman et al. (26) reported that resident macrophages have a plasma membrane area of 825 ± 80 $\mu m^2$; we (Table II) report a membrane area of 760 ± 100 $\mu m^2$ (2 × 380 $\mu m^2$) for resident macrophages. The close agreement between stereological and planimetric analyses of cell surface area of unstimulated resident macrophages indicates that the approximately threefold increase in surface area of PMA-treated resident macrophages (760–2,400 [2 × 1,200] $\mu m^2$, Table II) is not primarily attributable to a redistribution or flow of membrane from lamellopodia and ruffles into the flattened membrane extensions[1] of these highly spread cells. Rather, much of this additional 1,640 $\mu m^2$ (2,400 − 760) of membrane must be derived from intracellular membrane stores. The failure of resident macrophages to increase their pinocytic rate immediately after PMA stimulation may reflect their insertion of all available membrane stores into the plasma membrane. Time may be required to expand the membrane pool sufficiently to allow an increase in both surface membrane and the number of pinocytosis vesicles.

Several lines of evidence indicate that factors in addition to the observed increase in plasma membrane surface area are responsible for the pinocytosis-stimulating effect of PMA. (*a*) Resident macrophages are stimulated to spread maximally (Table II) immediately upon addition of PMA to the culture medium. However, these cells show no increase in pinocytic rate in response to PMA unless they have been maintained for 26 h in the presence of this compound (Fig. 1). Similar results were obtained with proteose-peptone-elicited macrophages. (*b*) Thioglycolate macrophages exhibit nearly maximal spreading when treated with 0.001 μg/ml PMA (Table II), yet they show far less than maximal stimulation of pinocytosis (Fig. 1) in response to this PMA concentration. A similar lack of congruence between the extent of cellular spreading and pinocytic stimulation is observed in thioglycolate macrophages treated with PMA at a concentration of 1 μg/ml (cf. Fig. 1 and Table II). (*c*) Both colchicine and cytochalasin B at concentrations of $10^{-5}$ M completely abolish the stimulatory effect of PMA on pinocytosis in thioglycolate macrophages (Figs. 3 and 4), but have only modest inhibitory effects on the extent of PMA-induced cellular spreading (Table III).

Steinman et al. (26) determined that at their basal pinocytic rate resident macrophages interiorize 26 $\mu m^2$ of membrane per minute. At this rate these cells interiorize an area of plasma membrane equivalent to the entire cell surface every 33 min. Planimetric data (Table III) indicate that thioglycolate macrophages have an ~17% greater surface area than resident macrophages. (This is much less than the figure calculated by Edelson et al. (16) from the protein and cholesterol contents of the two cell types.) The basal rate of HRP uptake by thioglycolate macrophages is about two to three times that of resident macrophages (reference 16, Figs. 1, 3, and 4, and Table I). Thus unstimulated thioglycolate macrophages internalize 52–78 $\mu m^2$ of membrane per minute. At this rate they internalize an area of plasma membrane equivalent to their cell surface area every 12–18 min (920 $\mu m^2$/52–78 $\mu^2$ $min^{-1}$). Correcting for the differences in cell surface areas, thioglycolate macrophages pinocytose 1.6–2.4 times more rapidly per unit of membrane area than resident macrophages. Because PMA stimulates the basal rate of pinocytosis about threefold (Fig. 1), we calculate that PMA-treated thioglycolate macrophages interiorize 156–234 $\mu m^2$ of membrane per minute, and thereby internalize an area of membrane equivalent to their cell surface area every 14–20 min (3200 $\mu m^2$/156–234 $\mu m^2$ $min^{-1}$). These figures are in good agreement with the values calculated by Edelson et al. (16). The finding that the maximal rate of membrane internalization in control and PMA-treated thioglycolate macrophages is roughly proportional to the membrane area suggests that there is a limit to the number of pinosomes that can be formed per unit area of cell surface per minute.

An important unresolved issue raised by our experiments is whether PMA increases the size of each pinosome, and/or the number of sites at which they form; and/or stimulates the rate of shuttling of pinocytic vesicles to and from the lysosomal compartment. (Wagner and Rosenberg [35] have suggested that microtubules regulate the movement of pinocytic vacuoles in Chang liver cells.) The capacity of colchicine and of cytochalasin to inhibit PMA-stimulated uptake of HRP is consistent with all of these possibilities. Careful measurements of the size and number of HRP-labeled pinosomes formed in response to PMA will be required to resolve this issue.

The capacities of cytochalasin B and of colchicine to inhibit membrane transport of glucose and nucleosides, respectively, appear unrelated to the effects of these drugs on PMA-stimulated pinocytosis. Cytochalasin D ($10^{-5}$ M) has little or no inhibitory effect on glucose transport in thioglycolate-elicited mouse macrophages (Loike and Silverstein, unpublished ob-

[1] The flattened and smooth-surfaced appearance of PMA-treated resident and thioglycolate macrophages has been confirmed by scanning electron microscopy (Phaire-Washington, unpublished observations).

servations), yet it has a greater inhibitory effect than cytochalasin B on basal level and PMA-stimulated pinocytosis (Fig. 1). Similarly, VP-16-213 inhibits nucleoside transport but not microtubule function (24) or PMA-stimulated pinocytosis (Table I), whereas podophyllotoxin inhibits all three processes (reference 24 and Table I). These findings suggest to us that cooperation between colchicine-sensitive structures (microtubules and 10-nm filaments [36]) and cytochalasin-sensitive structures (presumably actin filaments [37, 38]) is required to achieve a sustained increase in the rate of membrane internalization.

Aside from PMA, the only other known inducers of fluid-phase pinocytosis are concanavalin A (6) and epidermal growth factor (17). Both molecules bind to cell surface receptors, and both are internalized within pinocytic vesicles. Because pinocytosis is stimulated by native concanavalin A (tetravalent), but not by succinyl concanavalin A (bivalent) (39), the effect of this lectin on pinocytosis appears to be dependent upon the capacity of the tetravalent molecule to cross-link membrane glycoproteins. Epidermal growth factor also appears to cause aggregation of its receptors (40).

PMA both binds to specific cell surface receptors and intercalates into lipid bilayers (41, 42). In either location it may act as a general promotor of receptor aggregation and by this means may initiate a broad spectrum of hormonelike effects and transmembrane signals. (The finding that PMA stimulates removal of receptors for epidermal growth factor from the surface of HeLa cells (29) may be an indication of aggregation and internalization of these receptors.) Enhanced pinocytosis (6), membrane spreading (43), actin redistribution (44), and microtubule assembly (45) are known cosequences of such cell surface signals. The wide spectrum of cellular processes stimulated by PMA may reflect the number of qualitatively different "second messengers" that can be generated independently of one another by a cell surface perturbant that promotes receptor aggregation.

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*Note Added in Proof*: The presence of a large intracellular pool of plasma membrane proteins is not unique to macrophages. Doyle and colleagues (1978, *J. Biol. Chem.* 253:965–973, and 1979, *J. Biol. Chem.* 254:2542–2550) have identified an intracellular pool of plasma membrane proteins in hepatoma tissue culture cells.

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