PHORBOL MYRISTATE ACETATE STIMULATES PHAGOSOME-LYSOSOME FUSION IN MOUSE MACROPHAGES*

By MARGARET C. KIELIAN and ZANVIL A. COHN

From the Rockefeller University, New York 10021

Phorbol myristate acetate (PMA)[1] is a cocarcinogen that potentiates tumor formation by suboptimal doses of initiators and induces an intense inflammatory reaction in mammalian skin (1, 2). It has a number of pleiotropic effects on cells in culture, including their differentiation and enhanced replication and secretion (for review see [3, 4]). When added to explanted macrophages, it produces dramatic changes in cell shape, spreading, endocytosis, and the release of neutral proteinases and oxygen metabolites (5–8). These changes resemble some of the metabolic and structural correlates of macrophage activation, a process that has recently been shown to result in enhanced phagosome-lysosome (P-L) fusion (9).

Using a previously described fluorescence assay (10), we have now carried out a detailed study of the effects of PMA on macrophage membrane fusion. In this article we demonstrate that PMA in submicrogram doses markedly stimulates both the rate and extent of fusion of phagosomes with preexisting secondary lysosomes. The association of PMA with macrophages, the longevity of its affect on fusion, and the fate of macrophage-associated PMA have also been examined.

Materials and Methods

Cells and Culture Conditions. Resident peritoneal macrophages were obtained from female Nelson-Collins strain mice and cultured as previously described (10). J774 cells were maintained in spinner culture and were kindly provided by Dr. J. Unkeless, The Rockefeller University, New York. These cells were plated at a density of 1.3×10^6 per 35-mm culture dish for 2 h before being used in PMA studies. Primary rat embryo fibroblasts were from Microbiological Associates, Walkersville, Md., and were generously provided by Dr. R. Steinman, The Rockefeller University, as aliquots frozen in medium with 20% fetal calf serum (FCS) and 5% dimethyl sulfoxide (DMSO). Cells were thawed, washed, plated in 35-mm dishes, cultured overnight, and used the next day at densities just below confluence.

Assay of P-L Fusion. P-L fusion in mouse macrophages was assayed by monitoring the transfer of a fluorescent vital dye from lysosomes to phagocytic vacuoles as previously described (10). Briefly, cover slip cultures were labeled for 20 min with 5 μg acridine orange/ml, washed, and a dilute suspension of serum-opsonized, heat-killed yeast particles was centrifuged onto the cell monolayer at 4°C. Unbound yeast was washed away, and the cultures were rapidly warmed to 37°C to synchronize particle ingestion. Viable cells were examined by fluorescence microscopy at various times after an initial 10-min ingestion period. The presence of orange-stained intracellular yeast was considered positive for P-L fusion.

* Supported by grant AI-07012 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; FCS, fetal calf serum; [^{3}H]PMA, [20-^{3}H]-phorbol myristate acetate; MEM, modified Eagle's medium; P, phorbol, PA, phorbol-13-acetate; PBS, phosphate-buffered saline; P-L, phagosome-lysosome; PM, phorbol-12-myristate; PMA, phorbol myristate acetate; TLC, thin-layer chromatography.

Association of [20-^{3}H]Phorbol-12-Myristate-13-Acetate ([^{3}H]PMA) with Macrophages. Uptake and efflux studies were performed with [20-^{3}H]PMA at a specific activity of 4×10^6–5×10^6 cpm/μg. PMA in DMSO was added to 15% FCS-modified Eagle's medium (MEM) at a final concentration of 0.1 μg/ml PMA and a maximum of 0.5% DMSO. Cover slip cultures in Costar tissue-culture trays (Costar, Data Packaging, Cambridge, Mass.) were exposed to 0.5–1.0 ml of PMA-containing medium for the required times. This medium was then removed, cover slips washed once in phosphate-buffered saline (PBS) containing 1% FCS, and then washed sequentially in three beakers of ice-cold PBS. Cover slips were added to scintillation vials, the cells were solubilized in 200 μl 2% sodium dodecyl sulfate, and counted in 3 ml Hydrofluor (National Diagnostics, Somerville, N. J.). Under these conditions, the cover slips alone or the cell cultures at 2°C (time zero) bound from 100 to 400 cpm. This protocol was also used to follow the efflux of [^{3}H]PMA from prelabeled macrophages.

Metabolism of [^{3}H]PMA. 1 ml of [^{3}H]PMA in 15% FCS-MEM was added per 35-mm dish of cells, at a concentration of 0.1 μg PMA/ml and specific activity of 2×10^7 cpm/μg. After being pulsed with PMA, cells were washed three times with 5% FCS-PBS, scraped into normal saline, and extracted with three 1-vol washes ethyl acetate (11). Medium containing label released from cells was similarly extracted. For most samples, 30 μg each of unlabeled PMA, phorbol-12 myristate (PM), phorbol-13-acetate (PA), and phorbol (P) were added as standards just before extraction. Greater than 90% of the original radioactivity was recovered in the pooled ethyl acetate washes. Extracts were concentrated under N_2 and chromatographed on silica gel 60 plates using two developments in dichloromethane:acetone (3:1 vol:vol). Internal standards were detected by I_2 staining and lanes containing standards alone were visualized by I_2 or vanillin:H_2SO_4 (12). Both sets of standards showed the same migration. [^{3}H]PMA gave a single radioactive peak that corresponded to unlabeled PMA. Similar results were obtained using water-saturated diethyl ether or chloroform:acetone (3:1) as solvents, although the CH_2Cl_2:acetone system gave better separation of PA and PM.

Plates were sectioned into 1-cm strips, scraped, and fractions counted in 0.5 ml H_2O plus 3 ml Hydrofluor.

Reagents. [^{3}H]PMA was obtained from New England Nuclear, Boston, Mass. PMA was from Consolidated Midland Corp., Brewster, N. Y. P, PM, and PA were from Dr. P. Borchert at Chemical Carcinogenesis Co., Eden Prairie, Minn. All phorbol derivatives were stored as stock solutions at −70 in the dark. Precoated silica gel 60 plates (0.25 mm) were from E. Merck, Darmstadt, Federal Republic of Germany, and were prerun in ethyl ether before use. Superoxide dismutase (2,900 U/mg, from bovine blood), catalase (32,000 U/mg, from beef liver), indomethacin, hydrocortisone-21-phosphate (disodium salt), and puromycin dihydrochloride were from Sigma Chemical Co., St. Louis, Mo. Cycloheximide was from Boehringer und Soehn, Mannheim, Federal Republic of Germany.

Results

The Stimulation of P-L Fusion by PMA. The addition of PMA to macrophage cultures lead to a stimulation of P-L fusion within the first 15 min after particle ingestion (Fig. 1). This occurred with doses from 0.1 to 1.0 μg PMA/ml (1.6×10^{-7}–1.6×10^{-6} M) and lead to an increase in both the rate and extent of fusion comparable with that observed previously in activated populations (9) or long-term cultures (10). Neither the DMSO carrier alone nor 0.001 μg PMA/ml had a demonstrable effect on fusion. The addition of the nonesterified alcohol P or the monoesters PM or PA at concentrations of 1.6×10^{-7}–1.6×10^{-6} M were also without effect on fusion.

The dose-response determinations reported above were all performed in cells exposed to PMA for a 2-h preincubation period. This was required because of the pronounced lag between the addition of PMA and the expression of enhanced fusion (Fig. 2). These results contrast with the almost instantaneous effects of PMA on the spreading of macrophages (5) and the release of H_2O_2 (8).

The Longevity of PMA Stimulation. Cells pretreated with PMA as presented in Fig.

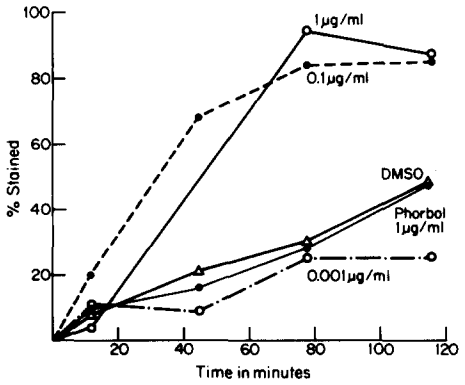

FIG. 1. The effect of pretreatment with PMA on P-L fusion. Rate of P-L fusion in cells cultured for 2 d and pulsed for 2 h with either PMA, P, or 0.33% DMSO (highest concentration used for PMA addition). (O—O) 1 μg PMA/ml; (●---●) 0.1 μg PMA/ml; (O·—·O) 0.001 μg PMA/ml; (●—●) 1 μg P/ml; (△) 0.33% DMSO.

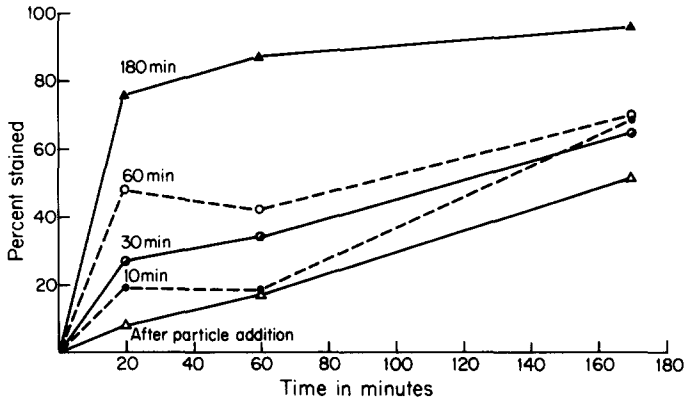

FIG. 2. Time of PMA pretreatment necessary for enhancement of P-L fusion. 2-d cultures were pulsed with 1 μg/ml PMA for the indicated times before particle addition and fusion assay. One set of cover slips (△) was given PMA after particle addition but just before being warmed to 37°C and was maintained in PMA-containing medium.

2 exhibit enhanced fusion throughout the period of assay. Because some of the other effects of PMA on macrophage physiology are short-lived, it was of interest to examine wash-out experiments carried out for longer time periods after pretreatment. Fig. 3 shows that the removal of PMA after 2 h of pretreatment results in a sustained enhancement of fusion for ≥20 h of additional culture in PMA-free medium. It appears therefore that after a relatively long induction period the presence of PMA is not required to maintain a maximum fusion response.

The Association of [^{3}H]PMA with Macrophages. The kinetics of PMA-stimulated fusion raised many questions concerning the association and fate of PMA in macrophages. For this reason, we obtained the tritiated compound and first examined the rates of its uptake and release by cultured macrophages.

The association of [^{3}H]PMA with macrophage monolayers showed a rapid and linear increase until 2–4 h (Fig. 4A). No significant increase above this plateau occurred with continued incubation up to 24 h. Thus the time-course of the uptake of PMA paralleled the time course of its effect on fusion.

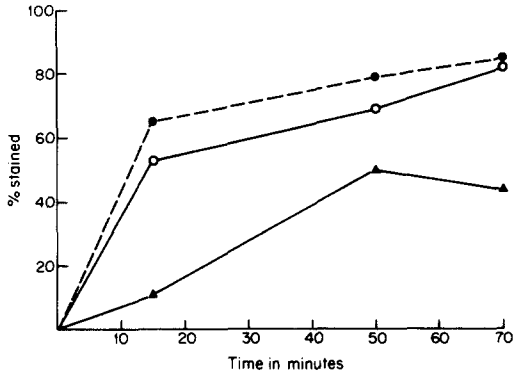

FIG. 3. Maintenance of PMA effect after treatment. Cultures were pulsed for 2 h with 0.1 μg/ml of PMA either immediately (●) or 20 hr before assay (○), washed, and returned to PMA-free medium. Controls received 0.03% DMSO (▲). All three cell populations have been in culture a total of 48 h.

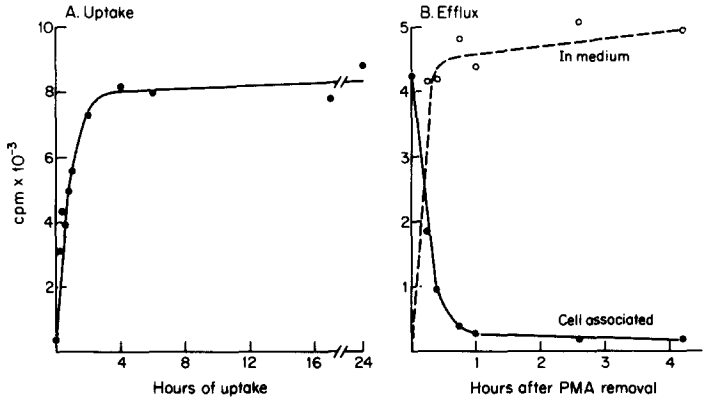

FIG. 4. Uptake and efflux of [^{3}H]PMA in macrophage cultures. (A) Association of [^{3}H]PMA with macrophages. 2-d cultures were exposed to medium containing 0.1 μg/ml PMA at 37°C for the indicated times. Points are the average counts per minute for triplicate cover slips at 5×10^5 peritoneal cells per cover slip. Maximum incorporation was 1.7% of the added label. (B) Efflux of [^{3}H]PMA from macrophages. 1-d cells were exposed to 0.1 μg/ml PMA for 3 h, washed, and returned to culture (time zero) in 15% FCS-MEM. At various times thereafter, triplicate cover slip cultures were assayed for cell-associated and released label. Incorporation at time zero was 2.2% of the added label.

After a pulse of labeled PMA, cell-associated label was rapidly released into the medium and returned to background levels within 1 h (Fig. 4 B), whereas the effect on fusion continued for ≥20 h (Fig. 3). Thus the continued physical association of PMA with macrophages is not necessary to maintain the enhanced fusion rate.

Prior uptake and release of PMA by macrophages did not alter the subsequent uptake of additional PMA. Label was released somewhat more slowly in medium without serum. Lower serum concentrations were used in the following chromatography experiments, both to facilitate extraction and to enable the comparison of released and cell-associated label. Results were qualitatively the same for 1–15% serum concentrations.

Metabolism of PMA by Macrophages. 90 min after a pulse of [^{3}H]PMA, cells and

medium were extracted in ethyl acetate and analyzed by thin-layer chromatography (TLC) (Materials and Methods). Label associated with cells at the start of the 90-min incubation comigrated with the PMA standard (data not shown). Label that was still cell-associated at the time of extraction chromatographed as shown in Fig. 5 B. The major peak of the material comigrated with the PMA standard, whereas some label ran as a more polar material between unesterified P and PM.

Label that was released from macrophages was almost exclusively the polar metabolite and little if any unmodified PMA remained (Fig. 5 A). In contrast, PMA incubated in medium without cells comigrated with the PMA standard after extraction and chromatography. Thus macrophages appear to metabolize and de-esterify PMA to a more polar product, with an R_f value not identical to either of the monoesters, and close but not identical to that of the parent alcohol. Because many oxidation products of PMA are possible (13), the metabolite could be an oxidized form of P or the monoester.

When the released label was extracted, concentrated, resuspended in fresh medium, and added to macrophages, little or no rebinding of the metabolite occurred. This implies that PMA remains cell-associated only when present as the intact molecule.

Metabolism of PMA by Other Cell Types. We wished to compare the metabolism of PMA by the highly endocytic mouse macrophage with that of other cell types. Accordingly, release and chromatography studies were performed for J774 cells, a macrophage-like murine cell line originally derived from a reticulum cell sarcoma (14), and for primary rat embryo fibroblasts. As shown in Fig. 6 A and B, J774 cells rapidly released labeled PMA after a pulse, and most of the label in the medium again migrated between P and PM. Label that was still cell-associated at the time of extraction mainly appeared to be unmodified PMA. Thus, the rapid release of metabolized PMA by J774 cells is similar to that of freshly explanted macrophages.

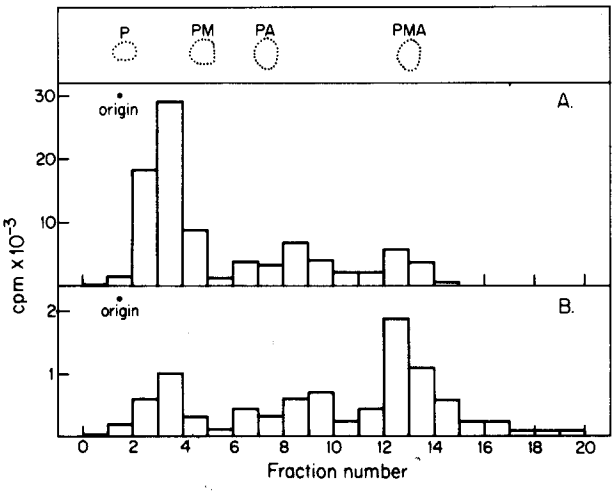

FIG. 5. TLC of released (A) and cell-associated (B) label from primary macrophage cultures treated with 0.1 μg/ml [^{3}H]PMA. Two 35-mm dishes of 24 h macrophages were treated with PMA for 4 h, washed, and recultured in 2% FCS-MEM without PMA for 90 min. Cells and medium were then extracted, chromatographed, and analyzed as described in Materials and Methods. Top: positions of standards (P, PM, PA, PMA) included on the same TLC. Solvent front was at 19 cm. for both developments in CH_2Cl_2:acetone.

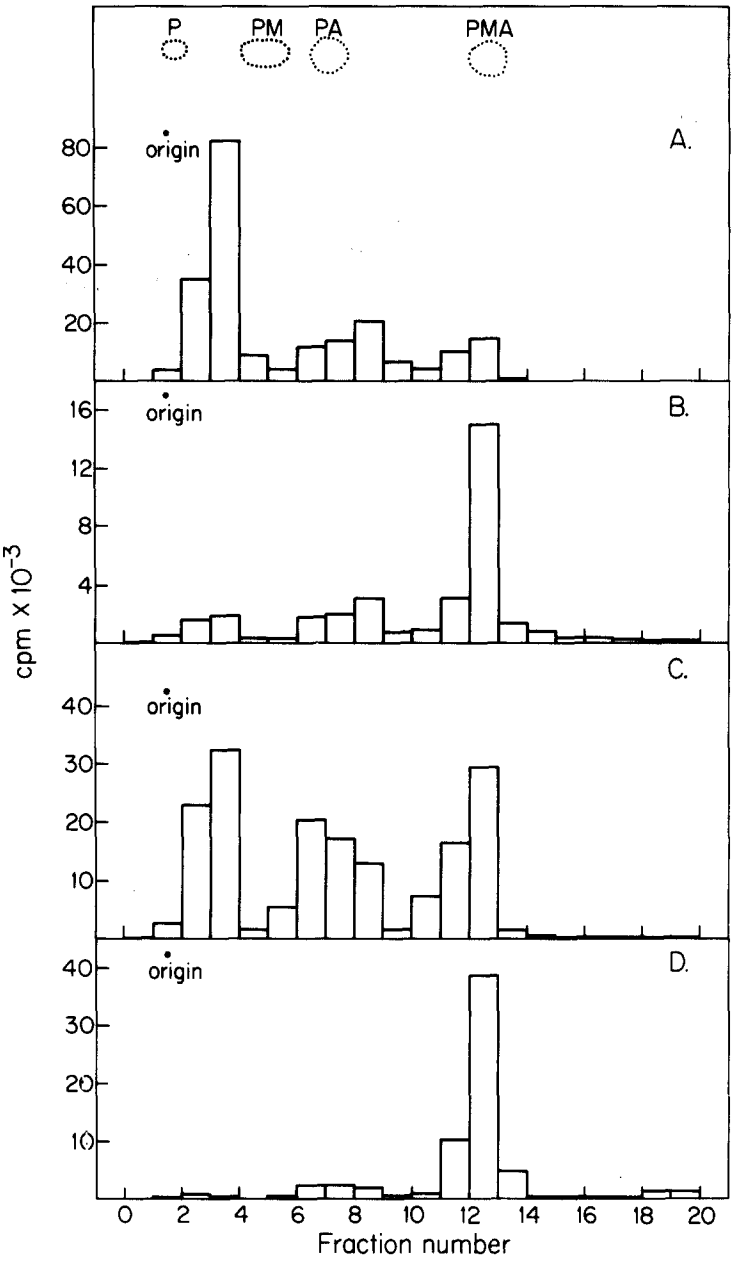

FIG. 6. TLC of released (A) and cell-associated (B) label from J774 cultures and released (C) and cell-associated (D) label from rat embryo fibroblast cultures treated with 0.1 μg/ml [^{3}H]PMA. Two 35-mm dishes of each cell type were treated with PMA for 2 h, washed, and recultured in 1% FCS-MEM without PMA for 90 min. Cells and medium were then extracted and chromatographed. Top: positions of standards (P, PM, PA, PMA) included on the same TLC. Solvent front was at 19 cm for both developments in CH_2Cl_2:acetone.

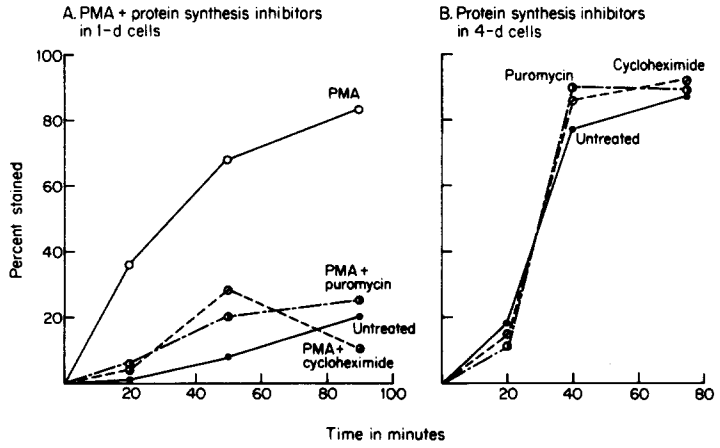

FIG. 7. (A) The effect of protein synthesis inhibitors on the enhancement of P-L fusion by PMA. 1-d cultures were incubated for 2 h in normal medium or medium containing 10 μg/ml puromycin or 5 μg/ml cycloheximide. PMA (0.2 μg/ml) was then added for 5 h to all except untreated cultures. Fusion was assayed as usual except that medium contained protein synthesis inhibitors where indicated. (B) The effect of protein synthesis inhibitors on P-L fusion in 4-d cells. Cells were preincubated for 5 h in medium alone or medium containing 10 μg/ml puromycin or 5 μg/ml cycloheximide. These concentrations were included during the fusion assay where indicated.

The results of a similar experiment with rat embryo fibroblasts were somewhat different from those with macrophages. The release of PMA was slower than for J774, and 37% remained cell-associated at 90 min. Cell-associated label (Fig. 6D) again was almost exclusively unmodified PMA. Released label (Fig. 6C) showed greater heterogeneity with three radioactive peaks, one of which comigrated with PMA standard, one of which ran as the usual metabolite seen with the other cell types, and a third broad intermediate peak that migrated as PA.

Fusion and the Other Metabolic Consequences of PMA. PMA is known to trigger the release of oxygen intermediates by macrophages (7, 8) and to stimulate the production of prostaglandins (15, 16). The enhancement of P-L fusion by PMA was not blocked by concurrent treatment throughout the assay with superoxide dismutase (1350 U/ml) or catalase (15,000 U/ml). These conditions inhibit microbicidal and tumoricidal activities in macrophages by hydrolyzing O_2^- or H_2O_2. Preincubation of macrophages with 10^{-5} indomethacin for 1 h and its presence during PMA stimulation block prostaglandin production (17), but were without effect on the increase in fusion.

The increased pinocytic rate seen in PMA-treated macrophages (5) can be reversed by 10^{-5} M colchicine, whereas enhanced fusion was unaffected by treatment of macrophages with colchicine at 10^{-5} M. Finally, the induction of plasminogen activator secretion by PMA is inhibited by glucocorticoids (6), whereas the fusion effect was not reduced by 10^{-5} M hydrocortisone, a concentration that shows potent inhibition of the secretion of this enzyme (18). These studies imply that the effect of PMA on fusion can be dissociated from several of its other effects on macrophages.

PMA-stimulated P-L Fusion Requires Protein Synthesis. The pronounced lag phase of PMA stimulation, its rapid deesterification and release from the macrophage and its prolonged influence when PMA was removed from the medium suggested that the molecule had induced the formation of a relatively stable product. Because blocking several of the known effects of PMA on macrophages did not affect the enhancement

of P-L fusion, we were interested to know if the enhancement was mediated by a protein product. For this purpose, we employed the inhibitors cycloheximide and puromycin in concentrations that inhibit [^{3}H]leucine incorporation by 90% (6) and the reexpression of surface receptors (19). The results are presented in Fig. 7. 1 d after explanting macrophages, the fusion rate is normally low (Fig. 7 A) and PMA showed a dramatic stimulatory effect. Pretreating the macrophages with inhibitors almost completely ablated this effect. Similar results were obtained 2 d after explant. However, 4 d after in vitro incubation, the fusion rate of untreated cells had already reached maximum levels (10) and the addition of PMA had little influence. Under these conditions, neither puromycin nor cycloheximide influenced the rate of fusion over a 5-h exposure (Fig. 7 B).

Discussion

PMA, a fatty acid diester of a tetracyclic diterpene alcohol, is the most active of several compounds purified from croton oil (1). It is an inflammatory agent and a very potent tumor promoter, although continued applications of promoter are required to produce tumors in the mouse skin carcinogenesis assay (2). Since this work, many activities of PMA on cultured animal cells have been described (for review see [3, 4]). These fall under two general headings: First, that PMA causes changes that mimic or accentuate the transformed phenotype, such as loss of cell-surface fibronectin or induction of plasminogen activator. Second, PMA appears to modify cell differentiation, for example, by inhibiting myogenesis.

In macrophages, the following sequence of events occurs after PMA treatment: within the first 10–20 min after the addition of PMA to resident macrophage cultures, the cells spread on the substrate (5), and within the first 30 min, a burst of prostaglandin and thromboxane release is observed (15, 16). Another rapid effect of PMA is the dramatically increased release of products of the reduction of molecular oxygen such as H_2O_2 (8) and O_2^- (7). This release is much greater when activated macrophages are treated, and unelicited cells show only a small increase in release. Within 1 h of PMA treatment, stimulation of fluid-phase pinocytosis and topological rearrangement of lysosomes into a radial pattern along microtubules occurs (20). Again, these two effects are much more pronounced in elicited rather than resident cells.

In contrast to these rapid effects, the induction of plasminogen activator in resident macrophages was much slower, had a lag period of several hours, and continued for at least 24 h in culture after induction (6). In these aspects, the effect of PMA on fusion resembles the induction of plasminogen activator, and is also expressed in unelicited cells. The requirement for the continued presence of PMA in these other macrophage responses has not been well-characterized. There is some evidence that the effect of PMA on fusion is dissociable from other effects because it is not blocked by catalase, superoxide dismutase, indomethacin, colchicine, or hydrocortisone.

Uptake and Metabolism of [^{3}H]PMA. PMA is a very lipophilic molecule and is known to interact with phosphatidyl choline monolayers, changing the monolayer surface pressure by physical intercalation, although having no apparent effect on model membrane permeability or fluidity (21). Using [^{3}H]PMA, the association of PMA with macrophages was examined to see if a physical intercalation might explain the effect on the ability of the membranes to fuse.

The results reported here indicate that mouse macrophages rapidly take up [^{3}H]-PMA from the culture medium, as has been described for several other cell types in culture (11, 22). Linear uptake levels off after ~3 h, at which time ~2% of the added label is cell-associated, or ~6.4×10^{-12} mol PMA/1×10^6 peritoneal cells plated. When cells are returned to PMA-free medium, this label is rapidly released. Cell-associated label appears to be unmodified PMA, whereas the released form migrates as a more polar compound in TLC. All of the label partitions into the organic phase during extraction, implying that the ^{3}H is still on the phorbol skeleton, and the compound migrates between P and PM. As others have reported (23, 24), we also found that PA migrated faster than PM, in spite of the shorter length of its acyl chain.

The release of metabolized PMA suggests that the plateau of uptake does not represent a saturation of specific binding sites for PMA. Rather, an equilibrium seems to be established between the uptake of native PMA and the release of the metabolized form. The macrophage cell line J774 showed similar rapid kinetics of PMA uptake and metabolism, producing a form that migrated in the same position as that from primary macrophages. Primary rat embryo fibroblasts had somewhat slower kinetics of release, and much of the label released was either unmodified PMA or ran at the same position as PA. Thus, results with rat fibroblasts are more similar to results in the literature in which either no metabolism of PMA was seen (11, 22, 23, 25) or in which PA was the only metabolite seen (23, 24). Human cells have been reported not to metabolize PMA (23, 26), and it would be interesting to know if this were true for human macrophages as well.

The effect of PMA on P-L fusion is maintained for hours after the release of PMA from treated cells, and thus this effect is not dependent on the physical association of PMA with membranes (21) or continued binding to a specific receptor (11, 27). The time-course of the PMA effect and its inhibition by puromycin and cycloheximide suggest that PMA is inducing the synthesis of a protein or proteins that mediate the effect.

Comparison with Other Modulators of P-L Fusion. P-L fusion is inhibited by several intracellular parasites (28), by lysosomal uptake of polyanions (29), and by decreased temperature (10). Fusion is dramatically increased by time in culture (10), by in vivo macrophage activation (9), and by PMA pretreatment, as reported here, while it is insensitive to a great number of other variables (10). Thus PMA pretreatment is one of a very limited number of experimental conditions able to modulate fusion. PMA may be inducing protein(s) in early cultures that are already present at higher levels in activated and 4-d cultures. This product could be an integral membrane protein of lysosomal or plasma membrane that directly influences fusion of its surrounding lipid domain. Another possibility is that a cytosolic or loosely associated membrane protein could be acting between the phagosome and lysosome membranes to affect their fusion. The lack of inhibition by puromycin and cycloheximide in 4-d cultures implies that if such molecules are involved, their turnover rate is slow. Slow turnover of a protein mediator would also explain the stability of the increased fusion rate for 20 h after the removal of PMA.

Summary

The effect of the tumor promoter phorbol myristate acetate (PMA) on phagosome-lysosome (P-L) fusion in mouse macrophages has been studied using a previously

described (10) fluorescence assay. Treatment with 0.1–1.0 μg PMA/ml caused a striking increase in the rate and extent of P-L fusion. Exposure of cells to phorbol, free myristate, or the monoesters of PMA did not reproduce this effect. Macrophages required from 2 to 3 h of pretreatment to express maximal P-L fusion, and this was maintained for at least 20 h when cells were returned to PMA-free medium.

Catalase, superoxide dismutase, indomethacin, and hydrocortisone, agents that are known to block the effect of PMA on H_2O_2, O_2^-, prostaglandins, or plasminogen activator, did not affect the stimulation of P-L fusion by PMA. The protein-synthesis inhibitors puromycin and cycloheximide did block the PMA effect under conditions in which the high fusion rate of 4-d cells was not affected.

Labeled PMA was rapidly taken up by macrophages, with a plateau of uptake at ~3 h. When cells were returned to PMA-free medium, cell-associated label was rapidly released, returning to background levels within 1 h. The released label was found to be a metabolite of PMA by thin-layer chromatography. This product migrated between the monoester phorbol-12-myristate and free phorbol. Rapid metabolism of PMA was also observed by a macrophage cell line, J774, and, to a lesser extent, by primary rat embryo fibroblasts.

We thank Richard Huntley for technical assistance, Grace Silvestri for typing the manuscript, and Dr. William Scott for advice on the chromatography.

Received for publication 16 March 1981.

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