Calcium Modulation of Phorbol Ester-induced Alterations in Murine Macrophage Morphology

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ABSTRACT

The phorbol ester tumor promoter phorbol-12-myristate-13-acetate (PMA) induced mouse resident peritoneal macrophage spreading in an in vitro system in a time- and dose-dependent manner; this process was modified by agents which alter intracellular calcium metabolism. After a 35-min incubation with PMA, 50% of the macrophages were spread (as classified by at least a 2-fold increase in cell surface area). Also at 35 min, the median effective concentration for PMA induction of spreading was 1.6 ng/ml. The intracellular calcium antagonist 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate inhibited PMA-induced cell spreading with a one-half maximal inhibitory concentration of 8.0 μM. The calcium ionophore A23187 enhanced PMA-induced spreading by 30% at 0.1 to 10 nM. The histological dye ruthenium red, which purportedly increases intracellular calcium by displacing membrane-bound calcium stores, enhanced PMA-induced spreading up to 75% at 1.0 μM. The cationic chelator ethyleneglycolbis(β-aminoethylether)-N,N,N,N-tetraacetic acid (1.8 and 3.6 μM) had no effect on PMA-induced spreading. Thus, PMA-induced spreading was independent of extracellular calcium but was modulated by agents altering intracellular calcium metabolism. Microfilament formation, a proposed mechanism of cell spreading, also depends on intracellular calcium availability. The microfilament inhibitor cytochalasin B inhibited PMA-induced spreading with a one-half maximal inhibitory concentration of 1 μM. Future experiments should investigate the hypothesis that calcium availability to the cytoskeletal elements regulates the morphological effects of PMA on macrophages.

INTRODUCTION

The tumor-promoting phorbol esters elicit or modify biological responses in numerous in vitro and in vivo systems (3, 4). In the macrophage, phorbol esters modify such functional responses as chemotaxis (25), phagocytosis (24), cytotoxicity (12, 23), prostaglandin synthesis and release (7), plasminogen activator production (51), and superoxide anion release (39). Furthermore, these effects are obtained at very low concentrations and with a structure activity relationship comparable to that obtained for tumor promotion in the mouse skin model. As such, this laboratory has found that the macrophage is an excellent model for examining underlying mechanisms of phorbol ester-induced effects, including biochemical modulation, receptor mediation, and interactions with cell membranes. Also pertinent to phorbol ester-macrophage biology is the possible role of the macrophage in the tumor promotion process itself (40) or as a mediator of phorbol ester-produced inflammation (18).

PMA, the most potent of the biologically active phorbol esters, induces a dramatic morphological change in macrophages called spreading. This change is characterized by the extension and thinning of peripheral cytoplasm, flattening of the nucleus, and 2- to 3-fold increase in cell surface area (24, 38, 51). Mononuclear phagocyte spreading observed under in vitro conditions has been correlated with increased cell activity in vivo, as evidenced by enhanced rates of phagocytosis (35). Indeed, mouse peritoneal macrophages stimulated with thioglycollate or endotoxin spread immediately after attachment to a substratum, while unstimulated (resident) macrophages spread only after 12 to 24 hr in culture (2). The effect of PMA on cell morphology is not limited to macrophages; PMA also causes profound changes in cell shape and structure in a variety of other cell types, including chick embryo fibroblasts (10), canine kidney cells (36), platelets (11), and WI-38 cells (9). In addition, PMA alters mouse epidermal basal cell morphology, although the relationship of this change to tumor promotion is not known (56).

Many agents have been reported to induce rapid spreading in unstimulated peritoneal macrophages: proteolytic enzymes, acidic conditions, and glassware covered with antigen-antibody complexes (4); ATP (34); and a factor isolated from normal serum, termed macrophage-stimulating protein (26, 27). Proteases of the alternative pathway of complement activation induce human monocytes to spread (15). In addition, the induction of cell spreading can be inhibited or enhanced by numerous factors. Tissue culture conditions such as temperature, osmolarity, cations, or serum present in the medium affect the spreading process (41). Exogenously applied pharmacological probes such as A23187 or dibutyryl cyclic adenosine 3′:5′-monophosphate (28) or glucocorticoids (16) also modify spreading. Other cell types also exhibit spreading; megakaryocytes can be induced to spread by ADP, thrombin, and arachidonic acid (28), while many tumor cell lines become adherent and/or flattened when exposed to various differentiating agents (8, 29).

Despite our general knowledge of agents, such as PMA, which induce cell spreading and of how factors modify the induction process, we have not yet delineated the intracellular biochemical messenger(s) which mediate the sequence of events resulting in morphological changes in the cell. Ultimately, the signals generated by these messengers may be essential for phenotypic transformation of a cell.

Calcium may be one such messenger and indeed has been implicated in a number of other systems as a modulator of phorbol ester effects. Movement of calcium from an intracellular
pool is thought to trigger or regulate many PMA-induced biological responses. For example, intracellular calcium was required for PMA-induced lysosomal enzyme release in neutrophils (45), and modulation of intracellular calcium altered the chemotaxis of mouse resident peritoneal macrophages to PMA (44). PMA modifies \(^{45}\text{Ca}\) flux in gial tumor cells and initiates a reduction in the calcium-dependent component of cyclic adenosine 3':5'-monophosphate accumulation (6). Recently, PMA was reported to induce changes in membrane potential and calcium flux in neutrophils as measured by a sensitive ion-selective electrode (32). Further evidence for the role of intracellular calcium in mediating phorbol ester-induced effects is that the calmodulin inhibitor trifluoperazine abolished concanavalin A capping in PMA-treated lymphocytes (22). Extracellular calcium is another calcium pool necessary for several effects of the phorbol esters, although many PMA-induced effects are independent of extracellular calcium. PMA stimulation of DNA synthesis in BALB/c 3T3 cells (5) was dependent on extracellular calcium as were PMA induction of ornithine decarboxylase in mouse skin explants (52) and release of protein in pancreatic acini (17). Recently, Pick et al. (40) have proposed an elegant model in macrophages depicting calcium as a pivotal regulator of PMA-induced production of superoxide anion, cyclic nucleotides, and arachidonic acid.

Although PMA modulation of macrophage function is well documented and the observation of PMA induction of macrophage spreading has been reported, the present study is the first characterization of PMA-induced morphological changes in macrophages and examination of the effects of pharmacological calcium probes on those changes.

**MATERIALS AND METHODS**

**Preparation of Mouse Resident Peritoneal Macrophages.** Peritoneal exudate cells were harvested from naive female ICR mice by lavage of the peritoneal cavity with 8 ml of DMEM (Grand Island Biological Co., Grand Island, N.Y.). Cells were centrifuged (500 × g) at \(4^\circ\) for 10 min in a Beckman TJ-6R centrifuge and resuspended in DMEM supplemented with 10% horse serum (Grand Island Biological Co.). Cells were counted with a hemacytometer, plated at 150,000 macrophages/well in 24-well Costar tissue culture plates (Costar, Cambridge, Mass.), and incubated for 1.5 hr at \(37^\circ\). Each well contained a piece of glass coverslip (4.5 sq mm) (Corning Glass Works, Corning, N.Y.) to which the macrophages adhered. After adherence, the macrophages were washed twice with DMEM. For the experimental protocol, 0.5 ml of medium or medium containing appropriate drugs was added to each well. The macrophages were incubated for various times in medium alone or in medium containing 100 ng PMA per ml. Cells were washed, fixed with Wright's stain, and stored in 100-μl aliquots in the freezer (−20°). Stock solutions of A23187 were prepared in 100% alcohol at \(10^{-3}\) M, and RR and TMB-8 were prepared (\(10^{-3}\) M) in DMEM and 10% horse serum.

**Photography.** Photographic documentation of changes in macrophage morphology was made with a Zeiss Model III photoscope using Kodak ASA 25 film. Magnification of cells was \(×160\) and \(×400\).

**RESULTS**

**Time and Concentration Dependency of PMA-induced Cell Spreading.** PMA induction of macrophage spreading was time dependent (Chart 1). By 60 min, over 85% of the macrophages exposed to PMA (100 ng/ml) were spread, and by 240 min, 95% were spread. The induction of spreading was seen as early as 5 min, where 7% of the treated cells exhibited spreading. The time point at which 50% of the cells were spread was approximately 35 min. The number of intermediate spread cells was greatest at 20 min and declined at later time points (Chart 1). The decline in the number of intermediate cells over time was evidence that these cells were truly in a stage between spreading and non-spreading and were not merely a subpopulation of cells, perhaps immature cells, that were capable only of a limited degree of spreading.

The degree of spontaneous spreading of resident peritoneal macrophages was very low in these experiments with only about 3% of control cells ever exhibiting spreading. Such low levels of spontaneous spreading can be attributed to the short time period between harvesting of cells and time of examination (Chart 1). PMA-induced spreading was seen as soon as 5 min, but the number of spreading cells declined after 60 min. This suggests that the effect of PMA on spreading is not sustained over time.

**Confocal Imaging.** Confocal imaging of calcium indicator dyes was used to determine the role of intracellular calcium in PMA-induced morphological changes. The calcium indicator dyes TMB-8, Quin 2, and Quin 3 were used to visualize changes in intracellular calcium. The calcium indicator dyes were excited with a 488-nm line of an argon laser and emitted fluorescence at 510-530 nm. The images were collected using a confocal laser scanning microscope (Olympus, Tokyo, Japan). The images were processed using a computer-aided image analysis system (Olympus, Tokyo, Japan).

**Calcium Probes.** Calcium probes were used to determine the role of intracellular calcium in PMA-induced morphological changes. The calcium probes were added to macrophages in the presence or absence of PMA. The calcium probes were excited with a 488-nm line of an argon laser and emitted fluorescence at 510-530 nm. The images were collected using a confocal laser scanning microscope (Olympus, Tokyo, Japan). The images were processed using a computer-aided image analysis system (Olympus, Tokyo, Japan).
that cells were incubated to perform the experiments described in Charts 2 to 6. The duration of incubation of cells for these experimental protocols was a maximum of 180 min (the 90-min adherence period plus the additional time necessary for performing the actual experiment). If resident macrophages were left in culture, they spread spontaneously over a 15- to 24-hr period (data not shown). This finding is in close agreement with Gotze et al. (15) who reported spontaneous spreading in unstimulated (resident) macrophages between 12 and 24 hr. By way of comparison, PMA (100 ng/ml) induced 50% spreading in the peritoneal macrophage population at about 0.5 hr (35 min), whereas 50% spontaneous spreading occurred at about 18 hr. The nuclei of cells which spread spontaneously were morphologically indistinguishable from the nuclei of PMA-incubated cells. However, the peripheral cytoplasm of spontaneously spread cells lacked the vacuoles observed in PMA-induced spread cells.

Phaire-Washington et al. (38) reported that, after 60 min of incubation, various concentrations of PMA induced equivalent amounts of macrophage spreading; that is, a concentration-dependent response was not evident. Our results concur with this observation (data not shown): in fact, doses as low as 1.0 ng PMA per ml induced maximal spreading at 60 min. However, a dose-response relationship in PMA induction of spreading was evident at the 35-min incubation time point (Chart 2). The maximum amount of spreading at the 35-min time point (about 50% of all macrophages adhered) was induced by 100 ng PMA per ml. Concentrations of 10.0 and 1.0 ng/ml induced relatively less spreading, while 0.1 to 0.01 ng PMA per ml had no effect. The median effective concentration was 1.6 ng/ml. Higher concentrations of PMA (1.0 and 5.0 µg/ml) induced less spreading than did 100 ng/ml. This decrease in spreading may be the result of a desensitization or down-regulation of receptors (19), if spreading is a receptor-mediated process as is chemotaxis. In summary, PMA-induced macrophage spreading is concentration-dependent at an early time point in the induction process (35 min). This finding suggests that the rate of PMA induction of spreading is a function of concentration; i.e., the rate is slower at lower levels of PMA.

The vehicle control of 0.1, 0.01, or 0.001% dimethyl sulfoxide did not induce spreading of macrophages, nor did the parent alcohol of PMA, phorbol.

Role of Calcium in PMA Induction of Macrophage Spreading. The significance of both extracellular and intracellular calcium in the induction of macrophage spreading was examined in the following studies.

The level of extracellular calcium was modified by adding the divalent ion chelator EGTA to the medium plus varying concentrations of calcium chloride. It was found that neither 1.8 nor 3.6 mM EGTA modified PMA induction of cell spreading. Also, when calcium chloride (0.45, 0.90, 1.8, or 3.6 mM) was added to medium containing 1.8 mM EGTA, no effect was seen on PMA-induced cell spreading.

The effect of the intracellular calcium antagonist TMB-8 on PMA-induced cell spreading was investigated. After 90 min of incubation, this agent (0.1 to 50 µM) inhibited PMA induction of spreading in a concentration-related manner (Chart 3). The concentration of TMB-8 which resulted in one-half the maximal inhibition of PMA-induced spreading was 8.0 µM.

Two other calcium-modulating agents which affected PMA induction of macrophage spreading were the histological dye RR and the ionophore A23187. RR modifies calcium binding (14, 21), calcium flux (47, 53), and calcium-dependent processes, such as neurotransmitter release (37) and ganglionic transmission (31). This agent is thought to increase intracellular calcium levels by displacing bound calcium stores within the cell. In the present investigation, RR produced a concentration-dependent stimulation of PMA-induced cell spreading at very low concentrations (<1.0 nM) (Chart 4). The concentration-response curve exhibited a broad peak and was biphasic with a maximum stimulation of 75% occurring at 1.0 µM.

The cationic ionophore A23187 transports calcium across cell membranes and thus elevates cytosolic calcium concentrations (1, 13). In the presence of 1.8 mM calcium, concentrations of 0.1 to 10 nM A23187 increased PMA induction of macrophage spreading by approximately 30% (Chart 5). This enhancement

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availability of calcium to the cytoskeletal elements. Therefore, therefore, another possible mechanism of PMA-induced spreading is that these calcium-modulating agents increase the ability of PMA to cause macrophages to spread? One plausible cellular calcium with EGTA removes the calcium available for spread, not spread, or intermediate. The number of spread cells incubated with PMA plus cytochalasin B was compared with the number of spread cells incubated with PMA alone and expressed as a percentage of change. Points, mean number of cells from each of 4 experiments; bars, S.E.

In contrast, EGTA had no effect on the RR enhancement or modulation of PMA-induced spreading. Peritoneal macrophages were harvested, adhered for 90 min at 37°C, and washed twice with DMEM. Either medium alone, medium containing PMA (100 ng/ml), or medium containing PMA (100 ng/ml) plus RR was added to the cells. Macrophages were incubated for 35 min, washed, fixed, and counted. The number of spread cells incubated with PMA plus RR was compared with the number of spread cells incubated with PMA alone. For example, if 48% of the PMA-treated cells were spread, and 76% of the PMA-plus-RR-treated cells were spread, then the percentage of stimulation was 58% of (76 - 48)/48. Points, mean number of cells from each of 6 experiments; bars, S.E.

The significance of microfilaments in PMA-induced spreading was examined. The data in Chart 6 show that the microfilament inhibitor cytochalasin B blocked PMA induction of macrophage spreading in a concentration-dependent fashion with a one-half maximal inhibitory concentration of 1.0 µM. Concentrations as low as 0.1 µM cytochalasin B inhibited PMA-induced spreading by 25%, while 50.0 µM cytochalasin inhibited this response up to 90%. When EGTA, A23187, RR, TMB-8, or cytochalasin B was tested in the absence of PMA, no changes in macrophage morphology were observed.

**DISCUSSION**

Biologically active phorbol esters are thought to exert their effects via receptor mediation (33, 42, 43); as a consequence, gene expression is altered, and ultimately functional or phenotypic changes are initiated in biological systems. However, the underlying biochemical mechanisms through which phorbol esters accomplish such transformations have not yet been identified. In the present study, a PMA-induced phenotypic change in macrophages has been characterized, and this change has been modulated by agents which can alter cellular calcium metabolism. Calcium appears to be a significant biochemical regulator of PMA-induced effects.

The calcium-modulating agents A23187 and RR increased PMA induction of mouse resident peritoneal macrophage spreading. The enhancement produced by A23187 was abolished when EGTA was added to the medium to deplete extracellular calcium. Therefore, it appears that A23187 is working via its purported mechanism of transporting extracellular calcium across the cell membrane and increasing cytosolic calcium levels (1, 13). Calcium may be important in inducing spreading in other cell types; A23187 in the presence of methylamine caused an increased number of megakaryocytes to spread (28). Although the precise site of action of RR has not yet been clearly established, there are numerous studies which document its interaction with calcium (14, 21, 31, 37, 47, 53). For example, RR was found recently to depress the rate of 45Ca flux in adrenal cortical cells (53) and to modify calcium-dependent neuronal transmission (31, 37). In the present study, the enhancement effect of RR on PMA-induced spreading was not affected by EGTA. This supports the premise that A23187 and RR are acting by different mechanisms.
to elevate levels of cytosolic calcium. Since neither A23187 nor RR increased basal cell spreading, it is reasonable to assume that increases in cytosolic calcium are not a sufficient signal for inducing cell spreading.

The intracellular calcium antagonist TMB-8 blocked PMA-induced cell spreading in the present study. This agent, originally found to block caffeine-induced skeletal muscle contractions (30), has been found also to block PMA-stimulated release of lysosomal enzymes in polymorphonuclear leukocytes (45) and to inhibit chemotaxis of mouse resident peritoneal macrophages to PMA (44). Also, TMB-8 has been found to block spreading in peritoneal macrophages induced by the protease subtilisin (50).

In contrast to the significant role that intracellular calcium appears to play in PMA-induced cell spreading, extracellular calcium appears to be of little importance. Depleting extracellular calcium with EGTA or adding calcium to EGTA-containing media did not affect the PMA induction of cell spreading. In agreement with this observation, Rabinovitch and DeStefano (41) found that the induction of macrophage spreading by subtilisin was not affected by extracellular calcium (although other inducers such as dithiothreitol required extracellular magnesium). In addition, there are a number of functional responses of cells of the immune system elicited by PMA which are independent of extracellular calcium (39, 44, 45).

An elevation of intracellular calcium increases the availability of this cation to any calcium-dependent system within the cell; however, a possible calcium-dependent target involved in cell spreading is the microfilaments of the cytoskeletal apparatus. In macrophages, calcium controls the size and gelation of actin filaments via a calcium-dependent regulatory protein, gelsolin (54, 55). The protein calmodulin is another potential calcium-dependent target associated with cytoskeletal function; the calmodulin antagonist trifluoperazine inhibits spreading and migration of WIRL cells (8).

Also, changes in localization and aggregation of microfilaments occur during spreading (28). Furthermore, the microfilament inhibitor cytochalasin B blocked PMA-induced cell spreading in a concentration-dependent fashion. Cytochalasin B also has been found to inhibit spontaneous spreading of macrophages (2) and spreading induced by trypsin, dithiothreitol, or acidic conditions (41). One study, however, found that only cytochalasin D (10^{-5} M), and not cytochalasin B, inhibited macrophage spreading (38). Cytochalasin B inhibits spreading in Chinese hamster ovary cells (20), WIRL cells (8), and megakaryocytes (28).

Perhaps, the most intriguing aspect of this study is the implied relationship between the induction of macrophage spreading and the chemotaxis of mouse resident peritoneal macrophages to PMA (see Ref. 44). Chemotaxis, a functional response of macrophages, is the directed movement of cells along a concentration gradient of a chemoattractant, and PMA is a potent chemoattractant for macrophages (25). Moreover, the first stage in the chemotactic process is a morphological change, a directed orientation of the cell toward the chemoattractant, thought to be produced by the differential occupancy of receptors along the cell membrane. The oriented cell exhibits a head-to-tail polarization with an anterior extension of cytoplasmic processes (lamellipodium) and a stubby posterior cytoplasmic pseudopod (uropod). A complex arrangement of actin microfilaments lies beneath the plasma membrane of the lamellipodium and uropod, with the greater concentration in the lamellipodium (46, 48). The later steps of chemotaxis are the transmission of the chemical signal to the motility apparatus and the actual cell locomotion. Thus, cell spreading and the first stage of chemotaxis (orientation) are both morphological changes induced by PMA, and both may occur by analogous changes in the cytoskeleton. The spread cell has been exposed to a uniform concentration of PMA, whereas the oriented cell has been exposed to a gradient of PMA.

The calcium-modulating agents A23187, RR, TMB-8, and EGTA altered the chemotaxis of macrophages to PMA in a qualitatively identical fashion to their effects on PMA-induced cell spreading (44). That is, A23187 and RR stimulated both PMA-induced spreading and PMA-stimulated chemotaxis; TMB-8 inhibited both of these phenomena, while EGTA had no effect. In addition, the shapes of the concentration-response curves were similar: RR exhibited a broad-peaked biphasic enhancement of both of these PMA-induced biological effects with a similar maximal stimulation (75% for PMA-induced spreading and 67% for PMA-stimulated chemotaxis). A23187 enhances PMA-induced spreading by about 31% and PMA-stimulated chemotaxis by about 40%, both phenomena occurring over a concentration range of 1000. Perhaps most important in this comparison is the dramatic quantitative difference between the effects of A23187 and RR on these 2 systems. Concentrations of 1.0 &times; 10^{-5} to 1.0 &times; 10^{-3} RR enhanced PMA-induced cell spreading, while 0.01 to 1.0 &times; 10^{-5} RR increased PMA-induced chemotaxis. Similarly, concentrations of 0.1 to 1.0 &times; 10^{-5} A23187 enhanced PMA-induced spreading, while 0.1 to 1.0 &times; 10^{-5} A23187 increased chemotaxis of macrophages to PMA. Thus, the effect of calcium-modulating agents on PMA-elicited biological responses in macrophages was shifted to the left when comparing a morphological response (spreading) to a functional response (chemotaxis). In short, the morphological response elicited by PMA was more sensitive to biochemical modulation than was a functional response, although both responses were affected in the same manner qualitatively. Perhaps, a sustained elevation in calcium levels is required for macrophages to exhibit a complex functional response such as chemotaxis, whereas morphological changes are less complex, occur earlier, require less expenditure of cellular energy, and, in fact, may represent the preliminary stage of a functional response like chemotaxis. Similar patterns of biochemical modulation of morphological and functional responses may help to elucidate the relationship between the 2 phenomena. In fact, the orientation (morphological) stage in the chemotactic process may be the most sensitive to pharmacological manipulation. Studies are presently underway to investigate the effects of these calcium-modulating agents on macrophage orientation.

The quantitative changes in calcium metabolism which occur following exposure to PMA have been investigated using 44Ca efflux studies. Preliminary results show that PMA increases both the rate constant and the exchangeable pool of calcium in the slow phase of 44Ca efflux. Furthermore, the calcium-modulating agents alter 44Ca efflux after PMA exposure; for example, A23187 dramatically increases the rate constant of the fast phase of calcium efflux. These results are consistent with the results presented herein that alterations in calcium metabolism are associated with exposure to PMA, and indeed, calcium may be a mediator of PMA-induced biological effects.

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Fig. 1. Photomicrographs of control (a and b) and PMA-treated (c to f) macrophages. Peritoneal macrophages were harvested and adhered for 90 min at 37°C. Cells were washed with DMEM and were then incubated in DMEM or in DMEM plus PMA (100 ng/ml). Cells were fixed with Wright's stain and mounted. a, control macrophages, 90-min incubation, × 160; b, control macrophages, 90-min incubation, × 400; c, "intermediate" macrophage (see arrow), 35-min incubation, × 160; d, intermediate macrophage (see arrow), 35-min incubation, × 400; e, spread macrophages, 90-min incubation, × 160; and f, spread macrophages, 90-min incubation, × 400.
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