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PHORBOL MYRISTATE ACETATE-INDUCED RELEASE OF GRANULE ENZYMES FROM HUMAN NEUTROPHILS: INHIBITION BY THE CALCIUM ANTAGONIST, 8-(N,N-DIETHYLAMINO)-OCTYL 3,4,5-TRIMETHOXYBENZOATE HYDROCHLORIDE

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SUMMARY: Phorbol myristate acetate (PMA) stimulated the extracellular release of the granule-associated enzyme lysozyme from human neutrophils. The extrusion of lysozyme was not accompanied by the release of β-glucuronidase or the cytosol enzyme lactate dehydrogenase. A time dependent PMA-induced release of lysozyme occurred in the absence of extracellular calcium and when neutrophils were preincubated with EGTA. 8-(N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an antagonist of intracellular calcium, caused a dose-dependent inhibition of lysozyme release from neutrophils exposed to PMA in a calcium-free medium. This effect of TMB-8 could be reversed by the addition of calcium to the extracellular medium. These studies indicate that TMB-8 represents a valuable pharmacologic tool used to define the dependence of a secretagogue such as PMA on intracellular as opposed to extracellular calcium.

INTRODUCTION

Human polymorphonuclear neutrophilic leukocytes (neutrophils) selectively release granule-associated enzymes under a variety of circumstances. Phagocytic release occurs during the internalization (endocytosis) of particulate material (1-4), whereas cell contact with a nonphagocytosable surface (1-5) or a soluble stimulus such as N-formyl-methionyl-leucyl-phenylalanine (6) results in the extracellular release of granule constituents. Electron microscopic, cytochemical, and isolation techniques have aided in the identification of essentially two populations of cytoplasmic granules in human neutrophils. Primary (azurophil) or peroxidase-positive granules contain acid hydrolases, lysozyme and all of the myeloperoxidase activity (7-10). Secondary (specific) or peroxidase-negative granules contain lactoferrin and lysozyme (7,9,10).

The release of granule-associated enzymes from neutrophils has been described as a "stimulus-secretion coupling" mechanism wherein calcium functions

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as the link between the stimulus (particulate or soluble) for and secretion of granule enzymes from these cells (11-14). Nevertheless, the concept of an absolute requirement of calcium for granule enzyme discharge remains controversial. It has become clear that when invoking a role for calcium one must be specific with reference to species and the stimulus employed to elicit release. It should also be noted that the induction of granule enzyme extrusion in the absence of extracellular calcium does not negate the possibility that intracellular calcium is involved in enzyme release.

The objective of this investigation, therefore, was to evaluate the mechanism by which PMA, a tumor promoting agent and the cocarcinogenic principle of croton oil (15), induces the selective discharge of granule enzymes from human neutrophils in the absence of extracellular calcium. To this end we employed the calcium antagonist TMB-8.

METHODS AND MATERIALS

Preparation of Neutrophils. Blood from normal human donors was drawn by venipuncture into one-tenth volume of 3.9% citrate in conical plastic tubes. Neutrophils were purified employing standard techniques of dextran sedimentation, centrifugation on Ficoll/Hypaque and hypotonic lysis. Final cell suspensions contained a minimum of 97% neutrophils. Viability of the neutrophils was always greater than 98% as determined by trypan blue exclusion.

Incubation Conditions. Neutrophils ($5x10^6$) in 2.0 ml of phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.1% glucose were incubated at 37°C in a Dubnoff shaker set at 130 excursions per minute according to the various procedures described under "Results." A 5 mM HEPES-138 mM NaCl buffer, pH 7.4, was used as a cell suspending medium in experiments where extracellular calcium ($CaCl_2$) concentrations exceeded 0.6 mM. This procedure was employed in order to avoid calcium precipitation as calcium phosphate. After incubation the samples were centrifuged at 750 x g (4°C) for two minutes and the clear supernatants were assayed for enzyme activities.

Enzyme Assays. β-glucuronidase (EC 3.2.1.31) and lactate dehydrogenase (EC 1.1.1.27) activities were determined as described previously (16).

Lysozyme (EC 3.2.1.17) activity was determined by the rate of hydrolysis of Micrococcus lysodeikticus (Sigma Chemical Co., St. Louis, Mo.) measured by decrease in absorbance at 450 nm according to the Worthington Enzyme Manual (17). Crystalline egg-white (Grade 1) lysozyme (Sigma Chemical Co.) was used as a standard. Activity was defined as μg lysozyme std/3 min/$5x10^6$ cells. Enzyme release is expressed as the percent of total activity released by 0.2% Triton X-100-0.04 M Tris acetate, pH 7.4.

TMB-8 was synthesized as described by Sastry and Lasslo (18) and Malagodi and Chiou (19).

Compounds. Phorbol myristate acetate (Sigma Chemical Co.) was dissolved in dimethylsulfoxide. TMB-8 was prepared in Tris buffer, pH 7.4. All compounds were soluble under the defined incubation conditions and they produced no alteration in pH of the incubation media. The small amounts of dimethylsulfoxide (final concentration of 0.05%) employed as vehicles did not alter cell viability or enzyme release.

RESULTS

Neutrophils incubated with PMA demonstrated a time-dependent release of lysozyme but not of β-glucuronidase (figure 1). There was no significant extrusion of cytoplasmic lactate dehydrogenase during 30 minutes of incubation which is indicative of selective granule enzyme release during cell contact with PMA.

The data in figure 2 shows that PMA stimulates the same degree of extracellular release of lysozyme from human neutrophils when these cells are suspended in calcium-containing (0.6 mM) or calcium-free PBS.

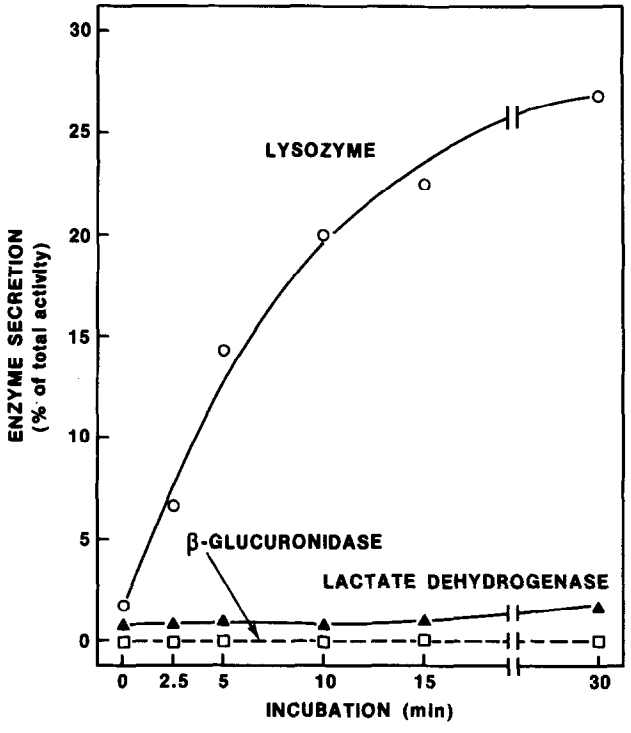

Figure 1

Phorbol myristate acetate-induced release of lysozyme, β-glucuronidase and lactate dehydrogenase from human neutrophils. Neutrophils ($5x10^6$) were incubated with phorbol myristate acetate (10 ng/ml) in PBS for the time periods indicated. Total cell enzyme activities were: 224.5±18.6 µg phenolphthalein/18 hr/$5x10^6$ cells for β-glucuronidase; 27.5 µg lysozyme std./3 min/$5x10^6$ cells for lysozyme; 428.8±34.3 absorbancy units/min/$5x10^6$ cells for lactate dehydrogenase. Data represent the mean of seven experiments.

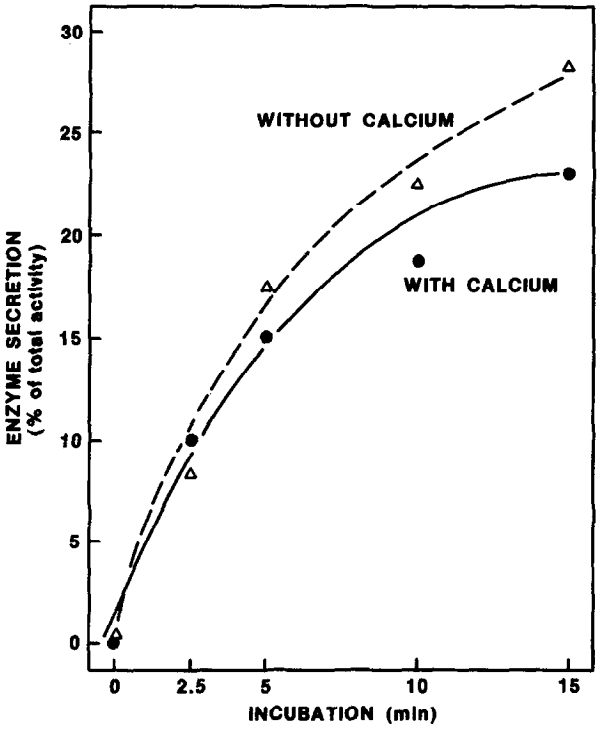

Figure 2

Effect of calcium on lysozyme release from human neutrophils in the presence of phorbol myristate acetate. Neutrophils ($5x10^6$) were incubated with phorbol myristate acetate (10 ng/ml) in PBS with (0.6 mM) or without extracellular calcium for the time periods indicated. Total cell lysozyme activity was: 26.9±2.4 lysozyme std./3 min/$5x10^6$ cells. Data represent the mean of four to six experiments.

The effect of increasing concentrations of extracellular calcium on the discharge of lysozyme from neutrophils is shown in Table 1. Calcium concentrations of 0.6 to 3 mM did not enhance the release of lysozyme from neutrophils in the presence of PMA. Preincubation of cells with EGTA before washing with PBS did not curtail the percent of total PMA-induced release of lysozyme.

TMB-8 caused a dose-dependent inhibition of lysozyme release from neutrophils incubated with PMA in calcium-free PBS (Table 2).

Recovery of total lysozyme, lactate dehydrogenase, and ß-glucuronidase activities in excess of 98% was obtained from cells and incubation media containing TMB-8 and PMA. Therefore, essentially all enzyme activities were accounted for after all incubations and the presence of various test agents did not alter enzyme activities appreciably.

TABLE 1

EFFECT OF EXTRACELLULAR CALCIUM AND EGTA ON PHORBOL MYRISTATE ACETATE-INDUCED RELEASE OF LYSOZYME FROM HUMAN NEUTROPHILS

EXPERIMENTAL CONDITION[a]	LYSOZYME SECRETION (% OF TOTAL ACTIVITY)
PMA (NO CALCIUM)	28.4 ± 2.3[c]
PMA + Ca^{++} (0.6 mM)	26.4 ± 1.8
PMA + Ca^{++} (1.0 mM)	29.3 ± 2.7
PMA + Ca^{++} (2.0 mM)	29.3 ± 2.5
PMA + Ca^{++} (3.0 mM)	28.6 ± 2.9
PMA (NO CALCIUM)[b]	22.9 ± 2.6
PMA + EGTA (2.0 mM)	24.3 ± 1.9

[a]NEUTROPHILS (5 x 10^6) WERE INCUBATED WITH OR WITHOUT EXTRACELLULAR CALCIUM IN THE PRESENCE OF PMA (10 ng/ml) FOR 15 MINUTES.

[b]NEUTROPHILS WERE PREINCUBATED WITH OR WITHOUT EGTA FOR 15 MINUTES IN CALCIUM-FREE HEPES-NaCl BUFFER. THE CELLS WERE SUBSEQUENTLY WASHED AND INCUBATED WITH PMA (10ng/ml) IN CALCIUM-FREE HEPES-NaCl BUFFER FOR 15 MINUTES. TOTAL CELL LYSOZYME ACTIVITY WAS: 27.6 ± 2.3 µg LYSOZYME STD/3 MIN/5 x 10^6 CELLS.

[c]DATA REPRESENT THE MEAN ± SEM OF THREE TO SIX EXPERIMENTS.

TABLE 2

EFFECT OF TMB-8 ON PHORBOL MYRISTATE ACETATE-INDUCED SECRETION OF LYSOZYME FROM HUMAN NEUTROPHILS

TMB-8 CONCN.[a]	LYSOZYME RELEASE (% OF TOTAL ACTIVITY)[b]
CONTROL (NO TMB-8)	30.0 ± 2.7 (0)[c]
176 µM	24.7 ± 2.3 (9.5)
264 µM	20.2 ± 1.7 (41.6)
352 µM	8.3 ± 0.7 (69.1)
528 µM	6.6 ± 0.6 (80.1)
704 µM	0 (100.0)

[a]NEUTROPHILS (5 x 10^6) WERE PREINCUBATED WITH OR WITHOUT TMB-8 FOR 10 MINUTES FOLLOWED BY A 15 MINUTE INCUBATION WITH PMA (10 ng/ml) IN CALCIUM-FREE PBS. TOTAL CELL LYSOZYME ACTIVITY WAS 28.8 ± 2.5 µg LYSOZYME STD/3 MIN/5 x 10^6 CELLS.

[b]DATA REPRESENT THE MEAN ± SEM OF THREE TO FIVE EXPERIMENTS.

[c]NUMBERS IN PARENTHESES INDICATE THE PERCENT INHIBITION OF RELEASE.

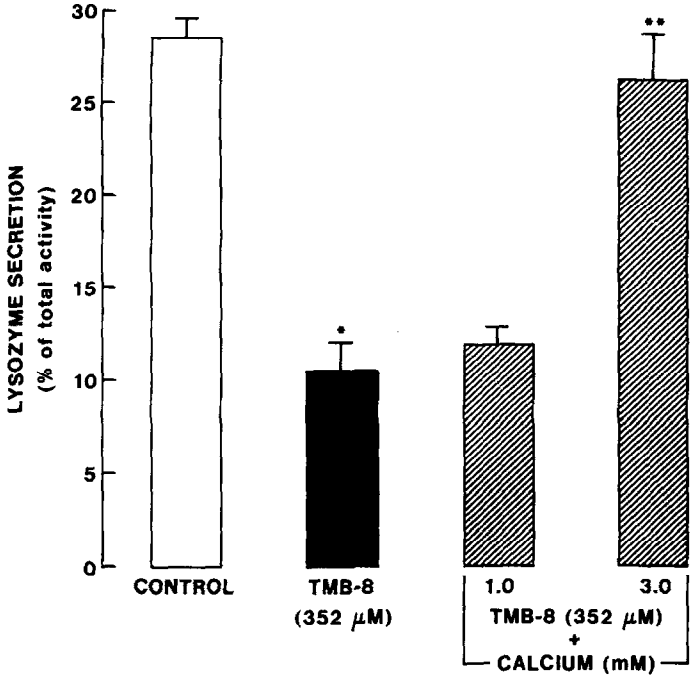

Figure 3

Effect of extracellular calcium on phorbol myristate acetate-induced release of lysozyme from TMB-8-treated human neutrophils. Control represents neutrophils ($5x10^6$) which were incubated with phorbol myristate acetate (10 ng/ml) for 15 minutes. Neutrophils were also preincubated with TMB-8 or TMB-8 and calcium for 10 minutes followed by a 15 minute incubation with phorbol myristate acetate. All reactions were carried out in calcium-free HEPES-NaCl buffer except in situations where calcium was added to the medium. Total cell lysozyme activity was: 26.5±1.9 μg lysozyme std./3 min/$5x10^6$ cells. Data represent the mean ± SEM of four experiments.

* Significant at $P < 0.01$ vs control

** Significant at $P < 0.01$ vs TMB-8

Pretreatment of human neutrophils with TMB-8 (352 μM) in calcium-free HEPES-NaCl buffer caused a significant curtailment of PMA-elicited release of lysozyme from these cells (figure 3). Addition of calcium (1-3 mM) to the extra cellular medium eventuated in a dose-related reversal of the TMB-8 inhibitory effect.

DISCUSSION

Human neutrophils exposed to PMA selectively secrete granule-associated lysozyme to the extracellular medium. The fact that PMA-treated cells do not discharge β-glucuronidase indicates that the extruded lysozyme was associated

with specific granules. This finding is consistent with reports from other laboratories concerning the selective release of specific granule components from neutrophils treated with PMA (20,21). The ability of PMA to elicit the release of specific but not azurophil granule-associated constituents indicates that different mechanisms may control the discharge of enzymes from the respective granules. In this regard, Bainton (22) and Bentwood (23) demonstrated the sequential degranulation of the two classes of granules utilizing particulate and soluble stimuli, respectively. The capacity of a stimulus such as PMA to induce specific but not azurophil granule exocytosis could be related to the reported differences in composition of the membranes of the respective granules (24,25). It is possible that the specific as opposed to the azurophil granule membranes are more amenable to interaction with the plasma membrane.

We report here that the mechanism by which PMA induces the discharge of specific granule-associated lysozyme from human neutrophils is independent of extracellular calcium. Furthermore, increasing the extracellular calcium up to 3 mM or preincubating the cells with EGTA neither enhances nor curtails, respectively, PMA-elicited release of lysozyme. These observations indicate that the action of PMA on exocytosis is either totally independent of calcium or requires intracellular calcium. The latter alternative would appear to be in order bacause TMB-8, a reported antagonist of intracellular calcium (19,26, 27), inhibits PMA-induced release of lysozyme from neutrophils in the absence of extracellular calcium. TMB-8 has been shown to curtail the caffeine-induced release of calcium from the sarcoplasmic reticulum (the principle site of intracellular calcium storage) of smooth and striated muscle (27). TMB-8, however, had no effect on the uptake of calcium by the sarcoplasmic reticulum (27). Furthermore, Charo et al. (28) and more recently Gorman et al. (29) demonstrated TMB-8 to inhibit platelet secretion which, like PMA-elicited enzyme release, occurs in the absence of extracellular calcium. In addition, we have shown that the inhibitory effect of TMB-8 could be reversed by addition of calcium to the extracellular medium. A similar finding was also reported

using platelets (28). The mechanism by which calcium reverses the TMB-8 effect, however, remains to be elucidated. Goldstein et al. (30) have shown that calcium alone can induce lysozyme release from neutrophils. We have also observed calcium to stimulate the selective extrusion of lysozyme (Smith, unpublished observations) from human neutrophils, but this effect is rather minimal and could not by itself account for the highly significant reversal of the TMB-8 effect demonstrated with 3 mM extracellular calcium. It is quite possible that PMA functions to mobilize a previously sequestered intracellular pool of calcium, thereby increasing the availability of cytoplasmic calcium needed for granule-associated enzyme release. TMB-8 may block calcium mobilization and/or antagonize the free calcium once it is released into the cytoplasm. In either situation, the addition of calcium to the extracellular medium would establish a gradient, facilitating increased calcium association with neutrophils resulting in the reversal of TMB-8 activity. Another possible mechanism of action of TMB-8 relative to enzyme release is that this agent may inhibit enzyme activity directly and have no effect on enzyme discharge per se. However, we have found TMB-8 to have no effect on lysozyme activity. Subsequent to calcium mobilization by PMA, there are several mechanisms by which calcium could mediate the "stimulus-secretion coupling" mechanism described for the extrusion of granule constituents from neutrophils. Calcium has been reported to act as an adhesive in promoting the sticking together of cell membranes (31). It has also been suggested that calcium may function to interact with anionic sites on plasma and granule membranes, respectively, thereby facilitating the fusion of these membranes (32) which represents one step in the sequelae of events resulting in exocytosis (33,34). In this regard, calcium has been demonstrated to stimulate membrane fusion (35). Therefore, the importance of calcium for exocytosis is well established, but the precise mode of action(s) of this cation remains conjectural at the present time.

Further investigation is obviously necessary in order to better understand the mechanism(s) by which calcium modulates the selective discharge of granule-

associated constituents induced by numerous stimuli. Nevertheless, by utilizing a secretagogue such as PMA, whose mode of action is independent of extracellular calcium, we are able to employ an agent such as TMB-8 as a pharmacologic tool which will aid in the elucidation of the true relevance of calcium to the granule enzyme secretory process which heretofore has not been possible.

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