CALCIUM-INDUCED LYSOZYME SECRETION FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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SUMMARY: Calcium ions, in the absence of other stimuli, are capable of provoking the release by exocytosis of the granule-associated enzyme, lysozyme, from human polymorphonuclear leukocytes. Calcium-induced extrusion of lysozyme occurs in a concentration, time and temperature-dependent fashion. It is enhanced in the presence of extracellular inorganic phosphate and the ionophore, A-23187, and is not accompanied by the release from cells of cytoplasmic or lysosomal marker enzymes.

INTRODUCTION

At least two major types of enzyme-containing granules have been identified in the cytoplasm of human polymorphonuclear leukocytes (PMN's)[1]. Primary, or azurophil, granules more closely resemble lysosomes of tissues such as liver and kidney and contain a variety of hydrolytic enzymes, predominantly with acid pH optima (1). Secondary, or specific, granules contain alkaline phosphatase, lysozyme and lactoferrin (2). Recently, Estensen et al (3,4) described the selective discharge by exocytosis of specific granule constituents from intact, viable human PMN's after exposure to the co-carcinogen, phorbol myristate acetate (PMA), and have related this effect to an accumulation of cGMP. We now report that a similar phenomenon occurs when human PMN's are exposed to Ca^{++} ions, in the absence of other stimuli.

METHODS

Preparation of leukocyte suspensions. Leukocyte suspensions containing approximately 85% PMN's were prepared from freshly drawn, heparinized venous blood utilizing standard techniques of dextran sedimentation and hypotonic lysis (5). Cells were suspended in a buffer containing 150 mM NaCl and 5 mM HEPES (Grand Island Biological Co., Grand Island, N.Y.) adjusted to pH 7.4 by addition of NaOH, after being washed once with this same buffer.

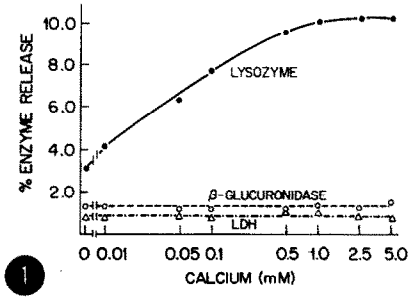
[1] Abbreviations: PMN's = polymorphonuclear leukocytes; cGMP = cyclic 3',5' - guanosine monophosphate; PMA = phorbol myristate acetate; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LDH = lactate dehydrogenase; DMSO = dimethyl sulfoxide.

Measurement of enzyme release. Aliquots (approximately 2 X 10^6 PMN's) of the leukocyte suspensions were dispensed into 10 X 75mm plastic tubes following which appropriate compounds in 150 mM NaCl and additional buffer were added to a final volume of 1.0ml. Following various periods of incubation, the reaction mixtures were centrifuged (755 X g for 10 minutes at 4°C) and cell-free supernatants removed for enzyme assays. Total enzyme activity was determined in simultaneously run duplicate samples containing 0.2% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.). Lysozyme (EC 3.2.1.17) was measured as previously described utilizing suspensions of Micrococcus lysodeikticus (Worthington Biochemical Corp., Freehold, N.J.) as substrate (6). β-glucuronidase (EC 3.2.1.31) was determined after 18 hours of incubation with phenolphthalein glucuronidate (Sigma Chemical Co., St. Louis, Mo.) as substrate (7). Lactate dehydrogenase (LDH) (EC 1.1.1.27) was measured by the method of Wacker et al (8). Enzyme release is expressed as the percent of total activity measured in reaction mixtures containing Triton X-100.

Compounds and other buffers. The ionophore A-23187 (kindly supplied by Dr. R. L. Hamill, Eli Lilly Co., Indianapolis, Ind.) was prepared as a stock solution in DMSO and was ultimately diluted in HEPES-NaCl buffer before use. Phorbol myristate acetate was the generous gift of Dr. Walter Troll of the Department of Environmental Medicine, New York University School of Medicine. This compound was similarly dissolved in DMSO and diluted to desired final concentrations in 150 mM NaCl. A buffer containing 100 mM NaH_2PO_4 - Na_2HPO_4, pH 7.4, was used as a source of inorganic phosphate. $CaCl_2$, $MgCl_2$, $MnCl_2$, $BaCl_2$, $CoCl_2$, $SrCl_2$ and $ZnCl_2$ salts were of reagent-grade or better.

RESULTS

Human leukocytes exposed to $CaCl_2$ in HEPES-NaCl buffer released lysozyme into the suspending medium in a dose, time, and temperature-dependent fashion (Figures 1 and 2). Cells suspended in this Ca^{++}-free medium released lysozyme with the addition of as little as 0.01 mM $CaCl_2$. Maximum release occurred with Ca^{++} concentrations of 1.0 mM and above.



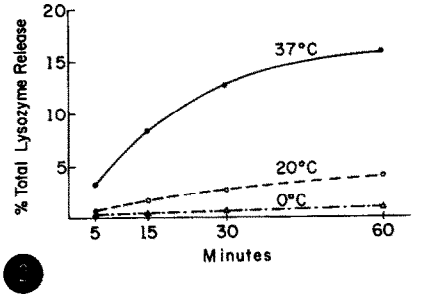


Figure 1: Calcium-induced secretion of lysozyme from human PMN's suspended in HEPES-NaCl buffer and incubated at 37°C for 30 minutes.

Figure 2: Time and temperature dependence of calcium (2.5mM)-induced lysozyme secretion from human PMN's.

Release of lysozyme was selective in that it was not accompanied by release of the lysosomal (azurophil granule) marker enzyme, β-glucuronidase. Cell viability was not altered as indicated by the lack of release of cytoplasmic LDH and by the absence of significant changes in the percentage of cells which excluded eosin Y. Ca^{++} per se, at the concentrations reported, did not influence the enzyme assays, and the slight alterations in medium pH and osmolality were insufficient to account for the observed results. Ca^{++}-induced lysozyme release was enhanced in the presence of inorganic phosphate and the ionophore A-23187 (Table I). As in the preceding experiments, this was not accompanied by enhanced release of LDH. There was, however, some release of β-glucuronidase from cells exposed to Ca^{++} in the presence of the ionophore. Appropriate control experiments failed to reveal evidence that DMSO alone could have accounted for these results. Similarly, these compounds had no effect on the enzyme assays.

Of the various other cations tested, only Mn^{++}, Sr^{++} and Zn^{++} were able to mimic the effects of Ca^{++}, albeit to a lesser degree. Mg^{++}, Ba^{++} and

TABLE I

ENHANCEMENT OF CALCIUM-INDUCED LYSOZYME SECRETION FROM HUMAN PMN's

Additions To PMN's*	Enzyme Release**		
	Lysozyme	β-Glucuronidase	LDH
PO_4^{---} (10mM)	2.1 ± 0.3	0.8 ± 0.2	1.1 ± 0.3
A-23187 (5μM)	3.9 ± 0.7	1.1 ± 0.3	1.2 ± 0.4
Ca^{++} (2.5mM)	10.1 ± 0.9	1.4 ± 0.4	1.0 ± 0.3
" + PO_4^{---} (10mM)	19.9 ± 1.5	0.9 ± 0.3	1.0 ± 0.2
" + A-23187 (5μM)	32.1 ± 4.1	4.1 ± 1.8	1.2 ± 0.3
" + " + PO_4^{---} (10mM)	62.7 ± 7.8	5.4 ± 1.6	1.4 ± 0.4

*PMN's suspended in HEPES-NaCl buffer and incubated for 30 min. at 37°C

**Expressed as percent of total (100%) enzyme activity released by Triton X-100. Totals (per 2 X 10^6 PMN's): lysozyme, 2.4 ± 0.4 μg; β-glucuronidase, 13.1 ± 0.9 μg phenolphthalein/18h; LDH, 231 ± 44 absorbance units. Mean ± SEM, n = 5-8

TABLE II

INHIBITION OF CALCIUM-INDUCED LYSOZYME SECRETION

Additions To PMN's	Percent Lysozyme Release
Ca^{++} (2.5mM) + A-23187 (5μM)	27.8
" + " + Ba^{++} (0.5mM)	27.8
" + " + " (2.5mM	18.9
" + " + " (5.0mM)	10.0
Ca^{++} (2.5mM) + A-23187 (5μM)	36.4
" + " + Mg^{++} (0.5mM)	25.9
" + " + " (2.5mM)	22.8
" + " + " (5.0mM)	12.9

PMN's in HEPES-NaCl buffer incubated at 37°C for 30 minutes

TABLE III

LYSOZYME RELEASE INDUCED BY PHORBOL MYRISTATE ACETATE (PMA)

	Enzyme Release*		
Additions To PMN's	Lysozyme	β-Glucuronidase	LDH
PMA (10ng/ml)	14.1	0.9	1.3
" + Ca^{++} (2.5mM)	20.2	1.1	1.1
PMA (20ng/ml)	25.3	1.2	1.1
" + Ca^{++} (2.5mM)	29.2	1.0	1.2

*Mean of 3 experiments
PMN's in HEPES-NaCl buffer incubated at 37°C for 30 minutes

Co^{++} were inactive. Furthermore, Mg^{++} and Ba^{++} produced a dose-dependent inhibition of lysozyme release from cells exposed to Ca^{++} and A-23187, indicating possible competition for the ionophore (Table II).

Leukocytes suspended in HEPES-NaCl buffer also released lysozyme when exposed to PMA (Table III). This occurred in the absence of added Ca^{++} and was enhanced little by the addition of this cation. Leukocytes incubated in HEPES-NaCl buffer containing 5 mM EDTA for 3 hours, washed, and suspended in the same buffer did not lose their responsiveness to PMA.

DISCUSSION

Secretory processes in a variety of mammalian cell types are Ca^{++}-dependent, particularly those involving the transport to extracellular fluid of proteins contained within cytoplasmic storage granules (exocytosis) (9-11). Evidence has recently appeared which suggests that this cation may directly mediate exocytosis in mast cells (9), peripheral sympathetic neurons (10) and in pancreatic exocrine cells (11). In each instance, Ca^{++} influx stimulated by the ionophore, A-23187, apparently was able to bypass surface stimuli and directly provoke the specific release of stored proteins. The experiments cited in this report provide evidence that this phenomenon can occur in yet another cell type, the human PMN. Ca^{++}-induced lysozyme release from this cell apparently occurs by exocytosis of the contents of a particular class (or classes) of granules. This is supported by the observations that this cation neither alters the permeability of the plasma membrane to cytoplasmic enzymes nor provokes the release of a lysosomal (azurophil granule) marker enzyme. The explanation for this specificity is unknown, but may relate to the physical and chemical properties of the limiting membranes of lysozyme-containing granules (12,13). A unique characteristic of this system is that secretion can be stimulated merely by increasing the extracellular concentration of Ca^{++}, and does not absolutely require the action of an ionophore. A-23187 and extracellular inorganic phosphate enhance secretion presumably by increasing Ca^{++} influx and, at least in the case of the latter, by decreasing Ca^{++} efflux (14,15).

Lysozyme secretion induced by phorbol myristate acetate, although similar to that provoked by Ca^{++}, appears not to be mediated by an influx of this cation. The possibility remains, however, that phorbol myristate acetate may act upon an as yet unknown storage pool of Ca^{++} in the PMN and in this fashion elevate intracellular (cytoplasmic) concentrations of this cation and thereby induce lysozyme secretion.

Ca^{++} is known to interact directly with membranes (16) and is a necessary cofactor for several cytoplasmic and membrane-associated enzyme

systems, including those associated with intracellular contractile proteins (17). It has been proposed that Ca^{++} ions interact with membrane phospholipids and thereby promote membrane-membrane adherence by acting as an "intermembrane bridge" (16). In at least one instance, Ca^{++} has been demonstrated as being capable of inducing membrane fusion (18). Which of these properties, if any, are related to Ca^{++}-mediated stimulus-secretion coupling is as yet unknown.

Whatever the exact mechanism, Ca^{++}-induced lysozyme secretion from human PMN's provides us with a unique experimental model system in which to study the role that divalent cations play in the phenomena of organelle translocation and membrane fusion.

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