EXOCYTOSIS IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES INDUCED BY A 23187 AND CALCIUM

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1. Introduction

Recently, both we [1] and Schell-Frederick [2] have found that ionophores for divalent cations can mimic the phagocytosis-associated stimulation of oxidative metabolism of polymorphonuclear leukocytes (PMNL). We now report that the ionophore for divalent cations A 23187 [3], in the presence of Ca^{2+}, stimulates another physiological response of PMNL to either phagocytosis or surface membrane perturbations [4], i.e. the selective discharge by exocytosis of granular constituents. This process has been studied both by biochemically assaying the distribution of enzymes between cells and the extracellular fluid, and by following the extrusion of granules by electron microscopic analysis.

2. Materials and methods

Peripheral blood was obtained from healthy volunteers and anticoagulated in ACD solution (0.48 g citric acid, 1.32 g Na citrate and 1.4 g glucose per 100 ml). Leukocytes were isolated in 2.5% dextran (mol. wt 250 000) and freed from contaminating erythrocytes by hypotonic lysis (30–90 sec in 0.2% NaCl) according to standard techniques. They were then suspended in a medium containing 123 mM NaCl, 5 mM KCl and 16 mM Tris-HCl, pH 7.4.

The ionophore A 23187, a generous gift of Dr R. L. Hamill (Eli Lilly Co.), was dissolved in dimethyl sulfoxide (DMSO); $CaCl_2$ and $MgCl_2$ salts were of reagent grade. PMNL (1×10^7/ml) were incubated in plastic tubes in the presence of 0.2 mM glucose and the indicated reagents. Following various periods of incubation, the reaction mixtures were centrifuged at $400 \times g$ for 10 min (4°C). The cell-free supernatants were carefully separated from the pellets, which were resuspended to the original volume with calcium-free Krebs-Ringer phosphate buffer. Disruption of the cells was achieved by sonication (Branson sonifier, 3 A, 10 sec). Both the supernatants and the disrupted cells were assayed for enzyme content. Peroxidase activity was measured as described by Romeo et al. [5]. β-Glucuronidase activity was determined after 4 hrs of incubation with phenolphtalein β-glucuronidate as substrate, in the presence of 0.05% Triton X-100 [6]. Lactate dehydrogenase (LDH) was assayed at 37°C according to Bergmeyer et al. [7] and protein according to Lowry et al. [8], with bovine serum albumin as standard. The average total activity of these enzymes and total protein content of the PMNL preparations used were as follows: peroxidase = 5.66 ± 2.45 μmol of tetraguaiacol/ min/1×10^7 cells: β-glucuronidase = 120.6 ± 5.9 μg of phenolphtalein/4 hr/1×10^7 cells; lactate dehydrogenase = 1.12 ± 0.12 μmol of NADH/min/1×10^7 cells; protein = 1.39 ± 0.16 mg/1×10^7 cells (10 or more determinations).

Oxygen uptake by PMNL was monitored at 37°C

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with a Clark-type electrode [1]. The rate of oxygen consumption was calculated from the recorded trace before the addition of the stimulant and 1–2 min thereafter, when it had reached its maximum value. The difference between the two rates is indicated in the text as ΔO_2.

Cells were fixed by mixing suspensions with equal volumes of chilled 6% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4), containing 0.06 M sucrose. After 90 min at 4°C, they were pelleted by centrifugation. The pellet was rinsed with 0.1 M cacodylate buffer, containing 0.2 M sucrose, and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 1 hr, dehydrated, and embedded in DER. Thin sections were double-stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

3. Results

Table 1 shows the distribution of protein, of two granular enzymes and of a cytoplasmic marker between cells and their suspending medium, after a short incubation of PMNL with Ca^{2+} and DMSO. Protein and enzyme activities which do not sediment with control cells have probably leaked out from damaged PMNL or, as in the case of LDH, from contaminating erythrocytes. While Ca^{2+} and DMSO alone have no effect on the enzyme distribution, the two reagents combined may cause a very slight increase of the leakage of cell constituents into the medium. If Ca^{2+} is permitted to act through the ionophore A 23187, a selective release of granular enzymes is promoted. The kinetics of this process are shown in fig.1. The release of peroxidase from

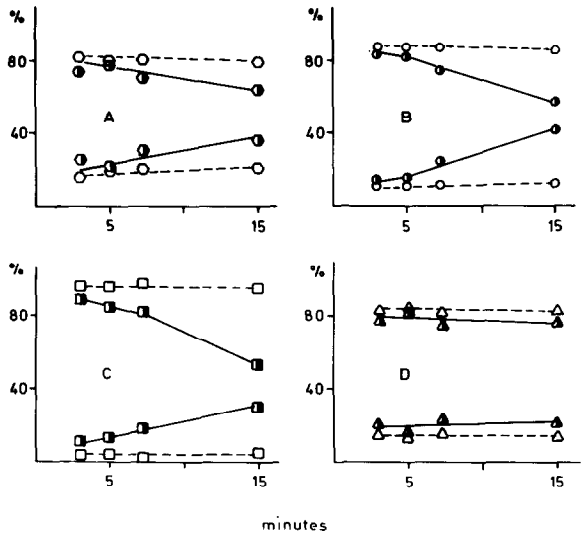

Fig.1. Kinetics of release of protein (A), β-glucuronidase (B) and peroxidase (C) from human PMNL by A 23187 and Ca^{2+}. The behaviour of the cytoplasmic marker lactic dehydrogenase (D) is reported by comparison. Dashed lines: 0.2% DMSO + 1 mM Ca^{2+}; solid lines: 10 μM A 23187 (in DMSO) + 1 mM Ca^{2+}. Each point is the average of two to five determinations. The two upper lines refer to the loss of activity from cells, whereas the two lower lines correspond to the activity recovered in the medium. 1 × 10^7 PMNL/ml were incubated for 15 min in the medium reported in table 1. The numbers shown in the abscissae refer to the time of incubation at 37°C and do not include the time required for separation of cells and medium by centrifugation.

Table 1
Percent of total protein and β-glucuronidase, peroxidase and LDH activity associated with PMNL after incubation with DMSO and Ca^{2+}

Additions	Protein	β-Glucur	Peroxidase	LDH
–	86.8 ± 1.7	92.6 ± 1.3	98.0 ± 0.5	89.2 ± 1.3
0.2% DMSO	87.8 ± 2.1	91.7 ± 0.9	97.8 ± 0.3	89.7 ± 2.1
1 mM Ca^{2+}	86.9 ± 1.7	91.8 ± 1.1	97.6 ± 0.2	89.7 ± 1.6
DMSO + Ca^{2+}	83.1 ± 2.3	90.1 ± 2.4	96.7 ± 0.8	87.7 ± 2.3

1 × 10^7 leukocytes/ml were incubated in the absence or in the presence of DMSO (0.2%) and Ca^{2+} (1 mM), for 5 min at 37°C, data are means of 4 experiments ± S.E. The amount of protein and enzyme activity not associated with cells was fully recovered in the medium.

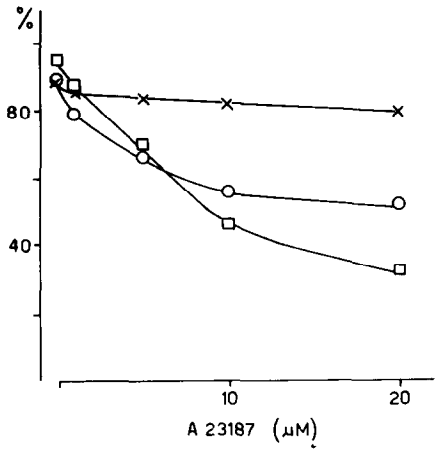

Fig.2. Effect of different concentrations of A 23187 on the Ca^{2+}-induced extrusion of β-glucuronidase (○—○) and peroxidase (□—□) from PMNL. The cell-associated activity of lactic dehydrogenase (X—X) is shown for comparison. 1 X 10^7 PMNL/ml were incubated for 15 min the medium reported in table 1. The final concentration of DMSO was always 0.2% and that of Ca^{2+} 1 mM. Each point is the average of two determinations.

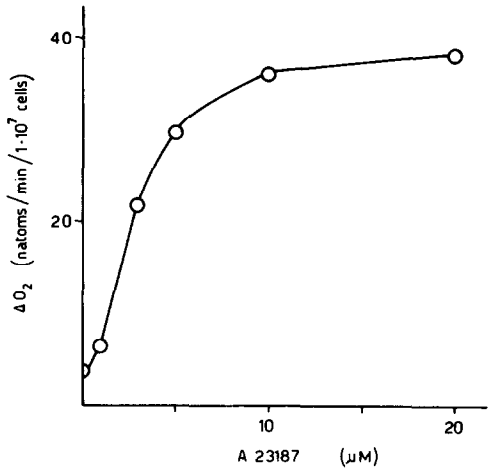

Fig.3. Stimulation of PMNL oxygen consumption by Ca^{2+} and A 23187. PMNL (1 X 10^7) were suspended in 2 ml of the medium indicated in table 1 and the oxygen consumption was continuously recorded by a Clark-type electrode. The rate of respiration was calculated before and after the addition of Ca^{2+} (1 mM final concentration) and A 23187. The data shown in the ordinates are respiratory increments.

PMNL is better indicated by the decrease of its activity in the cells. In fact, as reported also for other granular enzymes [9,10], peroxidase undergoes inactivation once extruded into the extracellular fluid. Under these conditions, after 15 min PMNL have lost about 50% of their β-glucuronidase and peroxidase activity. If Mg^{2+} replaces Ca^{2+} there is no promotion of exocytosis. On the contrary, Mg^{2+}, even at a five-fold lower concentration, inhibits the Ca^{2+}-dependent discharge of granular enzymes.

The effect of various concentrations of A 23187 on the release of β-glucuronidase and peroxidase, in the presence of Ca^{2+}, is shown in fig.2. The ionophore concentration-dependence of exocytosis correlates well with the stimulation of respiration (fig.3). An interesting observation is that in both cases the greatest stimulation by Ca^{2+} and A 23187 is obtained when the cation is added simultaneously with the ionophore.

The biochemical evaluation of exocytosis of granular content from leukocytes conforms to electron microscopic observation. While PMNL treated with DMSO and Ca^{2+} retain a normal ultrastructural appearance, the addition of A 23187 and Ca^{2+} leads to massive extrusion of granules (the ionophore alone does not promote exocytosis). After 5 min of incubation with these two reagents (fig.4a), most PMNL are vacuolated and have exocytosed primary and secondary granules both into vacuoles and into the surrounding medium. Later on, at 15 min (fig.4b), very few granules remain within the cells and those that do are usually very close to or attached to the inner face of the plasma membrane. Fig.5a shows a granule, with its intact membrane, approaching the cell periphery. As examplified by fig.5b, in the course of the exocytotic process, the granule loses its membrane, thereby becoming more prone to leak out its contents. Parallel experiments have shown that virtually all the released peroxidase is not sedimentable at 20 000 *g*.

4. Discussion

Calcium is generally recognized as an essential factor in promoting the secretory activity of various cells. This has led to the 'calcium-activated exocytosis' concept of stimulus-secretion [11]. This concept appears to apply also to the blood PMNL, a cell capable of discharging into the extracellular fluid a

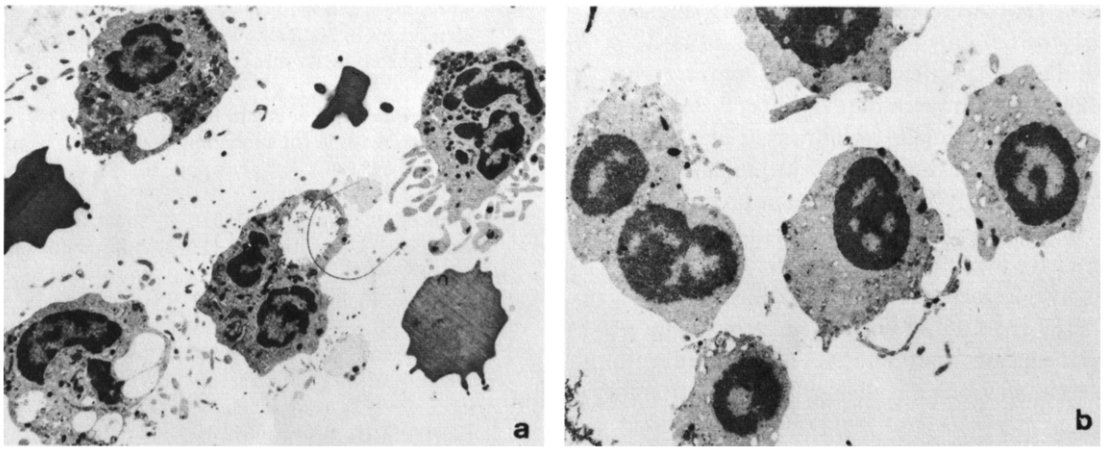

Fig.4. Ultrastructural appearance of PMNL treated with A 23187 and Ca^{2+}. a) 20 μM A 23187, 1 mM Ca^{2+}, 5 min; 4455 ×. b) 20 μM A 23187, 1 mM Ca^{2+}, 15 min; 4050 ×.

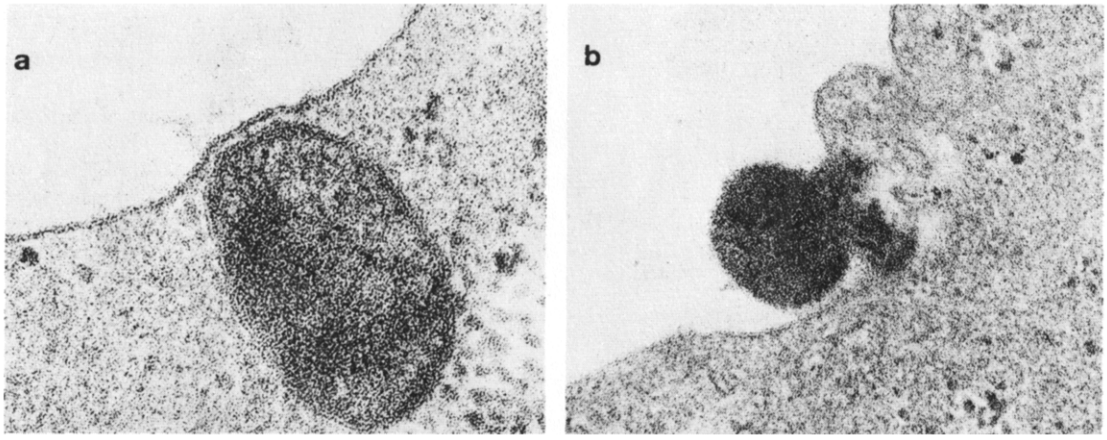

Fig.5. Exocytosis of a granule from PMNL treated with A 23187 (20 μM) and Ca^{2+} (1 mM). a) 141 300 ×; b) 135 900 ×.

battery of enzymes, normally stored in membrane-limited granules, when perturbed by appropriate stimuli [4,10]. The present experiments show that Ca^{2+} in the suspending medium leads to a massive exocytosis, which requires the presence of A 23187. Since this ionophore induces a redistribution of divalent ions across biological membranes, down their concentration gradient, the exocytotic process appears to be induced by a transient elevation of calcium concentration in special zones of the cytoplasm. This would suggest that Ca^{2+} influx from the cell exterior, or simply cell Ca^{2+} mobilization, is required for triggering exocytosis also in other conditions, such as phagocytosis or treatment with ligands interacting with the PMNL surface [4]. This hypothesis is in full agreement with the concepts expressed by Kagayama and Douglas [12], who have studied the Ca^{2+}-dependent degranulation of mast cells 'primed' with A 23187, and by Eimerl et al. [13], who have found that A 23187 and Ca^{2+} are capable of causing specific secretion of digestive enzymes from pancreatic slices.

Our results are contradictory with those of Gold-

stein et al. [14], who have shown that Ca^{2+} induces the exocytosis of only a particular class of PMNL granules, i.e. the specific ones. On the contrary, they are in full agreement with the very recent observations of Smith and Ignarro [15], who further suggest that cyclic GMP and calcium are involved in mediating, whereas cyclic AMP signals the inhibition of, the secretion of lysosomal enzymes from human PMNL. Since we have seen that low concentrations of Mg^{2+} can inhibit the extrusion of granular content provoked by A 23187 and Ca^{2+}, it would be interesting to analyze the influence of both these alkali-earth cations on the accumulation of cyclic nucleotides within the leukocyte.

Acknowledgement

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