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The Dissociation of Exocytosis and Respiratory Stimulation in Leucocytes by Ionophores

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By exploiting the unique characteristics of three ionophores, experimental conditions were found which permit the dissociation of respiratory stimulation from secretion in polymorphonuclear leucocytes. A marked stimulation of respiration was produced by ionophore X537A, which binds and transports both alkali-earth and alkali cations. The stimulatory activity of this ionophore was the same at either high or low Na^+/K^+ ratios in the medium and was virtually unaffected by extracellular Ca^{2+}. A slight stimulation of oxygen consumption was also caused by the K^+-selective ionophore valinomycin and by ionophore A23187, which complexes and transfers bivalent cations. Ionophore X537A and valinomycin were unable to stimulate selective release of granule-associated β-glucuronidase and gradually increased cell fragility, as monitored by increased leakage of lactate dehydrogenase. Ionophore A23187 slightly increased exocytosis of β-glucuronidase. In a Mg^{2+}-free medium, Ca^{2+}, added simultaneously with ionophore A23187, greatly enhanced respiration and secretion of the granule enzyme. If Ca^{2+} was added a few minutes after the ionophore, exocytosis occurred, but no respiratory burst was observed. If the latter experiment was repeated in the presence of extracellular Mg^{2+}, both secretion and respiration were stimulated. This effect was not produced by Mn^{2+} or Ba^{2+}. It is proposed that Ca^{2+} is required for triggering selective secretion of granule enzymes from leucocytes. It is suggested that the activation of oxygen uptake by leucocytes is caused by an intracellular redistribution of cations, which may involve Mg^{2+}-dependent mechanisms.

The process of endocytosis of foreign particles by polymorphonuclear leucocytes (hereafter referred to as 'leucocytes') induces a number of metabolic and structural changes in the phagocyte (Sbarra & Karnovsky, 1959; Hirsch & Cohn, 1960; Weissmann, 1967; Rossi *et al.*, 1972). Typically, the rate of oxygen uptake by leucocytes is enhanced several-fold, and enzyme-laden granules move towards phagocytic vacuoles. On membrane fusion the granule enzymes are secreted both into the vacuoles and into the extracellular fluid. Over the past years evidence has accumulated that these metabolic and structural changes are triggered by signal(s) originating at the level of the phagocyte plasma membrane. In fact, leucocytes can be stimulated in the manner just described by a number of substances (unable to be phagocytosed) interacting with their cell surface (Rossi *et al.*, 1974).

Thus far it has not been possible to establish whether the overall response of leucocytes to these stimuli is initiated by a single signal or by a spectrum of signals with specific and independent components activating a different process. A difficulty in clarifying this issue derives from a previously unresolved problem in separating one type of response from the others in normal leucocytes. Indeed, in general any agent that stimulates the respiration of leucocytes will also induce cell degranulation (Rossi *et al.*, 1974). We observed that exposure of leucocytes to certain antibiotic ionophores results in a stimulation of non-mitochondrial oxidative metabolism of the cells and in the extrusion of granules from them (Romeo *et al.*, 1975; Zabucchi *et al.*, 1975). Similar results have been reported by others (Schell-Frederick, 1974; Smith & Ignarro, 1975). A preliminary indication that ionophores may cause an enhancement of oxidative metabolism without inducing granule extrusion was obtained with the antibiotic X537A (Romeo *et al.*, 1975). This observation has now been further exploited, and by using several ionophores and a variety of experimental conditions, we were able to dissociate the respiratory stimulation from the specific release of granule enzymes. The ionophores used were the *Streptomyces* antibiotics A23187, which binds and transfers bivalent cations (Reed & Lardy, 1972), X537A, which complexes and transports alkaline-earth cations and a variety of

univalent cations as well (Pressman, 1973), and valinomycin, which specifically carries K^+ across membranes (Henderson *et al.*, 1969).

Materials and Methods

Ionophores A23187 and X537A were kindly provided by Dr. R. L. Hamill of Eli Lilly and Co., Indianapolis, IN, U.S.A., and by Dr. J. Berger of Hoffman–La Roche Inc., Nutley, NJ, U.S.A. respectively. Valinomycin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. They were dissolved in A.R.-grade dimethyl sulphoxide.

Leucocytes were obtained from guinea pigs and from the blood of healthy human volunteers. In the former case, animals were injected intraperitoneally with sterile 1.2% sodium caseinate (Sbarra & Karnovsky, 1959) and the peritoneal fluid was collected 16h after the injection. Cells were separated by centrifugation at 400*g* for 10min. Human blood was added to a one-third volume of ACD solution (22.8mM-citric acid, 44.8mM-sodium citrate and 74.1mM-glucose); the diluted blood samples were subsequently mixed with a one-sixth volume of 15% (w/v) high-molecular-weight dextran (Dextran type 200C; Sigma). After gravity sedimentation of erythrocytes for 45min at 37°C, the supernatant was centrifuged at 600*g* for 10min. Leucocytes isolated from both sources were freed from contaminating erythrocytes by hypo-osmotic lysis (30–90s in 0.2% NaCl), and finally suspended in a medium containing 123mM-NaCl, 5mM-KCl, 0.2mM-glucose and either 16mM-sodium/potassium phosphate, pH7.4 (KRP medium) or 16mM-Tris/HCl, pH7.4 (KRT medium). KRP medium contained in addition 1.2mM-$MgCl_2$. As judged by differential counts of May–Grünwald–Giemsa-stained smears, leucocytes were more than 85 and 92% of the total in cell preparations from human blood and guinea pig exudates respectively.

Oxygen uptake was measured with a Clark-type oxygen electrode (YSI, Yellow Springs, OH, U.S.A.) attached to a thermostatically controlled (37°C) plastic vessel. Each sample contained 2ml of cell suspension stirred magnetically. Additions of ionophores (max. 10μl) were made through a narrow puncture in the lid covering the vessel.

For experiments measuring enzyme release two procedures were adopted:

(*a*) Cell suspensions were incubated in disposable test tubes at 37°C in the presence of the appropriate reagents. After various periods of incubation, the reaction mixtures were centrifuged at 400*g* for 10min at 4°C. The cell-free supernatants were separated from the pellets, which were resuspended to the original volume with KRP medium. Disruption of the cells was achieved by sonication (3A, 10s; sonifier from Branson Instruments, Dambury, CT, U.S.A.). Both the supernatants and the disrupted cells were assayed for enzyme content. β-Glucuronidase activity was determined after 4h of incubation with phenolphthalein β-glucuronide as substrate, in the presence of 0.05% Triton X-100 (Gianetto & de Duve, 1955). Lactate dehydrogenase was assayed at 37°C by the method of Bergmeyer *et al.* (1965) with 0.16mM-NADH and 1mM-sodium pyruvate.

(*b*) Cells were incubated in the oxygen-electrode vessel (1×10^7cells/ml) and at suitable intervals portions (100μl) were withdrawn with a microsyringe and transferred to small tubes containing 0.9ml of ice-cold 0.9% NaCl. These tubes were centrifuged in an Eppendorf 3200 centrifuge (Eppendorf Geratebau Netheler und Hinz G.m.b.H., Hamburg, West Germany) at 1500*g* for 1min. Lactate dehydrogenase was assayed in the supernatants and β-glucuronidase in the pellets, treated as described above. This procedure permitted a simultaneous evaluation of oxygen consumption, selective release of granular enzymes (β-glucuronidase) and cell damage (leakage of lactate dehydrogenase).

Results

As illustrated in Fig. 1, the addition of ionophore X537A to guinea-pig leucocytes causes a rapid increase in oxygen uptake (the solvent dimethyl sulphoxide is without effect). The ionophore elicits the same respiratory enhancement whether the KRP medium contains 149mg-ions of Na^+ and 8mg-ions of K^+/litre or 8mg-ions of Na^+ and 149mg-ions

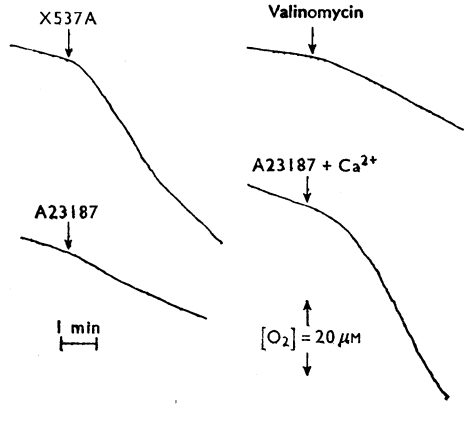

Fig. 1. *Polarographic traces of oxygen consumption by guinea-pig leucocytes after treatment with ionophores*

Leucocytes (2×10^7) were suspended in 2ml of KRP medium (X537A and valinomycin, 50μM) or KRT medium (A23187, 20μM). Oxygen uptake was measured with a Clark-type electrode at 37°C. The concentration of Ca^{2+} was 1mM.

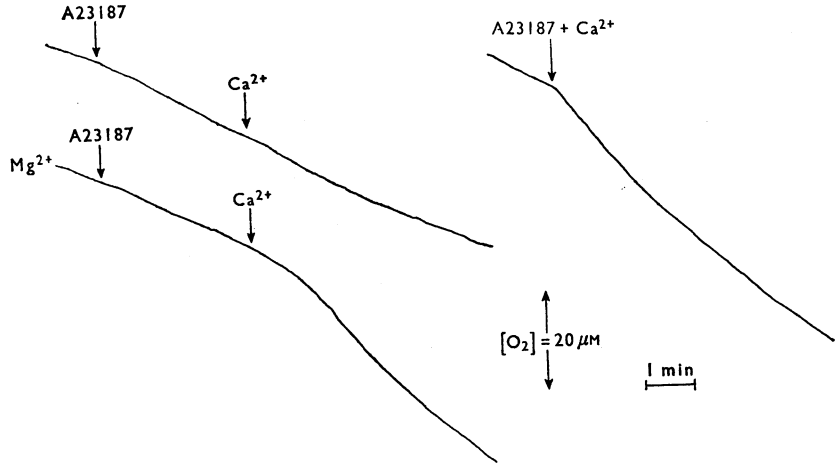

Fig. 2. *Polarographic traces of oxygen consumption by human leucocytes after treatment with A23187 and Ca^{2+}*

Leucocytes (2×10^7) were suspended in 2ml of KRT medium. Oxygen uptake was measured with a Clark-type electrode at 37°C. The concentration of ionophore A23187 and of Ca^{2+} were 10μM and 1mM respectively. Mg^{2+}, if present, was 0.3mM.

Table 1. *Effect of ionophores on release of β-glucuronidase and lactate dehydrogenase from guinea-pig leucocytes*

Leucocytes (1×10^7/ml) were incubated at 37°C in KRP medium (X537A and valinomycin, 50μM) or KRT medium (A23187, 20μM). Controls were run with the solvent dimethyl sulphoxide (0.5%, v/v). The Ca^{2+} concentration was 1mM. The percentage of enzyme activity recovered in the extracellular fluid was measured after separation of cells by centrifugation (400*g*, 10min). Values are means±S.E.M. of four experiments.

	Enzyme released (%)			
Time ...	10min		30min	
	β-Glucuronidase	Lactate dehydrogenase	β-Glucuronidase	Lactate dehydrogenase
Dimethyl sulphoxide	8.1±1.7	12.2±3.8	11.0±3.4	14.1±3.3
Ionophore X537A	17.1±3.4	18.4±3.7	22.3±4.4	31.9±7.6
Dimethyl sulphoxide	4.9±0.7	4.6±0.9	5.5±1.2	7.3±1.5
Valinomycin	6.4±1.5	7.0±0.5	9.4±0.7	13.9±2.7
Dimethyl sulphoxide	8.7±1.9	10.1±1.2	—	—
Ionophore A23187	15.6±3.0	12.0±2.5	—	—
Dimethyl sulphoxide+Ca^{2+}	9.3±1.5	10.1±1.2	—	—
Ionophore A23187+Ca^{2+}	40.7±3.5	12.3±2.7	—	—

of K^+/litre. The addition of 0.5mM-Ca^{2+} together with ionophore X-537A causes a 0–25% potentiation of the metabolic stimulation.

Fig. 1 also shows that the ionophores valinomycin and ionophore A23187 slightly stimulate oxygen consumption by leucocytes. Increasing the concentration of these ionophores induces no further enhancement of respiration. In the presence of extracellular Ca^{2+}, the stimulatory activity of ionophore A23187 is very much potentiated, and becomes similar to that of ionophore X537A. Mg^{2+} up to a concentration of 5mM does not exhibit this effect.

When Ca^{2+} is added to leucocytes 2–4min after the ionophore, no further stimulation of respiration is observed. This is shown in Fig. 2 for human blood leucocytes, but similar results are obtained with guinea-pig cells. If Mg^{2+} is present in the medium, then Ca^{2+} stimulates oxygen uptake by leucocytes 'primed' with ionophore A23187 to an extent comparable with that observed when ionophore and Ca^{2+} are added together. This effect of Mg^{2+} is not obtained with Ba^{2+} or Mn^{2+}.

The ionophores were also tested for their effect on secretion of granule-associated enzymes from leucocytes. As shown in Table 1, all three ionophores cause some release of β-glucuronidase into the medium. However, the release of the granule-associated enzyme induced by ionophore X537A and valinomycin

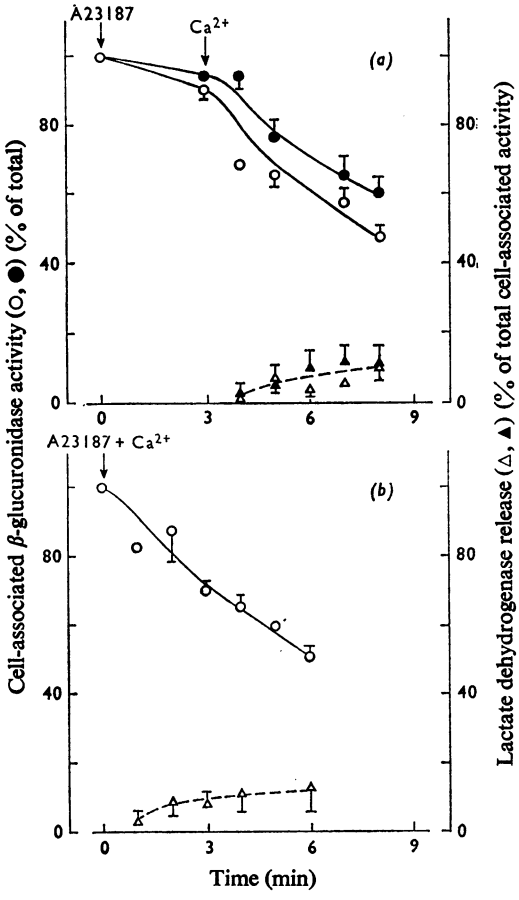

Fig. 3. *Release of β-glucuronidase and lactate dehydrogenase from human leucocytes treated with A23187 and Ca^{2+}*

Leucocytes (10^7/ml) were suspended in KRT medium. At different time-intervals, samples (100 μl) were withdrawn with a microsyringe and added to ice-cold 0.9% NaCl. After centrifugation β-glucuronidase was assayed in the pellets and lactate dehydrogenase in the cell-free medium. Values of β-glucuronidase activity are calculated as percentage of total cell-associated activity before the addition of ionophore A23187 (*a*) or A23187 and Ca^{2+} (*b*). Values for lactate dehydrogenase activity are calculated as percentage of total cell-associated activity released into the extra-cellular medium after addition of Ca^{2+}. The points given are means±S.E.M. of at least three experimental values for each point. Symbols: (*a*) without Mg^{2+} (○, △), with Mg^{2+} (●, ▲), β-glucuronidase (○, ●), lactate dehydrogenase (△, ▲); (*b*) ○, β-glucuronidase, △, lactate dehydrogenase. The concentrations of ionophore A23187 and of Ca^{2+} were 10 μM and 1 mM respectively.

is associated with a leakage of lactate dehydrogenase, which increases with increasing lengths of exposure to the ionophores. The addition of 0.5 mM-Ca^{2+} to the incubation medium, together with ionophore X537A, does not alter the non-selective pattern of enzyme release. Conversely, when added with ionophore A23187, Ca^{2+} causes a marked secretion of β-glucuronidase from leucocytes without significant leakage of lactate dehydrogenase.

The same experimental conditions of Fig. 2 were used to study the effect of ionophore A23187 on the secretion of granule-associated enzymes from human leucocytes. Fig. 3 shows that the ionophore alone has a very small effect, whereas the addition of Ca^{2+} either simultaneously with, or 3 min after, compound A23187 induces a remarkable secretion of β-glucuronidase from leucocytes. After addition of Ca^{2+}, some lactate dehydrogenase activity can also be found in the extracellular medium. This can probably be accounted for by the fact that the cell suspension was continuously stirred and damage to some cells cannot be completely prevented. However, after an initial increase, the leakage of lactate dehydrogenase from leucocytes virtually stops, whereas β-glucuronidase continues to be secreted.

Discussion

The results of the present studies clearly demonstrate that it is possible to stimulate respiration by leucocytes greatly, for example with ionophore X537A, without causing a selective release of granule enzymes. The opposite effect can also be observed, for example by delaying the addition of Ca^{2+} to leucocytes 'primed' with ionophore A23187.

At least some requirements for the stimulation of secretion and of oxygen uptake by leucocytes emerge from an analysis of our data. Ionophore A23187 induces a selective secretion of β-glucuronidase, which on addition of Ca^{2+} is greatly enhanced. It is conceivable that the effects caused by the ionophore alone are linked to a mobilization of Ca^{2+} from intracellular stores (Ashby & Speake, 1975). The role of Ca^{2+} in triggering secretion of granule enzymes from leucocytes is consistent with the known phenomenon of Ca^{2+}-dependent 'stimulus-secretion coupling' that has been observed in various tissues (Foreman *et al.*, 1973; Prince *et al.*, 1973; Cochrane & Douglas, 1974; Eimerl *et al.*, 1974; Grenier *et al.*, 1974; Smith & Ignarro, 1975; Ashby & Speake, 1975; Holz, 1975). Since phagocytosis or treatment with agents modifying the surface properties of leucocytes (Rossi *et al.*, 1974) may lead to granule extrusion in the absence of extracellular Ca^{2+}, it is conceivable that a displacement of this cation from intracellular stores usually provides the necessary signal for promoting granule secretion. The use of an ionophore such as A23187 and extracellular Ca^{2+} *in vitro* may merely amplify a phenomenon that under physiological conditions manifests itself in a more controlled manner.

The mechanism for the stimulation of the oxidative metabolism of leucocytes is more difficult to envisage. A marked and rapid stimulation is observed with ionophore X537A in the absence of extracellular Ca^{2+}. Valinomycin also stimulates respiration by leucocytes, although to a much lesser extent, and at concentrations higher than those usually required for K^+ transport across membranes (Henderson *et al.*, 1969). Whether the metabolic response elicited by these two ionophores depends on translocation of Ca^{2+} and/or alkaline ions from the extracellular fluid into the cytoplasm or vice versa is a question that cannot be fully answered at present. However, some experiments that we have performed to date with X537A offer no grounds for believing that this translocation is important. In fact, the stimulatory activity of ionophore X537A is the same at either high or low Na^+/K^+ ratios in the medium and is virtually unaffected by the presence of 0.5 mM-(extracellular Ca^{2+}). This suggests that the effects of ionophore X537A and valinomycin on respiration by leucocytes may be due to an intracellular redistribution of cations among different compartments.

The same redistribution of ions is apparently triggered by extracellular Ca^{2+} acting in conjunction with ionophore A23187. In fact, when added together these two agents provide a potent stimulus of respiration by leucocytes. However, if the cells, suspended in a Mg^{2+}-free medium, are first treated with ionophore A23187 and then with Ca^{2+}, no stimulation of respiration occurs. Conversely, an enhancement is observed if Mg^{2+}, but not Mn^{2+} or Ba^{2+}, is previously added to the medium before Ca^{2+}. It is likely that in a Mg^{2+}-free medium ionophore A23187 transfers this cation from the cytosol to the cell exterior down its concentration gradient, and that this transfer can be prevented or retarded significantly by low concentrations of extracellular Mg^{2+}. The loss of Mg^{2+} (presumably from specialized sites in the cytoplasm) might explain the decreased response of leucocytes to Ca^{2+}.

In chronic granulomatous disease of children, leucocytes secrete granule enzymes normally, but do not undergo a stimulation of respiration when challenged with bacteria or immune complexes that cannot be phagocytosed (Holmes *et al.*, 1967; Ulevitch *et al.*, 1974). This has been ascribed by Hohn & Lehrer (1975) to a lack of activation of the granule NADPH-oxidizing system, which is responsible for stimulation of respiration by leucocytes (Patriarca *et al.*, 1971; Rossi *et al.*, 1972). Our model system, in which Ca^{2+} is added to cells 'primed' with ionophore A23187, simulates *in vitro* this leucocyte defect. Thus it might be utilized to investigate the molecular mechanism(s) that control the activation of the oxidative metabolism of leucocytes under physiological and pathological conditions.

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