PHAGOCYTOSIS-INDUCED ^{45}CALCIUM EFFLUX IN POLYMORPHONUCLEAR LEUCOCYTES

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1. Introduction

The role of calcium ions in regulating the structure and function of non-muscle cells is a subject of intense study. Several lines of evidence suggest that calcium may be essential in the function of polymorphonuclear leucocytes (PMNL) and an important control element in the process of phagocytosis. We [1] and Romeo et al. [2] reported independently that the production of calcium flux by the ionophore A23187, in the presence of calcium, elicits a stimulation of the oxidative activities characteristic of phagocytic cells, consistent with, though not a proof of, the hypothesis that altered calcium distribution could be a signal or mediator of the phagocytic event. A23187, under the same conditions, has also been shown to induce granular enzyme release [3–5], a process which may be considered analagous to secretion. The calcium dependence of stimulus–secretion coupling has been demonstrated in a variety of cell types [6,7]. Contractile proteins have been demonstrated in PMNL [8,9] and implicated in normal cell movement and phagocytosis [10]. Although Stossel and Hartwig did not find calcium sensitivity in the contractile proteins isolated from alveolar macrophages [11], Shibata et al. reported Ca-sensitivity of equine PMNL actinomyosin [8]. In addition, glycerinated rabbit PMNL have been shown to contract in a Ca-dependent manner in the presence of ATP [12].

Abbreviations: PMNL, polymorphonuclear leucocyte(s). ATP, adenosine-5′-triphosphate

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Direct studies of calcium distribution and fluxes have only recently been undertaken. To our knowledge, no report of calcium movements during normal phagocytosis has been published. In the context of an overall study of calcium dynamics in the PMNL we report here initial studies on ^{45}Ca efflux in prelabelled guinea pig PMNL. The results demonstrate the energy dependence of resting calcium efflux and an increased efflux upon addition of phagocytic particles which is not dependent on particle internalization.

2. Methods

Leucocytes were isolated from the peritoneal fluid of guinea pigs as previously described [1]. The cells were washed twice in the incubation medium containing NaCl 124 mM, KCl 5 mM, Na Hepes, pH 7.3, at 20°C, 20 mM, $NaHCO_3$ 10 mM, $MgSO_4$ 1.25 mM, Na_2HPO_4 1.25 mM, glucose 8 mM and $CaCl_2$ 0.5 mM. Leucocytes (2.0×10^7/ml) were preincubated for 90 min at 37°C in medium containing ^{45}Ca 5–10 μCi/ml (The Radiochemical Centre Amersham). After centrifugation ($40 \times g$, 4 min) the cells were resuspended in fresh non-radioactive medium and further incubated at 37°C for 30 min to allow discharge of loosely associated radioactivity. Following recentrifugation, the cells were resuspended at a cell concentration approx. 1×10^7/ml and distributed in 1 ml aliquots to flasks for incubation. At various times the contents of duplicate flasks were centrifuged in a Fisher rapid-acceleration centrifuge Model 59 at 2000 rev/min for 1 min and the radioactivity measured in an

aliquot of the supernatant. The viability of the cells at the start of the efflux period was established in two ways. Trypan blue was excluded from 94–97% of the cells in all preparations. The rate of phagocytosis of suspensions of paraffin oil–Oil Red 0 particles was unchanged as compared to freshly prepared cells or cells kept at 0–4°C during the preincubation and wash periods. For measurement of ^{45}Ca efflux independent of particle internalization, labelled, washed cells were deposited on Millipore filters previously treated with aggregated γ-globulin or albumin according to the methods of Henson [13] and the appearance of radioactivity in the medium measured as described above. β-glucuronidase activity was measured in an aliquot of the supernatant medium according to Fishman after 18 h incubation [14]. Total β-glucuronidase was measured in an aliquot of cells suspended in medium containing Triton X-100 0.2%. Adenosine triphosphate was determined by the method of Stanley and Williams [15]. Zymosan (Sigma Chemical Co., St Louis, MO) was preopsonized by incubation in 20% pooled guinea pig serum for 20 min at 37°C, centrifuged and resuspended in Hepes medium. Iodoacetic acid (IAA) and antimycin A were purchased from Sigma (St. Louis, MO).

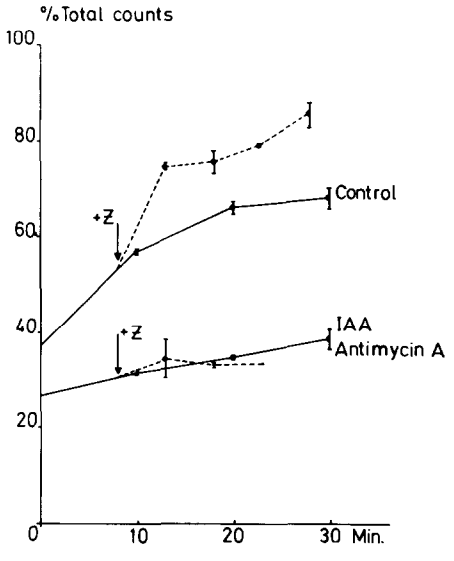

Fig.1. Release of ^{45}Ca from prelabelled guinea pig PMNL. Radioactivity recovered in the medium is expressed as percent of total counts present at the beginning of the efflux period (0 min). Total counts control curve: 8643 ± 353. Total counts in the presence of iodoacetate (IAA) 5 mM and Antimycin A 2 μg/ml: 4716 ± 92. Each point represents the mean and range of duplicate determinations in a representative of 5 experiments. Zymosan (- - - -) was added 8 minutes after the start of the efflux period.

3. Results

The release of ^{45}Ca into the medium under resting conditions is shown in fig.1. This efflux is nearly completely abolished when iodoacetate (5 mM) and antimycin A (2 μg/ml) are included in the wash medium. When these inhibitors of energy metabolism are added at the beginning of the efflux period, inhibition of basal ^{45}Ca efflux is observed after 15–20 min (data not shown). This is consistent with the time course of ATP depletion. After 15 min, ATP levels in treated leucocytes are decreased from 3.2 nmol/10^7 cells to 0.23 nmol and after 30 min to 0.046 nmol/10^7 cells.

Phagocytic particles provoke a sharp, short-lived increase in ^{45}Ca efflux within the first minutes following their addition (fig.1). Within 5–10 min the efflux curve in the presence of zymosan is again parallel to the control curve. Iodoacetate and antimycin A completely inhibit this effect. Simultaneous measurement of β-glucuronidase activity in the medium demonstrates a parallelism between the release of ^{45}Ca and enzyme activity.

However, when leucocytes are labelled at 0°C washed at 37°C and the efflux measured at 37°C, zymosan provokes a far smaller or no efflux of ^{45}Ca but retains its stimulatory effect on β-glucuronidase release (fig.2). IAA and Antimycin A are also less effective in inhibiting basal ^{45}Ca release. It should be noted that washing of cells at 0°C releases none of the label taken up by the cells during exposure to ^{45}Ca at either 37°C or 0°C.

The stimulation of ^{45}Ca efflux by phagocytic particles does not depend on phagosome formation and ingestion. Aggregated gamma globulins incorporated into Millipore filters provoke increased ^{45}Ca release when compared with filters pretreated with bovine serum albumin (fig.3).

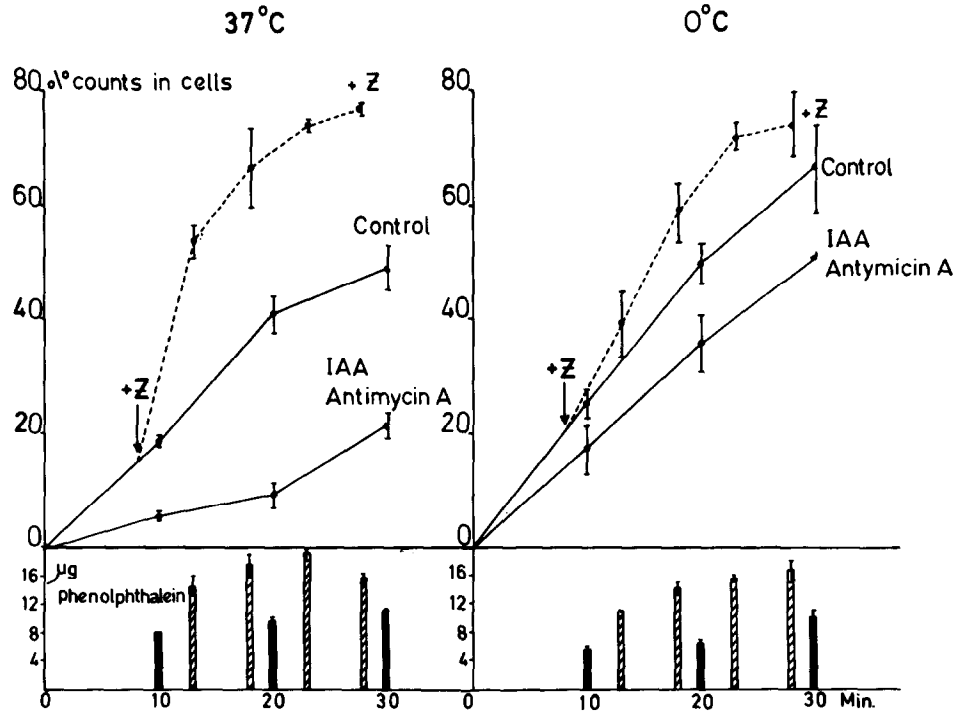

Fig.2. Comparison of ^{45}Ca release and β-glucuronidase activity. Radioactivity appearing in the medium is expressed as percent of counts in the cells at the beginning of the efflux period (0 min). Counts in cells are calculated by subtracting counts present in the medium at 0 min from total counts present in an aliquot of the cell suspension. In this representative of 5 experiments:

Preincubation	Total counts	Cell counts
37°C	4448 ± 122	2921
37°C + IAA Anti A	3339 ± 53	2316
0°C	2882 ± 14	1709
0°C + IAA Anti A	3036 ± 111	1838

β-glucuronidase activity is expressed as µg phenolphtalein/18 h incubation in an aliquot of resting cells (■) and cells phagocytizing zymosan particles (▯). Total activity: 57 + 2 µg phenolphthalein/18 h.

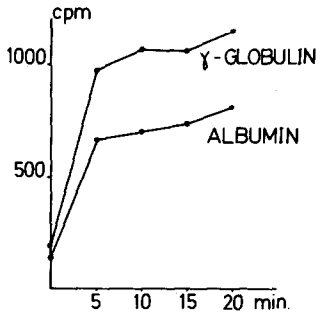

Fig.3. Release of ^{45}Ca from prelabelled guinea pig PMNL in the absence of phagosome formation. At the beginning of the efflux period aliquots of 0.5×10^6 cells were deposited on duplicate Millipore filters into which aggregated γ-globulin or albumin had been incorporated [13]. A representative of 3 experiments is shown.

4. Discussion

The evaluation of calcium dynamics poses important problems. Although the study of ^{45}Ca movements in intact cells at 37°C allows measurement of only two compartments, extracellular and 'cell-associated', it is erroneous to assume such a simple distribution in view of the ample evidence for one or more intracellular sites of active calcium sequestration in most if not all cell types. In addition, it is difficult to distinguish truly intracellular Ca^{2+} from calcium which may adhere non-specifically to the cell surface. We have used a long labelling period (90 min) and wash period (30 min) in an attempt to favor equilibrium of label among intracellular pools and to eliminate non-specific ^{45}Ca. Our results do not allow us to determine if true equilibrium has been reached. However, the nearly complete inhibition of basal ^{45}Ca release by energy inhibitors suggests that we are measuring calcium extrusion and not non-specific exchange at the cell surface (fig.1). This is the first demonstration that calcium efflux in the polymorphonuclear leucocyte is an active, energy-requiring process. As noted above, washing at 0°C removes no radioactivity, thus complicating and possibly invalidating efflux studies performed with this washing procedure.

Normal particle phagocytosis induces increased ^{45}Ca release into the medium (fig.2). However, this release does not require internalization of the particle (fig.3), suggesting again that the contact between particle and cell surface induces physicochemical changes in the membrane and/or the formation of one or more biochemical mediators, which, in turn, trigger the activation of the cell. The observed phagocytosis-induced calcium release undoubtedly represents one of several calcium fluxes and may be the resultant of a number of such movements. The origin and significance of this release are not yet established. It seems unlikely that calcium extrusion to the exterior of the cell serves as a trigger of the phagocytic event. However, other as yet unmeasured calcium movements, e.g., increased calcium entry or release of calcium from internal stores could play this role in the leucocyte.

When PMNL are labelled at 37°C, phagocytosis provokes a parallel increase in the appearance of ^{45}Ca and β-glucuronidase activity in the external medium (fig.2). These results have led to the tentative hypothesis that the calcium released by phagocytosis may originate in the leucocyte granules (at least the azurophil granules) and appear in the medium by exocytosis. If this hypothesis is valid, the observed calcium efflux could be analogous to the final step in a stimulus–secretion coupling which may in itself be calcium dependent.

When PMNL are labelled at 0°C two important differences are noted (fig.2): (1) Control ^{45}Ca release is poorly inhibited by iodoacetate and antimycin A. (2) The increase in ^{45}Ca release induced by phagocytosis is markedly diminished, but exocytosis persists.

These findings indicate an altered distribution of ^{45}Ca, a large fraction being associated with the cell surface and reappearing in the medium by non-energy-requiring exchanges. One would expect pools of energy-dependent calcium sequestration to be weakly labelled after preincubation at 0°C. The possibility that zymosan is releasing calcium from actively labelled leucocyte granules is currently being investigated.

Direct studies of the effects of chemotactic substances on ^{45}Ca movements have recently been reported [16–18]. Differences in experimental protocol, which may be important in evaluating the results render comparisons difficult both between these reports and in relation to the findings reported here. Nevertheless, these authors have all found increased ^{45}Ca efflux in response to a variety of chemotactic attractants.

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