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Single pulses of cytoplasmic calcium associated with phagocytosis of individual zymosan particles by macrophages

Laura C. Meagher, Baljit S. Moonga*, Christopher Haslett,
Christopher L.-H. Huang* and Mone Zaidi*[1]

Department of Medicine, Respiratory Unit, Hammersmith Hospital,
London W12 ONN UK

*Department of Cellular and Molecular Sciences, St. George's Hospital Medical School,
London SW17 ORE, UK

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We have measured cytosolic free calcium levels ($[Ca^{++}]_i$) in individual macrophages during the phagocytosis of single zymosan particles. We report here that the contact of a macrophage with zymosan results in a rapid transient elevation of $[Ca^{++}]_i$. Each $[Ca^{++}]_i$ pulse is symmetrical lasting for up to 30 seconds. In contrast, macrophage spreading is associated with repetitive $[Ca^{++}]_i$ spiking occuring in salvos of up to four smaller spikes, each lasting for between 8 and 18 seconds. These qualitative and kinetic differences might suggest that the role of $[Ca^{++}]_i$ in phagocytosis is distinct from its role in spreading. © 1991 Academic Press, Inc.

Intracellular calcium ($[Ca^{++}]_i$) is an important second messenger for a range of phagocytic functions, including phagocytosis itself (1), chemotaxis (2) and degranulation (3). In neutrophils, both temporal and spatial changes of $[Ca^{++}]_i$ occur during phagocytosis (4, 5). Although particle ingestion by the neutrophil is a $[Ca^{++}]_i$-independent step, $Ca^{++}{}_i$ transients triggered upon contact with particulate stimuli seem to be necessary for the control of events, such as phagosome-lysosome fusion (6). Thus, the increments of $[Ca^{++}]_i$ are often regional and can be strategically located (4). Kruskal and Maxfield (1986) have shown that macrophages, during "frustrated" phagocytosis, exhibit an oscillatory pattern of $[Ca^{++}]_i$ increment. As an extension of these earlier studies (7), we performed measurements of $[Ca^{++}]_i$ on single macrophages during the process of actual phagocytosis of particulate material. We report here, that each observed phagocytotic event in the macrophage was accompanied by a single rapid and transient pulse of $[Ca^{++}]_i$. The phenomenon we report, appears to be qualitatively and kinetically distinct from the oscillatory pattern of $[Ca^{++}]_i$ elevation observed during spreading.

[1]Corresponding author.

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MATERIALS AND METHODS

Culture method: Macrophages were derived from the bone marrow of excised murine femurs of Balb/c mice. The cells were flushed out from the bone and were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, Scotland, UK) containing foetal calf serum (FCS; Gibco; 25%, v/v) and L-929-conditioned DMEM (25%, v/v). The cells were then plated at an initial density of 0.5×10^6 ml^{-1} onto 21-mm glass coverslips placed in a 6-well plastic culture dish (Flow Laboratories, UK). The dishes were incubated at 37°C for between 7 and 10 days in humidified 7.5% CO_2. For the experiments, cells were washed free of culture medium and were incubated, at 37°C, with the calcium-sensitive dye indo 1-AM (1 μM) (Molecular Probes, San Diego, USA) diluted in Hank's Balanced Salt Solution (HBSS) for 40 minutes.

Measurement of $[Ca^{++}]_i$: The coverslips were transferred to a perspex bath mounted onto a heated stage of a dual emission microspectrofluorimeter constructed from an inverted microscope (Diaphot; Nikon, Telford, UK). The cells were exposed to an excitation wavelength of 340 nm and the fluorescent signal directed to the sideport which contained a 455 nm-dichroic mirror. The transmitted and reflected light was filtered at 480 nm and 405 nm, respectively, and the emission intensities recorded by separate photomultiplier tubes (PM28B, Thorn EMI, Middlesex, UK). Single photon currents were counted by a photon counter (Newcastle Photometric Systems, U.K.). Photons s^{-1} in each channel were recorded in an IBM microcomputer and the ratio of intensities (F405/F480) calculated and displayed. $[Ca^{++}]_i$ was then determined from a standard curve derived by a procedure used for extracellular calibration (8).

Phagocytosis: Zymosan (Sigma, Poole, Dorset, UK) was washed repeatedly, resuspended in HBSS without phenol red (100 mg l^{-1}) and maintained at 37°C for use in the experiments. Macrophages on coverslips were selected for $[Ca^{++}]_i$ measurements and the baseline $[Ca^{++}]_i$ recorded. Zymosan (50 μl) was then added to 1 ml of the bathing medium (HBSS) and observed whilst the particles settled next to the selected macrophage. Recording was commenced as the macrophage and zymosan particle were juxtaposed and was continued for between 1 and 5 min, after which phagocytosis was assessed microscopically. Cells containing one or more particles were also occasionally selected for further phagocytosis of juxtaposed particles. Macrophages were also monitored whilst spreading in the absence of zymosan.

RESULTS

Single $[Ca^{++}]_i$ pulses during phagocytosis: Well-spread macrophages were selected and repeated runs were made to look for episodes of spontaneous $[Ca^{++}]_i$ changes. Figure 1A shows that in resting macrophages, departures from baseline levels were not observed. Figure 1B shows two traces during phagocytosis of zymosan, in each case, of a single particle. One of these two selected macrophages did not (left trace), whilst the other (right trace) did contain a zymosan particle before recording was started. Under both conditions, we observed a rapid and transient elevation of $[Ca^{++}]_i$. When the pre-pulse, peak and post-pulse $[Ca^{++}]_i$ levels were computed and analysed by the Analysis of Variance, the mean peak $[Ca^{++}]_i$ was found to be about 1.8-fold greater than either the mean pre-peak or mean post-peak $[Ca^{++}]_i$ ($p = 0.012$ and 0.016, respectively) (Table 1). Each pulse was symmetrical and in each case, $[Ca^{++}]_i$ returned to a level that was not significantly different from basal ($p = 1.0$). Finally, no significant difference was noted between $[Ca^{++}]_i$ of cells containing zymosan particles compared with those that had failed to ingest zymosan. Zymosan particles themselves, did not fluoresce either in the presence or absence of the dye.

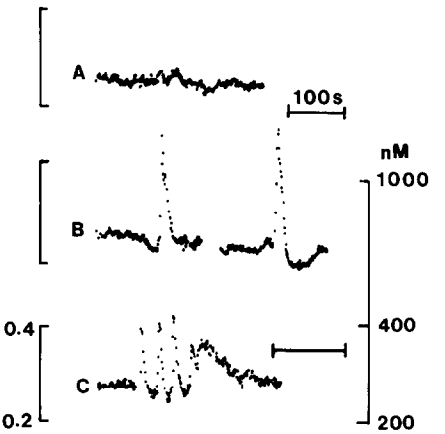

Figure 1.

Cytosolic free calcium ($[Ca^{++}]_i$) levels of macrophages during phagocytosis of zymosan particles or during spreading on non-opsonised surface. Panel A represents a $[Ca^{++}]_i$ trace of a fully spread macrophage not in contact with zymosan. Panel B shows two representative traces showing $[Ca^{++}]_i$ pulses during the early phase of phagocytosis by macrophages that either contained (right trace) or did not contain (left trace) another zymosan particle. Panel C represents the oscillatory pattern of $[Ca^{++}]_i$ increment during the spreading of a macrophage in the absence of a particulate stimulus. The left lower vertical axis represents the fluorescence ratio (F405/F480) which corresponds to the logarithmic $[Ca^{++}]_i$ scale on the right.

Oscillations in $[Ca^{++}]_i$ during cell spreading: When macrophages were observed during the process of spreading, periodic bursts of $[Ca^{++}]_i$ pulses were observed. Upto four spikes per cell were recorded, and in some instances, repetitive spikes fused to produce a more sustained rise of $[Ca^{++}]_i$ (Figure 1C). The lowest $[Ca^{++}]_i$ between peaks was often below the minimum $[Ca^{++}]_i$ for that cell, but when data was analysed by the Analysis of Variance, no significant differences were noted between mean pre-

TABLE 1

Function	Pre-peak (nM)	Peak (nM)	Post-peak (nM)	Duration (sec)
Cell spreading	219 ± 7.46	294 ± 18.8	217 ± 7.42	13.6 ± 1.49
Phagocytosis	228 ± 8.21	390 ± 43.1	228 ± 18.9	27.8 ± 3.83

Mean cytosolic free calcium ($[Ca^{++}]_i$) levels of macrophages during the processes of spreading on glass or during phagocytosis of zymosan. The mean minimal pre-peak, peak and minimal post-peak $[Ca^{++}]_i$ values (nM, ± standard error of mean) have been derived by computing the fluorescence ratio of individual pulses at these times. The mean pulse duration (sec, ± standard error of mean) is the mean time-difference between the minimal pre-peak and minimal post-peak value computed for each pulse. The p values for various comparisons, derived from Analysis of Variance, are indicated in the text (n = 13 and 5, respectively).

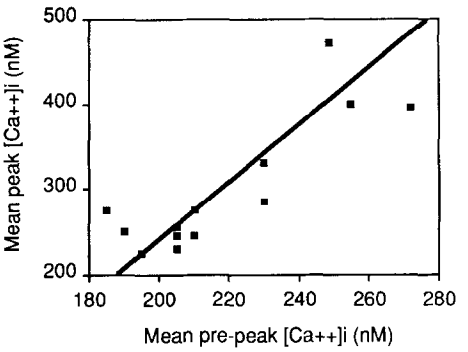

Figure 2.

Correlation between the mean peak $[Ca^{++}]_i$ and mean pre-peak $[Ca^{++}]_i$ (derived as in Legend to Table 1) of macrophages during spreading. The line is the least-square fit to the $[Ca^{++}]_i$ values (correlation coefficient: $r = 0.826$, $p < 0.01$).

peak and post peak $[Ca^{++}]_i$ ($p = 0.744$). Peak $[Ca^{++}]_i$ was approximately 1.4-fold higher than pre-peak $[Ca^{++}]_i$; this difference was highly significant ($p < 0.001$). No autocorrelation of peak $[Ca^{++}]_i$ was observed, for example, a tall peak was not followed by a short one or vice versa.

Further analysis of $[Ca^{++}]_i$ responses: There were some notable differences observed between the $[Ca^{++}]_i$ pulses due to phagocytosis and those observed during spreading. The mean height of the $[Ca^{++}]_i$ peak attained during phagocytosis was significantly ($p = 0.024$) greater than the peak height measured during cell spreading. The mean duration of the zymosan-associated phagocytotic pulses was also significantly greater ($p < 0.001$) than that of $[Ca^{++}]_i$ oscillations associated with spreading (Table 1). The peak and pre-peak $[Ca^{++}]_i$ values for pulses associated with phagocytosis did not significantly correlate ($r = 0.228$, $p = 0.718$), in contrast to those associated with spreading where a strong correlation was observed ($r = 0.826$, $p < 0.01$) (Figure 2). Finally, a weak, but statistically significant ($r = 0.4$, $p < 0.05$) correlation was also observed, in individual pulses, between peak height and pulse duration.

DISCUSSION

Earlier studies have reported that oscillatory increments of $[Ca^{++}]_i$ occur in periodic bursts during macrophage spreading (7). However, to our knowledge, it is for the first time, that single calcium pulses have been reported to occur during the phagocytosis by macrophages, of individual zymosan particles. Similar phenomenon have been widely reported for neutrophils. We found that a rapid and transient elevation of $[Ca^{++}]_i$ occurs during contact of a macrophage with a zymosan particle (4 - 6). Following this, the particle is ingested, whilst $[Ca^{++}]_i$ returns to levels indistinguishable from basal. In contrast, when $[Ca^{++}]_i$ was monitored whilst macrophages were

allowed to spread in a "frustrated" attempt to phagocytose the substrate without coming into contact with zymosan, we consistently observed bursts of repetitive $[Ca^{++}]_i$ spikes.

There are interesting differences between the two patterns of spiking behaviour. Zymosan-induced pulses are predictably elicited on particle contact, are always single and are of a longer duration and larger magnitude. In contrast, the oscillatory pattern associated with spreading is less predictable, consists generally of multiple bursts of spikes, each of which is of a relatively shorter duration and smaller magnitude. Secondly, whilst we observed a strong correlation between minimum (or pre-peak) $[Ca^{++}]_i$ and peak $[Ca^{++}]_i$ in the case of oscillations (confirming previous results, 7) no such correlation was seen in case of zymosan pulses. It has been suggested that the two values are correlated because each depends on the abundance of Ca^{++} in the intracellular pools (7). A lack of such a correlation could therefore mean the involvement, as in the neutrophil (4), of external calcium.

It is also notable that although repetitive $[Ca^{++}]_i$ spiking occurs widely (9), it has not been observed in any other phagocytic cell. In excitable cells including sinoatrial cells, pituitary cells, parabolic burster neurones and insulin-secreting β-cells, oscillations are thought to mediate response rythmicity (9). However, in non-excitable cells, including macrophages (7), hepatocytes (10), juxtaglomerular cells (11), fibroblasts (12) and endothelial cells (13), oscillations have a less defined role. Somewhat more generally, spiking has also been observed during cell motility, cell division and lymphocyte activation.

The significance of oscillations observed in the spreading macrophage is unknown, except for assumptions that spreading is akin to "frustrated" phagocytosis. As for phagocytosis, the role of receptor-mediated $[Ca^{++}]_i$ elevation has been extensively studied for the neutrophil (4 - 6). It has been clearly demonstrated that $[Ca^{++}]_i$ elevation is not necessary for the actual ingestion of particles (6). This is consistent with our observation that $[Ca^{++}]_i$ does not remain elevated during ingestion. Nevertheless, it now seems clear that early $[Ca^{++}]_i$ elevation achieved on particle contact is important in the phagocytic event that follows. It is therefore essential that we understand the temporal relationship between the $[Ca^{++}]_i$ pulse and events of functional significance which it may mediate during phagocytosis. One such event could be phagosome-lysosome fusion (6). The latter is known to be calcium-dependent and to be sensitive to alterations of $[Ca^{++}]_i$, probably from within distinct regions of the cell. Thus, further studies attempting to characterise spatial variations (15) of $[Ca^{++}]_i$ after particulate stimulation of the macrophage would seem necessary to fully understand the role of $[Ca^{++}]_i$ in phagocytosis.

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