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Localised and rapid Ca^{2+} micro-events in human neutrophils: Conventional Ca^{2+} puffs and global waves without peripheral-restriction or wave cycling

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Abstract

Ultra-localised and peripherally restricted zones of elevated Ca^{2+} (*z*-waves) have been reported to cycle around the periphery of neutrophils at low frequency (1/20 s) in the absence of conventional localised Ca^{2+} (puffs) and global Ca^{2+} (waves) signals. However, we report here that fast confocal laser scanning of human neutrophils loaded with either cytosolic fluo4 or its membrane associated analogue, MOMO reports both "conventional" stationary Ca^{2+} "puffs" (diameter c.3 μm) and global Ca^{2+} waves that sweep across the cell. The Ca^{2+} puff size and frequency of detection suggests that each neutrophil contained only a single release site and that its detection was limited by the location of the confocal plane relative to the event. Both formylated peptide receptor stimulation and cytosolic IP_3 uncaging generated Ca^{2+} puffs (c.6% of cells) and global Ca^{2+} signals (c.75% of cells). The Ca^{2+} puffs peaked at approx. 250 nM and had a duration of approx. 235 msec and remained at a single locus. This was similar to other Ca^{2+} events in other cell types but in direct contrast to the reported *z*-waves. It was concluded that the micro-events which underlie Ca^{2+} signalling in neutrophils are conventional and that the existence of novel Ca^{2+} *z*-waves is doubtful.

Keywords: Calcium; Neutrophils; Phagocytosis; Signalling

1. Introduction

Changes in cytosolic free Ca^{2+} plays an important role in the activation of a number of cells, including neutrophils. It is now well established that there are elemental events that underlie the Ca^{2+} signal. These include localized Ca^{2+} events restricted to part of the cell, such as the granular cytoplasm of pancreatic acinar cells [1–3] and also very restricted Ca^{2+} sparks and puffs. The latter can be best visualised when Ca^{2+} is released from storage sites in muscle cells and oocytes [4,5] and there are surprisingly few reports of similar micro-events in small and non-excitable cells. The most well characterised Ca^{2+} puff events in small cells are probably in HeLa cells [6–8] and in a rat basophilic cell line [9]. The scarcity of reported Ca^{2+} puff events in smaller non-excitable cells may be due to (i) the technical difficulties in rapid Ca^{2+} imaging in small cells or (ii) the absence or attenuation of such signals resulting from the higher density or closer proximity of organelles which release, sequester or impede diffusion of Ca^{2+} ions. The "simplified" microanatomy of human neutrophils, which have few mitochondria and only a single "vestigial" ER/Golgi organelle near the multi-lobed nucleus [10], should be beneficial in detecting these localised Ca^{2+} micro-events. It is therefore intriguing that conventional Ca^{2+} release "puffs" were reportedly absent in this cell type [11–13] and replaced by extremely localised and circularly mobile zones of elevated Ca^{2+} (called here zonal or *z*-waves). Using ultrafast imaging of indo-1 loaded neutrophils with acquisition times of 50 nsec, Petty's group have reported that there is an extremely localised zone of elevated Ca^{2+}, which travels around the periphery of unstimulated, polarised neu-

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trophils in a "clockwise" direction at regular intervals of 20 s [11–13]. When stimulated with the IP_3-generating, G-protein linked receptor agonist, f-mlp (f-met-leu-phe), the "*z*-wave" is reported to split into two opposite travelling zones. This group found no other Ca^{2+} signals in neutrophils, such as conventional stationary Ca^{2+} puffs or global cytosolic changes. The group have also reported that Ca^{2+} *z*-waves underlies regular Ca^{2+} spiking in a number of cell types with different micro-anatomical lay-outs including CHO cells [14] and HT1080 fibrosarcoma cells [15]. These data obviously present a major challenge to the conventional views of Ca^{2+}signal generation. Since neutrophils are the cell-type in which Ca^{2+} *z*-waves but not conventional Ca^{2+} signalling have been most commonly reported by this group [11–13], it is possible that its micro-anatomy may play a part in this type of Ca^{2+} signalling. We have therefore sought to extend the characterisation of the reported Ca^{2+} *z*-waves in neutrophils and to correlate them with the location of organelles within the cell. However, in contrast to the reports from Petty's group, we have found that neutrophils have both "conventional" stationary Ca^{2+} "puffs" and global Ca^{2+} signals in neutrophils. The Ca^{2+} puffs we have found were similar in magnitude and duration to those reported in HeLa and basophilic cells [6,9]. We were, however, unable to detect any evidence for the reported Ca^{2+} *z*-waves using either cytosolic mobile or membrane immobilised Ca^{2+} indicators.

2. Materials and methods

2.1. Cell preparation and probe loading

Human neutrophils, isolated from the blood of healthy volunteers as described previously [16] were suspended in Krebs medium (NaCl 120 mM, KCl, 4.9 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.3 mM, HEPES 25 mM and bovine serum albumin, 0.1% adjusted to pH 7.4 with NaOH) and loaded with the fluorescent Ca^{2+} probes fluo3, fluo4 (Molecular Probes) or MOMO (Teflabs) by incubation of with actoxyl methyl esters (1–5 μM) as described in detail elsewhere [17]. This produced cytosolic concentration of the probes at 50–100 μM. When loading neutrophils with both Ca^{2+} probe and (caged)-IP_3, the caged IP_3-PM ester (Alexis Biochem) was incubation was started before adding the acetoxylmethyl ester compound. Loading with caged compounds was done in the dark to prevent uncontrolled uncaging by ambient light. The times for loading of the fluorescent probes used in this paper were as follows: fluo4-AM, 40 min; IP_3 (caged)-PM, 1 h; MOMO, 3 h (37 °C); mitotracker Red (0.1 μM; Molecular Probes), 1 h; lysotracker green (0.1 μM, Molecular Probes), 30 min, and acridine orange (Sigma), 1 min. Neutrophils were also microinjected with Ca^{2+} probes (not esterified) and caged IP_3 using SLAM injection [18]. This method uses lipid coated microinjection pipette, which minimises damage to the neutrophil membrane and cytoskeleton [18,19].

2.2. Ca^{2+} measurement and manipulation

Cytosolic free Ca^{2+} was measured as described in detail elsewhere [20] using the resonant scanning head of the Leica RS confocal microscope, which had a frequency of approximately 40 MHz and was restricted to a field size of 32 × 512 pixels. Bi-directional data acquisition was used after manual adjustment of the phase to ensure synchronised data alignment in both directions. Neutrophils were allowed to adhere to the glass coverslip mounted onto a thermostatically control stage (37 ± 0.1 °C) in Krebs medium and soluble stimuli such as f-mlp (Sigma) were added to them during a data acquisition run. During image acquisition, uncaging was achieved by positioning of an additional dichroic mirror and band pass filter to illuminate the microscopic field with UV (330–300 nm) light (430 DCLPO2 dichroic; Omega Optics) from a 100 W mercury arc lamp [21]. It was shown, using caged fluorescein as a surrogate read-out, that this procedure generated photolytic product linearly with no initial time delay.

2.3. Data analysis

Adjustments to gain and offset were applied to whole time series data sets to allow genuine comparisons within a time sequence. Ratio images (*F*/*F*0) were taken to negate differences in Ca^{2+} probe loading, but this never revealed Ca^{2+} signals that were not evident in the raw fluorescent intensity data set. Localised Ca^{2+} puffs were identified visually and confirmed by statistical comparison of the "noise" in different locations in the cell. In some examples, the localised Ca^{2+} change at the "puff" location was plotted after subtraction of the intensity from the signal at "non-puff" cellular location. In this way, the effect of the rising background signal due to Ca^{2+} influx was nullified and the local Ca^{2+} signal alone recorded. All data shown were representative of at least three different experimental runs from at least three different blood donors as the source of neutrophils.

3. Results

3.1. Regular spontaneous Ca^{2+} spiking not detected in adherent neutrophils

The ability of Petty's group to detect the Ca^{2+} *z*-wave depended on the very regular cytosolic free Ca^{2+} "pulses" that were observed in neutrophils regularly at 20-s intervals. By beginning ultrafast (sub-microsecond) imaging immediately before the onset of a Ca^{2+} pulse, the Ca^{2+} *z*-wave was imaged as it travelled around the cell periphery. However, although sporadic Ca^{2+} spiking can occur during adhesion or in motile neutrophils on some surfaces [22,23], and occasional spontaneous Ca^{2+} spikes are recorded, we found no regular Ca^{2+} signals in unstimulated adherent neutrophils, whether spherical or spontaneously polarised. This is in agreement with