Biochemical and Biophysical Research Communications 457 (2015) 341–346

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Active calpain in phagocytically competent human neutrophils: Electroinjection of fluorogenic calpain substrate

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ARTICLE INFO

Article history:
Received 16 December 2014
Available online 7 January 2015

Keywords:
Neutrophils
Phagocytosis
Calpain
Electroinjection

ABSTRACT

Calpain has been implicated in the apparent expansion of neutrophil plasma membrane that accompanies cell spreading and phagocytosis. In order to test this hypothesis, an internally quenched fluorescent peptide substrate of calpain-1 which increased in fluorescence on cleavage, was microelectroinjected into neutrophils. The fluorescence intensity increased in a significant number of neutrophils, including those which appeared to be in a morphologically resting (spherical) state. In order to test whether calpain was activated by an elevation of cytosolic Ca^{2+} during the injection, Ca^{2+} chelators were added to the injectate and cytosolic free Ca^{2+} in the receiving neutrophil was simultaneously monitored. It was shown that this approach could be used without raising Ca^{2+} within the injected cell. Despite this, approximately 75% of individual neutrophils had calpain activity which consumed the substrate within approx. 100 s. It was found that all neutrophils had elevated calpain activity were phagocytically competent; whereas neutrophils with low or undetectable calpain activity failed to undergo phagocytosis. This association was consistent with the hypothesis that calpain activity within neutrophils was necessary for them to undergo efficient phagocytosis.

1. Introduction

Neutrophils are white blood cells which act as the first line of defence against invading pathogens. When “spreading “ on the endothelial cells which line blood vessels or undergoing phagocytosis, there is an apparent large expansion of the plasma membrane [1,2], increasing to 200% of it spherical (non-activated) area. It has been suggested that, since the plasma membrane has insufficient lateral stretch, there must be a reservoir of addition membrane which is called on to permit this expansion. There is accumulating evidence that cell surface wrinkles and “micro-ridges” represent this reservoir [3–5]. The wrinkles may be held in place by ezrin, which is also called cytovillin because of its location in “villi”, by linking the plasma membrane with the underlying cortical actin network [6–8]. Since ezrin has a calpain cleavage site between these two binding domains – an actin binding domain and a FERM domain [9], there may be a role for calpain in releasing the wrinkles and permitting phagocytosis. This is a particularly attractive proposal as it has been known for nearly 20 years that an elevation of cytosolic Ca^{2+} within neutrophils accompanies the process of spreading out [10]. More recently it has been shown that an elevation of Ca^{2+} alone, either by photolytically uncaging caged Ca^{2+} [11] or caged IP_3 [5], triggers neutrophil spreading. There is also a well-established link between Ca^{2+} signalling and phagocytosis by neutrophils [12–15]. It has been shown that neutrophils give a strong Ca^{2+} signal after the phagocytic cup has formed, which is obligatory for completion of phagocytosis [13]. Both neutrophil spreading induced by elevated cytosolic Ca^{2+} and completion of phagocytosis is prevented by inhibition of calpain activity [5,13].

Calpains are cytosolic cysteine proteases which are activated by Ca^{2+} [16]. They are involved in a number of cellular processes, including integrin-mediated cell migration, cytoskeletal remodelling, cell differentiation and apoptosis. μ-calpain is activated by Ca^{2+} concentrations of 5–50 μM, concentrations which can be detected just beneath the neutrophil plasma membrane during Ca^{2+} influx [17,18] and may be restricted to the intra-wrinkle space

Abbreviations: EGTA, ethylene glycol tetraacetic acid; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal.

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http://dx.doi.org/10.1016/j.bbrc.2014.12.113
0006-291X/

[19]. μ-calpain activation may thus be involved in linking Ca^{2+} influx to localised proteolysis of ezrin (or other cytoskeletal proteins) which maintains the wrinkled surface of neutrophils.

A major problem in testing this suggestion, is that these events cannot be studied in broken cells or cell lysates but require the cell by alive and functional. Although there are some reports of monitoring calpain activity in live cells (eg [20,21]), the calpain substrates used were necessarily membrane permeant and very short peptides or single amino acid fluor constructs which provide little discrimination between proteases and no discrimination between calpain-1 and calpain-2. An FRET-based approach has been reported in which a peptide with specificity for calpain-1 is labelled with a fluor and one end and a fluorescent quencher at the other [22]. This peptide has low fluorescence (due in FRET quenching), but on cleavage by calpain, the quenching is relieved and the molecule emits brightly. The problem for cell biology is, of course, that the peptide must be introduced into the cytosol of the living cell. Neutrophils are extremely difficult to microinject by traditional methods [23,24]. Here we use a method based on single-cell electroporation, which has previously been used for the delivery of genetic material into individual cells in intact tissue [25,26] and has been shown to be effective in neutrophils without disturbing their ability to undergo morphological changes, such as cell spreading [27]. We use this approach here to introduce a FRET calpain peptide substrate and demonstrate that calpain activity can be detected in some apparently morphologically resting neutrophils in the absence of an injection–induced Ca^{2+} signal and that all phagocytically competent neutrophils have elevated calpain activity.

2. Materials and methods

2.1. Human neutrophil isolation

Human neutrophils were isolated from blood donated by healthy volunteers, as previously described [13]. Neutrophils were resuspended in Krebs buffer (120 mM NaCl, 4.9 mM KCl 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM Hepes and 0.1% BSA, adjusted to pH 7.4 with NaOH stored at 4 °C) and allowed to sedimented onto a glass coverslip for observation by confocal microscopy.

2.2. Single cell electroinjection

Micropipettes were pulled from borosilicate glass capillaries (1.0 mm outer diameter, 0.5 mm inner diameter, 10 cm length) using a laser micropipettepuller (P-2000; Sutter Instrument, California, US). Pipettes were then loaded with 3–5 μl of the injectate solution and a silver wire of 0.25 mm diameter was passed down through the pipette and into the solution before the micropipette next to the cell of interest (InjectMan, Eppendorf, Hamburg Germany). The circuit was completed by a second wire immersed in the medium bating the cell. Pulses were applied (SD9 Square Pulse Stimulator, Grass, RI, USA)) at a frequency of 200 pps with a "voltage ramp" (20 V–50 V) being terminated when injection was successful. This ensured that the minimum voltage was applied for electroporation. Material to be injected were dissolved in the injection buffer HEPES (50 mM), EGTA (10 mM) pluronic (0.1%).

2.3. Fluorescent materials

Fluorogenic calpain-1 substrate (H-Lys(FAM)-Glu-Val-Tyr-Gly-Met-Met-Lys(Dabcyl)-OH) was purchased from Calbiochem (UK). Alexa633 hydrazide, bis(triethylammonium) salt and fluo4-AM were purchased from Life Technologies.

3. Results

3.1. Elevated calpain activity in spherical neutrophils

As we have previously reported, calpain activity could be detected in neutrophils following electroinjection of the FRET calpain substrate [27]. In order to establish whether the electroinjectin event could cause the activation, the dynamic of electroinjection were further studied.

3.2. Dynamics of neutrophil electroinjection

Alexa633 was electroinjected into neutrophils as a traceable, but inert, fluor, so that the dynamic characteristics of single cell electroporation and recovery could be demonstrated (Fig. 1). At low voltage, the fluor was ejected from the micropipette (by iontophoresis) to produce a "cloud" of extracellular fluorescence (Fig. 1A). At this voltage, the fluor failed to enter the cell as the voltage was below the threshold for electroporation. As the voltage was increased, more fluor was ejected (see Fig. 1B "extracellular" signal) and at a critical point fluor rapidly entered the cell. Indicating that electroporation had occurred. When the voltage was switched to zero, iontophoresis stopped and the extracellular concentration of fluor decreased due to diffusion. However, the concentration of fluor in the cell reached its equilibrium approximately 2 s later (Fig. 1C). This suggested that the pore generated by the voltage remained permeable to the extracellular fluor for about 2 s before closing. The total open time for the electroporation pore was estimated to be approximately 3 s in a typical experiment. While this is long enough to Ca^{2+} ions to enter the cell down its electrochemical

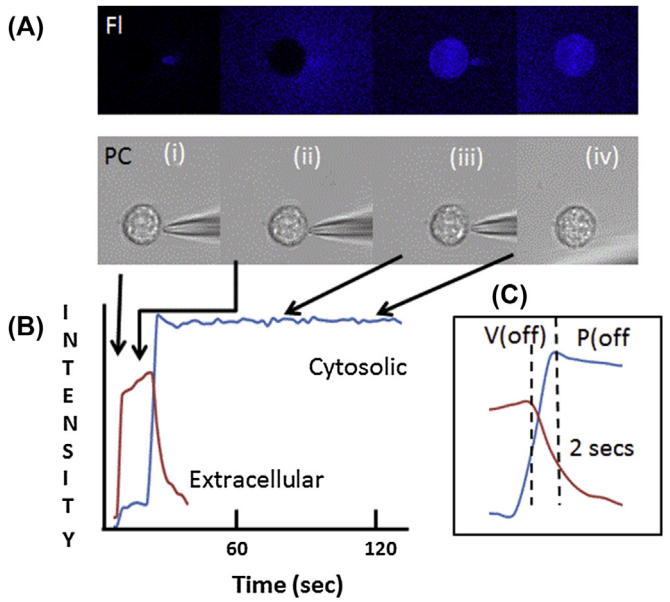

Fig. 1. Electroinjection of human neutrophils. The sequence of electroinjection inert fluor (Alexa 633) is shown in panel (A) where the lower row (labelled PC) shows the phase contrast image of the cell undergoing electroinjection with the position of the micropipette and the upper row (labelled FL) shows the fluorescent signal from Alex633 loaded into the micropipette. In image (i) no voltage was applied, (ii) the voltage ramp was begun and fluor is ejected into the extracellular space, (iii) shows breakthrough into the cell interior and (iv) shows repositioning of the micropipette away from the cell. In section (B) the time course of changes in intensity of fluor in the extracellular space and inside the cell is shown with the timing of the images shown in panel (A) indicated. In section (C) the detailed time course at the point at which the voltage ramp was switched off (Voff) showing the simultaneous decrease in extracellular fluorescence but the 2 s lag before the cytosolic fluorescence increase ceases as a result of closure of permeability pores (Poff).

concentration gradient. Since calpain would be activated by elevated levels of cytosolic Ca^{2+}, it was important to quantify the effect of electroporation on cytosolic Ca^{2+} before using this approach to establish the activity of calpain in resting cells.

3.3. Control of cytosolic Ca^{2+} during electroinjection

In order to monitor cytosolic Ca^{2+} in neutrophils undergoing electroinjection, cells were loaded with the Ca^{2+} indicator fluo4, before injection with alexa633. Without any Ca^{2+} buffering in the ejectate, there was a dramatic effect of electroinjection on elevating cytosolic Ca^{2+} (Fig. 2A). Following the injection pulse, cytosolic Ca^{2+} rapidly rose to a peak before returning an elevated equilibrium level. In experiments where a second injection was undertaken, cytosolic Ca^{2+} was elevated still higher and cell lysis is observed with the release of fluo4 (presumably due to the toxicity of high cytosolic Ca^{2+}). Clearly, Ca^{2+} homoeostasis was significantly disturbed and these conditions would be unsuitable for monitoring the activity of Ca^{2+} dependent processes (such as calpain activation).

However, the disturbance to cytosolic Ca^{2+} was minimised by the inclusion of the Ca^{2+} buffer EGTA in the ejectate, so that the cell would be temporarily bathed in a reduced Ca^{2+} medium immediately before electroporation. As the maximum concentration of chelator in the medium at the pore site will be the concentration within the pipette, the effect on reducing local Ca^{2+} can be estimated. Using Max chelator software [28,29], with the total extracellular Ca^{2+} at 1.3 mM and Mg^{2+} at 1.2 mM, EGTA (1 mM) the extracellular Ca^{2+} concentration would reduce to approximately 300 μM, thus lowering the inward driving gradient, but not eliminating it. Under these conditions, the cytosolic Ca^{2+} rose transiently, returning to the original equilibrium within 30 s (Fig. 2B). As an elevation in cytosolic Ca^{2+} remained, higher concentrations of EGTA were tested. At EGTA (10 mM), the extracellular Ca^{2+} was estimated to be reduced to 47 nM and under these conditions electroinjection caused a transient decrease in cytosolic Ca^{2+} (Fig. 1C), as expected because the extracellular Ca^{2+} would be below the resting cytosolic Ca^{2+} level in neutrophils, approximately 100 nM [30] and the concentration gradient would thus be reversed. Interestingly, with a single injection, the cytosolic Ca^{2+} returned to its pre-injection equilibrium within 15 s, whereas the recovery was slower to a subsequent injection (Fig. 1C). This suggests that EGTA had also entered the cell and increased its cytosolic Ca^{2+} buffering capacity. This conclusion was strengthened by the inclusion of EGTA (100 mM) in the injectate, which was expected to reduce the extracellular free Ca^{2+} further to only 3 nM. The injection again caused a decrease in cytosolic Ca^{2+} as expected. However, under these conditions, there was a significantly slower recovery time of approx 50 s. The longer recovery could be explained by the injection of significant amounts of EGTA into the cell under these conditions, so that passive Ca^{2+} influx would have to overcome the additional intracellular Ca^{2+} buffer before returning to the equilibrium level (100 nM Ca^{2+}).

It was concluded that the addition of EGTA (10 mM) to the injectate did not disturb cytosolic Ca^{2+} significantly and that, as cytosolic Ca^{2+} was not elevated during electroinjection, could not inadvertently activate calpain. It is unlikely that lowering cytosolic Ca^{2+} transiently (approx. 15 s) could activate calpain, as there is no known mechanism by which lowering Ca^{2+} below 100 nM would activate the enzyme which has a Kd of tens of micromolar [16].

3.4. Calpain activity in spherical neutrophils

Neutrophils were chosen for elctroinjection of fluorogenic calpain substrate peptide, which did not have a “spread” morphology, and were not motile or actively forming and retracting pseudopodia. These cells were approximately spherical and appeared at rest. However in the majority of cells chosen (approx. 75%), there was an increase in fluorescence from the calpain substrate on electroinjection, which was interpreted as calpain activity. The remaining cells injected had no detectable calpain activity. In the majority of neutrophils, nearly all the calpain substrate was converted to the fluorescent form within 100 s. By co-injecting alex633, it was confirmed that the increase in fluorescence of the FRET calpain substrate was not an optical artefact [31] as it occurred only with the substrate fluorescent signal and not the control fluor (Fig. 3A). Furthermore, the rise in fluorescence fitted the kinetics of a 1st

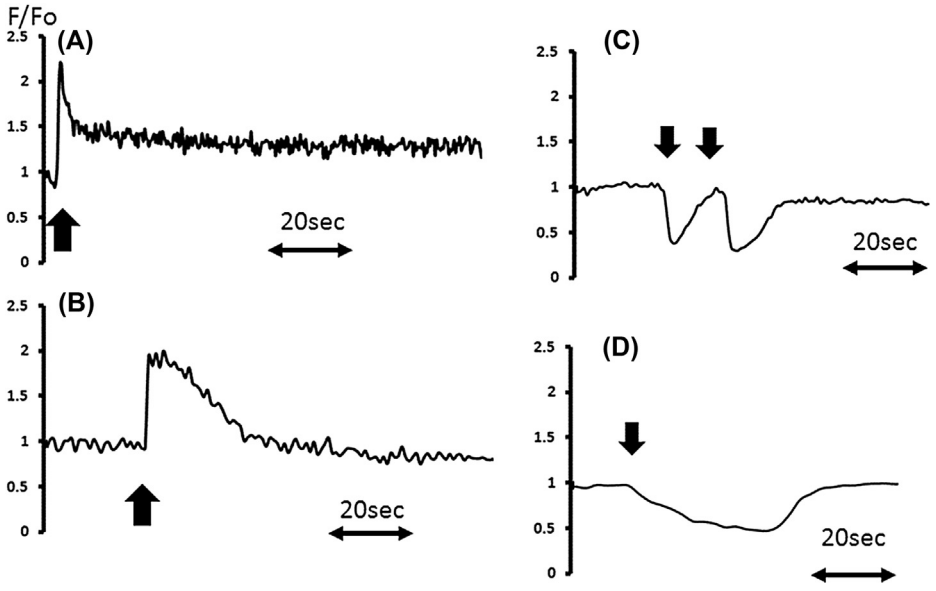

Fig. 2. Changes in cytosolic free Ca^{2+} during electroporation. The figures show the changes in fluo4 intensity loaded within neutrophils (as F/F0) during electroinjection. In all cases the incubation medium contained 1.3 mM Ca^{2+} but the solution ejected from the micropipette contained EGTA (A) 0 mM, (B) 1 mM), (C) 10 mM) and (iii) 100 mM. It is calculated the free Ca^{2+} in the extracellular space adjacent to the electroporation pore would be (A) 1.3 mM, (B) 300 μM, (C) 47 nM and (D) 3 nM. The point at which electroporation was achieved is indicated by the arrow. In (C) two injections were performed to demonstrate the robustness of the return to equilibrium after electroinjection.

order reaction; $I_t = I_{max}(1-e^{(-kt)})$, where k is the rate constant (Fig. 3C). The rate constant for calpain activity in these neutrophils was 0.034 s^{-1} ± 0.003. This was reduced by preteatment with conventional calpain inhibitors (Fig. 3D), including ALLN which reduced it to 0.0079 s^{-1} ± 0.0079. It was concluded that the increase in fluorescence from cytosolic calpain substrate was the result of proteolysis by calpain activity in many morphologically resting neutrophils.

3.5. Calpain activity and phagocytic neutrophils

In neutrophils, with elevated calpain activity it was possible to induce phagocytocytosis by delivery of iC3b-opsonised zymosan [13,15]. However, as 99% of the calpain substrate was consumed within 100 s, it was not possible to detect an additional elevation during a subsequent phagocytic event. In contrast, cells with little or no calpain activity (and hence having significant uncleaved calpain substrate remaining) phagocytosis could not be induced. This suggested the possibility that neutrophils with calpain activity were "primed and ready" for phagocytosis, whereas those without calpain activity were not. This proposal was tested by incubating neutrophil populations with opsonised zymosan particles and measuring calpain activity in neutrophils by electroinjection of calpain substrate subsequently. A comparison of the calpain activity in neutrophils which were phagocytically competent could thus be directly compared with those cells which were poor at phagocytosis (Fig. 4C). Non phagocytic neutrophils had detectable calpain activity with a mean rate of 0.02071 s^{-1} ± 0.001267, whereas cells which have undergone phagocytosis prior to injection had a calpain activity of 0.032 s^{-1} ± 0.003. Although this data could not be paired (ie measuring the rate before and after in the same cells), an unpaired t-test reached a significance level of p = 0.0565.

As the calpain activity measured in phagocytosis-competent cells was similar to the subset of neutrophils with elevated calpain activity in the "resting" state, these data suggested that elevated calpain activity was a prerequisite for phagocytosis-competency by neutrophils. This approach was unable to establish whether calpain activity was transiently elevated further by phagocytosis-induced Ca^{2+} signalling as these measurements were not possible by this approach.

4. Discussion

The data presented here relied on electroinjection to gain access to the interior of living neutrophils. We show that although Ca^{2+} chelators in the injectate prevented an elevation cytosolic Ca^{2+}, cytosolic calpain activity was detectable even in apparently resting spherical neutrophils. This observation was unexpected as it could be argued that cells which had not spread out prior to injection were expected to exhibit no calpain activity. However, calpain activation can result in auto-proteolysis, resulting in the generation of a catalytically active calpain fragment which is active independently of Ca^{2+} [16]. This could be generated in some neutrophils as a result of activation prior to commencement of the experiment. This activation could have occurred within the circulation, or on contact with the glass coverslip or inadvertently during the isolation procedure. Whatever the cause, if calpain were activated to generated the Ca^{2+}-independent form of calpain, these cells would retain a "biochemical memory" of this by having cytosolic calpain activity, as detected here. Constitutive calpain activity in neutrophil populations was also reported in an earlier study using flow cytometry [21]. This suggests (i) that generalised cytosolic calpain activity is not detrimental to neutrophil biology and (ii) that activation of calpain strategically located near its cytoskeletal

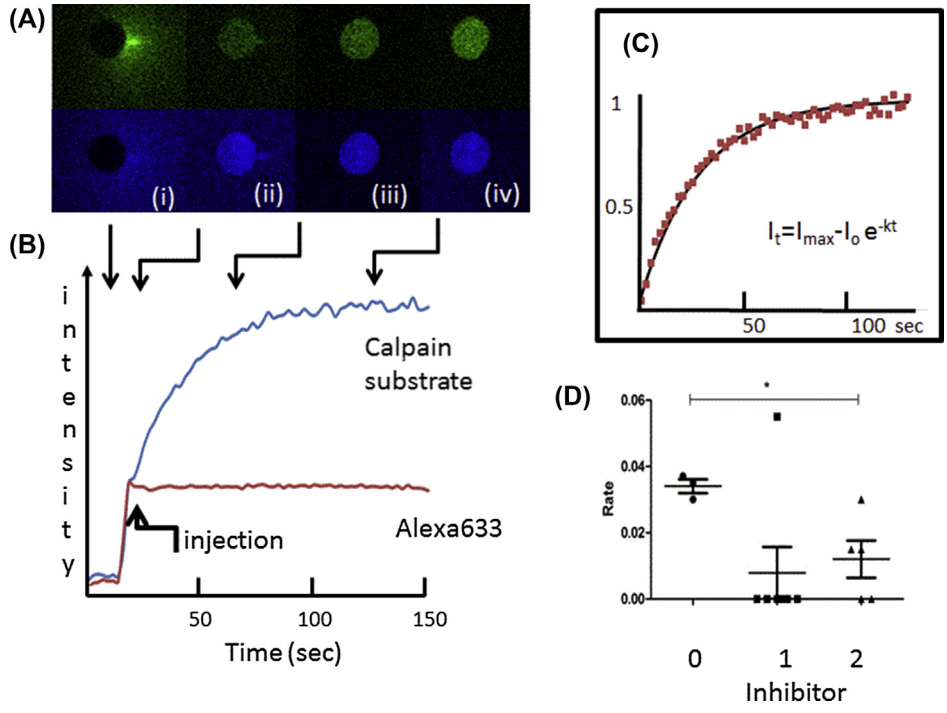

Fig. 3. Measurement of calpain activity in neutrophils. The figure shows the fluorescence intensity of cytosolic fluorogenic calpain peptide substrate as a measure of calpain activity in living neutrophils. (A) shows the sequence of images of a human neutrophil undergoing electroinjection with both Alexa633 (shown in blue) and calpain FRET substrate (shown in green). The injection solution included 10 mM EGTA so that cytosolic Ca^{2+} was held a lower than physiological during the injection. (B) show the time course of changes in the fluorescence intensity of the two fluors. In (C) the ratio of the intensity of the two fluors was calculated after injection and plotted with the theoretical curve for a first order reaction with the rate constant t set at (0.03 s^{-1}). The effect of calpain inhibition on the measured calpain activity (rate constant, s^{-1}) is shown in (d), where the neutrophils were pre-treated either without inhibitor (0) or with the calpain inhibitor ALLN (1) or ALLN and calpeptin (2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

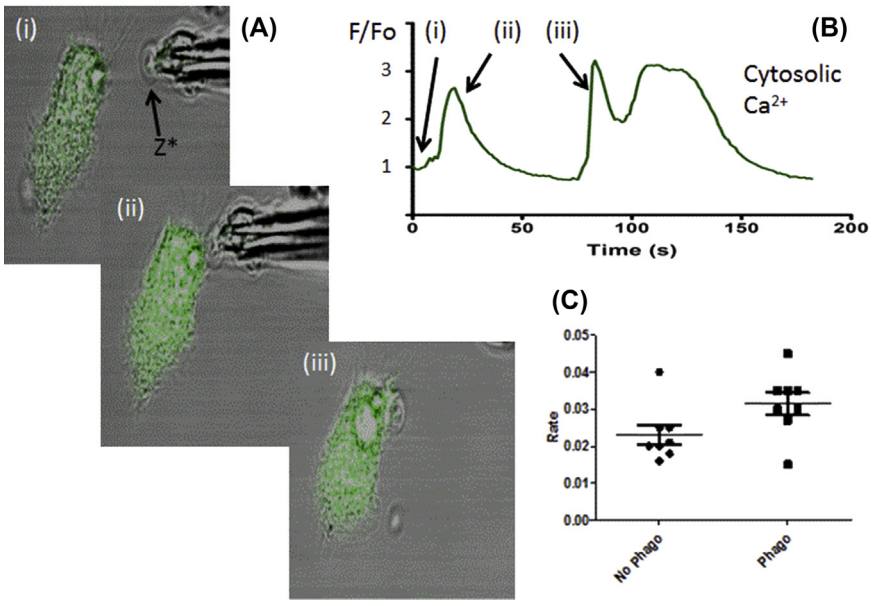

Fig. 4. Ca^{2+} signalling and calpain activity in human neutrophils undergoing phagocytosis. The figure shows the complex Ca^{2+} signal which accompany phagocytosis of C3bi-opsonsied zymosan particles and the effect on calpain activity. In panel (A) images are shown of the delivery of an opsonised zymosan particle (labelled Z*) by a micropipette with the intensity of fluo4 overlaid. In section (B) the complete time course of the changes in cytosolic Ca^{2+} (given as fluor4 F/F0) is shown with the location of the 3 images indicated where (i) is the before contact with the particles; (ii) is after contact and (iii) is during internalisation. In section (C) the calpain activity measured at the rate constant (s^{-1}) is shown for neutrophils which had not undergone phagocytosis ("No phago") and those cells which had undergone phagocytosis ("Phago").

substrates, perhaps within surface wrinkles, is the more important signalling component.

Unfortunately, active calpain within neutrophils cleaved the electroinjected calpain substrate at a fast rate, and the amount of injected cytosolic fluorogenic calpain substrate was limited by cell volume (diam approx. 10 μm), this study was unable to provide a temporal link between the elevation of cytosolic Ca^{2+} which accelerates phagocytosis [13]. However, the approach permitted demonstration of a clear association between elevated calpain activity and phagocytic competency of individual cells. This association was consistent with calpain activity in neutrophils being permissive for effective phagocytosis.

Conflict of interest

None.

Acknowledgments

We thank all blood donors and phlebotomists, particularly Maha Alujmaa and Gareth Willis, without whom this work would not have been possible; and also Iraj Laffafian for his help with cell isolations. JSC would also like to acknowledge the financial support of a WHRI Visiting Scholarship.

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