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Minimal impact electro-injection of cells undergoing dynamic shape change reveals calpain activation

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ABSTRACT

The ability of neutrophils to rapidly change shape underlies their physiological functions of phagocytosis and spreading. A major problem in establishing the mechanism is that conventional microinjection of substances and indicators interferes with this dynamic cell behaviour. Here we show that electroinjection, a "no-touch" point-and-shoot means of introducing material into the cell, is sufficiently gentle to allow neutrophils to be injected whilst undergoing chemokinesis and spreading without disturbing cell shape change behaviour. Using this approach, a fluorogenic calpain-1 selective peptide substrate was introduced into the cytosol of individual neutrophils undergoing shape changes. These data showed that (i) physiologically elevated cytosolic Ca^{2+} concentrations were sufficient to trigger calpain-1 activation, blockade of Ca^{2+} influx preventing calpain activation and (ii) calpain-1 activity was elevated in spreading neutrophil. These findings provide the first direct demonstration of a physiological role for Ca^{2+} elevation in calpain-1 activation and rapid cell spreading. Electroinjection of cells undergoing dynamic shape changes thus opens new avenues of investigation for defining the molecular mechanism underlying dynamic cell shape changes.

1. Introduction

An essential feature of the way in which motile and phagocytic cells behave, is their capacity for rapid changes in cell shape, often resulting in a doubling of the cell surface area [1]. It has been suggested that the additional membrane for this comes largely from the unwrinkling of cell surface folds [1,2] which are formed by proteins such as ezrin linking the plasma membrane to the underlying cortical actin network. Many of these proteins are sensitive to cleavage by the cytosolic Ca^{2+} activated protease calpain [3,4] and since an elevation of cytosolic Ca^{2+} can trigger rapid cell spreading [5–7], it has been speculated that calpain-1 activation provides the signalling link. However, calpain-1 activation requires 10–50 μM Ca^{2+}, yet cytosolic Ca^{2+} in spreading cells transiently only reaches a max of 1 μM. Theoretical modelling of Ca^{2+} within the wrinkles suggests that within this microdomain, Ca^{2+} concentration may reach sufficiently high for localised calpain activation [8]. Calpain inhibitor studies have pointed to a link between calpain activation and neutrophil shape change behaviour [9,10], and the possibility that calpain was constitutively active in these cells [10]. However, it is difficult to investigate whether this occurs physiologically as fluorogenic peptide substrates which are selective for calpain-1 must be micro-injected into the cytosol of cells. Conventional microinjection is prone to serious problems which hamper its use in this way for investigating dynamic cell shape changes, and usually results in an immediate retraction and cessation of cell motility [11,12]. Since the micropipette tip enters the cell during stabbing at velocities in the region of 700 μm/s [13], it is likely to displace, damage, or enter organelles. Excessive pressure required to expel the contents of the micropipette into the cell can also cause significant impairment of chemokinesis and phagocytosis, probably because the "inflation" of the cell reduces wrinkles on the cell surface. The SLAM (soft lipid-assisted microinjection) technique, where contact between a phospholipid coating on the micropipette and the plasma membrane allows fusion and results in transfer of material from within the micropipette into the cell cytosol, negating both the need for the micropipette to enter the cytosol and thus for high pressures, has been used successfully on cells which are otherwise difficult to microinject [14,15]. However, the fusion requires a close contact between the two bilayers for several (often tens of) seconds [13], and is not usable with rapidly moving cells or those undergoing other dynamic shape changes. We have therefore investigated a previously described "no touch" approach based on localised electroporation [16–18], which we found surprisingly benign and could be used in a number of cell types, including neutrophils engaged in chemokinesis without affecting their motile behaviour.

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2. Materials and methods

2.1. Electro-injection

Borosilicate glass capillaries (1 mm outer diameter and 0.5 mm inner diameter; with filament) were pulled with a Sutter Instrument P-2000 laser-operated micropipette puller creating a tip diameter of 0.8–1 μm (by optical inspection) and an inner opening diameter of 0.4–0.5 μm. The micropipette was back-filled with appropriate loading solution (approx 1–2 μl). A silver wire (0.25 mm diameter) was passed through the micropipette holder, into the micropipette and into the loading solution, and connected to a voltage stimulator terminal (Grass SD9). The opposite terminal was connected to a second silver wire, which was fixed in place in the cell-containing medium on a microscope slide. The micropipette was positioned next to the target cell (preferably within 1 μm) using a micromanipulator (InjectMan Eppendorf) and electroporation initiated by a 1 s train of pulses (1 ms square pulses; 10–50 V; 200 Hz).

2.2. Imaging and Ca^{2+} measurement

Cultured cells were grown on glass-bottomed petri dishes and neutrophils were attached to glass coverslips and maintained at 37 °C as previously described [31,32]. Human neutrophils, isolated from the blood of healthy volunteers as described previously [7,21,25] 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). Neutrophils were allowed to settle onto clean, non-coated glass coverslips maintained at 37 °C [19,20]. The imaging parameters were set before the electro-injection micropipette was manoeuvred into position and images were acquired using either convention or confocal microscopy. The relative amount of fluorescent material ejected from the micropipette and injected into the cell was monitored by the relative fluorescence intensities. The concentration of material ejected was also estimated by comparing the fluorescence intensity with the (known) concentration of material which is in the pipette. When required, formyl-met-leu-phe (1 μM) was added directly onto the cells to give a step change in concentration. Superimposed fluorescence and phase contrast images were created using Leica software and quantitative data was extracted using a measurement "region of interest" within the cell either using ImageJ or Leica software. The mean intensity of the cytosolic fluorogenic calpain-1 substrate was quantified by restricting the region of interest within the perimeter of the cell. When required, cells were pre-loaded with fura-red from its AM ester as previously described [30,31] and the change in Ca^{2+} concentration monitored from the intensity decrease using the standard Tsien equation [33]. For simultaneous Ca^{2+} and calpain measurement, images were acquired sequentially (acquisition parameters changed between lines) to avoid cross talk between the signals in each channel.

2.3. Materials

Lucifer yellow was purchased from Sigma-Aldrich. FuraRed-AM was purchased from Molecular Probes (Invitrogen). Calpain-1 substrate Fluorogenic calpain-1 substrate (H-Lys(FAM)-Glu-Val-Tyr-Gly-Met-Met-Lys(Dabcyl)-OH) was purchased from Calbiochem.

3. Results

The micro-injection technique used here involved passing controllable electrical voltage pulses from the open tip of a small bore micropipette (containing the molecules to be injected) through the cell to be injected as described by Haas et al. [16–18]. The voltage pulses will cause a localised and transient electroporation of the cell membrane and since many molecules also carry a charge, provided the electrical polarity is in the appropriate direction, the voltage pulses will also have an ionotophoretic effect forcing molecules out of the pipette synchronously with the opening of the electroporation pore. As the electroporation effect is dependent on the membrane curvature [22], it is selective for the larger radius of curvature of the cell membrane over the smaller curvatures of intracellular organelles. Single cell electroporation of this type was first described using two carbon fibre filaments [23] and GFP-expressing plasmid to show successfully transfection. Haas and Cline [16,17] extended this approach by using micropipettes as a method for transfecting neurones *in vivo* with GFP-expressing plasmids and for introducing macromolecules into cells *in vivo* [18] and *in vitro* [24]. This approach proved to be surprisingly gentle and simple, resulting in a no-touch (point and shoot) method for introducing material into the cell cytosol with minimal impact of cell shape change dynamics. For convenience, we have used the term "electro-injection" to describe the outcome of this procedure.

3.1. Transfer of material to the cytosol by electro-injection

In order to optimise the electro-injection transfer process, the cell impermeant dye, Lucifer yellow, was used as a marker of injection in the "stationary" cell line, 3T3 cells. With appropriate pulse parameters, the success rate of transfer into these immobile cells was very high, with 90–100% successfully injected cells. Since 3T3 cells are reasonably flat, the lateral distance between the cell and micropipette tip could be measured. It was estimated that effective electroinjection required the distance of micropipette tip from the cell to be within 1.25 μm (± 0.12 μm; n = 27). The cytosolic concentration as Lucifer yellow was approximately the same as ejectate (92–115%; n = 35) giving a final cytosolic concentration of 1% that within the micropipette (Fig. 1a).

3.2. Transfer to small and potentially motile neutrophils

Resting neutrophils, which were attached to the substrate but stationary, could also be easily electro-injected and were no obvious consequences of the injection process (Fig. 1b). In a study, electroinjection (10 V square pulse train; 1 ms 200 Hz; for 0.5 s) was 89.5% successful: 10.5% unsuccessful: 0% lysis (n = 19). The subsequent cell spreading behaviour in response to a formylated-met-leu-phe (f-mlp) of neutrophils which had been successfully electro-injected, was unaltered with symmetrical spreading showing no effect localised to the injection locus (Fig. 1b and Supplementary Movie 1). The fmlp-induced Ca^{2+} signal also remained intact and could be monitored in neutrophils previously loaded with fura red by acteoxymethyl ester loading (Fig. 1c).

3.3. Effect of electro-injection on cell changing shape dynamics

Given the apparently benign affect of electro-injection, human neutrophils were allowed to adhere, polarise and undergo spontaneous motility before attempting electro-injection. As we found that the micropipette need not touch the cell for effective electro-injection, it was possible that, provided the micropipette could be placed sufficiently close to the moving cells, motile neutrophils could also be injected. Although it was more difficult to estimate the tip cell distance in bright field images (due to the 3D character of the spherical cell and changing morphology of motile cells), it was estimated from confocal slice images that the maximum distance at which electroinjection could occur was about 3 μm. After fluorescent material was transferred to neutrophils, they remained motile and had similar characteristics to non-injected cells (Fig. 2 and Supplementary Movie 3). There was also no detectable effect on the motile behaviour of individual cells before and after electro-injection whilst in the process of chemokinesis, the rate of movement before (0.436 ± 0.19 μm/s; n = 14) and after (0.431 ± 0.069 μm/s; n = 14) microinjection not being significantly different ($p > 0.9$; paired *t*-test). Further no cells were observed to round up or

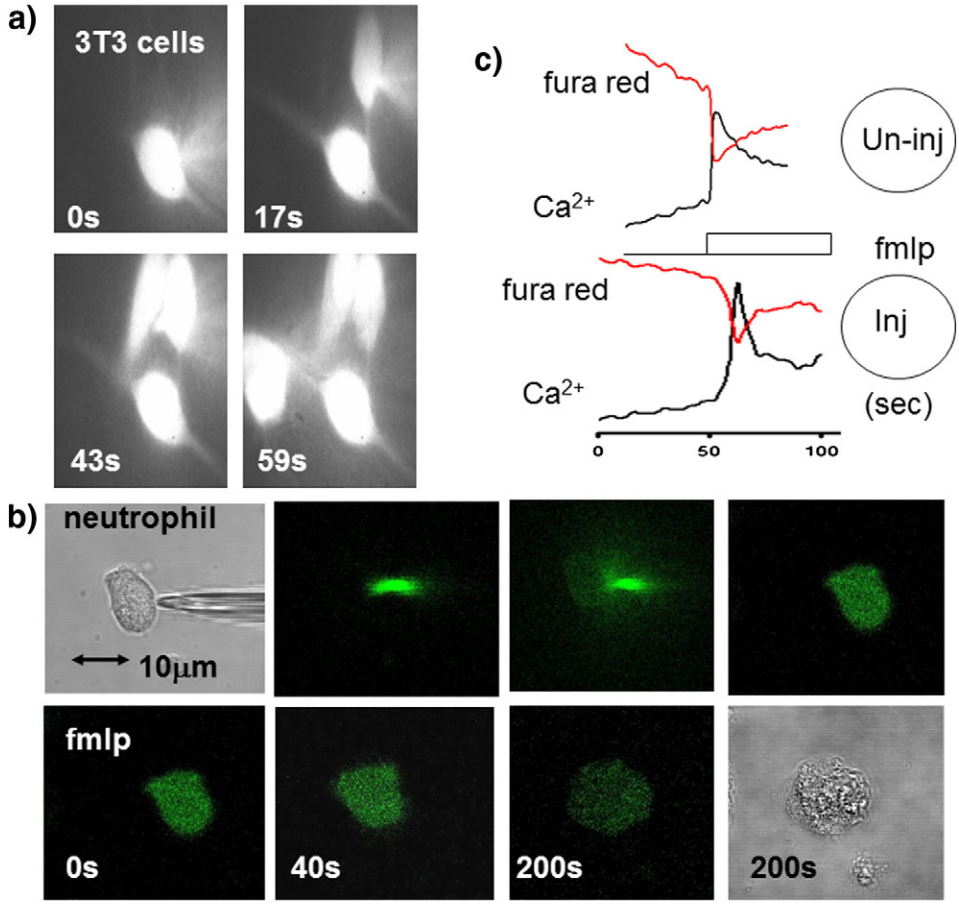

Fig. 1. Electro-injection of cells with the fluorescent marker Lucifer yellow (10 V square pulse train; 1 ms 200 Hz; for 0.5 s); (a) five 3T3 cells were electro-injected sequentially and the fluorescent images are shown for the times indicated; (b) a human neutrophil, which in response to fmlp (1 μM), was shown to spread symmetrically and at a similar rate to untreated neutrophils. The images show the fluorescent images for the neutrophil during the spreading response to fmlp at the times indicated. The phase contrast images show the same cell after injection, before and after the spreading event. The times at which the images are shown at given at the bottom of each image. (c) A comparison between the Ca^{2+} signalling in response to fmlp (1 μM) by non-injected (Uni-inj) and electroinjected (Inj) neutrophil is shown. In each pair of traces, the raw fluorescent signal from cytosolic fura red is shown and the estimated cytosolic Ca^{2+} change shown.

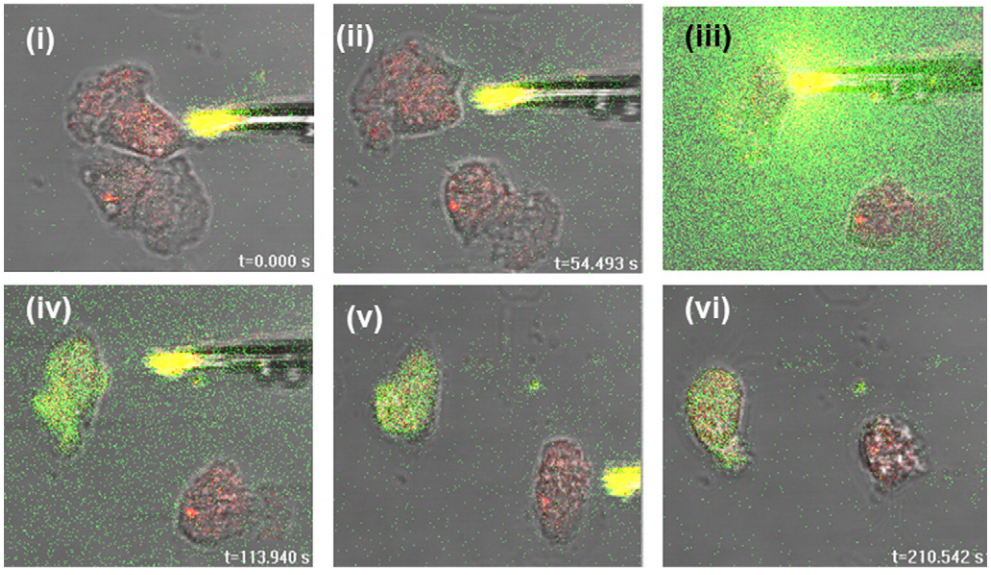

Fig. 2. (a) The sequence of images of a typical experiment and shows an overlay of the fluorescent signal (Lucifer yellow) and the phase contrast image of the cells. (i) The micropipette is positioned near the moving cell; (ii) the position of the cell at electro-injection; (iii) after electro-injection the cell continues to move as before; and (iv) the cell locations approx 150 s post injection. The exact times at which the images were taken is shown at the bottom of each image and the cell movements can be followed continuously in Supplementary Movie 1.

change direction after electro-injection (0/14 cells). In the example shown in Fig. 2, an uninjected motile cell is also shown next to an electro-injected cell for comparison of their cell motility. This comparison may be better seen in the entire imaging sequence which shows the cells moving randomly about the microscopic field during electro-injection (Supplementary Movie 3).

3.4. Electro-injection of fluorogenic peptide to monitor calpain activation

As electro-injected neutrophils retained the ability to respond to the chemotactic stimulus f-met-leu-phe by signalling Ca^{2+} and exhibiting the typical rapid cell spreading response (Fig. 1b), this opened the opportunity to investigate the relationship between Ca^{2+} signalling, calpain-1 activation and neutrophil spreading. As the rapid spreading of these cells is both accompanied by a large Ca^{2+} signal [4,5] and can be triggered by elevating cytosolic Ca^{2+} [7], it has consequently been proposed that the Ca^{2+} signal activates the cytosolic Ca^{2+}-activated protease calpain-1 [1,25]. Whilst measurement of cytosolic Ca^{2+} can be achieved using synthetic Ca^{2+} indicators, it is more difficult to measure calpain-1 activity within an individual living cell with specificity (especially discriminating between calpain-1 and calpain-2). However, α-spectrin is reported to be susceptible to μ-calpain degradation, yet resistant to cleavage by other proteases [26,27]. A peptide sequence from α-spectrin which includes the calpain-1 cleavage site has specificity for cleavage by calpain-1 [28]. This forms the basis for a fluorogenic peptide calpain substrate, the fluorescence from which is quenched by FRET between the fluor and quencher at either end of the peptide. After lysis at the calpain-1 specific cleavage site, the quenching is released [28]. A rise in fluorescence from the cleaved peptide thus indicates the appearance of proteolytic product and elevated calpain-1 activity [29]. The efficiency of electro-injection of the weakly fluorescent peptide was lower than Lucifer yellow, with the cytosolic concentration being approximately 40% of the injectate concentration, giving a cytosolic concentration 0.4% of the concentration within the micropipette. Electro-injecting the fluorogenic-calpain substrate peptide into spontaneously motile and spreading neutrophils showed that calpain-1 was active in these cells, as the cleavage of the peptide was observed by the progressive increase in cellular fluorescent signal after injection had terminated and the extracellular fluorescent signal had fallen back as a result of dilution (Fig. 3a). In polarising and spreading neutrophils, the fluorescent signal reached intensities approximately twice the initial level over the subsequent 30 s (6 out of 7 cells). This response was prevented by treatment of cells with mercaptoacrylate derivatives [29] which inhibit cytosolic calpain-1 activity. In non-motile or non-spreading neutrophils, the cellular fluorescent signal rose during electroinjection but was stable. Although calpain activity was enhanced, the cytosolic Ca^{2+} concentration in these cells was near the resting level. However, the Ca^{2+} signal during spreading is transient and these cells had probably experienced a large Ca^{2+} signal at the time of spreading [4,5]. In order to establish the temporal relationship between the cytosolic Ca^{2+} signal and activation of calpain-1, non-spreading neutrophils were pre-loaded with the Ca^{2+} indicator fura-red before electro-injection with fluorogenic calpain substrate to permit simultaneous measurement of changes in cytosolic free Ca^{2+} and calpain activity from the same cell. Rapid

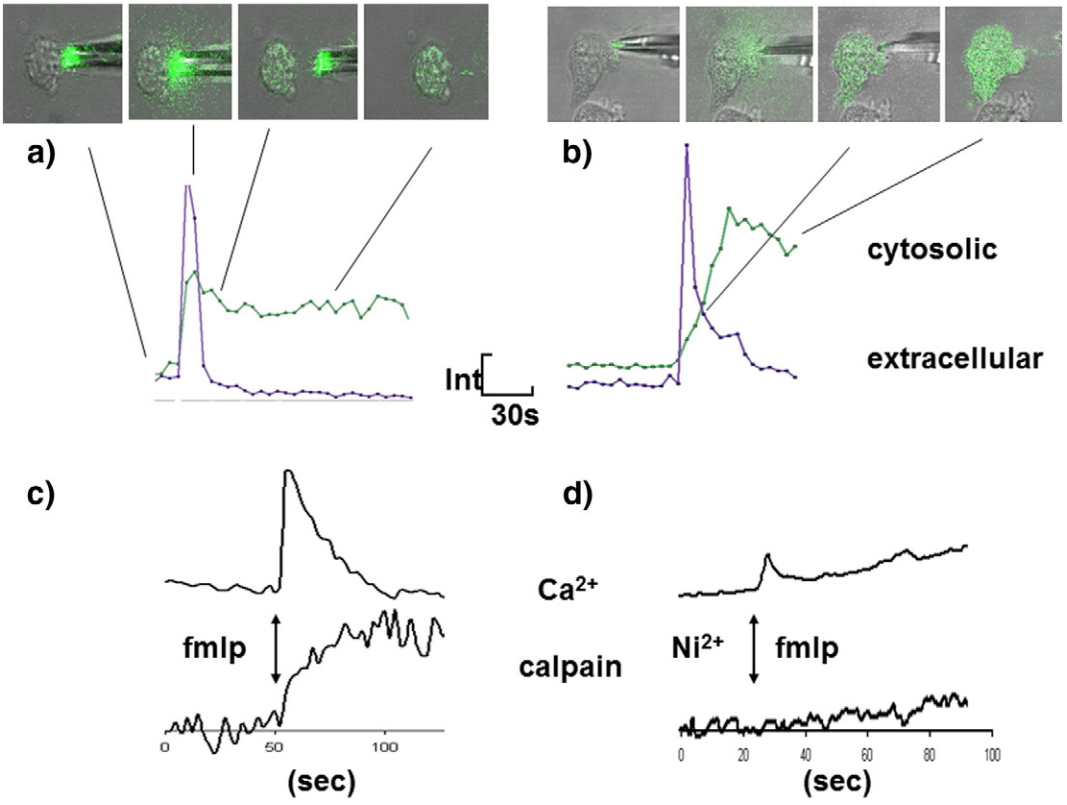

Fig. 3. (a) Electro-injection of a fluorogenic peptide substrate for calpain-1 into a quiescent neutrophil, showing the extracellular pulse which results from iontophoretic ejection (blue line) and the cytosolic intensity of the peptide (green line). It is seen that the cytosolic intensity of the substrate is stable in these cells: into a spread and actively shape changing neutrophil, showing the extracellular pulse which results from the iontophoretic ejection (blue line) and the cytosolic intensity of the peptide (green line). It is seen that the cytosolic fluorescence intensity of the substrate rises after the electroinjection pulse has subsided indicating calpain activity in these cells (6 out of 7 spreading/dynamically shape changing neutrophils electro-injected showed this effect). (c, d) Induced Ca^{2+} signalling and calpain activation was demonstrated by simultaneous imaging of the fura-red signal and the fluorescence intensity of the calpain substrate within the same electro-injected cell. The upper trace mark shows the cytosolic Ca^{2+} change (marked "Ca^{2+}") and the lower trace shows the intensity of the calpian substrate (marked "calpain"). At the point indicated in (c) and (d), the peptide f-mlp (1 μM) was added as a stimulus. In (d) Ni^{2+} (1 mM) was present through the experiment. These traces were typical of at least 5 similar experiments.

cell spreading was then induced by stimulation of the Ca^{2+} signal with f-met-leu-phe (Fig. 3b). This approach thus attempted to capture the temporal relationship between Ca^{2+} signalling and calpain-1 activation for the first time in spreading neutrophils. It has long been speculated that calpain-1 was activated by physiological rise in cytosolic Ca^{2+} even though it requires around 30 μM Ca^{2+} for activation [30,31] and the bulk cytosolic Ca^{2+} peaks at only 1 μM [32]. When triggered by the formylated peptide, fmlp, both an increase in cytosolic Ca^{2+} and an increase in calpain activity were recorded in 7 out of 9 cells. In the remaining two cells, a Ca^{2+} signal was not triggered and no calpain activation was observed. As the fluorescence increase reflects the accumulation of cleaved substrate, it is difficult to locate the precise instant at which calpain activation begins. There may be, therefore, an artefactual temporal displacement between the onset of the Ca^{2+} signal and apparent calpain activation. When Ca^{2+} influx into the neutrophils was blocked by extracellular Ni^{2+} ions (1 mM), fmlp failed to trigger calpain activation despite some small Ca^{2+} signals, which we have previously shown to be due to the release of stored Ca^{2+} from within the cell [34] (Fig. 3d). This is accompanied by inhibition of rapid cell spreading, as we have previously reported [35]. This data clearly demonstrates that physiological influx of Ca^{2+} is sufficient to trigger calpain activation in neutrophils.

4. Discussion

The data reported here showed a clear correlation between calpain activation and cell spreading by neutrophils. It was further shown that a physiological elevation in cytosolic free Ca^{2+} was correlated to an increase in calpain activity and that blockade of Ca^{2+} influx prevented the activation of calpain. Although calpain is well known as a Ca^{2+}-activated enzyme [30], the requirement for Ca^{2+} is in excess to that normally thought to be achieved within the cell. The kd for calpian-1 is within the 30–50 μM range, yet cytosolic free Ca^{2+} within neutrophils reach only approx. 1 μM [25,32]. When cytosolic Ca^{2+} is elevated by uncaging cytosolic (chelated) Ca^{2+}, it was previously found that extremely high levels of cytosolic Ca^{2+} are required to induce neutrophil spreading [7]. However, modelling Ca^{2+} levels within neutrophils during Ca^{2+} influx reveals that the cell surface topography can have an extreme influence on localised Ca^{2+}, and that microdomains of high Ca^{2+} are generated during physiological Ca^{2+} influx within the wrinkled region of the plasma membrane which reach levels of Ca^{2+} sufficient to activate calpain-1 [8]. High concentrations of Ca^{2+} can also be detected just beneath the plasma membrane of neutrophils using membrane attached fluorescent Ca^{2+} probes [34,36]. This suggests that calpain-1 within wrinkles at the cell surface are activated only at these cortical and intra-wrinkle regions of the cell. The fluorogenic peptide used here was not suitable to detect such localised sites of calpain activation as the peptide was free to diffuse within the cell. However, the data here showed that calpain was indeed activated by Ca^{2+} and was therefore consistent with that proposal that activation occurred in microdomains of high Ca^{2+}.

The no-touch electro-injection approach which we used here, and reported previously by Haas and Cline [16–18], was obviously key to the success of this work as the cytosolic processes involved in neutrophil spreading cannot be investigated by approaches which depended on conventional micro-injection. As electro-injection of dynamically active cells, had minimal impact on neutrophil morphological changes, it thus promises to open additional avenues for investigating dynamic shape change behaviour by motile cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.02.020.

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KJL, JSC and BM performed and developed the microinjection experiments and contributed equally to the data for this paper. IL manufactured the micropipettes and assisted with micromanipulation techniques. KJL, SD, BM and MBH analysed the data. MBH and SD devised and supervised the experiments and MBH wrote the paper.

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