MINIMAL IMPACT ELECTRO-INJECTION OF CELLS UNDERGOING DYNAMIC SHAPE CHANGE REVEALS CALPAIN ACTIVATION

Kimberley J Lewis+, Benjamin Masterman+, Iraj Laffafian, Sharon Dewitt1, Jennifer S. Campbell and Maurice B. Hallett*

Neutrophil Signalling Group, Institute of Molecular and Experimental Medicine. School of Medicine, and School of Dentistry1, Cardiff University, Heath Park, Cardiff University, Cardiff CF14 4XN, UK

+ contributed equally

*Corresponding author:

Maurice B Hallett   hallettmb@cf.ac.uk Tel ++44 (0)2920742748

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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 behavior.   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 behavior. 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.
Words 166

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 Ca2+ activated protease calpain [3,4] and since an elevation of cytosolic Ca2+ 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 ⬚M Ca2+, yet cytosolic Ca2+ in spreading cells transiently only reaches a max of 1 ⬚M. Theoretical modelling of Ca2+ within the wrinkles suggests that within this microdomain, Ca2+ 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 the velocities in the region of 700 um/s [13], and 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 and 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 was surprisingly benign and could be used in a number of cell types,

including neutrophils engaged in chemokinesis without affects their motile behaviour.

2. Materials and Methods

2.1 Electro-injection

Borosilicate glass capillaries (1mm outer diameter and 0.5mm inner diameter; with

filament) were pulled with a Sutter Instrument P-2000 laser-operated micropipette

puller creating a tip diameter of 0.8-1µm (by optical inspection) and an inner opening

diameter of 0.4-0.5 µm. The micropipette was back-filled with appropriate loading

solution (approx 1-2µl). A silver wire (0.25mm 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 1m) using a micromanipulator (InjectMan Eppendorf) and

electroporation initiated by a 1s train of pulses (1ms square pulses; 10-50V volts;

200Hz).

2.2 Imaging and Ca2+ measurement

Cultured cells were grown on glass-bottomed petri dishes and neutrophils attached to

glass coverslip and maintained at 37oC 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 120mM, KCl, 4.9mM KH2PO4, 1.2mM MgSO4, 1.2mM CaCl2, 1.3mM, HEPES 25mM 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 37oC. 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 within 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 (1M) 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 Ca2+ concentration monitored from the intensity decrease using the standard Tsien equation [33]. For simultaneous Ca2+ 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 ▯m (+ 0.12 ▯m; 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, electro-injection (10V square pulses train;1ms 200Hz; 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 Ca2+ 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 while in the process of chemokinesis. The rate of movement before (0.436 + 0.19µm/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 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 response to the chemotactic stimulus f-met-leu-phe by signalling Ca2+ and exhibiting the typical rapid cell spreading response (fig 1b), this opened the opportunity to investigate the relationship between Ca2+ signalling, calpain-1 activation and neutrophil spreading. As the rapid spreading of these cells is both accompanied by a large Ca2+ signal [4,5] and can be triggered by elevating cytosolic Ca2+ [7], it has consequently been proposed that the Ca2+ signal activates the cytosolic Ca2+ -activated protease calpain 1[1,25]. While measurement of cytosolic Ca2+ can be achieved using synthetic Ca2+ 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 specifity 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 30s (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 Ca2+ concentration in these cells was near the

resting level. However, the Ca2+ signal during spreading is transient and these cells

had probably experienced a large Ca2+ signal at the time of spreading [4.5]. In order

to establish the temporal relationship between the cytosolic Ca2+ signal and activation

of calpain-1, non-spreading neutrophils were pre-loaded with the Ca2+ indicator

fura-red before electro-injection with fluorogenic calpain substrate to permit

simultaneous measurement of changes in cytosolic free Ca2+ and calpain activity from the same cell. Rapid cell spreading was then induced by stimulation of the Ca2+ signal with f-met-leu-phe (fig 3b). This approach thus attempted to capture the temporal relationship between Ca2+ 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 Ca2+ even though it requires around 30μM Ca2+ for activation [30,31] and the bulk cytosolic Ca2+ peaks at only 1μM [32]. When triggered by the formylated peptide, fmlp, both an increase in cytosolic Ca2+ and an increase in calpain activity were recorded in 7 out of 9 cells. In the remaining two cells, a Ca2+ 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 Ca2+ signal and apparent calpain activation. When Ca2+ influx into the neutrophils was blocked by extracellular Ni2+ ions (1mM), fmlp failed to trigger calpain activation despite some small Ca2+ signals, which we have previously shown to be due to the release of stored Ca2+ 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 Ca2+ 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 Ca2+ was correlated to an increase in calpain activity and that blockade of Ca2+ influx prevented the activation of calpain. Although calpain is well known as a Ca2+-activated enzyme [30], the requirement for Ca2+ is in excess in that normally

thought to be achieved within the cell. The kd for calpian-1 is within the 30-50μM

range, yet cytosolic free Ca2+ within neutrophils reaching only approx. 1μM [25, 32].

When cytosolic Ca2+ is elevated by uncaging cytosolic (chelated) Ca2+, it was

previously found that extremely high levels of cytosolic Ca2+ are required to induce

neutrophil spreading [7]. However, modelling Ca2+ levels within neutrophils during

Ca2+ influx reveals that the cell surface topography can have a extreme influence on

localised Ca2+ ,and that microdomains of high Ca2+ are generated during

physiological Ca2+ influx within wrinkled region of plasma membrane which reach

levels of Ca2+ sufficient to activate calpain-1 [8]. High concentrations of Ca2+ can also

be detected just beneath the plasma membrane of neutrophils using membrane

attached fluorescent Ca2+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 Ca2+ and was therefore

consistent with that proposal that activation occurred in microdomains of high Ca2+.

  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.

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

References

[1]. Hallett MB, Dewitt S. Ironing out the wrinkles of neutrophil phagocytosis: membrane reservoirs for surface area expansion. Trends Cell Biol. (2001) 17: 209 214.

[2]. Houk AR, Jilkine A, Mejean CO, Boltyanskiy R, Dufresne ER, Angenent SB, Altschuler SJ, Wu LF, Weiner OD. Membrane tension maintains cell polarity by confining signals to the leading edge during neutrophil migration. Cell (2012) 148: 175-188

[3]. Wells A, Huttenlocher A, Lauffenburger DA. Calpain proteases in cell adhesion and motility. Int. Rev. Cytol. (2005) 245: 1-16.

[4]. Franco SJ, Huttenlocher A. Regulating cell migration: calpains make the cut. J. Cell Sci. (2005) 118: 3829-3838.

[5]. Kruskal BA, Shak S, Maxfield FR. Spreading of human neutrophils is preceded by a large increase in cytosolic free calcium. Proc. Natl. Acad. Sci. (USA) (1986) 83: 2919-2923.

[6]. Kruskal BA, Maxfield FR. Cytosolic free calcium increases before and oscillates during frustrated phagocytosis in macrophages. J. Cell Biol. (1987) 105: 2685-2693.

[7]. Pettit EJ, Hallett MB. Release of "caged" cytosolic Ca2+ triggers rapid spreading of human neutrophils adherent via integrin engagement. J. Cell Sci. (1998) 111: 2209-2215.

[8]. Brasen JC, Olsen LF, Hallett MB Cell surface topology creates high Ca2+signalling microdomains. Cell Calcium (2010 47: 339-349.

[9]. Lokuta MA, Nuzzi PA, Huttenlocher A. Calpain regulates neutrophil chemotaxis Proc. Natl. Acad. Sci. (USA) (2003)100: 4006-4011.

[10]. Wiemer A J, Lokuta MA. Surfus JC, Wernimont S A, Huttenlocher A. Calpain inhibition impairs TNF-alpha-mediated neutrophil adhesion, arrest and oxidative

burst. Mol. Immunol. (2010) 47: 894-902.

[11]. Graessmann M, Graessmann A. Microinjection of tissue culture cells. Meth. in Enzymol. (1983) 101: 482-492.

[12]. Jockusch BM, Temmgrove CJ. Microinjection of antibodies in the analysis of cellular architecture and motility. Meth. in Cell Biol. (1993) 37: 343-359.

[13]. Laffafian I, Hallett MB. Lipid-assisted microinjection: introducing material into the cytosol and membranes of small cells. Biophys. J.(1998) 75: 2558-2563.

[14]. Davies-Cox EV, Laffafian I, Hallett MB. Control of Ca2+ influx in human neutrophils by IP3 binding: differential effects of micro-injected IP3 receptor antagonists. Biochem. J.(2001) 355: 139-143.

[15]. Dewitt S, Laffafian I, Hallett MB. Phagosomal oxidative activity during ß2 integrin (CR3)-mediated phagocytosis by neutrophils is triggered by a non-restricted Ca2+ signal: Ca2+ controls time not space. J. Cell Sci. (2003) 116: 2857-2865.

[16]. Haas K, Sin WC, Javaherian A, Li Z, Cline HT. Single-cell electroporation for gene transfer in vivo. Neuron (2001) 29: 583-91.

[17]. Haas K, Jensen K, Sin WC, Foa L, Cline HT. Targeted electroporation in Xenopus tadpoles in vivo - from single cells to the entire brain Differentiation (2002) 70: 148-54.

[18]. Bestman JE, Ewald RC, Chiu SL, Cline HT. In vivo single-cell electroporation for transfer of DNA and macromolecules. Nat. Protocols (2006) 1: 1267-72.

[19]. Dewitt S, Tian W, Laffafian I, Hallett MB. Localised PtdIns(3,4,5)P-3 or PtdIns(3,4)P-2 at the phagocytic cup is required for both phagosome closure and Ca2+ signalling in HL60 neutrophils. J. Cell Sci. (2006) 119: 443-451.

[20]. Hillson, EJ, Dewitt S, Hallett MB. Optical methods for the measurement and Manipulation of cytosolic free Ca2+ in neutrophils. Methods in Molecular Biology (Neutrophil Methods and Protocols); Eds Mark Quinn, Frank DeLeo, & Gary Bokoch 2007

[21]. Dewitt S, Laffafian I, Morris MR, Hallett MB. Measurement of cytosolic free Ca2+ in human neutrophils. Methods in Mole. Biol. (2002) 225: 47- 60.

[22]. Zimmermann U. Electrical breakdown, electropermeabilization and electrofusion. Ann. Rev. Physiol. Biochem. (1986) 105: 175-256.

[23] . Lundqvist JA, Sahlin F, Aberg MAI, Stromberg A, Eriksson PS, Orwar O. Altering the biochemical state of individual cultured cells and organelles with ultramicroelectrodes. Proc. Natl. Acad. Sci. (USA) (1998) 95: 10356-10360.

[24]. Rae JL, Levis RA. Single cell electroporation. Pflugers Archiv. Eur. J. Physiol. (2002) 443: 664-670.

[25]. Dewitt S, Hallett MB. Cytosolic free Ca2+ changes and calpain activation are required for beta integrin-accelerated phagocytosis by human neutrophils J. Cell Biol. (2002) 159: 181-189.

[26]. Roberts-Lewis, J.M. Savage, M.J Marcy, V.R. Pinsker, L.R. Siman R. Immuno localization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain J. Neurosci., (1994) 14: 3934–3944

[27] Bednarski, E. Vanderklish, P. Gall, C. Saido, T.C. Bahr, B.A. Lynch G.Translational suppression of calpain-I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices Brain Res., (1995) 694: 147–157

 [28]. Mittoo S, Sundstrom LE, Bradley M. Synthesis and evaluation of fluorescent probes for the detection of calpain activity. Anal. Biochem. (2003) 319, 234-238.

[29]. Adams SE, Parr C, Miller DJ, Allemann RK, Hallett MB. Potent inhibition of Ca2+-dependent activation of calpain-1 by novel mercaptoacrylates. MedChemCommun (2012) 3: 566-570.

[30]. Goll DE, Thompson VF, Li HQ, Wei W, Cong JY. The calpain system Physiol. Rev. (2003) 83: 731-801.

[31]. Legendre JL, Jones HP. Purification and characterization of calpain from

human polymorphonuclear leukocytes. Inflammation (1998) 12: 51–65.

 [32]. Pettit EJ, Hallett MB. Early Ca2+ signalling events in neutrophils detected by rapid confocal laser scanning. Biochem J. (1995) 310: 445-448.

[33]. Hillson EJ, Hallett MB. Localised and rapid Ca2+ micro-events in human neutrophils: Conventional Ca2+ puffs and global waves without peripheral-restriction or wave cycling Cell Calcium (2007) 41: 525-536 .

[34]. Davies EV, Hallett MB. Near membrane Ca2+ changes resulting from store release in neutrophils: detection by FFP-18. Cell Calcium (1996) 19: 355-362 .

[35]. Dewitt S, Francis, RJ Hallett MB Ca2+ and calpain control membrane expansion during the rapid cell spreading of neutrophils J Cell Sci (2013) 126: 4627 4635

[36]. Davies EV, Hallett MB. High micromolar Ca2+ beneath the plasma membrane in stimulated neutrophils. Biochem. Biophys. Res. Commun. (1998) 248: 679-683 .

Figure Legends

Figure 1. Electro-injection of cells with the fluorescent marker lucifer yellow (10V square pulses train;1ms 200Hz; 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 (1μM), 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 shows 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 Ca2+ signalling in response to fmlp (1μM) 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 Ca2+ change shown.

Figure 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 position 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 150s 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.

Figure 3

(a) Electro-injection 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 marked shows the cytosolsic Ca^{2+} change (marked "Ca^{2+}") and the lower trace show the intensity of the calpian substrate (marked "calpain"). At the point indicated in (c) and (d), the peptide f-mlp ($1\square M$) was added as a stimulus. In (d) Ni^{2+} (1mM) was present through the experiment. These traces were typical of at least 5 similar experiments.

SUPPLEMENTARY MATERIAL

Supplementary Movies

1. Electro-injection and cell spreading (Spreading.mov).

The movie shows fluorescence image sequence of a human neutrophil being electro

injected with Lucifer yellow and then stimulated with fmlp (1µM) at the time

indicated. The spreading response is observed and it should be noted that spreading

was symmetrical with no evidence of localised cell damage or inhibition of spreading

at the electro-injection locus. The movie shows the complete data for fig 1b.

2. Electro-injection and spontaneous motility (inj-mov.mov).

The movie shows an overlay of the fluorescent signal (Lucifer yellow) and the phase

contrast image of the neutrophils undergoing spontaneous motility. The motility of

the non-injected and the electro-injected cell can be compared.

3. Moving target (electro-inj.mov) The movie shows a short sequence demonstrating the electro-injection of a human

neutrophil whilst undergoing rapid motility. The movie is an overlay of the

fluorescent signal (Lucifer yellow) and the phase contrast image of the cells. Each

attempted electro-injection is accompanied by the ejection of Lucifer yellow into the

medium (by iontophoresis) with a successful electroporation resulting in delivery of

fluorescence to the cytosol of a neutrophil which continues to move randomly after

micro-injection. The entire sequence shows about 200s of real time and is the

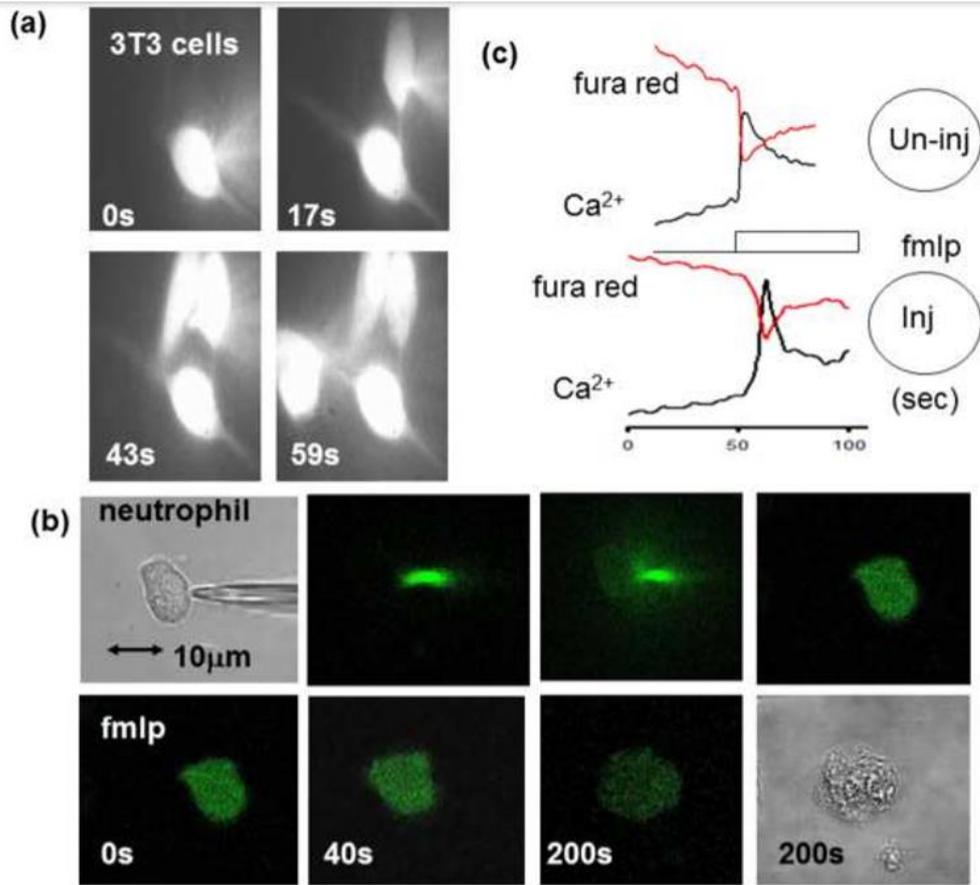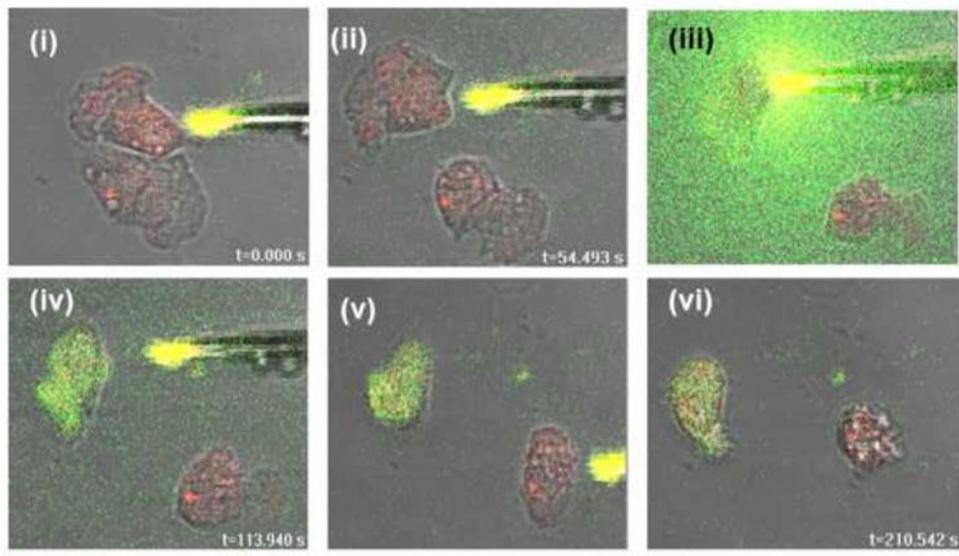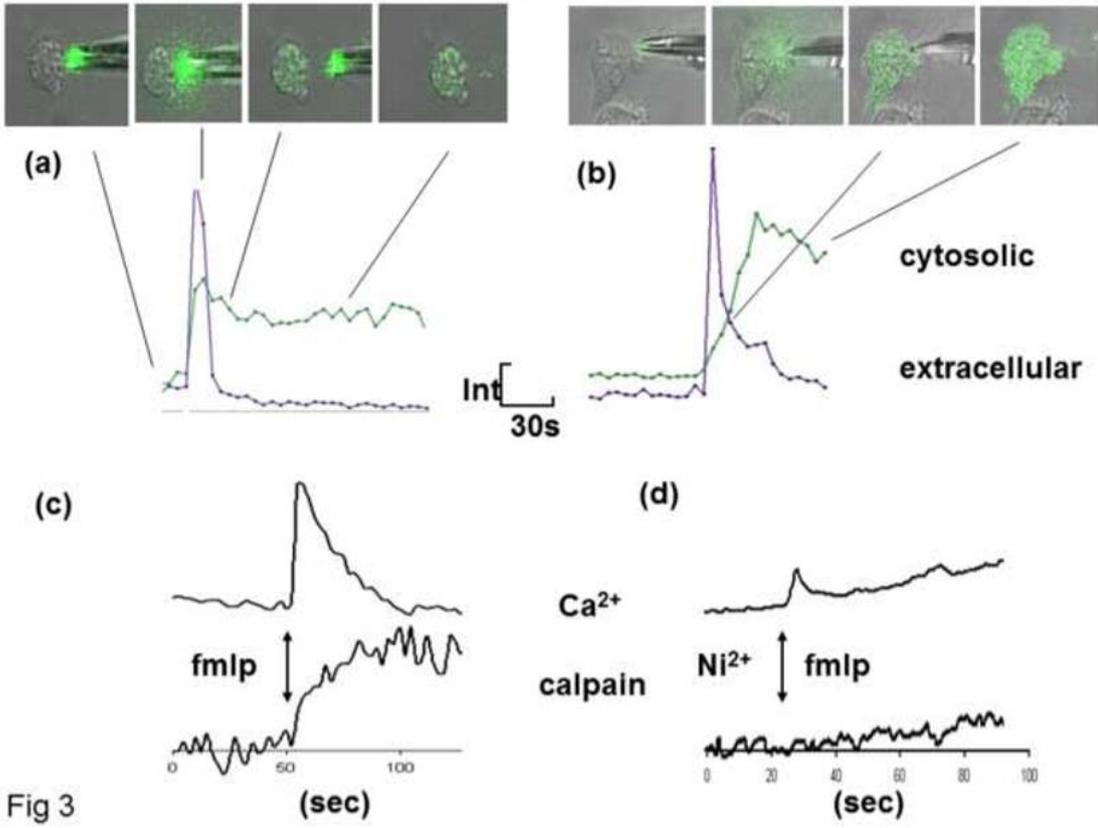complete data from which fig 2a was constructed

(a) 3T3 cells
0s   17s   43s   59s

(b) neutrophil
10μm
fmlp
0s   40s   200s   200s

(c) fura red   Ca²⁺   Un-inj
fmlp
fura red   Ca²⁺   Inj   (sec)
0   50   100

Fig 1

Figure 2

(a)

(b)

cytosolic

extracellular

Int
30s

(c)

(d)

Ca²⁺

fmlp

calpain

Ni²⁺ fmlp

Fig 3

(sec)

(sec)