Biochimica et Biophysica Acta 1843 (2014) 1182–1187

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

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

Kimberley J. Lewis [a,1], Benjamin Masterman [a,1], Iraj Laffafian [a], Sharon Dewitt [b], Jennie S. Campbell [a], Maurice B. Hallett [a,*]

[a] Neutrophil Signalling Group, Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, Heath Park, Cardiff University, Cardiff CF14 4XN, UK
[b] School of Dentistry, Cardiff University, Heath Park, Cardiff University, Cardiff CF14 4XN, UK

ARTICLE INFO

Article history:
Received 14 October 2013
Received in revised form 9 February 2014
Accepted 28 February 2014
Available online 7 March 2014

Keywords:
Cell spreading
Chemokinesis
Cytosolic Ca^{2+}
Neutrophil
Phagocytosis

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.

* Corresponding author. Tel.: +44 2920742748.
E-mail address: hallettmb@cf.ac.uk (M.B. Hallett).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.bbamcr.2014.02.020
0167-4889/

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