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

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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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[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

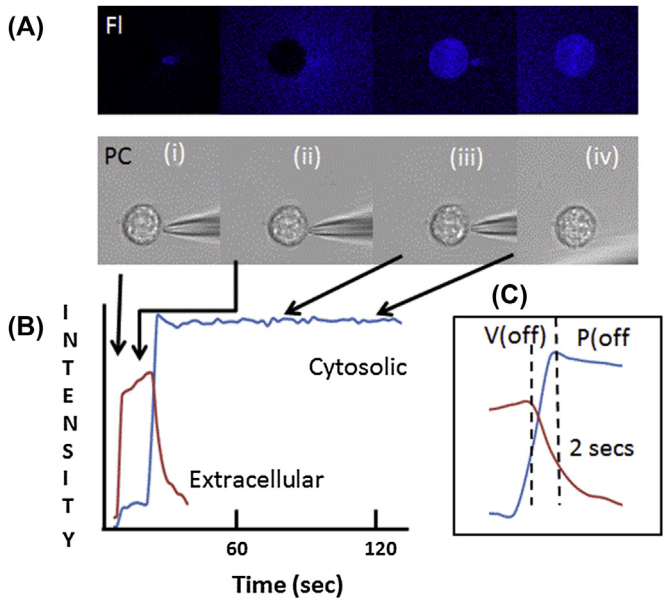

Fig. 1. Electroinjection of human neutrophils. The sequence of electroinjection inert fluor (Alexa 633) is shown in panel (A) where the lower row (labelled PC) shows the phase contrast image of the cell undergoing electroinjection with the position of the micropipette and the upper row (labelled FL) 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).