Control of Ca^{2+} influx in human neutrophils by inositol 1,4,5-trisphosphate (IP_3) binding: differential effects of micro-injected IP_3 receptor antagonists

Eryl V. DAVIES-COX, Iraj LAFFAFIAN and Maurice B. HALLETT¹

Molecular Signalling Group, University Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Neutrophils signal Ca^{2+} changes in response to occupancy of G-protein-linked receptors such as the formylated peptide receptor. This Ca^{2+} signal is composed of two parts, inositol 1,4,5-trisphosphate (IP_3)-triggered release of Ca^{2+} from an intracellular store and Ca^{2+} influx. In order to probe the relationship between these events, cytosolic free Ca^{2+} changes in neutrophils were monitored after micro-injection of agents which inhibit IP_3 binding. Micro-injection of heparin into neutrophils totally inhibited both formylmethionyl-leucylphenylalanine-induced Ca^{2+} release and the subsequent Ca^{2+} influx. This effect was not due to prior depletion of Ca^{2+} stores. Furthermore, micro-

injection with anti- IP_3 -receptor antibody also inhibited Ca^{2+} release. However, anti- IP_3 -receptor antibody and another high-molecular-mass IP_3 -binding antagonist, heparin–albumin conjugate, failed to inhibit the accompanying Ca^{2+} influx. It was concluded that two IP_3 -binding sites exist in neutrophils: one accessible by both heparin and the high-molecular-mass inhibitors of IP_3 binding and responsible for Ca^{2+} release, and another inaccessible to high-molecular-mass molecules and responsible for Ca^{2+} influx.

Key words: Ca^{2+} ion, micro-injection, signalling.

INTRODUCTION

In a number of electrically non-excitable cells, the influx of Ca^{2+} across the plasma membrane follows the release of Ca^{2+} from intracellular stores and is often termed store-operated or ‘capacitative’ Ca^{2+} entry [1]. The nature of the store-operated Ca^{2+} channel and the mechanism by which it opens is not clear, but inositol 1,4,5-trisphosphate (IP_3) has been implicated [2]. Although the role of IP_3 in releasing stored Ca^{2+} in non-excitable cells is well established, there remains uncertainty as to its role in mediating Ca^{2+} entry. Whereas there may be roles for IP_3 and the IP_3 receptor (IP_3R) as mediators of Ca^{2+} influx [2,3], other evidence goes against such a role [1,4]. Agents including GTP and calmodulin [1] and an unknown diffusible factor [5–8] have also been implicated as mediators of Ca^{2+} -channel opening. Using a new micro-injection technique [9], we have introduced heparin, albumin conjugated-heparin and anti- IP_3R antibody into the cytosol of neutrophils, a cell type that displays capacitative Ca^{2+} entry. These strategies were aimed at inhibiting IP_3 binding within the cell. However, while heparin inhibited both Ca^{2+} release and the accompanying Ca^{2+} influx, high-molecular-mass inhibitors (heparin–albumin conjugate and anti- IP_3R antibody) prevented Ca^{2+} release without impeding Ca^{2+} influx. The mechanism responsible for Ca^{2+} entry in neutrophils thus involves a heparin-sensitive step, which is not accessible to high-molecular-mass inhibitors.

EXPERIMENTAL

Neutrophil isolation

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously [10]. Essentially, the separation was a three-step process involving sedimentation (30–60 min, 1 g) with dextran, hypotonic lysis of red cells and centrifugation

through Ficoll Hypaque. The neutrophil were resuspended in Krebs buffer (120 mM NaCl, 4.8 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).

Cytosolic free Ca^{2+} measurement

Neutrophils were loaded with fura 2 from the acetoxymethyl ester and ratio-imaging measurements performed as described previously [11]. Excitation wavelengths (340 and 380 nm) selected using a rapid monochromator (Delta RAM; Photon Technologies International, Surbiton, Surrey, U.K.), and the image collected using an intensified charge-coupled device (CCD) camera (IC100 or IC200; Photon Technologies International). Ratio images were acquired using Image Master software (Photon Technologies International). Neutrophils that were not undergoing chemotaxis or shape changes, but which were loosely adherent and round, were chosen for experimentation. The stimulus, formylmethionyl-leucylphenylalanine (f-Met-Leu-Phe) (in DMSO), was diluted in Krebs medium to give the concentrations shown and added to the cells under view, giving a further dilution factor of 2–4. Experiments were repeated on different occasions with different neutrophil donors.

Simple lipid-assisted micro-injection (SLAM)

SLAM of loosely adherent neutrophils was achieved using lipid-coated micro-pipettes using an internal pressure of 0.5–2.0 MPa (5–20 mbar) as described previously [9]. Gentle contact between the micro-pipette and the neutrophil plasma membrane resulted in fusion of the lipid at the tip of the micro-pipette with the cell and transfer of the aqueous contents of the micro-pipette to the cytosol. The pressure was held constant and the contact time between the micro-pipette and the cell was used to control the amount of injected material, as this procedure had no effect on

Abbreviations used: f-Met-Leu-Phe, formylmethionyl-leucylphenylalanine; IP_3 , inositol 1,4,5-trisphosphate; IP_3R , IP_3 receptor; SLAM, simple lipid-assisted micro-injection.

¹ To whom correspondence should be addressed (e-mail hallettmb@cardiff.ac.uk).

cell viability [9]. Quantification of the amount injected was achieved by visualizing the transfer of Lucifer Yellow, which was used as an aqueous marker [9]. Micro-injection medium was either 150 mM KCl/25 mM Hepes, pH 7.0, or distilled water, with a free Ca^{2+} concentration of less than 200 nM. EGTA was not used to eliminate the possibility of additional Ca^{2+} buffering. Control micro-injections were of three types: (i) BSA, as a control for the presence of an irrelevant protein and specifically for heparin–albumin conjugate, used at the same concentration of the relevant protein, (ii) heat-denatured antibody, used at the same concentration of the relevant protein, as a control for non-protein constituents of the antibody preparation such as azide and (iii) micro-injection medium alone.

Materials

Monoclonal anti- IP_3R antibody raised against a short peptide (GGVGDVLRKPS; Calbiochem, Nottingham, U.K.) containing azide was stored at 4 °C before use. It was diluted 1:1 with micro-injection buffer on the day of the experiment to give a protein concentration of 250 $\mu\text{g}/\text{ml}$ and a cytosolic azide concentration (on injection) of less than 30 μM . Heat-denatured antibody was prepared by taking an aliquot of the active micro-injectate and heating at 70 °C for 30 min. Heparin conjugated to albumin was purchased from Sigma (Poole, Dorset, U.K.) and the highest-molecular-mass fractions were isolated on a Sephadex G-100 gel-filtration column. The apparent molecular mass of this heparin–albumin conjugate on PAGE was estimated to be above 200 kDa. Fura 2 acetoxymethyl ester was purchased from Molecular Probes (Junction City, OR, U.S.A.). Dry DMSO and other reagents were from Sigma.

RESULTS

Ca^{2+} release and Ca^{2+} -channel opening are separately demonstrable

The peptide f-Met-Leu-Phe may signal via classical capacitative Ca^{2+} influx in neutrophils [12]. Previously, it has been shown that f-Met-Leu-Phe causes ‘store-operated Ca^{2+} channels’ to open in whole cells, but not on isolated membrane patches [13]. Furthermore, manoeuvres which reduce stored Ca^{2+} , such as inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (‘SERCA’) pumps by thapsigargin, increase the influx of Ca^{2+} and other ions such as Mn^{2+} [14]. Thus, when neutrophils are activated in the absence of extracellular Ca^{2+} , the rise in cytosolic free Ca^{2+} concentration results from release of Ca^{2+} from intracellular storage sites. We show here that subsequent re-admission of extracellular Ca^{2+} caused elevations of cytosolic free Ca^{2+} concentration as a result of Ca^{2+} influx into stimulated cells in a microscopic field (e.g. Figure 1). This rise in cytosolic free Ca^{2+} concentration had two components; the major part was inhibited by Ni^{2+} ions and attributable to influx through open Ca^{2+} channels. There was also a small rise in cytosolic free Ca^{2+} , which occurred in the presence of Ni^{2+} and was presumably due to the re-establishment of a non-specific leak-pump equilibrium. The rate (and extent) of Ni^{2+} -inhibitable Ca^{2+} influx increased dramatically after stimulation of Ca^{2+} -store release by f-Met-Leu-Phe (see for example Figure 1). The rate of increase of cytosolic free Ca^{2+} concentration was used as a measure of the number of Ca^{2+} channels open. The strategy of stimulating the cell in the absence of extracellular Ca^{2+} to detect release of stored Ca^{2+} followed by addition of extracellular Ca^{2+} to monitor the extent of Ca^{2+} -channel opening was used in subsequent experiments to visualize the two events separately.

Figure 1 Cytosolic heparin inhibits Ca^{2+} release and influx

The Figure shows a series of cytosolic free Ca^{2+} measurements (a–f) taken from neutrophils in the same microscopic field, one of which was micro-injected with heparin (100 $\mu\text{g}/\text{ml}$). The abscissa in each time course is time and the ordinate is cytosolic free Ca^{2+} concentration (with the common scales marked in the inset). Cells (a–d) were uninjected and cell (f) was SLAM-injected with heparin (estimated intracellular concentration, 100 $\mu\text{g}/\text{ml}$) before the experiment. At the time marked CP, f-Met-Leu-Phe (1 μM) was added in the absence of extracellular Ca^{2+} (EGTA, 1 mM) and then the extracellular Ca^{2+} concentration was increased to 1.3 mM (normal Krebs medium) at the time indicated by Ca^{2+} . This experiment was typical of 10 other experiments performed on different occasions with different neutrophil donors.

Cytosolic heparin inhibits both Ca^{2+} release and Ca^{2+} influx

To establish the role of IP_3 in coupling receptor stimulation to Ca^{2+} entry, an inhibitor of IP_3 binding, heparin, was micro-injected into the cytosol of fura 2-loaded neutrophils, using the lipid-assisted technique. Control micro-injections had no effect on the resting cytosolic free Ca^{2+} concentration or responsiveness to stimulation, whereas micro-injection of heparin totally inhibited both Ca^{2+} release and Ca^{2+} influx (Figure 1f). The inhibition of the release of Ca^{2+} by heparin could not be explained by prior emptying of the Ca^{2+} stores, as there was no significant reduction in the amount of Ca^{2+} released by the addition of ionomycin or thapsigargin after micro-injection of heparin (Table 1). It was also unlikely that heparin had a direct effect on the opening of Ca^{2+} channels as the Ca^{2+} influx induced by thapsigargin was insensitive to intracellular heparin [61 ± 7 nM/s ($n = 13$) and 75 ± 19 nM/s ($n = 4$) in the presence and absence of intracellular heparin, respectively]. These data were therefore consistent with the mechanism for Ca^{2+} influx being via store-operated Ca^{2+} channels with heparin inhibiting IP_3 binding and the release of Ca^{2+} from stores. However, significant Ca^{2+} influx was often observed in neutrophils in which no or little Ca^{2+} release was detectable in response to f-Met-Leu-Phe (e.g. Figures 1d and 1e). These data together suggested that although there was a heparin-inhibitable binding step that was responsible for triggering Ca^{2+} influx, only a low level of Ca^{2+} release (if any) was required. This called into question whether Ca^{2+} influx in neutrophils truly triggered a store-operated mechanism, and whether the inhibition caused by heparin was solely due to its effect on IP_3Rs on Ca^{2+} stores.

Cytosolic anti- IP_3R antibody prevents Ca^{2+} release but not Ca^{2+} influx

The important issue of the specificity of heparin as an inhibitor of IP_3 binding on to Ca^{2+} stores has been raised previously (see [15]). Indeed, it has been reported that heparin can inhibit a range of other Ca^{2+} -release agents, including cADP and nicotinic acid–adenine dinucleotide phosphate [16]. In order to address

Table 1 Comparison of the effects of intracellular inhibitors of IP₃ binding on the release of stored Ca²⁺ and the accompanying capacitative Ca²⁺ entry

The agents were added to neutrophils in the absence of extracellular Ca²⁺ (1 mM EGTA). The initial rate of cytosolic free Ca²⁺ rise in response to the agent was taken as a measure of the amount of Ca²⁺ in the Ca²⁺ store, as apparent from Fick's law. Data are the means $\pm$ S.D. of Ca²⁺ release rate (nM/s), with numbers in parentheses indicating the number of cells (*n*).

Micro-injection conditions	Stored Ca ²⁺ released by ...	Rate of Ca ²⁺ release (nM/s)	
		Ionomycin (4 μ M)	Thapsigargin (150 nM)
Control		43 $\pm$ 5 (15)	9 $\pm$ 6 (15)
Heparin		52 $\pm$ 14 (4)	8 $\pm$ 4 (4)
Anti-IP ₃ R antibody		47 $\pm$ 17 (4)	11 $\pm$ 5 (4)
Albumin–heparin conjugate		42 $\pm$ 9 (4)	12 $\pm$ 6 (4)

Table 2 Comparison of the effects of intracellular inhibitors of IP₃ binding on the release of stored Ca²⁺ and the accompanying capacitative Ca²⁺ entry

The Table shows data from a series of experiments designed to compare the four conditions shown. The total number of cells (shown by the value *n*) were from three separate donors, repeated on three occasions. In each experiment, non-injected neutrophils in the same microscopic field were used as controls. The data show the change in cytosolic free Ca²⁺ concentration (means $\pm$ S.D.) in response to stimulation of neutrophils with f-Met-Leu-Phe (1 μ M) in the absence of extracellular Ca²⁺ (Ca²⁺-store release), and the effect of re-admission of extracellular Ca²⁺ (Ca²⁺ entry rate). The ratio of these two parameters is also given for comparison. **P* < 0.05, as compared with the control value (Students *t* test).

Micro-injection conditions	Ca ²⁺ -store release (nM)	Ca ²⁺ entry rate (nM/s)	Ratio (mean influx rate/mean release, s ⁻¹)
Control	210 $\pm$ 162 (86)	83 $\pm$ 72	0.4
Heparin	15 $\pm$ 14* (27)	12 $\pm$ 3*	1.25
Anti-IP ₃ R antibody	46 $\pm$ 10* (27)	130 $\pm$ 79	2.83
Albumin–heparin conjugate	14 $\pm$ 6* (26)	145 $\pm$ 6	10.36

this issue, we micro-injected an antibody to the IP₃R [17]. This antibody recognizes the C-terminus of all three IP₃Rs and has been shown previously to prevent IP₃-induced Ca²⁺ release from mouse T-lymphoma microsomes [17]. As this antibody did not bind to other neutrophil proteins on Western blots, we used this reagent as a more specific inhibitor of IP₃Rs in neutrophils. Micro-injection of the antibody into neutrophils, reduced or inhibited the release of stored Ca²⁺ induced by f-Met-Leu-Phe (Table 2). As with heparin, the inhibition of the release of Ca²⁺ by anti-IP₃R antibody could not be explained by emptying the Ca²⁺ stores, as there was no significant reduction in the amount of Ca²⁺ released by the addition of ionomycin or thapsigargin (Table 1). However, in contrast to heparin-injected cells, Ca²⁺ influx in neutrophils injected with anti-IP₃R antibody was readily demonstrable after stimulation (Figure 2). In neutrophils in which f-Met-Leu-Phe-induced Ca²⁺-store release was totally inhibited, subsequent challenge with extracellular Ca²⁺ produced rates of Ca²⁺ influx higher than the expected rate of Ca²⁺ entry (Figure 2 and Table 2). In order to eliminate the possibility that the increased Ca²⁺ influx was due to a direct effect of the antibody, or a result of 'cell damage' caused by the antibody, cytosolic free Ca²⁺ concentration was monitored while micro-injecting with the antibody. Under these conditions, a transient increase in cytosolic free Ca²⁺ concentration was observed during micro-injection as a result of the unbuffered Ca²⁺ within the micro-pipette, which returned quickly to the resting concentration (Figure 2e). No evidence for gross 'cell damage', which would have resulted in fura 2 leakage, was observed. Furthermore, there was no increased Ca²⁺ leakage or Ca²⁺-channel opening, as revealed by a lowering of cytosolic free Ca²⁺ concentration (as would have resulted from efflux into the extracellular medium containing almost no Ca²⁺ and 1 mM EGTA). However, the amount of stored Ca²⁺ released in response to f-Met-Leu-Phe was reduced, whereas the subsequent rate of Ca²⁺ influx was enhanced (Figure 2e). It was also unlikely that the anti-IP₃R antibody had a direct effect on Ca²⁺-channel

Figure 2 Cytosolic anti-IP₃R antibody inhibits Ca²⁺ release but not Ca²⁺ influx

The Figure shows a series of cytosolic free Ca²⁺ measurements taken from neutrophils in the same microscopic field, in which anti-IP₃R antibody (5–25 μ g/ml) was SLAM-injected into one cell (e). The graphs (a–e) show the changes in response to f-Met-Leu-Phe (1 μ M, marked by CP), in the absence of extracellular Ca²⁺, and restoration of extracellular Ca²⁺ (1.3 mM, marked by Ca²⁺) of all cells in the field. Cells (a–d) were uninjected and cell (e) was micro-injected with the anti-IP₃R antibody, as indicated by the arrow in (e). Graph (f) shows the ratio the level of cytosolic free Ca²⁺ concentration after restoration of extracellular Ca²⁺ (to monitor capacitative Ca²⁺ entry) to the peak of released Ca²⁺ for all the cells in the field. The mean $\pm$ S.E.M. of the ratios for the non-injected cells is also shown (■) and compared with the ratio from the micro-injected cell (□). The microscopic field and the relative cytosolic free Ca²⁺ changes shown as pseudocolour images can be seen at <http://www.BiochemJ.org/bj/355/bj3550139add.htm>. This effect was observed in four other experiments performed on different occasions with different neutrophil donors but not with heat-denatured antibody or BSA.

opening as Ca²⁺ influx induced by thapsigargin was unaffected [72 $\pm$ 5 nM/s (*n* = 10) and 68 $\pm$ 15 nM/s (*n* = 3) in the presence and absence of intracellular antibody, respectively].

Figure 3 Cytosolic heparin delays Ca^{2+} release in maximally stimulated cells

The Figure shows a series of cytosolic free Ca^{2+} measurements taken from neutrophils in the same microscopic field, in which heparin was micro-injected into three cells. Cell (a) was uninjected and cells (b–d) were SLAM-injected with intracellular heparin concentrations of 50, 30 and 12.5 $\mu\text{g/ml}$ respectively. The intracellular concentrations were estimated from the concentration of heparin within the micro-pipette and the contact time between the micro-pipette and the cell, using Lucifer Yellow as a marker. The graphs (a–d) show the time courses of responses to f-Met-Leu-Phe (4 μM , added at CP) in the absence of extracellular Ca^{2+} and the effect of addition of Ca^{2+} (1.3 mM, added at Ca^{2+}). Graph (e) shows the up-phase of the responses to f-Met-Leu-Phe of the cells (marked a–d) on the same expanded time axis to show more clearly the delay in Ca^{2+} signalling. The delay is also clearly seen in the pseudocolour Ca^{2+} images at <http://www.BiochemJ.org/bj/355/bj3550139add.htm>. In panel (f) the relationships between cytosolic heparin concentration in these cells and the mean cytosolic free Ca^{2+} concentration reached on replacing extracellular Ca^{2+} (upper panel) and the delay before release of stored Ca^{2+} (lower panel) are also shown. The experiment shown here was a demonstration of the delaying effect of heparin, which was also observed in three similar experiments on performed on different occasions with different neutrophil donors ($n = 12$ individual cells).

Cytosolic heparin–protein conjugate prevents Ca^{2+} release but not Ca^{2+} influx

The possibility that the discrepancy between the effects of IP_3R blockade by heparin and the antibody were due to the large difference in molecular mass between heparin (14–17 kDa) and antibody (250 kDa) was investigated by the use of a heparin–albumin conjugate with an estimated molecular mass of > 200 kDa. Micro-injection of the heparin–albumin conjugate, as with low-molecular-mass heparin, inhibited the release of stored Ca^{2+} in response to f-Met-Leu-Phe (Table 2). This effect was not attributable to a prior depletion of Ca^{2+} stores (Table 1). As with the anti- IP_3R antibody, heparin–albumin conjugate did not inhibit Ca^{2+} influx (Table 2). There was enhancement of Ca^{2+} entry similar to that achieved with maximum blockade by the anti- IP_3R antibody (Table 2). The failure of very high-molecular-mass heparin and the antibody to inhibit Ca^{2+} influx was consistent with their exclusion from a binding site. It was thus concluded that the mechanism responsible for Ca^{2+} entry in neutrophils involved a heparin-sensitive step, which was not

accessible to the high-molecular-mass heparin–albumin conjugate or inhibited by anti- IP_3R antibody.

Relationship between IP_3R occupancy and Ca^{2+} -response delay

The evidence presented suggests that, in neutrophils, Ca^{2+} influx triggered by f-Met-Leu-Phe was controlled by IP_3 binding at a site other than that coupled to Ca^{2+} release from stores. In order to test this proposal, a feature of the Ca^{2+} responses in neutrophils was exploited. Stimulation with f-Met-Leu-Phe results in the marked individual Ca^{2+} -response delays ([18], and see Figure 1). It was thought that this arose from differences in the rates of IP_3 generation in different cells, such that the time required to reach a critical number of occupied IP_3Rs on the Ca^{2+} store varied from cell to cell [19]. In order to test this, maximally stimulated neutrophils, in which micro-delays of only 75–1000 ms occur [20], were micro-injected with heparin at different concentrations. At maximal stimulation with f-Met-Leu-Phe it was not possible to inhibit either Ca^{2+} influx or the extent of Ca^{2+} release. However, there were marked delays in the onset of the Ca^{2+} response of up to 6 s, which correlated with the concentration of intracellular heparin (Figure 3). Thus the Ca^{2+} -response delay provided an indicator of the time required for IP_3R occupancy on Ca^{2+} stores (critical time $\propto 1/\text{delay time}$) to reach a critical level within an individual cell. There was a good correlation between this measure of IP_3R occupancy on the Ca^{2+} store and the extent of Ca^{2+} release ($r = 0.83$), suggesting the validity of the approach (see examples in Figure 1). However, for the same data set, the correlation between Ca^{2+} release and subsequent Ca^{2+} influx was poor ($r = 0.016$), and not expected for classic store-operated Ca^{2+} influx. The correlation between IP_3R occupancy and Ca^{2+} influx was also poor ($r = 0.35$), but a better predictor of subsequent Ca^{2+} influx than was the amount of released Ca^{2+} . These trends were consistent with the hypothesis that IP_3 binding was an important step in Ca^{2+} influx independently of its effect on Ca^{2+} release.

DISCUSSION

The inhibition of both Ca^{2+} influx and Ca^{2+} -store release by heparin reported here suggests a role for IP_3 binding in these events in neutrophils. However, the ability of high-molecular-mass heparin and the IP_3R -blocking antibody to inhibit only Ca^{2+} release points to two separate IP_3 -binding locations. One location was at a site that was accessible to both the high- and low-molecular-mass inhibitors and was responsible for Ca^{2+} release. This was consistent with the previously identified Ca^{2+} release site on the 'vestigial' endoplasmic reticulum near the multi-lobed nucleus of the neutrophil [21–23]. The other IP_3 -binding site was inhibitable only by the low-molecular-mass heparin and was involved in Ca^{2+} influx. The demonstration that Ca^{2+} influx occurred when IP_3 -mediated Ca^{2+} release was abolished by the high-molecular-mass inhibitors of IP_3 binding (heparin–albumin conjugate and anti- IP_3R antibody) calls into question the role of a diffusible Ca^{2+} -influx factor in neutrophils [5–8] and of a Ca^{2+} sensor within the Ca^{2+} store.

A similar relationship between IP_3 binding and Ca^{2+} entry to that suggested here has previously been proposed from observations on other cell types [24–27]. It has been suggested that the linkage between IP_3 binding and store-operated Ca^{2+} -channel opening results from a very close conjugation between the IP_3R and the Ca^{2+} channel [28]. The data presented here for neutrophils was consistent with this model of 'capacitative' Ca^{2+} influx. The

data presented here would be explained if either the site of the relevant IP₃Rs or their proximity to the store-operated Ca²⁺ channel presented a steric-hindrance barrier to high-molecular-mass molecules, such as the antibody and high-molecular-mass heparin, but allowed inhibition by small heparin molecules. However, neutrophils have no recognizable endoplasmic reticulum near the cell plasma membrane [23,29]. This raises the question of whether there are specialized organelles with IP₃Rs that are important in Ca²⁺ entry and which do not constitute part of the conventionally recognized endoplasmic-reticular system.

Another question that this study raises relates to the mechanism by which inhibition of Ca²⁺-store release by the high-molecular-mass IP₃-binding inhibitors caused an increase in the subsequent Ca²⁺ influx (Figure 2 and Table 2). This effect may be explained by negative feedback on store-operated Ca²⁺-channel opening provided by the release of the Ca²⁺-store contents. Several possibilities exist that may provide an explanation for this phenomenon, including (i) the release of an inhibitory factor ('anti-CIF', or anti-Ca²⁺-influx factor) together with Ca²⁺ and (ii) that raised cytosolic free Ca²⁺ inhibited the ability of IP₃ to open store-operated Ca²⁺ channels. The latter possibility is attractive, as the effect of IP₃ on its receptor at the store release site is modulated by the ambient Ca²⁺ concentration [30]. Recently, Broad et al. [31] have also provided evidence that cytosolic free Ca²⁺ may modulate Ca²⁺ entry by inhibiting opening of the store-operated channel. They propose that Ca²⁺ entry through the open channel inactivates the IP₃R responsible for the influx. Such a mechanism may provide the explanation for the enhancing effect observed in this paper, since releasing stored Ca²⁺ elevates cytosolic free Ca²⁺ at the plasma membrane (see [31], Figures 1–4 and [32]).

It was thus concluded that two IP₃-binding sites exist in neutrophils, one accessible to heparin and high-molecular-mass inhibitors of IP₃ binding (such as the anti-IP₃R antibody and albumin–heparin conjugate), which is responsible for Ca²⁺ release, and another IP₃-binding site inaccessible to the antibody or high-molecular-mass heparin, which is responsible for Ca²⁺ influx. Our data, however, do not shed light on the mechanism by which pharmacological 'emptying' of Ca²⁺ stores by thapsigargin is coupled to Ca²⁺ influx. In this study, no inhibitory effect of intracellular heparin on IP₃-independent thapsigargin-induced Ca²⁺ entry could be found. This is in contrast to HEK-293 cells [3], and may suggest that neutrophils have more than one mechanism by which store-operated Ca²⁺ entry is signalled. Further work is now required to establish (i) whether the two IP₃-binding sites are of the same or different IP₃R isotype, and (ii) the mechanism by which non-IP₃-generating stimuli also trigger Ca²⁺ influx.

We thank the Arthritis Research Campaign, U.K. (ARC; grant nos H0553 and H0558) and the Maxime Hanss Fund (Biotechnology and Biological Sciences Research Council) for support.

REFERENCES

- Putney, J. W. and McKay, R. R. (1999) Capacitative calcium entry channels. *Bioessays* **21**, 38–46
- Berridge, M. J. (1995) Capacitative calcium entry. *Biochem. J.* **312**, 1–11
- Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L. and Muallem, S. (1998) Functional interaction between IP₃ receptors and store operated channels. *Nature (London)* **396**, 478–482
- Sugawara, H., Kurosaki, M., Takat, M. and Kurosaki, T. (1997) Genetic evidence for involvement of type-1, type-2 and type-3 IP₃ receptors in signal transduction through the B-cell antigen receptor. *EMBO J.* **16**, 3078–3088
- Randriamampita, C. and Tsein, R. Y. (1993) Emptying of intracellular Ca²⁺ stores releases a novel small messenger that stimulates Ca²⁺ influx. *Nature (London)* **364**, 809–814
- Parekh, A. B., Terlau, H. and Stuhmer, W. (1993) Depletion of InsP₃ stores activates a Ca²⁺ and K⁺ current by means of a phosphatase and a diffusible messenger. *Nature (London)* **364**, 814–818
- Davies, E. V. and Hallett, M. B. (1995) A soluble factor directly stimulates Ca²⁺ entry in neutrophils. *Biochem. Biophys. Res. Commun.* **206**, 348–354
- Csutora, P., Zhengchang, S., Kim, H. K., Bugrim, A., Cunningham, K. W., Nuccitelli, R., Keizer, J. E., Hanley, M. R., Blalock, J. E. and Marchase, R. B. (1999) Calcium influx factor is synthesised by yeast and mammalian cells depleted of organellar calcium stores. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 121–126
- Laffalian, I. and Hallett, M. B. (1998) Lipid-assisted microinjection: introducing material into the cytosol and membranes of small cells. *Biophys. J.* **75**, 2558–2563
- Davies, E. V. and Hallett, M. B. (1995) A novel pathway for Ca²⁺ signalling in neutrophils by immune complexes. *Immunology* **85**, 538–543
- Hallett, M. B., Davies, E. V. and Pettit, E. J. (1996) Fluorescent methods for measuring and imaging the cytosolic free Ca²⁺ in neutrophils. *Methods* **9**, 591–606
- Montero, M., Alvarez, J. and Garcia-Sancho, J. (1991) Agonist-induced Ca²⁺ influx in human neutrophils is secondary to the emptying of intracellular calcium stores. *Biochem. J.* **277**, 73–79
- von Tscharnner, V., Prod'homme, B., Baggiolini, M. and Reuter, H. (1986) Ion channels in human neutrophils activated by a rise in free cytosolic Ca²⁺ concentration. *Nature (London)* **324**, 369–373
- Foder, B., Scharff, O. and Thastrup, O. (1989) Ca²⁺ transients and Mn²⁺ entry in human neutrophils induced by thapsigargin. *Cell Calcium* **10**, 477–490
- Taylor, C. W. and Broad, L. M. (1998) Pharmacological analysis of intracellular Ca²⁺ signalling: problems and pitfalls. *Trends Pharmacol. Sci.* **19**, 370–375
- Cancela, J. M., Churchill, G. C. and Galione, A. (1999) Coordination of agonist-induced Ca²⁺-signalling patterns by NAADP in pancreatic acinar cells. *Nature (London)* **398**, 74–76
- Bourguignon, L. Y. W., Jin, H. T., Iida, H., Brandt, N. R. and Zhang, S. H. (1993) The involvement of ankyrin in the regulation of inositol 1,4,5-trisphosphate receptor-mediated internal Ca²⁺ release from Ca²⁺ storage vesicles in mouse T-lymphoma cells. *J. Biol. Chem.* **268**, 7290–7297
- Hallett, M. B., Davies, E. V. and Campbell, A. K. (1990) Oxidase activation in individual neutrophils is dependent on the onset and magnitude of the Ca²⁺ signal. *Cell Calcium* **11**, 655–663
- Hallett, M. B. and Pettit, E. J. (1997) Stochastic events underlie Ca²⁺ signalling in neutrophils. *J. Theor. Biol.* **186**, 1–6
- Pettit, E. J. and Hallett, M. B. (1995) Early Ca²⁺ signalling events in neutrophils detected by rapid confocal laser scanning. *Biochem. J.* **310**, 445–448
- Davies, E. V., Hallett, M. B. and Campbell, A. K. (1991) Localised superoxide release by neutrophils can be provoked by a cytosolic calcium 'cloud'. *Immunology* **73**, 228–234
- Pettit, E. J. and Hallett, M. B. (1998) Two distinct Ca²⁺ storage and release sites in human neutrophils. *J. Leukocyte Biol.* **63**, 225–232
- Pettit, E. J., Davies, E. V. and Hallett, M. B. (1997) The microanatomy of Ca²⁺ stores in neutrophils. *Histol. Histopathol.* **12**, 479–490
- Hartmann, J. and Verkhratsky, A. (1998) Relations between intracellular Ca²⁺ stores and store-operated Ca²⁺ entry in primary cultured human glioblastoma cells. *J. Physiol. (London)* **513**, 411–424
- Huang, Y. and Putney, J. W. (1998) Relationship between intracellular calcium store depletion and calcium release-activated calcium current in a mast cell line (RBL-1). *J. Biol. Chem.* **273**, 19554–19559
- Parekh, A. B., Fleig, A. and Penner, R. (1997) The store-operated calcium current I-CRAC: nonlinear activation by InsP(3) and dissociation from calcium release. *Cell* **89**, 973–980
- Putney, J. W. (1999) TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14669–14671
- Putney, J. W. (1999) Kissin' cousins: intimate plasma membrane–ER contact induces capacitative Ca²⁺ entry. *Cell* **99**, 5–8
- Hallett, M. B., Pettit, E. J. and Davies, E. V. (1996) Capacitative Ca²⁺ influx and a diffusible influx factor. *Biochem. J.* **314**, 1054–1055
- Bezprozvanny, I., Watras, J. and Ehrlich, B. E. (1991) Bell-shaped calcium-response curves of IP₃ and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature (London)* **351**, 751–754
- Broad, L. M., Armstrong, D. L. and Putney, J. W. (1999) Role of the inositol 1,4,5-trisphosphate receptor in Ca²⁺ feedback inhibition of calcium release-activated calcium current (I-crac). *J. Biol. Chem.* **274**, 32881–32888
- Davies, E. V. and Hallett, M. B. (1996) Near membrane Ca²⁺ changes resulting from store release in neutrophils: detection by FFP-18. *Cell Calcium* **19**, 355–362