A role for calcium and protein kinase C in agonist-stimulated adhesion of human neutrophils

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Stimulated adherence of human neutrophils to plastic and changes in cytosolic free Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$) were measured in the same cell preparations. $[\text{Ca}^{2+}]_i$ -activation curves were constructed to compare the relation between $[\text{Ca}^{2+}]_i$ and adhesion in response to ionomycin and formylmethionyl-leucyl-phenylalanine (FMLP). This showed that FMLP-stimulated adhesion required less increase in $[\text{Ca}^{2+}]_i$ than did ionomycin's effect, a result suggesting that an additional stimulatory component might be involved in the response to FMLP. Protein kinase C activation was a possibility, and activation of protein kinase C with a phorbol ester (PMA) was found to stimulate adhesion with no change in $[\text{Ca}^{2+}]_i$. A low concentration of PMA was found to synergize with ionomycin to stimulate a greater adhesion response than with each alone, and the $[\text{Ca}^{2+}]_i$ -activation curve for ionomycin in the presence of PMA was shifted towards that for FMLP. Thus, synergy between $[\text{Ca}^{2+}]_i$ and protein kinase C (each of which is sufficient alone) probably explains the stimulatory effects of FMLP on adhesion of neutrophils.

INTRODUCTION

Neutrophils constitute a primary defence mechanism against microbial infection. In response to chemotactic stimuli at the site of infection, a sequence of cellular responses is triggered; neutrophils adhere to the endothelium, migrate to the site of infection and then release superoxide radicals and hydrolytic enzymes (for reviews see Cybulsky *et al.*, 1988; Uhing *et al.*, 1988). Chemotactic stimuli, including FMLP, bind to specific receptors which are coupled to activation of phospholipase C through a G-protein. Phospholipase C catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (which releases Ca^{2+} from intracellular stores) and diacylglycerol (which activates protein kinase C) (Prentki *et al.*, 1984; Nishizuka, 1984; Berridge & Irvine, 1984; Cockcroft & Gomperts, 1985; Rider & Niedel, 1987). These signalling pathways can be activated independently of receptor occupation by means of Ca^{2+} ionophores, such as ionomycin, or with exogenous activators of protein kinase C, such as phorbol esters (including PMA). The contributions of Ca^{2+} and protein kinase C in activating enzyme secretion and superoxide formation have been studied extensively (Pozzan *et al.*, 1983; Sha'afi *et al.*, 1983; Korchak *et al.*, 1984; Lew *et al.*, 1986). It has been shown that either pathway alone is sufficient to support a response, and that synergy between these two messenger systems can promote a greater response (White *et al.*, 1984; DiVirgilio *et al.*, 1984).

In vivo, adhesion of neutrophils to the endothelial lining of post-capillary venules (before chemotaxis) is an early functional response to stimulation by chemotactic factors (Cybulsky *et al.*, 1988; Webster *et al.*, 1986). To date, little is known about the second messengers involved in stimulation of adhesion, although specific cell-surface adhesion molecules have been characterized (Hickstein *et al.*, 1987). Activation of protein kinase C by PMA has been found to stimulate adhesion of neutrophils to endothelium or plastic (Webster *et al.*, 1986), and use of a $[\text{Ca}^{2+}]_i$ imaging system has shown that a rise in $[\text{Ca}^{2+}]_i$ precedes spreading of neutrophils on a glass surface (Jacob, 1990).

The aim of the experiments reported here was to investigate the role of $[\text{Ca}^{2+}]_i$ and protein kinase C in the stimulation of neutrophil adhesion (to albumin-coated plastic) in order to gain further knowledge of the mechanism of agonist (FMLP)-stimulated adhesion. Ionomycin and PMA, respectively, were used to activate the two pathways independently of agonist. Their effects on both adhesion and $[\text{Ca}^{2+}]_i$ in the same cell preparation, were compared with that of FMLP. $[\text{Ca}^{2+}]_i$ was measured with fura-2, and adhesion was measured by using Acridine Orange to label the cells. The spectral characteristics of the two dyes permit each to be measured independently when the cells are co-loaded with both dyes. These results demonstrate the contributions of $[\text{Ca}^{2+}]_i$ (distinguishing between Ca^{2+} influx and release from intracellular stores) and protein kinase C in mediating FMLP-stimulated adhesion. Some of these results have been published in abstract form (Davies *et al.*, 1989).

EXPERIMENTAL

Percoll and dextran T-500 were from Pharmacia (Uppsala, Sweden); fura-2 acetoxymethyl ester was from Molecular Probes Inc. (Eugene, OR, U.S.A.); Acridine Orange, FMLP and PMA were from Sigma; Hepes (ultrapure) and ionomycin were from Calbiochem.

Human peripheral neutrophils were prepared as previously described (Merritt *et al.*, 1989). The method essentially consists of collection of fresh blood into citrate anticoagulant, sedimentation of erythrocytes with dextran, concentration of the leucocyte-rich plasma, and purification of the neutrophils on a discontinuous density gradient of Percoll. This preparation contains > 95 % neutrophils, with a viability of > 99 % (assessed by Trypan Blue exclusion). It should be noted that a very high degree of viability is required in order to maintain a low level of unstimulated adhesion. Neutrophils were resuspended at 6×10^6 cells/ml in medium containing 145 mM-NaCl, 5 mM-KCl, 1 mM- MgCl_2 , 10 mM-Hepes (pH 7.4), 10 mM-glucose, 1 % BSA and 1 mM- CaCl_2 . For experiments involving depletion of the

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; FMLP, formylmethionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate.

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intracellular Ca^{2+} stores, the CaCl_2 was replaced with 1 mM-EGTA. For loading with dyes, the cells were incubated for 45 min at 37 °C in the above medium supplemented with fura-2 acetoxymethyl ester (2 μM) and Acridine Orange (1 μM). The cells were then resuspended at 2×10^6 cells/ml in medium containing 145 mM-NaCl, 5 mM-KCl, 1 mM- MgCl_2 , 10 mM-Hepes (pH 7.4), 10 mM-glucose, 0.1 % BSA and 0.2 mM- CaCl_2 . For experiments involving depletion of the intracellular Ca^{2+} stores, the CaCl_2 was replaced with 50 μM -EGTA. All measurements of both $[\text{Ca}^{2+}]_i$ and adhesion were carried out at room temperature.

For measurement of $[\text{Ca}^{2+}]_i$, fura-2 fluorescence was measured in a Perkin-Elmer LS-5 fluorimeter. Fluorescence was measured at 340 nm excitation and 500 nm emission, and $[\text{Ca}^{2+}]_i$ was calculated as previously described (Gryniewicz *et al.*, 1985; Pollock *et al.*, 1986). Since all measurements were made at room temperature, the K_d for Ca^{2+} binding to fura-2 was taken to be 135 nM at 20 °C, as reported by Gryniewicz *et al.* (1985).

For measurement of adhesion, Acridine Orange fluorescence was used as a measure of cell number. Incubations were carried out at room temperature for 15 min in plastic tissue-culture wells (Costar 24-well plates) in a total volume of 1 ml (0.1 % BSA was required in order to maintain a low level of basal adhesion). After the 15 min incubation, non-adherent cells were washed out, the adherent cells were solubilized in Triton X-100 (10 %), and the fluorescence of the adherent cell lysate was measured with a Perkin-Elmer LS-5 fluorimeter (495 nm excitation, 530 nm emission). The fluorescence of a total cell lysate was also measured, and adherence was expressed as percentage of total cells. To correct for leakage of label during the course of the experiment, cells were incubated in suspension under parallel conditions, then centrifuged to remove any dye that had leaked into the supernatant, and the fluorescence of the lysed cell pellet was taken as a measure of total cell fluorescence for each condition.

All results are expressed as means $\pm$ S.E.M. from at least three separate experiments, each carried out in duplicate.

RESULTS AND DISCUSSION

Fig. 1(a) shows that FMLP stimulated a dose-dependent elevation in $[\text{Ca}^{2+}]_i$ and stimulated adhesion of neutrophils over the same dose range. In these and all subsequent experiments, $[\text{Ca}^{2+}]_i$ and adhesion were measured in samples of the same cell preparation co-loaded with the two dyes (see the Experimental section). In each case, the effective concentrations of FMLP were in the range of 0.1–10 nM. Platelet-activating factor also stimulated rises in $[\text{Ca}^{2+}]_i$ and adhesion (results not shown). The observation that receptor occupation by agonists evokes rises in $[\text{Ca}^{2+}]_i$ and adhesion suggests that rises in $[\text{Ca}^{2+}]_i$ may have a role in stimulating adhesion. In order to address this question more directly, the Ca^{2+} ionophore ionomycin was used to elevate $[\text{Ca}^{2+}]_i$ independently of receptor agonists. Fig. 1(b) shows that ionomycin also stimulated adhesion over the same range of concentrations that stimulated increases in $[\text{Ca}^{2+}]_i$. This result shows that simply elevating $[\text{Ca}^{2+}]_i$ is a sufficient stimulus for adhesion of neutrophils. Similar results have been reported for stimulation of superoxide and enzyme secretion from neutrophils (Pozzan *et al.*, 1983; Lew *et al.*, 1986).

Stimulation of neutrophils with FMLP causes a rise in $[\text{Ca}^{2+}]_i$ that is due to both release of Ca^{2+} from intracellular stores and influx of Ca^{2+} from the extracellular medium (Pozzan *et al.*, 1983; Andersson *et al.*, 1986; Von Tscharner *et al.*, 1986; Merritt *et al.*, 1989). The following experiments were designed to determine the source of the Ca^{2+} required for FMLP-stimulated adhesion. Fig. 2(a) shows that loading cells with fura-2 under different conditions permits manipulation of the contributions of store release and influx to the FMLP-stimulated rise in $[\text{Ca}^{2+}]_i$. When cells were loaded with fura-2 in the presence of extracellular Ca^{2+} , the intracellular Ca^{2+} store remained full; stimulation of cells with FMLP in the absence of extracellular Ca^{2+} (1 mM-EGTA added just before FMLP) resulted in a rise in $[\text{Ca}^{2+}]_i$, owing to store release. Stimulation of cells in the presence of extracellular Ca^{2+} resulted in a larger increase in $[\text{Ca}^{2+}]_i$, the

Fig. 1. Effect of FMLP and ionomycin on $[\text{Ca}^{2+}]_i$ and adhesion in human neutrophils

Neutrophils were co-loaded with fura-2 and Acridine Orange to allow $[\text{Ca}^{2+}]_i$ and adhesion to be measured in the same preparation: (a) shows the effects of FMLP, with data represented as means $\pm$ S.E.M. from three separate experiments; (b) shows the effects of ionomycin in a typical experiment.

Fig. 2. Effect of manipulating $[\text{Ca}^{2+}]_i$ with fura-2

Neutrophils were loaded with fura-2, such that the intracellular Ca^{2+} store would remain full ('normal') or be depleted ('depleted'): (a) shows typical traces of $[\text{Ca}^{2+}]_i$ for neutrophils, loaded under each of these conditions, stimulated with FMLP (10 nM) in the presence (1 mM- CaCl_2) or absence (1 mM-EGTA) of extracellular Ca^{2+} ; (b) shows the adhesion responses (means $\pm$ S.E.M.; $n = 3-4$ separate preparations) of cells with 'normal' or 'depleted' Ca^{2+} stores. All responses are shown in the absence ($\square$) or presence ($\blacksquare$) of FMLP (10 nM) under the conditions indicated.

difference being attributed to Ca^{2+} entry. When cells were loaded with the same amount of fura-2 in the presence of EGTA (see the Experimental section), the intracellular Ca^{2+} stores were partially depleted; under these conditions, the FMLP-stimulated rise in $[\text{Ca}^{2+}]_i$ was very small in the absence of extracellular Ca^{2+} , and the response was restored in the presence of extracellular Ca^{2+} . In all of the experiments reported here, the cells were loaded with fura-2 to a cytosolic concentration of 500 μM , which is sufficient to buffer partially release of intracellular Ca^{2+} stores. Loading cells to a cytosolic concentration of 50–100 μM -fura-2 (Merritt *et al.*, 1989) results in little buffering of the $[\text{Ca}^{2+}]_i$ rises, such that the initial rise in $[\text{Ca}^{2+}]_i$ stimulated by FMLP is similar in the presence or absence of extracellular Ca^{2+} . The adhesion response of neutrophils to FMLP in the presence or absence of extracellular Ca^{2+} was no different when the cytosolic concentration of fura-2 was 50 or 500 μM , and this was no different from the responses in the absence of fura-2 (results not shown). Fig. 2(b) shows the adhesion responses of neutrophils loaded with fura-2, such that the intracellular Ca^{2+} stores are 'normal' or 'depleted'. In each case, basal and FMLP-stimulated adhesions have been measured in the presence or absence of extracellular Ca^{2+} , and in the presence of Ca^{2+} with 5 mM- Ni^{2+} (this concentration of NiCl_2 is sufficient to block Ca^{2+} influx; Merritt *et al.*, 1989). When the Ca^{2+} stores of neutrophils were full ('normal'), FMLP-stimulated adhesion was no different in the presence or absence of extracellular Ca^{2+} or when Ca^{2+} influx was blocked by Ni^{2+} . This result shows that extracellular Ca^{2+} is not a requirement for adhesion of neutrophils, and that Ca^{2+} influx is not normally required for FMLP-stimulated adhesion. Ca^{2+} release from intracellular stores alone would seem to be sufficient; this result

is consistent with adhesion being an acute response of neutrophils, which is unlikely to require a maintained rise in $[\text{Ca}^{2+}]_i$. Fig. 2(b) also shows that, when the Ca^{2+} stores are depleted, FMLP-stimulated adhesion becomes partially dependent on extracellular Ca^{2+} and therefore Ca^{2+} influx. However, even when the Ca^{2+} stores were partially depleted and FMLP was only able to stimulate a small rise in $[\text{Ca}^{2+}]_i$, owing to store release, adhesion was stimulated to some extent. These results suggest that $[\text{Ca}^{2+}]_i$ has a role in FMLP-stimulated adhesion, but that it may not be the only mediator.

Fig. 2 shows that FMLP can stimulate adhesion, partially, with very little increase in $[\text{Ca}^{2+}]_i$. Comparison of Figs. 1(a) and 1(b) shows that, for the same degree of stimulation of adhesion, ionomycin evokes considerably greater increases in $[\text{Ca}^{2+}]_i$ than does FMLP. Fig. 3 shows ' $[\text{Ca}^{2+}]_i$ -activation curves' for adhesion stimulated by both ionomycin and FMLP. For these experiments, both $[\text{Ca}^{2+}]_i$ and adhesion were measured in the same cell preparations in response to various concentrations of FMLP and ionomycin. For each concentration of stimulus, the results are plotted as $[\text{Ca}^{2+}]_i$ against adhesion. It is clear that an increase in $[\text{Ca}^{2+}]_i$ is associated with an increase in adhesion. However, the $[\text{Ca}^{2+}]_i$ -activation curve for FMLP is shifted to the left of that for ionomycin, showing that FMLP can stimulate adhesion at lower $[\text{Ca}^{2+}]_i$ than for ionomycin. This result suggests that an additional stimulatory component may be involved in the response to FMLP. This result contrasts with previous results with endothelial cells, where the $[\text{Ca}^{2+}]_i$ -activation curves for prostacyclin production stimulated by thrombin and ionomycin are superimposable, showing that in this case a rise in $[\text{Ca}^{2+}]_i$ alone is sufficient to explain the stimulatory effects of thrombin (Hallam *et al.*, 1988). It is known that FMLP activates phospholipase C (through a G-protein), which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (Cockcroft & Gomperts, 1985; Uhing *et al.*, 1988). Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from intracellular stores (Berridge & Irvine, 1984; Prentki *et al.*, 1984), and is probably responsible for the component of the adhesion response to FMLP that is associated with Ca^{2+} release from intracellular stores. Diacylglycerol activates protein kinase C (Nishizuka, 1984). The next experiment was therefore to determine whether activation of protein kinase C may have a role in the stimulatory effect of FMLP.

Phorbol esters, such as PMA, are able to activate protein kinase C directly, by-passing receptor stimulation by agonists

Fig. 3. $[\text{Ca}^{2+}]_i$ -activation curves for adhesion of neutrophils stimulated with FMLP or ionomycin

Both $[\text{Ca}^{2+}]_i$ and adhesion were measured in response to various concentrations of FMLP (0.1–100 nM) or ionomycin (10–500 nM) in the same cell preparations. For each concentration of FMLP ($\blacksquare$) and ionomycin ($\bullet$), as well as the control ($\circ$), adhesion was plotted against $[\text{Ca}^{2+}]_i$. Results are the means $\pm$ S.E.M. from three separate preparations.

Fig. 4. Effect of PMA on adhesion of neutrophils

Adhesion was measured in response to PMA (0.2–10 nM). The response to a maximal concentration of FMLP (10 nM) is included for comparison. $[Ca^{2+}]_i$ was unchanged by all concentrations of PMA. Results are means $\pm$ S.E.M. from four separate cell preparations.

Fig. 5. $[Ca^{2+}]_i$ -activation curves for adhesion showing synergy between ionomycin and PMA

$[Ca^{2+}]_i$ -activation curves were constructed as described in the legend to Fig. 3. Points are means $\pm$ S.E.M. ($n = 3$ separate preparations) for control (□), FMLP (▲), ionomycin (■) and ionomycin plus 0.15 nM-PMA (●).

(Nishizuka, 1984). Fig. 4 shows that PMA caused dose-dependent stimulation of adhesion, with the maximal stimulation being similar to that evoked by a maximal concentration of FMLP. The effective concentration range was 0.2–10 nM-PMA, concentrations similar to those required for activation of protein kinase C *in vitro* (Nishizuka, 1984). $[Ca^{2+}]_i$ was unchanged from the basal level of around 100 nM at all concentrations of PMA. Activation of protein kinase C can therefore stimulate adhesion with no increase in $[Ca^{2+}]_i$.

Since FMLP stimulates both the $[Ca^{2+}]_i$ and protein kinase C signalling pathways, experiments were carried out to see whether synergy between the two signalling pathways might explain the stimulatory effects of FMLP. Fig. 5 shows that incubation of neutrophils with a low concentration of PMA (0.15 nM), which itself does not stimulate adhesion, shifts the $[Ca^{2+}]_i$ -activation curve for ionomycin to the left towards that for FMLP. This results shows that, when protein kinase C is activated, adhesion can be stimulated by a smaller increase in $[Ca^{2+}]_i$. It is therefore

likely that FMLP stimulates adherence of neutrophils through activation of both the $[Ca^{2+}]_i$ and protein kinase C pathways, which act in synergy to promote the response. Synergy between $[Ca^{2+}]_i$ and protein kinase C has been reported for other functional responses of neutrophils; the two signalling systems have been shown to synergize in the stimulation of superoxide formation and enzyme secretion (White *et al.*, 1984; DiVirgilio *et al.*, 1984). Similar results have also been reported for a variety of functional responses of different cells (Nishizuka, 1984), including pancreatic enzyme secretion (Merritt & Rubin, 1985) and platelet secretion (Rink *et al.*, 1983).

In conclusion, these results have shown that either an elevation in $[Ca^{2+}]_i$ or activation of protein kinase C alone is sufficient to stimulate adherence of neutrophils to plastic, and that the two signalling pathways synergize to promote a greater response. This synergy probably explains the stimulatory effect of FMLP on adherence of neutrophils.

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