Activation of protein kinase C in human neutrophils attenuates agonist-stimulated rises in cytosolic free Ca^{2+} concentration by inhibiting bivalent-cation influx and intracellular Ca^{2+} release in addition to stimulating Ca^{2+} efflux

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Stimulation of fura-2-loaded human neutrophils with formylmethionyl-leucyl-phenylalanine (FMLP) or ionomycin elevated the cytosolic free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, to a maintained elevated level. Activation of protein kinase C (C-kinase) with phorbol 12-myristate 13-acetate, 4β -phorbol 12,13-didecanoate or dioctanoylglycerol caused decreases in $[\text{Ca}^{2+}]_i$ from this level. 4α -Phorbol didecanoate, which does not activate C-kinase, had no effect. These results confirm previous reports that C-kinase activation decreases neutrophil $[\text{Ca}^{2+}]_i$ by stimulating removal of Ca^{2+} from the cytosol. Further experiments showed that activation of C-kinase attenuated the component of the FMLP-stimulated $[\text{Ca}^{2+}]_i$ rise that was dependent on external Ca^{2+} . C-kinase activation also inhibited FMLP-stimulated entry of the quenching cation, Mn^{2+} , used as an indicator of bivalent-cation entry. In contrast, C-kinase activation caused only a partial inhibition of FMLP-stimulated release of Ca^{2+} from intracellular stores. 4α -Phorbol didecanoate was ineffective in inhibiting Ca^{2+} entry, Mn^{2+} entry and intracellular Ca^{2+} release. Addition of FMLP also stimulated a decrease in the ionomycin-elevated $[\text{Ca}^{2+}]_i$, and this effect was blocked by staurosporine, a protein kinase inhibitor. These results show that, in addition to stimulating Ca^{2+} efflux, C-kinase activation in neutrophils inhibits FMLP-stimulated entry of bivalent cations, and partially inhibits intracellular release of Ca^{2+} . Further, FMLP itself can modulate $[\text{Ca}^{2+}]_i$ by activation of C-kinase.

INTRODUCTION

In many cell types, including neutrophils, agonists activate phosphoinositidase C, which catalyses the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, yielding $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (Berridge & Irvine, 1984; Nishizuka, 1984; Cockcroft *et al.*, 1985). After this, $[\text{Ca}^{2+}]_i$ rises rapidly as $\text{Ins}(1,4,5)\text{P}_3$ stimulates the discharge of Ca^{2+} from intracellular stores. In the continued presence of agonist, $[\text{Ca}^{2+}]_i$ is maintained at an elevated level as Ca^{2+} enters from the extracellular medium (Hallam *et al.*, 1984; Hallam & Pearson, 1986; Merritt & Rink, 1987; Merritt *et al.*, 1989). The precise nature of the stimulus for Ca^{2+} influx is unclear, and there may be more than one mechanism (for review, see Hallam & Rink, 1989). Diacylglycerol activates C-kinase, which itself activates a range of cellular processes (Nishizuka, 1984).

Elevation of $[\text{Ca}^{2+}]_i$ is an early event in the response of neutrophils to many agonists, including the chemotactic peptide FMLP, the complement fragment C5a, platelet-activating factor and leukotriene B_4 (Sha'afi *et al.*, 1983; Pozzan *et al.*, 1983; Lew *et al.*, 1987; Hartiala *et al.*, 1987). The rise in $[\text{Ca}^{2+}]_i$ is due to both release of Ca^{2+} from intracellular stores and influx from the extracellular medium (Korchak *et al.*, 1984a,b; Andersson *et al.*, 1986; von Tscharner *et al.*, 1986; Merritt *et al.*, 1989). Activation of C-kinase with tumour-promoting phorbol

esters and synthetic diacylglycerols is known to attenuate agonist-stimulated elevations of $[\text{Ca}^{2+}]_i$ in neutrophils (Naccache *et al.*, 1985), and also in other cell types (MacIntyre *et al.*, 1985; Rosoff *et al.*, 1985; Vicenti *et al.*, 1985; Dubyak *et al.*, 1988). In contrast, activation of C-kinase stimulates neutrophil 'end-responses' such as lysosomal enzyme secretion and superoxide formation (Sha'afi *et al.*, 1983; Korchak *et al.*, 1984a,b). These findings have led to the suggestion that C-kinase may regulate neutrophil function in a bidirectional manner, on the one hand inhibiting, or controlling, agonist-stimulated elevations of $[\text{Ca}^{2+}]_i$, while on the other stimulating the respiratory burst and degranulation (Drummond & MacIntyre, 1985).

A number of previous studies have dealt with C-kinase-stimulated efflux of Ca^{2+} from neutrophils. A clear stimulatory effect of C-kinase activation on the plasma-membrane Ca^{2+} -ATPase of neutrophils has been demonstrated (Lagast *et al.*, 1984), and the phorbol ester PMA has been shown to cause a decrease in both agonist- and ionophore-elevated $[\text{Ca}^{2+}]_i$ in neutrophils (Lagast *et al.*, 1984; Perianin & Snyderman, 1989). Similar observations of the effects of PMA on $[\text{Ca}^{2+}]_i$ have been made in human platelets (Rink & Sage, 1987; Pollock *et al.*, 1987). Further, direct C-kinase-mediated stimulation of the plasma-membrane Ca^{2+} -ATPase of erythrocytes has been demonstrated, suggesting that in this

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; C-kinase, protein kinase C; DMSO, dimethyl sulphoxide; DOG, 1,2-dioctanoyl-sn-glycerol; FMLP, formylmethionyl-leucyl-phenylalanine; 4α -PDD, 4α -phorbol 12,13-didecanoate; 4β -PDD, 4β -phorbol 12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate.

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cell type the pump can be activated by direct phosphorylation (Smallwood *et al.*, 1988). These studies have indicated that a major mechanism by which C-kinase activation may decrease agonist-stimulated elevations of $[Ca^{2+}]_i$, or depress maintained elevated levels of $[Ca^{2+}]_i$, is by stimulated Ca^{2+} efflux. A second mechanism by which C-kinase may antagonize agonist-stimulated elevations of $[Ca^{2+}]_i$ is by feedback inhibition of phosphoinositidase C, leading to decreased $Ins(1,4,5)P_3$ formation (Della Bianca *et al.*, 1986; Smith *et al.*, 1987), and therefore decreased release of Ca^{2+} from intracellular stores.

In the present study, we have attempted to define the precise effects of C-kinase activation on agonist-stimulated changes in $[Ca^{2+}]_i$ in suspensions of human neutrophils loaded with the fluorescent Ca^{2+} -indicator dye fura-2. To study Ca^{2+} efflux, we have used the Ca^{2+} ionophore, ionomycin, to elevate $[Ca^{2+}]_i$ independently of receptor stimulation, a modification of the method described by Pollock *et al.* (1987). FMLP-stimulated Ca^{2+} influx has been studied in neutrophils heavily loaded with fura-2 to buffer $[Ca^{2+}]_i$ and thus decrease the contribution of intracellular release of Ca^{2+} to the overall elevation of $[Ca^{2+}]_i$, as reviewed by Tsien *et al.* (1984). Stimulated influx of the quenching cation Mn^{2+} has been used as a more direct measure of bivalent-cation influx (Merritt *et al.*, 1989). In addition to confirming that C-kinase activation stimulates removal of Ca^{2+} from the cytosol, our results show that C-kinase can act in two further ways to inhibit agonist-stimulated elevations of $[Ca^{2+}]_i$ in neutrophils; by causing blockade of stimulated influx of bivalent cations from the extracellular medium, and by partially inhibiting release of Ca^{2+} from intracellular stores.

METHODS

Percoll and dextran T500 were from Pharmacia (Uppsala, Sweden); fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, U.S.A.); staurosporine, HEPES (ultrapure) and ionomycin were from Calbiochem; PMA, 4 α -PDD and 4 β -PDD were from Sigma; DOG was from BCL; DMSO (anhydrous, 99% pure) was from Aldrich.

Neutrophils were prepared from freshly drawn blood of healthy volunteers by dextran sedimentation followed by Percoll-density-gradient centrifugation, as previously described (Merritt *et al.*, 1989). The cells were washed and suspended in HEPES-buffered saline (145 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 10 mM-HEPES, pH 7.4 at 37 °C) supplemented with 10 mM-glucose, 1 mM-CaCl₂ and 1% bovine serum albumin, to a cell density of approx. 6×10^6 cells/ml. 'Light' fura-2 loading was achieved by incubating cells with 0.5 μ M-fura-2-AM for 30 min at 37 °C, resulting in a cytosolic dye concentration of approx. 100 μ M. 'Heavy' loading was achieved by incubating cells with 2 μ M-fura-2-AM for 45 min at 37 °C, resulting in a cytosolic dye concentration of approx. 500 μ M. After dye loading, the cells were washed, resuspended in the same medium to a cell density of approx. 2.5×10^6 cells/ml and kept at room temperature until use.

Fura-2 fluorescence of neutrophil suspensions (500 nm emission) was measured at 37 °C, either in a dual-excitation single-emission-wavelength Spex fluorimeter or in a single-excitation single-emission-wavelength Perkin-Elmer LS-3 fluorimeter. Samples (0.7 ml) of cell

suspensions were centrifuged (Microfuge; 6500 rev./min for 30 s) and the cells resuspended in buffer containing no added Ca^{2+} or albumin. $CaCl_2$ (1 mM) or EGTA (1 mM) was added to the cuvettes as required. $[Ca^{2+}]_i$ was measured either as the ratio of fura-2 fluorescence at excitation wavelengths of 340 and 380 nm (Fig. 1) or as changes in fluorescence at a single excitation wavelength of 340 nm (Figs. 2, 3, 6 and 8), in accordance with Grynkiewicz *et al.* (1985). Dual-wavelength measurements of Mn^{2+} influx were made at excitation wavelengths of 340 nm and 360 nm as described by Merritt *et al.* (1989), with traces shown as actual fluorescence (Fig. 4). All other measurements of Mn^{2+} influx were made at a single excitation wavelength of 360 nm. Mn^{2+} influx was quantified by measuring both the initial rate and the final extent of the stimulated quench of fura-2 fluorescence. All traces are typical of at least three experiments. Dose-inhibition curves show means $\pm$ S.E.M. ($n = 3$).

RESULTS

Effect of PMA on the elevated steady-state $[Ca^{2+}]_i$ in response to FMLP and ionomycin

Fig. 1 shows the effect of PMA on the steady-state $[Ca^{2+}]_i$ seen after treatment of neutrophil suspensions, in

Fig. 1. Effect of PMA on elevated steady-state $[Ca^{2+}]_i$ in fura-2-loaded neutrophils

$[Ca^{2+}]_i$ was calculated from the ratio of fluorescence at two excitation wavelengths (340 nm and 380 nm). Fura-2 loading was light (cytosolic dye concn. approx. 100 μ M). Neutrophils were incubated in the presence of 1 mM- $CaCl_2$. $[Ca^{2+}]_i$ was elevated from resting levels by (a) stimulation with FMLP (10 nM) or (b) addition of ionomycin (IONO; 50 nM). PMA (100 nM) was added at the times indicated.

Fig. 2. Effect of preincubation with PMA before addition of ionomycin

Fura-2 loading was the same as in Fig. 1. $[Ca^{2+}]_i$ was measured at a single excitation wavelength of 340 nm. (a) Partial inhibition by PMA of the rise in $[Ca^{2+}]_i$ evoked by 50 nM-ionomycin. (b) Dose-inhibition curve for effect of PMA on the ionomycin-evoked elevation of $[Ca^{2+}]_i$. All points represent means $\pm$ S.E.M. ($n = 3$).

the presence of 1 mM extracellular Ca^{2+} , with either FMLP or ionomycin. Stimulation of neutrophils lightly loaded with fura-2 (cytosolic dye concn. approx. 100 μM) with FMLP (10 nM) evoked an increase in $[Ca^{2+}]_i$ from a

resting value of 150 nM to a peak value in excess of 1 μM (Fig. 1a). After the initial transient peak, $[Ca^{2+}]_i$ declined to an elevated steady-state level of approx. 650 nM. Addition of PMA (100 nM) caused an immediate and sustained depression of this steady-state $[Ca^{2+}]_i$. Treatment of neutrophils with ionomycin (50 nM) in the presence of 1 mM extracellular Ca^{2+} (Fig. 1b) raised $[Ca^{2+}]_i$ from a resting level of 100 nM to a steady-state level comparable with the maintained elevation seen in response to FMLP. Addition of PMA (100 nM) caused an immediate depression of the ionophore-elevated $[Ca^{2+}]_i$, although the decrease was only transient. Similar decreases in $[Ca^{2+}]_i$ were observed in response to 4 β -PDD (100 nM) and DOG (0.5 μM), whereas DMSO vehicle (0.1%) or 4 α -PDD (100 nM), which does not activate C-kinase, had no effect (results not shown). Preincubation of cells with the protein kinase inhibitor staurosporine (100 nM) partially blocked the effects of PMA, 4 β -PDD and DOG on the ionomycin-elevated $[Ca^{2+}]_i$ (results not shown). When cells were incubated with PMA for 2 min before addition of ionomycin (50 nM), in the presence of 1 mM extracellular Ca^{2+} , a partial, dose-dependent, inhibition of the peak $[Ca^{2+}]_i$ was observed (Fig. 2). Maximal inhibition of 40% occurred at a dose of 100 nM-PMA. This partial inhibition corresponds to the partial decrease in $[Ca^{2+}]_i$ seen when PMA was added after treatment of cells with ionomycin (Fig. 1).

Effect of C-kinase activation on FMLP-stimulated Ca^{2+} and Mn^{2+} entry

To study the effects of C-kinase activation on FMLP-stimulated Ca^{2+} influx, neutrophils were heavily loaded with fura-2 (cytoplasmic dye concn. approx. 0.5 mM), to buffer release of Ca^{2+} from intracellular stores, thus exaggerating the component of the stimulated elevation of $[Ca^{2+}]_i$ caused by Ca^{2+} influx. Under these conditions the peak rise in fluorescence in response to FMLP in the presence of 1 mM extracellular Ca^{2+} was approximately twice that in the absence of external Ca^{2+} . Fig. 3 shows the effect of PMA on FMLP-stimulated elevations of

Fig. 3. Effect of PMA on the FMLP-stimulated increase in $[Ca^{2+}]_i$ in neutrophils heavily loaded with fura-2

Single-excitation-wavelength measurements of $[Ca^{2+}]_i$ showing the effects of various concentrations of PMA on the FMLP-stimulated rise in $[Ca^{2+}]_i$ in neutrophils with approx. 0.5 mM cytosolic fura-2. Neutrophil suspensions were incubated in either the presence (1 mM $CaCl_2$ present) or the absence (1 mM-EGTA present) of external Ca^{2+} . PMA (or DMSO vehicle, 0.1%) was added 2 min before stimulation of cells with 10 nM-FMLP.

Fig. 4. Effect of PMA on FMLP-stimulated Mn^{2+} entry in fura-2-loaded neutrophils

Changes in fura-2 fluorescence stimulated by FMLP (a) in the absence of PMA and (b) in the presence of PMA (2 nM). Fluorescence was monitored at two excitation wavelengths (340 nm and 360 nm). Fura-2 loading was the same as in Fig. 1. Arrows indicate the time of addition of PMA (or DMSO vehicle, 0.1%), MnCl_2 (100 μM), FMLP (10 nM) and ionomycin (IONO; 2 μM).

$[\text{Ca}^{2+}]_i$ in neutrophils heavily loaded with fura-2. Cells were incubated with PMA or DMSO vehicle for 2 min before addition of agonist. PMA caused dose-dependent blockade of the response to FMLP (10 nM) in the presence of external Ca^{2+} . PMA caused a partial inhibition of the response to FMLP in the absence of extracellular Ca^{2+} (extracellular Ca^{2+} was removed by chelation with EGTA). Because PMA inhibited the response to FMLP in the absence as well as the presence of external Ca^{2+} , this method prevented clear dose-inhibition studies being carried out on Ca^{2+} influx. To study the effects of C-kinase activation on FMLP-stimulated bivalent-cation influx independently of release from intracellular stores, influx of the quenching cation Mn^{2+} was measured (Merritt *et al.*, 1989). Fig. 4 shows actual fluorescence traces at two excitation wavelengths (340 nm and 360 nm) from suspensions of neutrophils (lightly loaded with fura-2) incubated in the presence of MnCl_2 (100 μM) with no added Ca^{2+} . At 340 nm, fura-2 fluorescence increases as $[\text{Ca}^{2+}]_i$ increases, whereas the fluorescence at 360 nm is insensitive to changes in $[\text{Ca}^{2+}]_i$. Fura-2

fluorescence at both wavelengths is quenched by Mn^{2+} . In the absence of FMLP, addition of Mn^{2+} caused a slow but steady decrease in fluorescence at both wavelengths, owing to the basal rate of leakage of Mn^{2+} into the cells (see control trace at 360 nm, Fig. 5a). Fig. 4(a) shows that, in the absence of PMA, FMLP evoked a rapid increase in fluorescence at 340 nm, owing to release of Ca^{2+} from intracellular stores. This rise was followed by a sharp decrease in fluorescence to well below pre-stimulated levels as Mn^{2+} entered the cells and quenched the intracellular dye. At 360 nm, only the stimulated quench of fura-2 fluorescence was seen. Addition of ionomycin (which can translocate Mn^{2+} in addition to Ca^{2+}) 8 min after FMLP caused little further change in fluorescence, indicating that most of the intracellular dye had already been quenched. Preincubation of cells with PMA (2 nM) before addition of FMLP had little effect on the stimulated rise in fluorescence at 340 nm, but abolished the

Fig. 5. Dose-dependency of C-kinase-mediated inhibition of FMLP-stimulated Mn^{2+} entry

(a) Overlaid fluorescence traces recorded from separate neutrophil suspensions, at an excitation wavelength of 360 nm. PMA (or DMSO vehicle, 0.1%), MnCl_2 (100 μM), FMLP (10 nM) and ionomycin (Iono; 2 μM) were added at the times indicated. Lower trace, FMLP-stimulated Mn^{2+} entry in the absence of PMA; upper trace, basal rate of Mn^{2+} entry in the absence of PMA and FMLP; middle traces, dose-dependent inhibition of FMLP-stimulated Mn^{2+} entry by increasing doses of PMA (0.1, 0.2, 0.5, 0.7 and 1.0 nM). (b) Dose-inhibition curves for inhibition of the final extent of the FMLP-stimulated Mn^{2+} entry into neutrophils by PMA (○), 4β-PDD (▲), DOG (△) and 4α-PDD (●). All points represent means $\pm$ S.E.M. ($n = 3$).

Fig. 6. Effect of PMA on FMLP-stimulated release of Ca^{2+} from intracellular stores

Neutrophil suspensions were incubated in the presence of 1 mM-EGTA. Fura-2 loading was the same as in Fig. 1. PMA (or DMSO vehicle, 0.1%) was added 2 min before FMLP. (a) Single-excitation-wavelength measurements of $[Ca^{2+}]_i$ showing effect of various doses of PMA on the FMLP (10 nM)-stimulated rise in $[Ca^{2+}]_i$. (b) Dose-inhibition curve for inhibition by PMA of the FMLP-stimulated peak rise in $[Ca^{2+}]_i$. All points represent means $\pm$ S.E.M. ($n = 3$).

FMLP-stimulated Mn^{2+} entry (Fig. 4b). The remaining slow but steady decrease in fluorescence was due to the basal rate of leakage of Mn^{2+} into the cells. Addition of ionomycin (2 μ M) 8 min after FMLP caused complete quenching of the intracellular dye, showing that PMA does not interfere with ionomycin-mediated bivalent cation influx. Fig. 5(a) shows that PMA blocked FMLP-stimulated Mn^{2+} influx in a dose-dependent manner; it shows typical fluorescence traces recorded at 360 nm from suspensions of neutrophils exposed to different concentrations of PMA. The control trace shows the basal rate of leakage of Mn^{2+} into the cells in the absence of FMLP. Fig. 5(b) shows dose-response curves for inhibition of FMLP-stimulated Mn^{2+} entry by PMA [IC_{50} (concn. giving 50% inhibition) = 0.65 nM], 4 β -PDD (IC_{50} = 16 nM), DOG (IC_{50} = 180 nM) and 4 α -PDD (inactive at concentrations up to 100 nM). Each compound inhibited the initial rate and final extent of stimulated Mn^{2+} entry with similar IC_{50} values. Staurosporine (100 nM) completely blocked the inhibition of FMLP-stimulated Mn^{2+} entry caused by 2 nM-PMA (result not shown).

Effect of C-kinase activation on release of Ca^{2+} from intracellular stores

To determine the effect of C-kinase activation on FMLP-stimulated release of Ca^{2+} from intracellular stores, neutrophils (lightly loaded with fura-2) were suspended in medium containing 1 mM-EGTA. PMA or DMSO vehicle (0.1%) was added 2 min before FMLP (10 nM). PMA only partially inhibited FMLP-stimulated internal Ca^{2+} release under these conditions. A maximally effective concentration of PMA (100 nM) caused approx. 40% inhibition of the stimulated peak rise in $[Ca^{2+}]_i$ (Fig. 6). Further experiments showed that C-kinase activation with PMA had no effect on ionomycin-mediated release of Ca^{2+} from intracellular stores (results not shown).

All the above dose-inhibition studies were carried out with a fixed preincubation time of 2 min with PMA. More detailed investigation of the time-dependence of these effects showed a far more rapid onset of inhibition of FMLP-stimulated Mn^{2+} influx compared with inhibition of intracellular release of Ca^{2+} . In Fig. 7, cells were preincubated for various times with a dose of PMA (1 nM) that gave approx. 60% inhibition of stimulated Mn^{2+} entry at 2 min preincubation. This dose of PMA caused complete inhibition of stimulated Mn^{2+} entry after 5 min. In contrast, FMLP-stimulated intracellular Ca^{2+} release was only 40% inhibited after 5 min, and 60% inhibited after 10 min.

Effect of FMLP on ionomycin-elevated $[Ca^{2+}]_i$

To determine whether FMLP could also stimulate removal of Ca^{2+} from the cytosol, analogous experiments to those shown in Fig. 1 were carried out. Ionomycin (100 nM) was used to elevate $[Ca^{2+}]_i$ to a new steady-state, and addition of FMLP (10 nM) 4 min after ionomycin caused an immediate but transient decrease in $[Ca^{2+}]_i$ (Fig. 8a). Pretreating cells with the protein kinase in-

Fig. 7. Time course of PMA inhibition of FMLP-stimulated Mn^{2+} entry and intracellular Ca^{2+} release

Neutrophils were incubated with a fixed dose of PMA (1 nM, or DMSO vehicle) for various times before addition of FMLP (10 nM). For measurement of Mn^{2+} entry, fluorescence was measured at a single excitation wavelength of 360 nm. Intracellular Ca^{2+} release was measured as described in Fig. 6. The curves show percentage inhibition by PMA (compared with 0.1%-DMSO control) of the final extent of the Mn^{2+} quench ($\circ$), and percentage inhibition of the peak rise in $[Ca^{2+}]_i$ ($\bullet$), stimulated by FMLP. All points represent means $\pm$ S.E.M. ($n = 3$).

Fig. 8. Effect of FMLP on ionomycin-elevated $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured at a single excitation wavelength of 340 nm. Fura-2 loading was the same as in Fig. 1. Neutrophil suspensions were incubated in the presence of 1 mM- $CaCl_2$, and treated with staurosporine (ST, 100 nM) or DMSO vehicle (0.1%) before addition of ionomycin (50 nM). FMLP was added at the times indicated. (a) Effect of FMLP (100 nM) on the ionomycin-evoked steady-state $[Ca^{2+}]_i$ in the absence of staurosporine. (b) Effect of FMLP (100 nM) in the presence of staurosporine.

hibitor staurosporine (100 nM) completely blocked the decrease caused by 10 nM-FMLP (Fig. 8b). However, the response to 100 nM-FMLP was only partially blocked (result not shown).

DISCUSSION

C-kinase activation is known to inhibit agonist-stimulated elevations of $[Ca^{2+}]_i$ in neutrophils, and evidence suggests this may be due to stimulation of Ca^{2+} efflux and feedback-inhibition of phosphoinositidase C (see the Introduction). The possibility that C-kinase activation may also inhibit agonist-stimulated entry of Ca^{2+} in neutrophils has not previously been considered. Studies to date have failed to discriminate between the effects of C-kinase activation on intracellular Ca^{2+} release and Ca^{2+} entry from the external medium. The experiments described here have investigated these possibilities in more detail.

Addition of PMA caused a rapid decrease in the

maintained elevated $[Ca^{2+}]_i$ stimulated by FMLP. The maintained elevated $[Ca^{2+}]_i$ observed after agonist stimulation is thought to reflect a steady-state balance between stimulated influx of Ca^{2+} from the extracellular medium, efflux of Ca^{2+} via plasma-membrane Ca^{2+} -ATPases and re-sequestration of Ca^{2+} into intracellular stores. This effect of PMA could therefore have been due to stimulated Ca^{2+} efflux/re-sequestration, or inhibited Ca^{2+} influx, or a combination of these. Ionomycin raises $[Ca^{2+}]_i$ independently of receptor stimulation, and PMA did not interfere with ionomycin-mediated bivalent-cation influx. When ionomycin was used to elevate $[Ca^{2+}]_i$ to a steady-state level, comparable with that seen after FMLP stimulation, PMA caused a similar decrease in $[Ca^{2+}]_i$, showing that PMA stimulated removal of Ca^{2+} from the cytosol. 4β -PDD and DOG also decreased the FMLP- or ionomycin-evoked steady-state $[Ca^{2+}]_i$, whereas 4α -PDD was ineffective. Staurosporine, a potent but non-specific protein kinase inhibitor (Tamaoki *et al.*, 1986), blocked the effects of PMA, 4β -PDD and DOG on the FMLP- or ionomycin-evoked steady states. These results strongly implicate C-kinase in the regulation of neutrophil $[Ca^{2+}]_i$. Further, addition of PMA before ionomycin caused a partial, dose-dependent, inhibition of peak $[Ca^{2+}]_i$. This partial inhibition correlated well with the partial decreases in the steady-state $[Ca^{2+}]_i$ seen when PMA was added after ionomycin, and suggested that C-kinase-mediated inhibition of elevations of $[Ca^{2+}]_i$ can be caused by activation of Ca^{2+} -efflux/re-sequestration processes. These results are consistent with previously published results showing that C-kinase stimulates Ca^{2+} efflux from neutrophils (Lagast *et al.*, 1984; Rickard & Sheterline, 1985).

After stimulated release of Ca^{2+} from intracellular stores, and in the continued presence of agonist, $[Ca^{2+}]_i$ is maintained at an elevated level as Ca^{2+} enters from the extracellular medium (Hallam *et al.*, 1984; Hallam & Pearson, 1986; Merritt & Rink, 1987; Merritt *et al.*, 1989). We investigated in detail the effects of C-kinase activation on FMLP-stimulated entry of Ca^{2+} . When neutrophils are heavily loaded with fura-2, the buffering capacity of the cytosol is increased. This decreases the peak rise in $[Ca^{2+}]_i$ owing to intracellular Ca^{2+} release, such that the final stimulated peak $[Ca^{2+}]_i$ is more dependent on external Ca^{2+} . At low doses, PMA blocked the component of the rise in $[Ca^{2+}]_i$ that was dependent on extracellular Ca^{2+} , and therefore was presumably due to Ca^{2+} influx. At higher doses of PMA, however, intracellular Ca^{2+} release was also partially inhibited. This prevented clear dose-inhibition studies being carried out on Ca^{2+} influx with this method. This method is also further limited because it only gives information concerning the balance between the rates of Ca^{2+} influx and extrusion. The inhibitory effect of PMA might therefore have been due to C-kinase-stimulated efflux/re-sequestration of Ca^{2+} from the cytosol, leading to decreased peak responses to FMLP, rather than inhibited Ca^{2+} entry. Similar inhibitory effects of PMA on pig neutrophils have been observed and attributed solely to stimulated efflux of Ca^{2+} (Rickard & Sheterline, 1985). To distinguish between these possibilities, we studied the effect of C-kinase activation on FMLP-stimulated Mn^{2+} influx.

When Mn^{2+} is added extracellularly, FMLP stimulates quenching of fluorescence of fura-2-loaded neutrophils, indicating stimulated Mn^{2+} influx. This technique was

first demonstrated in human platelets loaded with quin2 (Hallam & Rink, 1985), but has since been modified for dual-wavelength studies with fura-2 (Merritt *et al.*, 1989). Since neutrophils contain no releasable store of Mn^{2+} , and fura-2 has a high affinity for Mn^{2+} (approx. 40-fold greater than for Ca^{2+}), this method measures unidirectional flux of Mn^{2+} into the cytosol. It appears that Mn^{2+} enters neutrophils by similar mechanisms to Ca^{2+} (Merritt *et al.*, 1989).

Activation of C-kinase inhibited FMLP-stimulated Mn^{2+} influx. The relative potencies of PMA, 4 β -PDD, DOG and 4 α -PDD in inhibiting Mn^{2+} entry paralleled their potencies in activating isolated C-kinase enzyme activity (Castagna *et al.*, 1982). Further, PMA inhibition of stimulated Mn^{2+} entry was blocked by preincubation of cells with staurosporine. These results suggest that activation of C-kinase inhibits bivalent-cation influx, in addition to stimulating Ca^{2+} efflux.

C-kinase activation was also found to inhibit partially release of Ca^{2+} from intracellular stores. More detailed dose-inhibition studies showed that concentrations of PMA that caused complete blockade of both Ca^{2+} and Mn^{2+} influx had a negligible effect on intracellular release of Ca^{2+} . These results contrast with studies with human platelets, where C-kinase activation inhibited platelet-activating-factor-stimulated intracellular release of Ca^{2+} more potently than Mn^{2+} influx (Valone & Johnsen, 1987). The time-dependency of these effects confirmed that C-kinase activation in neutrophils caused more rapid and extensive blockade of stimulated bivalent-cation entry than of intracellular release of Ca^{2+} . Further investigation of C-kinase-mediated inhibition of FMLP-stimulated intracellular Ca^{2+} release in neutrophils suggested that the partial inhibition observed was not due to stimulated efflux of Ca^{2+} , because PMA had no effect on the peak $[Ca^{2+}]_i$ seen after treating neutrophils with ionomycin in Ca^{2+} -free medium. PMA has been reported to inhibit phosphoinositide formation in neutrophils (Della Bianca *et al.*, 1986) and in other cell types (Rosoff & Cantley, 1985; Vicentini *et al.*, 1985; MacIntyre *et al.*, 1985; Zavoico *et al.*, 1985; Dubyak *et al.*, 1988). Inhibition of $Ins(1,4,5)P_3$ formation may explain the inhibition of stimulated release of Ca^{2+} from intracellular stores caused by PMA.

Although activation of C-kinase with pharmacological agents caused inhibition of FMLP-stimulated Ca^{2+} transients in neutrophils, the physiological significance of this effect was not clear. FMLP stimulation of neutrophils causes intracellular diacylglycerol levels to rise, which activates C-kinase (Nishizuka 1984; Truett *et al.*, 1988; Korchak *et al.*, 1988). We therefore investigated the effect of FMLP on the steady-state $[Ca^{2+}]_i$ seen after treatment of neutrophils with ionomycin. FMLP stimulated a rapid but transient decrease in $[Ca^{2+}]_i$ when added to neutrophils pretreated with ionomycin. Recently it has been reported that PMA- and DOG-stimulated, but not FMLP-stimulated, decreases in $[Ca^{2+}]_i$ in neutrophils pretreated with ionomycin could be blocked by staurosporine (Perianin & Snyderman, 1989). From this result, the last authors went on to suggest that FMLP lowered neutrophil $[Ca^{2+}]_i$ by a C-kinase-independent mechanism. Our results, however, show that the FMLP-stimulated decrease in $[Ca^{2+}]_i$ can be blocked by staurosporine, suggesting that this effect is due to C-kinase activation. However, we have shown that decreases in $[Ca^{2+}]_i$ caused by PMA, 4 β -PDD, DOG and

FMLP can all be blocked by staurosporine, but that the extent of the inhibition varies considerably between cell preparations. Despite this, staurosporine clearly blocks what we assume to be C-kinase-mediated removal of Ca^{2+} from the cytosol. Taken together with the lack of specificity of staurosporine for C-kinase (Tamaoki *et al.*, 1986), the existence of multiple forms of the enzyme (Coussens *et al.*, 1986) and the ever-increasing number of reports of non-specific effects of C-kinase activators (Hockberger *et al.*, 1989; Restrepo *et al.*, 1989), we consider that these results argue against the existence of multiple pathways for lowering $[Ca^{2+}]_i$ in neutrophils. Our data are consistent with the hypothesis that FMLP lowers $[Ca^{2+}]_i$ in neutrophils by a C-kinase-dependent mechanism.

In conclusion, these results show that activation of C-kinase with pharmacological agents decreases the agonist-stimulated elevation of $[Ca^{2+}]_i$ in neutrophils by inhibiting bivalent-cation influx, stimulating Ca^{2+} efflux and partially inhibiting release of Ca^{2+} from intracellular stores. Further, in addition to stimulating increases in $[Ca^{2+}]_i$, FMLP stimulation of neutrophils can decrease $[Ca^{2+}]_i$ from maintained elevated levels through activation of C-kinase. These data suggest that receptor-mediated C-kinase activation is an important factor in the control of agonist-stimulated changes in neutrophil $[Ca^{2+}]_i$.

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