Involvement of calcium in modulation of neutrophil function by phorbol esters that activate protein kinase C isotypes and related enzymes

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In this study, the effects of a series of phorbol esters with different spectra of biological activities and different patterns of activation of the isoenzymes of protein kinase C (PKC) have been studied in human neutrophils. The aim was to gain more information on which isoenzymes of PKC are involved in neutrophil activation, specifically inhibition of fMet-Leu-Phe (fMLP)-stimulated bivalent cation influx and stimulation of $O_2^{\cdot-}$ release (either alone or potentiation of the response to fMLP). Prior addition of both phorbol 12-myristate 13-acetate (PMA) and sapintoxin A (SAPA) inhibited fMLP-stimulated Mn^{2+} influx. Higher concentrations of resiniferatoxin (RX) were also inhibitory, inhibition being more apparent at longer preincubation times. However, 12-deoxyphorbol 13-O-phenylacetate (DOPPA) showed only a slight inhibitory effect and required a prolonged preincubation. PMA, SAPA and RX, but not DOPPA, stimulated $O_2^{\cdot-}$ release by themselves. Lower concentrations of PMA, SAPA and RX, which were ineffective alone, considerably potentiated $O_2^{\cdot-}$ release stimulated by fMLP, whereas DOPPA had little or no effect. These results rule out a major role for PKC- δ (not activated by SAPA) and PKC- β_1 (activated by DOPPA), but suggest the involvement of RX kinase in addition to PKC in the inhibition of fMLP-stimulated Mn^{2+} influx and potentiation of fMLP-stimulated $O_2^{\cdot-}$ release. However, when the cytosolic free Ca^{2+}

concentration ($[Ca^{2+}]_i$) was elevated with the Ca^{2+} ionophore ionomycin, DOPPA was able to stimulate $O_2^{\cdot-}$ release, which probably reflects the known Ca^{2+} requirement for activation of PKC- β_1 by DOPPA *in vitro*. The effects of the other phorbols were also enhanced when $[Ca^{2+}]_i$ was elevated; all of the phorbols synergize, to variable extents, with Ca^{2+} to activate PKC *in vitro*. Enhancement of RX-stimulated $O_2^{\cdot-}$ release by elevation of $[Ca^{2+}]_i$ was unexpected, since RX kinase has been reported to be inhibited by high concentrations of Ca^{2+} *in vitro*. Finally, use of fura-2 and SK&F 96365 to manipulate the fMLP-stimulated rise in $[Ca^{2+}]_i$ showed that when fMLP was able to evoke its normal rise in $[Ca^{2+}]_i$ (to a peak of 700–900 nM), $O_2^{\cdot-}$ release was potentiated by PMA, SAPA and RX. However, when fMLP was only able to evoke a small increase in $[Ca^{2+}]_i$ (to a peak of 400 nM), potentiation by PMA was unaffected but potentiation by SAPA and RX was considerably reduced. This observation agrees with published data demonstrating that activation of PKC *in vitro* by SAPA is more Ca^{2+} -dependent than activation by PMA. In conclusion, these results show that activation of PKC by phorbols in neutrophils correlates with their Ca^{2+} -dependence for activation of PKC *in vitro*. These results also suggest a role for RX kinase, which appears to synergize with Ca^{2+} under physiological conditions.

INTRODUCTION

Activation of protein kinase C (PKC) is thought to play a major role in activation of neutrophils. Stimulation of neutrophils with receptor agonists, including fMet-Leu-Phe (fMLP), results in activation of phospholipase C with the production of $InsP_3$ and diacylglycerol (Cockcroft et al., 1985; Di Virgilio et al., 1985; Della Bianca et al., 1986), and diacylglycerol is known to activate PKC (for reviews see Farago and Nishizuka, 1990; Bell and Burns, 1991). Phorbol esters have been widely used as a means to activate PKC independently of receptor agonists (for review see Evans et al., 1991a). In neutrophils, activation of PKC by phorbol esters such as phorbol 12-myristate 13-acetate stimulates superoxide formation (Sha'afi et al., 1983; Korchak et al., 1984a,b; Della Bianca et al., 1986) and inhibits agonist-stimulated bivalent cation influx (McCarthy et al., 1989).

PMA is one of the most widely used phorbol esters in studies of PKC activation, and it has a broad spectrum of biological

effects, including tumour promotion (Evans & Taylor, 1983). The phorbol ester range of plant products contains a number of chemically related substances with similar biological activities, although not all of the biologically active compounds are tumour promoters (Evans and Taylor, 1983; Evans & Edwards, 1987). PMA exhibits a complete range of biological activities, including complete tumour promotion, induction of inflammation, T cell mitogenesis, HL60 cell differentiation and platelet aggregation (Evans et al., 1991a). Other phorbol esters that also activate PKC have a more limited range of biological activities. Sapintoxin A (SAPA) is a 4-deoxyphorbol analogue that was isolated from ripe fruits of the Indian plant *Sapium indium* (Taylor et al., 1989). SAPA is not a tumour-promoting agent, but it does have other biological activities similar to those of PMA, including T cell mitogenesis and HL60 cell differentiation (Evans et al., 1991a). SAPA and PMA are equipotent in a crude cell-free PKC activation assay (Ellis et al., 1987). 12-Deoxyphorbol 13-O-phenylacetate (DOPPA) was isolated from the latex of the West

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DOPPA, 12-deoxyphorbol 13-O-phenylacetate; fMLP, formylmethionine-leucine-phenylalanine; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RX, resiniferatoxin (9,13,14-o-phenylacetylresiniferoyl-20-O-homovanillate); SAPA, sapintoxin A (12-O-2-methylaminobenzoyl-13-O-acetyl-4-deoxyphorbol); SK&F 96365, 1- β -(3-[4-methoxyphenyl]propoxy)-4-methoxyphenethyl-1H-imidazole hydrochloride.

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Indian plants *Euphorbia unispina* and *E. poissoni* (Evans et al., 1975). DOPPA is not a tumour promoter and does not induce HL60 cell differentiation, but it is proinflammatory and is a weak PKC activator (Evans et al., 1991a). Resiniferatoxin (RX), isolated from the fresh latex of *Euphorbia unispina* and *E. poissoni* (Evans et al., 1975), is a very weak activator of mammalian brain PKC (Ellis et al., 1987) and shows few of the activities of PMA, although RX does stimulate superoxide production by macrophages (Evans et al., 1990) and is a very potent irritant (Evans and Schmidt, 1979). RX has been shown to activate a Ca^{2+} -inhibited kinase, RX kinase, which is distinct from the known PKC isoenzymes (Ryves et al., 1989) and is found only in inflammatory cells (Evans et al., 1991b).

The effects of this series of phorbol esters were initially studied using crude preparations of brain PKC. However, multiple subspecies of PKC have now been described (Parker et al., 1986; Coussens et al., 1986; Farago & Nishizuka, 1990; Bell and Burns, 1991). The isotypes α , β_1 , β_2 and γ require Ca^{2+} and phosphatidylserine for activation, but the isotypes δ , ϵ , ζ and η do not require Ca^{2+} . There is little information on the tissue distribution of the isoenzymes of PKC. Most isotypes appear to be widely distributed, with the exception of PKC- γ , which is found only in the central nervous system. To date, the limited studies of PKC isotypes in human neutrophils have demonstrated the presence of α , β (β_1 and β_2 have not been distinguished in these studies) and ζ , but not γ , and activation of PKC by PMA causes translocation of PKC- β but not PKC- α from cytosol to membranes (Pontremoli et al., 1990; Stasia et al., 1990; Majumdar et al., 1991; Obel et al., 1991). When the cell-free activation of NADPH oxidase in macrophage membranes was studied, purified PKC isoforms α , β , γ , δ and ϵ were all able to activate the enzyme, resulting in production of superoxide (Sharma et al., 1991).

The series of phorbol esters described above has recently been examined in more detail with regard to effects on the different isotypes of PKC (Ryves et al., 1991). The isotypes studied were α , β_1 , γ , δ and ϵ . PMA activates all five isotypes; activation of α , β_1 and γ is slightly enhanced in the presence of Ca^{2+} , while activation of δ and ϵ is independent of Ca^{2+} . SAPA activation of PKC- α , - β_1 and - γ is considerably increased in the presence of Ca^{2+} , there is no activation of PKC- δ , and PKC- ϵ is slightly activated independently of Ca^{2+} . DOPPA activates only PKC- β_1 and activation is absolutely dependent on Ca^{2+} . RX causes a small Ca^{2+} -dependent activation of PKC- γ , and causes Ca^{2+} -dependent activation of PKC- β_1 at high concentrations, in addition to its potent activation of RX kinase.

Since PMA, SAPA, DOPPA and RX show different activities *in vitro* with isolated PKC isotypes, the aim of the present study was to examine their effects on two PKC-mediated responses of human neutrophils in order to gain information on the isotypes of PKC involved. The PKC-mediated responses studied were inhibition of fMLP-stimulated bivalent cation influx and stimulation of $\text{O}_2^{\cdot-}$ formation. The effects of the compounds alone on $\text{O}_2^{\cdot-}$ and the effects of lower concentrations (ineffective alone) on priming of the fMLP response were studied. In addition, since the phorbol esters have different Ca^{2+} requirements for activation of the PKC isoenzymes, and the isoenzymes differ in their requirement for Ca^{2+} , we have investigated the effect of manipulating the cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$).

METHODS

Percoll and Dextran T500 were from Pharmacia (Uppsala, Sweden); PMA, fMLP and luminol were from Sigma; fura-2 acetoxymethyl ester was from Molecular Probes Inc. (Eugene,

OR U.S.A.); Hepes (ultrapure) and ionomycin were from Calbiochem. SAPA was isolated from the ripe fruits of *Sapium indicum* (Taylor et al., 1989). DOPPA and RX were isolated from the fresh latex of *Euphorbia poissonii* and *Euphorbia unispina* (Evans et al., 1975). SK&F 96365 was synthesized as previously described (Merritt et al., 1990a).

Neutrophils were prepared from freshly drawn human blood by dextran sedimentation of erythrocytes followed by Percoll density gradient centrifugation to separate the leucocytes, as previously described (Merritt et al., 1989). The cells were washed and suspended in medium containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 10 mM Hepes (pH 7.4 at 37 °C), plus 1 mM CaCl_2 and 1% BSA. The cell density was approx. 6×10^6 cells/ml. Cells were loaded with fura-2 for all experiments so that fura-2 fluorescence (Mn^{2+} influx or $[\text{Ca}^{2+}]_i$) and luminol chemiluminescence ($\text{O}_2^{\cdot-}$ release) could be measured in the same cell preparations under the same conditions. For loading with fura-2, the cell suspension was incubated for 45 min at 37 °C with fura-2 acetoxymethyl ester at either 0.3 μM (all experiments except the heavily buffered cells in Figures 6 and 7) or 2 μM (the heavily buffered cells in Figures 6 and 7). Incubation with 0.3 μM fura-2AM results in a cytosolic fura-2 concentration of 50–100 μM ('low fura-2'), and incubation with 2 μM fura-2AM results in a cytosolic dye concentration of approx. 500 μM ('high fura-2'). After loading with dye, the cells were resuspended at 2×10^6 /ml in the Hepes-buffered medium containing 1 mM CaCl_2 and 1% BSA, and maintained at room temperature until required. For use in the experiments, samples of the cell suspension were centrifuged (Microfuge; 6500 rev./min for 30 s) and resuspended at 2×10^6 cells/ml in the Hepes-buffered medium containing no BSA. For the luminol chemiluminescence studies the medium contained 1 mM CaCl_2 , and for the fura-2 fluorescence studies 1 mM CaCl_2 or 100 μM MnCl_2 was added as required.

Fura-2 fluorescence (500 nm emission) was measured at 37 °C in a dual-excitation wavelength fluorimeter (Spex) with additions as indicated in the Figures. In Figure 6, $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fura-2 fluorescence at two excitation wavelengths (340 and 380 nm), as described by Grynkiewicz et al. (1985). In Figure 1, fluorescence was monitored at excitation wavelengths of 340 and 360 nm; at 340 nm excitation, fura-2 fluorescence increases with increasing $[\text{Ca}^{2+}]_i$, while the 360 nm fluorescence is insensitive to $[\text{Ca}^{2+}]_i$; however, the fluorescence at both wavelengths is quenched by Mn^{2+} . Quenching of fura-2 fluorescence at 360 nm excitation therefore provides a direct measure of bivalent cation (Mn^{2+}) influx (Merritt et al., 1989).

Superoxide generation was monitored as the enhancement of luminol chemiluminescence (Englberger et al., 1987). Luminol (15 $\mu\text{g}/\text{ml}$) was added to a suspension of neutrophils, which were then preincubated for 5 min at 37 °C before addition of test substances and measurement of chemiluminescence. Chemiluminescence was measured in a lumiaggregometer (Chrono-Log). Because of the complexity of the responses, it was not possible to quantify them; typical traces are therefore shown. It should be noted that no cytochalasin B was used in these studies.

All the results are typical of at least three or four others obtained from different cell preparations.

RESULTS

Effects of phorbols on fMLP-stimulated bivalent cation influx

Figure 1 shows the effects of SAPA, DOPPA and RX on fMLP (10 nM)-stimulated Mn^{2+} influx in fura-2-loaded human neutrophils incubated in the presence of 100 μM MnCl_2 . At 340 nm excitation, fMLP evoked a rapid and transient increase

Figure 1 Effects of SAPA, DOPPA and RX on fMLP-stimulated Mn^{2+} influx in neutrophils

Fura-2 fluorescence (500 nm emission) was monitored simultaneously at excitation wavelengths of 340 nm (solid lines) and 360 nm (broken lines). Additions were made as indicated: Mn^{2+} (100 μM MnCl_2), fMLP (10 nM), IONO (2 μM ionomycin) and phorbols as shown. The phorbols were solubilized in dimethyl sulphoxide (DMSO), so an equivalent amount (0.1%) was included in the control experiment.

in fluorescence, reflecting an increase in $[\text{Ca}^{2+}]_i$ due to release of Ca^{2+} from intracellular stores (fluorescence at 360 nm excitation is unaffected by $[\text{Ca}^{2+}]_i$). The rise in fluorescence at 340 nm excitation was followed by a rapid decline in fluorescence to well below prestimulated levels as Mn^{2+} entered the cells and quenched the intracellular dye. At 360 nm excitation, only the stimulated quench in fluorescence (due to stimulated Mn^{2+} entry) is seen. Subsequent addition of ionomycin (translocates Mn^{2+} as well as Ca^{2+}) caused little further change in fluorescence, indicating that most of the intracellular dye had already been quenched. When SAPA (20 nM) was added 5 min before fMLP, the initial fMLP-stimulated rise in fluorescence at 340 nm excitation was unaffected. This result shows that SAPA (at this concentration) did not affect release of Ca^{2+} from intracellular stores. However, SAPA totally inhibited the fMLP-stimulated quench in fluorescence at both wavelengths, which shows that SAPA totally inhibited fMLP-stimulated bivalent cation influx. Subsequent addition of ionomycin then caused complete quenching of the fura-2. A similar effect of PMA has already been reported (McCarthy et al., 1989). The inhibitory effects of both SAPA and PMA can be reversed by the non-specific protein kinase inhibitor staurosporine (100 nM; results not shown). Figure 1 also shows that another phorbol ester, DOPPA (1 μM) had no inhibitory effect on fMLP-stimulated Mn^{2+} influx after a 5 min preincubation, although some inhibition was seen after a longer preincubation (30% inhibition after a 45 min preincubation; results not shown). Figure 1 shows that RX (1 μM) inhibited fMLP-stimulated Mn^{2+} influx by 30% after a 5 min preincubation. This inhibition was increased in a time-dependent manner upon prolonged preincubation (30% at 5 min, 35% at 10 min, 41% at 15 min, 62% at 20 min, 68% at 30 min, 74% at

Figure 2 Dose-response curves for inhibition of fMLP-stimulated Mn^{2+} influx by the phorbols

Mn^{2+} influx was measured as shown in Figure 1, with phorbols added 5 min before fMLP. Points represent means $\pm$ S.E.M. of four experiments.

45 min and 80% at 60 min). Figure 2 shows dose-response curves, following 5 min preincubations, for the inhibition of fMLP-stimulated Mn^{2+} influx by the four phorbols studied. PMA was the most potent inhibitor of Mn^{2+} influx, with an IC_{50} of 48 ± 4 pM ($n = 4$), inhibition by SAPA had an IC_{50} of 4.0 ± 0.3 nM ($n = 4$), and little or no inhibition was seen with high concentrations of DOPPA and RX (the maximal concentrations used were limited by solubility). Preincubations of 5 min were used here to allow comparison between these

Figure 3 Effects of maximal concentrations of the phorbols alone on $O_2^{\cdot -}$ release from neutrophils

$O_2^{\cdot -}$ was measured as enhancement of luminol chemiluminescence following a 5 min preincubation with luminol. Additions were made at the times shown. fMLP ($1 \mu M$) is included for comparison. DMSO, dimethyl sulphoxide.

Figure 4 Effect of adding phorbols 5 min before fMLP on stimulation of $O_2^{\cdot -}$ release

$O_2^{\cdot -}$ was measured as enhancement of luminol chemiluminescence. Additions were made at the times shown. The fMLP concentration was $1 \mu M$, which was maximal alone or with the phorbols.

studies and the 5 min preincubations for the priming of $O_2^{\cdot -}$ release described below.

Effects of phorbols on $O_2^{\cdot -}$ release

Figure 3 shows the effects of maximally effective concentrations of the phorbols, alone, on $O_2^{\cdot -}$ release from neutrophils. The traces show $O_2^{\cdot -}$ monitored as enhancement of luminol chemiluminescence (no cytochalasin B is required). A maximal concentration of fMLP ($1 \mu M$), which alone evokes a small rapid transient response, is included for comparison. Both PMA (250 pM) and SAPA (20 nM), at concentrations that totally inhibited Mn^{2+} influx (Figure 2), evoked large increases in chemiluminescence ($O_2^{\cdot -}$ release) after a short delay. DOPPA ($1 \mu M$), which had no effect on Mn^{2+} influx after a 5 min

preincubation, did not stimulate $O_2^{\cdot -}$ release (no response was seen at incubation times longer than that shown). RX ($1 \mu M$) inhibited Mn^{2+} influx by 35% when preincubated for 10 min and caused a large increase in $O_2^{\cdot -}$ release after a delay of about 10 min. Stimulation of $O_2^{\cdot -}$ release by PMA, SAPA and RX was totally inhibited by the non-specific protein kinase inhibitor staurosporine (10 nM; results not shown).

Figure 4 shows fMLP ($1 \mu M$)-stimulated $O_2^{\cdot -}$ release and the effect of preincubating with the phorbols 5 min before the addition of fMLP. The concentrations of the phorbols used in these experiments had no stimulatory effect, by themselves, on $O_2^{\cdot -}$ release even after prolonged incubation periods. In each case, the fMLP response following preincubation with a phorbol was considerably enhanced compared with that of fMLP alone. The concentrations of PMA and SAPA used here caused 60–80% inhibition of Mn^{2+} influx, RX caused 5% inhibition and DOPPA had no inhibitory effect (Figure 2). The potentiation seen with DOPPA was far less than that seen with the other phorbols, and in most experiments little or no potentiation was seen (see Figures 5 and 7). The greatest effect was always seen with PMA. Although the effect of RX alone on $O_2^{\cdot -}$ release was only seen after a 10 min lag (Figure 3), potentiation of the fMLP response was seen after a 5 min preincubation (Figure 4).

Effect of manipulating $[Ca^{2+}]_i$ on phorbol-mediated $O_2^{\cdot -}$ release

Since the different isotypes of PKC vary in their Ca^{2+} -dependency for activation, and the phorbols have different Ca^{2+} requirements for activation (Ryves et al., 1991), we have examined the effect of manipulating $[Ca^{2+}]_i$ on phorbol-mediated $O_2^{\cdot -}$ release. For the experiments shown in Figure 5, the Ca^{2+} ionophore ionomycin was used to raise $[Ca^{2+}]_i$ to known levels before examining the effects of phorbols alone (Figure 5a) or potentiation of the fMLP response (Figure 5b). Before carrying out measurements of $O_2^{\cdot -}$ release, $[Ca^{2+}]_i$ was measured in the same preparation of fura-2-loaded cells in response to various concentrations of ionomycin (2.5–15 nM). Addition of ionomycin evoked an increase in $[Ca^{2+}]_i$ which was maintained throughout the incubation. For the experiment shown in Figure 5, the $[Ca^{2+}]_i$ rises evoked by ionomycin were as follows: unstimulated $[Ca^{2+}]_i = 90$ nM; with 2.5 nM ionomycin, $[Ca^{2+}]_i = 146$ nM; with 5 nM ionomycin, $[Ca^{2+}]_i = 233$ nM; with 10 nM ionomycin, $[Ca^{2+}]_i = 284$ nM; with 15 nM ionomycin, $[Ca^{2+}]_i = 396$ nM. Addition of ionomycin alone, at 2.5–15 nM, had no effect on $O_2^{\cdot -}$ release, as shown in Figure 5. Figure 5(a) shows stimulation of $O_2^{\cdot -}$ release by phorbols (no fMLP) in the presence of a minimally effective concentration of ionomycin for each phorbol. These concentrations of the phorbols were ineffective in the absence of ionomycin (Figure 4) when the $[Ca^{2+}]_i$ was 90 nM. Elevation of $[Ca^{2+}]_i$ from 90 nM to 146 nM (2.5 nM ionomycin) was sufficient to promote large responses to PMA (100 pM) and SAPA (5 nM). Once $[Ca^{2+}]_i$ was elevated to 233 nM (5 nM ionomycin), RX (500 nM) was able to evoke a large and rapid (immediate) response (a slower response of RX alone was also evident at 146 nM $[Ca^{2+}]_i$ in Figure 5b before fMLP was added). The response with RX was greater than that seen with the other phorbols. This immediate response to 500 nM RX when $[Ca^{2+}]_i$ is elevated contrasts to the delayed (10 min lag) response to $1 \mu M$ RX at basal $[Ca^{2+}]_i$ (Figure 3). In order to see a response to DOPPA ($1 \mu M$), it was necessary to elevate $[Ca^{2+}]_i$ to 396 nM (15 nM ionomycin). Figure 5(b) shows that elevation of $[Ca^{2+}]_i$ had little effect on fMLP ($1 \mu M$)-stimulated $O_2^{\cdot -}$ release, but it did slightly potentiate the enhanced response to fMLP seen in the presence of DOPPA, and greatly potentiated the enhanced response to fMLP in the presence of RX under conditions when

Figure 5 Effect of ionomycin on $O_2^{\cdot -}$ release stimulated by phorbols alone (a) or phorbols plus fMLP (b)

Ionomycin (IONO) was used to elevate $[Ca^{2+}]_i$ to known levels which were measured in the same cell preparation (see text for $[Ca^{2+}]_i$ levels obtained). Additions were made as shown (the fMLP concentration was 1 μM), and $O_2^{\cdot -}$ was measured as enhancement of luminol chemiluminescence. These concentrations of phorbols were ineffective alone at basal $[Ca^{2+}]_i$, and (a) shows the responses at a minimally effective concentration of ionomycin for each phorbol; (b) compares the responses to fMLP alone or plus DOPPA or RX at basal $[Ca^{2+}]_i$, or in the presence of a minimally effective concentration of ionomycin.

the phorbols alone had little or no effect. (This experiment could not be carried out with PMA or SAPA because they were active alone at the lowest concentration of ionomycin used.)

An alternative approach to studying interactions between the phorbols and $[Ca^{2+}]_i$ is shown in Figures 6 and 7. In these experiments the fMLP-stimulated rise in $[Ca^{2+}]_i$ was manipulated (as shown in Figure 6), and the potentiating effect of the phorbols on fMLP-stimulated $O_2^{\cdot -}$ release was examined under conditions where fMLP evoked either a normal or a small increase in $[Ca^{2+}]_i$ (as shown in Figure 7). Figure 6 illustrates the protocol used to manipulate the fMLP-stimulated rise in $[Ca^{2+}]_i$. Neutrophils were loaded with fura-2 by incubation with either 0.3 μM fura-2AM (low fura-2, as used in all the previous experiments described here) or with 2 μM fura-2AM (high fura-2). All measurements were carried out in the presence of 1 mM $CaCl_2$.

Figure 6 Effect of SK&F 96365 on the fMLP-stimulated rise in $[Ca^{2+}]_i$ when neutrophils contain low or high levels of fura-2

Neutrophils were loaded with fura-2 by incubation with either 0.3 μM fura-2AM (low fura-2, as used in all the previous experiments) or 2 μM fura-2AM (high fura-2). $[Ca^{2+}]_i$ was calculated from the ratio of fura-2 fluorescence (500 nm emission) at excitation wavelengths of 340 nm and 380 nm. All the incubations contained 1 mM $CaCl_2$, and SK&F 96365 (7.5 μM) was added 1.5 min before fMLP where indicated. Addition of fMLP (10 nM) is shown by the arrow.

When neutrophils contain a low cytosolic concentration of fura-2 (50–100 μM), fMLP evoked a rapid and transient increase in $[Ca^{2+}]_i$ to a peak of 820 nM, which then declined to a maintained elevated level; this response is due to Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} . SK&F 96365 inhibits influx of Ca^{2+} with little effect on release from intracellular stores (Merritt et al., 1990a). When SK&F 96365 (7.5 μM) was added to cells containing a low level of fura-2, 1.5 min before the addition of fMLP, fMLP evoked a rapid transient increase in $[Ca^{2+}]_i$ to a peak of 730 nM which then declined; this response is largely due to Ca^{2+} release from intracellular stores, since SK&F 96365 blocks Ca^{2+} influx (the response to fMLP was similar in the absence of extracellular Ca^{2+} ; results not shown). Incorporation of higher levels of fura-2 into the cytosol results in buffering of the $[Ca^{2+}]_i$ response (Merritt et al., 1990b). When neutrophils contained a high cytosolic concentration of fura-2 (500 μM), fMLP evoked an increase in $[Ca^{2+}]_i$ to a peak of 890 nM which only slowly declined. When SK&F 96365 (7.5 μM) was added to these cells 1.5 min before the addition of fMLP, fMLP evoked a small transient increase in $[Ca^{2+}]_i$ to a peak of 400 nM; this smaller rise in $[Ca^{2+}]_i$ is due to increased buffering of the finite amount of Ca^{2+} released from intracellular stores when influx of the infinite amount of extracellular Ca^{2+} (which overcomes the buffering) is blocked (a similar response to fMLP was seen in the absence of extracellular Ca^{2+} ; results not shown). Thus, in cells with either low or high concentrations of fura-2, the initial rise in $[Ca^{2+}]_i$ evoked by fMLP is similar; in cells with low fura-2, SK&F 96365 has little effect on the initial rise in $[Ca^{2+}]_i$ evoked by fMLP; and in cells with high fura-2, SK&F 96365 considerably reduces the initial rise in $[Ca^{2+}]_i$ evoked by fMLP. The potentiating effects of phorbols on fMLP (1 μM)-stimulated $O_2^{\cdot -}$ release were studied

Figure 7 Priming of fMLP-stimulated $O_2^{\cdot -}$ release by phorbols in the presence or absence of SK&F 96365 when neutrophils contain low (a) or high (b) levels of fura-2

Neutrophils were loaded with low (a) or high (b) levels of fura-2, as described in the legend to Figure 6. $O_2^{\cdot -}$ release was monitored as enhancement of luminol chemiluminescence. Additions were made as follows: fMLP (1 μM), SK&F 96365 (7.5 μM), dimethyl sulphoxide (vehicle; 0.1%) and phorbols as shown.

in cells with low (Figure 7a) or high (Figure 7b) levels of fura-2 in the presence or absence of SK&F 96365 (7.5 μM). Figure 7(a) shows fMLP-stimulated $O_2^{\cdot -}$ release from neutrophils containing a low level of fura-2, with potentiation of the response in the presence of PMA, SAPA and RX, but not DOPPA (as in Figure 4). Under these conditions, SK&F 96365 (7.5 μM) had no effect on the response to fMLP alone or on potentiation by any of the phorbols. Figure 7(b) shows fMLP-stimulated $O_2^{\cdot -}$ release from neutrophils containing a high level of fura-2, with potentiation of the response in the presence of PMA, SAPA and RX, but not DOPPA; these results are similar to those with cells containing a low level of fura-2 (although the actual level of chemiluminescence was lower). When cells contained a high level of fura-2, the response to fMLP alone and the potentiation by PMA were not reduced by SK&F 96365. However, under these conditions, SK&F 96365 inhibited the potentiation seen with SAPA and RX. Thus when the fMLP-stimulated rise in $[Ca^{2+}]_i$ is limited, the potentiating effects of SAPA and RX, but not that of PMA, on $O_2^{\cdot -}$ release are decreased.

DISCUSSION

Two PKC-mediated activities of human neutrophils have been monitored: inhibition of fMLP-stimulated bivalent cation influx, and stimulation of $O_2^{\cdot -}$ release (either alone or potentiation of the fMLP response). Phorbols with different ranges of biological activity and different patterns of PKC isoenzyme activation *in vitro* were used to activate PKC in the intact neutrophils to evoke

functional responses. Neutrophils are known to contain PKC- α , - β (β_1 and β_2 not distinguished) and - ζ , but not - γ (see Introduction); PKC- δ and - ϵ have not been examined in neutrophils.

The first response studied was inhibition of fMLP-stimulated bivalent cation influx, which has already been shown to be inhibited on PKC activation with PMA (McCarthy et al., 1989). SAPA also inhibited fMLP-stimulated Mn^{2+} influx, and the inhibition was reversed by the non-specific protein kinase inhibitor staurosporine. SAPA activates PKC isotypes α , β , γ and ϵ but not δ (Ryves et al., 1991), which rules out a major role for PKC- δ in this response. DOPPA (up to 1 μM) had no inhibitory effect on fMLP-stimulated Mn^{2+} influx after a 5 min preincubation and only caused 30% inhibition after a 45 min preincubation. DOPPA activates only PKC- β_1 (and PKC- γ at high concentrations, but PKC- γ is not present in neutrophils), which would appear to rule out a role for PKC- β_1 in modulating agonist-stimulated bivalent cation influx. However, activation of PKC- β_1 by DOPPA is absolutely dependent on Ca^{2+} *in vitro* (Ryves et al., 1991), and the $[Ca^{2+}]_i$ under these conditions may not be sufficient to support PKC- β_1 activation (the $[Ca^{2+}]_i$ dependency of neutrophil activation by DOPPA is discussed below). RX (1 μM) inhibited fMLP-stimulated Mn^{2+} influx by 30% after a 5 min preincubation and inhibition was greater after longer preincubations (74% after 45 min). This smaller effect of RX compared with PMA and SAPA suggests that RX kinase, in addition to PKC, could be involved in modulating fMLP-stimulated bivalent cation entry.

There is considerable evidence that activation of PKC in neutrophils stimulates $O_2^{\cdot -}$ release (see Introduction). PMA and SAPA, at concentrations that totally inhibited fMLP-stimulated Mn^{2+} influx, stimulated $O_2^{\cdot -}$ release after short delays. Lower concentrations of PMA or SAPA alone were ineffective. DOPPA (1 μM), which had no effect on fMLP-stimulated Mn^{2+} influx (after a 5 min preincubation), did not, itself, stimulate $O_2^{\cdot -}$ release even on prolonged incubation. RX (1 μM), which caused 35% inhibition of Mn^{2+} influx after a 10 min preincubation, stimulated a large increase in $O_2^{\cdot -}$ release after a delay of about 10 min. This delayed response to RX might reflect poor cell permeability, but this is unlikely in view of the immediate effects under different conditions as discussed below. Stimulation of $O_2^{\cdot -}$ release by RX suggests that RX kinase is involved in this response. Stimulation of $O_2^{\cdot -}$ by PMA, SAPA and RX is inhibited by the non-specific protein kinase inhibitor, staurosporine, which confirms the involvement of a protein kinase. Evidence that RX acts through a protein kinase (RX kinase) is an important result, because RX has also been reported to act as a potent analogue of the neurotoxin capsaicin to induce pain responses (DeVries & Blumberg, 1989), but this is unlikely to explain its protein kinase-mediated stimulation of $O_2^{\cdot -}$ release from neutrophils.

fMLP-stimulated $O_2^{\cdot -}$ release contrasts with the phorbol-stimulated response in that a maximal concentration of fMLP (1 μM) evokes an immediate small and transient response, compared with the delayed large response seen with the phorbols. However, preincubation of neutrophils with PMA, SAPA or RX for 5 min before addition of fMLP resulted in major potentiation of the fMLP response, which is still immediate and transient. For these experiments low concentrations of phorbols were used that had no stimulatory effect alone even on prolonged incubation. PMA and SAPA were effective at priming the fMLP response at concentrations that caused 60–80% inhibition of fMLP-stimulated Mn^{2+} influx. Preincubation of neutrophils with RX (500 nM) for 5 min inhibited Mn^{2+} influx by only 5%, but caused a significant potentiation of fMLP-stimulated $O_2^{\cdot -}$ release. This concentration of RX alone was ineffective even on prolonged

incubation. Incubation of neutrophils with a higher concentration of RX (1 μ M) alone stimulated $O_2^{\cdot-}$ release, but only after a 10 min delay; this delay cannot be due to poor cell penetration, since the lower concentration of RX was effective at priming the fMLP response following a 5 min preincubation. DOPPA did not inhibit Mn^{2+} influx after a 5 min preincubation and had little or no potentiating effect on fMLP-stimulated $O_2^{\cdot-}$ release. These results rule out a major role for PKC- δ (not activated by SAPA) and PKC- β_1 (activated by DOPPA), but suggest the involvement of RX kinase in addition to PKC for inhibition of fMLP-stimulated Mn^{2+} influx and potentiation of fMLP-stimulated $O_2^{\cdot-}$ release. However, these results cannot identify the precise stage in the cascade of events controlling these agonist-dependent responses that is modulated by the phorbols. Although it is apparent that phorbols are able to stimulate directly the $O_2^{\cdot-}$ -generating enzyme NADPH oxidase (Sharma et al., 1991), they could also act indirectly, for example by stimulating phospholipase D-mediated hydrolysis of phosphatidylcholine (Exton, 1990).

In vitro the phorbols have different requirements for Ca^{2+} to activate PKC, and the isoenzymes vary in their Ca^{2+} -dependency for activation (Ryves et al., 1991). In the present study, two different protocols were used to investigate interactions between the phorbols and $[Ca^{2+}]_i$ in neutrophils. In one series of experiments the Ca^{2+} ionophore ionomycin was used to elevate $[Ca^{2+}]_i$ to known levels in the physiological range. Elevation of $[Ca^{2+}]_i$ from the basal level of 90 nM to 230 nM was sufficient to support $O_2^{\cdot-}$ generation evoked by low concentrations of PMA, SAPA or RX that were ineffective alone at basal $[Ca^{2+}]_i$. The immediate response to RX at elevated $[Ca^{2+}]_i$ shows that the delayed response at basal $[Ca^{2+}]_i$ cannot be due to poor cell penetration of RX. Stimulation of $O_2^{\cdot-}$ release by DOPPA alone was only seen when $[Ca^{2+}]_i$ was elevated to 400 nM. This greater $[Ca^{2+}]_i$ requirement for activation by DOPPA reflects the greater degree of Ca^{2+} -dependency for PKC- β_1 activation *in vitro* by DOPPA compared with PMA (Ryves et al., 1991). The different $[Ca^{2+}]_i$ -dependency of the phorbols was also illustrated in experiments where a combination of different $[Ca^{2+}]_i$ buffering with fura-2 and inhibition of Ca^{2+} influx with SK&F 96365 was used to manipulate the fMLP-stimulated $[Ca^{2+}]_i$ (see the Results section for details). When fMLP was able to evoke a normal large rise in $[Ca^{2+}]_i$ (to a peak of 700–900 nM), fMLP-stimulated $O_2^{\cdot-}$ release was potentiated by PMA, SAPA and RX. This potentiation was seen when cells were lightly or heavily loaded with fura-2, and in the presence of SK&F 96365 in the lightly loaded cells. When fMLP could only evoke a small rise in $[Ca^{2+}]_i$ (to a peak of 400 nM) in the heavily loaded cells in the presence of SK&F 96365, potentiation by PMA was unaffected, but potentiation by SAPA or RX was considerably reduced or abolished. Taken together, these results are consistent with the observation that activation of PKC by PMA *in vitro* is less Ca^{2+} -dependent than activation by SAPA (Ryves et al., 1991). The clear synergy between RX and Ca^{2+} was an unexpected result since RX kinase has been reported to be inhibited by 1 mM Ca^{2+} *in vitro* (Ryves et al., 1989). However, nothing is known about the effects of more physiological Ca^{2+} concentrations on RX kinase activity *in vitro*, and our results suggest that physiological levels of $[Ca^{2+}]_i$ could synergize with RX to activate RX kinase. An alternative explanation could be that RX is activating PKC- β_1 when $[Ca^{2+}]_i$ is elevated; PKC- β_1 can be activated by high concentrations of RX in the presence of Ca^{2+} *in vitro* (Ryves et al., 1991). However, it should be noted that DOPPA was generally ineffective at modulating fMLP-stimulated responses in the absence of ionomycin, whereas RX was effective under all conditions. Previous reports have shown that SAPA requires

more extracellular Ca^{2+} than does PMA for stimulation of hepatocyte mitogenesis (Menapace et al., 1991), and that stimulation of platelet p47 phosphorylation can be seen with PMA alone, whereas DOPPA only evokes phosphorylation in the presence of a Ca^{2+} ionophore (Brooks et al., 1990). These reports are consistent with the results obtained here with neutrophils and with the *in vitro* studies of Ryves et al. (1991) showing that PKC activation with SAPA and DOPPA is more Ca^{2+} -dependent than that with PMA.

In conclusion, activation of PKC with PMA, SAPA and DOPPA in human neutrophils correlates with activation of PKC *in vitro* in terms of the dependency of the phorbols on Ca^{2+} ; however, activation of neutrophils by RX has also been found to synergize with Ca^{2+} . The data also suggest that both PKC- α and RX kinase may function in the regulation of the neutrophil respiratory burst.

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