Chemotactic peptide down-regulation of calcium mobilization induced by platelet-activating factor and by leukotriene B₄ in human neutrophils is uncovered by protein phosphatase inhibitors

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When human neutrophils were incubated in the presence of the protein phosphatase inhibitors calyculin A or okadaic acid, the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) produced a sustained (> 5 min) inhibition of the Ca²⁺ mobilization from intracellular stores induced by platelet-activating factor (PAF) or by leukotriene B₄ (LTB₄). No effect on Ca²⁺ mobilization by PAF or LTB₄ was observed 2 min after the addition of fMLP alone or only in the presence of phosphatase inhibitors, but a similar inhibition was produced by high (> 50 nM) concentrations of phorbol 12,13-dibutyrate (PDB). However, inhibition by PDB was sensitive to the protein kinase C (PKC) inhibitors staurosporin and Ro 31-8220, while inhibition by fMLP and calyculin A was not. These results suggest that fMLP induces a transient phosphorylation not mediated by PKC

which interferes at some point with the transduction pathway leading from the plasma membrane receptors for PAF and LTB₄ to the release of Ca²⁺ from the stores. Protein phosphatases 1 and/or 2A revert the inhibition effected by fMLP within less than 2 min. PAF and LTB₄ were also able to activate this mechanism to a smaller extent. Phosphatase inhibitors also delayed by 1–2 s the start of agonist-induced rises in [Ca²⁺]_i, and this delay was further increased by previous addition of any other agonist. Finally, given that both phosphatase inhibitors and low concentrations of PDB (2–10 nM) strongly inhibit Ca²⁺ entry, we conclude that phosphorylation down-regulates both agonist-induced Ca²⁺ entry and Ca²⁺ mobilization, but with different potency.

INTRODUCTION

Polymorphonuclear leucocytes are activated by a variety of receptor-dependent stimuli, including the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF) and leukotriene B₄ (LTB₄) [1–6]. Binding of any of these agonists to its specific receptor in the plasma membrane induces a transient increase in cytosolic Ca²⁺ ([Ca²⁺]_i) lasting about 1–2 min. This increase is due both to a fast inositol 1,4,5-trisphosphate (InsP₃)-mediated Ca²⁺ mobilization from the intracellular Ca²⁺ stores and to a late Ca²⁺ entry from the extracellular medium [1–6]. This late Ca²⁺ entry appears to be mostly a consequence of the emptying of the intracellular Ca²⁺ stores which leads to the activation of a store-operated Ca²⁺ entry pathway in the plasma membrane (SOCP) [6–9].

It has been reported that phorbol esters inhibit both the binding and the [Ca²⁺]_i increase induced by PAF and LTB₄ in neutrophils [10–13], and that they partially inhibit the [Ca²⁺]_i increase induced by fMLP [14]. fMLP has also been reported to decrease binding of PAF and LTB₄ to neutrophils [13]. These results point to a possible heterologous interaction of physiological relevance between different agonists acting on different receptors. We have recently reported that fMLP reduces the width of the PAF-induced [Ca²⁺]_i peak when both agonists are added simultaneously, which was proposed to be a consequence of the transient inhibition by fMLP of Ca²⁺ entry through an SOCP [8]. A possible additional explanation, which we have investigated in this paper, would be a more direct inhibitory action of fMLP on PAF receptors or on the transduction

pathway leading from the receptor to the Ca²⁺-mobilizing mechanisms.

We find here that, in the presence of the serine/threonine protein phosphatase inhibitors calyculin A or okadaic acid [15–18], fMLP inhibits PAF- and LTB₄-induced [Ca²⁺]_i increase, the inhibition being sustained for at least 5 min. Although PDB produced a similar effect, the inhibition by fMLP and phosphatase inhibitors was not sensitive to protein kinase C (PKC) inhibitors, suggesting that fMLP transiently interferes with the transduction pathway leading to PAF- and LTB₄-induced [Ca²⁺]_i responses through a mechanism involving protein phosphorylation not mediated by PKC. A similar effect was also produced by PAF and LTB₄ although with less potency.

MATERIALS AND METHODS

Human neutrophils were obtained as described previously [8] from blood of healthy volunteers. Cells were finally suspended at 1–2% cytocrit in standard medium containing (in mM): NaCl, 145; KCl, 5; MgCl₂, 1; CaCl₂, 1; sodium-Hepes, 10; glucose, 10; pH 7.4.

Neutrophils were loaded with fura-2 by incubation with 2–4 μM fura-2/AM for 30 min at room temperature in standard incubation medium. Cells were then washed twice and re-suspended at 2% cytocrit in standard medium. [Ca²⁺]_i was measured in 0.5 ml aliquots of this cell suspension, kept at 37 °C and under magnetic stirring, using a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.), which allows simultaneous excitation

Abbreviations used: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; LTB₄, leukotriene B₄; SOCP, store-operated Ca²⁺ entry pathway; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; PKC, protein kinase C; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; LTB₄, leukotriene B₄; 4α-PDD, 4α-phorbol 12,13-didecanoate.

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of fluorescence at 340 nm, 360 nm and 380 nm. Fluorescence emission above 520 nm was recorded and integrated at 1 s intervals. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescences excited at 340 nm and 380 nm [19].

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. PAF and ionomycin were purchased from Calbiochem. Okadaic acid and calyculin A were obtained from Alomone Labs., Jerusalem, Israel. LTB_4 , fMLP, PDB and staurosporin were obtained from Sigma, Madrid, Spain. Ro 31-8220 was provided by Dr. T. J. Hallam, Roche Research Centre, Welwyn Garden City, U.K. Other chemicals were obtained either from Sigma, Madrid or Merck, Darmstadt, Germany.

RESULTS

Figure 1 illustrates the effects of stimulation with fMLP on the subsequent response to other agonists, PAF (a) and LTB_4 (b), added 2 or 5 min later. Results obtained with or without

Figure 1 Inhibition by fMLP of the $[Ca^{2+}]_i$ increase induced by PAF (a) and LTB_4 (b) in cells treated with phosphatase inhibitors

Fura-2-loaded human neutrophils suspended in medium containing 1 mM Ca^{2+} were incubated either for 10 min with 20 nM calyculin A (+Cal), for 30 min with 1 μ M okadaic acid (+Oka) or with no additions, as indicated, and the $[Ca^{2+}]_i$ was monitored. Then, either 36 nM PAF, 1 μ M fMLP or 20 nM LTB_4 was added, as indicated by the arrows. The experiments shown are representative of 6–17 similar ones.

treatment with protein phosphatase inhibitors (calyculin A and okadaic acid, see Figure legend for details of the treatment) are shown. A summary of the results obtained in many similar experiments is shown in Table 1. The effects were very different depending on whether or not the cells had been treated with phosphatase inhibitors. In cells not treated with phosphatase inhibitors, pretreatment with fMLP had little effect on the subsequent response to LTB_4 and increased somewhat the height of the $[Ca^{2+}]_i$ peak elicited by PAF (trace 3, Figures 1b and 1a, cf. trace 1 and see also Table 1). In contrast, in cells treated with the phosphatase inhibitors, either calyculin A or okadaic acid, prestimulation with fMLP, produced a strong inhibition of the $[Ca^{2+}]_i$ increase elicited by the subsequent addition of either PAF or LTB_4 (traces 4 and 5 in Figure 1, see also Table 1). In trace 5 of Figure 1a, PAF was added only 2 min after the addition of fMLP. Control experiments showed that in the absence of phosphatase inhibitors this time was sufficient to allow for a full cell $[Ca^{2+}]_i$ response to PAF (data not shown). The overlapping of the $[Ca^{2+}]_i$ peaks and the incomplete refilling of intracellular stores prevented the study of the inhibition by fMLP in the absence of phosphatase inhibitors at shorter times. The concentrations of both calyculin A and okadaic acid used, 20 nM and 1 μ M respectively, as well as the required pre-incubation times, are consistent with previous reports on the effects of these phosphatase inhibitors in neutrophils [20–23].

Calyculin A alone did not modify the height of the $[Ca^{2+}]_i$ peaks induced by PAF or LTB_4 (trace 2, Figure 1a and 1b), but narrowed the PAF-induced $[Ca^{2+}]_i$ peak (see also Fig. 4b). This effect may be due to the previously documented [20] inhibition by calyculin A or Ca^{2+} entry through the SOCP. Removal of extracellular Ca^{2+} had the same effect, the height of the PAF-induced $[Ca^{2+}]_i$ peak was not modified but its width was reduced [6] (see also Figure 4a).

The above results clearly suggest that treatment with phosphatase inhibitors uncovers an inhibitory effect of pre-stimulation with fMLP on the subsequent cell response to other agonists. This inhibition must be due to a diminished mobilization of Ca^{2+} from the $[Ca^{2+}]_i$ stores, which may reflect either poor refilling after the fMLP treatment or decreased releasing capacity of the agonists. In the experiments shown in Figure 2, we measured the degree of filling of the intracellular stores after stimulation with fMLP. This was estimated from the size of the $[Ca^{2+}]_i$ peak elicited by ionomycin in Ca^{2+} -free medium [6,7]. Five minutes after treatment with fMLP the stores had refilled somewhat above the resting level, as indicated by the fact that the ionomycin-induced $[Ca^{2+}]_i$ peak was larger than before treatment with fMLP (trace 2 in Figure 2, cf. trace 1 and see also Table 1). This may explain why the response to PAF was somewhat larger in the cells first stimulated with fMLP than in the controls (Figure 1a). In cells treated with calyculin A or with okadaic acid (traces 3 and 4 in Figure 2), the degree of filling of the stores 5 or 2 min, respectively, after stimulation with fMLP was about the same as in the control cells, not treated with fMLP (trace 1). These results indicate that, since the stores are adequately refilled, the inhibition of the response to PAF and LTB_4 is due to a decrease of the store-releasing effect of these agonists in the cells first treated with fMLP in the presence of phosphatase inhibitors.

We have also studied the effect of pretreatment with LTB_4 and PAF on the subsequent $[Ca^{2+}]_i$ peak induced both by other agonists and by ionomycin. The results are summarized in Table 1. The response to ionomycin was affected little in the absence of phosphatase inhibitors, suggesting that refilling of the stores was quite complete 5 min after treatment with any of the agonists. In fact, an increase in the level of filling of the stores over the control values was observed both after fMLP and after PAF

Table 1 Effects of prestimulation with fMLP, PAF or LTB_4 in the presence or in the absence of calyculin A on both the height and the $t_{1/2}$ of the $[\text{Ca}^{2+}]_i$ peaks induced by ionomycin or by the other agonists

Experiments were similar to those shown in Figures 1–3. Pretreatment was added 5 min before the agonist, except for the effect of fMLP on the PAF-induced $[\text{Ca}^{2+}]_i$ peak in the presence of okadaic acid (Oka), where fMLP was added 2 min before PAF. $\Delta[\text{Ca}^{2+}]_i$ was calculated as peak $[\text{Ca}^{2+}]_i$ minus basal $[\text{Ca}^{2+}]_i$, and $t_{1/2}$ was calculated as the time required to increase $[\text{Ca}^{2+}]_i$ by 50%. Data are expressed as mean $\pm$ S.E.M. (number of data given in parentheses).

Pre-treatment	Agonist	Peak $\Delta[\text{Ca}^{2+}]_i$ (nM)			Lag, $t_{1/2}$ (s)		
		Control	+ Calyculin	+ Oka	Control	+ Calyculin	+ Oka
None	Iono	810 $\pm$ 60 (13)	1150 $\pm$ 20 (3)		1.8 $\pm$ 0.2	1.5 $\pm$ 0.2	
fMLP	Iono	1060 $\pm$ 110 (9)	680 $\pm$ 60 (15)	990 $\pm$ 70 (3)	1.3 $\pm$ 0.2	1.7 $\pm$ 0.2	1.1 $\pm$ 0.1 (3)
PAF	Iono	1140 $\pm$ 60 (7)	690 $\pm$ 70 (9)		1.2 $\pm$ 0.2	1.3 $\pm$ 0.2	
LTB_4	Iono	770 $\pm$ 180 (7)	400 $\pm$ 40 (7)		1.1 $\pm$ 0.3	1.0 $\pm$ 0.1	
None	fMLP	770 $\pm$ 40 (36)	750 $\pm$ 60 (36)	1020 $\pm$ 70 (13)	2.0 $\pm$ 0.1	4.5 $\pm$ 0.2	3.6 $\pm$ 0.2 (13)
PAF	fMLP	910 $\pm$ 140 (5)	850 $\pm$ 100 (8)		2.2 $\pm$ 0.3	6.5 $\pm$ 0.2	
LTB_4	fMLP	800 $\pm$ 70 (4)	410 $\pm$ 20 (6)		1.8 $\pm$ 0.3	5.9 $\pm$ 0.3	
None	PAF	700 $\pm$ 30 (29)	680 $\pm$ 40 (27)	680 $\pm$ 180 (2)	2.1 $\pm$ 0.1	3.4 $\pm$ 0.2	3.2 $\pm$ 0.1 (2)
fMLP	PAF	860 $\pm$ 100 (14)	260 $\pm$ 30 (17)	160 $\pm$ 30 (5)	3.5 $\pm$ 0.2	8.6 $\pm$ 0.6	9.8 $\pm$ 0.8 (5)
LTB_4	PAF	860 $\pm$ 50 (4)	290 $\pm$ 49 (6)		2.1 $\pm$ 0.2	6.3 $\pm$ 0.6	
None	LTB_4	660 $\pm$ 30 (22)	610 $\pm$ 30 (24)	750 $\pm$ 4 (3)	1.7 $\pm$ 0.1	4.3 $\pm$ 0.2	2.5 $\pm$ 0.2 (3)
fMLP	LTB_4	580 $\pm$ 40 (6)	9 $\pm$ 3 (6)	140 $\pm$ 50 (3)	3.1 $\pm$ 0.4	N.D.*	5.2 $\pm$ 0.2 (3)
PAF	LTB_4	690 $\pm$ 110 (5)	72 $\pm$ 15 (7)		2.6 $\pm$ 0.4	9.3 $\pm$ 1.1	

* N.D. = Not determined.

Figure 2 Effects of fMLP and calyculin A and the Ca^{2+} content of the intracellular stores

Fura-2-loaded human neutrophils suspended in medium containing 1 mM Ca^{2+} were incubated either for 10 min with 20 nM calyculin A (+ Cal), for 30 min with 1 μM okadaic acid (+ Oka) or with no additions, as indicated, and the $[\text{Ca}^{2+}]_i$ was monitored. Then, either 5 mM EGTA + 2 mM Tris followed by 200 nM ionomycin (EGTA + IONO) or 1 μM fMLP was added as indicated by the arrows. The experiments shown are representative of 3–15 similar ones.

pretreatment. In cells treated with calyculin A, the refilling tended to be somewhat smaller after agonist treatment, this being most marked after LTB_4 . This may be due in part to the inhibition of SOCP by calyculin A [20] which slows down Ca^{2+} entry, thus slowing store refilling. The height of the agonist-induced $[\text{Ca}^{2+}]_i$ peaks was not modified by preincubation with calyculin A, and prestimulation with an agonist in the absence of

phosphatase inhibitors produced either no change or even an increase in the subsequent $[\text{Ca}^{2+}]_i$ peak induced by another agonist, probably as a consequence of the increased refilling. However, prestimulation with an agonist in the presence of phosphatase inhibitors produced a decrease in size of the subsequent agonist-induced $[\text{Ca}^{2+}]_i$ peak, which was dependent on both the agonists used for the prestimulus and for the stimulus. The most potent inhibitory effect was obtained when prestimulation was performed with fMLP (54% and 98% inhibition for PAF and LTB_4 , respectively; values calculated taking into account the decrease in the ionomycin-induced Ca^{2+} peak produced by fMLP). fMLP was also the least sensitive to the inhibition. Taking into account the decrease of refilling produced by prestimulation with PAF and LTB_4 in the presence of calyculin A, the height of the fMLP-induced $[\text{Ca}^{2+}]_i$ peak was not significantly decreased by prestimulation with these agonists. Among PAF and LTB_4 , PAF was more potent and less sensitive (see the effects of fMLP above) than LTB_4 . Pretreatment with PAF produced 83% inhibition of the LTB_4 -induced $[\text{Ca}^{2+}]_i$ peak, while pretreatment with LTB_4 only produced 35% inhibition of the PAF-induced $[\text{Ca}^{2+}]_i$ peak, in both cases taking into account the effects of these agonists on refilling.

Pretreatment with agonists and/or phosphatase inhibitors also modified the kinetics of the $[\text{Ca}^{2+}]_i$ response to the subsequent addition of other agonists. Figure 3 illustrates the effects of these treatments on PAF and fMLP responses. Pretreatment with calyculin A alone delayed both the PAF-induced (Figure 3a) and the fMLP-induced (Figure 3b) $[\text{Ca}^{2+}]_i$ responses by introducing a lag time previous to the $[\text{Ca}^{2+}]_i$ rise. The delays are quantified in Table 1 as $t_{1/2}$ (time from the addition of the agonist until the $[\text{Ca}^{2+}]_i$ level reached 50% of the maximum value). Note that the delay was due to an increase in the lag time previous to the $[\text{Ca}^{2+}]_i$ increase, which then took place at a similar rate as in the control and reached similar maximum values. Figure 3c shows that, while pretreatment with fMLP alone did not modify the kinetics of the subsequent PAF-induced $[\text{Ca}^{2+}]_i$ peak (open symbols), pretreatment with fMLP in the presence of calyculin A (closed symbols) produced not only a decrease in the height of the PAF-

Figure 3 Effect of calyculin A and/or fMLP or PAF pretreatment on the kinetics of the PAF- and fMLP-induced $[Ca^{2+}]_i$ increase

Fura-2-loaded human neutrophils suspended in medium containing 1 mM Ca^{2+} were incubated for 10 min either with no additions (open symbols) or with 20 nM calyculin A (closed symbols). Then, at $t = 0$, either 36 nM PAF (a) or 1 μ M fMLP (b) was added, as indicated by the arrows. In (c) and (d) either 1 μ M fMLP or 36 nM PAF respectively, was added 5 min before. These experiments are representative of 5–36 similar ones.

induced $[Ca^{2+}]_i$ peak (as shown in Figure 1), but also introduced a long delay before the $[Ca^{2+}]_i$ rise was initiated. This effect was also clear when we pretreated the cells with PAF and then stimulated them with fMLP. Figure 3d shows that, even though PAF pretreatment in the presence of calyculin A did not significantly modify the height of the fMLP-induced $[Ca^{2+}]_i$ peak (Table 1), a long delay in the response to fMLP was introduced. This delay was larger than that produced by calyculin A alone (see Figure 3b and Table 1), suggesting that PAF pretreatment potentiates the effect of calyculin A. In the absence of calyculin A, PAF pretreatment did not modify the kinetics of the fMLP-induced $[Ca^{2+}]_i$ peak (see Figure 3b and d and Table 1).

Table 1 also shows the $t_{1/2}$ obtained after all the possible interactions between the three agonists and ionomycin. The lag time for the $[Ca^{2+}]_i$ increase induced by ionomycin was not significantly modified by any of the treatments. Calyculin A produced a discrete increase in the lag for all the three agonists, the least sensitive being PAF. In the absence of calyculin A, prestimulation with fMLP, but not with PAF or LTB_4 , also produced a discrete increase in the lag for $[Ca^{2+}]_i$ increase after the subsequent addition of PAF or LTB_4 . Finally, prestimulation with any agonist of calyculin-treated cells produced in all cases a much larger increase in the lag for a subsequent agonist-induced $[Ca^{2+}]_i$ increase. Okadaic acid had the same effects as calyculin A (Table 1) on the inhibition by prestimulation with fMLP of Ca^{2+} mobilization induced by PAF and LTB_4 in neutrophils.

The results shown in Figures 1–3 show the presence of heterologous interactions between the fMLP, PAF and LTB_4 receptors, leading to inhibition of Ca^{2+} mobilization. Uncovering of this effect by protein phosphatase inhibitors suggests that these interactions are mediated by phosphorylation. We therefore decided to compare this inhibition with the effects of phorbol

esters, which have been reported to strongly inhibit PAF-induced Ca^{2+} mobilization [10–13] and to inhibit partially fMLP-induced Ca^{2+} mobilization [14]. Figure 4 illustrates some representative experiments and more extensive results are summarized in Table 2. The first trace in Figure 4a compares the effects of PAF in Ca^{2+} -free and Ca^{2+} -containing medium. As reported previously [6], the height of the $[Ca^{2+}]_i$ peak is not modified by Ca^{2+} removal, since it is mainly due to the release of Ca^{2+} from the stores. The width of the $[Ca^{2+}]_i$ peak decreased on removal of Ca^{2+} , reflecting the contribution of delayed Ca^{2+} entry through the SOCP. The origin of the unusual shape of the PAF-induced $[Ca^{2+}]_i$ peak, showing either a shoulder or a secondary-peak after the initial one, is unknown. These peculiarities are even observed in single-cell imaging experiments (our unpublished work) and they are always removed in the absence of extracellular Ca^{2+} . Treatment with 2 nM PDB did not decrease the height of the $[Ca^{2+}]_i$ peak (in fact it was increased, see Table 2), but prevented Ca^{2+} entry almost completely, as shown by the fact that the effect of PAF was almost the same in Ca^{2+} -containing and in Ca^{2+} -free media (second trace in Fig. 4a). At 10 nM PDB the height of the $[Ca^{2+}]_i$ peak decreased only a little, but Ca^{2+} entry was completely prevented (third trace). These results confirm our previous report [8] that PDB inhibits Ca^{2+}/Mn^{2+} entry through the SOCP with an IC_{50} of about 1 nM. At 50 nM PDB the $[Ca^{2+}]_i$ increase induced by PAF was almost fully abolished (fourth trace), indicating that these concentrations inhibit Ca^{2+} release from the intracellular stores. The results obtained with the low concentrations of PDB are very similar to those obtained with calyculin A alone (cf. Fig. 4b, second trace) and are consistent with the previously determined IC_{50} for inhibition of the SOCP, 1 nM [8]. The inactive 4 α -phorbol 12,13-didecanoate (4 α -PDD, fifth trace) did not have an inhibitory effect on the PAF-induced $[Ca^{2+}]_i$ increase (last trace in Fig. 4a).

Table 2 Effects of PDB both in the presence and in the absence of calyculin A on both the height and the $t_{1/2}$ of the $[\text{Ca}^{2+}]_i$ peaks induced by ionomycin, fMLP, PAF or LTB_4

Experiments were similar to those shown in Figure 4. The phorbol ester was always added 2 min before the agonist. Other details as in Table 1.

Pre-treatment	Agonist	Peak $\Delta[\text{Ca}^{2+}]_i$ (nM)		Lag, $t_{1/2}$ (s)	
		Control	+ Calyculin	Control	+ Calyculin
None	Iono	810 ± 60 (13)	1150 ± 20 (3)	1.8 ± 0.2	1.5 ± 0.2
PDB 2	Iono	950 ± 150 (4)	850 ± 60 (4)	1.8 ± 0.2	1.4 ± 0.1
PDB 10	Iono	890 ± 60 (4)	755 ± 17 (4)	1.7 ± 0.3	1.2 ± 0.2
PDB 50	Iono	690 ± 60 (6)	860 ± 70 (3)	1.1 ± 0.2	1.2 ± 0.3
None	fMLP	770 ± 40 (36)	750 ± 60 (36)	2.0 ± 0.1	4.5 ± 0.2
PDB 2	fMLP	700 ± 100 (3)	N.D.*	1.9 ± 0.5	N.D.
PDB 10	fMLP	630 ± 60 (3)	540 ± 20 (3)	2.0 ± 0.1	5.6 ± 0.2
PDB 50	fMLP	497 ± 13 (6)	570 ± 40 (5)	2.6 ± 0.2	5.9 ± 0.1
4 α -PDD	fMLP	596 ± 11 (3)	N.D.	1.7 ± 0.2	N.D.
None	PAF	700 ± 30 (29)	680 ± 40 (27)	2.1 ± 0.1	3.4 ± 0.2
PDB 2	PAF	930 ± 60 (9)	590 ± 40 (10)	2.8 ± 0.2	3.8 ± 0.2
PDB 10	PAF	610 ± 40 (8)	192 ± 16 (5)	4.0 ± 0.3	7.6 ± 0.3
PDB 50	PAF	190 ± 40 (13)	N.D.	5.9 ± 0.5	N.D.
4 α -PDD	PAF	890 ± 110 (4)	N.D.	2.8 ± 0.3	N.D.
None	LTB_4	660 ± 30 (22)	610 ± 30 (24)	1.7 ± 0.1	4.3 ± 0.2
PDB 2	LTB_4	638 ± 19 (3)	473 ± 5 (3)	1.6 ± 0.4	4.3 ± 0.2
PDB 10	LTB_4	530 ± 30 (7)	170 ± 20 (3)	2.5 ± 0.2	6.2 ± 0.2
PDB 50	LTB_4	120 ± 30 (11)	N.D.	4.7 ± 0.3	N.D.
4 α -PDD	LTB_4	680 ± 10 (3)	N.D.	2.2 ± 0.1	N.D.

*N.D. = Not determined.

Figure 4 Effects of PDB and/or calyculin A on the PAF-induced $[\text{Ca}^{2+}]_i$ peak

Human neutrophils were loaded with fura-2 and suspended in medium containing 1 mM Ca^{2+} . Then 36 nM PAF was added as indicated by the arrows either to control cells or to cells treated for 2 min with either 2 nM PDB, 10 nM PDB, 50 nM PDB or 100 nM 4 α -PDD, as indicated. In the curves labelled 'EGTA', 2 mM EGTA and 2 mM Tris were added 15 s before the addition of PAF. In curves labelled '+ Cal', the cells were incubated for 10 min with 20 nM calyculin A before the addition of PAF. The experiments shown are representative of 4–27 similar ones.

Figure 4b shows the interactions between the effects of calyculin A and those of PDB. Treatment with calyculin A alone did not modify the height of the $[\text{Ca}^{2+}]_i$ peak but decreased its width (second trace in Figure 4b, compare with the first one). This is consistent with the inhibitory effect of calyculin A on SOCP reported previously [20]. The effects were about the same as those of 2 nM PDB (third trace) and not additive to them (fourth trace). The fifth trace shows the effects of 10 nM PDB, which were about the same as those achieved at 2 nM, but they were largely potentiated by calyculin A (sixth trace).

Table 2 summarizes the effects of PDB on the $[\text{Ca}^{2+}]_i$ increase induced by ionomycin and the three agonists, and on the lag between agonist addition and the $[\text{Ca}^{2+}]_i$ response. We did not find any significant effect of PDB either on the height or on the $t_{1/2}$ of the ionomycin-induced $[\text{Ca}^{2+}]_i$ peaks. Neither did PDB modify the width of the ionomycin-induced $[\text{Ca}^{2+}]_i$ peaks (not shown), indicating that the rate of Ca^{2+} extrusion through the plasma membrane was not significantly modified. This reinforces the idea that the narrowing effect of low concentrations of PDB on the PAF-induced $[\text{Ca}^{2+}]_i$ peaks is due to inhibition of Ca^{2+} entry. The higher concentration of PDB (50 nM) strongly decreased the height of the $[\text{Ca}^{2+}]_i$ peaks induced by PAF and LTB_4 , and increased the lag time. A concentration of 10 nM PDB did not decrease the height of the $[\text{Ca}^{2+}]_i$ peaks induced by PAF or LTB_4 , but increased slightly the lag time and its effect on both the height of the $[\text{Ca}^{2+}]_i$ peaks and the lag time was strongly potentiated by calyculin A. The effect of fMLP was more resistant to the inhibition, 50 nM PDB producing only a 35% reduction in the height of the $[\text{Ca}^{2+}]_i$ peak. This is consistent with previous results showing only partial inhibition by phorbol ester of the fMLP-induced Ca^{2+} mobilization [14]. This effect was not further potentiated in the presence of calyculin A. The lag time of the fMLP-induced $[\text{Ca}^{2+}]_i$ peak was also affected only a little by PDB. However, in the presence of calyculin A, PDB potentiated the increase in lag time induced by calyculin A.

Figure 5 Effect of staurosporin on the inhibition by PDB (a) and by fMLP and calyculin A (b and c) of the $[Ca^{2+}]_i$ peaks induced by PAF (b) or LTB₄ (c)

Human neutrophils were loaded with fura-2 and suspended in medium containing 1 mM Ca^{2+} . In (a), either 36 nM PAF or 20 nM LTB₄ was added, as indicated by the arrows, either to control cells or to cells treated for 2 min with 50 nM PDB (labelled PDB50+PAF or PDB50+LTB₄). In the traces marked '+Stau', 100 nM staurosporin was added 5 min before the addition of PAF. In (b and c) cells were incubated for 5 min with 100 nM staurosporin and for 10 min either with (labelled +Cal) or without 20 nM calyculin A. Then, either 1 μ M fMLP, 36 nM PAF, 20 nM LTB₄ or 5 mM EGTA + 2 mM Tris followed by 200 nM ionomycin (EGTA+IONO) were added, as indicated by the arrows. The experiments shown are representative of three similar ones.

Finally, Figures 5 and 6 illustrate the sensitivity to PKC inhibitors of the inhibition of Ca^{2+} release from the stores effected by phorbol esters or by prestimulation with fMLP and calyculin A. The inhibition by 50 nM PDBu of the $[Ca^{2+}]_i$ increases induced by PAF and LTB₄ was reversed by staurosporin, a non-specific PKC inhibitor (Figure 5a). In contrast, staurosporin did not prevent the inhibition by prestimulation with fMLP, in the presence of calyculin A, of Ca^{2+} mobilization from the stores induced by either PAF (Figure 5b) or LTB₄ (Figure 5c). In the last trace of Figure 5c it can be seen that a final addition of EGTA and ionomycin produced a $[Ca^{2+}]_i$ peak, demonstrating that the stores were still filled with Ca^{2+} by that time. Identical results were obtained with the more specific PKC inhibitor Ro 31-8220. Figure 6a shows that Ro 31-8220 prevented

Figure 6 Effect of the PKC inhibitor Ro 31-8220 on the inhibition by PDB (a) and by fMLP and calyculin A (b and c) of the $[Ca^{2+}]_i$ peaks induced by PAF (b) or LTB₄ (c)

Human neutrophils were loaded with fura-2 and suspended in medium containing 1 mM Ca^{2+} . In (a), either 36 nM PAF or 20 nM LTB₄ was added, as indicated by the arrows, either to control cells or to cells treated for 2 min with 50 nM PDB (labelled PDB50+PAF or PDB50+LTB₄). In the traces marked '+Ro', 3 μ M Ro 31-8220 was added 20 min before the addition of PAF. In (b) and (c) cells were incubated either for 20 min with 3 μ M Ro 31-8220 (labelled +Ro, first trace), for 10 min with 20 nM calyculin A (labelled +Cal, third trace) or with both together (middle trace). Then, either 1 μ M fMLP, 36 nM PAF or 20 nM LTB₄ were added, as indicated by the arrows. The experiments shown are representative of three similar ones.

the inhibition by PDB of Ca^{2+} mobilization by PAF and LTB₄. In contrast, Ro 31-8220 had no effect on the inhibition by prestimulation with fMLP of PAF- (Figure 6b) and LTB₄- (Figure 6c) induced Ca^{2+} mobilization.

DISCUSSION

The results shown in this paper strongly suggest that the chemotactic peptide fMLP induces a transient protein phosphorylation which interferes at some point with the transduction pathway going from the binding of PAF and LTB₄ to their receptor in the plasma membrane, to the production of $InsP_3$ and the subsequent Ca^{2+} release from the intracellular stores. Dephosphorylation by protein phosphatases 1 or 2A quickly reactivates the transduction pathway, thus allowing a

new $[\text{Ca}^{2+}]_i$ response to PAF or LTB_4 . The time course of this inhibition is difficult to calculate precisely from $[\text{Ca}^{2+}]_i$ records. We only can say that the inhibition reverses completely within less than 2 min after the addition of fMLP. It was not possible to study shorter times because of the overlapping between the $[\text{Ca}^{2+}]_i$ peaks induced by both agonists (fMLP and PAF or LTB_4) and of the incomplete store refilling at times shorter than 2 min after fMLP addition.

PAF and LTB_4 were also able to activate a similar heterologous interaction in the presence of calyculin A, but with less potency. The order of activity for the inhibition of other agonist-induced Ca^{2+} mobilization was fMLP > PAF > LTB_4 , and the order of sensitivity to the inhibition was the reverse, LTB_4 > PAF > fMLP. These sequences should probably reflect the different abilities to activate the protein kinase involved, and the different sensitivities of the corresponding target in the transduction pathway to phosphorylation by that protein kinase.

The inhibition of the PAF and LTB_4 -induced $[\text{Ca}^{2+}]_i$ mobilization by fMLP resembles very much the inhibition of these responses by phorbol esters. The lack of effect of the inactive 4 α -PDD suggests that PDB is in fact acting through activation of PKC. However, the effect of PDB was fully reversed by both staurosporin and Ro 31-8220, while the effect of fMLP and calyculin A was insensitive to these inhibitors. This suggests that the effect of fMLP is mediated by a PK other than PKC, which is insensitive to both staurosporin and Ro 31-8220, even though PKC appears to be able to phosphorylate the same substrate. Low concentrations of PDB (2 nM) did not decrease the height of the $[\text{Ca}^{2+}]_i$ peak, but considerably reduced its width (Figure 4a). This effect can be attributed to the inhibition of Ca^{2+} entry produced by phorbol esters, which has an IC_{50} of about 1 nM [8]. The same effect was produced by phosphatase inhibitors [20] (see also Figure 4b). Higher concentrations of PDB (50 nM) were required to decrease the height of the $[\text{Ca}^{2+}]_i$ peak, which must be attributed to inhibition of Ca^{2+} release from the intracellular stores. We can therefore differentiate between two distinct effects of the phorbol esters with different IC_{50} values. The reason for the differences in IC_{50} is unclear. In both cases, dephosphorylation is mediated by protein phosphatases 1 and/or 2A, and similar concentrations of the inhibitors calyculin A and okadaic acid were necessary to produce inhibition (compare data in this paper and in our previous report on the inhibition of SOCP [20]). The fact that the phosphatase inhibitors are able to inhibit the SOCP in the absence of fMLP [20] (see Figures 1 and 4b in this paper), indicates that a constitutively active kinase is able to phosphorylate the SOCP or a possible SOCP regulatory protein, even in the resting condition. In contrast, the kinase responsible for inhibiting the PAF- and LTB_4 -induced Ca^{2+} mobilization from the stores reported here seems to be only active in the presence of fMLP or to require an increase in activity to produce detectable effects.

Treatment with protein phosphatase inhibitors introduced a lag time of 1–2 s for the start of agonist-induced $[\text{Ca}^{2+}]_i$ rise. This lag time was further increased by pretreatment with phorbol esters or with agonists and was present even under conditions in which the height of the agonist-induced $[\text{Ca}^{2+}]_i$ peak was not significantly reduced by the pretreatment (see Figure 3). The significance of this lag time is unclear. It may represent an intermediate step between normal agonist-induced Ca^{2+} release and its full inhibition after phosphorylation of a component of the transduction pathway. This component could be involved in a co-operative all or none step, strictly dependent on a threshold level of activation of that particular component. Partial phosphorylation could then lead to an increase in the time necessary to reach the threshold. This component could be

tentatively assigned to a G-protein, as it has been reported that a threshold of coupled G-proteins is required to transduce neutrophil responses [24]. On the other hand, it has also been reported that phorbol 12-myristate 13-acetate (PMA) is able to inhibit the PAF-induced hydrolysis of phosphatidylinositol biphosphate without affecting the PAF-induced deacylation in rat Kupffer cells [25]. Similarly, PMA inhibits the PAF-induced activation of phospholipase C and enhances the PAF-induced phospholipase D activity in differentiated U937 cells [26]. Finally, it has been shown in platelets that the PMA-induced inhibition of agonist-induced Ca^{2+} mobilization had a time and concentration dependence similar to that of the PMA-induced phosphorylation of $\text{G}_i\alpha$ protein [27]. Overall, these results suggest that, in different cell types, agonists or phorbol esters could inhibit activation of phospholipase C by other agonists, perhaps through phosphorylation of the $\text{G}_i\alpha$ protein connecting the receptor and the phospholipase C, without affecting other receptor-mediated responses to these agonists.

The physiological significance of the complex interaction between the fMLP, PAF and LTB_4 receptors described here is unknown. It may perhaps be considered as a way to moderate the $[\text{Ca}^{2+}]_i$ increase when the cell is exposed simultaneously to several agonists. The inhibition by fMLP of the SOCP [3,20], which helps to rapidly reduce $[\text{Ca}^{2+}]_i$ after stimulation, may have the same role. In fact, our previously reported inhibition by fMLP of PAF-induced Ca^{2+} and Mn^{2+} entry when both agonists are added simultaneously [8] reflected probably not only the inhibition of SOCP by fMLP but also the inhibition of PAF-induced Ca^{2+} release.

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