Transient Inhibition by Chemotactic Peptide of a Store-operated Ca\textsuperscript{2+} Entry Pathway in Human Neutrophils*

Mayte Montero, Javier García-Sancho, and Javier Alvarez

From the Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005-Valladolid, Spain

(Received for publication, December 28, 1992, and in revised form, March 5, 1993)

Emptying the intracellular calcium stores of fura-2-loaded human neutrophils by treatment with the endomembrane ATPase inhibitor thapsigargin leads to a maintained increase of [Ca\textsuperscript{2+}], by Ca\textsuperscript{2+} entry through a store-operated Ca\textsuperscript{2+} entry pathway. Under these conditions, [Ca\textsuperscript{2+}], was reduced transiently by N-formylmethionyl-leucyl-phenylalanine (fMLP) and permanently by phorbol 12,13-dibutyrate (PDB). Platelet-activating factor (PAF) had no effect. The fMLP- and PDB-induced [Ca\textsuperscript{2+}], decreases were not due to stimulated Ca\textsuperscript{2+} efflux but to inhibition of store-operated Ca\textsuperscript{2+} entry pathway. PDB and fMLP, but not PAF, inhibited the entry of Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Be\textsuperscript{2+} in thapsigargin-treated cells. This inhibition was dependent on [Ca\textsuperscript{2+}], barely detectable at [Ca\textsuperscript{2+}], of 50 nM and increasingly strong and fast to appear at 170 and 630 nM. Inhibition of entry by fMLP was complete within 5–10 s, disappeared within 2–3 min, and was partially prevented by staurosporin (100 nM). Inhibition by PDB was equally fast, but no recovery was detected within 5 min, and it was fully prevented by staurosporin. The inhibitory effect of fMLP had similar characteristics when PAF was used instead of thapsigargin to induce the entry of Ca\textsuperscript{2+} or Mn\textsuperscript{2+}. We conclude that fMLP, but not PAF, is able to produce a transient inhibition of store-operated Ca\textsuperscript{2+} entry pathway, probably mediated by protein kinase C. This action could be part of a general homeostatic mechanism designed to moderate [Ca\textsuperscript{2+}], increases induced by some agonists.

The agonist-induced increase of cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in human neutrophils is often composed of two phases: (i) an early and transient [Ca\textsuperscript{2+}], peak due to inositol 1,4,5-trisphosphate-mediated Ca\textsuperscript{2+} release from the intracellular Ca\textsuperscript{2+} stores, and (ii) a sustained [Ca\textsuperscript{2+}], increase due to increased Ca\textsuperscript{2+} entry through the plasma membrane (1–5). The mechanism for the late Ca\textsuperscript{2+} entry is still obscure, although there is strong evidence that the opening of this plasma membrane Ca\textsuperscript{2+} entry pathway is signaled by the emptying of the intracellular Ca\textsuperscript{2+} stores (5, 6). We shall use the term store-operated Ca\textsuperscript{2+} entry pathway (SOCP), to refer to this Ca\textsuperscript{2+} entry mechanism.

In addition to the agonist-induced rise in [Ca\textsuperscript{2+}], there is evidence that some agonists also induce homeostatic processes, which tend to restore [Ca\textsuperscript{2+}], to the basal level. An agonist-induced [Ca\textsuperscript{2+}], decrease has been described in thrombin-stimulated platelets (11), vasopressin-stimulated hepatocytes (12), thrombin-stimulated endothelial cells (13), and fMLP-stimulated neutrophils (14, 15). It has also been shown that fMLP reduces [Ca\textsuperscript{2+}], in neutrophils permeabilized to Ca\textsuperscript{2+} with ionomycin (14, 15). A similar effect has been obtained with phorbol esters (14, 15), suggesting that the [Ca\textsuperscript{2+}], decrease could be mediated by protein kinase C. The fMLP-induced reduction of [Ca\textsuperscript{2+}], in ionomycin-treated human neutrophils has been attributed to a stimulation of the plasma membrane Ca\textsuperscript{2+} pump, leading to increased Ca\textsuperscript{2+} efflux (14, 15). Stimulation of the Ca\textsuperscript{2+} pump by phorbol esters has been demonstrated in neutrophil's inside-out plasma membrane vesicles (16) and cytoplasts (17), although this effect was evident only about 2 min after the addition of the phorbol ester. Additionally, it has been shown that phorbol esters inhibit fMLP-induced Mn\textsuperscript{2+} entry, although the physiological significance of this effect was not clear (15).

We have used here thapsigargin-treated human neutrophils to investigate the mechanism of the fMLP-induced decrease in [Ca\textsuperscript{2+}],. Thapsigargin is a very specific inhibitor of the sarcoplasmic and endoplasmic reticulum Ca\textsuperscript{2+} pump (18), which induces a permanent elevation of [Ca\textsuperscript{2+}], in intact cells. This increase of [Ca\textsuperscript{2+}], is initiated by slow release of Ca\textsuperscript{2+} from the intracellular Ca\textsuperscript{2+} stores, and it is maintained by a stimulation of Ca\textsuperscript{2+} entry due to activation of SOCP once the stores are emptied of Ca\textsuperscript{2+} (19). We show here that, in thapsigargin-treated human neutrophils, fMLP and phorbol ester, but not platelet activating factor (PAF), decrease [Ca\textsuperscript{2+}], by

---

1 The abbreviations used are: SOCP, store-operated Ca\textsuperscript{2+} entry pathway; PAF, platelet-activating factor (a) 1-O-alkyl-2-α-glycerol-3-phosphorylcholine; fMLP, N-formylmethionyl-leucyl-phenylalanine; PDB, phorbol 12,13-dibutyrate.

2 The name SOCP was chosen by analogy to voltage-operated (VOCC), receptor-operated (ROCC), or second messenger-operated (SMOCC) Ca\textsuperscript{2+} channels. Although SOCP could be a subclass of SMOCC, we want to stress with this name that this Ca\textsuperscript{2+} entry pathway opens as a consequence of the emptying of the intracellular calcium stores rather than by the action of a second messenger generated directly by binding of an agonist with its membrane receptor. SOCP is preferred to the term "capacitative Ca\textsuperscript{2+} entry" originally proposed by Putney (7), as it is generally thought at present that the entry from the extracellular medium to the cytoplasm does not take place "capacitatively" across the calcium stores (8). The term store-operated Ca\textsuperscript{2+} channel was not used, as the data presented here cannot distinguish between a channel and some other kind of entry mechanism, even though available evidence on the effects of membrane potential (9) and electrophysiological recordings (10) fits better to a "channel" than to a "carrier" mechanism.

---

* This work was supported by the Spanish Dirección General de Investigación Científica y Técnica Grant PB89-0359. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: Tel.: 34-83-423084; Fax: 34-83-423085.

13055
inhibition of SOCP and not by activation of the Ca\(^{2+}\) pump. Inhibition of SOCP by fMLP is transient and precedes the better known fMLP-induced activation of this entry pathway (4, 5, 15). Similarities with the effect of phorbol ester suggest that this inhibition may be mediated by protein kinase C, although a clear difference in sensitivity to staurosporine between fMLP- and PDB-induced decrease of [Ca\(^{2+}\)], was apparent.

**MATERIALS AND METHODS**

Human neutrophils were obtained from blood of healthy volunteers anticoagulated by mixing 6:1 (v/v) with acid citrate-dextrose. Dextran (T500, Pharmacia LKB Biotechnology Inc.) was then added to obtain a final concentration of 1.3%. After 45 min at room temperature, the upper phase containing no red cells was removed and centrifuged (300 \(\times\) g, 10 min). The cell pellet was resuspended, layered on a Ficoll gradient (lymphocyte separation medium; Flow Laboratories, Irvine, Scotland), and centrifuged for 20 min at 400 \(\times\) g. The cells were resuspended, and contaminating red cells were disrupted by hypotonic lysis (20). Neutrophils were finally suspended at 1-2% cytocrit in standard medium containing (in mM): NaCl, 145; KCl, 5; MgCl\(_2\), 1; CaCl\(_2\), 0.2; sodium-HEPES, 10; glucose, 10 (pH 7.4).

Neutrophils were loaded with fura-2 by incubation with 2-4 \(\mu\)M fura-2/AM for 30 min at room temperature in standard incubation medium. Cells were then washed twice and resuspended at 2% cytocrit in nominally Ca\(^{2+}\)-free standard medium. [Ca\(^{2+}\)] was measured in Fura-Z/AM was obtained from Molecular Probes, Eugene, OR.

**RESULTS**

Fig. 1 (left panels) shows the effects of fMLP (1 \(\mu\)M), PAF (36 nm), and PDB (100 nm) on [Ca\(^{2+}\)], of human neutrophils. The first two produced an increase of [Ca\(^{2+}\)], and PDB did not modify [Ca\(^{2+}\)]. fMLP produced an early and transient (30-s duration) [Ca\(^{2+}\)], peak due to Ca\(^{2+}\) release from the stores followed by a sustained plateau due to increased Ca\(^{2+}\) entry (5). The [Ca\(^{2+}\)], increase produced by PAF was composed of a wider early [Ca\(^{2+}\)], peak followed by a sustained plateau. Fig. 1 (right panels) shows that thapsigargin induced an increase of [Ca\(^{2+}\)], up to a level of about 500 nm, which remained stable for at least 10 min. This sustained increase of [Ca\(^{2+}\)], is due to the activation of the SOCP as a consequence of the emptying of the intracellular Ca\(^{2+}\) stores. Addition of 1 \(\mu\)M fMLP to thapsigargin-treated cells (upper right panel) induced a fast decrease in [Ca\(^{2+}\)], which then returned to the previous high [Ca\(^{2+}\)], level within about 2 min. Addition of 100 nm PDB (center right panel) induced a similar decrease in [Ca\(^{2+}\)], but it remained stable at this lower level. Finally, addition of 36 nm PAF (lower right panel) had only minor effects on [Ca\(^{2+}\)].

The decrease of [Ca\(^{2+}\)], elicited by fMLP and PDB (Fig. 1, right panels) could, in principle, be due either to stimulation of Ca\(^{2+}\) efflux or to inhibition of Ca\(^{2+}\) entry. The possible stimulation of a Ca\(^{2+}\) efflux mechanism by fMLP or PDB was investigated in two ways. In a first series of experiments, we followed the efflux of Ca\(^{2+}\) from thapsigargin-treated neutrophils on removal of external Ca\(^{2+}\) by addition of EGTA. Fig. 2 compares the rates of [Ca\(^{2+}\)], decrease after addition of either EGTA, fMLP, EGTA + fMLP, or EGTA + PDB to thapsigargin-treated cells. Fura-2-loaded cells incubated in 1 mM Ca\(^{2+}\)-containing medium were treated with 0.5 \(\mu\)M thapsigargin for 10 min, and the [Ca\(^{2+}\)], was monitored. In the left panel, either 1 \(\mu\)M fMLP (upper panel), 100 nm PDB (center panel), or 36 nm PAF (lower panel) were added at t = 0 in the figure. In the right panels, 0.5 \(\mu\)M thapsigargin was added at t = 0 in the figure and, 10 min later, either 1 \(\mu\)M fMLP (upper panel), 100 nm PDB (center panel), or 36 nm PAF (lower panel) were added. Traces representative of 4 to 12 similar experiments are shown. The mean [Ca\(^{2+}\)], decreases induced by fMLP and PDB at 30 s were (mean ± S.D.) 78 ± 5% (n = 12) and 80 ± 10% (n = 7), respectively, 100% was the difference between the maximum and the basal [Ca\(^{2+}\)].
thapsigargin-treated cells at any [Ca\(^{2+}\)], (results not shown).

Mn\(^{2+}\) has been shown to be a good Ca\(^{2+}\) surrogate in human neutrophils (5, 6) but, in other cells, the adequacy of Mn\(^{2+}\) to follow unidirectional Ca\(^{2+}\) movements has been questioned (24–27). We have also used Ba\(^{2+}\) to trace the Ca\(^{2+}\) entry pathway opened by emptying the intracellular Ca\(^{2+}\) stores with thapsigargin. As with Mn\(^{2+}\), Ba\(^{2+}\) is not pumped out by the Ca\(^{2+}\) pump, and its uptake can be followed by the increase of the fluorescence of fura-2 at the Ca\(^{2+}\)-insensitive excitation wavelength of 360 nm (22) (see spectrum of fura-2-Ba\(^{2+}\) complex in Ref. 23). Fig. 5 shows that Ba\(^{2+}\) uptake by thapsigargin-treated cells was also transiently inhibited by fMLP in a [Ca\(^{2+}\)]-dependent manner. No inhibition was detected in this case at the low [Ca\(^{2+}\)], (mean ± S.D., 52 ± 11 nM, n = 17) (left panel), whereas at an intermediate [Ca\(^{2+}\)], (mean ± S.D., 228 ± 15 nM, n = 9) (right panel), the inhibition by fMLP was evident.

The time course of the inhibition of Mn\(^{2+}\) entry by fMLP was studied by adding fMLP at different times before starting Mn\(^{2+}\) uptake measurements. The results of such experiments are shown in Fig. 6. Inhibition was maximum 10 s after the addition of fMLP, and then it decreased with time, 50% recovery being reached between 1 and 2 min after the addition of fMLP. The time course of the inhibitory effect of fMLP was also tested directly on Ca\(^{2+}\) entry. Fig. 7 shows the effect of fMLP on the increase in [Ca\(^{2+}\)] induced by the addition of 1 mM Ca\(^{2+}\) to thapsigargin-treated cells incubated previously in 0.1 mM Ca\(^{2+}\). Ca\(^{2+}\) entry was completely prevented 5–10 s after the addition of fMLP, and then it recovered with time. In similar experiments (not shown), PDB also inhibited the [Ca\(^{2+}\)], rise induced by addition of 1 mM Ca\(^{2+}\) to thapsigargin-treated cells. Inhibition by PDB was complete 10 s after its addition, and no recovery was observed for at least 5 min. PAF did not reveal any inhibitory effect in similar experiments (results not shown).

Fig. 8 shows the concentration dependence of the inhibition by fMLP and PDB of the Mn\(^{2+}\) entry. With fMLP, the inhibition was complete at 100 nM and half-maximal at about 10 nM. With PDB, inhibition was complete at 10 nM and half-maximal at about 1 nM. When [Ca\(^{2+}\)], was kept at lower levels (thapsigargin-treated cells suspended in 0.1 mM Ca\(^{2+}\), [Ca\(^{2+}\)], = 170–230 nM), the inhibitory effects were smaller, but the concentration dependence was similar (results not shown).

To test for the possible involvement of protein kinase C in the inhibition of SOCP, we have investigated the effects of the protein kinase C inhibitor staurosporin on the fMLP- and PDB-induced inhibition of the increase in [Ca\(^{2+}\)], in experiments similar to those of Fig. 7. Fig. 9 shows that 100 nM staurosporin prevented the inhibition of the [Ca\(^{2+}\)], increase by PDB but blocked only partially the inhibition induced by fMLP. Similarly, 100 nM staurosporin prevented the inhibition of Mn\(^{2+}\) entry in thapsigargin-treated cells by PDB but had only a partial effect on the inhibition produced by fMLP (results not shown).

Inhibition of SOCP by fMLP in cells not treated with thapsigargin was investigated in the experiments shown in Fig. 10. Mn\(^{2+}\) was used to trace activation of SOCP, which was achieved by PAF. PAF produces a Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores, which is followed by an increased plasma membrane permeability (5). PAF accelerated the entry of Mn\(^{2+}\) after a small delay, corresponding to the time required to empty the intracellular Ca\(^{2+}\) stores. This delay was very short in experiments of Fig. 10, because the cells had been first incubated in medium containing only 0.2 mM Ca\(^{2+}\) in order to obtain a submaximal filling of the stores, but this delay would be considerably larger in cells first incubated with

\[\text{[Ca}^{2+}]_{i} \text{(nM)} \]

\[\text{Time (s)}\]

\[\text{[Ca}^{2+}]_{i} \text{ (nM)} \]

\[\text{fMLP-induced Inhibition of Ca}^{2+} \text{ Entry} \]

\[\text{FIG. 3. Comparison of the effects of fMLP and PDB on the} \]

\[\text{ionomycin-induced [Ca}^{2+}]_{i} \text{ peak in Ca}^{2+}-\text{free medium. Pura-2-}

\[\text{loaded cells were incubated in 1 mM Ca}^{2+}-\text{containing medium and the}

\[\text{[Ca}^{2+}]_{i}\]

\[\text{were added (see also Table I). Inhibition was also tested directly on Ca}^{2+}\text{ entry. Fig. 7 shows the}

\[\text{effect of fMLP on the increase in [Ca}^{2+}] \text{ induced by the addition}

\[\text{of 1 mM Ca}^{2+}\] to thapsigargin-treated cells incubated previously in

\[\text{0.1 mM Ca}^{2+}. \text{Ca}^{2+} \text{ entry was completely prevented 5–10 s}

\[\text{after the addition of fMLP, and then it recovered with time. In}

\[\text{similar experiments (not shown), PDB also inhibited the [Ca}^{2+}],

\[\text{rise induced by addition of 1 mM Ca}^{2+}\] to thapsigargin-

\[\text{treated cells. Inhibition by PDB was complete 10 s after its}

\[\text{addition, and no recovery was observed for at least 5 min. PAF}

\[\text{did not reveal any inhibitory effect in similar experiments}

\[\text{(results not shown).}\]

\[\text{Fig. 8 shows the concentration dependence of the inhibition}

\[\text{by fMLP and PDB of the Mn}^{2+} \text{ entry. With fMLP, the}

\[\text{inhibition was complete at 100 nM and half-maximal at about}

\[\text{10 nM. With PDB, inhibition was complete at 10 nM and}

\[\text{half-maximal at about 1 nM. When [Ca}^{2+}], was kept at lower}

\[\text{levels (thapsigargin-treated cells suspended in 0.1 mM Ca}^{2+}, [Ca}^{2+}],

\[\text{= 170–230 nM), the inhibitory effects were smaller, but the}

\[\text{concentration dependence was similar (results not shown).}\]

\[\text{To test for the possible involvement of protein kinase C in}

\[\text{the inhibition of SOCP, we have investigated the effects of the}

\[\text{protein kinase C inhibitor staurosporin on the fMLP- and}

\[\text{PDB-induced inhibition of the increase in [Ca}^{2+}], in}

\[\text{experiments similar to those of Fig. 7. Fig. 9 shows that 100 nM}

\[\text{staurosporin prevented the inhibition of the [Ca}^{2+}], increase}

\[\text{by PDB but blocked only partially the inhibition induced by}

\[\text{fMLP. Similarly, 100 nM staurosporin prevented the inhibition}

\[\text{of Mn}^{2+} \text{ entry in thapsigargin-treated cells by PDB but}

\[\text{had only a partial effect on the inhibition produced by fMLP}

\[\text{(results not shown).}\]

\[\text{Inhibition of SOCP by fMLP in cells not treated with}

\[\text{thapsigargin was investigated in the experiments shown in}

\[\text{Fig. 10. Mn}^{2+} \text{ was used to trace activation of SOCP, which}

\[\text{was achieved by PAF. PAF produces a Ca}^{2+} \text{ release from the}

\[\text{intracellular Ca}^{2+} \text{ stores, which is followed by an increased}

\[\text{plasma membrane permeability (5). PAF accelerated the entry of}

\[\text{Mn}^{2+}\] after a small delay, corresponding to the time required}

\[\text{to empty the intracellular Ca}^{2+} \text{ stores. This delay was very}

\[\text{short in experiments of Fig. 10, because the cells had been}

\[\text{first incubated in medium containing only 0.2 mM Ca}^{2+}

\[\text{in order to obtain a submaximal filling of the stores, but this}

\[\text{delay would be considerably larger in cells first incubated with}

\[\text{We have show before (5) that small concentrations of ionomycin}

\[\text{permeabilize much more strongly the endomembranes (releasing Ca}^{2+}

\[\text{to the cytosol) than the plasma membrane. Most of Ca}^{2+} \text{ exit from}

\[\text{the cells is mediated by the active extrusion mechanisms, not by}

\[\text{the ionophore. Complete permeabilization of the intracellular stores by}

\[\text{200 nM ionomycin was indicated by the observation that addition of}

\[\text{1 mM ionomycin 1 min after adding 200 nM ionomycin did not induce}

\[\text{any increase in the [Ca}^{2+}], in cells suspended in Ca}^{2+}-\text{free medium}

\[\text{(results not shown).}\]
fMLP-induced Inhibition of Ca\(^{2+}\) Entry

**TABLE I**

| [Ca\(^{2+}\)]\(_i\), nM | Percent inhibition | Time (s) |
|------------------------|-------------------|----------|
|                        | Control + fMLP    | Control + PDB |
| 0                     | 15 ± 2            | 8 ± 1     |
| 0.1                   | 55 ± 7            | 48 ± 7    |
| 1                     | 633 ± 3           | 68 ± 6    |

**FIG. 4.** Effects of fMLP and PDB on the uptake of Mn\(^{2+}\) by thapsigargin-treated neutrophils. Fura-2-loaded cells were treated with 0.5 μM thapsigargin for 10 min either in nominally Ca\(^{2+}\)-free medium (left panel), 0.1 mM Ca\(^{2+}\)-containing medium (center panel), or 1 mM Ca\(^{2+}\)-containing medium (right panel). Then either 0.2 mM Mn\(^{2+}\) (traces labeled Control) or 0.2 mM Mn\(^{2+}\) + 1 μM fMLP (traces labeled +fMLP, upper panels) or 0.2 mM Mn\(^{2+}\) + 100 nM PDB (traces labeled +PDB, lower panels) were added. The [Ca\(^{2+}\)]\(_i\), just before Mn\(^{2+}\) addition are indicated at the top of each panel. The uptake of Mn\(^{2+}\) was followed from the quenching of fura-2 fluorescence excited at 360 nm. In similar experiments in which fMLP or PDB was added 10 s before Mn\(^{2+}\), the inhibition of Mn\(^{2+}\) entry was already near maximal from the beginning at [Ca\(^{2+}\)]\(_i\) = 172 nM but increased only slightly at [Ca\(^{2+}\)]\(_i\) = 55 nM (results not shown). Traces shown are representative of three similar experiments (inhibition percentages shown in Table I).

**FIG. 5.** Effect of fMLP on the uptake of Ba\(^{2+}\) by thapsigargin-treated human neutrophils. Fura-2-loaded cells incubated either in nominally Ca\(^{2+}\)-free medium (left panel) or in 0.1 mM Ca\(^{2+}\)-containing medium (right panel) were treated with 0.5 μM thapsigargin for 10 min. the [Ca\(^{2+}\)]\(_i\), just before Ba\(^{2+}\) addition are indicated at the top of each panel. At t = 0 in the figure, 10 mM Ba\(^{2+}\) was added either to control cells or to cells incubated with 1 μM fMLP, as indicated, and the increase in fura-2 fluorescence excited at 360 nm was recorded. Traces shown are representative of two similar experiments.

**FIG. 6.** Time dependence of the inhibition of Mn\(^{2+}\) entry by thapsigargin-treated cells by fMLP. Fura-2-loaded cells incubated in 1 mM Ca\(^{2+}\)-containing medium were treated with 0.5 μM thapsigargin for 10 min. Then, 1 μM fMLP was added either 10 s, 30 s, 1 min, or 2 min before the addition of 0.2 mM Mn\(^{2+}\), as indicated in the figure. The trace labeled Control shows the effect of the addition of 0.2 Mn\(^{2+}\) in the absence of fMLP. The uptake of Mn\(^{2+}\) was followed from the quenching of fura-2 fluorescence excited at 360 nm.

1 mM Ca\(^{2+}\) (3). The addition of fMLP together with PAF produced an additional delay of the start of the PAF-induced acceleration of Mn\(^{2+}\) entry. This effect of fMLP was concentration-dependent, half-maximal inhibition being reached between 4 and 10 nM. Since fMLP itself mobilizes Ca\(^{2+}\) and accelerates Mn\(^{2+}\) entry (4, 5), it should in principle cooperate with and make faster the effect of PAF. Therefore, the results shown in Fig. 10 strongly support that fMLP induces an early and transient inhibition of SOCP, which prevents the effects of PAF during the first 1–2 min of incubation.

Further evidence for this hypothesis was provided by the experiment shown in Fig. 11. We show that PAF induces a long-lasting increase of [Ca\(^{2+}\)]\(_i\) (upper panel), which typically shows secondary peaks dependent on the entry of extracellular Ca\(^{2+}\) (4, 5). Simultaneous addition of fMLP and PAF induced a [Ca\(^{2+}\)]\(_i\) transient whose width was less than half that obtained with PAF alone. This inhibitory effect was observed
fMLP-induced Inhibition of Ca\(^{2+}\) Entry

**Fig. 7.** Effects of fMLP on the [Ca\(^{2+}\)]\(i\) increase induced by addition of Ca\(^{2+}\) to thapsigargin-treated cells. Fura-2-loaded cells incubated in 0.1 mM Ca\(^{2+}\)-containing medium were treated with 0.5 \(\mu\)M thapsigargin for 10 min, and the [Ca\(^{2+}\)]\(i\) was monitored. Then either 1 mM Ca\(^{2+}\) (A), 1 mM Ca\(^{2+}\) together with 1 \(\mu\)M fMLP (B), 1 \(\mu\)M fMLP followed 10 s later by 1 mM Ca\(^{2+}\) (C), or 1 \(\mu\)M fMLP followed 1 min later by 1 mM Ca\(^{2+}\) (D) were added. Traces shown are representative of three similar experiments.

**Fig. 8.** Effects of different concentrations of fMLP and PDB on the uptake of Mn\(^{2+}\) induced by thapsigargin-treated human neutrophils. Fura-2-loaded cells were incubated with 0.5 \(\mu\)M thapsigargin for 10 min in 1 mM Ca\(^{2+}\)-containing medium. At \(t = 0\) in the figure, 3.2 mM Mn\(^{2+}\) was added either to control cells, to cells treated for 10 s with different concentrations of fMLP as indicated (upper panel), or to cells treated for 30 s with different concentrations of PDB as indicated (lower panel). The uptake of Mn\(^{2+}\) was monitored by the quenching of fura-2 fluorescence excited at 360 nm. Traces shown are representative of two similar experiments.

**Fig. 9.** Comparison of the effect of staurosporin on the inhibition by fMLP and PDB of the [Ca\(^{2+}\)]\(i\) increase induced by addition of Ca\(^{2+}\) to thapsigargin-treated cells. Fura-2-loaded cells incubated in 0.1 mM Ca\(^{2+}\)-containing medium were treated with 0.5 \(\mu\)M thapsigargin for 10 min, and the [Ca\(^{2+}\)]\(i\) was monitored. At \(t = 0\) in the figure, 1 mM Ca\(^{2+}\) was added either to control cells, to cells treated for 20 s with 100 nM PDB (upper panel, PDB), to cells treated for 10 s with 20 \(\mu\)M fMLP (lower panel, fMLP), or to cells preincubated with 100 nM staurosporin for 5 min before addition of either 100 nM PDB (20 s before Ca\(^{2+}\)) or 20 \(\mu\)M fMLP (10 s before Ca\(^{2+}\)), as indicated (PDB+st or fMLP+st, respectively). With larger concentrations of fMLP (50 and 100 nM) the effect of staurosporin preventing inhibition of the [Ca\(^{2+}\)]\(i\) increase was still evident but smaller (results not shown).

**Fig. 10.** Effects of fMLP on the uptake of Mn\(^{2+}\) induced by PAF. Fura-2-loaded cells were incubated in 0.2 mM Ca\(^{2+}\)-containing medium. 0.2 mM Mn\(^{2+}\) was then added, and 1 min afterwards (\(t = 0\) in the figure, shown by the arrow), either 36 nM PAF (A), 36 nM PAF + 4 nM fMLP (B), 36 nM PAF + 10 nM fMLP (C), 36 nM PAF + 100 nM fMLP (D), or 36 nM PAF + 1 \(\mu\)M fMLP (E) were added. The uptake of Mn\(^{2+}\) was followed from the quenching of fura-2 fluorescence excited at 360 nm.

**DISCUSSION**

It is widely known that fMLP activates Ca\(^{2+}\) influx from the extracellular medium in human neutrophils, and there is strong evidence that this activated Ca\(^{2+}\) influx is a consequence of the emptying of the intracellular Ca\(^{2+}\) stores (5, 6). We report here that the fMLP-induced activation of Ca\(^{2+}\) influx through SOCP is preceded by a transient inhibition contributing to delay the onset of activated Ca\(^{2+}\) entry. This inhibition is maximum 10 s after the addition of 1 \(\mu\)M fMLP and gradually decreases with a half-time of about 1 min. fMLP-induced stimulation of Ca\(^{2+}\) (Mn\(^{2+}\)) entry (as a consequence of the emptying of the Ca\(^{2+}\) stores) becomes...
phor-mediated emptying of the Ca2+ stores and activation of induced Ca2+ (Mn2+) entry (see Figs. 10 and 11). Refilling of enter and refill the stores. This is why fMLP is able to induce gradually apparent as inhibition is relieved, allowing Ca2+ to concentrations of fMLP Fura-2-loaded cells were incubated in 1 mM Ca2+-containing medium, may therefore reduce the [Ca2+]i increase in the ionophore-free MLP on the [Ca2+]i increase, which activates the plasma membrane Ca2+ pump. The effects cannot be explained by inhibition of the ionophoric ionomycin (14, 15). Stimulation of 46Ca loss by fMLP could such activation, at least within the initial 30 s after the addition of any of these agents. Previous evidence for activation of Ca2+ efflux by fMLP relies on the stimulation by fMLP of 46Ca loss by phorbol esters has also been reported (16, 17), even though phorbol esters do not increase [Ca2+]. This effect, however, was only evident about 2 min after the addition of the phorbol ester, whereas PDB-induced inhibition of Ca2+/Mn2+ uptake starts in less than 10 s (see Fig. 4, and Table I).

The fMLP-induced early inhibition of the SOCP is very much paralleled by the phorbol ester PDB, suggesting the involvement of protein kinase C in the mechanism of the inhibition. Both agents, fMLP and PDB, inhibited Ca2+ entry with a delay of 10 s or less (Figs. 2 and 4 and Table I), in both cases the inhibition was strongly dependent on [Ca2+], (Fig. 4 and Table I), and the decrease of [Ca2+]i, in thapsigargin-treated cells took place at the same rate for both agents (see Fig. 2). Inhibition by fMLP was transient, while that induced by PDB was permanent, as it would be expected for a non-metabolizable protein kinase C activator. Another important difference was the smaller sensitivity to staurosporin of the fMLP-induced inhibition, as compared with that induced by PDB. A similar discrepancy had been reported for the decrease of [Ca2+]i, induced by fMLP and PDB in ionomycin-treated neutrophils (14, 15). The different sensitivity to this inhibitor may be due to a different mechanism for activation of protein kinase C in both cases. If, as suggested above, fMLP-induced inhibition of SOCP was mediated by protein kinase C, the inability of PAF to produce such inhibition might seem rather puzzling. PAF is able to produce inositol 1,4,5-trisphosphate (28) and to release Ca2+ from the intracellular stores more strongly than fMLP does (5). Then, if activation of protein kinase C were due to diacylglycerol derived from phophatidylinositol 4,5-bisphosphate by activation of phospholipase C, PAF should be at least as strong as fMLP to inhibit SOCP. An alternative view would be that the activation of protein kinase C produced by fMLP in human neutrophils took place by a different pathway, perhaps through phospholipase D (29) and that PAF were unable to activate this pathway.

It has been recently reported that thrombin inhibits Ca2+ entry induced by emptying the Ca2+ stores of endothelial cells with histamine or ionomycin (13). This effect seems rather similar to the fMLP-induced inhibition of SOCP reported here. However, several differences exist that make it difficult to decide at present whether both effects share a common mechanism. In the first place, phorbol esters do not modify Ca2+ entry in endothelial cells, and staurosporin does not affect the thrombin-induced inhibition of Ca2+ entry (13). Additionally, the thrombin-induced effect is much more long lasting than the effect of fMLP; no signs of recovery were still detected 5 min after the addition of thrombin (13). Despite these differences, phenomenological similarities between the effects of thrombin in endothelial cells and of fMLP in neutrophils suggests that agonist-induced inhibition of Ca2+ entry may be a more general phenomenon involved in Ca2+ homeostasis.

The physiological significance of the fMLP-induced inhibition of Ca2+ entry could be to prevent coincidence between the Ca2+ mobilization and the Ca2+ influx induced by the emptying of the stores, allowing for a fast return of [Ca2+], to basal levels. The subsequent delayed activation of Ca2+ influx would then allow refilling of the intracellular Ca2+ stores, leaving the cell ready to respond to a new stimulus. Alternatively, inhibition of SOCP might allow the cell to distinguish between agonists that induce initially only mobilization of stored Ca2+ and a late Ca2+ influx, such as fMLP, and agonists

* M. Montero, J. Garcia-Sancho, and J. Alvarez, unpublished results.
that induce almost simultaneously Ca\(^{2+}\) mobilization and Ca\(^{2+}\) influx, such as PAF. In the first case, the [Ca\(^{2+}\)]\(_{\text{i}}\) transient is narrow and is followed by a late elevated [Ca\(^{2+}\)]\(_{\text{i}}\) level; in the second case, the [Ca\(^{2+}\)]\(_{\text{i}}\) transient is much wider (4, 5) (see also Figs. 1A and 11). fMLP could also act by moderating the [Ca\(^{2+}\)]\(_{\text{i}}\) transients induced by PAF or other agonists.

Acknowledgments—We thank Jesús Fernandez for excellent technical assistance and the University Hospital Blood Bank for blood supply.

REFERENCES

1. Pozzan, T., Lew, D. P., Wollheim, C. B. & Tsien, R. Y. (1983) Science 221, 1413-1415
2. Andersson, T., Dahlgren, C., Pozzan, T., Stendahl, O. & Lew, D. P. (1986) Mol. Pharmacol. 30, 437-443
3. Korchak, H. M., Rutherford, L. E. & Weissmann, G. (1984) J. Biol. Chem. 259, 4070-4075
4. Merritt, J. E., Jacob, J. & Hallam, T. J. (1989) J. Biol. Chem. 264, 1522-1527
5. Monoero, M., Alvarez, J. & Garcia-Sancho, J. (1991) Biochem. J. 277, 73-79
6. Montero, M., Alvarez, J. & Garcia-Sancho, J. (1992) Biochem. J. 288, 519-525
7. Putney, J. W., Jr. (1986) Cell Calcium 7, 1-12
8. Putney, J. W., Jr. (1990) Cell Calcium 11, 611-624
9. Di Virgilio, F., Lew, P. D., Andersson, T. & Pozzan, T. (1987) J. Biol. Chem. 262, 4574-4579
10. Hoth, M. & Penner, R. (1992) Nature 355, 353-356
11. Thastrup, O., Foder, B. & Scharff, O. (1987) Biochem. Biophys. Res. Commun. 142, 664-669
12. Kass, G. E. N., Duddy, S. K., Moore, G. A. & Orrenius, S. (1989) J. Biol. Chem. 264, 15192-15198
13. Nefy, C. E. & Irvine, R. F. (1991) J. Biol. Chem. 266, 4251-4256
14. Periasin, A. & Snyderman, R. (1989) J. Biol. Chem. 264, 1006-1009
15. McCarthy, S. A., Hallam, T. J. & Merritt, J. E. (1989) Biochem. J. 254, 357-364
16. Lagast, H., Pozzan, T., Waldvogel, F. A. & Lew, P. D. (1984) J. Clin. Invest. 73, 878-883
17. Rickard, J. E. & Sheterline, P. (1985) Biochem. J. 231, 623-628
18. Lytton, J., Westlin, M. & Hanley, M. R. (1991) J. Biol. Chem. 266, 17067-17071
19. Takemura, H., Hughes, A. R., Thastrup, O. & Putney, J. W., Jr. (1989) J. Biol. Chem. 264, 12266-12271
20. Goldstein, I. M., Brai, M., Oster, A. G. & Weissmann, G. (1973) J. Immunol. 111, 3-17
21. Gronkiewicz, G., Poenie, M. & Tsien, R. (1986) J. Biol. Chem. 260, 3440-3450
22. Alonso, M. T., Sanchez, A. & Garcia-Sancho, J. (1990) Biochem. J. 272, 435-443
23. Kwan, C. Y. & Putney, J. W., Jr. (1990) J. Biol. Chem. 265, 678-684
24. Llopa, J., Chow, S. B., Kass, G. E. N., Gahm, A. & Orrenius, S. (1991) Biochem. J. 277, 553-556
25. Llopa, J., Kass, G. E. N., Gahm, A. & Orrenius, S. (1992) Biochem. J. 284, 243-247
26. Clementi, E., Scheer, H., Zacchetti, D., Fusolato, C., Pozzan, T. & Meldolesi, J. (1992) J. Biol. Chem. 267, 2164-2172
27. Sage, S. O., Sargent, P., Merritt, J. E., Mahaut-Smith, M. P. & Rink, T. J. (1992) Biochem. J. 285, 341-343
28. Naccache, P. H., Molaki, M. M., Volpi, M., Becker, E. L. & Sha'afi, R. I. (1985) Biochem. Biophys. Res. Commun. 130, 677-684
29. Geiss, P., von Schinnerer, V., Record, M., Bagnolimin & Chap, H. (1992) Biochem. J. 287, 67-72