This study addresses the role of store-operated \(Ca^{2+}\) influx in the regulation of exocytosis in inflammatory cells. In HL-60 granulocytes, which do not possess voltage-operated \(Ca^{2+}\) channels, the chemotactic peptide N-formyl-methionyl-leucyl-phene (fMLP) was able to stimulate store-operated \(Ca^{2+}\) influx and to trigger exocytosis of primary granules. An efficient triggering of exocytosis by fMLP required the presence of extracellular \(Ca^{2+}\) and was inhibited by blockers of store-operated \(Ca^{2+}\) influx. However, receptor-independent activation of store-operated \(Ca^{2+}\) influx through thapsigargin did not trigger exocytosis. fMLP was unable to stimulate exocytosis in the absence of cytosolic free \(Ca^{2+}\) concentration \([Ca^{2+}]_c\) elevations. However, a second signal generated by fMLP synergized with store-operated \(Ca^{2+}\) influx to trigger exocytosis and led to a left shift of the exocytosis/[\(Ca^{2+}]_c\] relationship in ionomycin-stimulated cells. The synergistic fMLP-generated signaling cascade was long-lasting, involved a pertussis toxin-sensitive G protein and a phosphatidylinositol 3-kinase. In summary, store-operated \(Ca^{2+}\) influx is crucial for the efficient triggering of exocytosis in HL-60 granulocytes, but, as opposed to \(Ca^{2+}\) influx through voltage-operated \(Ca^{2+}\) channels in neurons, it is not a sufficient stimulus by itself and requires synergistic receptor-generated signals.

Many types of inflammatory cells, in particular granulocytes, possess secretory granules that contain microbiidal and proinflammatory substances. Exocytosis of these granules plays an important role not only in the host defense, but also in the development of inflammatory tissue damage. Thus, understanding the regulation of granule release from these cells is crucial for the understanding of the inflammatory response. However, while research over the last years has greatly advanced our knowledge about the mechanisms of exocytosis in neuronal cells, the mechanisms of exocytosis in inflammatory cells are still poorly understood. Exocytosis in neurons is intimately linked to the activation of voltage-operated \(Ca^{2+}\) channels (1). A low affinity \(Ca^{2+}\)-sensitive step in the exocytotic machinery of neurons (EC\(_{50}\) up to 200 \(\mu\)M in the synapse) has been demonstrated. It is thought that only \(Ca^{2+}\) influx through \(Ca^{2+}\) channels is able to achieve submembraneous \([Ca^{2+}]_c\) which are sufficiently high to activate this low affinity step (2, 3). In contrast to excitatory cells, inflammatory cells secrete in response to receptor activation, not membrane depolarization. Voltage-activated \(Ca^{2+}\) channels are absent in inflammatory cells. Previous reports suggested that the cytosolic free \(Ca^{2+}\) concentration ([\(Ca^{2+}]_c\)] induces exocytosis in inflammatory cells with a high affinity (EC\(_{50}\) 0.5–3 \(\mu\)M in human neutrophils) (4). Thus, at this point there is no compelling evidence that \(Ca^{2+}\) influx through ion channels plays the same role in exocytosis by inflammatory cells as it does in excitable cells.

Inflammatory cells, however, do have plasma membrane \(Ca^{2+}\) channels, and \(Ca^{2+}\) influx occurs in response to stimulation with receptor agonists (5). The predominant type of \(Ca^{2+}\) influx in inflammatory cells is the so-called store-operated \(Ca^{2+}\) influx (or capacitative \(Ca^{2+}\) entry) (6), and the underlying channels are referred to as store-operated \(Ca^{2+}\) channels (or \(I_{\text{CRAC}}\) channels) (7). As opposed to voltage-operated \(Ca^{2+}\) channels, stored-operated \(Ca^{2+}\) channels are found in virtually all cell types, and their most obvious function is the refilling of intracellular \(Ca^{2+}\) stores after stimulated \(Ca^{2+}\) release. In neuronal cells, it has been suggested that store-operated \(Ca^{2+}\) channels are important for refilling of \(Ca^{2+}\) stores but do not play a relevant role in shaping the \([Ca^{2+}]_c\) transients during cell activation. In inflammatory cells however, store-operated \(Ca^{2+}\) influx is likely to play an important role for cell activation, with the best documented example being lymphocyte proliferation (8). The role of store-operated \(Ca^{2+}\) influx in the exocytotic secretion by inflammatory cells has hitherto received little attention. It has previously been noted that the \(Ca^{2+}\) sensitivity of exocytosis in inflammatory cells is modulated by other agonist-generated signals (4). However, the identity of these signals and their relationship to store-operated \(Ca^{2+}\) influx have not been revealed.

In this study we demonstrate that store-operated \(Ca^{2+}\) influx plays a premier role in the regulation of receptor-stimulated exocytosis in HL-60 granulocytes. However, as opposed to the situation in neuronal cells and as opposed to previous results obtained with \(Ca^{2+}\) ionophores in granulocytes, even maximal activation of store-operated \(Ca^{2+}\) influx is not sufficient to induce exocytosis in HL-60 granulocytes by itself, and synergistic signals generated through receptor activation are required for exocytosis to occur. The synergistic signaling cascade involves pertussis toxin sensitive G proteins and a phosphatidylinositol 3-kinase (PI 3-kinase).

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\(^{1}\) Abbreviations used are: \([Ca^{2+}]_c\), cytosolic free \(Ca^{2+}\) concentration; boc-MLP, \(N\)-tert-butoxycarbonyl-methionyl-leucyl-phenylalanine; fMLP, formyl-methionyl-leucyl-phenylalanine; HL-60, human leukemia cell line 60; LY294,002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PI 3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLD, phospholipase D; SK&F96365, 1-[(β3-(4-methoxyphenyl)propoxy]-4-methoxyphenethenyl)-1H-imidazole hydrochloride; GTPγS, guanosine 5′-O-(thiotriphosphate).
EXPERIMENTAL PROCEDURES

Materials—Cell culture media were obtained from Life Technologies, Inc. (Paisley, Scotland), H-platelet-activating-factor from Amersham (Little Chalfont, UK), silica plates from Merck (Darmstadt, Germany), erastinan analog (methyl 2,5-dihydroxycinnamate) and LY294,002 (2,4-morpholiny1)-8-phenyl-1H-1-benzopyran-4-one) from Alexis (Laulsfenningen, Switzerland), U73122 and U73342 from Calbiochem, and fura-2 AM from Molecular Probes (Eugene, OR). SK&F96365 (1-(β-[4-(3 equivalents), and 1 unit/ml horseradish peroxidase, 50 μM HEPES, pH 7.4. The free Ca2+ concentration of this medium was about 5 μM as determined with a Ca2+ sensitive electrode (9). The “Ca2+ free medium” consisted of Ca2+ free medium supplemented with 1 mM CaCl2.

Cell Culture—HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and l-glutamine (2 mM). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO2, 95% air. The culture was passaged twice weekly, and granulocytic differentiation was initiated by addition of dimethyl sulfoxide (final concentration 1.3% for 3 days, then 0.65% for 1 or 2 days).

Measurement of Exocytosis—2.5 × 106 cells were suspended in 2.4 ml of the indicated medium including 5 μM Cytochalasin B. Fura-2 fluorescence was recorded using an excitation wavelength of 380 nm and an emission wavelength of 500 nm.

RESULTS

Store-operated Ca2+ Influx Is Necessary, but Not Sufficient, to Mediate the fMLP Activation of Primary Granule Release in HL-60 Granulocytes—We first studied the effect of extracellular Ca2+ on the [Ca2+]i, signal and on primary granule release in response to the chemotactic peptide fMLP (for the definition of granule subpopulations in neutrophil granulocytes, see Borregaard et al. (16)). In the absence of extracellular Ca2+, the [Ca2+]i signal showed a marked decrease in duration, but only a slight decrease in amplitude (Fig. 1, A and C). The initial rapid rise from basal [Ca2+]i, ~100 nM to over 1 μM, was the result of Ca2+ release from intracellular stores. In Ca2+-containing medium, the initial peak was followed by a prolonged influx of Ca2+ from the extracellular medium. In the absence of extracellular Ca2+, only the release from intracellular stores occurred. Peak [Ca2+]i was reached about 15 s after stimulation in both conditions. fMLP stimulated exocytosis was completed in 30 s. The amplitude of exocytosis was four times higher in the presence of extracellular Ca2+ (Fig. 1B), whereas peak [Ca2+]i, was only 25% higher (Fig. 1C). Thus Ca2+ influx was necessary for efficient triggering of exocytosis. To further study the importance of Ca2+ influx for fMLP-induced exocytosis, we analyzed the effect of inhibitors of Ca2+ influx in granulocytes (6, 17). Two classes of inhibitors, trivalent metal ions and imidazole derivatives, reduced exocytosis to levels that were obtained in the absence of extracellular Ca2+ (Fig. 1D).

Thus, Ca2+ influx is a necessary signal for fMLP-stimulated exocytosis in HL-60 granulocytes. However, is it also sufficient to induce exocytosis? To separate the effect of fMLP on [Ca2+]i, from its effect on other intracellular signals, we designed protocols to control [Ca2+]i, independently of receptor stimulation. We used thapsigargin, an inhibitor of the Ca2+-ATPase that provokes release of Ca2+ from intracellular stores (18). Inhibiting the refilling of the stores (18). The empty stores generate a yet unknown signal that activates store-operated Ca2+ channels in the plasma membrane (19). These channels are the physiological pathway for Ca2+ entry after fMLP stimulation (6). Without extracellular Ca2+, thapsigargin induced a slow, transient elevation of [Ca2+]i, which returned to baseline levels within 5 min (Fig. 2A). Because thapsigargin acts irre-
versibly, the stores can not be refilled, and the plasma membrane Ca\(^{2+}\) channels remain permanently activated. Thus, subsequent addition of Ca\(^{2+}\) to the extracellular medium caused a sustained Ca\(^{2+}\) influx, and [Ca\(^{2+}\)], reached high plateau levels (Fig. 2, A and B). Despite its essential role in

MLP-stimulated exocytosis (see above), store-operated Ca\(^{2+}\) influx did not efficiently trigger exocytosis by itself (Fig. 2B, right side, compared with Fig. 1B). Addition of fMLP did not alter [Ca\(^{2+}\)]\(_i\) in this protocol (Fig. 2, C and D). 5 min after thapsigargin treatment, the stores were almost empty, and fMLP induced only minimal Ca\(^{2+}\) release. Under these conditions, fMLP was unable to stimulate exocytosis underlining the essential role of Ca\(^{2+}\) in fMLP-stimulated exocytosis (Fig. 2D, center). However, together with store-operated Ca\(^{2+}\) influx, fMLP markedly stimulated exocytosis (Fig. 2D, right side, compared with B, right side). Thus, fMLP activates additional signaling pathways that synergize with [Ca\(^{2+}\)]\(_i\) elevations.

**In Contrast to Store-operated Ca\(^{2+}\) Influx, Ionophore-induced Ca\(^{2+}\) Elevations Stimulate Primary Granule Release**—The inefficiency of thapsigargin-induced Ca\(^{2+}\) influx to stimulate exocytosis contrasts with the well known stimulation of neutrophil exocytosis by the Ca\(^{2+}\) ionophore ionomycin (see, for example, Lew et al. (10)). To investigate this discrepancy, we stimulated HL-60 granulocytes with either ionomycin or thapsigargin in the presence of increasing extracellular Ca\(^{2+}\) concentrations and measured [Ca\(^{2+}\)]\(_i\) elevations (Fig. 3A), exocytosis (Fig. 3B), and superoxide production (Fig. 3C). The results show that the Ca\(^{2+}\) ionophore raised average [Ca\(^{2+}\)]\(_i\), to levels substantially higher than thapsigargin-induced store-operated Ca\(^{2+}\) influx. They also show that the Ca\(^{2+}\) ionophore induced substantial exocytosis, while thapsigargin, even at the highest extracellular Ca\(^{2+}\) concentration tested, did not trigger exocytosis. However, the lack of thapsigargin-induced exocytosis cannot solely be explained by the lower average [Ca\(^{2+}\)]\(_i\), levels. Indeed, when average [Ca\(^{2+}\)]\(_i\), values of ~500 nM were obtained with ionomycin, significant exocytosis occurred, while store-operated Ca\(^{2+}\) influx did not induce exocytosis even when it raised average [Ca\(^{2+}\)]\(_i\), levels above 1 \(\mu\)M. The absence of exocytosis in thapsigargin-stimulated cells was not due to an inhibition of the exocytotic machinery through thapsigargin, as costimulation of cells with thapsigargin did not diminish fMLP-induced exocytosis (data not shown). To better understand the discrepancy between the thapsigargin effect and ionomycin effect, we studied O\(_2^−\) generation under the same experimental conditions (Fig. 3C). Both thapsigargin and ionomycin were able to stimulate O\(_2^−\) generation in the presence of extracellular Ca\(^{2+}\). However, the extracellular Ca\(^{2+}\) concentrations necessary to induce maximal O\(_2^−\) generation were much lower for ionomycin than for thapsigargin. This was particularly striking with 0.2 mM extracellular Ca\(^{2+}\), where ionomycin induced maximal O\(_2^−\) generation, while thapsigargin had a very small effect. Thus, even under conditions where the fura-2 measurements did not reveal differences in thapsigargin- and ionomycin-stimulated average [Ca\(^{2+}\)]\(_i\), elevations, the measurements of Ca\(^{2+}\)-dependent O\(_2^−\) generation clearly suggests that ionomycin leads to higher [Ca\(^{2+}\)]\(_i\) at the site of Ca\(^{2+}\) action. This discrepancy most likely reflects a different spatial organization of the thapsigargin- and the ionomycin-induced Ca\(^{2+}\) signal (see “Discussion”). Note also the inhibition of the ionomycin-induced O\(_2^−\) generation by increasing Ca\(^{2+}\) concentrations. This probably is an in vivo reflection of the previously described inhibition of the assembled NADPH oxidase activity in membrane preparations by high Ca\(^{2+}\) concentrations (20).

The fMLP-generated Signaling Pathway That Acts in Synergy with Store-operated Ca\(^{2+}\) Influx Is Long Lasting and Requires Continuous Receptor Occupation—It appears that fMLP is able to activate additional signals at resting [Ca\(^{2+}\)]\(_i\), (Fig. 2) and that these signals have a synergistic action when store-operated Ca\(^{2+}\) influx is allowed to occur. To investigate the kinetics of these synergistic signals, we used the Ca\(^{2+}\) store.
depletion protocol as outlined above (Fig. 2), varied the delay between fMLP and Ca\(^{2+}\) readdition from 0 to 15 min, and compared exocytosis and Ca\(^{2+}\) influx (Fig. 4A). The level of [Ca\(^{2+}\)], that was reached under these conditions was similar at all time points of Ca\(^{2+}\)-addition, albeit a small transient decrease during the first minutes after fMLP was observed. Exocytosis was highest, when Ca\(^{2+}\) and fMLP were given at the same time (0 min). When Ca\(^{2+}\) was added 30 s to 2 min after fMLP, exocytosis was reduced by 50%. With longer delays between fMLP stimulation and Ca\(^{2+}\) addition, exocytosis reached almost the same level as at time 0. This suggests at least three possible explanations: (i) the synergistic signaling pathway has complex kinetics, including a transient inactivating component; (ii) there are several distinct synergistic signals with different kinetics; and (iii) the small transient decrease in average [Ca\(^{2+}\)], reflects a large decrease in submembraneous [Ca\(^{2+}\)], which accounts for the large decrease in exocytosis. Given the possibility that the submembraneous [Ca\(^{2+}\)] rather than the average cellular [Ca\(^{2+}\)] is crucial for the induction of exocytosis, we favor the last possibility.

The fMLP receptor is known to be rapidly inactivated upon ligand binding (21). It was therefore surprising to see that, under our experimental conditions, the synergistic fMLP-stimulated signals were maintained for as long as 15 min. To understand whether this long lasting effect was due to a long lasting receptor activity, or rather the persistence of activated downstream signals, we studied the effect of the fMLP antagonist N-tert-butoxycarbonyl-methionyl-leucyl-phenylalanine, boe-MLP (Fig. 4B). Experiments were performed as shown in Fig. 2; however, where indicated, boe-MLP was added to the medium 3 min after the addition of fMLP. As shown in Fig. 4B, exocytosis was almost completely inhibited by boe-MLP. Thus, the long lasting fMLP effect to synergize with store-operated Ca\(^{2+}\) influx was due to a long lasting receptor/ligand interaction and not to the persistence of activated downstream signals.

The Synergistic Signaling Pathway Involves a Pertussis Toxin-sensitive G Protein—We then investigated the nature of the synergistic signal by generating steady-state [Ca\(^{2+}\)], between 100 nm and 10 \(\mu\)M with low levels of ionomycin (0.5 \(\mu\)M) in the presence of 0–2 \(\mu\)M extracellular Ca\(^{2+}\). For any given average [Ca\(^{2+}\)], value from 100 nm to 10 \(\mu\)M, fMLP enhanced exocytosis (Fig. 5A). A fit of the data in Fig. 5A with a logistic equation suggested that fMLP enhanced both maximal exocytosis (\(Y_{\text{max}}\): 18.2 ± 0.9 to 24.2 ± 0.5% release in the absence and presence of fMLP, respectively) and its apparent affinity for [Ca\(^{2+}\)], (\(EC_{50}\) for average [Ca\(^{2+}\)], values: 1000 ± 150 nm and 670 ± 50 nm in the absence and presence of fMLP, respectively). This suggests that the role of the synergistic signals generated by fMLP is not only an enhancement of exocytosis, but also a shift of its Ca\(^{2+}\) sensitivity to [Ca\(^{2+}\)], values that can be achieved by physiological Ca\(^{2+}\) influx. However, while a shift of the Ca\(^{2+}\) sensitivity can be reliably detected with the fura-2 method, the discrepancy between the submembraneous [Ca\(^{2+}\)], values (seen by the exocytotic machinery) and the average [Ca\(^{2+}\)], values (measured by the fluorescent dye) precludes calculations of the absolute values of the Ca\(^{2+}\) sensitivity of the exocytotic process (see also Fig. 3). Loading of cells with GTPyS (for details, see Jaconi et al. (14)) induced an increase in the amplitude and the Ca\(^{2+}\) sensitivity of the ionomycin-induced exocytosis similar as seen for fMLP (Fig. 5, A and B). This suggests that GTP-dependent processes are involved in the second signal pathway. To understand whether these GTP-dependent processes act along the same synergistic signaling pathway as fMLP, we investigated whether fMLP stimulation of GTPyS-loaded cells leads to a further enhancement of exocytosis. As shown as in Fig. 5C, no additivity between fMLP and GTPyS was detectable compatible with the concept that both act along the same pathway. To date most, if not all, fMLP-activated signaling cascades were found to be mediated by pertussis toxin-sensitive G proteins, presumably by members of the \(G_i\) family of heterotrimeric G proteins. To analyze whether such proteins might be involved in the activation of the synergistic signal by fMLP, we investigated the effect of pertussis toxin on the synergistic signal generated by fMLP. A complete suppression of the synergistic signal was observed (Fig. 5D). Taken together, these results suggest that fMLP
stimulates a synergistic signal via a pertussis toxin-sensitive G protein to increase the amplitude and the affinity of Ca\(^{2+}\)-activated exocytosis.

**Store-operated Ca\(^{2+}\) Influx Is Sufficient to Activate Phospholipase D**—Phospholipase D (PLD) has been implicated in the regulation of exocytosis in neutrophils (22). To investigate, whether PLD activation could account for the synergistic signaling pathway, we investigated activation of the enzyme under our experimental conditions (Fig. 6). As described above, cells were similarly suspended in Ca\(^{2+}\)-free medium and store-depleted by the addition of thapsigargin (0 min), followed by addition of fMLP or Me\(_2\)SO (5 min). Under these conditions, fMLP did not stimulate PLD activity, demonstrating that there is an absolute requirement for [Ca\(^{2+}\)]\(_c\) elevations in the fMLP stimulation of PLD activity. When store-operated Ca\(^{2+}\) influx was allowed to occur (1 mM CaCl\(_2\); 10 min), a significant increase of PLD activity was observed, both in the absence (6-fold) or in the presence (12-fold) of fMLP (Fig. 6A). Thus, store-operated Ca\(^{2+}\) influx by itself is a good activator of PLD. Ethanol is an inhibitor of PLD-mediated signaling. Pretreatment with ethanol diminished both fMLP- and ionomycin-induced exocytosis in a dose-dependent manner (Fig. 6C). Thus, PLD activity may have a role downstream from store-operated Ca\(^{2+}\) influx. A major role of PLD in the synergistic signals elicited by fMLP as shown in Fig. 2 appears less likely but cannot be excluded. There might be a high threshold (\(>6\%\)).
phosphatidylethanol per total lipid, Fig. 6A) above which PLD activity could synergize with Ca\(^{2+}\) to induce exocytosis.

**Evidence for the Involvement of PI-3 Kinase, but Not Phospholipase C in the Synergistic Signaling Pathway**—A phosphatidylinositol bisphosphate-specific phospholipase C (PLC) is activated by fMLP stimulation of granulocytes via pertussis toxin-sensitive G proteins. The PLC reaction generates inositol 1,4,5-trisphosphate, which is the mediator of the Ca\(^{2+}\) signal. In addition, PLC also generates the protein kinase C activator diacylglycerol and diminishes the phosphatidylinositol bisphosphate content of the plasma membrane (although the latter effect is relatively small and short lived) (23). Thus, PLC could also be involved in the synergistic signaling pathway. We therefore investigated the effect of the phospholipase C inhibitor U73122 (24). As expected, U73122 inhibited fMLP-induced [Ca\(^{2+}\)] \(_i\) increase with an IC\(_{50}\) of 250 nM, while the thapsigargin-induced [Ca\(^{2+}\)] \(_i\) increase was not affected (Fig. 7B). Next, we investigated whether U73122 had an additional effect on fMLP-induced exocytosis, independent of the inhibition of [Ca\(^{2+}\)] \(_i\) increase. To obtain the obligatory rise in [Ca\(^{2+}\)], even in the presence of PLC inhibitor, the cells were pretreated with thapsigargin in the presence of 1 mM Ca\(^{2+}\) and then stimulated with fMLP (Fig. 7A). Under these conditions, U73122 did inhibit exocytosis. However, as (i) exocytosis was less sensitive to U73122 than was the [Ca\(^{2+}\)] \(_i\), increase (IC\(_{50}\), 754 ± 104 nM versus 256 ± 11 nM, \(p < 0.01\), for exocytosis and [Ca\(^{2+}\)] \(_i\), respectively), and (ii) ionomycin-induced exocytosis was also affected, the inhibitory effect of U73122 on exocytosis is probably unrelated to PLC inhibition.

The tyrosine kinase inhibitor genistein moderately reduced fMLP- but not ionomycin-induced exocytosis at higher concentrations (Fig. 8A). However, at these concentrations genistein also reduced fMLP-mediated Ca\(^{2+}\) release and Ca\(^{2+}\) influx (Fig. 8B; inhibition of Ca\(^{2+}\) influx by genistein has been reported previously (25)). Another tyrosine kinase inhibitor, the erbstatin analog methyl 2,5-dihydroxycinnamate, did not inhibit fMLP-induced exocytosis (with 1, 3, and 10 \(\mu\)M erbstatin analog exocytosis was at 95.3 ± 3.0%, 89.5 ± 7.8%, and 93.9 ± 5.0% of the control level, mean ± S.E. of four determinations from two independent experiments).

Wortmannin is a potent inhibitor of PI 3-kinases and of granulocyte superoxide generation (26). Fig. 9A shows that wortmannin inhibited fMLP- but not ionomycin-induced exocytosis. The inhibition of fMLP-induced exocytosis was incomplete (maximal inhibition 62 ± 4%; however, its affinity was relatively high (IC\(_{50}\) 70 nM), compatible with an effect on a PI-3-kinase. No relevant effects of wortmannin on Ca\(^{2+}\) release and Ca\(^{2+}\) influx were observed. These results would be compatible with an involvement of PI-3 kinase in the synergistic signaling pathway, as wortmannin inhibited fMLP-induced exocytosis, but neither ionomycin-induced exocytosis nor fMLP-induced [Ca\(^{2+}\)] \(_i\), elevations. To obtain additional evidence, we tested a second PI-3-kinase inhibitor, namely LY294,002, which inhibits the enzyme with 250-fold lower potency (27). LY294,002 also inhibited fMLP-induced exocytosis (10, 30, and 100 \(\mu\)M LY294,002 reduced exocytosis to 76.5 ± 5.2%, 58.3 ± 6.9%, and 46.4 ± 15.4% of control, mean ± S.E. of four determinations from two independent experiments). Thus, taken together, our results suggest that PI 3-kinase activation is a part of the synergistic signaling pathway toward granulocyte exocytosis. Ptasznik et al. (28) recently reported that tyrosine kinases were involved in the stimulation of PI 3-kinases by fMLP in neutrophils. The inhibition of exocytosis at high genistein concentrations (see Fig. 8), might therefore involve an effect on tyrosine kinase-mediated PI 3-kinase activation.

**DISCUSSION**

Our results demonstrate that store-operated Ca\(^{2+}\) influx plays a key role in the regulation of exocytosis of primary granules in HL-60 granulocytes. Thus, as seen in neuronal cells, there is a similar tight correlation between Ca\(^{2+}\) channel activation and activation of exocytosis.

**Why Is Store-operated Ca\(^{2+}\) Not Sufficient to Activate Exocytosis?**—Unlike the situation in neuronal cells, Ca\(^{2+}\) influx through Ca\(^{2+}\) channels is by itself not sufficient to induce exocytosis in HL-60 granulocytes. Nevertheless, exocytosis of primary granules has been activated by Ca\(^{2+}\) alone in ionomycin-stimulated intact granulocytes (29), in permeabilized gran-
ulocytes (30, 31), as well as in whole cell patch-clamped granulocytes. A high [Ca^{2+}]_c affinity of primary granule release (EC_{50} ≈ 2.6 μM) has been determined using the “ionophore clamp” technique in combination with fluorescence measurements of average [Ca^{2+}]_c values (4). Our ionophore-clamp experiments (Fig. 5A) yield similar results; however, the data obtained with thapsigargin-induced Ca^{2+} influx are not compatible with such a high affinity for Ca^{2+}. In digitonin-permeabilized and in electroperemeabilized cells, a much higher [Ca^{2+}]_c (between 10 and 100 μM) was needed to achieve primary granule release (31, 32), and in patch-clamp studies pipette Ca^{2+} concentrations up to 4 μM were unable to induce substantial exocytosis (33). Thus, most likely, the Ca^{2+} affinity of primary granule exocytosis in granulocytes is underestimated by the ionophore experiments. In addition, [Ca^{2+}]_c elevations at the site of exocytosis achieved through store-operated Ca^{2+} influx in granulocytes are probably lower than those achieved through voltage-operated Ca^{2+} influx in neurons. When normalized for the cell surface, peak Ca^{2+} currents through store-operated channels in inflammatory cells are at least 10 times smaller than currents through voltage-operated Ca^{2+} channels in excitable cells (current density ~1–3 pA/pF in inflammatory cells (8, 34), versus 30–90 pA/pF in excitable cells (35, 36)). The requirement for additional signals that activate exocytosis in synergy with store-operated Ca^{2+} influx may be a direct consequence of the relatively low amplitude of submembrane Ca^{2+} increases generated through store-operated Ca^{2+} influx.

2 O. Nüsse, manuscript in preparation.

How Can the Difference in the Ionomycin and the Thapsigargin Response be Explained?—Both thapsigargin and ionomycin stimulate store operated Ca^{2+} influx by emptying intracellular Ca^{2+} stores. In addition, ionomycin induces Ca^{2+} influx through ionophore pores in the plasma membrane. This difference is witnessed by the saturation of the steady-state [Ca^{2+}]_c levels with increasing extracellular [Ca^{2+}] in thapsigargin-treated, but not ionomycin-treated, cells (Fig. 3A).

Ionomycin, but not thapsigargin, stimulated exocytosis, and ionomycin activated a maximal respiratory burst response at a 10 times lower extracellular Ca^{2+} concentration than did thapsigargin. Thus ionomycin appears to induce much higher Ca^{2+} concentrations at the site of Ca^{2+} action (i.e. the submembrane space) than does thapsigargin. These local Ca^{2+} elevations are poorly reflected by the average [Ca^{2+}]_c, measurements probably because (i) the submembraneal space represents a minor portion of the cellular volume and (ii) fura-2 is already saturated below the [Ca^{2+}]_c values that can be reached in the submembrane space.

At least two mechanisms might contribute to the generation of much higher submembrane Ca^{2+} concentrations by ionomycin compared with thapsigargin: (a) ionomycin insertion into the plasma membrane and (b) Ca^{2+} buffering by a ionomycin-sensitive, thapsigargin-resistant Ca^{2+} store. High capacity low affinity Ca^{2+} stores that might buffer [Ca^{2+}]_c, elevations in a thapsigargin-resistant fashion include mitochondria (37), and recently described endoplasmatic reticulum subcompartments (38).

Properties of the Synergistic Signals—The synergistic signals enhance exocytosis and promote a left-shift of the [Ca^{2+}]_c sensitivity of exocytosis. Such a shift through receptor agonists or various pharmacological activators has been observed in intact cells (Fig. 5C) (4), as well as in permeabilized cells (39, 40).

Our results do not favor a role of phospholipase C in the synergistic signaling pathway. Phospholipase D could have a role when its activated above a high threshold. Previous studies have already argued against a role of protein kinase C enzymes in this context, as primary granules release in response to fMLP is not inhibited by various protein kinase C inhibitors and activation of protein kinase C by phorbol ester does not induce primary granule release (41, 42).

fMLP receptor-dependent signaling has been shown to occur in many instances through pertussis toxin-sensitive G proteins. However, biochemical analysis has also suggested that the fMLP receptor may interact with low molecular weight (i.e. pertussis-toxin insensitive) GTP-binding proteins (43). Small GTP-binding proteins are thought to be involved in the regulation of exocytosis in various cellular systems (44). Our results (Fig. 5) show that the activation of pertussis toxin-sensitive G

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**FIG. 8.** The effect of the tyrosine kinase inhibitor genistein on fMLP stimulated Ca^{2+} rise and exocytosis. HL-60 cells were preincubated for 3 min with 3 to 100 μM genistein before stimulation with fMLP (1 μM) or ionomycin (1 μM). A, exocytosis was measured 5 min after stimulation and is shown as mean ± S.E. of four determinations from two independent experiments. B, fMLP-induced Ca^{2+} release was measured in Ca^{2+}-free medium and Ca^{2+} influx was measured by Ca^{2+} readdition (1 mM CaCl₂) 5 min after fMLP. Basal [Ca^{2+}]_c, was subtracted, mean ± range, n = 2, is shown. Results in A and B are shown as percent of control (i.e. no genistein).

**FIG. 9.** The effect of the PI 3-kinase inhibitor wortmannin on fMLP stimulated Ca^{2+} rise and exocytosis. HL-60 cells were preincubated with 1 nM to 10 μM wortmannin for 7 min before stimulation with fMLP (1 μM) or ionomycin (1 μM) in Ca^{2+}-containing medium. A, exocytosis was measured 5 min after stimulation. The results were fitted with a logistic equation (dotted line). B, fMLP-induced Ca^{2+} release was measured in Ca^{2+}-free medium and Ca^{2+} influx was measured by Ca^{2+} readdition (1 mM CaCl₂) 5 min after fMLP. Basal [Ca^{2+}]_c, was subtracted. Results in A and B are shown as percent of control (i.e. no wortmannin).
proteins (presumably Gαq or Gα11) (22) is an obligatory step in the activation of the second signaling pathway. These results do not exclude the downstream involvement of other G proteins in the exocytotic process.

PI 3-kinases are likely to be involved in the regulation of exocytosis (26). This was supported by the recent observation that synaptotagmin, a potential Ca2+ sensor in neuronal exocytosis, is able to bind phosphatidylinositol 3,4,5-trisphosphate alone cannot fully account for the synergistic signal inhibitors did not exceed 60%. Therefore, activation of PI 3-kinase (70 nM for wortmannin, 15 μM for LY294002) is not sufficient stimulus by itself and requires the concomitant activation of synergistic signals to activate the exocytotic machinery. Our results suggest that PI 3-kinase is involved in the synergistic signaling pathway. However, maximal inhibition obtained through PI 3-kinase inhibitors did not exceed 60%. Therefore, activation of PI 3-kinases alone cannot fully account for the synergistic signal pathway and additional mechanisms are likely to be involved.

Relative Importance of the Ca2+ Influx, Ca2+ Release, and the Synergistic Signals in Different Cellular Systems—To our knowledge, this study is the first in-depth analysis of the role of store-operated Ca2+ influx in the regulation of exocytosis in granulocytes. In previous studies, the consequence of omission of extracellular Ca2+ during chemoattractant-stimulated exocytosis in granulocytes has varied from a minor reduction in exocytosis (26), to a profound inhibition (29, 47, 48). However, in all studies, chemoattractant stimulation of exocytosis was Ca2+-dependent. Thus, it appears that Ca2+ release from intracellular stores under certain circumstances substitute for Ca2+ influx. Two explanations may be considered. (i) Synergistic receptor-generated signals (e.g. priming) might lead to a more pronounced left-shift of the exocytosis/[Ca2+]i curve, and Ca2+ release from intracellular stores might then suffice to trigger exocytosis. (ii) Positioning of Ca2+ stores close the exocytotic machinery similar to their accumulation around phagosomes (49) might generate localized high [Ca2+]i by Ca2+ release.

In summary, store-operated Ca2+ influx is necessary for efficient stimulation of HL-60 exocytosis by fMLP, but it is not a sufficient stimulus by itself and requires the concomitant activation of synergistic signals to activate the exocytotic machinery. Our results suggest that the synergistic signaling pathway is initiated by the interaction of the fMLP receptor with a pertussis toxin-sensitive G protein and it involves PI 3-kinase activation of synergistic signals to activate the exocytotic machinery. Our results suggest that the synergistic signaling pathway is initiated by the interaction of the fMLP receptor with a pertussis toxin-sensitive G protein and it involves PI 3-kinase activity. The IC50 for the inhibition of exocytosis by the PI 3-kinase inhibitors wortmannin and LY294,002. The IC50 for the inhibition of exocytosis (70 nM for wortmannin, 15 μM for LY294,002) was in the range of PI 3-kinase inhibition. Thus, our results suggest that PI 3-kinase is involved in the synergistic signaling pathway. However, maximal inhibition obtained through PI 3-kinase inhibitors did not exceed 60%. Therefore, activation of PI 3-kinases alone cannot fully account for the synergistic signal pathway and additional mechanisms are likely to be involved.
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