Chemoattractant-induced respiratory burst: increases in cytosolic Ca^{2+} concentrations are essential and synergize with a kinetically distinct second signal

Reyhaneh FOYOUZI-YOUSSEFI*, Fredric PETERSSON, Daniel P. LEW, Karl-Heinz KRAUSE and Oliver NÜSSE
Division of Infectious Diseases, University Hospital, 1211 Geneva 14, Switzerland

The role of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) and its relationship to other second messengers in the signalling between chemoattractant [e.g. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)] receptors and the NADPH oxidase is still poorly understood. In this study, we have used thapsigargin, an inhibitor of the Ca^{2+}-ATPase of intracellular stores, as a tool to selectively manipulate Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane. We thereby temporarily separated the Ca^{2+} signal from other signals generated by fMLP and analysed the consequences on the respiratory burst. Under all conditions investigated, the extent of fMLP-induced respiratory burst activation was critically determined by $[Ca^{2+}]_c$ elevation. fMLP was unable to activate the respiratory burst without $[Ca^{2+}]_c$ elevation. Thapsigargin-induced Ca^{2+} influx activated the respiratory burst in the absence of fMLP, but only to approx. 20 % of the values observed in the presence of fMLP. The second signal generated by fMLP did not activate the respiratory burst by itself, but acted in synergy with $[Ca^{2+}]_c$ elevation. The second signal was long lasting (> 15 min) provided that there was no rise in $[Ca^{2+}]_c$ and that the receptor was continuously occupied. The second signal was inactivated by high $[Ca^{2+}]_c$ elevation. Our results demonstrate that $[Ca^{2+}]_c$ elevations are an essential step in the signalling between the fMLP receptor and NADPH oxidase. They also provide novel information about the properties of the second Ca^{2+}-independent signal that activates the respiratory burst in synergy with $[Ca^{2+}]_c$.

INTRODUCTION

Neutrophil granulocytes are able to produce reactive oxygen species via NADPH oxidase. This so-called respiratory burst is a crucial bactericidal mechanism, but also a major cause of pathological inflammation and tissue damage when inappropriately triggered. It can be elicited through a variety of stimuli, including chemoattractants. Chemoattractant receptors, such as the *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) receptor, elicit intracellular signalling through the activation of associated heterotrimeric G-proteins, which in turn activate enzyme effectors to generate intracellular messenger molecules. The fMLP receptor activates in a G-protein-dependent manner the phospholipase C {and thereby the Ins(1,4,5)P_3/cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) signalling system}, phosphatidylinositol 3-kinase (PI-3-kinase), and possibly also phospholipase A_2 and phospholipase D (reviewed in [1]).

The signalling steps from the fMLP receptor to the activation of the NADPH oxidase are still poorly understood. Ca^{2+} is thought to play a role, on the basis of the following experimental evidence: (i) the fMLP-induced respiratory burst is diminished in the absence of extracellular Ca^{2+} [2–4]; (ii) Ca^{2+} ionophores, albeit weak stimuli by themselves, can synergize with other agonists (e.g. exogenously added diacylglycerol [5], low PMA concentration [6] or granulocyte/macrophage colony-stimulating factor [7]) to activate the respiratory burst; (iii) the fMLP-induced respiratory burst is suppressed in Ca^{2+}-depleted neutrophils (i.e. neutrophils loaded with high concentrations of cell-permeant Ca^{2+} buffers in the absence of extracellular Ca^{2+} [6,8]).

However, none of these points conclusively resolves the role of $[Ca^{2+}]_c$ elevation in the fMLP-induced respiratory burst activation. The decreased respiratory burst in the absence of extracellular Ca^{2+} might hint at a modulatory rather than an essential role for $[Ca^{2+}]_c$, and the synergy with other agonists might be unrelated to fMLP receptor signal transduction. The strongest argument for Ca^{2+}-dependence of the respiratory burst comes from the Ca^{2+}-depleted cells. However, Ca^{2+}-depletion protocols (i) lower $[Ca^{2+}]_c$ to unphysiologically low values and therefore also block processes that do not require $[Ca^{2+}]_c$ elevation but simply depend on basal $[Ca^{2+}]_c$ (see also the Discussion), and (ii) depend on the use of high cytosolic concentrations of Ca^{2+} buffers, which are known to have considerable non-specific effects (see for example [9]). Finally, in human neutrophils, the effect of Ca^{2+} on the activation of the respiratory burst is at least partially due to exocytotic insertion of NADPH oxidase components from secondary granules into the plasma membrane. Thus the above observations might reflect $[Ca^{2+}]_c$-dependence of exocytosis rather than signalling NADPH oxidase activation [10–12].

In addition to a role for $[Ca^{2+}]_c$, previous studies suggested the presence of additional Ca^{2+}-independent signals generated by fMLP [2]. The precise nature of this second signal remains elusive; candidates include phospholipase D, phospholipase A_2, protein kinase C, tyrosine kinases and PI-3-kinase. Many of these signals are also $[Ca^{2+}]_c$-activated; however, for several of them at least, it appears that the fMLP activation occurs via other mechanisms in addition to $[Ca^{2+}]_c$. The arguments implicating or refuting a given signal in the respiratory burst activation rely heavily on the use of inhibitors and therefore remain ambiguous.

Abbreviations used: boc-MLP, *N*-t-butoxycarbonyl-L-methionyl-L-leucyl-L-phenylalanine; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HRP, horseradish peroxidase; L-NAME, N^G-L-nitroarginine methyl ester; PI-3-kinase, phosphatidylinositol 3-kinase; fura 2/AM, fura 2 acetoxymethyl ester.

* To whom correspondence should be addressed.

We have designed protocols that allow us (i) to selectively manipulate the amplitude of $[Ca^{2+}]_c$ elevations during fMLP stimulation of the respiratory burst, and (ii) to temporarily separate the $[Ca^{2+}]_c$ signal from the second fMLP-generated signal. Our results demonstrate that $[Ca^{2+}]_c$ elevations are essential in the activation of respiratory burst by fMLP. They also reveal novel and unexpected properties of the second fMLP-generated signal.

MATERIALS AND METHODS

Materials

fMLP, *N*-t-butoxycarbonyl-L-methionyl-L-leucyl-L-phenylalanine (boc-MLP), EGTA, luminol and horseradish peroxidase (HRP) type II were purchased from Sigma, thapsigargin and cell culture medium from Gibco (Paisley, Scotland), and fura 2 acetoxymethyl ester (fura 2/AM) from Molecular Probes (Eugene, OR, U.S.A.). N^G-l-Nitroarginine methyl ester (L-NAME) was from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade and were obtained from Sigma, Merck or Fluka. The medium referred to as 'Ca^{2+} medium' contained (in mM): NaCl 143; KCl 6; $CaCl_2$ 1; $MgSO_4$ 1; glucose 0.1 %; Hepes 20; pH 7.4. The 'Ca^{2+}-free medium' had the same ionic composition, but $CaCl_2$ was omitted. Since no Ca^{2+} chelator was added, this medium had a free Ca^{2+} concentration of approx. 5 μM [13].

Culture of HL-60 cells

The human promyelocytic cell line HL-60 was cultured in the tissue culture medium RPMI 1640 supplemented with 10 % fetal calf serum, penicillin (5 units/ml) and streptomycin (50 μg/ml). The cells were passaged twice per week and differentiated by adding DMSO (final concentration 1.3 %, v/v) to the cell suspension 5–6 days before the experiments.

Measurement of $[Ca^{2+}]_c$

$[Ca^{2+}]_c$ was measured with the fluorescent Ca^{2+} indicator fura 2. Cells (2×10^7/ml) suspended in Ca^{2+} medium containing 0.1 % BSA were loaded for 45 min at 37 °C with 2 μM fura 2/AM,

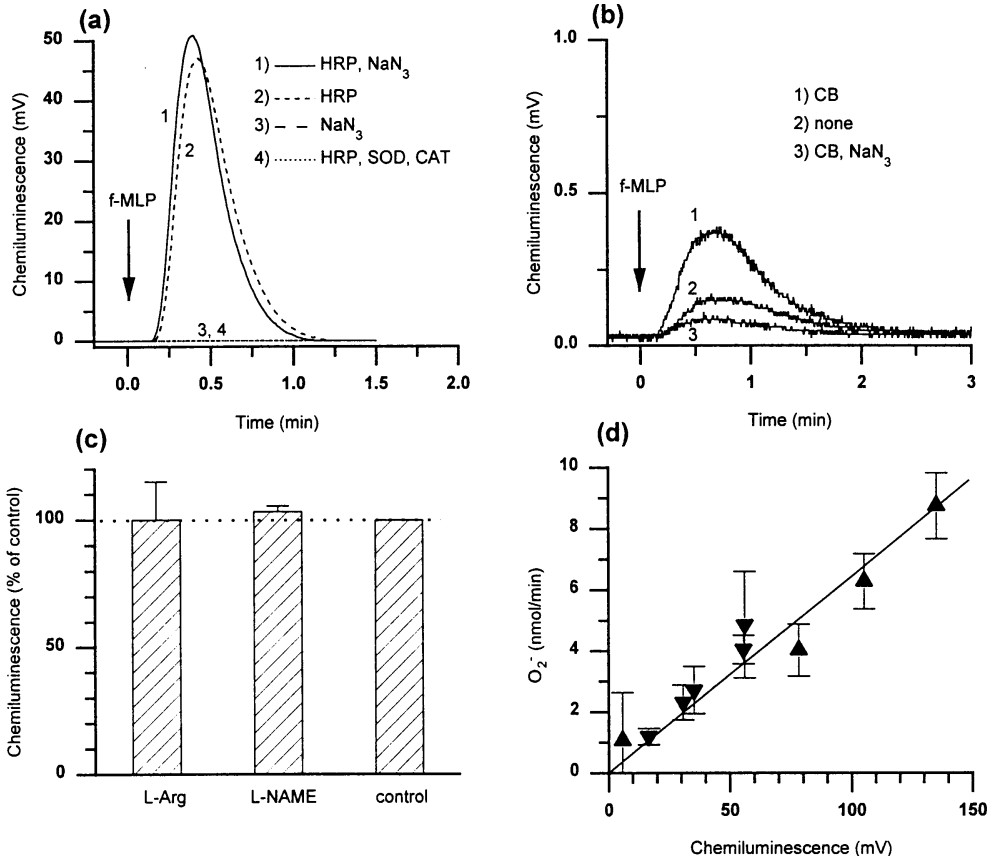

Figure 1 Specificity and calibration of the chemiluminescence signal

HL-60 neutrophils were stimulated with fMLP in Ca^{2+}-free medium and the respiratory burst was measured as luminol-enhanced chemiluminescence. (**a**) To determine the specificity and localization (intra- or extra-cellular) of the signal, four conditions were compared: (1) presence of 1 unit/ml HRP and 90 μM NaN_3 (standard conditions); (2) standard conditions without NaN_3; (3) no HRP but 90 μM NaN_3; (4) HRP, 50 units/ml superoxide dismutase (SOD), 2000 units/ml catalase (CAT). (**b**) Cells were pretreated with 5 μg/ml cytochalasin B (CB) for 5 min in Ca^{2+} medium. Respiratory burst was stimulated with fMLP with (3) and without (1) 90 μM NaN_3, no HRP. For comparison, cells stimulated without CB pretreatment are shown (2). (**c**) Chemiluminescence of fMLP-stimulated cells in standard conditions after pretreatment for 45 min with 300 μM L-arginine, 300 μM L-NAME or buffer only as control. Results are shown as means ± S.D. from two experiments. (**d**) HL-60 neutrophils at concentrations of (0.5–4) $\times 10^6$/ml were stimulated with fMLP in Ca^{2+} medium. The maximal rate of superoxide (O_2^-) production, calculated from cytochrome *c* reduction, was plotted against chemiluminescence. Results are shown as means ± S.D. from two determinations from two cell preparations (▲, ▼). The data were fitted by linear regression.

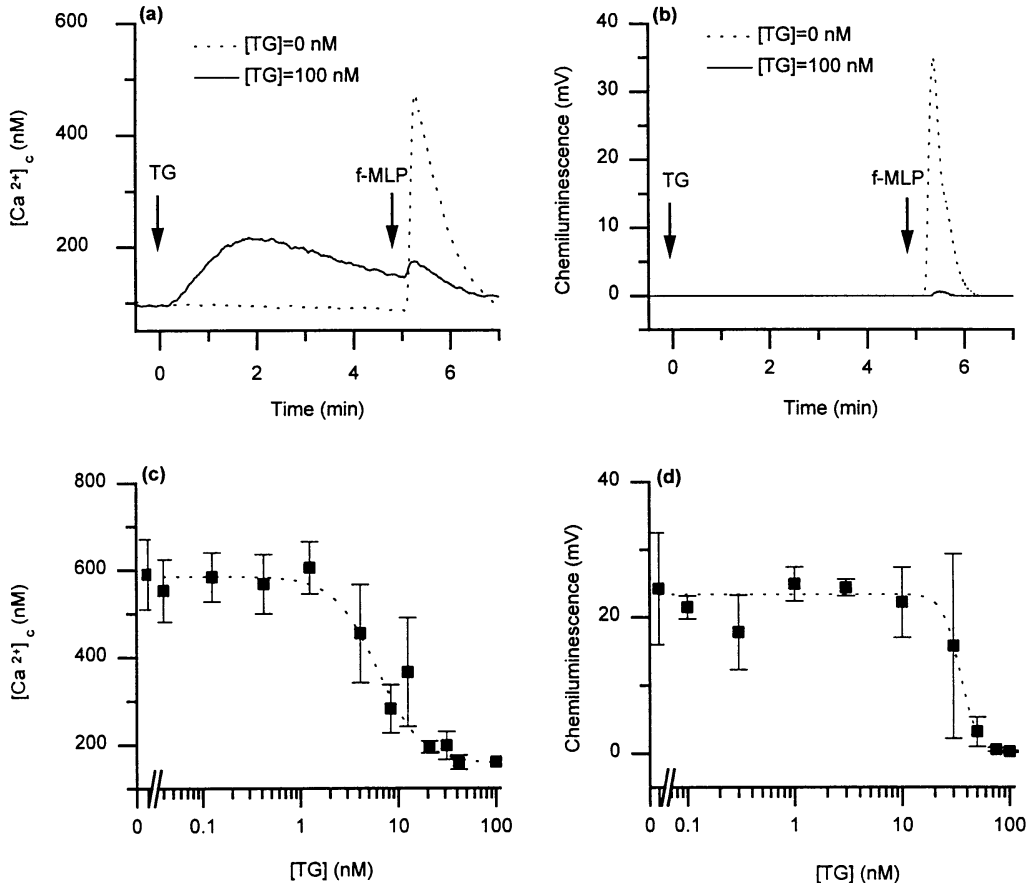

Figure 2 In the absence of Ca^{2+} influx, the amplitude of fMLP-induced Ca^{2+} release from intracellular stores critically determines the respiratory burst

HL-60 neutrophils were loaded with fura 2/AM and suspended in a Ca^{2+}-free medium without EGTA (i.e. in the absence of Ca^{2+} influx). $[Ca^{2+}]_c$ was measured as fura 2 fluorescence, and the respiratory burst as luminol-enhanced chemiluminescence. To modulate the amplitude of the fMLP-induced Ca^{2+} release, cells were incubated in the presence of various concentrations of thapsigargin (TG) for 5 min before stimulation with 1 μM fMLP. (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevation and superoxide production respectively. (**c**) and (**d**) Dose–response effect of thapsigargin pretreatment on peak $[Ca^{2+}]_c$ (mean $\pm$ S.D., $n = 2$) and on maximal chemiluminescence after fMLP stimulation (mean $\pm$ S.E.M., $n = 3$). A sigmoidal curve was fitted to the data [dotted lines in (**c**) and (**d**)].

then diluted to 10^7/ml and kept on ice. Just before use, a sample of loaded cells (2×10^6/ml) was centrifuged and resuspended in the indicated medium. Fluorescence measurements were performed on a Perkin–Elmer fluorimeter (LS3, Perkin–Elmer Cetus) maintained at 37 °C. Excitation and emission wavelengths were 340 and 505 nm respectively. Calibration was performed for each cuvette by sequential addition of 2 mM Ca^{2+} (for Ca^{2+}-free medium), 1 μM ionomycin to measure Ca^{2+}-saturated fura 2 (F_{max}), followed by 8 mM EGTA, 3 mM Tris (pH 9.3) and 0.1% Triton X-100 to measure Ca^{2+}-free fura 2 (F_{min}). Free Ca^{2+} concentrations were calculated from the fura 2 fluorescence (F) according to the formula of Grynkiewicz et al. [14]: $[Ca^{2+}]_c = K_d(F-F_{min})/(F_{max}-F)$, where $K_d = 224$ nM.

Chemiluminescence measurements

Respiratory burst activity was measured by chemiluminescence originating from HRP-catalysed luminol oxidation in a Wallac LKB Luminometer 1250. Cells (2×10^6/ml in Ca^{2+}-free medium supplemented with 1 unit/ml HRP, 50 μM luminol and 90 μM NaN_3) were preincubated at 37 °C before stimulation as indicated in the Figure legends. Chemiluminescence was measured continuously at 37 °C [15]. When superoxide dismutase (50 units/ml) and catalase (2000 units/ml) were added (in the absence of NaN_3), chemiluminescence was less than 2% of our standard conditions (fMLP, NaN_3 and HRP present) (Figure 1a). In addition, the chemiluminescence was dependent on HRP (Figure 1a). Without HRP (fMLP stimulation, NaN_3 present), chemiluminescence was less than 1% of the standard level. These results are in agreement with a previous report [16] that HL-60 cells exhibit only extracellular and no intracellular production of superoxide. When secretion of endogenous myeloperoxidase was stimulated with fMLP in the presence of cytochalasin B (5 μg/ml) in Ca^{2+} medium, we obtained maximal 20% HRP-independent chemiluminescence. However, more than 70% of this response was inhibited by 90 μM NaN_3 (Figure 1b). Thus, under our experimental conditions, the contribution of endogenous peroxidase activity to the chemiluminescence signal was negligible.

NO was shown to regulate the chemiluminescence from human neutrophils [17]. L-NAME is an inhibitor of NO synthase with an IC_{50} of 340 μM in human neutrophils [18]. In our experimental system, 300 μM L-NAME had no effect on the chemiluminescence (Figure 1c). L-arginine, the substrate of NO synthase which should increase NO production, was equally ineffective. Therefore NO synthesis appears to have no importance in chemiluminescence measurements.

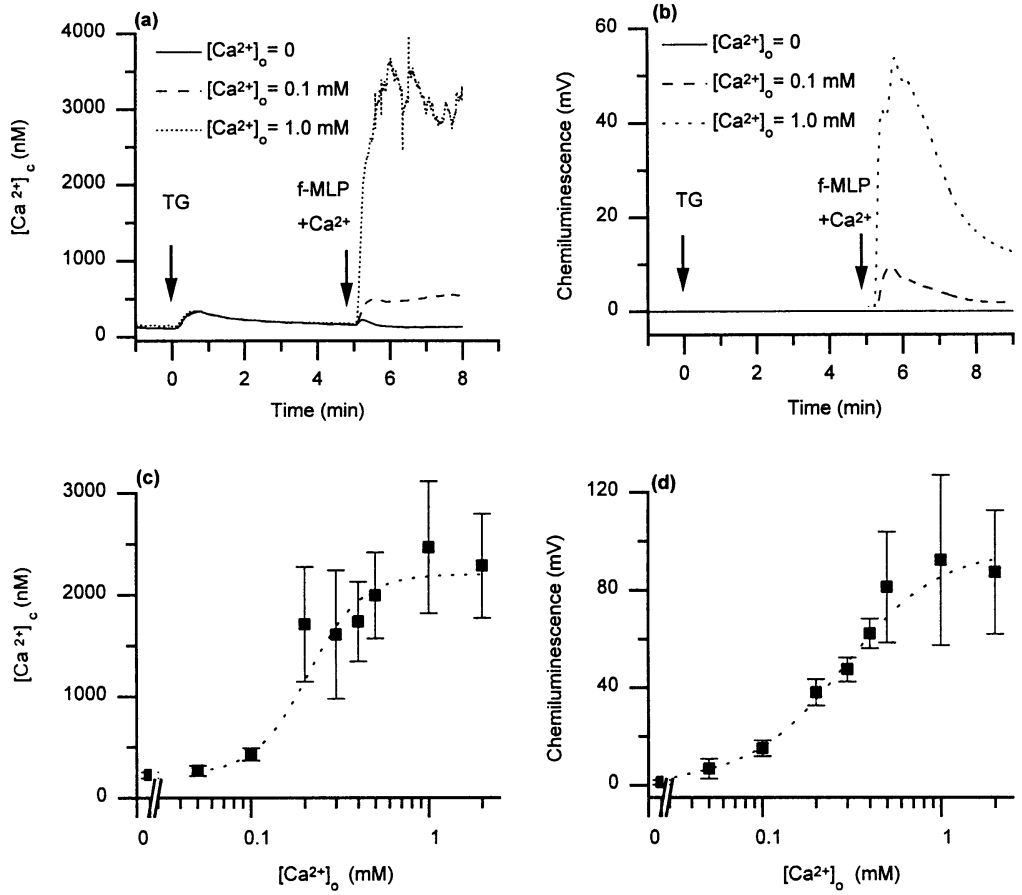

Figure 3 In the absence of Ca^{2+} release, Ca^{2+} influx across the plasma membrane is essential for the activation of the respiratory burst

HL-60 neutrophils were loaded with fura 2/AM and suspended in a Ca^{2+}-free medium. $[Ca^{2+}]_c$ was measured as fura 2 fluorescence, and the respiratory burst as luminol-enhanced chemiluminescence. To abolish fMLP-induced Ca^{2+} release completely, cells were preincubated with 100 nM thapsigargin (TG) for 5 min before addition of 1 μM fMLP and the indicated Ca^{2+} concentration to the extracellular medium. (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevation and superoxide production respectively. (**c**) and (**d**) Dose–response of extracellular Ca^{2+} on peak $[Ca^{2+}]_c$ and maximal chemiluminescence respectively (mean $\pm$ S.E.M. from three independent experiments). A sigmoidal curve was fitted to the data [dotted lines in (**c**) and (**d**)].

It was recently shown that two methods of superoxide measurement, luminol-dependent chemiluminescence and cytochrome *c* reduction, gave comparable results with activated neutrophils [19]. Thus the luminometer output in mV may be calibrated by a direct comparison of chemiluminescence and cytochrome *c* reduction. Different concentrations of differentiated HL-60 cells [$(0.5–4) \times 10^6$/ml] were stimulated with 1 μM fMLP in Ca^{2+} medium in the presence of 1 unit/ml HRP, 90 μM NaN_3 and either 150 μM cytochrome *c* or 50 μM luminol. The maximal rate of superoxide production was calculated from the increase in A_{550} on reduction of cytochrome *c* ($\Delta\epsilon_{550} = 21000\ cm^{-1} \cdot M^{-1}$) [20]. Maximal luminol-dependent chemiluminescence was measured under identical conditions. Superoxide production for each cell concentration from two cell preparations was plotted against chemiluminescence (Figure 1d). The slope 0.064 ± 0.003 nmol/(min $\times$ mV) of the linear fit ($r = 0.972$) may be used as a calibration factor for the chemiluminescence signals. In a typical experiment (Figure 5b), we obtained peak chemiluminescence values of 60 mV with 2×10^6 cells. According to our calibration, this corresponds to 1.92 nmol of superoxide/(10^6 cells $\times$ min). Similar values for superoxide generation from fMLP-stimulated HL-60 cells [0.75 and 1.24 nmol/(10^6 cells $\times$ min)] have been obtained with two different techniques [21,22]. The differences may reflect differences in this experimental protocols as well as the degree of differentiation of the cells.

Data analysis and fitting

For both $[Ca^{2+}]_c$ and chemiluminescence measurements, data were digitized at 2–10 Hz on an 8-bit A/D converter, and recorded on a PC-based computer using the acquisition software DaQSys (SICMU, Geneva, Switzerland). Data were then transferred to Origin (MicroCal, Southampton, Hants., U.K.). For presentation, $[Ca^{2+}]_c$ curves were filtered by adjacent averaging (25–49 points). Sigmoidal curves were fitted using logistic equations.

RESULTS

In this study, we have analysed the fMLP activation of the respiratory burst in HL-60 neutrophils using a quantitative chemiluminescence technique [15]. Given (i) its very high sensitivity and (ii) the fact that it measures the rate rather than the cumulative value of the respiratory burst, this chemiluminescence method turned out to be largely superior to the classical

cytochrome *c* reduction measurements for our quantitative analysis. The choice of HL-60 neutrophils, rather than peripheral blood neutrophils, was due to the following considerations: HL-60 neutrophils express functional fMLP receptors [23] and all the NADPH oxidase components required for respiratory burst activity [24]. As opposed to blood neutrophils, however, HL-60 neutrophils do not have secondary granules and therefore no intracellular pool of oxidase components that may be inserted into the plasma membrane through exocytosis. Thus analysis of the Ca^{2+}-sensitivity of respiratory burst activation in these cells will not have a superimposed contribution from exocytotic delivery of oxidase components.

In the first series of experiments, we analysed the role of extracellular Ca^{2+} in the fMLP activation of the respiratory burst in our experimental system. Stimulation of HL-60 neutrophils by the chemoattractant fMLP led to $[Ca^{2+}]_c$ elevations and respiratory burst activation, in both the presence and absence of extracellular Ca^{2+} (not shown). However, in the absence of extracellular Ca^{2+}, the duration (measured at half-maximal response) of $[Ca^{2+}]_c$ elevation and respiratory burst was shortened by $69 \pm 3\%$ (mean $\pm$ S.E.M., $n = 3$) and $55 \pm 21\%$ (mean $\pm$ S.E.M., $n = 5$) respectively. Similarly, in the absence of extracellular Ca^{2+}, the peaks of $[Ca^{2+}]_c$ elevation and superoxide production were diminished by $31 \pm 9\%$ (mean $\pm$ S.E.M., $n = 3$) and $43 \pm 7\%$ (mean $\pm$ S.E.M., $n = 5$) respectively. Thus the omission of extracellular Ca^{2+} led to a similar decrease in $[Ca^{2+}]_c$ elevation and respiratory burst. These results resemble previous observations in human neutrophils [2].

To investigate the contribution of Ca^{2+} release from internal stores to the respiratory burst activation in the absence of extracellular Ca^{2+}, we used thapsigargin, an inhibitor of the Ca^{2+}-ATPase of intracellular Ca^{2+} stores. Ca^{2+}-ATPase inhibition by thapsigargin causes a relatively slow dose-dependent release of Ca^{2+} from agonist-sensitive intracellular Ca^{2+} stores [25]. This is due to a leak of Ca^{2+} across the Ca^{2+} store membrane, which has to be compensated for by a permanent Ca^{2+}-ATPase activity [13]. When maximal concentrations of thapsigargin (100 nM) were added to HL-60 cells in the absence of extracellular Ca^{2+}, a relatively long-lasting $[Ca^{2+}]_c$ elevation of low amplitude (200–400 nM) was observed (Figure 2a). This thapsigargin-induced Ca^{2+} release did not lead to a detectable activation of the respiratory burst (Figure 2b). When fMLP was added 5 min after TG, the fMLP-induced Ca^{2+} release was almost completely abolished (Figure 2a, solid line). Thus agonist-sensitive Ca^{2+} stores were depleted by thapsigargin and therefore fMLP could not release any more Ca^{2+}. Under these conditions, fMLP was unable to activate the respiratory burst (Figure 2b). This suggests that, in the absence of extracellular Ca^{2+}, Ca^{2+} release from intracellular stores is a necessary signal for the activation of the respiratory burst. To study further the relationship between Ca^{2+} release and respiratory burst activation, we achieved partial emptying of agonist-sensitive Ca^{2+} stores through the use of submaximal thapsigargin concentrations. After the addition of thapsigargin at concentrations of less than 1 nM, cells showed no detectable $[Ca^{2+}]_c$ increase, and a subsequent stimulation with fMLP led to $[Ca^{2+}]_c$ elevation (Figure 2c) and to a respiratory burst activation (Figure 2d) similar to that seen in cells not exposed to thapsigargin. Thapsigargin concentrations between 1 and 50 nM caused a dose-dependent reduction in the amplitude of the fMLP-induced Ca^{2+} release (Figure 2c) and in the amplitude of the respiratory burst (Figure 2d). Thus, in the absence of Ca^{2+} influx, the respiratory burst showed a strict dependence on Ca^{2+} release from intracellular stores. In fact, only a small elevation in $[Ca^{2+}]_c$ was required to obtain superoxide production in fMLP-stimulated cells. Further $[Ca^{2+}]_c$ elevation between 350 nM and 600 nM did not increase the superoxide production any further. The shift between the dose–response curves to thapsigargin of $[Ca^{2+}]_c$ and superoxide production (Figures 2c and 2d respectively) reflects the non-linear Ca^{2+}-dependence of the superoxide production (see below).

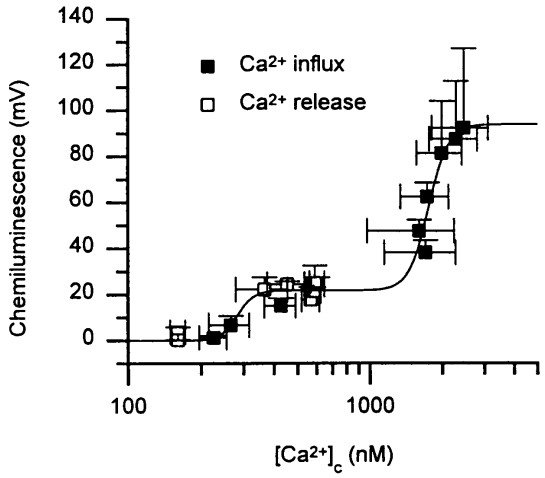

Figure 4 Apparent affinity of the respiratory burst activation for $[Ca^{2+}]_c$

The Ca^{2+}-dependence of superoxide production deduced from experiments shown in Figures 2 and 3. ■, Intracellular Ca^{2+} stores were depleted through a 5 min preincubation with 100 nM thapsigargin in the absence of extracellular Ca^{2+}; then 1 μM fMLP and various concentrations of extracellular Ca^{2+} (see Figure 3) were added. □, Stores were depleted with different concentrations of thapsigargin. Subsequent stimulation with fMLP caused release of remaining Ca^{2+} from the stores and stimulated the respiratory burst (Figure 2). The activation of the respiratory burst through a given extracellular Ca^{2+} concentration or after treatment with a given concentration of thapsigargin is plotted as a function of $[Ca^{2+}]_c$ achieved through the respective treatment. Data were fitted using a double logistic equation (——). The $[Ca^{2+}]_c$ necessary to obtain half-maximal activation of the first phase of the respiratory burst (EC_{50}-I) was 280 nM. The second phase showed an EC_{50}-II of 1760 nM $[Ca^{2+}]_c$.

The experiments shown in Figure 2 were performed in the absence of extracellular Ca^{2+}, and thus $[Ca^{2+}]_c$ changes could only occur through Ca^{2+} release from intracellular stores. The Ca^{2+}-ATPase inhibitor thapsigargin, however, does not only deplete intracellular Ca^{2+} stores, but also leads to the activation of the so-called store-operated Ca^{2+} influx. Store-operated Ca^{2+} influx, which is activated through the depletion of intracellular Ca^{2+} stores, is the predominant, if not the only, Ca^{2+} influx pathway in neutrophils [26]. Figure 3(a) shows this activation of Ca^{2+} influx through store depletion by thapsigargin. Ca^{2+} stores of HL-60 cells were depleted by incubation with 100 nM thapsigargin in a Ca^{2+}-free medium and after 5 min fMLP together with 0.05–2 mM Ca^{2+} was added to the extracellular medium. $[Ca^{2+}]_c$ increased immediately up to 2.5 μM as the result of maximal activation of the Ca^{2+}-influx pathway through store depletion. $[Ca^{2+}]_c$ elevations were a function of the Ca^{2+} concentration added to the extracellular medium (Figure 3c). The respiratory burst was measured under identical conditions. fMLP-stimulated superoxide production showed a strict correlation with the extracellular Ca^{2+} concentration (Figures 3b and 3d). In the absence of Ca^{2+} influx, no superoxide production occurred.

When the amplitude of the respiratory burst (derived from Figures 2d and 3d) is plotted against $[Ca^{2+}]_c$ elevation (derived from Figures 2c and 3c), the Ca^{2+}-dependence of the fMLP-activated respiratory burst can be clearly seen (Figure 4). Interestingly, a linear fit or a simple sigmoidal curve fits the data only poorly. In contrast, a fit allowing two superimposed

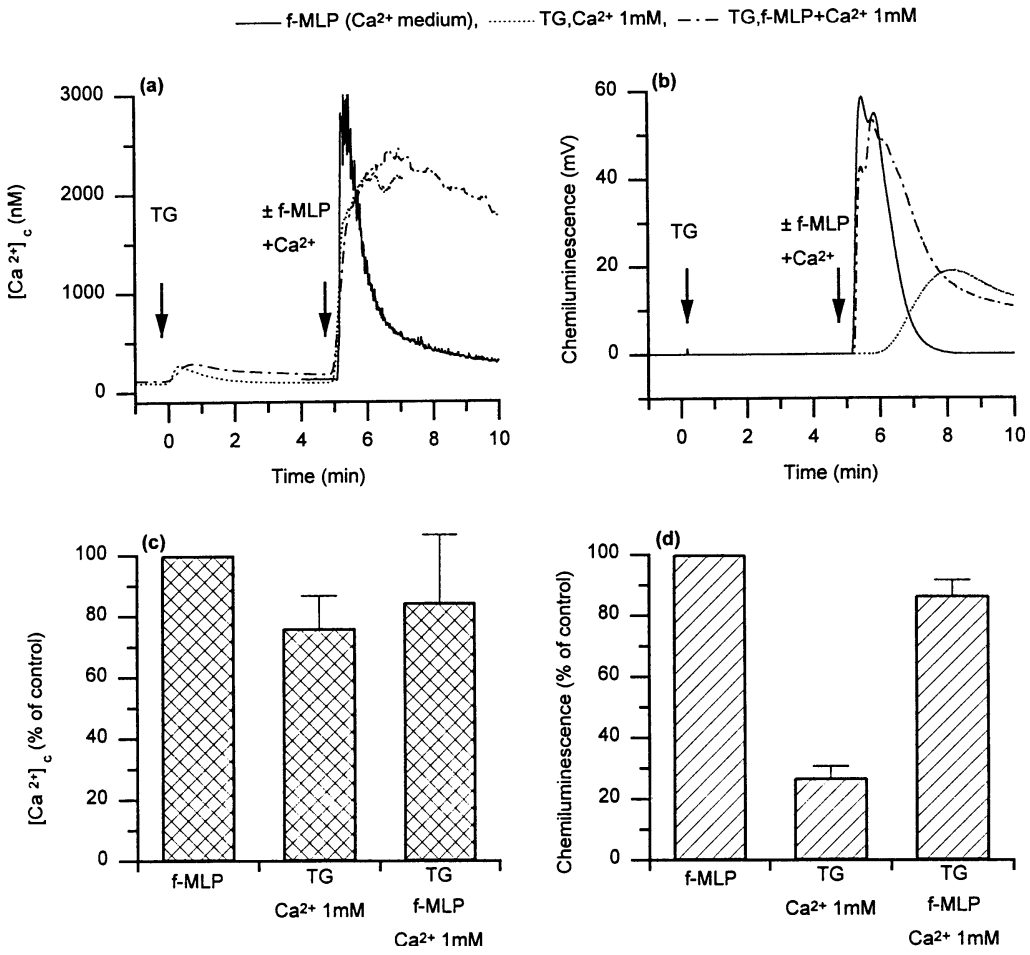

Figure 5 fMLP affects the respiratory burst, but not thapsigargin-induced Ca^{2+} influx

HL-60 neutrophils were loaded with fura 2/AM and suspended in a Ca^{2+}-free medium. $[Ca^{2+}]_c$ was measured as fura 2 fluorescence, and the respiratory burst as luminol-enhanced chemiluminescence. Cells were incubated with thapsigargin (100 nM) for 5 min before stimulation with 1 μM fMLP and/or by addition of 1 mM Ca^{2+} (–·–· and ···· respectively). For comparison, untreated cells in Ca^{2+} medium were stimulated with 1 μM fMLP (——). (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevation and chemiluminescence respectively. (**c**) and (**d**) Peak $[Ca^{2+}]_c$ and maximal chemiluminescence after stimulation with fMLP and/or Ca^{2+} (mean ± S.E.M., $n = 3$) are shown as a percentage of the control (fMLP in Ca^{2+} medium).

sigmoidal curves yielded a good fit (solid line in Figure 4), raising the possibility that two Ca^{2+}-dependent processes with different $[Ca^{2+}]_c$ affinities are involved in the fMLP activation of the respiratory burst. However, given the limitations of fura 2 measurements at $[Ca^{2+}]_c$ above 1 μM, the analysis of the shape of the $[Ca^{2+}]_c$–response curves has to be interpreted with caution. It is remarkable that Ca^{2+} release from intracellular stores (Figure 4 open squares) and Ca^{2+} influx (Figure 4 closed squares) appear to be equally efficient in stimulating the respiratory burst. There is no indication that subcellular differences in $[Ca^{2+}]_c$ might play a role. This is in contrast with the effect of Ca^{2+} on secretion, where stimulation by Ca^{2+} influx is much more efficient than Ca^{2+} release (O. Nüsse, L. Serrander, R. Foyouzi, A. Monod, D. P. Lew and K.-H. Krause, unpublished work).

To determine the contribution of fMLP to the stimulation of the respiratory burst, the store-depletion/Ca^{2+}-readdition protocol (Figure 3) was used with and without fMLP. The amplitude of the $[Ca^{2+}]_c$ elevations in thapsigargin-treated cells was identical in the presence or absence of fMLP (Figures 5a and 5c). Thus fMLP has no effect on thapsigargin-induced store-operated Ca^{2+} influx. The $[Ca^{2+}]_c$ values were similar to the peak values of fMLP-induced Ca^{2+} transients in Ca^{2+} medium. However, the respiratory burst under these conditions was markedly different (Figures 5b and 5d). Induction of maximal Ca^{2+} influx by thapsigargin alone led not only to a relatively small but a long-lasting activation of the respiratory burst. Without thapsigargin, Ca^{2+} addition did not induce a detectable response. However, when fMLP was added simultaneously with Ca^{2+}, a respiratory burst of large amplitude, similar to that with fMLP in Ca^{2+} medium, was observed. Thus fMLP generates a signal in addition to Ca^{2+} which is important for the activation of the respiratory burst.

The results shown so far demonstrate that $[Ca^{2+}]_c$ elevations are (i) absolutely required for fMLP activation of the respiratory burst and (ii) determine its amplitude. They also suggest that fMLP generates a second signal relevant for the activation of the respiratory burst as shown previously in human neutrophils [2]. This signal is not sufficient to activate the respiratory burst by itself (Figure 2b), but it strongly enhances the respiratory burst generated by $[Ca^{2+}]_c$ elevation (Figure 5b). To study the time course of the Ca^{2+}-independent signal generated by fMLP, cells in Ca^{2+}-free medium were exposed to thapsigargin (−5 min), fMLP (0 min) and finally 1 mM Ca^{2+} at different times (0, 0.5, 1, 2, 5, 10 and 15 min). The subsequent $[Ca^{2+}]_c$ elevations were

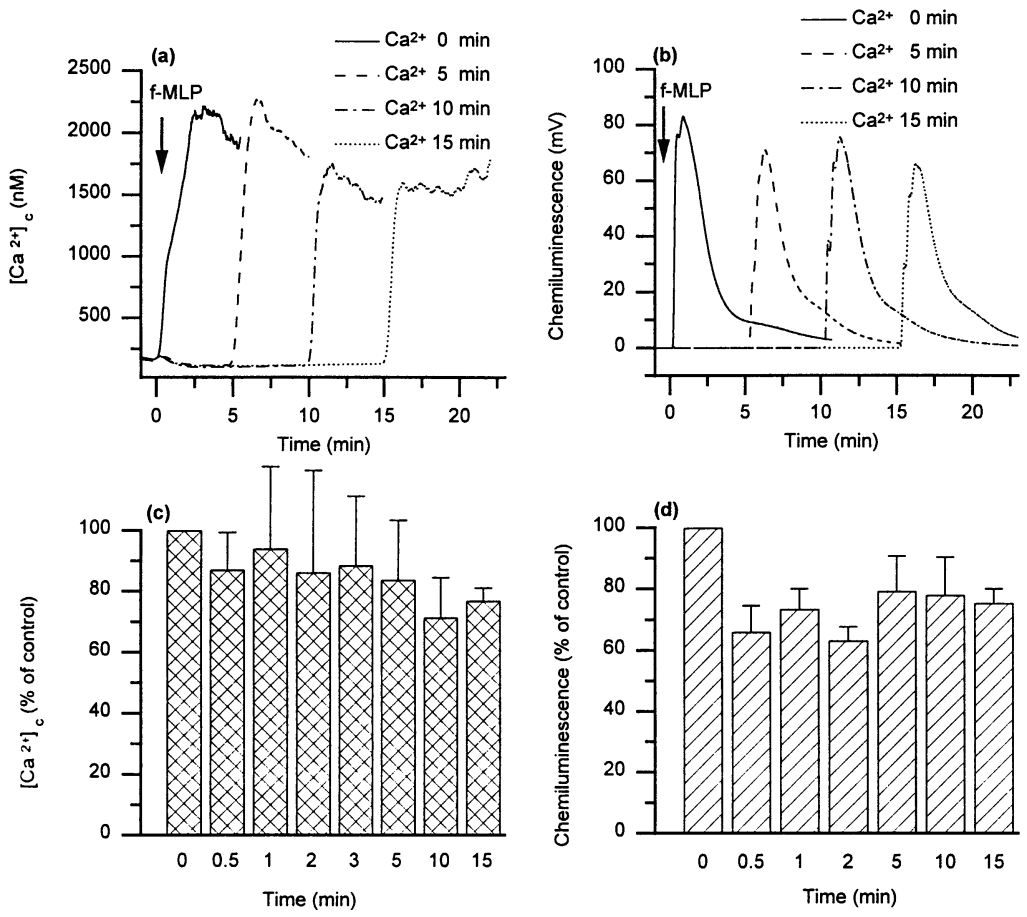

Figure 6 The Ca^{2+}-independent signal does not inactivate in the absence of extracellular Ca^{2+}

HL-60 neutrophils were loaded with fura 2/AM and suspended in a Ca^{2+}-free medium. $[Ca^{2+}]_c$ was measured as fura 2 fluorescence, and the respiratory burst as luminol-enhanced chemiluminescence. Cells were preincubated with 100 nM thapsigargin (TG) for 5 min before stimulation with 1 μM fMLP. Then 1 mM $CaCl_2$ was added at the indicated times after fMLP stimulation. (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevation and chemiluminescence respectively. (**c**) and (**d**) Peak $[Ca^{2+}]_c$ (mean $\pm$ S.D., $n = 2$) and integrated chemiluminescence during 10 min after Ca^{2+} readdition (mean $\pm$ S.E.M., $n = 3$). Data in (**c**) and (**d**) are expressed as a percentage of control (fMLP and Ca^{2+} at the same time).

essentially unchanged when Ca^{2+} was added late (Figures 6a and 6c). This confirms that the store-operated Ca^{2+} influx persists in thapsigargin-treated cells. Surprisingly, the activation of the respiratory burst by Ca^{2+} readdition was also essentially preserved over the entire period of 15 min (Figures 6b and 6d). This suggests that the fMLP-activated second signal shows almost no inactivation under our experimental conditions (i.e. in the absence of $[Ca^{2+}]_c$ elevation).

To investigate whether the long duration of the second signal generated by fMLP requires continuous receptor–ligand interaction, we performed experiments using the competitive fMLP receptor antagonist boc-MLP. Cells in Ca^{2+}-free medium were exposed to thapsigargin (0 min), fMLP (1 μM) or solvent (5 min), boc-MLP (200 μM) or solvent (15 min) and 1 mM Ca^{2+} (20 min). The addition of Ca^{2+} to the extracellular medium led to comparable $[Ca^{2+}]_c$ elevations under all conditions (Figures 7a and 7c). However, in the absence of fMLP, only a small amplitude respiratory burst was observed; when fMLP was added, the respiratory burst was enhanced by a factor of 5–10; this enhancement by fMLP could be entirely abolished by the addition of boc-MLP (Figures 7b and 7d). Thus the second signal for the fMLP-induced respiratory burst activation entirely depends on continuing receptor–ligand interaction.

To analyse the relationship between the Ca^{2+} signal and the second fMLP-generated signal further, we studied the respiratory burst response to Ca^{2+} influx in the presence of very high $[Ca^{2+}]_o$. For these experiments, cells were incubated in a Ca^{2+}-free medium and the following compounds were added: thapsigargin (-10 min), fMLP or solvent (-5 min) and 0.1–100 mM Ca^{2+} (0 min). In the absence of fMLP, almost maximal stimulation of the respiratory burst was achieved with 1 mM Ca^{2+} and a further increase in $[Ca^{2+}]_o$ had only a small effect (Figures 8a and 8c). The effect of increasing $[Ca^{2+}]_o$ in the presence of fMLP was biphasic (Figures 8b and 8c). Between 0.1 and 1 mM, increased $[Ca^{2+}]_o$ enhanced the respiratory burst. However, between 1 and 100 mM, increased $[Ca^{2+}]_o$ inhibited the respiratory burst to values seen in the absence of fMLP. These experiments suggest that the second signal generated by fMLP does not simply act by increasing the affinity of the respiratory burst activation for Ca^{2+} (i.e. through a left shift of the activation curve), but through a truly synergistic effect. The inhibition of the fMLP-stimulated, but not of the Ca^{2+}-stimulated, respiratory burst activation by high Ca^{2+} concentrations suggests that high $[Ca^{2+}]_c$ inactivate the second fMLP-generated signal.

In addition to the difference in amplitude, striking differences between the thapsigargin/Ca^{2+} activation and the fMLP activation of the respiratory burst are seen in the kinetics of activation and deactivation. Under the conditions shown in

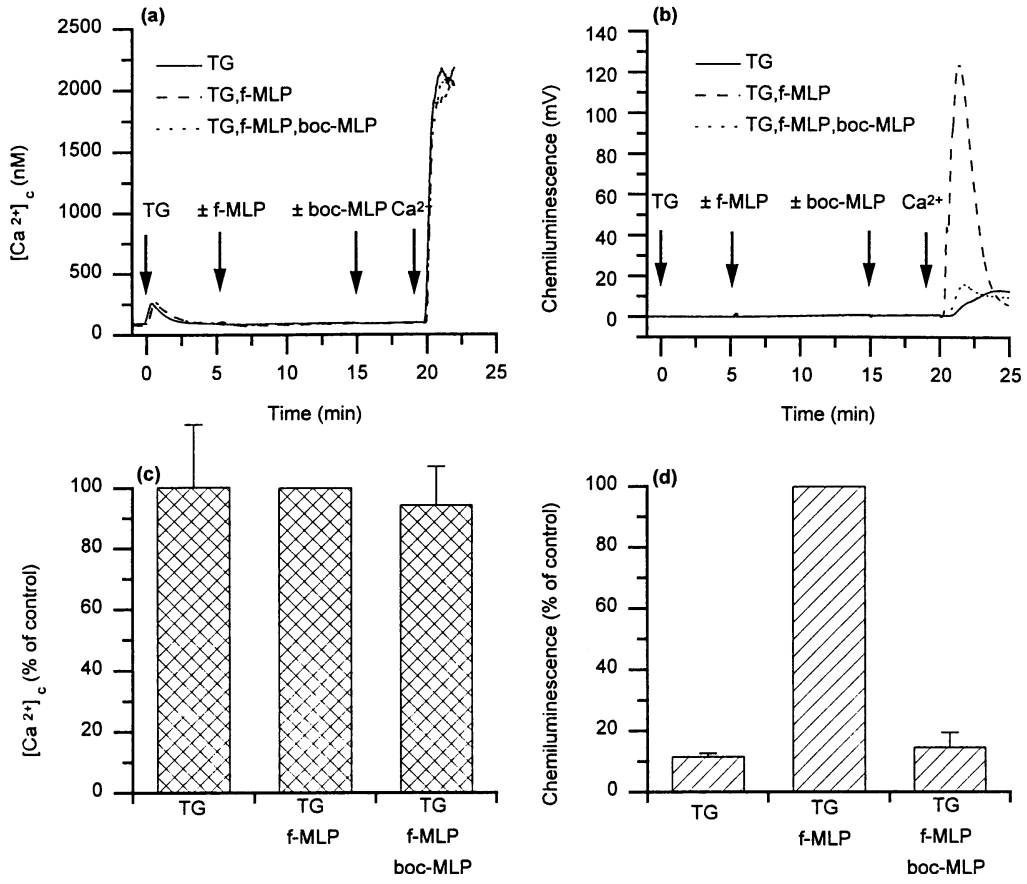

Figure 7 The persistence of the second signal generated by fMLP requires continuous fMLP receptor occupation

HL-60 neutrophils were loaded with fura 2/AM and suspended in a Ca^{2+}-free medium. $[Ca^{2+}]_c$ was measured as fura 2 fluorescence, and the respiratory burst as luminol-enhanced chemiluminescence. The following compounds were added: 100 nM thapsigargin (0 min); 1 μM fMLP (5 min); 200 μM boc-MLP (15 min); 1 mM $CaCl_2$ (20 min). (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevations and superoxide production respectively. (**c**) and (**d**) Peak $[Ca^{2+}]_c$ (mean $\pm$ S.D., $n = 2$) and maximal chemiluminescence (mean $\pm$ S.E.M., $n = 3$) after Ca^{2+} readdition. Data are shown as a percentage of control (thapsigargin preincubation and fMLP stimulation).

Figure 5, the fMLP activation in Ca^{2+} medium is fast, with a time to peak of 35 ± 10 s (mean $\pm$ S.E.M., $n = 5$), whereas the thapsigargin/Ca^{2+} activation is slow, with a time to peak of 214 ± 24 s (mean $\pm$ S.E.M., $n = 3$). Similarly, the deactivation is fast for the fMLP-stimulated burst, but slow for the thapsigargin/Ca^{2+}-stimulated burst [superoxide production decreased in the 2 min after the peak by $99 \pm 1\%$ and $42 \pm 5\%$ respectively (mean $\pm$ S.E.M., $n = 5$ and 3 respectively)]. The fast inactivation of the fMLP-stimulated respiratory burst is not simply due to the transient nature of the $[Ca^{2+}]_c$ increase after fMLP stimulation. When cells were exposed to thapsigargin before fMLP, the $[Ca^{2+}]_c$ elevation was long-lasting, but still the respiratory burst showed a rapid inactivation (see Figure 5). In the thapsigargin-pretreated cells, the phase of rapid inactivation was, however, followed by a sustained phase of slowly inactivating respiratory burst. This phase was of a similar amplitude to that of the thapsigargin/Ca^{2+}-activated respiratory burst.

DISCUSSION

Our results demonstrate that $[Ca^{2+}]_c$ elevations are essential for the activation of the respiratory burst by the chemotactic peptide fMLP. However, $[Ca^{2+}]_c$ elevations by themselves only weakly activate the respiratory burst, and a second signal generated by fMLP acts in synergy with $[Ca^{2+}]_c$ elevation to achieve full activation. The second signal has the following properties: (i) it is not sufficient to stimulate the respiratory burst in the absence of $[Ca^{2+}]_c$ increase; (ii) it does not inactivate unless either $[Ca^{2+}]_c$ elevation and respiratory burst activation occur or fMLP is displaced from its receptor; (iii) it is inactivated by high $[Ca^{2+}]_c$.

As discussed in the Introduction, the precise role of $[Ca^{2+}]_c$ elevation in the activation of the respiratory burst remains poorly defined. The fact that Ca^{2+}-depleted cells (i.e. cells with $[Ca^{2+}]_c$ well below basal [8]) do not respond to fMLP might indicate a necessity for permissive Ca^{2+} concentrations, rather than a necessity for $[Ca^{2+}]_c$ elevation. Also, $[Ca^{2+}]_c$ might modulate a basically Ca^{2+}-independent process. The example of the fMLP stimulation of phospholipase C illustrates the latter points: the enzyme can be blocked by Ca^{2+} depletion of cells, and is positively modulated by Ca^{2+} and inhibited at high Ca^{2+} concentrations; still its physiologically relevant activation occurs through receptor G-protein coupling independently of a preceding $[Ca^{2+}]_c$ increase [27]. Our results clearly demonstrate that the activation of the respiratory burst by fMLP is different: it has an absolute requirement for $[Ca^{2+}]_c$ elevation, and its amplitude is governed, albeit in a biphasic manner, by $[Ca^{2+}]_c$ elevation. Also, the observed effects of Ca^{2+} are not due to a Ca^{2+}-

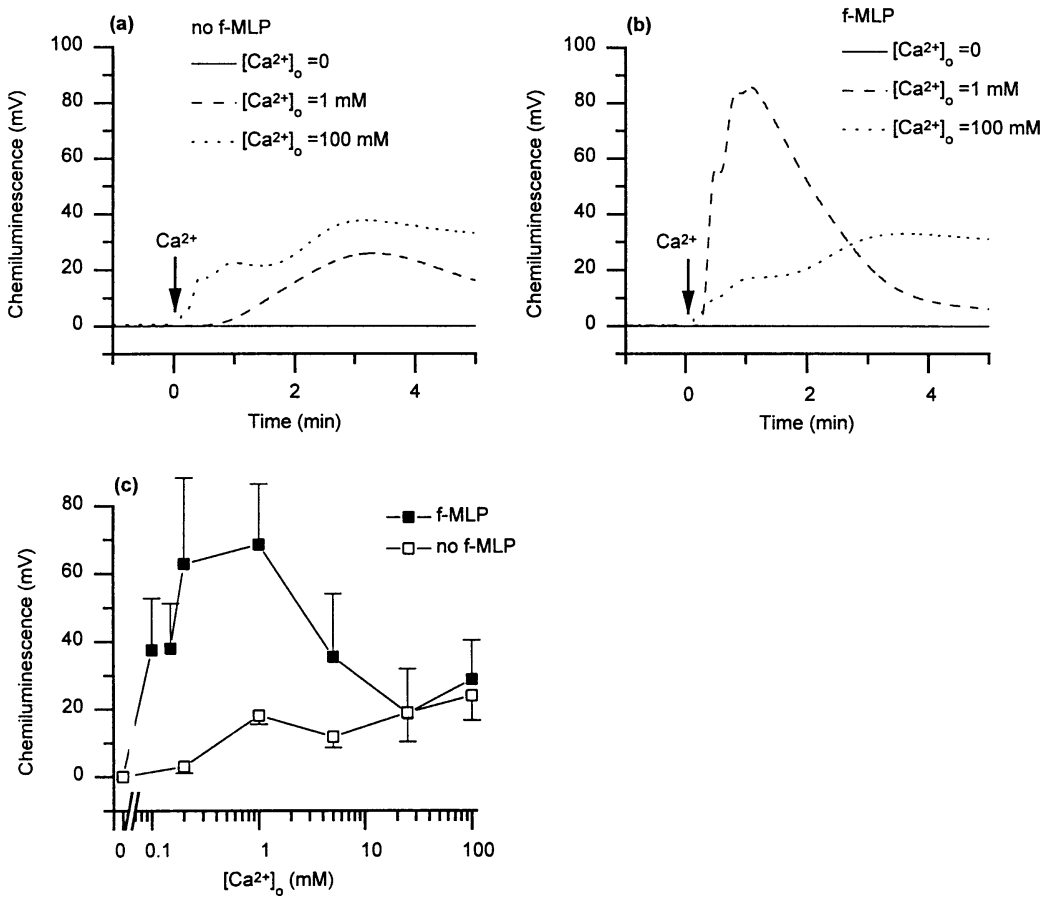

Figure 8 Extracellular Ca^{2+} has a biphasic effect on the second signal generated by fMLP

HL-60 neutrophils were suspended in a Ca^{2+}-free medium. The following compounds were added: 100 nM thapsigargin (−10 min); 1 μM fMLP or the corresponding amount of solvent DMSO (−5 min); the indicated concentration of $CaCl_2$ (0 min). The respiratory burst was measured as luminol-enhanced chemiluminescence. (**a**) and (**b**) Typical experiments showing $[Ca^{2+}]_c$ elevations and chemiluminescence respectively. (**c**) Dose–response effects of extracellular Ca^{2+} on maximal chemiluminescence with and without fMLP stimulation (mean ± S.E.M., $n = 5$).

dependent exocytotic plasma-membrane insertion of an intracellular pool of membrane-bound oxidase components, as HL-60 neutrophils do not have such a pool.

What might be the Ca^{2+} target(s) essential for oxidase activation? In systems of cell-free NADPH oxidase activation and in membranes isolated from PMA-prestimulated neutrophils, the respiratory burst is not Ca^{2+}-sensitive [28]. Thus the assembled and activated oxidase is not a Ca^{2+}-sensitive enzyme, and the Ca^{2+}-sensitive step should be at the level of oxidase assembly and/or oxidase activation. Potential Ca^{2+} targets that might be involved in oxidase activation include Ca^{2+}-dependent protein kinase, protein kinase C, tyrosine kinases and phospholipase A_2. Note that the complex relationship between $[Ca^{2+}]_c$ and the activation of the respiratory burst (Figure 4) might hint at the involvement of several Ca^{2+}-dependent processes with different Ca^{2+} affinities. Phospholipase D is another Ca^{2+}-sensitive enzyme that has been implicated in the oxidase activation. We have, however, performed our experiments without cytochalasin B, a condition where no fMLP-induced phospholipase D activation can be detected ([29]; R. Foyouzi-Youssefi, L. Serrander, D. P. Lew, K.-H. Krause and O. Nüsse, unpublished work). Therefore an involvement of phospholipase D in the mechanisms studied here is unlikely.

Through the precise control of $[Ca^{2+}]_c$ in our experiments, we could obtain new and unexpected results concerning the nature of the second signal involved in the activation of the respiratory burst. The most striking property of this signal is its lack of inactivation under defined experimental conditions. If fMLP is added to cells that are unable to increase $[Ca^{2+}]_c$ above basal, the signal appears to put the neutrophil in a 'Ca^{2+}-sensitive waiting state'. This waiting state persists for at least 15 min. The persistence of the signal, however, depends on permanent receptor–ligand interaction, as demonstrated by the loss of the 'Ca^{2+}-sensitive waiting state' after addition of an fMLP receptor antagonist. This long-lasting effect of fMLP is unique. To our knowledge, all previously reported responses mediated by the fMLP receptor were found to be transient, with a time course in the minute range. This relatively rapid termination of fMLP responses is thought to be mediated by at least three different mechanisms: (i) rapid internalization of the fMLP receptor in response to ligand binding ($t_{1/2} = 15$–20 s [30]); (ii) receptor desensitization through phosphorylation by receptor kinases [31]; (iii) lateral segregation of the fMLP receptor into membrane domains that are devoid of G-proteins [32]. The lack of inactivation under our experimental conditions suggests that Ca^{2+} is involved in one or several of these mechanisms.

PI-3-kinase and its reaction product PtdInsP_3 have become leading candidates for the second-signal pathway based on studies using the PI-3-kinase inhibitor wortmannin [33]. However, our present knowledge of the activation of this enzyme fits as-

tonishingly little with the 'second signal profile'. PI-3-kinase activation has been described as (i) transient (approx. 3 min) and (ii) Ca^{2+}-independent [34]. Thus, in the protocols described in this paper (e.g. Figure 6), PI-3-kinase activation would be expected to occur during the 3 min after fMLP stimulation, and no residual PtdInsP_3, able to synergize with Ca^{2+} in the respiratory burst activation, would be expected at time points as late as 15 min. Studies of PI-3-kinase activity using experimental protocols similar to those described in this study will be necessary to clarify the point.

Taken together, our results would be most compatible with a central effector molecule in fMLP-mediated respiratory burst activation which (i) is sensitive to $[Ca^{2+}]_c$, (ii) is strongly potentiated by receptor–ligand interaction and (iii) becomes desensitized to activation by the receptor–ligand complex at high $[Ca^{2+}]_c$. Phospholipase A_2 might be a good candidate: it is activated by fMLP [35], blocked by wortmannin [36], its product arachidonic acid is a potent activator of NADPH oxidase in the cell-free system (reviewed in [37]) and circumstantial evidence also links its activation to respiratory burst activation in intact cells [38]. Activation of phospholipase A_2 requires activated G-proteins and $[Ca^{2+}]_c$ elevation [1,39]. Since G-proteins constantly inactivate themselves through GTP hydrolysis, continued receptor occupation would be required to keep phospholipase A_2 in a 'Ca^{2+}-sensitive waiting state'. However, the evidence for phospholipase A_2 involvement remains circumstantial and there might be another effector molecule that shows a double regulation similar to phospholipase A_2. Alternatively, two different effector molecules (one activated by $[Ca^{2+}]_c$, the other by the second signal) that converge at the level of NADPH oxidase might account for the results of our study.

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