Reactivation of Formyl Peptide Receptors Triggers the Neutrophil NADPH-oxidase but Not a Transient Rise in Intracellular Calcium*

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Human neutrophil granulocytes constitute an important part of our innate immune defense against microbial infections, and the bactericidal activities performed by these cells rely on the ability to recognize and be guided by chemoattractants and other inflammatory mediators (1). The molecular basis for recognition and signaling in response to chemoattractants such as C5a, platelet-activating factor (PAF), interleukin-8 (IL-8), and formylated peptides is the binding to and activation of specific receptors (2), resulting in directed migration, granule mobilization, and activation of the neutrophil NADPH-oxidase. The chemoattractant receptors can be recruited to the cell surface from intracellular storage organelles (3–5), but the responses are not only influenced by the degree of receptor exposure on the cell surface but also by the survival time of its activated state and the time required for termination of the signals from the occupied receptor (6).

The classical chemoattractant receptors in phagocytes include the formyl peptide receptor (FPR). The FPR belongs to the pertussis toxin-sensitive, G-protein-linked receptor family that is defined by a seven-transmembrane domain structure (2, 7). Ligand binding to seven-transmembrane domain receptors creates a receptor-ligand complex that through a G-protein activates several downstream signaling pathways, including the phospholipase C/inositol phosphate pathway responsible for inositol 1,4,5-trisphosphate generation and a transient elevation of intracellular calcium (8–10). Following activation, deactivation mechanisms are put into action, and the resulting non-signaling, deactivated, receptor-ligand complex is endocytosed and either degraded or recirculated back to the plasma membrane (11–14).

Termination of the chemoattractant-induced response is rapid and renders the cells refractory to further stimulation with the same agonist (7, 15). This loss of cellular responsiveness, commonly termed homologous desensitization, is present in many hormonal and neurotransmitter signaling systems (16, 17). Phosphorylation of the agonist-occupied receptor by specific receptor kinases has been suggested to be an important mechanism for both the termination of signaling and internalization of the occupied receptors (18). In addition, the neutrophil response to FPR agonists is both augmented and prolonged in the presence of cytochalasin B (a drug that inhibits reorganization of actin polymers) (19), suggesting that the cytoskeleton also takes part in terminating receptor signaling. The molecular basis for such cytoskeleton-dependent termination of FPR signaling is suggested to be a direct cessation of the transmembrane signals when the ligated receptor binds to the cytoskeleton, an event known to follow shortly after binding of the chemoattractant to its receptor (20, 21). In fact, the association of the agonist-occupied receptor to cytoskeletal components induces a physical segregation of the G-protein and the receptor into different plasma membrane domains (20, 22). If binding of the ligand takes place at a nonphysiological temperature (at or below 15 °C), the chronological order in which the signaling/activation/deactivation steps normally occur is broken. The signaling events leading to receptor internalization, secretion, and activation of the NADPH-oxidase are by-passed at 15 °C (20, 23), and the receptor is directly associated with...
the cytoskeleton and deactivated/desensitized. This process occurs without triggering a rise in intracellular Ca\(^{2+}\) (24).

As outlined above, activation, deactivation/desensitization, and possibly reactivation/resensitization of chemotactant receptors are complex and highly regulated processes. In the literature, desensitization (as well as resensitization) is commonly defined by the level of receptor phosphorylation, but clearly there are other mechanisms for switching the receptors on and off. From here on we therefore use the word “deactivation” to define the transfer of a receptor into a nonsignaling mode and the word “reactivation” to designate the shift of the deactivated receptor into an actively signaling state. These definitions are used without reference to the phosphorylation state of the receptors.

We show here that deactivated FPR can be reactivated by cytochalasin B-induced uncoupling from the cytoskeleton, leading to NADPH-oxidase activation. The signaling by reactivated FPR is unique in that no transient elevation of intracellular calcium accompanies the NADPH-oxidase activation. Following the reactivation the cells are transferred into a novel deactivated state. The same type of cytoskeleton-dependent receptor activation occurs with some, but not all, of the other neutrophil seven-transmembrane domain chemotactant receptors.

EXPERIMENTAL PROCEDURES

Isolation of Human Neutrophils—Human peripheral blood neutrophils were isolated from buffy coats obtained from apparently healthy blood donors using dextran sedimentation and Ficoll-Hypaque centrifugation (25). The isolated neutrophils were washed and subsequently suspended in Krebs-Ringer phosphate buffer (containing 10 mM glucose, 1 mM Ca\(^{2+}\) and 1.5 mM Mg\(^{2+}\) at pH 7.3 (KRG)) to a final concentration of 10\(^{-6}\) M. The cell suspensions were stored on melting ice until use.

Chemoattractants and Receptor Antagonists—The hexapeptide Trp-Lys-Tyr-Met-Val-o-Met-NH\(_2\) (WKYMVm) was synthesized and high pressure liquid chromatography-purified by Alta Bioscience (University of Birmingham, Birmingham, UK). The formylated peptide N-formyl-Met-Leu-Ph (fMLF), C\(_5\)a, PAF, IL-8, and PAF were dissolved in water, whereas the other peptide agonists and antagonists were dissolved in dimethyl sulfoxide to 10\(^{-2}\) M and stored at -70 \(^\circ\)C until use. Further dilutions were made in KRG.

Neutrophil NADPH-oxidase Activity—Neutrophil production and release of superoxide anions was measured by means of an isoluminol-enhanced chemiluminescence assay (26). The chemiluminescence activity was measured in a six-channel Biolumat LB 9505 apparatus (Berthold Co., Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 900-\(\mu\)l reaction mixture containing 10\(^{-10}\)–10\(^{-6}\) neutrophils, horseradish peroxidase (4 units), and isoluminol (20 \(\mu\)M). When deactivated cells were used, the vials also contained fMLF (10\(^{-7}\) M). The measuring tubes were equilibrated for at least 5 min (or sometimes incubated for extended periods) at 37 \(^\circ\)C, the cells were activated either by addition of a peptide agonist or cytochalasin B, and the light emission was recorded continuously. By a direct comparison of the superoxide dismutase-inhibitable reduction of cytochrome c and superoxide dismutase-inhibitable chemiluminescence, 7.2 \(\times\) 10\(^{-6}\) counts were found to correspond to production of 1 nmol of superoxide (a millimolar extinction coefficient for cytochrome c of 21.1 was used). Details about the chemiluminescence technique are given in Ref. 26.

Determination of Changes in Cytosolic Calcium—Neutrophils at a density of 2 \(\times\) 10\(^{11}\) neutrophils/ml in Ca\(^{2+}\)-free KRG supplemented with bovine serum albumin (0.1%), were incubated with acetoxymethylated fura-2 (2 \(\mu\)M) at 37 \(^\circ\)C for 30 min. The cells were washed twice and resuspended in KRG, adjusted to 2 \(\times\) 10\(^{11}\) cells/ml, and kept protected from light on ice until use. The same loading protocol was used for MAPT/actinometry with a final concentration of 20 \(\mu\)M. The fura-2 fluorescence was measured with a luminescence spectrometer (LS50B, PerkinElmer Life Sciences) at 37 \(^\circ\)C (27). Cells (2 \(\times\) 10\(^{10}\) cells/ml) with or without fMLF (10\(^{-7}\) M) were equilibrated for 5 min at 37 \(^\circ\)C, after which cytochalasin B (5 \(\mu\)g/ml) was added. The fluorescence change was followed using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm, and the Ca\(^{2+}\) was calculated as described earlier (28).

RESULTS

Deactivation of the Formyl Peptide Receptor—Resting neutrophils challenged with the chemotactic peptide fMLF at 37 \(^\circ\)C were rapidly activated to generate superoxide anion (Fig. 1A). The maximal production was reached within the first minute, and the response was terminated within 3–4 min.

Neutrophils that had first been incubated with fMLF at 15 \(^\circ\)C and then transferred to 37 \(^\circ\)C did not respond with an oxidative burst (data not shown and Ref. 13). Neither did these cells respond to further fMLF stimulation at 37 \(^\circ\)C, i.e. they...
were deactivated (Fig. 1A). The hexapeptide WKYMVm can activate cells through the FPR but is a more potent agonist for the formyl peptide-like receptor 1 (FPRL1) (27), and, as illustrated by the fact that the neutrophils deactivated to fMLF responded to WKYMVm (Fig. 1A), the deactivation was stimulus/receptor-specific. Uncoupling of the Deactivated FPR with Cytochalasin B (Reactivation)—The deactivation at 15 °C is achieved through binding of the receptor-ligand complex to the cytoskeleton, subsequently leading to a lateral segregation of the occupied receptor from the signaling heterotrimeric G-protein (13, 23). We and others have shown that neutrophil production of reactive oxygen species in response to chemoattractants such as fMLF or C5a is both prolonged and increased when induced in the presence of the cytoskeleton-disrupting drug cytochalasin B (19, 20, 29–31). We have investigated the effect of cytochalasin B as a receptor-uncoupler on cells deactivated using the protocol described above. In untreated, nondeactivated cells, no NADPH-oxidase activity was induced by cytochalasin B (Fig. 2A). However, when added to fMLF-deactivated neutrophils, cytochalasin B induced a robust burst of oxidase activity (Fig. 2A), i.e. the cells were reactivated. The duration and magnitude of the response was dependent on the concentration of cytochalasin B and on the time point for addition (Fig. 2, C and D). Typically, when using a final cytochalasin B concentration of 5 μg/ml and a desensitization time of 10 min (equilibration time at 37 °C), the response reached a peak of superoxide production after 3–4 min and returned to a resting value after 5–8 min. The fMLF-induced oxidative burst occurs extracellularly (26), and the same applies to the reactivation-induced burst, illustrated by the fact that the activity was reduced by more than 90% in the presence of superoxide dismutase (a cell-impermeable scavenger of superoxide anion).

When fMLF-deactivated cells were reactivated by cytochalasin B, the oxidative burst was blocked by the competitive FPR antagonists Boc-FLFLFL (Fig. 3) and cyclosporin H (data not shown), showing that a signaling fMLF/FPR complex is needed for the radical production. The response was inhibited when the antagonists were added at the same time as cytochalasin B as well as when added at the peak of the cytochalasin B-induced cellular response (shown for Boc-FLFLFL in Fig. 3). The concentration of the antagonists was 100 times higher than that of the agonist (fMLF), and when added during the response a reduction of 50% was reached in around 20 s (20.9 ± 4.5 and 20.3 ± 1.6 s for Boc-FLFLFL and cyclosporin H, respectively; mean ± S.D., n = 4). Because removal of superoxide as soon as it is formed (by addition of superoxide dismutase) reduces the chemiluminescence activity immediately (less than 2 s; not shown by figure), the obtained curves reflect the instantaneous levels of superoxide at any given time. This indicates that the time needed for the antagonists to abolish the response really reflects the survival time for the signaling receptor-ligand complex.

HL-60 Cells and Receptor Mobilization—In neutrophils, a
fraction of the FPR is stored in mobilizable granules (32). A direct determination of the exposure of FPR during deactivation and reactivation revealed that the number of receptors exposed (determined as specific binding to the cell surface of a fluorescent labeled formylated peptide) decreased during the deactivation process. The level of receptor exposure was low also in reactivated cells, but the presence of cytochalasin B gave rise to a small increase in receptor exposure compared with the level for desensitized cells (Fig. 4).

To determine whether up-regulation of new receptors is required for the reactivation by cytochalasin B, we used the cell line HL-60 that lacks the secretory granules and by that the reserve pool of mobilizable receptors (33, 34). Differentiated HL-60 cells are equipped with surface exposed FPR and an activable NADPH-oxidase. The same activation and deactivation patterns were obtained when normal neutrophils were replaced with differentiated HL-60 cells, i.e. also these cells were activated to generate reactive oxygen species when challenged with fMLF at 37 °C but deactivated when the peptide was added at 15 °C (data not shown). We found cytochalasin B to be a receptor-uncoupler also in deactivated HL-60 cells, i.e. when cytochalasin B was added to fMLF-deactivated HL-60 cells using the protocol described above, a robust oxidative burst was induced (Fig. 2B).

Uncoupling of Other G-protein-coupled, Deactivated Chemotaxtractant Receptors with Cytochalasin B—The neutrophil receptors that bind the chemoattractants C5a (C5aR), PAF, IL-8 (CXCR1 and 2), and WKYMVm (FPRL1) all belong to the seven-transmembrane domain G-protein-linked receptor family (2). Binding to these receptors of their respective ligand at 37 °C resulted in activation of the neutrophil NADPH-oxidase. In agreement with the results obtained with fMLF, neutrophils that were allowed to interact with either of the other agonists at 15 °C and then transferred to 37 °C became deactivated, i.e. there was no burst in oxidase activity, and the cells did not respond to further stimulation with the same chemoattractant. Furthermore, cytochalasin B induced a robust burst of oxidase activity in C5a, as well as in WKYMVm-deactivated cells, indicating that also C5aR and FPRL1 could be reactivated by cytoskeletal uncoupling. No such reactivation was, however, induced by cytochalasin B in IL-8- or PAF-deactivated cells.

Induction of a New Deactivated/Desensitized State—The reactivation response was terminated within 5–8 min after the addition of cytochalasin B. After cessation of the response the cells could not be reactivated again, by adding either a new dose of fMLF or a new dose of cytochalasin B (Fig. 5, A and B), suggesting that the cells enter a second deactivated state. This state of deactivation is receptor-specific, because the cells are fully responsive to the PKC activator phorbol 12-myristate 13-acetate (data not shown), as well as to the FPRL1 agonist WKYMVm (Fig. 5C).

The Reactivated FPR Signals via a G-protein without Elevating Intracellular Ca2+—The cytochalasin B-reactivated response was found to be pertussis toxin-sensitive (Fig. 6). This is in agreement with the well known fact that FPR is a G-protein-linked receptor (35). Downstream of the G-protein, activation of the phosphoinositide-specific phospholipase C (PLC) leads to an increase in inositol 1,4,5-trisphosphate and diacylglycerol, two second messengers that induce release of Ca2+ from intracellular stores and translocate/activate PKC, respectively. Accordingly, when untreated, nondeactivated cells were activated by fMLF, intracellular Ca2+ levels were clearly elevated in a transient manner (Fig. 7A). Such a Ca2+ transient was observed only when cytochalasin B was added to the cells prior to the chemoattractant (not shown). Surprisingly, signaling from the cytochalasin B-reactivated FPR did not induce a rise in intracellular Ca2+ (Fig. 7B). This inability to respond with a...
**Fura-2 (2/H9262 activated (A) and fMLF-deactivated (B) neutrophils were loaded with Ca$$^{2+}$$ production without any Ca$$^{2+}$$ transient.** Figure 3. The calcium rise was not due to a general down-regulation of the Ca$$^{2+}$$ responsiveness because addition of the FPRL1 agonist WKYMVM to the reactivated cells induced a large Ca$$^{2+}$$ transient (Fig. 1B).

Although the reactivation of FPR resulted in superoxide production without any Ca$$^{2+}$$ transient, the process was clearly Ca$$^{2+}$$-dependent, because no oxidase activity was triggered by cytochalasin B when extracellular Ca$$^{2+}$$ was removed through an addition of EGTA (final concentration, 2 mM) or when intracellular Ca$$^{2+}$$ was buffered through loading of the cells with MAPTAM (data not shown).

**Signal Transduction Inhibitors and Activation of the NADPH-oxidase**—To investigate whether the signaling by the reactivated FPR shows unique characteristics apart from the absence of a Ca$$^{2+}$$ transient, different signal transduction inhibitors were employed. As previously shown, the products formed by the phosphatidylinositol 3-kinase (PI3K) were involved as second messengers in the fMLF-induced signaling leading to NADPH-oxidase activation because the PI3K inhibitor wortmannin dose-dependently inhibited both direct fMLF activation and cytochalasin B-induced reactivation of fMLF-deactivated cells (Fig. 8A). This wortmannin inhibition of the reactivation was achieved at much lower concentrations than the direct fMLF-induced activation of nondeactivated cells.

Activation of MEK (the MAPK/extracellular signal-regulated kinase), the kinase upstream of the extracellular signal-regulated kinase and mitogen-activated protein kinase (p38 MAPK), has also been suggested to be involved in fMLF-induced activation of the NADPH-oxidase (36). The MEK inhibitor PD98059 was utilized to determine whether this pathway is involved also in cytochalasin B-induced reactivation. The response in reactivated cells was partly inhibited by PD98059, whereas no inhibitory effect was seen for the fMLF-induced response in nondeactivated cells (Fig. 8B). The same type of inhibition profile was obtained with the p38 MAPK inhibitor SB203580 (Fig. 8C). When the time of incubation with the inhibitors was increased from 5 to 30 min, the response was inhibited also in nondeactivated cells, but the inhibition in reactivated cells was more pronounced for PD98059-treated (10$$^{-5}$$ M) cells (46 ± 13 and 83 ± 8% inhibition for nondeactivated and reactivated cells, respectively; means ± S.D., n = 4) and for SB20358-treated (10$$^{-5}$$ M) cells (73 ± 6% and 82 ± 9% for nondeactivated and reactivated cells, respectively; means ± S.D., n = 3). Despite the fact that no Ca$$^{2+}$$ transient occurred during reactivation of the deactivated FPR, the PLC-specific inhibitor Ro318220 had a slight inhibitory effect on the NADPH-oxidase activity (Fig. 8D).

**DISCUSSION**

The classical chemotactic receptors in phagocytes are all members of the G-protein-coupled receptor superfamily. The signal transduction pathways to the various effector systems in the cell have been more extensively and thoroughly studied for FPR than for any other leukocyte GPCR (2, 9, 10, 37). Coupling of FPR (and all other GPCRs) to a heterotrimeric G-protein activates multiple downstream phospholipases and protein kinases that pass the signal on to several transduction pathways working in parallel. The signaling is highly regulated, allowing cellular adaptation to a wide range of conditions, and receptor deactivation results in termination of signaling. Our data (summarized in Fig. 9) show that at least two different mechanisms can induce a refractory state in the cells, making them nonresponsive to fMLF. Deactivation without triggering the NADPH-oxidase was achieved through a procedure during which fMLF was allowed to bind FPR at 15°C. A shift in temperature to 37°C leads to an up-regulation of the storage pool of granule localized receptors, but the fact that no triggering of the NADPH-oxidase occurs during the shift in temperature suggests that the newly mobilized receptors are rapidly deactivated (24), and the fact that the desensitization process is associated with a strong reduction in the number of binding sites for formylated peptides suggests that the process of re-

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**Fig. 6.** Effect of pertussis toxin on NADPH-oxidase activity induced by cytochalasin B-mediated reactivation. The figure shows the time courses of neutrophil superoxide production with cytochalasin B added to cells incubated for 20 min with (broken line) or without (solid line) pertussis toxin (Ptx; 500 ng/ml). The curves are representative of at least four experiments.

**Fig. 7.** Induction of an intracellular calcium response. Nondeactivated (A) and fMLF-deactivated (B) neutrophils were loaded with Fura-2 (2/H9262 and analyzed with respect to mobilization of intracellular Ca$$^{2+}$$ induced by direct fMLF stimulation (10$$^{-7}$$ M) (A) or cytochalasin B (CB) reactivation (5 μg/ml) (B) followed by stimulation with WKYMVM (10$$^{-7}$$ M). The curves are representative of at least four experiments.
ceptor deactivation/uptake is more rapid than that for mobilization. Addition of the cytoskeleton-disrupting agent cytochalasin B leads to a rapid reactivation of the deactivated FPR, suggesting that one of the deactivation mechanisms relies on an intact cytoskeleton. Even though the level of FPR exposure remains low also after cytochalasin B-induced reactivation, it is evident (Fig. 4) that the process leads to an increased binding of labeled formyl peptide to the surface. This could be due to a mobilization of new receptors from intracellular stores, but it is clear that a cytochalasin-mediated change in affinity of the receptors and a decreased receptor uptake/removal can also contribute to the change in binding. An increased mobilization of receptors from intracellular stores could possibly contribute to the burst activity induced, but still, activation occurs without any transient Ca$^{2+}$ rise. It should also be noticed that cytochalasin B-induced reactivation was obtained also with HL-60 cells. These cells lack secondary granules and by that also the mobilizable pool of FPR (33, 34), and accordingly the reactivation process occurs without any increased number of binding sites for formyl peptides. These data suggest that in neutrophils, the reactivation is not due to up-regulation of new FPR but rather due to the fact that the deactivated plasma membrane-localized receptors are reactivated by the addition of cytochalasin B. A model for such a deactivation/reactivation mechanism has earlier been presented, suggesting that the signaling from the occupied receptor is terminated through a lateral segregation of the G-protein from the receptor-ligand complex in the plasma membrane (13, 22). This physical separation of the components is achieved through a direct binding of the ligand-receptor complex to the cytoskeleton.

Among the family members of chemoattractant receptors, there are differences regarding ability to mediate various cellular responses, and these differences are reflected in their respective signaling properties (31). We found that all of the GPCRs investigated were homologously deactivated when incubated with their respective agonist at 15°C. However, only two of these receptors, C5aR and FPRL1, could be reactivated in a similar manner as FPR, whereas the CXCR1/2 and PAF receptor showed no reactivation potential. This is in line with recently presented data describing distinct signaling pathways for GPCRs that mediate directional migration by chemoattractants guiding the neutrophils out of the vasculature (e.g. interleukins and lipid mediators acting through CXCR1/2 and PAF receptor) versus those guiding the cells through the interstitium (e.g. bacterial chemoattractants and activated complement factors acting through FPR and C5aR) (38).

After cytochalasin B-induced reactivation of FPR, the cells rapidly enter a new deactivated state in which they become refractory to further stimulation by either fMLF or cytochalasin B. The mechanisms of induction of this new homologously deactivated state is independent of cytoskeletal binding of FPR (because the cytoskeleton is already disrupted by the presence of cytochalasin B) and probably involves phosphorylation of the agonist-occupied, cytoskeleton-uncoupled receptor (18). The carboxyl terminus of FPR (as well as of
other GPCRs) contains serine and threonine residues that may be phosphorylated by various kinases. Removal of eight possible phosphorylation sites in the cytoplasmic tail of FPR has been shown to block ligand-induced phosphorylation of the receptor, a loss that correlates to a lack of receptor desensitization and receptor internalization (39). These two processes are regulated separately, illustrated by the fact that an inability to phosphorylate some residues (but not others) completely abolishes receptor desensitization but is without effect on internalization (40). In line with this, we show here that there are two different deactivation mechanisms in neutrophils, one involving cytoskeletal coupling and one that does not, and these two mechanisms can operate independently of each other.

The duration and magnitude of the reactivation response declined if the preincubation time of the deactivated cells exceeded 20 min. This could be due to a partial induction of the second (nondefined) deactivation mechanism but could equally well be the result of receptor internalization during the preincubation. The fact that two mechanisms for deactivation may work in parallel makes it hard to determine their relative contribution to a given activation and/or deactivation state. It should be noticed that all studies focusing on the role of receptor phosphorylation in deactivation/desensitization have been performed with the wild-type receptor or mutants thereof expressed in a new host cell, and the outcome and interpretation of experiments in such a system depend on if and how other alternative desensitization systems are present in the new host cell.

The intracellular parts of FPR contain important determinants for G-protein interaction, and studies aiming at defining the basic characteristics of this interaction and downstream signaling events have been performed using a number of dif-
different approaches. These include creation of chimeric receptors, site-directed mutagenesis, and construction of peptides with abilities to directly block the receptor-G-protein interaction (41). Taken together, these studies suggest that activation of the FPR causes the \( \alpha \) subunit of a heterotrimeric G-protein to dissociate from the \( \beta \gamma \) subunit. Both subunits possess signaling capacity, and when dissociation is initiated an extensive phospholipid remodeling in the leukocyte membranes is rapidly induced. These activities are mediated by phosphoinositide kinases, phospholipase D, phospholipase A\(_2\), and a phosphoinositide-specific PLC. An immediate consequence of FPR-initiated, G-protein-mediated activation of PLC is the production of inositol 1,4,5-triphosphate and a subsequent transient elevation of intracellular Ca\(^{2+}\). The Ca\(^{2+}\) elevation has been suggested to activate downstream effectors, directly or in synergy with other signals (31). Accordingly, Ca\(^{2+}\) has been shown to be of prime importance in the signaling steps from the occupied FPR to assembly and activation of the NADPH-oxidase (42–44). However, the experimental evidence relies on methods that cannot distinguish a dependence of basal Ca\(^{2+}\) levels from a requirement for a Ca\(^{2+}\)-transient, because the experiments were performed on Ca\(^{2+}\)-depleted cells, using depletion protocols that will also affect processes that simply depend on basal levels of the ion. A Ca\(^{2+}\) transient has been claimed to be required but not sufficient for the generation of an NADPH-oxidase activating signal from FPR (45). The rise in intracellular Ca\(^{2+}\) has also been shown to be required for granule mobilization (46). Our data showing that neutrophils can be activated without any cytosolic Ca\(^{2+}\) transient are thus in contradiction with the prevailing view. We also show that no cytochalasin B-induced reactivation can occur when the basal Ca\(^{2+}\) levels are chelated by MAPTAM and/or EGTA, further indicating the difference between the availability of the ion and the transient elevation as an intracellular signal. In murine neutrophils, fMLF-mediated oxidase activation, but not chemotaxis, has been shown to be dependent on a transient elevation of cytosolic Ca\(^{2+}\) (as well as on PLC) (47). This discrepancy could be due to important differences between human and murine neutrophils with respect to the signaling properties of their formyl peptide receptors. It should, however, be noticed that the concentrations of fMLF necessary and sufficient to mobilize Ca\(^{2+}\) are significantly lower than those required to induce functional responses in murine neutrophils (48, 49).

As stated above, reactivation of human FPR activates the NADPH-oxidase via a G-protein-linked signaling pathway that is not associated with a Ca\(^{2+}\) transient. Normally, a Ca\(^{2+}\) transient is dependent on PLC activation, and because the reactivation occurs without a transient, it appears that PLC is by-passed. The \( \beta\gamma \)-subunit of the activated G-protein not only activates PLC\(\beta2\) (the main PLC in neutrophils) but also PI3K, and the fact that reactivation is inhibited by wortmannin indicates that signaling to the oxidase utilizes this pathway instead. Multiple downstream phospholipases and protein kinases are activated and passes the signal on to several transduction pathways working in parallel. Accordingly, the p38 MAPK inhibitor SB203580 has in earlier studies been shown to inhibit the fMLF-induced oxidase activity (50). We found this inhibitor to be without effect on the fMLF-induced burst in nondeactivated cells, but more importantly, we show that reactivation of FPR is sensitive to the short time incubation, reducing the response more than 50%. To induce an inhibitory activity on nondeactivated cells, the incubation time with the inhibitor has to be increased, which is in agreement with earlier published data (50), but the difference in sensitivity to the inhibitor between nonactivated and reactivated cells was maintained. The inhibitory effect of the MEK inhibitor PD98059 showed the same pattern as that described for SB203580. The increased sensitivity to the inhibitors may be related to a change of the target molecules in the deactivated cells or that adequate inhibitory concentrations are reached more rapidly in these cells. Taken together, we show that the reactivation of FPR through cytoskeletal uncoupling uses a signaling route to the NADPH-oxidase that involves PI3K, p38 MAPK, and MEK but is unique in that it is independent of PLC and a transient rise in intracellular Ca\(^{2+}\).
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