Role of the actin cytoskeleton during respiratory burst in chemoattractant-stimulated neutrophils

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The role of the neutrophil actin cytoskeleton during chemoattractant stimulated respiratory burst

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ABSTRACT

The aim of this study was to clarify the role of the actin cytoskeleton during chemotactic peptide fMet-Leu-Phe (fMLF)-stimulated respiratory burst in human neutrophil granulocytes. Reactive oxygen species (ROS) was measured as luminol-amplified chemiluminescence (CL) and F-actin content as bodipy phallacidin fluorescence in neutrophils treated with latrunculin B or jasplakinolide, an inhibitor and activator of actin polymerization, respectively. Latrunculin B markedly decreased, whereas jasplakinolide increased, the F-actin content in neutrophils, unstimulated or stimulated with fMLF. Latrunculin B enhanced the fMLF-triggered ROS production more than tenfold. Jasplakinolide initially inhibited the fMLF-induced CL-response, however, caused a potent second sustained phase (>400% of control). Both actin-drugs triggered a substantial CL-response when added 5-25 min after fMLF. This was also valid for chemotactic doses of fMLF, where latrunculin B and jasplakinolide amplified the ROS-production 5-10 times. By using specific signal transduction inhibitors, we found that the NADPH-oxidase activation triggered by destabilization of the actin cytoskeleton occurs downstream of phospholipase C and protein kinase C but is mediated by Rho GTPases and tyrosine phosphorylation. In conclusion, this study indicates that rearrangements of the actin cytoskeleton are a prerequisite in connecting ligand/receptor activation, generation of second messengers and assembly of the NADPH oxidase in neutrophil granulocytes.

1. INTRODUCTION

The generation of reactive oxygen species (ROS) by neutrophil granulocytes plays a crucial role in the host defense system against bacterial infection. Neutrophils engulf microorganisms and destroy them by releasing superoxide and the contents of cytoplasmatic granules into the phagosome (Faurschou and Borregaard, 2003; Roos et al., 2003). Superoxide is generated by the NADPH oxidase, which oxidizes NADPH and passes the electrons across the membrane to reduce oxygen. The oxidase is a multicomponent enzyme consisting of three membrane-bound ($p22^{phox}$, $gp91^{phox}$ and Rap 1a) and four cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and p21*rac*) (Bokoch and Knaus, 2003; Roos et al., 2003). This enzyme is dormant in resting neutrophils and become activated upon stimulation trough assembly of the different components, including translocation of the cytosolic phox proteins to the membrane where they dock with the flavocytochrome. The assembly of NADPH oxidase components may occur at the membrane of intracellular organelles leading to intracellular ROS-production, or at the plasma membrane causing a release of radicals into the phagosome or the extracellular space (Karlsson and Dahlgren, 2002; Seguchi and Kobayashi, 2002; Vaissiere et al., 1999).

Actin is one of the major components of the cytoskeleton in neutrophils, as well as in other non-muscle cells (Stossel, 1999). Upon neutrophil stimulation, the actin cytoskeleton is reorganized through reversible cycles of polymerization and depolymerization, thereby consisting the driving motor for formation of lamellipodia and pseudopodia in migrating and phagocytosing cells (Niggli, 2003; Stossel, 1999). Several

cell and cell-free studies indicate that the actin cytoskeleton has an important role in regulating the assembly and the stability of the NADPH oxidase (Al-Mohanna and Hallett, 1987; Bengtsson et al., 1991; Norgauer et al., 1988; Tamura et al., 2000a; Tamura et al., 2000b). Following observations support a role for actin in the regulation of NADPH oxidase activity: i) stimulation of ROS-production is mostly associated with rearrangements of the actin cytoskeleton, including reversible actin polymerization (Bengtsson et al., 1991); ii) disruption or stabilization of actin filaments by e.g. cytochalasin B, botulinum C2 toxin or phalloidin, markedly interfere with the oxidative response (Bengtsson et al., 1991; Norgauer et al., 1988; Wiles et al., 1995); iii) cells with impaired ß-actin gene have an impaired ROS production (Li et al., 2004); iv) several components of the NADPH oxidase (e.g. $p47^{phox}$, $p67^{phox}$ and p21*rac*) and associated signalling systems directly or indirectly interact with actin or actin binding proteins (Grogan et al., 1997; Nauseef et al., 1991; Wientjes et al., 2001; Woodman et al., 1991); v) cell-free activation of neutrophil NADPH oxidase is enhanced by actin (Morimatsu et al., 1997; Tamura et al., 2000a); and, vi) neutrophils from CGD patients lacking $p47^{phox}$ and $p67^{phox}$ express disturbed dynamics of the actin cytoskeleton (Grogan et al., 1997).

Chemotactic peptide-induced NADPH oxidase activation and actin rearrangements are complex processes involving multiple interrelated signalling pathways (Cicchetti et al., 2002; Niggli, 2003; Quinn and Gauss, 2004). It is generally considered that activation of phospholipase C, Rho-GTPases, tyrosine kinases and protein kinase C is involved. How these signaling components interplay with the actin cytoskeleton and activation of the oxidase is, however, incompletely understood.

In this study, we have used latrunculin B and jasplakinolide that inhibits and stimulates, respectively, actin polymerization, in combination with different inhibitors of signal transduction, to evaluate the relationship between the actin cytoskeleton, intracellular signaling and NADPH oxidase activity in chemotactic peptide-stimulated human neutrophils.

2. MATERIALS AND METHODS

2.1 Materials and buffers

The materials and their sources were as follows: fMet-Leu-Phe (fMLF), latrunculin B, 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), lysophosphatidylcholine (Sigma Chemical Co., St. Louis, MO); Lymphoprep™, Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway); catalase, horseradish peroxidase, superoxide dismutase (SOD) (Boehringer Mannheim GmbH, Mannheim, Germany); N-acetyl-S-farnesyl-L-cysteine (AFC), 4′,5,7-trihydroxyisoflavone (genistein), 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X), 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U-73122) (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.); bodipy phallacidin, jasplakinolide (Molecular Probes, Eugene, OR, U.S.A.). The buffers were phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.7 mM Na_2HPO_4 x $2H_2O$ and 1.5 mM KH_2PO_4, pH 7.3), and Krebs-Ringer phosphate buffer (KRG; 120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4x7H_2O$, 1.7 mM KH_2PO_4, 8.3 mM $Na_2HPO_4x2H_2O$, 1.1 mM $CaCl_2$ and 10 mM glucose, pH 7.3).

2.2 Neutrophils

Peripheral whole blood was drawn daily from apparently healthy non-medicated volunteering blood donors at the Linköping University Hospital and was anti-coagulated with 5 U/mL heparin. Neutrophil granulocytes (neutrophils) were isolated from the same

donor for each individual experiment. Blood and neutrophils were handled using plastic utensils exclusively, and calcium was not present in buffers during isolation.

The neutrophils were prepared essentially as pioneered by Böyum (Böyum, 1968), a methodology that was later optimized by others (Ferrante and Thong, 1980). A density separation medium was prepared by careful layering of one part of Lymphoprep™ over four parts of Polymorphprep™. Whole blood was layered on an equal volume of separation liquid and the tubes centrifuged in a swing-out rotor for 40 min (480 x *g*, room temperature), yielding an upper band containing mononuclear cells, a middle band containing neutrophils and a pellet of red blood cells. The neutrophils were harvested and the separation liquid removed by dilution in PBS and another centrifugation for 10 min at 480 x *g*. The remaining erythrocyte contamination was lysed by 35 seconds of exposure to ice-cold distilled water, followed by washing of the cells twice in KRG (200 x *g* at 4°C). The neutrophils were counted in a Coulter Counter ZM Channelyser 256 (Coulter-Electronics Ltd., Luton, U.K.) and stored on ice.

2.3 Chemiluminescence

The neutrophil generation of ROS was studied by luminol-amplified chemiluminescence (CL) in a six-channel Biolumat (LB 9505 C, Berthold Co., Wildbaden, Germany), essentially as previously described by Dahlgren *et al*. (Dahlgren et al., 1991). Neutrophils were pre-incubated at least 5 min at 37°C with 4 U/mL horseradish peroxidase and 50 μM luminol. With these reagents, both intra- and extracellulary produced ROS are detected since luminol is membrane permeable and peroxidase is sufficiently available in

both intra- and extracellular compartments. The intracellular ROS generation was registered after exchange of extra peroxidase for 200 U/mL superoxide dismutase and 2,000 U/mL catalase that scavenges extracellular superoxide anion and hydrogen peroxide (Dahlgren et al., 1991).

The dynamics of the actin cytoskeleton were disrupted by treating the neutrophils with the membrane-permeable natural compounds jasplakinolide of the marine sponge *Jaspis johnstoni* (stimulates actin polymerization (Scott et al., 1988)) and latrunculin B of the marine sponge *Latrunculia magnifica* (inhibits actin polymerization (Spector et al., 1983)). The role of different intracellular signalling components in fMLF-induced CL were studied by preincubating the neutrophils for 5 min with the phospholiase C inhibitor U-73122, the Rho GTPase inhibitor AFC, the protein kinase C inhibitor GF109203X or the tyrosine kinase inhibitor genistein.

2.4 Quantification of F-actin in single cells

Stimulated cells were fixated in 4% ice-cold paraformaldehyde in PBS for 30 minutes. The samples were then washed twice in PBS and stained for F-actin through 30 minutes of incubation in a solution containing 0.6 μg/ml bodipy phallacidin and 100 μg/ml lysophosphatidylcholine in PBS at room temperature and protected from light. The F-actin content in single, adherent neutrophils was quantified by registration of the fluorescence in a cytofluorometer (Bengtsson et al., 1988). In brief, cells were observed and individual neutrophils selected in the light-microscopy mode of a Leitz MPV II microscope photometer connected to a computer system. For each sample, ≥30 individual

cells were excited for <0.8s and the fluorescence recorded. The background fluorescence values were routinely subtracted.

3. RESULTS

3.1. Disrupted actin dynamics change NADPH oxidase activity

The importance of actin reorganization in the regulation of NADPH oxidase activity was investigated by registering fMLF-induced changes in luminol-amplified CL in neutrophils untreated or treated with the actin drugs latrunculin B or jasplakinolide. Exposure of human neutrophils to the chemotactic peptide fMLF (10^{-9}-10^{-7}M) caused a dose-dependent CL-response, peaking after 1-2 min and lasting for 10-15 min. Pretreatment with the actin polymerization inhibitor latrunculin B markedly amplified the fMLF-induced CL-response in a dose-dependent manner (both the peak and the duration), with a maximum effect (more than tenfold amplification) at 100-1000 nM of the drug (Fig 1A). In contrast, the actin polymerization stimulator jasplakinolide inhibited the fMLF-triggered ROS-production during the first period of 10 min, where after a huge respiratory burst was obtained lasting for at least 40 min (Fig 1B). Testing different concentrations of jasplakinolide revealed a strong inhibition of the fMLF-induced CL-response at 5-10 µM, whereas lower concentration, 250-1000 nM, markedly elevated (2-5 times) the response, peaking after approximately 20 min.

Neither latrunculin B nor jasplakinolide triggered ROS-production by themselves. However, addition of these actin drugs at the end of a fMLF-induced CL-response caused an additional extensive ROS-production (Fig. 2A and 2B). Latrunculin B induced a CL-

response within seconds (Fig. 2A), whereas a jasplakinolide-triggered ROS-production was obtained after a 5 min lag phase (Fig. 2B). The magnitude of the latrunculin B- and jasplakinolide-induced CL-response, respectively, was dependent on the fMLF-concentration. Interestingly, the latrunculin B- and jasplakinolide-induced CL-responses were several fold higher compared to the preceding fMLF-stimulated CL-response, especially when low, almost chemotactic doses, of fMLF were used. The extent of the jasplakinolide-induced CL-response after fMLF-stiumulation was similar whether jasplakinolide was introduced 10 min or 30 min after fMLF (Fig. 3B). In contrary, the latrunculin B-triggered ROS-production declined when the time between addition of fMLF and latrunculin B increased (Fig. 3A).

To separate the intracellular activity of the CL-response, we used superoxide dismutase (SOD; 200 U) and catalase (2 000 U), which are membrane-impermeable O_2^- and H_2O_2 scavengers, respectively. The intracellular part of the fMLF-induced CL-response was about 20 % in the untreated control. Latrunculin B enhanced the extracellular ROS production (85 % of total), whereas the oxidase activity was mainly located intracellularly (60 % of total) in jasplakinolide-treated neutrophils. Consequently, assembly of actin filaments by jasplakinolide leads to an increased intracellular accumulation of ROS in fMLF-stimulated neutrophils, whereas disruption of the actin cytoskeleton by latrunculin B enhances the extracellular release of oxygen metabolites.

3.2 The effects of latrunculin B and jasplakinolide on the F_actin content in neutrophils

The major part of neutrophil functions is dependent on a dynamic reorganization of the actin cytoskeleton. As previously reported, and shown in figure 4B, the chemotactic peptide fMLF induces a transient actin polymerization in neutrophils. Jasplakinolide (0.5 µM) slightly raised the F-actin content by itself (approximately 120 % of control) and markedly potentiated a subsequent actin polymerization in fMLF-stimulated neutrophils (Fig 3A). In contrast, latrunculin B (0.1 µM) dramatically decreased the F-actin level to around 30 % of control after 5 min and inhibited a following fMLF-induced actin polymerization (Fig 4A). Addition of jasplakinolide (0.5 µM) or latrunculin B (0.1 µM) 10 min after fMLF-stimulation induced an actin polymerization and depolymerization response, respectively (Fig 4B). Jasplakinolide inuced a peripheral accumulation of actin filaments and formation of lamellipodia in neutrophils, whereas latrunculin B disrupted the membrane actin cytoskeleton and antagonized shape changes.

3.3 Actin reorganization interferes with fMLF-induced intracellular signaling

To evaluate the role of intracellular signalling pathways in the latrunculin B- and jasplakinolide-induced modulation of the fMLF-induced respiratory burst, different inhibitors were used at concentrations close to their IC_{50} values (Bleasdale et al., 1990; Goldman et al., 1992; Molony et al., 1996; Yun et al., 2001).

The phospholipase C inhibitor U-73122 effectively blunted the fMLF-induced ROS-production in neutrophils in a dose-dependent manner (0.05-5 μM), with a total abolishment at 1 μM of the drug and with a CL-response of around 7 % of the control at 0.5 μM (Fig. 5). In neutrophils pretreated with jasplakinolide or latrunculin B, the effects of the phospholipase C inhibitor were much less pronounced, with CL-responses of 6% and 44% at 1 and 0.5 μM of U-73122 in jasplakinolide-tretaed cells and 18% and 63 %, respectively, in latrunculin B-treated cells (Fig. 5).

Similar pattern was observed when comparing the effects of the protein kinase C inhibitior GF109203X (0.5-50 μM) in untreated versus jasplakinolide or latrunculin B-treated neutrophils. As shown in Fig 6, 10 μM of GF109203X almost abolished the fMLF-induced ROS-production. However, the actin drugs antagonized the effects of protein kinase C-inhibition revealed by responses of 25 % and 78 % of control in jasplakinolide- and latrunculin B-treated cells, respectively. The IC_{50} values for GF109203X were approximately 1 μM, 5 μM and 50 μM in untreated, jasplakinolide-treated and latrunculin B-treated neutrophils, respectively.

We also tested a possible involvement of tyrosine kinases in jasplakinolide- and latrunculin B-induced ROS-production. Treatment with the tyrosine kinase inhibitor genistein dose-dependently (1-100 μM) inhibited the fMLF-induced CL-response (Fig. 7). In contrast to the other signal transduction inhibitors tested, genistein also efficiently repressed the fMLF-triggered ROS-production in neutrophils treated with the actin drugs. The effects of genistein were most prominent in jasplakinolide-treated cells with an IC_{50} around 1 μM compared to IC_{50} values of 10 μM in control and latrunculin B-treated cells.

The Rho GTPases control actin redistribution to membrane ruffles and lamellipodia, as well as activation of the NADPH-oxidase. Targeting of Rho GTPases to the plasma membrane during cellular activation is promoted by a post-translational modification, involving attachment of farnesyl groups to cysteine residues followed by a reversible methylation (Zhang and Casey, 1996). To investigate the possible role of these processes in the interaction between the actin cytoskeleton and the NADPH-oxidase, the neutrophils were treated with N-acetyl-S-farnesyl-L-cysteine (AFC), a specific inhibitor of farnesyl methyltransferases, in the absence or presence of jasplakinolide or latrunculin B. As shown in Fig. 8, AFC dose-dependently (1-100 μM) inhibited the fMLF-induced CL-response, both in the absence and presence of the actin disturbing agents. However, the effects of low concentrations (1-10 μM) of AFC were less pronounced in pretreated (especially jasplakinolide-treated) neutrophils compared to the untreated control.

4. DISCUSSION

The actin cytoskeleton has a central role in driving migration and phagocytosis of the neutrophil granulocyte. This study provides further evidence that reorganization of the actin cytoskeleton is also a prerequisite for an assembly and activation of the NAPDH-oxidase in human neutrophils.

We and others have previously presented data suggesting that disruption of actin barriers at the plasma membrane and around intracellular granules markedly enhance chemotactic peptide-induced ROS-production (Bengtsson et al., 1991; Jesaitis et al., 1986). These observations are further supported by the present study showing that depolymerization of actin by latrunculin B clearly potentiates fMLF-induced CL in neutrophils. Furthermore, the actin stabilizing or polymerizing drug jasplakinolide inhibited the stimulated ROS-production. However, the marked suppression of the early fMLF-induced rise in CL was followed by a substantial longlasting generation of ROS. Consequently, we found that both depolymerization and polymerization of actin enhanced fMLF-triggered ROS-production in neutrophils, indicating that a rearrangement of the actin cytoskeleton per se is a crucial event in the activation of the NADPH oxidase.

Newly formed actin filaments are suggested to stabilize the NADPH-oxidase and thereby regulate its durability (Tamura et al., 2000b). It has been shown that actin can directly interact with components of the oxidase. For example $p47^{phox}$, p21*rac* and $p67^{phox}$ have been localized in the detergent-insoluble cytoskeletal fraction of stimulated neutrophils (Knaus et al., 1992; Nauseef et al., 1991; Wientjes et al., 2001; Woodman et al., 1991).

Furthermore, the actin-binding proteins coronin and moesin have been found associated with the cytosolic phox proteins (Grogan et al., 1997; Wientjes et al., 2001). It is suggested that the moesin-$p47^{phox}$ interaction keep the $p47^{phox}$ and possibly the $p40^{phox}$/$p67^{phox}$ complex in an inactive state to release it upon activation (Wientjes et al., 2001).

Functional assembly of NADPH-oxidase and generation of ROS is accomplished essentially within intracellular compartments (Karlsson and Dahlgren, 2002; Seguchi and Kobayashi, 2002; Vaissiere et al., 1999). By using SOD and catalase in the CL system, the intracellular generation of ROS was determined. Interestingly, we found that latrunculin B mainly stimulated an extracellular release of oxygen metabolites in fMLF-stimulated neutrophils, whereas the enhanced ROS-production in jasplakinolide-treated cells was localized intracellularly. The effects of latrunculin B are similar to those obtained by cytochalasin B, which is recognized to facilitate the secretion of granule constituents (Henson et al., 1988). Disruption of the peripheral, submembraneous actin barrier and the actin meshwork surrounding granules markedly increase the fusion of granules with the plasma membrane (Aunis and Bader, 1988; Ryder et al., 1988). This leads to an extracellular release of the granular contents and an increased expression of NADPH-oxidase and fMLF receptors in the plasma membrane. Furthermore, these agents antagonize receptor desensitization by unabling a cytoskeletal association and subsequent internalization of the ligand-receptor complex (Jesaitis et al., 1986; Jesaitis et al., 1985; Särndahl et al., 1989). This means that the time during which the active ligand-receptor complex triggers production of second messengers, leading to increased NADPH-oxidase activity, is prolonged. Indeed, we have previously reported that cytochalasin B markedly

potentiate the fMLF-induced formation of diacylglycerol, which is the endogenous activator of protein kinase C (Bengtsson et al., 1991). The coupling of the ligand-receptor complex to the cytoskeleton causes a physical segregation of the receptor and the G-protein associated signaling system into different domains of the plasma membrane (Klotz and Jesaitis, 1994). We have previously demonstrated that cytochalasin B and the local anesthetics tetracaine (polymerizes actin) separately potentiate fMLF-induced signal transduction and oxidase activation (Bengtsson et al., 1991). Consequently, this suggests that destabilization of the actin cytoskeleton through latrunculin B and jasplakinolide, respectively, eliminates the actin-based control of the NADPH-oxidase, leading to an enhanced and prolonged ROS-production. The amount and the distribution of actin filaments appear to determine whether ROS is released extra- or intracellularly.

Latrunculin B and jasplakinolide did not by themselves activate the NADPH oxidase of resting neutrophils. However, addition of these drugs 15 min after fMLF-stimulation caused a second dramatic oxidative burst, manifold higher compared to the preceeding response triggered by fMLF. The level of ROS production triggered by latrunculin B and jasplakinolide was dependent on the fMLF concentration. In neutrophils stimulated with low (5-10 nM) concentrations of fMLF, latrunculin B induced a NADPH oxidase response 10 times higher compared to the prior fMLF-triggered ROS production. Thus, activation of the NADPH oxidase by actin deorganizing agents requires a prior priming or activation of the neutrophil. In correlation, Bylund *et al.* have shown that TNF-α-treated neutrophils are primed for oxidase activation by the actin-disrupting drug cytochalasin B (Bylund et al., 2004).

Low doses of fMLF induce chemotaxis-related events, including polymerization of actin. Omann and Sklar have shown that the fMLF-induced actin polymerization is saturated when less than 1 % of the receptors are occupied, whereas the ROS-production is nearly proportional to the number of receptors occupied reaching maximum at 100% occupation (Omann and Sklar, 1988). Occupation of few receptors causes a sparse activation of intracellular signalling pathways that are turned off after association of the receptor/ligand complexes to the cytoskeleton. This intracellular signalling is sufficient for triggering chemotaxis and associated events (e.g. actin polymerization), but insufficient for causing a sustained ROS production (McPhail and Harvath, 1993; Omann and Sklar, 1988). Activation of the NADPH oxidase requires generation of a relatively large pool of diacylglycerol and prolonged stimulation of protein kinase C (Rider and Niedel, 1987). However, by disturbing the dynamics of the cortical actin network with cytochalasin B (Bylund et al., 2004), latrunculin B or jasplakinolide (this study), the receptor-ligand complexes are uncoupled from the actin filament system resulting in re-established signalling function of the receptor. Consequently, by blocking the termination of receptor-induced signalling only a restricted number of fMLF-receptors have to be activated in order to stimulate an extensive generation of oxygen radicals.

The activation of NADPH-oxidase is regulated by multiple interrelated signalling pathways more or less associated with the cytoskeleton (Karlsson and Dahlgren, 2002; Quinn and Gauss, 2004; Roos et al., 2003). Stimulation of neutrophils with chemoattractants such as fMLF leads to activaton of phospholipase C, increased activity of protein kinase C and mobilization of intracellular free calcium (Bokoch, 1995). As previously shown, inhibition of phospholipase C markedly impeded the fMLF-induced

ROS-production. Whereas the phospholipase C inhibitor U-73122 suppressed the CL-response below 10 % compared to the untreated control, the effects on neutrophils preatreated with latrunculin B or jasplakinolide were less pronounced (approx. 50% of control). We and others have previously reported that stimulation of neutrophils with chemotactic peptides causes a rapid and transient polymerization of actin through mechanisms involving G-proteins and the phospholipase C pathway (Bengtsson et al., 1990; Bengtsson et al., 1986; Shefcyk et al., 1985; Therrien and Naccache, 1989). We believe that a re-organized actin cytoskeleton provides a scaffold for assembly and activation of the NADPH-oxidase. Consequently, bypass of the early actin polymerizing steps of phospholipase C signalling by using actin reorganizing drugs, may explain the lower sensitivity to phospholipase C inhibition.

Stimulation of neutrophils with fMLF leads to a rapid and transient increase in the GTP-bound state of the Rho family of small GTPases, including Rho, Rac and Cdc42 (Cicchetti et al., 2002; Niggli, 2003). These proteins emerge as key regulators of actin polymerization and reorganization involved in cell shape change and motility. Rac2 controls cytoskeletal rerrangements leading to membrane ruffling and is also essential for activation of the NADPH-oxidase through binding of $p67^{phox}$ (Bokoch, 1995). Rac2 is thus suggested to be a link between the actin cytoskeleton and the cytosolic components of the oxidase. Rap1a is another abundant RhoGTPase in neutrophils closely associated with the NADPH oxidase (Bokoch, 1995). It has been shown that post-translational processing, including prenylation, proteolysis and carboxyl methylation, of Rac2 is necessary for its translocation to the plasma membrane and activation of the NADPH oxidase (Ando et al., 1992). In correlation, we found that the farnesylcysteine analogue

AFC dose-dependently repressed fMLF-induced ROS-production, both in the absence and presence of latrunculin B or jasplakinolide. This suggests that the actin-driven assembly of the NADPH-oxidase is dependent on, or occurs in conjunction with, enzymatic modifications of Rho GTPases.

Activation of NADPH-oxidase constrains phosphorylation of its components by several protein kinases, including protein kinase C (Quinn and Gauss, 2004). We found that the protein kinase C-inhibitor GF109203X effectively and dose-dependently impeded the fMLF-triggered ROS-production in neutrophils. However, reorganization of the actin cytoskeleton with jasplakinolide or latrunculin B antagonized the inhibitory effects of GF109203X on oxidase activity. This suggests that destabilization of the actin cytoskeleton overpasses protein kinase C-dependent steps in NADPH-oxidase activation. Previous studies have reported a translocation of protein kinase C and $p47^{phox}$ from the cytosol to the plasma membrane in activated human neutrophils (Nixon and McPhail, 1999; Suzuki et al., 2003). Possibly protein kinase C regulates an actin-driven assembly of the NADPH-oxidase at cellular membranes, which is mimicked by latrunculin B and jasplakinolide in GF109203X-treated neutrophils.

Tyrosine phosphorylation also participates in the signalling pathways of NADPH oxidase activation (Quinn and Gauss, 2004; Roos et al., 2003). We used the tyrosine kinase inhibitor genistein to elucidate the relationship between tyrosine phosphorylation, actin reorganization and ROS production. This study shows that genistein dose-dependently repressed fMLF-induced oxidase activity in neutrophils irrespective if they were untreated or treated with the actin modifying drugs. In fact genistein was most effective

in inhibiting the fMLF-stimulated ROS-production in neutrophils pretreated with jasplakinolide. These results imply a role for tyrosine phosphorylation that is directly associated with the NADPH oxidase and more or less independent of the extent of the preceeding actin reorganisation.

In conclusion, this study supports a central role of the actin cytoskeleton in the regulation of NADPH oxidase activity in neutrophil granulocytes. A dynamic actin filament system appear to be a scaffold at the plasma membrane upon which activated receptors, intracellular signalling pathways and the NADPH oxidase are associated. The actin cytoskeleton has to be carefully regulated to direct the neutrophil to the site of infection before it has a role in assembling the oxidase complex leading to generation of bactericidal reactive oxygen radicals.

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Legends to Figures

Fig. 1. fMLF-triggered (100 nM) chemiluminescence profiles for isolated neutrophils (1 x 10^6/ml) pre-incubated for 5 min at 37°C with 0-1000 nM of latrunculin B (A) or jasplakinolide (B), respectively. Chemiluminescence was recorded in a six-channel Biolumat in KRG buffer at 37°C in the presence of 50μM luminol and 4U/ml horseradish peroxidase. (CPM = counts per minute). The figure shows representative recordings of five separate experiments.

Fig. 2. Neutrophil (1 x 10^6/ml) chemiluminescence recordings after initial stimulation with 0-100 nM fMLF, followed by a secondary stimulation after 15 min with 100 nM latrunculin B (A) or 500 nM jasplakinolide (B), respectively. Chemiluminescence was recorded in a six-channel Biolumat in KRG buffer at 37°C in the presence of 50μM luminol and 4U/ml horseradish peroxidase. (CPM = counts per minute). The figure shows representative recordings of five separate experiments.

Fig. 3. Temporal resolution of the effects exerted by 100 nM latrunculin B (A) and 500 nM jasplakinolide (B) on the neutrophil respiratory burst triggered by 10 nM fMLF. The actin drugs were given either before (-) or after (+) stimulation of cells with fMLF. Representative chemiluminescence recordings in a six-channel Biolumat of five separate experiments performed in KRG buffer at 37°C in the presence of 50μM luminol and 4U/ml horseradish peroxidase. (CPM = counts per minute)

Fig. 4. Quantification of the filamentous actin (F-actin) in fMLF-stimulated (100nM) neutrophils, subsequent (A) or prior (B) to treatment with jasplakinolide (500nM) or latrunculin B (100nM). The neutrophils were fixated after 30 minutes in 4% paraformaldehyde, permeabilized with lysophosphatidylcholine and the F-actin stained with bodipy-phallacidin. Data are from 2-3 separate experiments, each including values of ≥30 cells.

Fig. 5. Effects of the phospholipase C inhibitor U-73122 on the fMLF-triggered neutrophil respiratory burst. Neutrophils (1 x 10^6/ml) were treated with or without (control) latrunculin B (lat B; 100 nM) or jasplakinolide (jasp; 500 nM) for 5 min followed by exposure to various concentrations (0.05-5 μM) of U-73122 for 5 min and then stimulation with fMLF (10 nM). The experiments were performed in a six-channel Biolumat at 37°C in KRG buffer in the presence of 50μM luminol and 4U/ml horseradish peroxidase. The data are based on integral values of chemiluminescence over 30 min and are expressed as percent of the untreated control; mean ± SEM of five separate experiments.

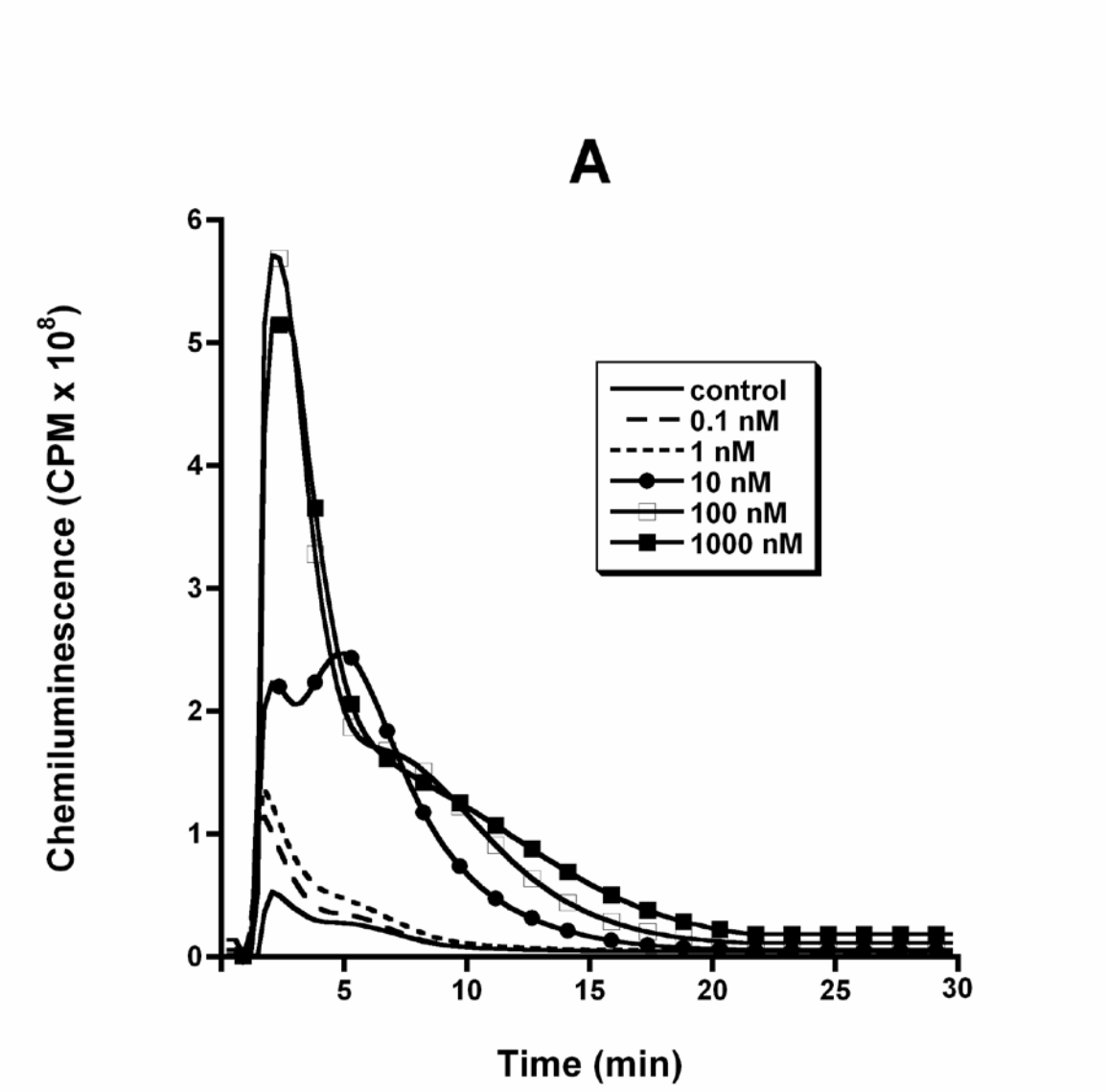
Fig. 6. Effects of the protein kinase C inhibitor GF109203X on the fMLF-triggered neutrophil respiratory burst. Neutrophils (1 x 10^6/ml) were treated with or without (control) latrunculin B (lat B; 100 nM) or jasplakinolide (jasp; 500 nM) for 5 min followed by exposure to various concentrations (0.5-50 μM) of GF109203X for 5 min and then stimulation with fMLF (10 nM). The experiments were performed in a six-channel Biolumat at 37°C in KRG buffer in the presence of 50μM luminol and 4U/ml

horseradish peroxidase. The data are based on integral values of chemiluminescence over 30 min and are expressed as percent of the untreated control; mean ± SEM of five separate experiments.

Fig. 7. Effects of the protein tyrosine kinase inhibitor genistein on the fMLF-triggered neutrophil respiratory burst. Neutrophils (1 x 10^6/ml) were treated with or without (control) latrunculin B (lat B; 100 nM) or jasplakinolide (jasp; 500 nM) for 5 min followed by exposure to various concentrations (1-100 μM) of genistein for 5 min and then stimulation with fMLF (10 nM). The experiments were performed in a six-channel Biolumat at 37°C in KRG buffer in the presence of 50μM luminol and 4U/ml horseradish peroxidase. The data are based on integral values of chemiluminescence over 30 min and are expressed as percent of the untreated control; mean ± SEM of five separate experiments.

Fig. 8. Effects of the Rho GTPase inhibitor AFC on the fMLF-triggered neutrophil respiratory burst. Neutrophils (1 x 10^6/ml) were treated with or without (control) latrunculin B (lat B; 100 nM) or jasplakinolide (jasp; 500 nM) for 5 min followed by exposure to various concentrations (1-100 μM) of AFC for 5 min and then stimulation with fMLF (10 nM). The experiments were performed in a six-channel Biolumat at 37◦C in KRG buffer in the presence of 50μM luminol and 4U/ml horseradish peroxidase. The data are based on integral values of chemiluminescence over 30 min and are expressed as percent of the untreated control; mean ± SEM of five separate experiments.

Figure 1 A-B


A
Chemiluminescence (CPM x 10^8)
Time (min)
control
0.1 nM
1 nM
10 nM
100 nM
1000 nM
0
1
2
3
4
5
6
5
10
15
20
25
30

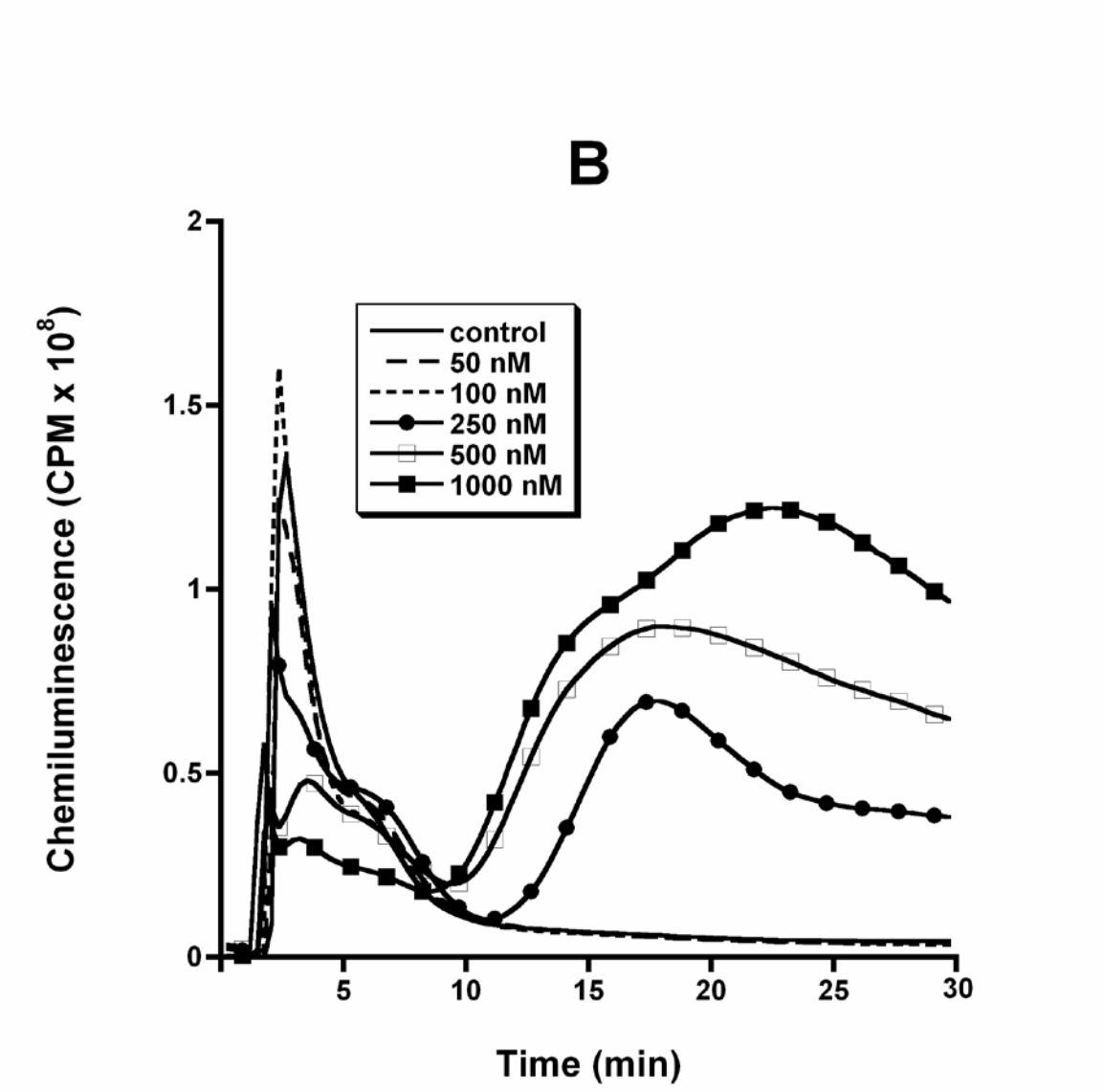
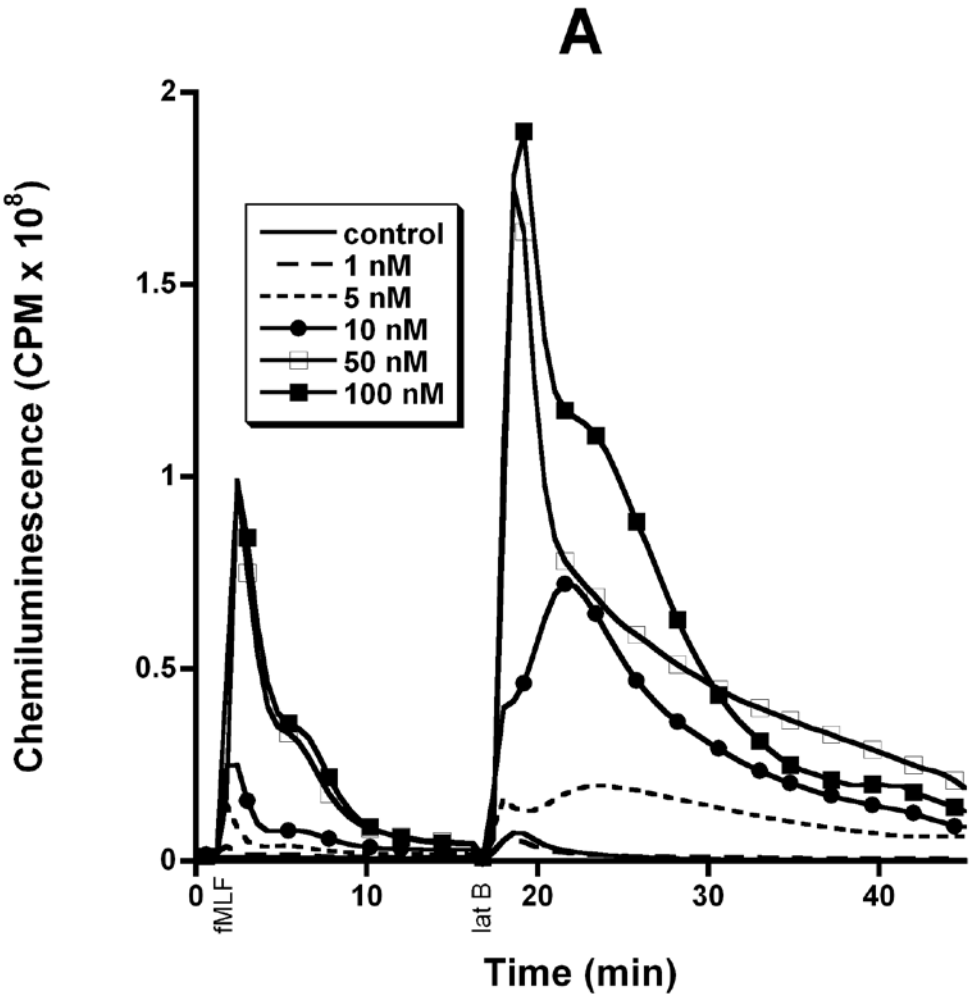

B
Chemiluminescence (CPM x 10^8)
Time (min)
control
50 nM
100 nM
250 nM
500 nM
1000 nM
0
0.5
1
1.5
2
5
10
15
20
25
30

Figure 2 A-B


A
Chemiluminescence (CPM x 10^8)
2
1.5
1
0.5
0
control
1 nM
5 nM
10 nM
50 nM
100 nM
0
fMLF
10
lat B
20
30
40
Time (min)

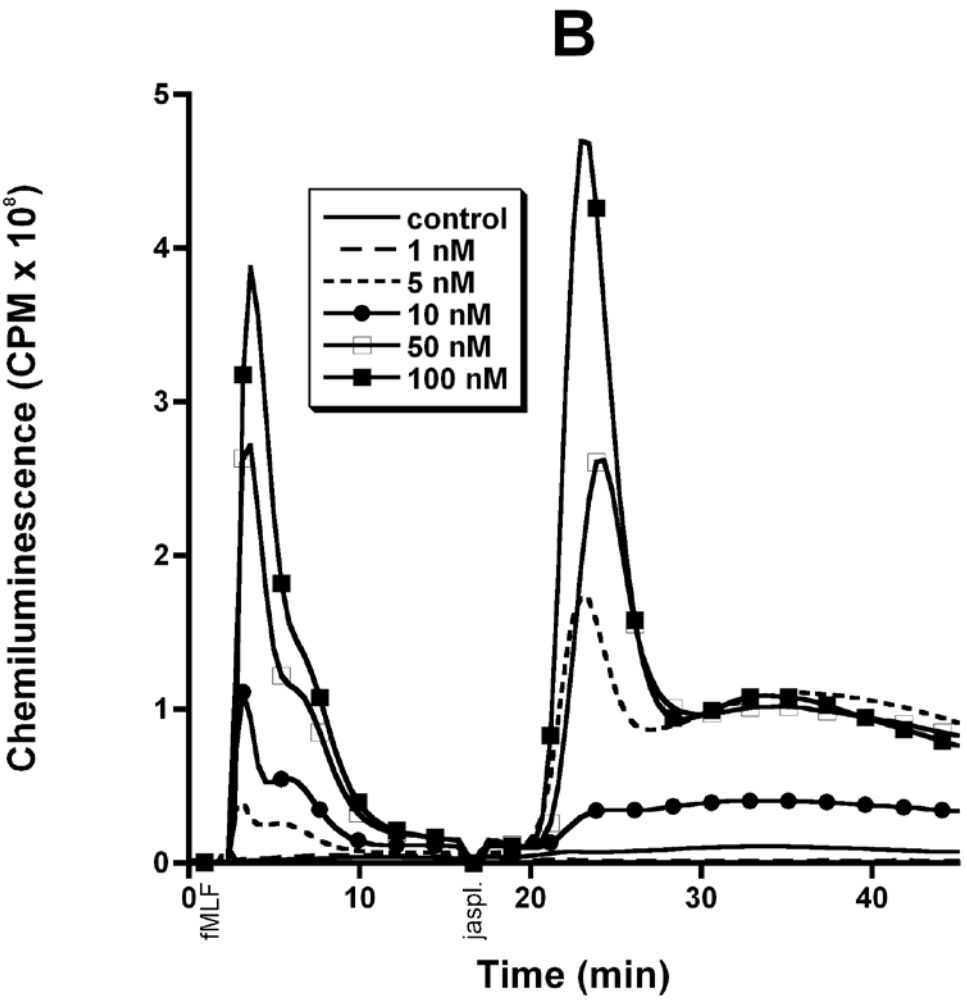
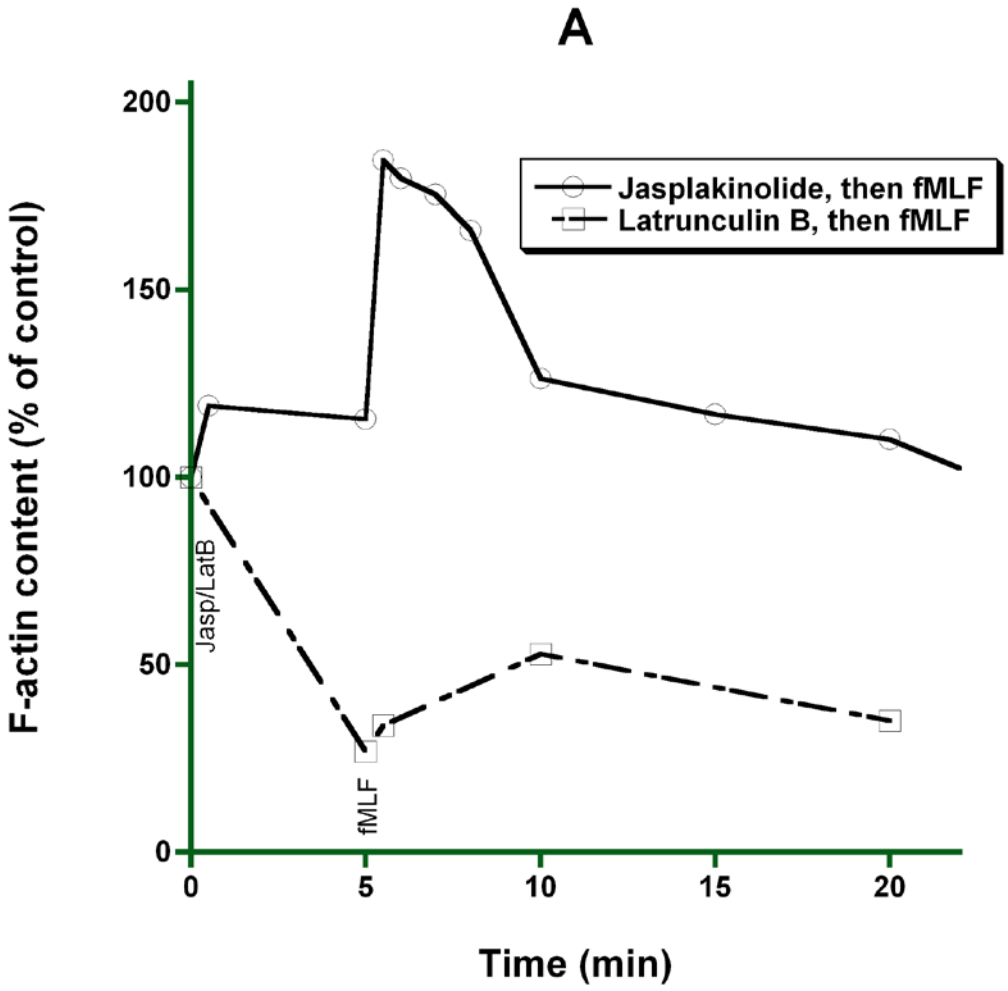

B
Chemiluminescence (CPM x 10^8)
control
1 nM
5 nM
10 nM
50 nM
100 nM
fMLF
jaspl.
Time (min)
0
1
2
3
4
5
0
10
20
30
40

Figure 3 A-B


A
F-actin content (% of control)
200
150
100
50
0
Jasplakinolide, then fMLF
Latrunculin B, then fMLF
Jasp/LatB
fMLF
0
5
10
15
20
Time (min)

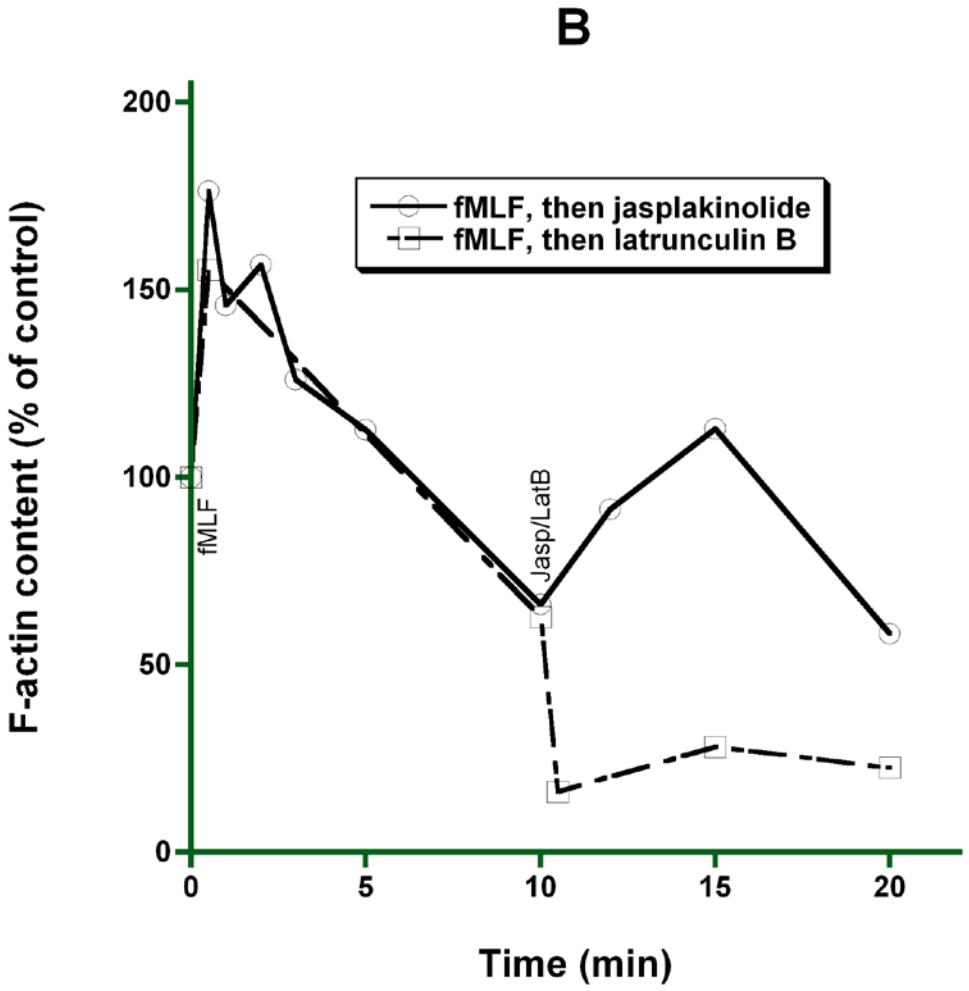
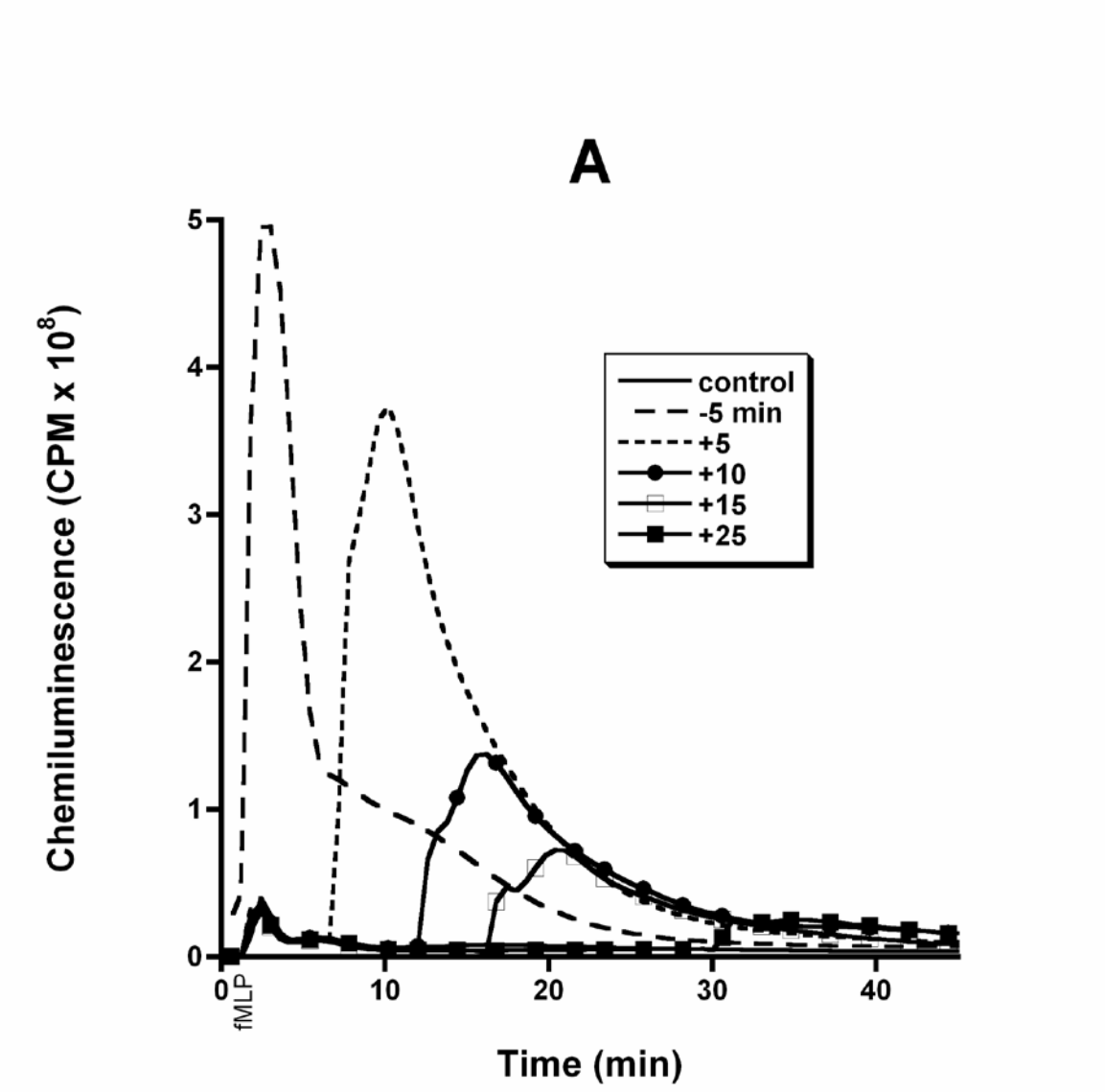

B
F-actin content (% of control)
200
150
100
50
0
fMLF
Jasp/LatB
fMLF, then jasplakinolide
fMLF, then latrunculin B
0
5
10
15
20
Time (min)

Figure 4 A-B


A
Chemiluminescence (CPM x 10^8)
5
4
3
2
1
0
0
fMLP
10
20
30
40
Time (min)
control
-5 min
+5
+10
+15
+25

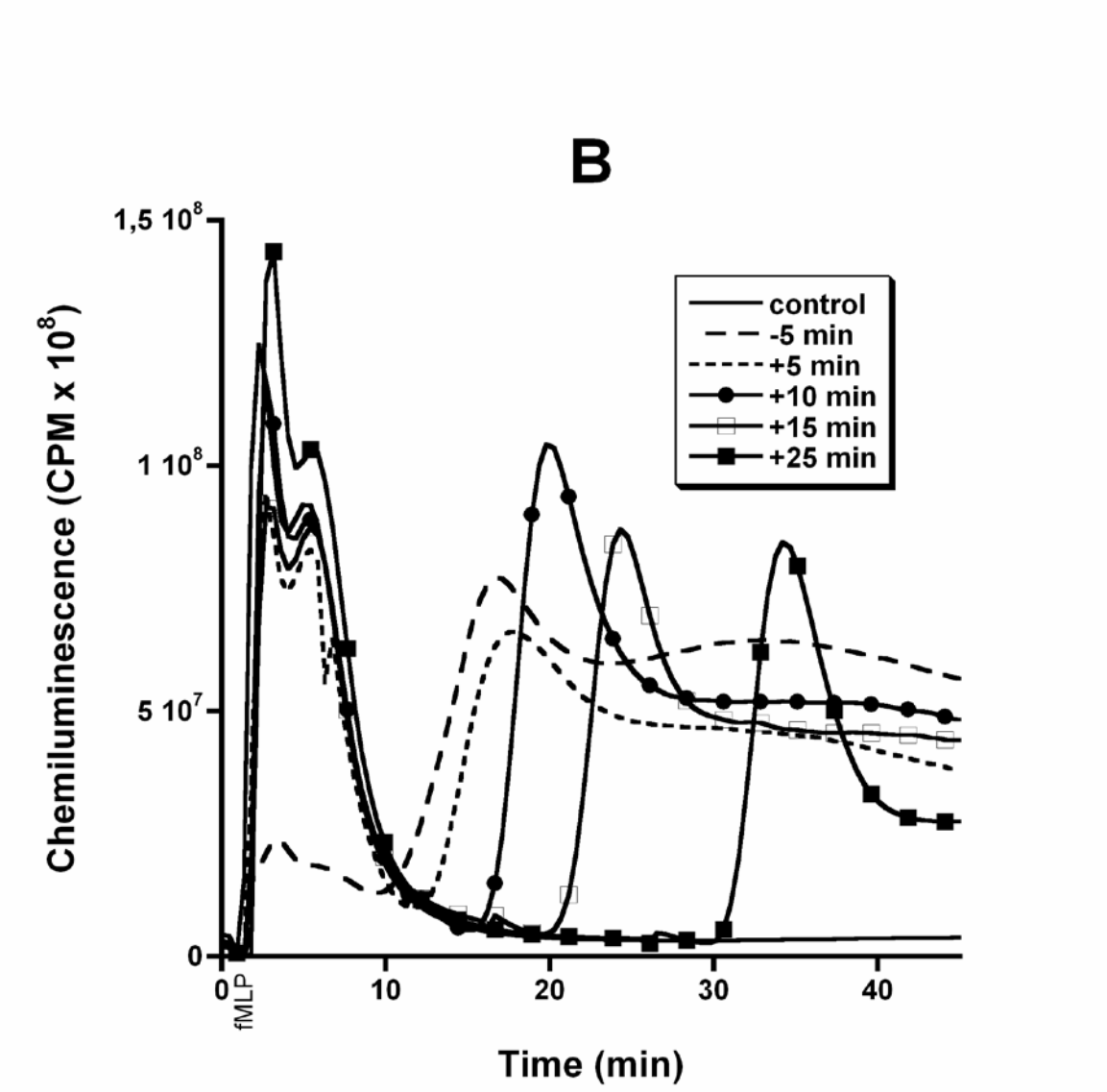

B
1,5 10^8
1 10^8
5 10^7
0
Chemiluminescence (CPM x 10^8)
control
-5 min
+5 min
+10 min
+15 min
+25 min
0
fMLP
10
20
30
40
Time (min)

Figure 5

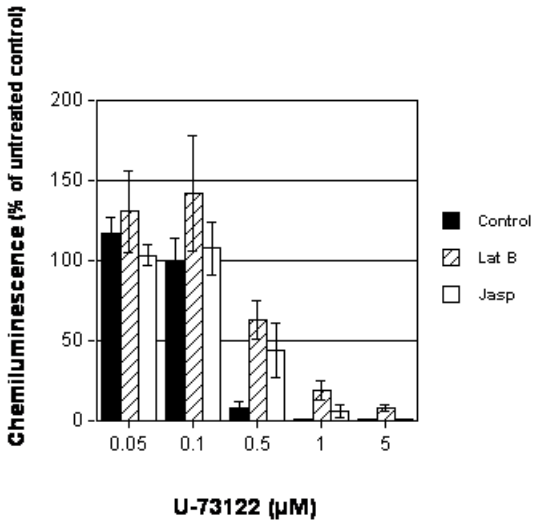

Chemiluminescence (% of untreated control)
200
150
100
50
0
0.05
0.1
0.5
1
5
U-73122 (µM)
Control
Lat B
Jasp

Figure 6

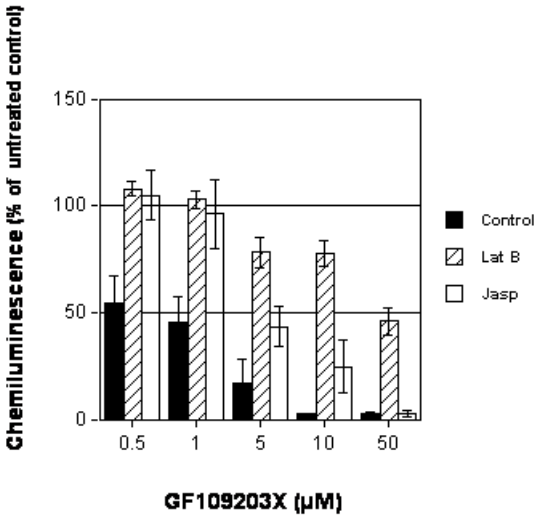

Chemiluminescence (% of untreated control)
150
100
50
0
0.5
1
5
10
50
GF109203X (µM)
Control
Lat B
Jasp

Figure 7

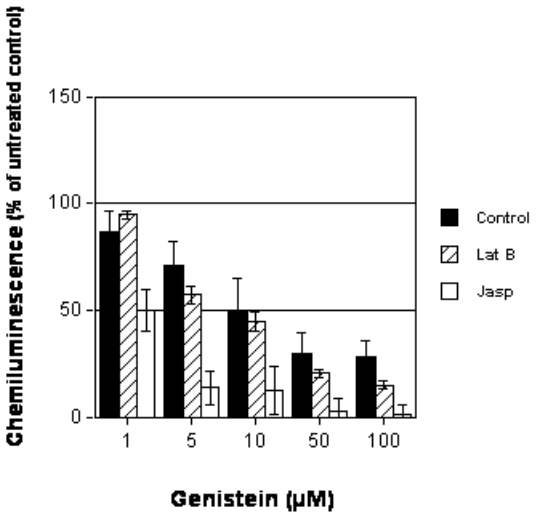
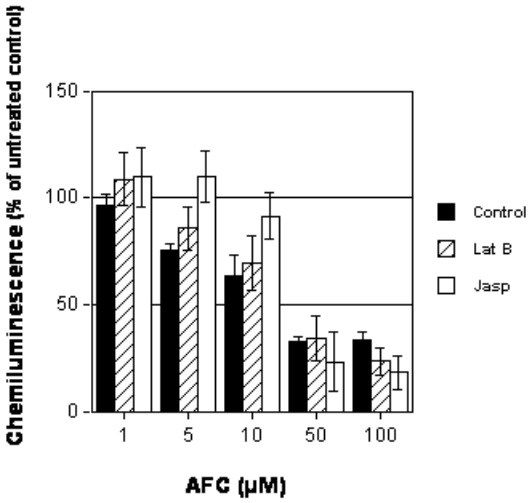

Chemiluminescence (% of untreated control)
150
100
50
0
1
5
10
50
100
Genistein (µM)
Control
Lat B
Jasp

Figure 8


Chemiluminescence (% of untreated control)
150
100
50
0
1
5
10
50
100
AFC (µM)
Control
Lat B
Jasp