To see a World in a Grain of Sand
And a Heaven in a Wild Flower,
Hold Infinity in the palm of your hand
And Eternity in an hour.

William Blake's "Auguries of Innocence"

University of Alberta

The role of Rac signalling and actin remodeling in the regulation of neutrophil exocytosis

by

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Dedication

I would like to dedicate this thesis to my family. They have been the driving force behind much of my success in life. Without their constant support, love and guidance I would have not accomplished as much as I have. I would especially like to honor my parents who provided me with the fundamentals needed to succeed in life. My father who taught me to not let the world bring you down and to try to look toward the brighter things in life. My mother who was taken from me too soon, I feel her absence everyday but the strength and ambition she exhibited in her life taught me to strive for the best in my own. Last but not least I would like to thank my brother who kept me grounded whenever I drifted too far from reality. Thank you for these gift I will cheish you always.

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Abstract

Neutrophils form part of the first line of defence in the innate immune system. Previous studies have shown a role for Rac in F-actin polymerization and primary granule exocytosis in neutrophils. This thesis describes how actin remodeling regulates Rac mediated primary granule exocytosis in human neutrophils and the potential role for select kinases in this process.

We performed experiments to determine the role Rac plays in the remodeling of actin for primary granule exocytosis. Using microscopic and biochemical analyses to examine various actin altering drugs, we found that there was a requirement for both depolymerization and polymerization of F-actin. Through the use of a small molecule Rac inhibitor we demonstrated that Rac regulates F-actin remodeling for granule translocation. We also examined the role Rac plays in modulating kinases. We found that numerous kinases had altered levels of phosphorylation that could be attributed to a lack of Rac activity.

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List of Abbreviations

A23187 - Calcium ionophore

BSA - Bovine serum albumin

CAMKII - Calmodulin kinase II

CB - Cytochalasin B

CHAPS - 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

COPD - Chronic obstructive pulmonary disease

DAB - 3,3'-Diaminobenzidine

DMSO - Dimethyl sulfoxide

EDTA - Ethylenediaminetetraacetic acid

ERK1 - Extracellular signal-regulated kinases 1

FBS - Fetal bovine serum

fMLF - Formyl-Met-Leu-Phe

FPR - Formyl peptide receptor

G-buffer - 5 mM Tris-Cl, pH 8, 0.2 mM $CaCl_2$, 0.2 mM ATP

G-CSF - Granulocyte-colony stimulating factor

GDP - Guanosine 5'-diphosphate

GEF - Guanine nucleotide exchange factor

GST-PBD - Glutathione S transferase-p21- binding domain

GTP - Guanosine 5'-triphosphate

HBSS - Hanks balanced salt solution

HBSS BG - Hanks balanced salt solution with BSA and glucose

H-buffer - 20 mM HEPES-KOH, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 60 mM NaCl, 1% Triton X-100 + PIC: 1 μg/ml each of leupeptin, pepstatin, antipain and aprotinin, 1 mM phenylmethylsulfonyl fluoride

HOCl - Hypochlorous acid

JP - Jasplakinolide

KN-93 - 2-[*N*-(2-Hydroxyethyl)]-*N*-(4 methoxybenzenesulfonyl) amino-*N*-(4 chlorocinnamyl)-*N*-methylbenzylamine

Lat B - Latrunculin B

LTF - Lactoferrin

MAP kinase - p38 mitogen-activated protein kinase

MEK1/2 - MAP-ERK kinase 1/2

MMP-9 - Matrix metalloproteinase 9

MPO - Myeloperoxidase

NSC23766 - Small molecule Rac inhibitor

PAK1/2/3 - p21 activated kinase 1/2/3

PBS - Phosphate buffered saline

PBS+ - Phosphate buffered saline with 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose and 0.1% BSA

PKC - Protein kinase C

PMA - phorbal 12-myristate 13-acetate

RBL-2H3 - Rat basophil leukemia cell line 2H3

TMB - 3, 3', 5, 5'- tetramethylbenzidine

1.0 Introduction

1.1 The polymorphonuclear neutrophil

Neutrophils, the most abundant human white blood cells, play an important role in an innate immune response, our primary defence against microbial infection. Upon activation, neutrophils rapidly migrate to sites of infection where they aid in pathogen clearing. Neutrophils can launch a two-pronged attack: 1. secretion of potent immune and inflammatory mediators, which are stored in abundant intracellular granules; this is also a means to sequester potentially harmful cytolytic enzymes; 2. phagocytosis of pathogens and infected cells.

Neutrophils are phagoctyic cells which are capable of ingesting micro-organisms to aid in defense (Nauseef *et al.,* 2007). During internalization of microbes, each of these phagocytic events results in creation of a phagolysosomes after intracellular fusion with granules which contains reactive oxygen species and various hydrolytic enzymes that aid in digestion of the microbes (Nauseef *et al.,* 2007). Therefore granules also are important in defence as they not only assist in formation of a phagolysosome but also assist in inate immunity. They achieve this by release of their antimicrobial and highly reactive mediators mediators during granule exocytosis

Neutrophils also undergo "respiratory burst" which is the consumption of oxygen to generate reactive oxygen species (Nauseef *et al.,* 2007). Respiratory bursts result in the activation of the enzyme NADPH oxidase, which produces large quantities of superoxide. Superoxide dismutases either spontaneously or through catalysis via an enzyme known as catalase, to hydrogen peroxide. Once hydrogen peroxide is produced it is then converted

to hypochlorous acid (HOCl) by the enzyme myeloperoxidase. Hypochlorous acid can than go on to cause tissue damage.

This thesis examines the mechanism of granule translocation to the plasma membrane and exocytosis, a process called degranulation in myeloid cells. Degranulation is highly regulated (Lacy, 2005); dysfunctional regulation or hyperactivation of degranulation in neutrophils can lead to many inflammatory disorders and is a prominent cause of immune dysfunction (Skubitz, 1999).

1.2 Neutrophils and disease

Neutrophilia (or neutrophil leukocytosis) is a condition of elevated numbers of neutrophil granulocytes in the blood. The accumulation and hyperactivation of neutrophils can result in fatal disorders such as in septic shock or acute respiratory distress (Elliott *et al.*, 2001). An example in which hyperactivation of neutrophils that can lead to deleterious effects is in the case of myocardial ischemia reperfusion injury. Myocardial ischemia-reperfusion induces an intense inflammatory condition in the myocardium that results in tissue destruction and compromised pump function (Braunwald *et al.*, 1985). Although the ischemic insult is initially responsible for the myocardial injury, additional damage occurs as a result of the reinstatement of coronary blood flow. The additional insult and propagation of inflammation occurs due to activation of multiple cell types, including endothelial cells and leukocytes such as neutrophils. Myocardial ischemia reperfusion injury attenuates the production of certain cardioprotective factors, such as nitric oxide. Under normal circumstances the inflammatory response is a result of intensified leukocyte-endothelial cell interactions via

enhanced adhesion molecule expression subsequent to myocardial reperfusion injury. Once at the site of inflammation, the activated neutrophils release reactive oxygen species and proteolytic enzymes.

Neutropenia is the absence of neutrophils. There are various forms of neutropenia; severe congenital, cyclic, idiopathic, myelokathexis, autoimmune as well as drug induced. Neutropenia often leads to overgrowth of normally resident skin and gut bacteria and fungi at sites of injury. It can also lead to opportunistic infections in exposed mucosal tissues. There are few ideal treatments for neutropenias however recombinant granulocyte-colony stimulating factor (G-CSF) can be effective in concert with chemotherapy for treatment of patients with congenital forms of neutropenia including severe congenital neutropenia, autosomal recessive Kostmann's syndrome, cyclic neutropenia and myelokathexis (Furukawa *et al.,* 1991).

1.3 Potential role in asthma and allergy

Neutrophils also play a role in asthma and allergy which makes their functionality important to study. Specifically, neutrophils like other leukocytes such as lymphocytes, eosinophils and macrophages are involved in the late phase response of allergy (Sampson, 2000). Chemoattractants such as histamine, various cytokines, interleukins, leukotrienes, and prostaglandins are released by activated mast cells and basophils during the acute phase. The release of these chemical mediators induces the migration of neutrophils to sites of allergen exposure. Knowledge of the role neutrophils have in the propagation of severe asthma as well as chronic obstructive pulmonary disease (COPD) has increased in recent years. During COPD exacerbations and cases of severe asthma the

release of IL-8 and leukotriene B_4 (LTB_4) are highly responsible for neutrophil recruitment to airways (McNulty *et al.,* 1999; Adams *et al.,* 1997). Neutrophils in the airways are implicated in severe damage. Among the many cytotoxic factors and enzymes released from neutrophils, MPO is released via primary granule exocytosis. MPO, although not the only component to induce tissue damage, does lead to both epithelial and lung parenchymal damage through formation of toxic hypochlorous acids and increases the production and release of cytokines (Klebanoff *et al.,* 2005). Release of elastase from primary granules facilitates tissue elastolysis, emphysema, and leads to mucus secretion from bronchial glands (Nadel *et al.,* 2000). Elastase along with matrix metalloproteinase (MMP)-9 contributes to airway remodeling that is often associated with fixed airflow limitation in COPD, as well as in long-standing asthma (Atkinson *et al.,* 2003).

1.4 Neutrophil exocytosis / degranulation

Human neutrophils contain four granule subtypes: primary/azurophilic granules, secondary/specific granules, tertiary/gelatinase granules, and secretory vesicles. Granule subtypes contain unique luminal contents (Table 1) and of these, primary granules sequester the most potent cytolytic enzymes such as elastase and myeloid peroxidase. These aid in digestion of pathogens, but also significantly contribute to host tissue damage (Hogg, 1987; Jaeschke; Smith, 1997). The mechanisms that control exocytosis of these granule subtypes appear to be unique to each population. Granules are released in a hierarchical fashion in a graded response to secretagogue and calcium

Mediator Release in Neutrophils

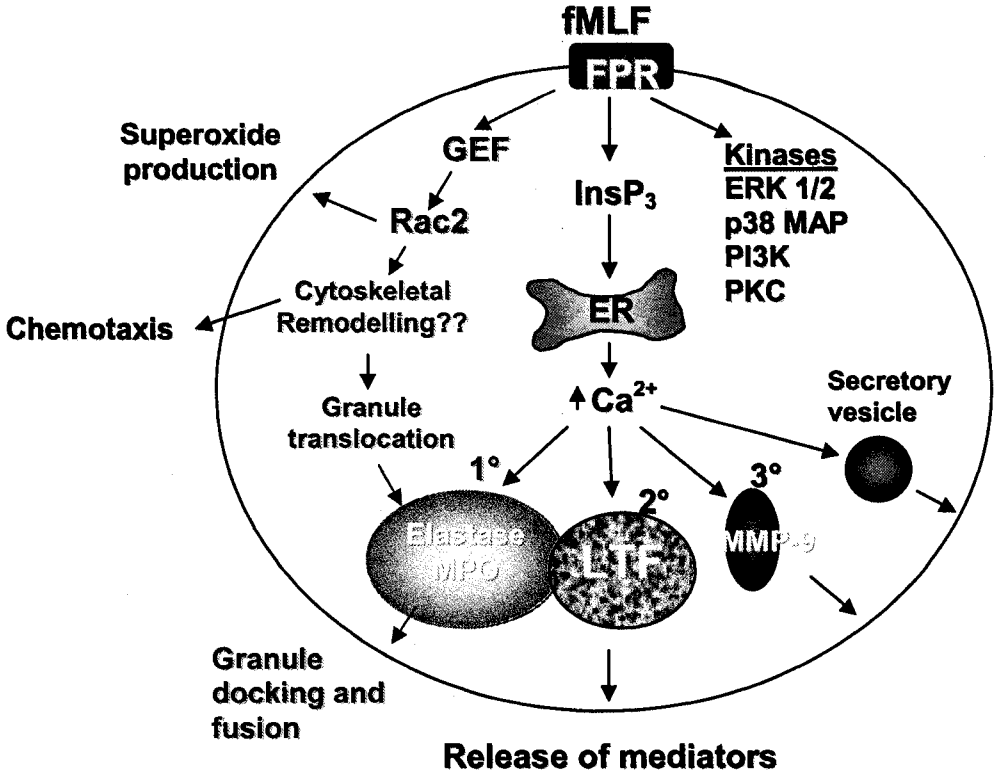

Figure 1 Schematic diagram of neutrophil mediator release signalling pathways
This diagram details possible and proposed signalling pathways involved in stimulation of the formyl peptide receptor (FPR) of the neutrophil. When a bacterial peptide (fMLF) binds to the FPR it results in the activation of a signalling cascade. This leads to proposed activation of GEFs, various kinases and the calcium signalling pathway. Activation of GEFs results in the activation of Rac2 which is able to trigger superoxide production as well as cytoskeletal remodelling. The activation of cytoskeletal remodelling not only is necessary for chemotaxis, but also proposed to be necessary for granule translocation. How other signalling pathways triggered by the initial binding of fMLF to the receptor feed into the granule translocation pathway is unclear, however it has been reported that these pathways are integral to the regulation of granule exocytosis.

Table 1

NEUTROPHIL GRANULE CONSTITUENTS

Primary (azurophilic)	Secondary (specific)	Tertiary (gelatinase)	Secretory vesicles
Myeloperoxidase	Lysozyme	Gelatinases (MMP-9)	Complement receptor 1
Lysozyme	Lactoferrin	Plasminogen activator	Complement receptor 3
Defensins	Collagenase	Cathepsin B	CD11b/CD18
Bacterial permeability increasing protein	Complement activator	Cathepisn D	Formyl peptide receptor
Azurocidin	Phospholipase A2	β-D-Glucyronidase	CD14
Elastase	CR3	α-mannosidase	CD16
Cathepsin G	CR4	cytochrome b558	
Proteinase 3	fMLF receptors	Leukolysin	
Cathepsin B	Laminin receptors	Lysozyme	
β-D-Glucuronidase	Cytochrome *b*558	Natural-resistance-associated macrophage protein 1 (NRAMP)	
α-mannosidase	Monocyte-chemotactic factor		
Phospholipase A2	Histaminase		
Chondroitin-4-sulphate	Vitamin B12 binding protein		
	Leukolysin		

(Borregaard *et al.*, 1997)

levels. Initially, secretory vesicles are released, followed by tertiary granules, secondary granules, and finally primary granules (Sengelov *et al.,* 1993). Primary granule release is specifically regulated by the Rho GTPase, Rac2 (Abdel-Latif *et al.,* 2004) (Fig.1).

1.5 Regulation of neutrophil exocytosis

1.5.1 Role of Rho GTPases

Rho GTPases have been shown to regulate exocytosis in a variety of immune cells including mast cells (Brown *et al.,* 1998; Khurana *et al.,* 2003), cytotoxic T lymphocytes (Billadeau *et al.,* 1998) and neutrophils (Lacy, 2005). However, the precise role that Rho proteins plays in exocytosis remains unclear. Rho GTPases, such as Rac2, are key regulators of cytoskeletal remodelling. This was clearly demonstrated in their ability to activate cytoskeletal remodelling for chemotaxis (Norman *et al.,* 1996). More recently, the Rho GTPase, Cdc42p, was shown to facilitate exocytosis in PC12 cells by promoting actin polymerization through WASP (Gasman *et al.,* 2004). Importantly, neutrophils deficient in Rac2 have defects in filamentous (F-) actin assembly which prevents cell migration, and this was distinct from Rac1 deficiency which led to inhibition of cell spreading (Gu *et al.,* 2003; Filippi *et al.,* 2004). Recently, a membrane permeable small molecule inhibitor of Rac1 NSC23766 was discovered (Gao *et al.,* 2004). Its discovery was based on structural features of the activator binding sites of the guanine exchange factors (GEFs) Trio and Tiam1. Rac activation assays of mouse bone marrow neutrophils showed this drug effectively disrupted Rac1 and Rac2 activation (Cancelas *et al.,* 2005).

1.5.2 Role of actin remodelling in neutrophils

Actin remodelling is implicated as a downstream reaction catalyzed by effector molecules activated during receptor-mediated exocytosis. Actin seems to form a mesh-like network around the periphery in many different kinds of secretory cells such as neutrophils, mast cells, neurons and endocrine cells. This may act as a barrier preventing aberrant granule docking and fusion at the plasma membrane. Thus, in order for exocytosis to occur, it is hypothesized that this actin network must be disassembled (Norman *et al.,* 1994; Muallem *et al.,* 1995; Takao-Rikitsu *et al.,* 2004). Contrary to this, some studies in neutrophils suggest that F-actin is normally diffuse in resting neutrophils, and only assembles into a cortical ring upon FPR stimulation (Downey *et al.,* 1997; Fillipi *et al.,* 2004). However, these studies used live neutrophils that were first adhered to poly-L-lysine-coated glass slides, a process that may have led to neutrophil activation before stimulation with fMLF and cell fixation. Thus it is likely that this procedure may induce F-actin remodelling prior to stimulation.

There are numerous studies that identify actin remodelling as being responsible for directing neutrophil migration (Affolter *et al.,* 2005). Coordinated neutrophil chemotaxis towards sites of infection is guided by the formation of polarized F-actin. Cells appear to have a "sidedness" with the leading edge highly concentrated in F-actin. Interestingly, neutrophils produce a polarized response even when subjected to uniform concentrations of chemoattractants, and maintain this polarized stated via continued production of activated effector molecules such as 3'-phosphoinositol lipids in the leading edge of the cell membrane (Zigmond *et al.,* 1981; Xu *et al.,* 2003).

A comprehensive study of neutrophil exocytosis revealed actin associations for all major granule types (Jog *et al.,* 2007). Indeed, primary, secondary and tertiary granule

exocytosis from isolated neutrophils is stimulated several fold by pre-incubation of cells with cytochalasin B, which favors an actin-barrier hypothesis (Jog *et al.,* 2007). The actin-barrier hypothesis suggests that in the resting state, neutrophils have a network of cortical actin that may prevent unregulated exocytosis of specific granule types. Depolymerization of this network would therefore be needed to allow large granules to dock and fusion at the plasma membrane.

1.5.3 Role of kinases

Phosphorylation is a key event in neutrophil activation leading from plasma membrane receptor stimulation to granule exocytosis. Stimulation through the FPR by fMLF leads to phosphorylation of a wide range of kinases, which then activate their respective effector pathways such as *src* family kinases which have been implicated in neutrophil exocytosis. There are three *src* family members, Hck, Fgr, and Lyn that are expressed in neutrophils and activated by fMLF receptor stimulation. Hck translocates to the primary granule population following cell activation (Mohn *et al.,* 1995), while Fgr translocates to secondary granules during exocytosis (Gutkind *et al.,* 1989). Human neutrophils exposed to the *src* family inhibitor PP1 show reduced exocytosis of primary granules, secondary granules, and secretory vesicles in response to fMLF (Mocsai *et al.,* 2000). Neutrophils isolated from $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice also showed deficient secondary granule release of lactoferrin, however primary granule release of β-glucuronidase from murine cells could not be determined (Mocsai *et al.,* 2000). The deficiency that was seen in secondary granule release in neutrophils isolated from the triple kinase knockout mice correlated with a reduction in p38 mitogen-activated protein (MAP) kinase activity. Also, treatment of human neutrophils with the p38 MAP kinase

inhibitor SB203580 led to reduced primary and secondary granule exocytosis in response to fMLF (Mocsai *et al.,* 2000). These findings indicate that *src* kinases Hck, Fgr, and Lyn, along with p38 MAP kinases play a role in regulating the release of granules in response to fMLF stimulation in neutrophils, and probably act at an early signalling step proximal to the FPR in this process.

The role of calmodulin kinase II (CAMKII) has been studied in mast cells and shown to be important in exocytosis from these cells. CAMKII is a serine/threonine kinase. It has an N-terminal catalytic domain, a regulatory domain, and C-terminal protein association domain. In the absence of calcium/calmodulin, the catalytic domain is autoinhibited by the regulatory domain, which contains a pseudo-substrate sequence. Autophosphorylation of threonine-286 occurs in the presence of calcium and calmodulin and activates CAMKII (Takeuchi-Suzuki *et al.,* 1992; Yang *et al.,* 1999). The specific CAMKII inhibitor, KN-93, inhibited exocytosis (Funaba *et al.,* 2003).

1.5.4 Role of Calcium

It is possible to elicit granule release from neutrophils when the levels of intracellular calcium are increased (graded response). This alone is sufficient to induce the exocytosis of granules from neutrophils, and is often achieved by using calcium ionophores such as A23187 or ionomycin to obtain the high concentration required. The activation of many receptors on the neutrophil surface, including the FPR, can lead to elevated calcium levels. Several candidates have been suggested to be targets of calcium signalling such as annexins, protein kinase C, and calmodulin, all of which bind calcium to modulate their activities (Brown *et al.,* 1991; Sjolin *et al.,* 1994; Steadman *et al.,*1996;

Donnelly and Moss, 1997; Haribabu *et al.,* 2000). Neutrophils have been shown to require these calcium binding proteins for granule translocation and exocytosis. Although these are potential candidates, the exact role of this crucial messenger in exocytosis has yet to be fully elucidated.

1.6 Regulation of exocytosis by actin and Rho in other secretory cells

Studies of exocytosis in many different kinds of secretory cells have defined a regulatory role for the actin cytoskeleton in granule translocation (Valentijn *et al.,* 1999; Burgoyne and Morgan, 2003; Malacombe *et al.,* 2006). Myeloid cells contain an F-actin-rich cortical region which is deemed to be a barrier preventing granule docking and fusion at the plasma membrane, and existing in equilibrium with cytoplasmic G-actin. However, other studies have shown that actin depolymerization inhibits exocytosis, suggesting that actin polymerization instead facilitates exocytosis (Lang *et al.,* 2000; Jahraus *et al.,* 2001; Eitzen *et al.,* 2002; Gasman *et al.,* 2004; Yu and Bement, 2007). This hypothesis seems quite plausible in neutrophils since their migration towards a stimulus (i.e. microbial peptide gradient) is triggered by polarized F-actin assembly, which could similarly drive polarized mobilization of granules on this actin network (Norman *et al.,* 1996; Glogauer *et al.,* 2000).

1.7 Guanine exchange factors (GEFs)

GEFs are upstream molecules which activate Rho proteins such a Rac by stimulating the exchange of GDP for GTP. GEFs are believed to provide a direct link between Rho activation and cell-surface receptors for various cytokines, growth factors, adhesion molecules, and G protein–coupled receptors (Zheng *et al.,* 2001). Therefore,

understanding how GEF signalling to Rac activation accounts for its various role within the neutrophil is of great importance. There is evidence to suggest that different GEFs may direct specific Rac function. With regards to neutrophil chemotaxis, it has been shown that the GEF, DOCK2, is increasingly important. In DOCK2 deficient neutrophils, chemoattractant-induced activation of Rac was diminished. Loss of polarized accumulation of F-actin and phosphatidylinositol 3,4,5-triphosphate (PIP_3) at the leading edge was also observed (Kunisaki *et al.,* 2006). The Rac GEF, P-Rex1, serves as a critical regulator of chemoattractant-stimulated neutrophil motility and ROS production. Knockout studies as well as the use of antisense oligonucleotides to P-Rex1 have shown a severe deficiency in chemoattractant-stimulated ROS formation as well as reduction of cell speed during chemotaxis (Welch *et al.,* 2002 and 2005, Dong *et al.,* 2005). Vav GEFs have been shown to be required for multiple $ß_2$ integrin-dependent functions, including sustained adhesion, spreading, and complement-mediated phagocytosis in neutrophils (Gakidis *et al.,* 2004). Also fMLP-mediated NADPH oxidase generation in neutrophils was shown to be partially dependent on Vav1 (Kim *et al.,* 2003). Although, no specific GEF has been shown to regulate neutrophils exocytosis, it seems reasonable to hypothesize that one does exist. Recent studies using the small molecule Rac inhibitor, NSC23766, suggest that it likely blocks binding sites of the GEFs Trio and Tiam-1 in human prostate cancer PC-3 cells (Gao *et al.,* 2004). Although Trio and Tiam-1 have yet to be identified in neutrophils the possibility exists that this inhibitor may effect Rac-mediated functions within neutrophils. Identifying a Rac GEF in neutrophils responsible for exocytosis would significantly contribute to our understanding of this complicated process.

1.8 Rationale for this study

A hypothesis that suggests a role for both actin depolymerization and polymerization during exocytosis - perhaps within specific sub-reactions of the exocytosis mechanism - would best encompass all of the current information on how actin regulates exocytosis. For example, actin polymerization may be required in the cell cytoplasm to direct granules to the cell membrane, while depolymerization must occur concurrently at the cell cortex to allow exocytosis. In this study we examined the hypothesis that specific regions of actin remodelling are required for exocytosis through a comprehensive examination of pharmacological reagents that disrupt actin remodelling.

2.0 Material and Methods

2.1 Materials

Material	Source
0.1% bovine serum albumin (BSA)	Sigma-Aldrich, Oakville, ON
0.25 M sucrose	Sigma-Aldrich, Oakville, ON
2% paraformaldehyde	Sigma-Aldrich, Oakville, ON
A23187	Sigma-Aldrich, Oakville, ON
Alexa Fluor 488 succinimidyl ester	Invitrogen, Burlington, ON
Anti-CD63 mouse monoclonal	Serotec, Raleigh, NC
Anti-Rac1 mouse monoclonal	BD Bioscience, San Jose, CA
Anti-Rac2 rabbit polyclonal	Gift from Dr. Gary Bokoch
Beckman DU 640 spectrophotometer	Beckman Instruments, Mississauga, ON
C57Bl/6 (WT) mice	Charles River Canada Saint-Constant, PQ
CB	Sigma-Aldrich, Oakville, ON
CHAPS	Sigma-Aldrich, Oakville, ON
DMSO	Sigma-Aldrich, Oakville, ON
EDTA	Invitrogen, Burlington, ON
FBS	Invitrogen, Burlington, ON
Ferricytochrome *c*	Sigma-Aldrich, Oakville, ON
fMLF	Sigma-Aldrich, Oakville, ON
G-buffer	Cytoskeleton Inc., Denver, CO

HBSS	Invitrogen, Burlington, ON
Histopaque-1119	Sigma-Aldrich, Oakville, ON
IRDye800 secondary antibody	Rockland Immunochemicals, Gilbertsville, PA
JP	Calbiochem, San Diego, CA
KN-93	Calbiochem, San Diego, CA
Lat B	Calbiochem, San Diego, CA
mortar and pestle	Canadawide Scientific, Ottawa, ON
NSC23766	Calbiochem, San Diego, CA
Odyssey image analysis system	LiCor, Seattle, WA
Olympus FV1000 confocal microscope	Olympus Canada, Markham, ON
100% Percoll	GE Healthcare, Baie d'Urfe, PQ
Osmium tetroxide	Sigma-Aldrich, Oakville, ON
PBS	Fisher Scientific, Toronto, ON
PMA	Sigma-Aldrich, Oakville, ON
RPMI-1640	Invitrogen, Burlington, ON
Spectrophotometer	Molecular Devices, Sunnyvale, CA
Spectrofluorimeter	Photon Technologies Inc., London ON
TMB	Sigma-Aldrich, Oakville, ON
Trypan Blue	VWR, Mississauga, ON

2.2 Animals

Rac2 knockout (*rac2* $^{-/-}$) mice were previously generated by targeted disruption of the *RAC2* gene (1) and were backcrossed into C57Bl/6 mice for more than 11 generations. Wild type C57Bl/6 (WT) mice were purchased from Charles River Canada (Saint-Constant, PQ). Animals were housed under specific pathogen-free conditions and fed autoclaved food and water *Ad libitum*. Mice used in experiments were between 4-8 weeks of age.

2.3 Isolation of murine bone marrow neutrophil

Isolation of bone marrow neutrophils (BMN) was performed via removal of the femur and tibia of WT and *rac2* $^{-/-}$ mice. Bones were immersed in a solution of Hanks' balanced salt solution with 0.1% BSA and 5 mM glucose (HBSS-BG). Following immersion in HBSS-BG, cells were flushed from the femurs and tibias with 3 ml of HBSS-BG using a 22-gauge needle. The filtrate was pelleted at 300*g* for 10 min at 4°C. Ninety percent Percoll stock solution was made by mixing 9 vol of Percoll to 1 vol of 10X HBSS-BG to adjust osmolarity of the Percoll. Following preparation of Percoll the following gradients were prepared by mixing with HBSS-BG to obtain Percoll layers of 45%, 50%, 55%, 62% and 81% from the diluted stock. After centrifugation the pelleted cells were resuspended in 45% Percoll and then layered onto successive gradients consisting of 3 ml of 81%, 2 ml of 62%, 2 ml of 55% and 2 ml of 50% Percoll and centrifuged at 600*g* for 30 min at 10°C. The neutrophil-containing layer contaminated with red blood cells located between the 81% and 62% steps were harvested using a plastic Pasteur pipette and washed twice in 10 ml of HBSS-BG by centrifugation at 300*g* for 10 min at 4°C. Following the wash step, cells were resuspended in 3 ml of HBSS-BG

and were layered over 3 ml of Histopaque-1119 and centrifuged at 600*g* for 30 min at 10°C to remove remaining red blood cells. The purified neutrophil cell layer was located between Histopaque-1119 and HBSS-BG was collected and washed twice in 10 ml HBSS-BG at 300*g* for 10 min at 4°C. The neutrophils were resuspended in colour-free RPMI-1640 and counted by using Kimura stain (11 ml of 0.05% toluidine blue, 5 ml of PBS pH 6.4, 0.8 ml of 0.03% light green dye and 0.5 ml of saturated saponin solution) which allows for differential lymphocyte counting. Neutrophil purity was between 80-85% as assessed by nuclear morphology with the remaining contaminating cells being mononuclear cells.

2.4 Isolation of human peripheral blood neutrophils

Human polymorphonuclear neutrophils were isolated from healthy donors (Lacy et al., 2003). Briefly, 50-100 ml of whole blood was drawn from donors and mixed with 6% dextran in RPMI 1640 and incubated at room temperature for 30 min to allow for separation of red blood cells from leukocytes. The leukocyte suspension was then layered onto a 100% Ficoll cushion (15 ml) and centrifuged at room temperature at 400*g* for 30 min to separate leukocytes, monocytes and granulocytes. Following centrifugation, the leukocyte, monocyte and Ficoll layers were removed and the cell pellet with granulocytes was exposed to 1.5 ml of sterile deionized water for 20 s to lyse red blood cells. Cell pellets containing neutrophils were quickly placed into excess buffer A (RPMI-1640 and 5 mM EDTA) and centrifuged at room temperature at 300*g* for 5 min. Following centrifugation the supernatant was removed and the cell pellet was resuspended in buffer B (RPMI-1640, 5 mM EDTA and 2% FBS). Cells were then allowed to rest on ice for 1

h before experiments, allowing cells to return to a resting state in case activation occurred during isolation.

2.5 Secretion assays

Secretion assays were performed as previously described (Abdel-Latif et al., 2004). Briefly, cells were resuspended at 1 x 10^6 cells/ml in phenol red–free RPMI 1640, and 50 µl of cell suspension was added to each well of a 96 v-well plate containing the various drugs to be examined in RPMI 1640 to a total volume of 250 µl. Latrunculin B (Lat B; destabilizes F-actin), jasplakinolide (JP; stabilizes F-actin) and the small molecule Rac inhibitor NSC23766 were dissolved in DMSO. Neutrophils were pretreated with these drugs for 15 min at 37°C prior to stimulation with 2.5 µM calcium ionophore A23187 or 10 µM cytochalasin B (CB) for 5 min followed by 5 µM fMLF for 15 min (CB/fMLF) for 15 min at 37°C to induce degranulation. Following this, the microplate was centrifuged at 300*g* at 4°C for 6 min to pellet cells. The supernatant was removed for determination of degranulation. Degranulation of neutrophils was determined by measurement of granule-derived mediator release.

2.6 Rac activation assays

Activated (GTP-bound) Rac1 and Rac2 were affinity precipitated from neutrophil lysates using GST-PBD (Benard et al., 1999). This is essentially a pull down assay in which the Rac binding domain of p21 activated kinase (PBD) is used as a probe. This binding domain only interacts with activated Rac. Lysates were prepared from 8 x 10^6 neutrophils by sonication in 400 µl of H-buffer (20 mM HEPES-KOH, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 60 mM NaCl, 1% Triton X-100 + PIC: 1 µg/ml each of leupeptin,

pepstatin, antipain and aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell debris was then removed using centrifugation and 200 μg of lysate was incubated with 30 μg of immobilized GST-PBD. These were then incubated in 400 μl H-buffer for 30 min at 4°C. The bead pellet was then washed four times with H-buffer and suspended in 45 µl of Laemmli sample buffer. 15 µl of each sample was analyzed by immunoblot for Rac1 (AB4202) and Rac2 (a gift from G. Bokoch) specific antibodies. Immunoblots were detected using IRDye800 fluorescently-tagged secondary antibodies and an Odyssey image analysis system (LiCor, Seattle, WA).

2.7 Measurement of O_2^- release from neutrophils

Generation of extracellular O_2^- from cells in suspension was measured as previously described (Lacy et al., 2003). Briefly, cells (1-2 x 10^6) were suspended in 1 ml microcuvettes containing supplemented PBS (PBS^+, pH 7.4, with 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose, and 0.1% BSA) and 50 µM ferricytochrome *c* at 25°C. The mixture was blanked at 550 nm in a Beckman DU 640 spectrophotometer before adding PMA at a dose of 10 ng/ml or fMLF at a dose of 5 µM. This solution should be slightly red due to oxidized ferricytochrome *c*, which is detected spectrophotometrically. As O_2^- production occurs, ferricytochrome *c* is reduced detected as the loss of signal at 550 nm. To test the effect of NSC23766 on O_2^- production, 160 µM of NSC23766 was added to 2 x 10^7 cells/ml in RPMI 1640, incubated at 37°C for 15 min before treatment with PMA or fMLF.

2.8 Measurement of primary granule exocytosis

Myeloperoxidase release (MPO), an enzyme specific to primary granules, was assayed by using tetramethylbenzidine (TMB) as previously described (Lacy et al., 1999). Briefly, 150 µL TMB substrate solution was added to 50 µL of sample and incubated at room temperature for 15- 30 min prior to termination of the reaction with 50 µL 1 M H_2SO_4. Plates were read spectrophotometrically at 450 nm. Absorbance values for released MPO were divided into the average of values from an equivalent number of 0.5% CHAPS-lysed cells to give percentage of total cellular mediator released.

2.9 Confocal microscopy

Samples for confocal analysis were prepared by treatment of cells in suspension with drugs for 15 min, followed by stimulation of secretion. Cells were then fixed in freshly prepared 2% paraformaldehyde in 0.25M sucrose while still in suspension to maintain cell integrity. Cells were adhered to glass slides coated with poly-L-lysine, permeabilized by incubation with 0.5% Triton-X100 in PBS and stained using a 1:500 dilution of anti-CD63 conjugated to Alexa Fluor 488 to detect primary granules. F-actin was detected using a 1:1000 dilution of rhodamine-phalloidin. Images were acquired on an Olympus FV1000 confocal laser scanning microscope, with a 63X/1.4 N.A. plan apochromat objective and processed using Fluoview software.

2.10 Actin polymerization assay

To assay cellular activity inducing actin polymerization, we used an established pyrene-actin polymerization assay (Cooper and Pollard, 1982). Briefly, 12 µg of

neutrophil lysate in 70 µl lysis buffer (5 mM Tris-Cl, pH 8, 17 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.17% NP-40, 0.35 mM $MgCl_2$ + PIC) was mixed with 50 µl of 10 µM actin polymerization stock mixture containing 35% pyrene-labeled actin in G-buffer (5 mM Tris-Cl, pH 8, 0.2 mM $CaCl_2$, 0.2 mM ATP). Fluorescence intensity readings were taken every 18 s using a PTI QM-4SE spectrofluorimeter (Ex 360 nm/Em 407nm, 10 nm bandwidth, 2 s integration), with a four-position heated sample holder set to 30°C. Baseline fluorescence of the pyrene-actin stock mixture was taken for ~5 min, test samples were then added and measurements continued for 1 h. Actin polymerization activity (A.P.A.) was calculated from polymerization curves by determining the average rate of fluorescence intensity increase for 3000 s of reaction time (Isgandarova et al., 2007), divided by the micrograms of test sample protein (µFI/µg). A.P.A. values were normalized to untreated resting cells for each experiment with the A.P.A. of the lysis buffer alone subtracted.

2.11 Electron microscopy

Neutrophils were examined by electron microscopy after brief stimulation or drug treatments as described in the results. Treated cells were cooled on ice, re-isolated by centrifugation (1000g, 1 min) and fixed by incubation overnight in 2.5% glutaraldehyde, 0.1 M cacodylate, pH 7.2 at 4°C. Fixed cells were stained by DAB method to enhance peroxidase containing vesicles (5 min incubation in 2.5 mM diaminobenzidine, 0.02% hydrogen peroxide, 0.1 M cacodylate, pH 7.2 at room temperature). Samples were washed three times in 0.1 M cacodylate, pH 7.2, embedded in 1% ultrapure agarose and post-fixed in 0.2% aqueous osmium tetroxide for 1 h at 4°C. Samples were embedded in Epon resin after serial dehydration by incubation in 60%, 80%, 95% and 100% ethanol,

propylene oxide and 50% propylene oxide Epon resin. Ultrathin sections were cut from cured resin-embedded cells, mounted on copper coated grids and stained with saturated uranyl acetate and lead citrate. Samples were viewed on a Philips 410 transmission electron microscope and images were acquired using an AnalySIS camera and software.

2.12 Flow cytometry

Samples for flow cytometry were prepared and stimulated in similar fashion to those prepared for the *in vitro* actin polymerization. Samples were fixed in 4% paraformaldehyde for 30 min on ice. Following fixation samples were permeabilized by incubation with 0.5% Triton-X100 in PBS and stained using a 1:500 dilution of anti-CD63 conjugated to Alexa Fluor 488 to detect primary granules. F-actin was detected using a 1:1000 dilution of rhodamine-phalloidin. Sample volume was approximately 500 µl. Samples were then measured for differences F-actin fluorescence as well as granularity via detection of anti-CD63 labelled primary granules.

2.13 Calculations and statistical analysis

Data was analyzed by one-way statistical analysis of variance (ANOVA) and *post-hoc* analysis was determined by Tukey's post test. The data are depicted in figures as means plus or minus the standard error of the mean (± SEM).

3.0 Results

3.1 Stimulation of neutrophil exocytosis via actin remodelling

Bacterially-derived N-formyl methionyl derivatives (i.e. fMLF) are potent stimuli for human neutrophils, however, *in vitro* stimulation of primary granule exocytosis from neutrophils requires "priming" with an actin depolymerizing agent such as CB. CB blocks the barbed ends of actin filaments (Cooper, 1987) and leads to actin meshwork disassembly, mimicking surface binding in neutrophils and enhancing fMLF-induced primary granule release (Showell *et al.,* 1976; Henson *et al.,* 1978). This well-known observation in neutrophils suggests that primary granules require F-actin depolymerization for their secretion. We hypothesized that similar drugs which destabilize F-actin will stimulate primary granule exocytosis when combined with fMLF, and that stabilizing F-actin should have the opposite effect. We found that latrunculin B (Lat B), a marine sponge toxin that acts by sequestering monomeric actin and consequently stimulates actin depolymerization (Spector *et al.,* 1989), stimulated primary granule exocytosis at much lower doses than CB (Fig. 2). However, at high doses of Lat B or CB (> 10 μM), primary granule exocytosis was inhibited. This result suggests that partial actin depolymerization is needed via low drug dosage, while complete F-actin depolymerization blocks exocytosis. Cell viability was not altered at high dosages as determined by trypan blue staining and confocal microscopy. Jasplakinolide (JP), which binds to and stabilizes F-actin (Bubb *et al.,* 1994), did not stimulate exocytosis at any dose tested (Fig. 2).

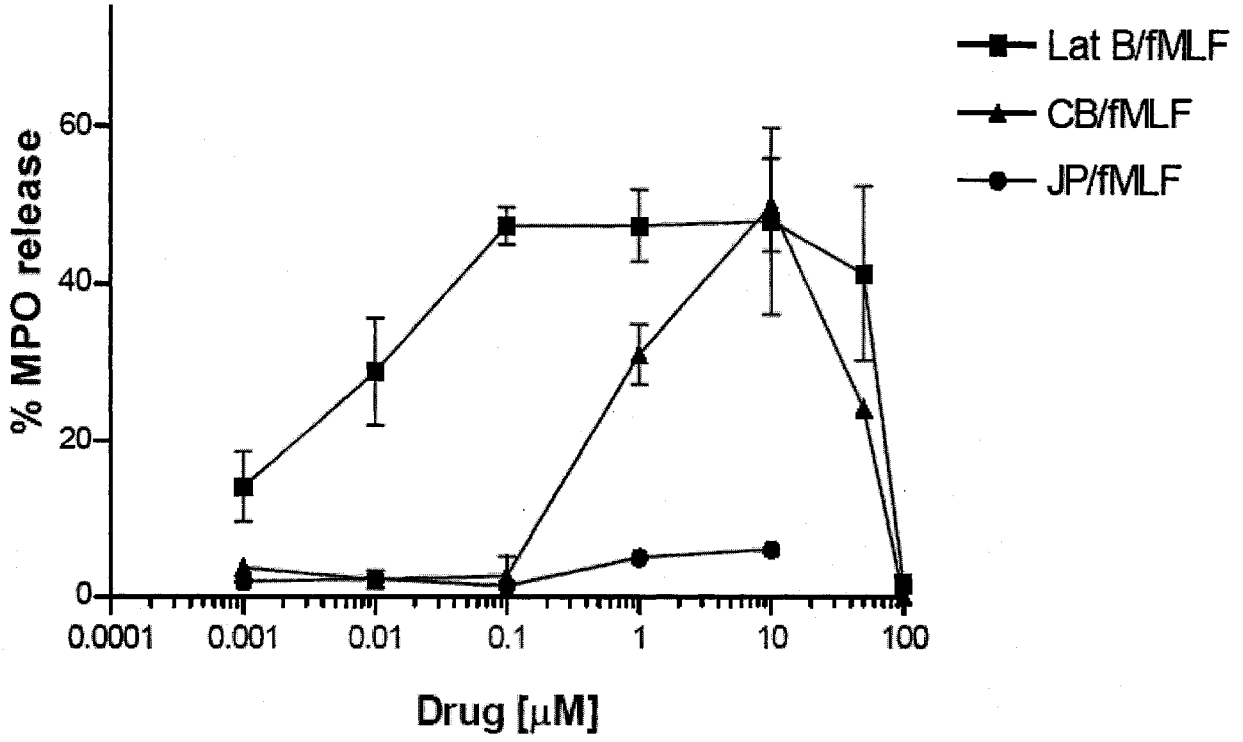

Figure 2 Comparison of the effects of actin drugs on fMLF-stimulated neutrophil exocytosis.
Neutrophils were preincubated with increasing concentrations of either CB, Lat B, or JP for 5 min followed by stimulation with 5 μM fMLF for 15 min at 37°C. Extracellular supernatants were collected and assayed for MPO activity. Released MPO was calculated as a percentage of total activity (± SEM) from at least 3 independent experiments (except for JP, where $n = 1$). $P < 0.01$ for all repeated experiments compared to fMLF alone.

3.2 Inhibition of neutrophil exocytosis via actin remodelling

To further investigate the role that the actin cytoskeleton plays in the regulation of primary granule exocytosis, we examined the effect of drug combinations on either CB/fMLF or calcium ionophore (A23187)-stimulated exocytosis. Based on data indicating the importance of actin depolymerization for exocytosis via CB priming (Fig. 2; Showell *et al.,* 1976; Henson *et al.,* 1978; Abdel-Latif *et al.,* 2004), we hypothesized that further destabilization of F-actin might increase primary granule exocytosis, while stabilization of F-actin would suppress exocytosis. We found that addition of 0.5 - 10 μM Lat B to CB/fMLF- or A23187-stimulated neutrophils further augmented MPO release (Fig. 3, A and C). Lat B was stimulatory for A23187 at doses of up to 10 μM, while for CB/fMLF, it was only stimulatory at doses up to 0.5 μM. Addition of CB to A23187-stimulated neutrophils also increased MPO release over A23187 stimulation alone (data not shown). However, at higher concentrations of Lat B, above 50 μM for CB/fMLF and 100 μM for A23187, MPO secretion was inhibited (Fig. 3, A and C). The wider range of stimulatory concentrations for Lat B in combination with A23187 is likely due to the presence of only one drug that directly affected actin depolymerization.

Our data suggest the existence of an actin depolymerization "threshold" for exocytosis, which when surpassed, is inhibitory to exocytosis. Treatment of cells with Lat B in combination with CB had only a small window of stimulatory concentration for exocytosis compared to Lat B in combination with A23187 (Fig. 3A vs 3C). Lat B and CB both synergistically promote actin depolymerisation which may shift F-actin to G-actin, thus pass the F-actin threshold needed to promote exocytosis. However, stabilization of F-actin with JP, inhibited both CB/fMLF- and A23187-stimulated

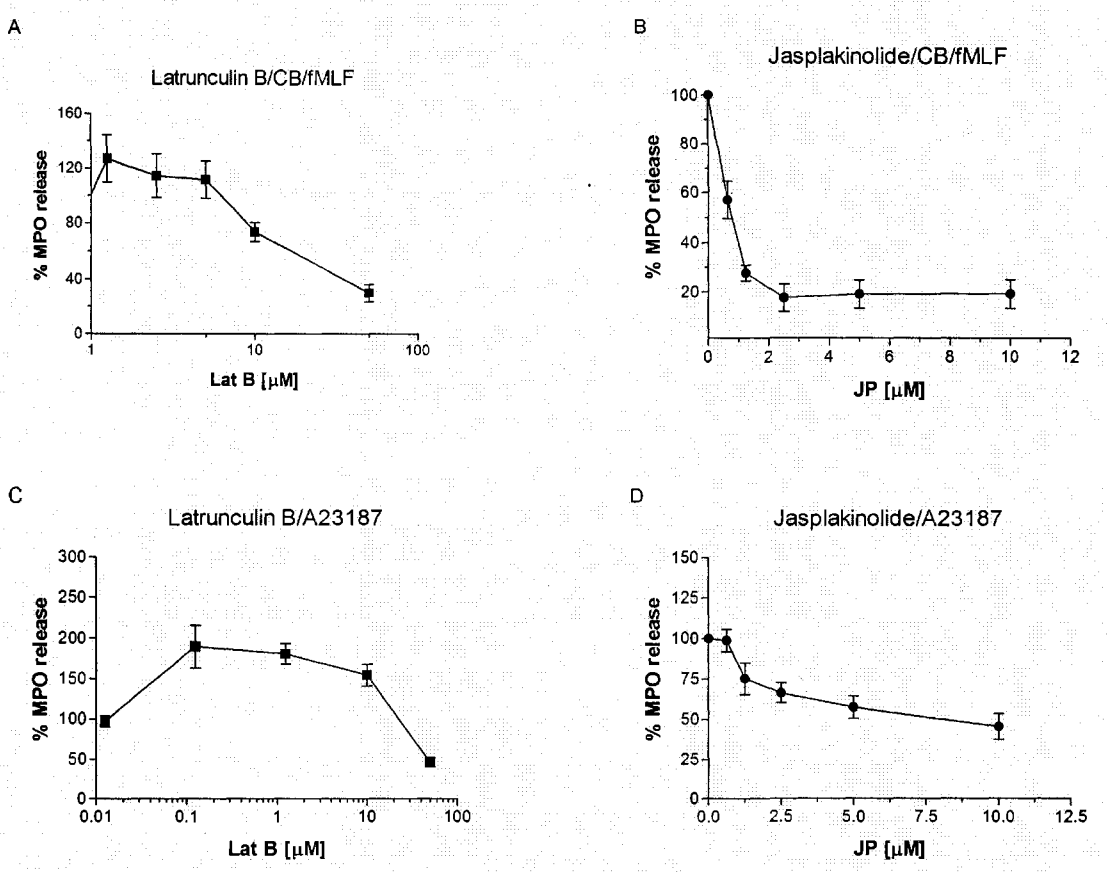

Figure 3 Effects of actin drugs on CB/fMLF- and A23187-induced primary granule exocytosis.

Neutrophils were preincubated with increasing concentration of either Lat B or JP for 15 min followed by stimulation with CB/fMLF or A23187 for 15 min at 37°C. Supernatants were collected from each condition and assayed for MPO activity. Released MPO was calculated as a percentage of total activity (± SEM) of lysed cells and then normalized to stimulus alone for each of at least three independent experiments. $P < 0.001$ for all experiments except, $P < 0.01$ for A, Lat B at 50 μM; C, Lat B 0.125 – 2.5 μM; D, JP 5 μM compared to stimulus alone.

exocytosis (Fig. 3B and 3D), although the effect was not complete. At least 20% of maximal MPO release was still obtained in response to CB/fMLF, while as much as 50% of the response persisted in response to A23187 when cells were pre-treated with JP. A possible explanation for the persistence of exocytosis in the JP treated and A23187 stimulated condition may be due to the fact that A23187 is a robust stimulus that triggers high levels of exocytosis. Therefore, it may be that higher concentrations of JP are required to obtain more complete inhibition.

To confirm our biochemical findings and to visualize the effects of actin altering drugs, we examined human neutrophils by confocal microscopy. Neutrophil primary granules were labelled with anti-CD63 antibodies conjugated to Alexa Fluor 488 and F-actin with rhodamine-phalloidin. Cells were treated similarly to those used in the secretion assay, fixed while still in suspension and adhered to poly-L-lysine-coated glass slides for staining. We first examined if the individual components of the stimuli we used (CB/fMLF) had any effect on actin remodelling and primary granule distribution. As shown in Figure 4, the addition of CB appears to induce the dissipation of the cortical acting ring seen in resting cells however there is no indication of polarization of F-actin to one side of the cell or reorganization as seen in the CB/fMLF condition. Addition of fMLF appeared similar to resting cells (Fig. 4). Granule translocation was not observed in any of these cases. These results strongly indicate that in order to observe granule translocation and polarization of actin, the action of both drugs in concert are required.

We next examined cells treated with various combinations of actin altering. Resting neutrophils once again exhibited diffuse primary granule staining with an intact F-actin ring-like structure (Fig. 5). Intensity profiling (Fig. 5, right column) showed sharp

peaks at the cell periphery for rhodamine-phalloidin staining, corresponding to the actin ring, but relatively even distribution of the primary granule marker CD63. Upon stimulation with CB/fMLF, F-actin polarization occurred along with redistribution of primary granules to the same sites, which is also shown as co-localization of peaks on the intensity profile. Pre-treatment with JP resulted in diffuse cytoplasmic F-actin staining accompanied by reduced cortical actin staining (Fig. 5). Granule staining in the JP treated cells exhibited an interesting distribution. There appeared to be larger than normal clusters of granules throughout the cell suggesting the possibility that homotypic granule fusion occurred, which was not obstructed by JP pre-treatment. Also although granule distribution was relatively diffuse, there was some translocation to the cell periphery after CB/fMLF stimulation (Fig. 5). These observations suggest that stabilization of F-actin by JP may not completely obstruct granule translocation or homotypic fusion, but stabilization of the cortical ring likely prevents the final docking/fusion steps required for exocytosis, as evident in the secretion assay which used the same JP treatment.

We next examined the morphology of cells treated with stimulatory versus inhibitory doses of Lat B. Interestingly, low doses of Lat B (1.25 μM) together with CB/fMLF increased primary granule translocation to the cell periphery. However, it dramatically reduced cytoplasmic F-actin staining, with a few faint patches of F-actin localized to sites of polarized granule translocation remaining (Fig. 5). High Lat B doses (50 μM) together with CB/fMLF induced a pattern of F-actin staining that resembled the intensity profile of low Lat B and CB/fMLF. However, CD63 granule staining was strongly enhanced throughout the cytoplasm, suggesting retention of primary granules in

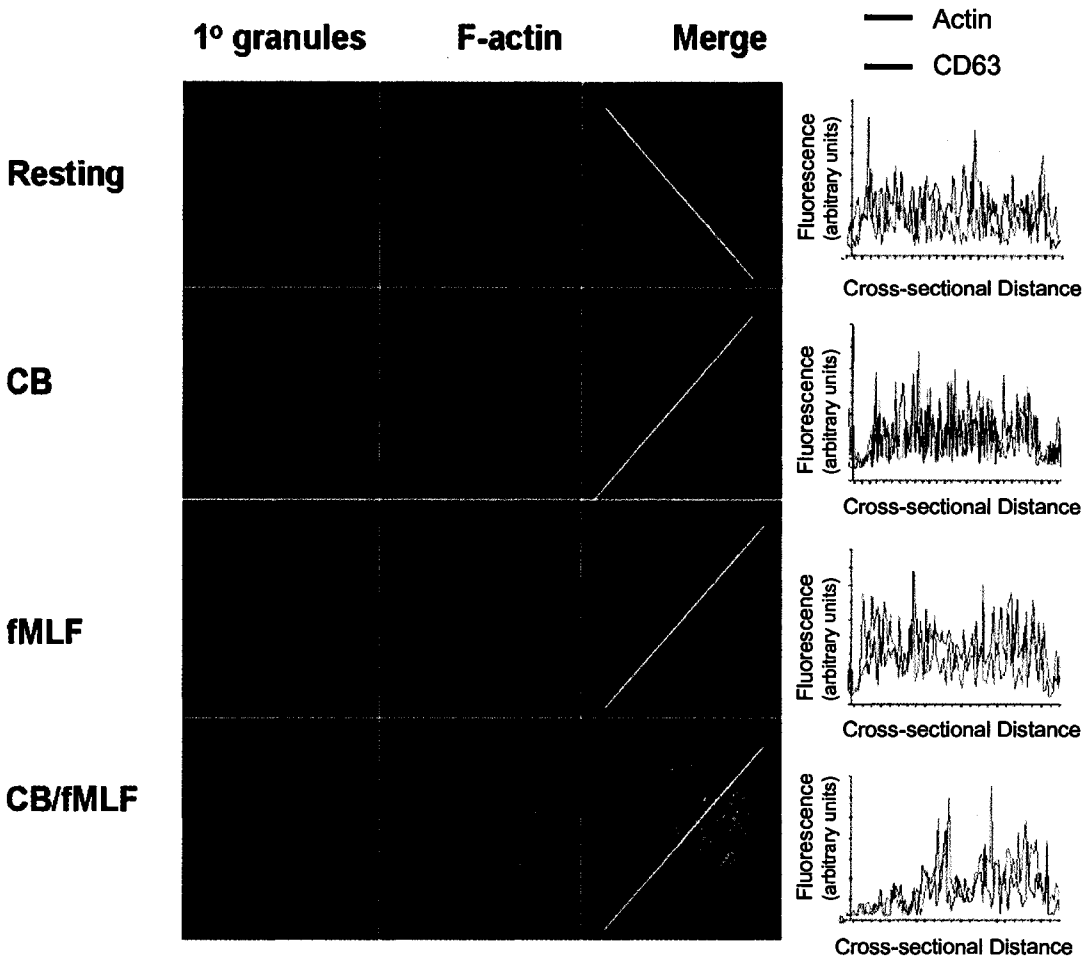

Figure 4 Morphological analysis of stimulated neutrophils with CB and fMLF controls

Samples were prepared for confocal analysis by treatment of cells in suspension with either 10 µM CB or 5 µM fMLF or both for 15 min at 37°C. Cells were then fixed while still in suspension to maintain cell integrity. Cellular F-actin was stained with rhodamine-phalloidin (red) and primary granules were stained with Alexa Fluor 488-conjugated CD63 antibodies (green). Cross-sectional intensity profiles for F-actin (*red line*) and primary granules (*green line*) are shown on the right. Scale: each panel is 12 µm x 12 µm. These images are representative from two experiments, and show typical results for > 65% of cells.

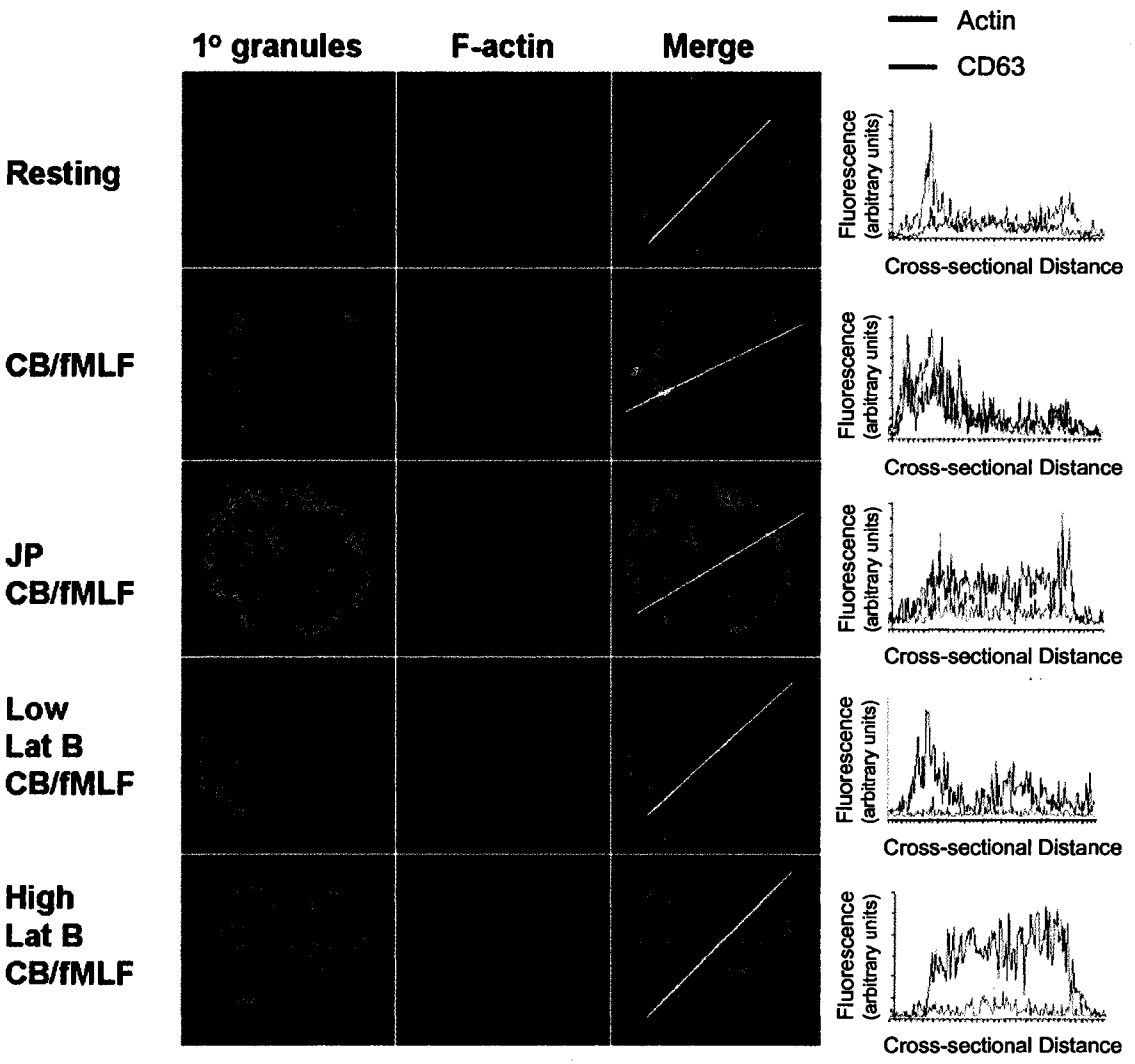

Figure 5 Morphological analysis of stimulated neutrophils treated with a combination of actin altering drugs.

Samples were prepared for confocal analysis by treatment of cells in suspension with either 1.25 µM Lat B (low dose), 50 µM Lat B (high dose) or 10 µM JP for 15 min, followed by stimulation with CB/fMLF for 15 min at 37°C. Cells were then fixed while still in suspension to maintain cell integrity. Cellular F-actin was stained with rhodamine-phalloidin (red) and primary granules were stained with Alexa Fluor 488-conjugated CD63 antibodies (green). Cross-sectional intensity profiles for F-actin (*red line*) and primary granules (*green line*) are shown on the right. Scale: each panel is 12 µm x 12 µm. These images are representative from four experiments and show typical results for > 65% of cells.

3.3 Role of Rac in exocytosis

It was recently shown that primary granule exocytosis was reduced in bone marrow neutrophils isolated from *rac2* $^{-/-}$ mice, which was associated with a lack of primary granule translocation to the cell membrane during stimulation (Abdel-Latif *et al.,* 2004). Therefore, we investigated whether Rac regulates primary granule exocytosis by altering actin cytoskeleton dynamics in human neutrophils. The small molecule Rac inhibitor, NSC23766, has been shown to inhibit the binding of GTP to both Rac1 and Rac2, which is required for small G-protein activation and signalling (Gao *et al.,* 2004; Cancelas *et al.,* 2005). NSC23766 blocks Rac function by binding to Trp56 and specifically inhibiting the binding of the GEFs Trio and Tiam1 (Gao *et al.,* 2004). However, it has yet to be shown to inhibit GTP binding to Rac1 and Rac2 in stimulated human neutrophils. Therefore, we examined the effects of this inhibitor on Rac activation in stimulated neutrophils. To do this, we used a pull-down assay where GST was conjugated to the Rac-binding domain of p21 activated kinase (PAK). This fusion protein specifically associates with GTP-bound Rac and Cdc42 (Benard *et al.,* 1999). Cells stimulated with fMLF, CB/fMLF, or Lat B/fMLF showed increased levels of GTP-bound Rac1 (Fig. 6A) and Rac2 (Fig. 6B). Pre-treatment with NSC23766 for 15 min prior to stimulation with CB/fMLF reduced GTP-Rac1 and Rac2, however, the inhibitory effect was less evident in Lat B/fMLF-stimulated neutrophils. This suggests that the Lat B/fMLF stimulus may result in secondary effects that cause a unique GEF to bind Rac; one which is not inhibited by NSC23766. However, this GEF, although able to activate Rac, is not able to induce Rac-mediated exocytosis since NSC23766 still inhibits

exocytosis in this condition (see below, Fig. 7). This also suggests that specific GEFs are needed to activate specific downstream reactions as previously shown (Zhou *et al.,* 1998).

Our experiments allowed us to compare levels of GTP-bound Rac at different times of stimulation. High levels of Rac-GTP were present in fMLF-stimulated samples after 1 min of stimulation, but were significantly reduced by 15 min. However, CB/fMLF and Lat B/fMLF stimulated samples showed no reduction in Rac-GTP levels after 15 min of stimulation (Fig. 6 A and B, compare *fMLF* with *CB/fMLF* and *Lat B/fMLF* at *15 min*). Thus, the combined effect of CB/fMLF or Lat B/fMLF caused sustained Rac activation which might be important to the molecular mechanism of exocytosis. This result also supports our microscopic observations, which showed that neutrophil treatment with a single drug (i.e. fMLF alone) does not promote actin polarization and primary granule translocation.

We next examined whether NSC23766 affects primary granule exocytosis using a biochemical secretion assay. Human neutrophils were pre-treated with NSC23766 for 15 min and then stimulated with CB/fMLF. This brief pre-treatment significantly reduced the secretion of primary granule MPO (Fig. 7), which is in accord with inhibition of Rac activation (Fig. 6). Inhibition was evident at 10 μM NSC23766 and we saw maximal inhibition at concentrations greater than 40 μM. Secretion of MPO in response to A23187 was unaffected by NSC23766, even at high doses (up to 160 μM). However, Lat B/fMLF-stimulated cells also showed reduced primary granule exocytosis when pre-treated with NSC23766 (Fig. 7), even though Rac was activated under these conditions (Fig. 6).

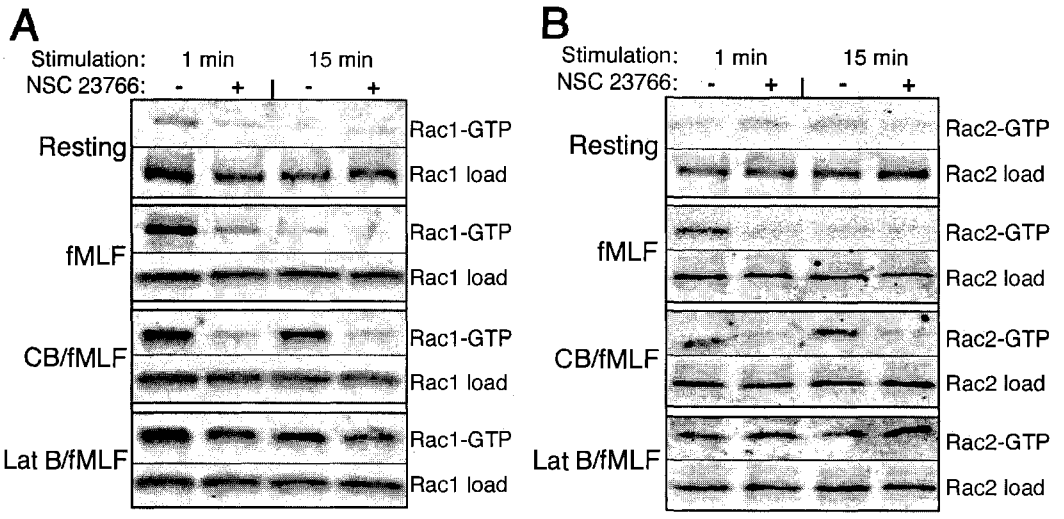

Figure 6 Detection of activated Rac1 and Rac2 in stimulated neutrophils.
Neutrophils were preincubated with 40 μM NSC 23766 or vehicle for 15 min followed by stimulation for 1 min (left columns) or 15 min (right columns) with fMLF, CB/fMLF, or Lat B/fMLF. Control samples were not stimulated (Resting). Activated Rac1-GTP (A) or Rac2-GTP (B) was determined by incubating of 300 μg of lysates prepared from human neutrophils with 30 μg of GST-PBD beads (in 500 μl) and immunoblotting for Rac1 and Rac2 in the bound fraction. Rac1 load and Rac2 load is immunoblot analysis of 30 μg lysate. Typical results from three experiments are shown.

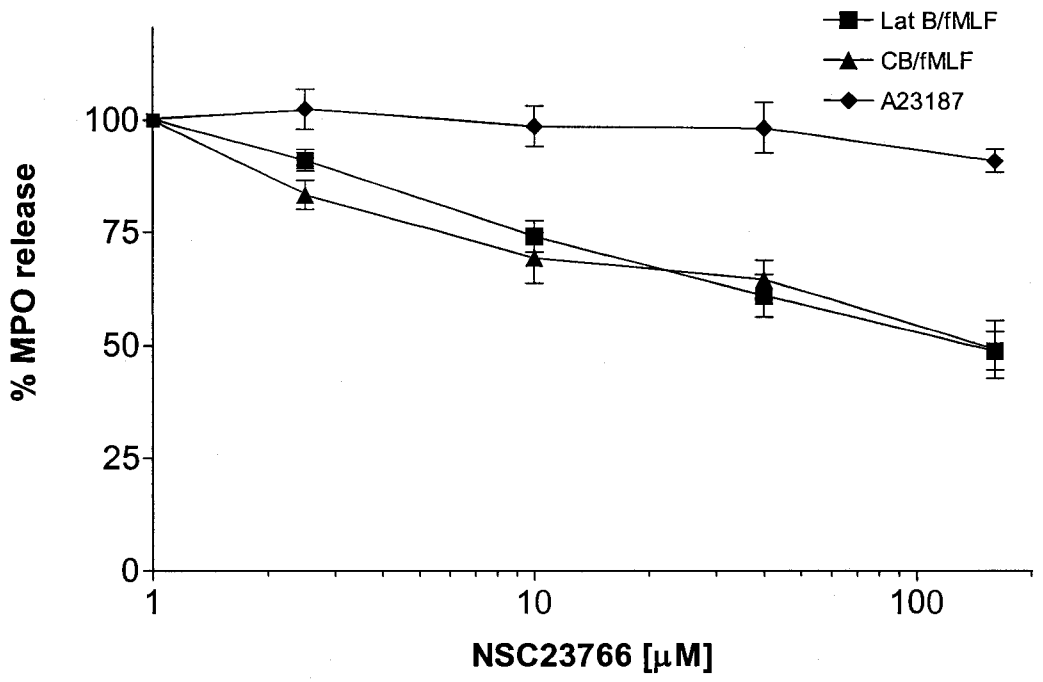

Figure 7 Effect of NSC23766 pre-treatment on neutrophil exocytosis.
Neutrophils were preincubated with increasing concentration of NSC23766 for 15 min followed by stimulation with CB/fMLF, Lat B/fMLF or A23187 for 15 min at 37°C. Supernatants were collected from each condition and assayed for MPO activity. Released MPO was calculated as a percentage of total activity (± SEM) of lysed cells and normalized to stimulus alone for at least three independent experiments. $P < 0.001$ for all experiments except, $P < 0.01$ for Lat B/fMLF at 40 μM NSC23766 and CB/fMLF at 10 μM and NSC23766 compared to stimulus alone.

To determine whether the small molecule Rac inhibitor was specifically inhibiting exocytosis we examined the effects of NSC23766 on respiratory burst induced by PMA or fMLF. Pre-treatment of neutrophils with NSC23766 for 15 min had no effect on O_2^- release from neutrophils stimulated with either PMA (Fig. 8A) or fMLF (Fig. 8B). Stimuli that activate G protein-coupled receptors, such as fMLF, have been shown to activate the GEFs Vav1 and P-Rex1, leading to Rac activation required for respiratory burst (Kim *et al.,* 2003; Welch *et al.,* 2005). Since NSC23766 was unable to block O_2^- release from PMA stimulated neutrophils, a different GEF, such as Trio or Tiam1, may be responsible for activating Rac-mediated exocytosis of primary granules. It also suggests that actin alterations are also not required for this process due to the fact that CB and Lat B are not needed.

Our findings indicate that primary granule exocytosis was specifically inhibited by NSC23766 in a dose-dependent fashion when stimulated with CB/fMLF or Lat B/fMLF. We confirmed these results via confocal microscopy by examining neutrophils treated with secretagogue and NSC23766. Upon stimulation with CB/fMLF or Lat B/fMLF, neutrophils showed primary granule translocation to the periphery (Fig. 9) which is depicted in the intensity profile as large peaks located at the cell periphery (Fig. 9, right column). However, unlike CB/fMLF stimulation, we did not observe significant polarization of F-actin at the cell periphery when cells were stimulated with Lat B/fMLF. Pre-treatment of cells with NSC23766 prior to stimulation also modestly reduced F-actin polarization at the cell periphery, and reduced primary granule translocation to the cell periphery. Taken together our results suggest that Rac plays a vital role in primary granule exocytosis via stimulation of actin polymerization to aid in granule mobilization

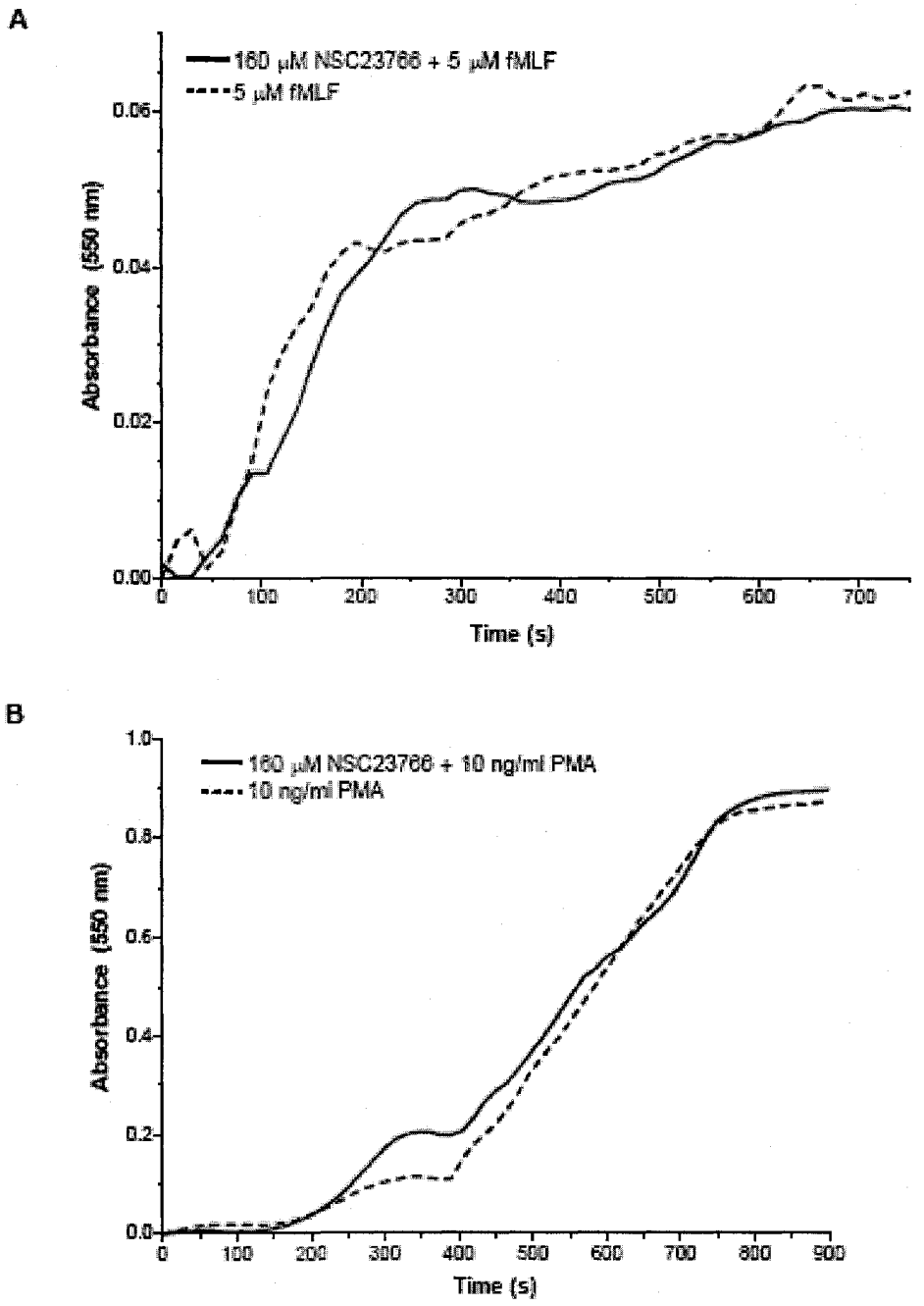

Figure 8 Effect of NSC23766 on respiratory burst.
Neutrophils (2 x 10^7/ml) were preincubated with 160 μM NSC23766 for 15 min at 37°C. Following this incubation step, 2 x 10^6 neutrophils, either NSC23766 or non-treated, were suspended in 1 ml microcuvettes containing PBS^+ and 50 μM ferricytochrome *c* at 25°C. The mixture was blanked at 550 nm. Stimulation of respiratory burst was achieved using either 5 μM fMLF (**A**) or 10 ng/ml of PMA (**B**). Readings were collected every 15 s for a total running time of 15 min.

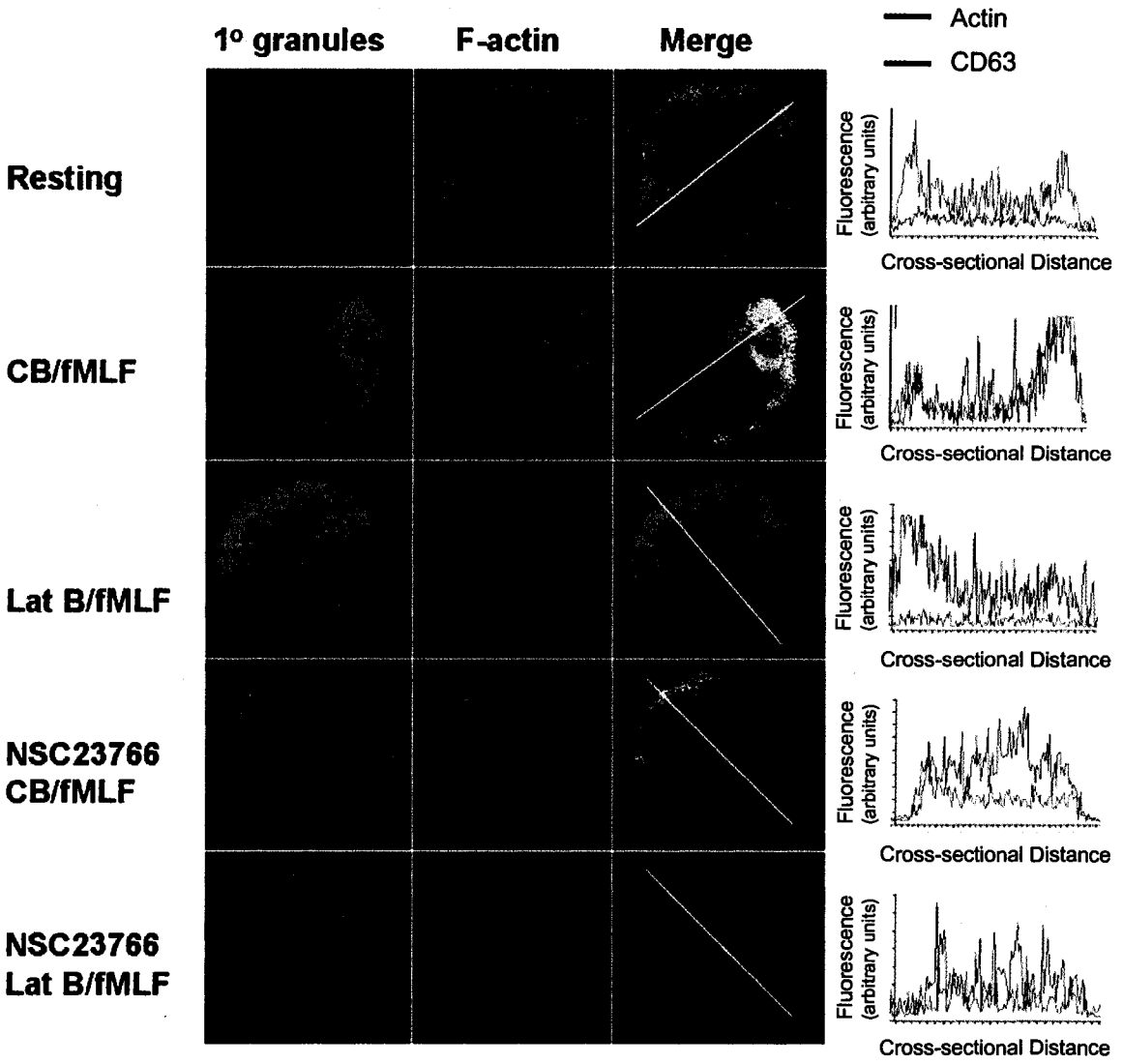

Figure 9 Morphological analysis of stimulated neutrophils pretreated with NSC 23766, via actin and primary granule staining.
Samples for confocal analysis were prepared by treatment of cells in suspension with either 40 μM of NSC 23766 for 15 min, followed by stimulation 10 μM CB 5 min/5 μM fMLF or 10 μM Lat B 5 min/5 μM fMLF for 15 min at 37°C. Cells were then fixed while still in suspension to maintain cell integrity. Cells were adhered and to glass slides and than stained accordingly. Slides were visualized using the 60X objective. Scale: each panel is 12 μm x 12 μm. Intensity profiles were generated by taking a cross section of the cell and then analyzing fluorescent intensity for both primary granules (*green line*) and actin (*red line*). These images are representative from two experiments and show typical results for < 65% of cells.

to the cell periphery. Importantly, it seems that, although little F-actin staining was observed in the Lat B/fMLF condition, there is likely sufficient levels to allow for granule exocytosis.

3.4 Assay of actin remodelling in neutrophil

To link the role of Rac to F-actin formation, we investigated whether inhibition of Rac via NSC23766 would affect F-actin. We used an *in vitro* actin polymerization assay that measures the capacity of a sample to stimulate the polymerization of exogenously added pyrene-actin, which undergoes a fluorescence intensity increase when incorporated into F-actin. Lysates prepared from fMLF- or CB/fMLF-stimulated cells exposed to NSC23766 showed reduced stimulation of F-actin formation as compared to those unexposed (Fig. 10). Interestingly, there was negligible differences in lysates prepared from cells that were stimulated via A23187.

3.5 Summary of neutrophil exocytosis

Treatment of neutrophils with low doses ($\leq$10 μM) of Lat B or CB promoted fMLF- and A23187-triggered secretion of MPO. Increased concentrations (> 10 μM) of Lat B or CB inhibited exocytosis. The F-actin stabilizing drug, JP, inhibited release of MPO at doses > 1 μM. This suggests the necessity for F-actin depolymerization prior to primary granule exocytosis. Interestingly, lysates from stimulated neutrophils enhanced actin polymerization *in vitro* as seen in the actin polymerization assay results. The small molecule Rac inhibitor, NSC23766, inhibited actin polymerization and MPO secretion induced by Lat B/fMLF or CB/fMLF, but not A23187. However this same inhibitor had

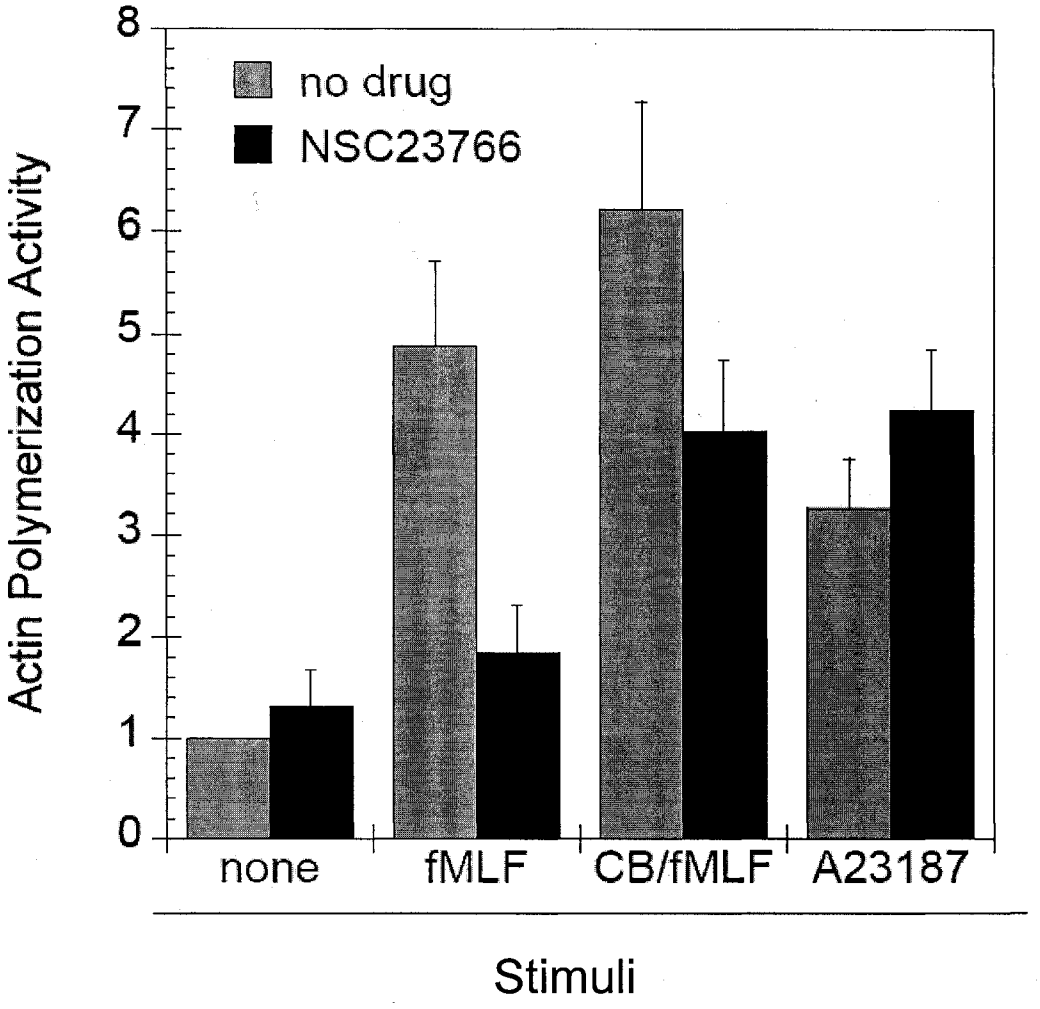

Figure 10 Determination of actin polymerization activity of neutrophil lysates. Actin polymerization stimulated by neutrophil lysates was determined by pyrene-actin polymerization assay as described in the Materials and Methods. Polymerization reactions contained 5 μM pyrene-actin and 0.1 mg/ml neutrophil lysate prepared from resting cells, or fMLF, CB/fMLF or A23187 stimulated cells. Lysates prepared from fMLF or CB/fMLF stimulated neutrophils showed enhanced polymerization activity (grey bars), which was reduced when cells were preincubated with NSC23766 (black bars). Shown are the average activities (± SEM) calculated from at least three experiments normalized to unstimulated samples (*none*).

no effect on PMA or fMLF-induced superoxide release. Visualization of neurophils via confocal microscopy analyses showed significant actin reorganization in Lat B/fMLF- and CB/fMLF-stimulated cells, accompanied by primary granule translocation to the cell membrane, while NSC23766 inhibited these changes. Therefore, we propose that one role for Rac is the stimulation of F-actin polymerization to facilitate granule translocation to the cell periphery. *In vivo* this process must be coupled with cortical actin depolymerization, induced by an unknown signalling protein in response to extracellular stimuli such as formylated bacterial peptides or possibly other cytokines. Our results suggest that the actin cytoskeletons role in receptor-mediated exocytosis of primary granules in human neutrophils is a dynamic process that involves both polymerization and depolymerization reactions.

3.6 Flow cytometry

We attempted to confirm our results which showed enhanced actin polymerization activity in stimulated samples using flow cytometry. Stimulated cells were fixed, F-actin and primary granules were stained and analyzed by flow cytometry. Samples were prepared and stimulated in similar fashion to those prepared for the *in vitro* actin polymerization. Samples were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton-X100 in PBS and stained with anti-CD63 conjugated to Alexa Fluor 488 to detect primary granules and rhodamine-phalloidin to detect F-actin. Ideally, we had hoped to detect an increase in F-actin signal in samples such as CB/fMLF, Lat B/fMLF, and fMLF. This would have corroborated results seen with the *in vitro* actin polymerization assay, however not reproduced in intact cells. We also hoped to observe that pre-treatment

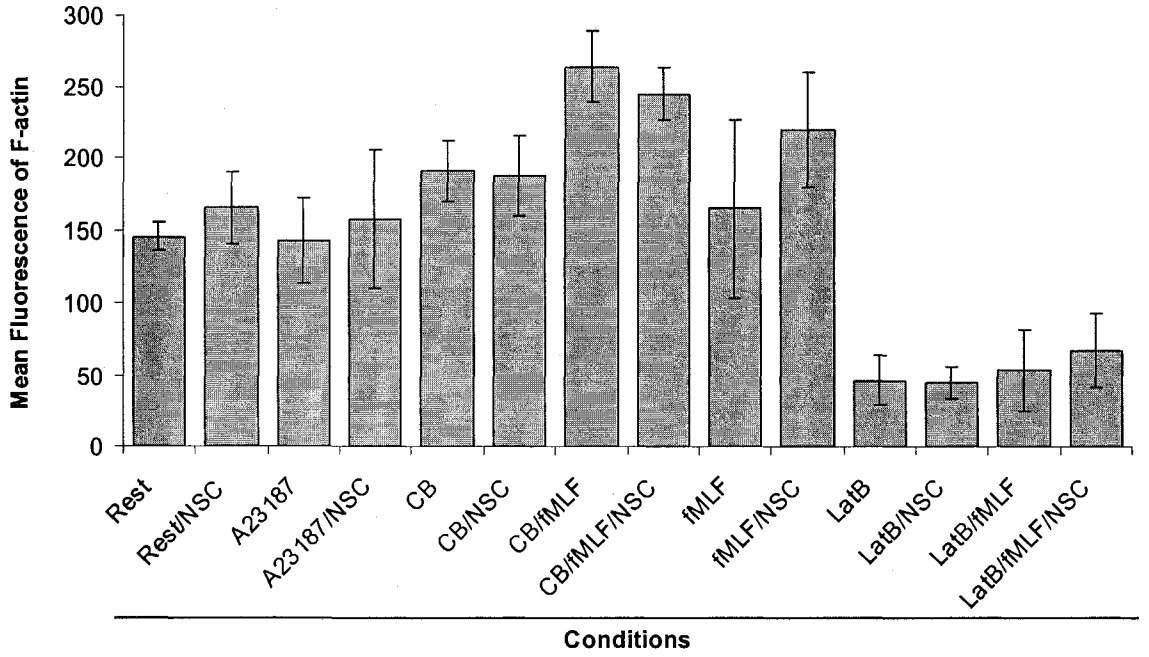

Figure 11 Changes F-actin mean fluorescence in response to varying stimuli and the small molecule Rac inhibitor as determined by Flow cytometry.
Samples for FLOW analysis were prepared by treatment of cells in suspension with either 40 μM of NSC 23766 for 15 min, followed by stimulation 10 μM CB 5 min/5 μM fMLF, 10 μM Lat B 5 min/5 μM fMLF, 2.5 μM A23187 or 10 μM CB, 10 μM Lat B and 5 μM fMLF alone for 15 min at 37°C. Samples were then fixed in 4% paraformaldehyde for 30 min on ice. Following fixation samples were permeabilized by incubation with 0.5% Triton-X100 in PBS and stained using a 1:500 dilution of anti-CD63 conjugated to Alexa Fluor 488 to detect primary granules and F-actin was detected using rhodamine-phalloidin. Sample volume was approximately 500 µl containing 100,000 cells. Values were determined from taking the average mean fluorescence from triplicate samples.

under these same conditions with the Rac inhibitor NSC23766 would have shown reduced F-actin fluorescence, also supporting the findings of the *in vitro* actin polymerization assay. However, comparison of the mean fluorescence of F-actin from the various samples showed negligible differences between the various conditions assayed (Fig. 11). One plausible explanation for this is that actin remodelling causes changes to actin polymerization activity, however, steady-state levels of F-actin remain relatively unchanged. Therefore actin remodelling could not be detected in our hands by flow cytometry since it lacks the sensitivity to observe these changes. Another possibility for this experiments failure to detect these changes may be due to the use of an inappropriately long time interval. It is likely that at 15 min we may have missed the window in which changes in F-actin are observable, this notion is supported by Roberts et al., (1999) who showed increases in F-actin using flow cytometry after 15 sec in response to fMLF.

3.7 Electron microscopy

Although confocal microscopy allowed us to examine changes in actin dynamics by observing alterations in F-actin staining, as well as primary granule dispersion, the resulting images were of low resolution. Therefore, we next examined human neutrophils, both stimulated and drug treated, by transmission electron microscopy (EM) since this technique provided the highest resolution for optimal morphological analysis. Isolated neutrophils were treated in suspension with the normal secretagogue, CB/fMLF, other combinations of actin modifying drugs (Lat B and JP), and the small molecule Rac inhibitor NSC23766 followed by fixation and sectioning for EM. Resting cells showed

numerous DAB-stained primary granules dispersed throughout the cytoplasm, which were unchanged when stimulated with fMLF alone (Fig. 12, a and b). Cells stimulated with CB/fMLF showed significantly fewer primary granules, indicative of their exocytosis (Fig. 12c, Fig 14). Pre-treatment with JP prior to CB/fMLF stimulation resulted in clustering of primary granules in the centre of the cell (Fig. 12d) with similar granule count to resting (Fig. 14). Interestingly, a low dose of Lat B (1.25 μM) together with CB/fMLF showed polarized granule localization at cell membranes (Fig. 12f), while Lat B alone did not (data not shown). High Lat B doses in combination with CB/fMLF showed retention of granules within the cell body similar to that of resting (Fig. 12e).

Next we repeated morphological examination after NSC23766 drug treatment. Human neutrophils were again stimulated with CB/fMLF or Lat B/fMLF, which showed reduced primary granule counts as compared to resting (Fig. 13). Pre-treatment of cells with NSC23766 prior to stimulation with CB/fMLF or Lat B/fMLF inhibited primary granule exocytosis and the translocation step which is indicated by similar primary granule distribution (Fig. 13) and counts (Fig. 15) as resting cells. Taken together our results using EM confirm observation seen in the secretion assay as well as confocal microscopy which showed that inhibition of Rac or altering the balance of remodelling actin via excessive stabilization (JP treatments) or excessive destabilization (High Lat B treatments) severely reduces primary granule exocytosis.

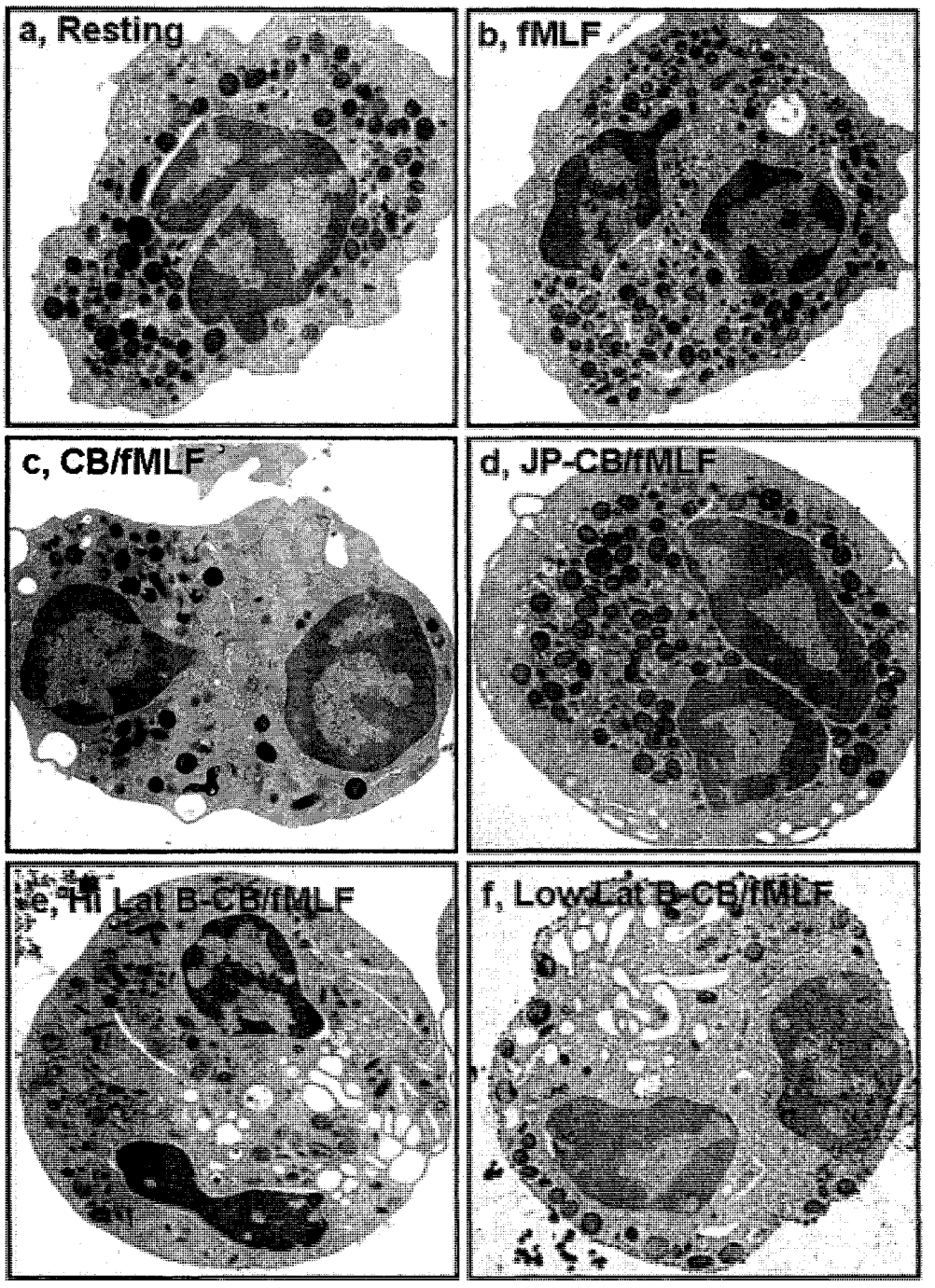

Figure 12 Examination of granularity of human neutrophils exposed to various stimulatory conditions and actin altering drugs.
Samples were prepared for EM analysis by treatment of cells in suspension with either 1.25 μM Lat B (*low Lat B*), 50 μM Lat B (*high Lat B*) or 10 μM JP for 15 min, followed by stimulation with CB/fMLF for 15 min at 37°C. Cells were then fixed and sectioned as described in the EM method section. The window size is 7 μm and magnification was 9100X.

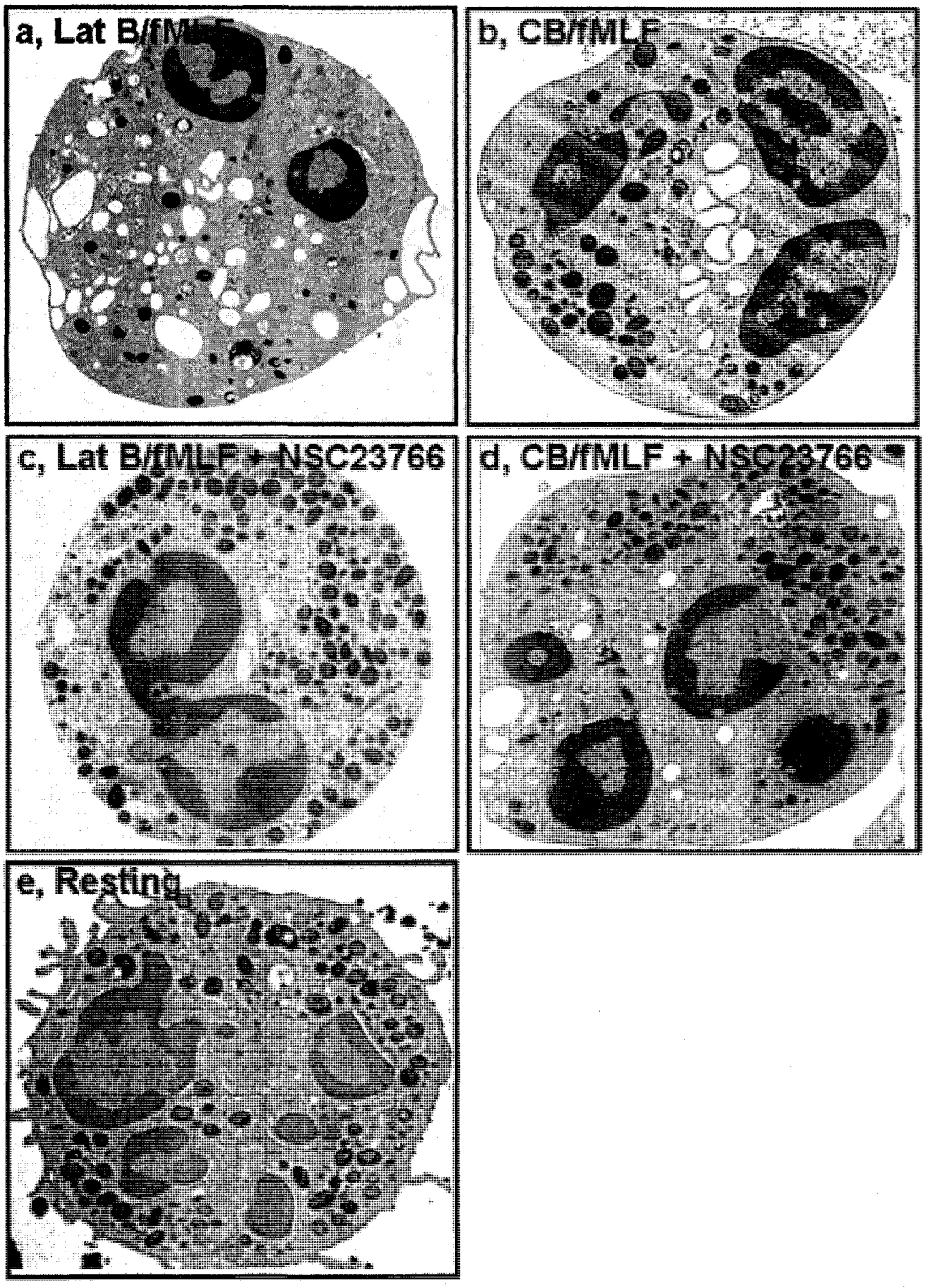

Figure 13 Examination of granularity of human neutrophils exposed to various stimulatory conditions and NSC23766.

Samples for confocal analysis were prepared by treatment of cells in suspension with either 40 μM of NSC 23766 for 15 min, followed by stimulation 10 μM CB 5 min/5 μM fMLF or 10 μM Lat B 5 min/5 μM fMLF for 15 min at 37°C. Cells were then fixed and sectioned as described in the EM method section. The window size is 7 μm and magnification was 9100X.

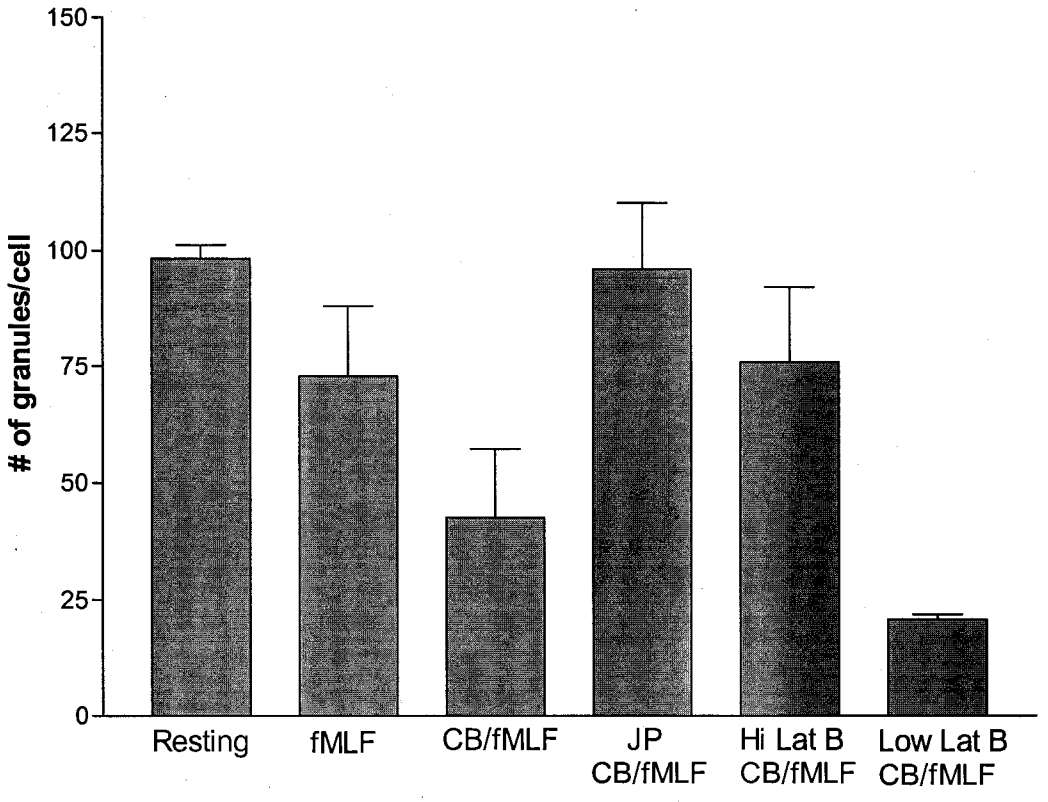

Figure 14 Granular count from stimulated neutrophils exposed to actin altering drugs

Graphs were generated by DAB positive primary granule count of at least three separate cells in EM that underwent treatment.

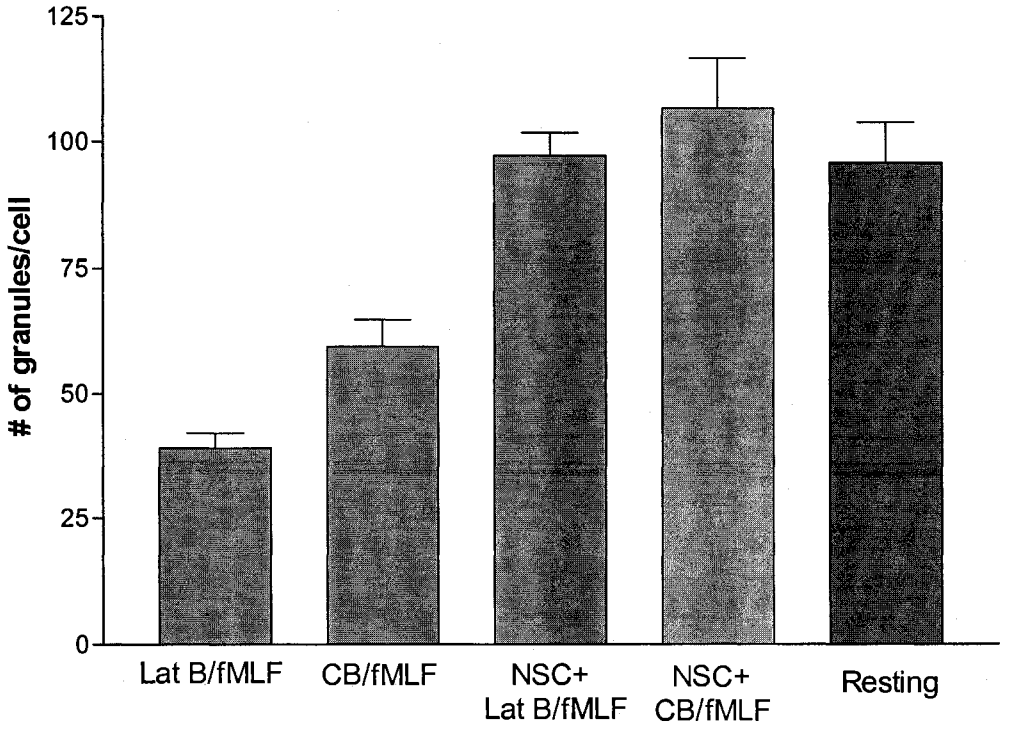

Figure 15 Granular count from stimulated neutrophils exposed to NSC23766. Graphs were generated by DAB positive primary granule count of at least three separate cells in EM that underwent treatment.

3.8 Phosphosite screen for downstream kinase involved in Rac2 stimulated exocytosis

Phosphorylation is a key event following neutrophil receptor activation leading to exocytosis. Receptor stimulation through the FPR receptor by fMLF leads to phosphorylation of numerous kinases which then activate their respective effector pathways. Using this rationale potential kinase candidates identified in literature to play a role in exocytosis in various secretory cells were selected to be examined for their role in neutrophil exocytosis by phosphosite screening. Kinexus is a biotech company that has obtained an enormous library of kinase and phosphatase antibodies. This company takes samples prepared by researchers and perform quantitative immunoblot analysis with requested phospho-specific and whole protein antibodies. The kinases and phosphatases we selected for analysis are listed in Table 2. Bone marrow neutrophils (BMN) were isolated from wild-type and *rac2*$^{-/-}$ mice (20 million cells, necessary to yield ~500 μg of protein require by Kinexus). CB, CB/fMLF stimulated and untreated control samples were prepared as instructed by Kinexus, then shipped to them for phospho-site screening.

Using the Kinexus phospho-site screen we were able to identify four different protein kinases that showed significantly decreased phosphorylation in *rac2*$^{-/-}$ compared with WT neutrophils after stimulation for degranulation. CB/fMLF induced the phosphorylation of calmodulin kinase II (CAMKII), MAP kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1), and these were all significantly reduced in *rac2*$^{-/-}$ mice as compared to their WT counterparts (Fig. 16). A fourth kinase, PAK1/2/3, showed reduced phosphorylation in both resting and stimulated *rac2*$^{-/-}$ BMN.

Table 2

Densitometry values of immunoblot bands from selected proteins for the phospho-site screen

FULL NAME OF PROTEIN	ABBREV	Resting WT	CB WT	CB/fMLF WT	CB/fMLF Rac2 KO	CB Rac2 KO	Resting Rac2 KO
Calcium/calmodulin-dependent kinase II (T286)	CaMK2		269	727	387	354	
Cofilin 2 (S3)	Cofilin 2						
Extracellular signal-regulated kinase 1 (T202/Y204)	ERK1			131	76		
Extracellular signal-regulated kinase 2 (T185/Y187)	ERK2		162	112	215	214	94
Focal adhesion kinase (S722)	FAK						
Focal adhesion kinase (S910)	FAK						
Focal adhesion kinase (Y576)	FAK	323	168	263	281	152	411
Lyn (Y507) (44)	Lyn	1671	1127	638	908	785	954
Lyn (Y507) (46)	Lyn	2205	1300	949	1917	2021	1722
MAP kinase kinase 1/2 (S217/221)	MEK1/2	511	985	1679	848	440	344
p21-activated kinase 1/2/3 (S144/141/154) (54)	PAK1/2/3	4865	4885	4215	2126	1359	1569
p21-activated kinase 1/2/3 (S144/141/154) (56)	PAK1/2/3	5547	5824	5620	4845	3657	3786
p38 alpha MAP kinase (T180/Y182)	p38a MAPK	2201	5669	5839	9229	8802	5012
Protein kinase C alpha (S657)	PKCa	1974	1892	3474	2037	2969	2868
Protein kinase C alpha/beta (T638)	PKCa/b	633	865	892	1329	952	1717
Protein kinase C delta (T507)	PKCd	2386	2480	2828	2480	2365	3144
Protein kinase C delta (Y313)	PKCd	6422	7487	5624	4717	4040	5526
protein kinase C epsilon (S729)	PKC e						
Stress-activated protein kinase (JNK) (T183/Y185) (39)	JNK (SAPK)	371	789	1067	1761	1796	973
Stress-activated protein kinase (JNK) (T183/Y185) (47)	JNK (SAPK)	385	563	502	996	1276	1035
The mammalian target of Rapamycin (S2448)	mTOR						
Type1 protein phosphatase alpha (T320)	PP1a	168	141	95	161	150	247

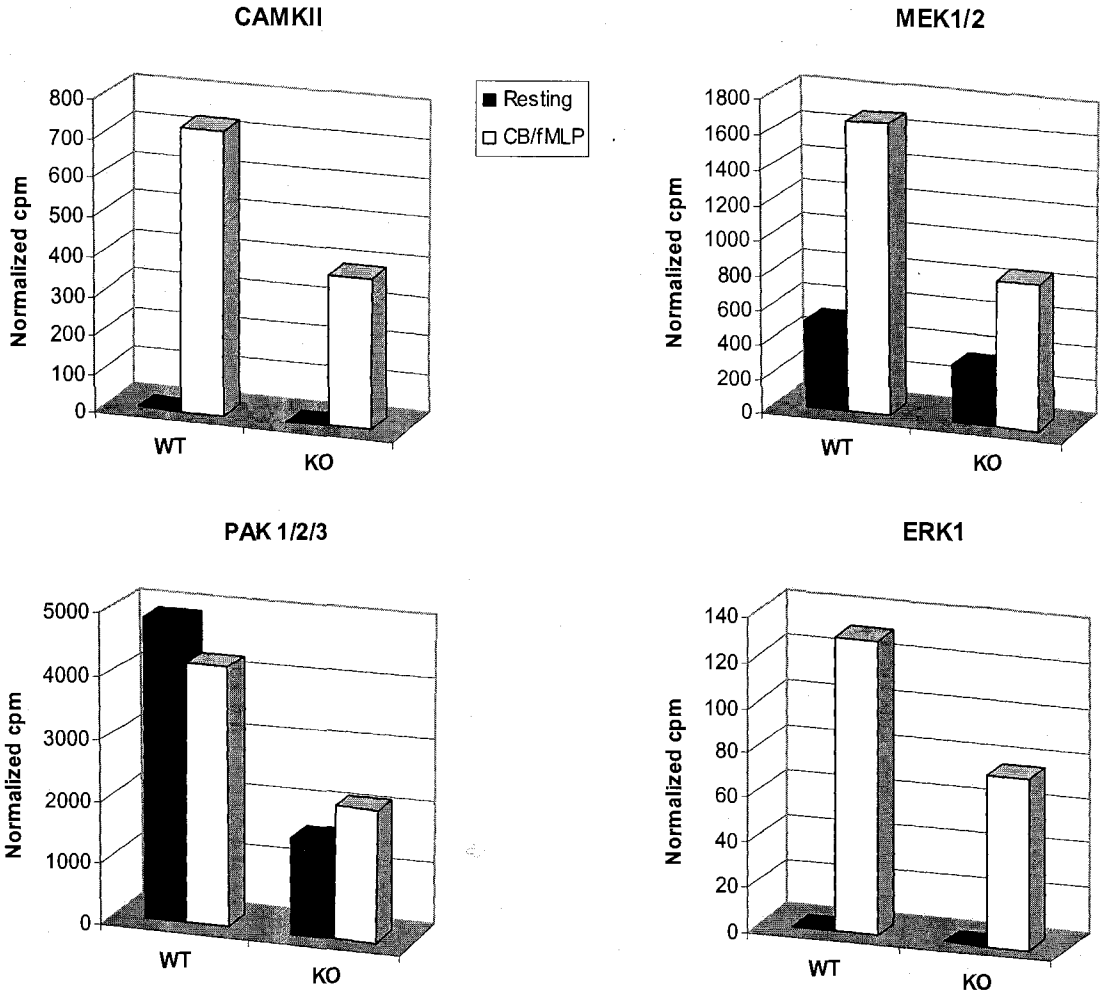

Figure 16 Proteins showing decreased phosphorylation in Rac2 KO neutrophils using Kinexus phospho-site screen service.

Cells were prepared as described in bone marrow neutrophil isolation method section. 20 million cells from either WT or *rac2*$^{-/-}$ mice (necessary to yield ~500 μg of protein require by Kinexus) were either treated with CB or CB/fMLF or left untreated and then prepared as instructed by Kinexus for shipment. Optical density measurements of phosphorylated serine, threonine, and/or tyrosine residues were screened by Western blot analysis. Changes of more than 25% were considered significant. CB/fMLF-treated WT and *rac2*$^{-/-}$ BMNs were treated at maximally stimulating doses for degranulation. *Note that these graphs show results from one set of samples, and are therefore subjective findings and require confirmation.*

3.9 Potential role for calmodulin kinase II in neutrophil exocytosis

Various kinases whose phosphorylation state differed between WT and *rac2*$^{-/-}$ during stimulation were identified via the phosphosite screen performed by Kinexus. We chose to further investigate CAMKII since this kinase had already been shown to be important in degranulation from RBL-2H3 cells (Funaba *et al.,* 2003). We examined whether CAMKII is needed for neutrophil exocytosis using a specific CAMKII inhibitor, KN-93. The results show that KN-93 also inhibited primary granule exocytosis from CB/fMLF stimulated neutrophils (Fig. 17). Therefore, CAMKII may also play an important regulatory role in neutrophil primary granule exocytosis. It is has been shown that Rac plays a critical role in regulating calcium flux required for calcium mediated exocytosis in RBL-2H3 cells (Hong-Geller *et al.,* 2000; Hong-Geller *et al.,* 2001), and thus it is reasonable to assume that CAMKII may be an important kinase that is involved in this pathway. Whether this is part of the actin cytoskeletal remodeling mechanism has yet to be determined.

3.10 Summary of kinase activation during neutrophil exocytosis

Using the Kinexus screen, we have identified four kinases that may be important in neutrophil exocytosis. These results indicated that these kinases may play integral roles in Rac2 mediated exocytosis. Further studies and confirmation of phosphorylation states of these kinases will be required. As well, determination of their respective roles in the Rac signalling pathway will need to be deduced.

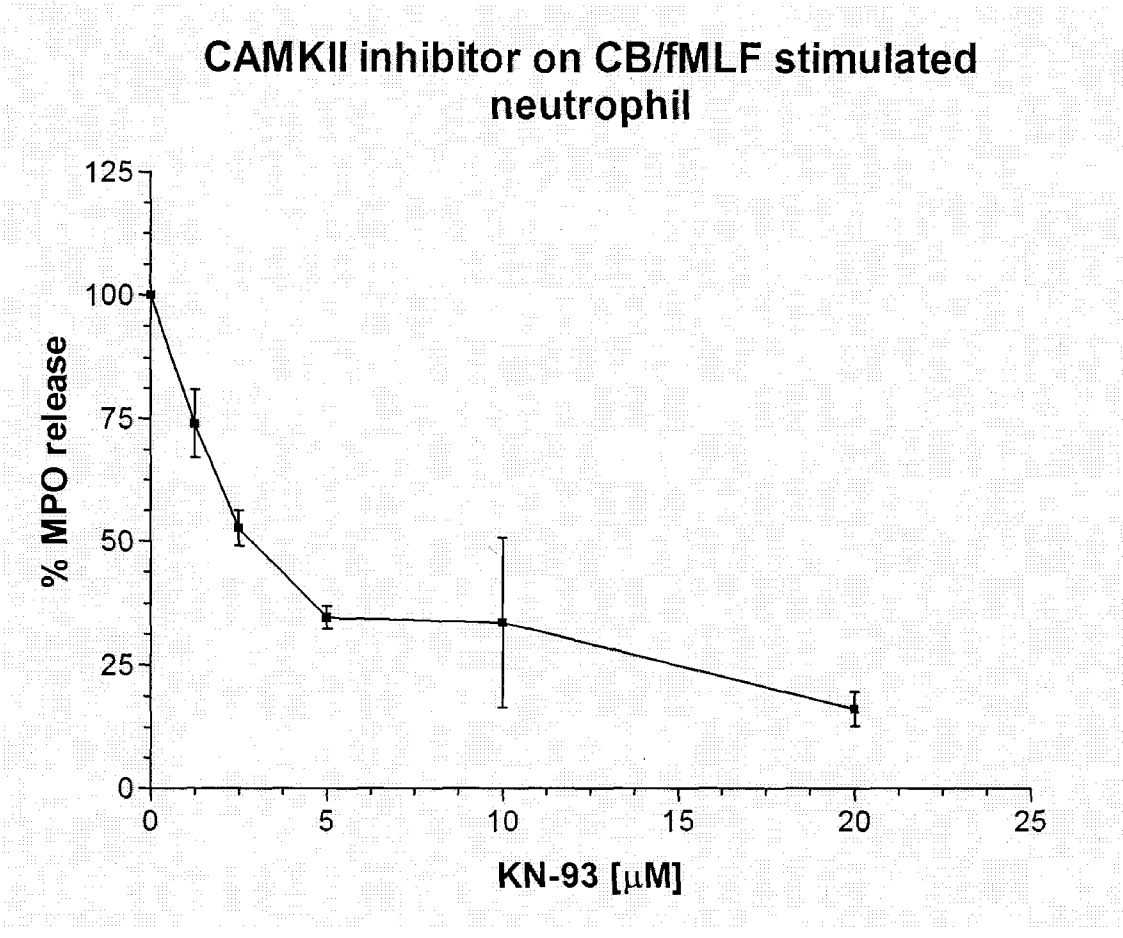

Figure 17 Neutrophil secretion assay using CB/fMLF and KN-93
Peripheral blood neutrophils were preincubated with increasing concentration of KN-93 for 15 min followed by stimulation with 10 μM CB 5min/5 μM fMLF for 15 min at 37°C. Supernatants were collected from each condition and assayed for MPO activity using the substrate TMB. Release of MPO was calculated as a percentage of total cellular mediator activity by dividing the corrected absorbance of supernatants into the sum of supernatants and average corrected values for lysed pellets

4.0 Discussion and Perspectives

4.1 Role of actin in neutrophil exocytosis

Actin remodelling is considered to be integral to the mechanism of exocytosis of neutrophil granules (Rizoli *et al.,* 2000), although the specific mechanisms have not yet been fully elucidated. In this thesis, we show a requirement for both actin polymerization and depolymerization in primary granule exocytosis. While low concentrations of actin depolymerization agents promoted exocytosis, high concentrations or combinations of these reagents inhibited this process. Confocal imaging of neutrophils showed that cortical actin was remodelled during CB/fMLF stimulation into polarized patches at the cell periphery that colocalized with primary granules. Lat B/fMLF-stimulated neutrophils also showed an increase in granule translocation, but clearly there was less polarization of actin to sites of granule translocation. The F-actin stabilizing drug, JP, was inhibitory to granule exocytosis as determined by the MPO secretion assay, although, intriguingly, it did not completely inhibit granule translocation as indicatied by the confocal images of neutrophils exposed to JP prior to stimulation. These results were similar in cells stimulated with A23187.

In resting cells, we observed an F-actin cortical ring, similar to that observed in resting mast cells (Norman *et al.,* 1994), which dissipated upon stimulation by CB/fMLF and more prominently by Lat B/fMLF. This is distinct from previous studies which showed diffuse F-actin in resting neutrophils that assembled into a cortical ring upon stimulation by fMLF (Downey *et al.,* 1991; Filippi *et al.,* 2004). A possible reason for this difference is likely in the methods used. Whereas in previous studies, live neutrophils

were allowed to adhere to poly-L-lysine-coated glass slides (which itself leads to activation) before stimulation with fMLF, our method of visualizing neutrophils involved fixation of neutrophils in suspension immediately following stimulation. Cytospin is another commonly used technique to mount neutrophils using centrifugation at high speeds to flatten the cells against glass slides; however, this may lead to changes their morphology. Our cell fixation protocol thus likely provides a more accurate representation of neutrophil morphology during stimulation and treatment with various reagents.

Our results also show that drugs affecting the stability of F-actin, either through strongly stimulating or preventing depolymerization, inhibit neutrophil primary granule exocytosis. However, we also found that a very low concentration of Lat B, together with CB/fMLF, increased granule translocation and exocytosis, indicating a requirement for some actin depolymerization, possibly through reduction of the actin polarization patch observed with CB/fMLF alone. Our findings suggest that there may be two pools of actin involved in regulation of neutrophil granular exocytosis, especially in the case of primary granules in that you require depolymerization of cortical F-actin for granules to fuse with the membrane but you also require some F-actin assembly to allow for granule translocation to sites of fusion, similar to that reported for exocytosis in neuroendocrine cells (Malacombe *et al.,* 2006). When neutrophils encounter a chemoattractant, they undergo actin remodelling steps required for chemotaxis (Filippi *et al.,* 2004), and it is likely that actin remodelling also facilitates polarized granule translocation and exocytosis. A shift from G-actin to F-actin would be required to assist in granule mobilization to the cell periphery. However, this conflicts with the need for

depolymerization of F-actin in primary granule exocytosis, since depolymerizing agents are required to achieve this *in vitro* (Showell *et al.,* 1976; Henson *et al.,* 1978; Abdel-Latif *et al.,* 2004). Furthermore, it is possible that in resting neutrophils the cortical actin ring-like structure we observed acts as a physical barrier to prevent uncontrolled granule docking and fusion, similar to mast cells (Norman *et al.,* 1994). Our results confirm that upon stimulation with CB/fMLF there is a preferential reorganization of the F-actin. We observed that, although the cortical ring-like structure dissipates upon stimulation with CB/fMLF, F-actin was not fully converted into G-actin. Rather, it was reorganized and polarized to sites of primary granule translocation at the cell edge. Our results suggest that CB/fMLF activation of Rac induced rearrangement and polarization of F-actin and that neither of these drugs individually was able to induce this pattern. In comparison, Lat B was a more potent stimulator of F-actin depolymerization as seen in our confocal images, which correlated with its more potent effect on primary granule exocytosis (Figs. 2 and 7). This is likely due to the high affinity that Lat B has for actin monomers (Kd of 200 nM; Coue *et al.,* 1987) resulting more specific depolymerization as compared to CB (up to 100 μM for actin severing; Urbnik *et al.,* 1989).

4.2 Proposed role of Rac in regulation of actin for neutrophil exocytosis

We showed for the first time that the specific small molecule Rac inhibitor NSC23766 attenuated primary granule exocytosis in response to CB/fMLF or Lat B/fMLF stimulation. Here we found that using NSC23766, we could create similar results on primary granule translocation as observed with gene-deletion of Rac2 in mice (Abdel-Latif *et al.,* 2004). This suggests that Rac also plays an important role in regulation of primary granule exocytosis in human neutrophils. However, it should be

noted that when examining Rac activation using Lat B/fMLF as a stimulus, we observed sustained Rac1- or Rac2-GTP formation when neutrophils were treated with a dose of NSC23766 that inhibited secretion. Our explanation of this is that a sensor may exist which can sense increases in G-actin which then promotes continued activation of Rac by a GEF which is not inhibited by NSC23766. Currently, two Rac GEFs, Trio and Tiam1, are known to be blocked by NSC23766, while this inhibitor has no effect on Vav1 binding to Rac (Gao *et al.,* 2004). It is also important to note that we have not observed a lack of Rac-GTP formation in the presence of MPO secretion. Thus, Rac-GTP may be necessary but not sufficient for primary granule exocytosis, and Rac-GTP formation may be dissociated from primary granule exocytosis.

Our findings from image and biochemical analysis of granule exocytosis indicated that NSC23766 inhibited cytoplasmic F-actin polymerizing activity, as well as primary granule translocation for subsequent exocytosis (Fig. 9 and 10). This indicates that Rac signals through F-actin polymerization to cause granule translocation to the cell membrane. In contrast, NSC23766 had no effect on calcium ionophore-induced exocytosis, suggesting that calcium acts downstream of Rac to induce granule translocation and secretion. It has been shown in RBL-2H3 cells that calcium signalling is highly important in granule exocytosis, and that calcium levels were reduced when cells are exposed to dominant-negative constructs of Rac proteins, as well as restored when exposed to constitutively active forms of Rac (Hong-Geller *et al.,* 2001). It therefore is reasonable to assume that Rac-mediated signalling, and specifically Rac2 signalling in neutrophils since Rac2 is the most abundant isoform of Rac within

neutrophils (Clark *et al.,* 1990), exploits calcium signalling as a mode of primary granule translocation and exocytosis.

The role of Rac and actin in primary granule translocation was further supported by EM analysis. We observed that inhibiting Rac severely reduced primary granule exocytosis, and treatment of neutrophils with drugs that induced excessive stabilization or depolymerization of actin also inhibit primary granule exocytosis.

Taken together, our results illustrate a possible mechanism by which Rac regulates exocytosis and release of primary granule content, in which Rac controls favourable F-actin formation necessary for granule translocation to sites of exocytosis. It is clear from these results that there is a necessity for F-actin depolymerization at the cell cortex, which is achieved by reagents such as CB or Lat B (Fig. 18). During neutrophil activation in response to infection or inflammation, multiple signalling cascades are activated in these cells leading to the necessary depolymerization steps required to remove cortical F-actin barriers concurrently with central F-actin polymerization to promote granule movement to the cell periphery. Our results provide strong evidence for a role for Rac in actin-mediated granule translocation, as well as a dynamic process that involves continuous actin remodelling rather than simple static polymerization or depolymerization. Our experimental approach will allow us to further characterize key regulatory molecules involved in the Rac signalling cascade, which in turn will lead to the development of new therapeutic strategies for treatment of inflammatory diseases.

4.3 Proposed role of calmodulin kinase and other kinases in neutrophil exocytosis

It is clear that kinases play an important role in the regulation of many cellular processes and several labs have defined their role in the regulation of primary granule exocytosis in neutrophils (Gutkind *et al.,* 1989, Mocsai *et al.,* 2000, Funaba *et al.,* 2003, Mohn *et al.,* 2005). We have identified four kinases whose phosphorylation state is altered in *rac2*$^{-/-}$ murine neutrophils as compared to WT ; CAMKII, ERK1, MEK1/2 and PAK1/2/3. Although the role of these kinases in exocytosis may not be novel, the fact that their phosphorylation state is altered in *rac2*$^{-/-}$ neutrophils has not been previously observed. How each of these impact primary granule exocytosis still needs to be determined. However, we have some evidence with preliminary inhibitor studies. As the results indicated (Fig. 17) inhibition of CAMKII by KN-93 correlated with a reduction in primary granule exocytosis from stimulated neutrophils. It is premature to suggest exactly what CAMKII is targeting in this process, and thus observation of possible downstream targets would be essential as well as mapping upstream signalling molecules. One possible scenario is that Rac is governing calcium flux, as suggested by Hong-Geller et al. (2001). Calcium flux is necessary for calcium levels to reach sufficient levels for the activation of CAMKII. Thus one could speculate that activation of Rac may lead CAMKII activation, which may in turn aid in regulating granule exocytosis. The methodology we used to verify a role for CAMKII can be applied to the other kinases. One could obtain inhibitors to these kinases and observe their effect on primary granule exocytosis. After determining if these inhibitors have an effect in primary granule exocytosis, one could search for their targets of phosphorylation as well as their direct effector molecules.

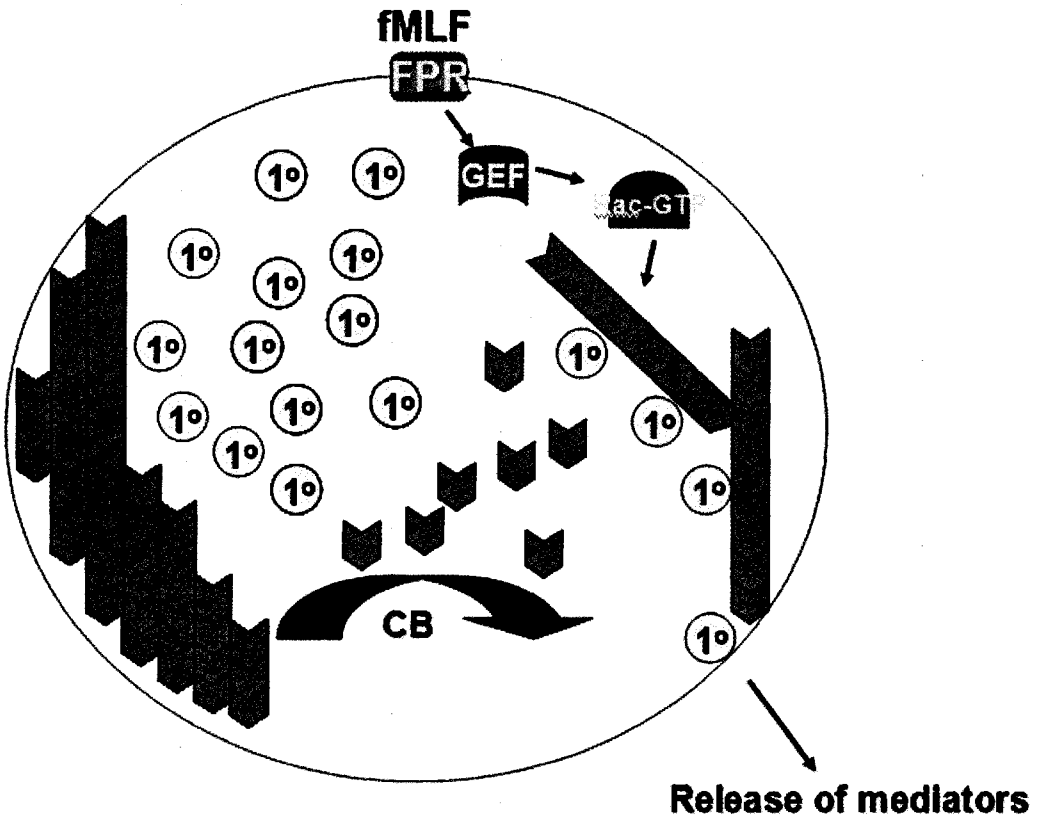

Figure 18 Schematic figure of the roles that Rac and actin play in modulating primary granule release.
This diagram details proposed signalling pathway based on the results form this thesis. Stimulation of the formyl peptide receptor (FPR) receptor of the neutrophil by a bacterial peptide (fMLF) binding results in the activation of a signalling cascade. This leads to proposed activation of GEFs. Activaton of GEFs results in the activation of Rac2, by inducing the binding of GTP and release of GDP. This triggers signalling that resultsin cytoskeletal remodelling, in the from of actin polymerization. The polymerization of actin than allows for granule translocation to the periphery. CB is require for in vitro removal of cortical F-actin, however it is likely that activation of other signalling pathways during neutrophil exposure to various chemokines and other signalling molecules is responsible for this depolymerization step.

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