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Stable adhesion and migration of human neutrophils requires phospholipase D-mediated activation of the integrin CD11b/CD18

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Abstract

The pathways regulating integrin-mediated adhesion during neutrophil migration are incompletely defined. Using a flow-based model in which human neutrophils rolling on P-selectin were activated to migrate by the chemoattractant peptide fMLP, we investigated the role of phospholipase D (PLD). fMLP-stimulated PLD generation of phosphatidate (PtdOH); while inhibition of PtdOH production with butan-1-ol had no effect on the initial immobilisation of rolling neutrophils (supported by activation of constitutively surface-expressed β_2-integrin CD11b/CD18) it impaired longer-term stability of adhesion and reduced the rate of migration (supported by activation of de novo-exocytosed CD11b/CD18). PtdOH regulated these processes by controlling activation of exocytosed CD11b/CD18, and appeared to act by directly stimulating phosphatidylinositol 4-phosphate 5-kinase type I to generate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Cell-permeable PtdIns(4,5)P_2 recovered migration of neutrophils after PLD inhibition; PtdIns(4,5)P_2 appeared to act by promoting talin binding to CD18 and hence activating CD11b/CD18, as migration was inhibited when neutrophils were loaded with peptides previously shown to block the interaction between PtdIns(4,5)P_2 and talin or talin and CD18. Thus, these data indicate that PLD-synthesised PtdOH stimulates the generation of PtdIns(4,5)P_2, which in turn mediates talin binding to, and activation of, CD11b/CD18 required for neutrophil stable adhesion and migration.

Keywords: Migration; Adhesion; Integrin; CD11b/CD18; Phospholipase D; Phosphatidate; Alcohol; Phosphatidylinositol 4,5-bisphosphate

1. Introduction

The migration of neutrophils into tissue is an essential part of the first line of host defence against invading pathogens such as bacteria and fungi. In infected tissue, local endothelial cells lining blood vessels are stimulated to express members of the selectin family (E- and/or P-selectin) (Simon and Goldsmith, 2002). These receptors are able to capture neutrophils from the bloodstream, and support a rolling form of adhesion along the endothelium, driven by the shear force applied by the flow (Simon and Goldsmith, 2002). Subsequent stimulation of the neutrophils by specific chemoattractants induces activation of CD18 (β_2)-integrins (Simon and Goldsmith, 2002; Springer, 1995). The activated integrins support stable adhesion by binding to endothelial ligands such as intercellular adhesion molecule-1. It is known that successive cycles of integrin binding and unbinding, coupled to the generation of actomyosin-based protrusive and contractile force, enable neutrophil migration to the site of infection (Anderson et al., 2003, 2000; Sheikh and Nash, 1996). However, the pathways linking signals from chemoattractant receptors to integrin activation and cytoskeletal re-arrangements are incompletely defined.

Chemoattractants such as the bacterial peptide analogue, fMLP, initiate signals at the leading edge and the rear of a migrating neutrophil via a G protein-linked receptor. At the leading edge following G_i-coupled receptor occupation, a major migratory signal is initiated by $\beta\gamma$ receptor-subunit stimulation of

Abbreviations: PLD, phospholipase D; PtdOH, phosphatidate; PtdIns-(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PIPkin, phosphatidylinositol 4-phosphate 5-kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FERM domain, band 4.1, ezrin, radixin, moesin domain

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phosphatidylinositol 3-kinase γ to generate phosphatidylinositol 3,4,5-trisphosphate (Hirsch et al., 2000; Sasaki and Firtel, 2006). Downstream of this, Rac2 activation mediates WAVE-dependent stimulation of the Arp2/3 complex and thus, protrusive actin polymerisation (Srinivasan et al., 2003; Van Keymeulen et al., 2006). At the rear of the cell $G_{12/13}$-coupled receptor occupation provides another major signal required for migration; the α12/13 receptor-subunit mediates RhoA activation necessary for actin re-arrangements and integrin-based adhesion disassembly (Xu et al., 2003). Although changes in the binding of talin and α-actinin to the cytoplasmic tail of CD18 integrins have been linked to changes in integrin activation necessary for migration (Ginsberg et al., 2005; Tadokoro et al., 2003), the signals upstream of these responses have not been investigated in neutrophils.

A possible effector system, shown to regulate migration and integrin activation in various cell types is the phospholipase D (PLD) family of enzymes (PLD1 and PLD2) (Santy and Casanova, 2001; Zheng et al., 2006; Zhou et al., 1995; Zouwail et al., 2005). These enzymes operate through the generation of the messenger lipid, phosphatidate (PtdOH), resulting from phosphatidylcholine hydrolysis (Yang et al., 1967). Currently there are no available inhibitors of PLD family enzymes, although the generation of PtdOH by PLD1 or PLD2 can be attenuated by short chain primary aliphatic alcohols such as ethanol, propan-1-ol and butan-1-ol; both PLD1 and PLD2 preferentially transphosphatidylate these alcohols to generate non-signalling phosphatidylalcohols, instead of PtdOH (Yang et al., 1967). In contrast, secondary or tertiary isomers of these alcohols (where available) are not transphosphatidylated by PLD1 or 2 and thus, in experimental scenarios may be used as controls for the non-PLD-dependent effects of alcohol on cells.

PLD-generated PtdOH has previously been identified as a key messenger in inflammatory processes and its importance in the regulation of neutrophil migration is suggested by several lines of evidence; firstly, it has been shown that the anti-inflammatory agonist, lipoxin A_4 (Bonnans and Levy, 2006), inhibits neutrophil migration by generating a specific, direct intracellular inhibitor of both PLD1 and 2 (Levy et al., 1999); secondly, cAMP-specific phosphodiesterase 4D, a potent amplifier of neutrophil migration (Smith and Spina, 2005), is a direct target of PtdOH (Baillie et al., 2002; Grange et al., 2000); and thirdly recreational alcohol consumption (ethanol) is well-known to suppress immune-system responses (Happel and Nelson, 2005), with neutrophils from alcohol-fed humans and mice having reduced migration rates (Gluckman et al., 1977; Zhang et al., 1998). Nevertheless, the molecular mechanisms of PLD as a regulator of migration in neutrophils remain poorly defined.

The objective of this study was, for the first time, to investigate the role(s) of PLD-generated PtdOH in the regulation of adhesion and migration in primary human neutrophils. To do this we used a well-characterised system mimicking physiological venular flow conditions (Anderson et al., 2000; Rainger et al., 1997b; Sheikh and Nash, 1996) to study the kinetics of integrin-mediated neutrophil adhesion and migration, when neutrophils rolling on P-selectin were activated by superfusion with fMLP.

2. Materials and methods

2.1. Reagents

Phosphate-buffered saline (PBS) contained 1 mM Ca^{2+} and 0.5 mM Mg^{2+} pH 7.4 (Gibco, Paisley, UK) and 0.15% bovine serum albumin (BSA) (PBSA) (Sigma, Poole, UK). $MnCl_2$ was made up in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline instead of PBS to eliminate Mn^{2+} precipitation with phosphate. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) was dissolved in ethanol at 1 mM and diluted to 100 nM working concentration in PBSA. Butan-1-ol (Fisher, Loughborough, UK) was used to attenuate PLD signalling as, unlike ethanol, this form of alcohol has secondary (butan-2-ol) and tertiary (*tert*-butanol) isomers that are not transphosphatidylated by PLD and can therefore be used as controls to determine the non-PLD effects of alcohol on cells. Aqueous-soluble, cell-permeable PtdOH, DAG (both Sigma) and PtdIns(4,5)P_2 (A.G. Scientific, San Diego, USA) have acyl chains of eight carbons while the major physiological species of LPA (Sigma) (18 carbons and one double bond) is aqueous-soluble and cell-permeable. These lipids were prepared as previously described (Powner et al., 2005a). The monoclonal antibody against CD11b (KIM249) was used at 10 μg/ml and was a gift from Dr. Martyn Robinson, Celltech, UK. The following peptides were dissolved in PBS at 10 mg/ml (all from Alta Bioscience, University of Birmingham, UK): talin binding peptide PTDERSWVYSPLHYSAQ (SWVY) (Di Paolo et al., 2002; Ling et al., 2002). Control peptide PTDERS**A**VYSPLHYSAQ (SAVY). PtdIns(4,5)P_2-binding region of talin (385–406) GEQIAQLIAGYIDIILKKKKSK (KKKKSK) (Seelig et al., 2000).

2.2. Isolation and treatment of neutrophils

Venous blood was collected into 1.5 mg/ml ethylenediaminetetraacetic acid (EDTA). Neutrophils were isolated using a two-step density-gradient of Histopaque 1077 and 1119 (Sigma) as previously described (Sheikh and Nash, 1996). Neutrophils were washed twice and diluted to 10^6 cells/ml in PBSA.

Butan-1-ol or *tert*-butanol were added to cells for 5 min prior to perfusion and were present in all solutions throughout the adhesion assays. Lipids were added to cells with 0.5% butan-1-ol 5 min prior to perfusion and were present in all solutions throughout. Dose-response experiments were performed for each lipid and the optimal effective concentration was used in subsequent experiments.

Neutrophils were loaded with proteins and peptides using hypotonic shock as described (Anderson et al., 2003, 2000). In those studies, proteins up to 64 kD were loaded using this procedure, and loading was verified using fluorescently-tagged peptides or albumin. Briefly, neutrophils were incubated with peptides for 10 min at room temperature. Six volumes of sterile water were added, followed 30 s later by two volumes of 4x-concentrated PBSA. The neutrophils were then washed and resuspended at 10^6 cell/ml. All relevant controls were prepared in the same way but using PBSA in place of the peptide solution.