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An essential role for phospholipase D in the recruitment of vesicle amine transport protein-1 to membranes in human neutrophils

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ABSTRACT

Although phosphatidic acid (PA) regulates a wide variety of physiological processes, its targets remain poorly characterized in human neutrophils. By co-sedimentation with PA-containing vesicles we identified several PA-binding proteins including vesicle amine transport protein-1 (VAT-1), Annexin A3 (ANXA3), Rac2, Cdc42 and RhoG in neutrophil cytosol. Except for ANXA3, protein binding to PA-containing liposomes was calcium-independent. Cdc42 and RhoG preferentially interacted with PA whereas VAT-1 bound to PA or phosphatidylserine with the same affinity. VAT-1 translocated to neutrophil membranes upon N-formyl-methionyl-leucyl-phenylalanine (fMLF) stimulation. Inhibition of fMLF-induced PLD activity with the Src kinase inhibitor PP2, the selective inhibitor of PLD FIPI, or of PA formation with primary alcohols reduced VAT-1 translocation. In contrast, inhibition of PA hydrolysis with propranolol enhanced fMLF-mediated VAT-1 recruitment to membranes. PMA also redistributed VAT-1 to membranes in a PKC- and PLD-dependent manner. Though fMLF and PMA increased VAT-1 phosphorylation, different kinases appear to be involved. Cell fractionation revealed that a pool of VAT-1 was co-localized with primary, secondary and tertiary granules and plasma membrane markers in resting neutrophils. Stimulation with fMLF enhanced VAT-1 co-localization with CD32a, a plasma membrane marker. Confocal microscopy revealed that VAT-1 decorates granular structures at the cell periphery and double labeling with VAT-1/lactoferrin antibodies showed a partial co-localization with secondary granules in control and fMLF-stimulated cells. Characterization of these putative PA-binding proteins constitutes another step forward for a better understanding of the role of PLD-derived PA in neutrophil physiology.

1. Introduction

Polymorphonuclear leukocytes or neutrophils represent the most abundant type of white blood cell in peripheral blood. They express a wide variety of cell surface receptors, which allow them to respond to bacterial products, inflammatory cytokines and complement cleavage products. Neutrophils constitute therefore the first line of cellular defence against infection. Activation of cell surface receptors such as G protein-coupled receptors (GPCRs), Fc receptors, or receptors for tyrosine kinases induces a cascade of signaling events including the activation of phospholipase D (PLD), which is crucial for the regulation of many neutrophil functions [1,2].

Two mammalian PLDs (PLD-1 and PLD-2) have been described as well as several splice variants. PLD-1 is activated by conventional protein kinase C (PKC), ADP-ribosylation factors (ARFs), Rho small GTPases (RhoA, Cdc42, Rac1), phosphatidylinositol 4,5-bisphosphate (PIP_2) [1,3] and the small GTPase Sar1 [4]. In contrast, the mechanism of activation of PLD-2 remains elusive even though a weak activation of PLD-2 by ARFs and PKC has been reported [1]. PLD-2 is likely the PLD isoform that is sensitive to activation by unsaturated fatty acids [1,5]. The hydrolysis of the major membrane phospholipid phosphatidylcholine (PtdCho) by PLD results in the production of choline and phosphatidic acid (PA). Moreover, PLD has the propensity to catalyze a trans-esterification reaction called transphosphatidylation in the presence of short-chain primary alcohols such as ethanol or 1-butanol, resulting in the generation of phosphatidylethanol (PEt) or phosphatidylbutanol (PBut), respectively [1]. In neutrophils, PLD activation has been implicated in agonist-induced cell migration, exocytosis, phagocytosis of opsonized particles and activation of NADPH oxidase [1,6].

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PA is a lipid second messenger involved in the regulation of the activity of the enzyme such as type I PIP5-kinase as well as in the recruitment of specific proteins to cell membranes. Raf-1 kinase was the first protein reported to be translocated to membranes by its binding to PA. Other proteins were then discovered to be modulated by PA such as protein phosphatase-1, sphingosine kinase 1 [7], mTOR [8], the tyrosine kinase Fgr [9], Dock2 [10], and more recently the tyrosine kinase Fer [11]. Other studies have identified putative PA-binding proteins from rat brain cytosol [12,13]. Several of these proteins, including coatomer, ARFs, N-ethylmaleimide-sensitive factor (NSF), and kinesin are known to be involved in intracellular membrane traffic and in regulation of actin cytoskeleton dynamics [8].

PA is an important bioactive lipid but its molecular targets remain poorly investigated in neutrophils. Using a co-sedimentation method with liposomes containing PA, we identified several putative PA-binding proteins including Rac2, Cdc42, Annexin A3 (ANXA3), RhoG and vesicle amine transport protein-1 (VAT-1) from neutrophil cytosol. We show for the first time that VAT-1 is phosphorylated and recruited to membranes in response to stimulation with phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLF). Manipulation of the cellular levels of PA using propranolol, a non-specific inhibitor of PA phosphatase, a primary alcohol, butanol, and the PLD inhibitor FIPI suggests a role for PLD-derived PA in fMLF-mediated translocation of VAT-1 to intracellular membrane compartments in neutrophils.

2. Materials and methods

2.1. Reagents

Dextran T-500 and 1-O-[^{3}H]alkyl-2-lyso-phosphatidylcholine were purchased from Amersham Biosciences (Baie d'Urfé, QC, Canada) and adenosine deaminase (ADA) was from Roche (Mississauga, ON, Canada). PP2 and PP3 were purchased from Calbiochem (San Diego, CA, USA). Brain phosphatidylcholine, phosphatidylethanolamine (PtdEtn), phosphatidylserine (PS), egg phosphatidic acid and phosphatidylethanol were from Avanti Polar Lipids (Alabaster, AL, USA). SYPRO® Ruby protein gel staining, silver staining kits and Fura2/AM were from Molecular Probes (Eugene, OR, USA) and Bio-rad (Hercules, CA, USA), respectively. Propranolol and GF109203X were obtained from Biomol (Plymouth, PA, USA), di-isopropyl-fluorophosphate (DFP) was from Helix Technologies Inc (Scarborough, ON, Canada) and all other reagents including 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), 1-butanol, 2-butanol, PMA, fMLF and cytochalasin B (CB) were from Sigma–Aldrich (Oakville, ON, Canada). Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) and lymphocyte separation medium were obtained from Wisent Inc (St-Bruno, QC, Canada). Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA, USA). HiTrap NHS-activated Sepharose HP columns were from GE Healthcare (Baie d'Urfe, QC, Canada).

2.2. Antibodies

Anti-Rac2 and anti-RhoG antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Cdc42 Ab was from Becton Dickinson Biosciences (Rockville, MD, USA). Anti-MPO and anti-lactoferrin (LF) were obtained from Dako Canada (Mississauga, ON, Canada) and Sigma–Aldrich Canada, respectively. Anti-MMP9 was from Abcam (Cambridge, MA, USA). Anti-CD32a (FcRIIA) is an IgG fraction purified from a rabbit antiserum against the cytoplasmic domain of CD32a [14]. Rabbits were immunized with the N-terminal peptide MSDEREVAEAATGEDA (synthesized by the Centre de Protéomique de l'Est du Québec, Québec, QC, Canada) or the His-tagged C-terminal domain of VAT-1 (amino acids 317–393) as immunogen to produce N- or C-terminal Abs, respectively. The N-terminal Ab was used for blotting and the C-terminal Ab for immunoprecipitation. Anti-ANXA3 was purchased from Aviva Antibody Corp (San Diego, CA, USA). Rabbit polyclonal antibody against PLD-1, affinity purified from serum using HiTrap NHS-activated Sepharose HP columns coupled with antigens, has been described previously [15]. Texas Red-conjugated anti-lactoferrin and Texas Red-conjugated anti-IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Alexa Fluor 488-conjugated goat anti-rabbit was from Invitrogen (Carlsbad, CA, USA).

2.3. Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. After centrifugation at $180 \times g$ for 10 min, platelet-rich plasma was discarded from whole blood. Leukocytes were then isolated following sedimentation in 2% dextran T-500. Mononuclear cells were discarded after a centrifugation on Ficoll-Paque gradient and erythrocytes were removed by a 20s-hypotonic lysis in water. Neutrophils were finally resuspended in HBSS (pH 7.4) containing 1.6 mM Ca^{2+} but no Mg^{2+}.

2.4. Measurement of intracellular calcium concentration

Neutrophils (10×10^6 cells/ml) pre-treated or not with PP2 or PP3 for 10 min were incubated with 1 μM fura 2-AM for 30 min at 37 °C. Extracellular probe was removed by washing in HBSS, and cells were resuspended at 5×10^6 cells/ml. Cell suspensions were stimulated with 100 nM fMLF at the time indicated by the arrow. Fluorescence was monitored in a fluorescence spectrophotometer (Fluorolog-SPEX from Jobin Yvon, Edison, NJ, USA) using two excitation wavelengths at 340 and 380 nm and an emission wavelength of 510 nm. The internal calcium measurement was calculated as the ratio of the fluorescence values obtained at 340 and 380 nm.

2.5. PA protein-binding assay

Neutrophils (4×10^7/ml) were incubated with 1.1 mM DFP for 30 min at room temperature (RT), centrifuged, resuspended in cold buffer B (buffer B: 100 mM KCl, 50 mM Hepes, 5 mM NaCl, 3.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM PMSF and 2.5 μg/ml of both leupeptin and aprotinin, pH 6.8) at 6×10^7/ml, and sonicated using a Branson Sonifier (Danbury, CT, USA). Lysates were centrifuged at $700 \times g$ and the post-nuclear fractions were centrifuged ($175{,}000 \times g$, 45 min) at 4 °C. Lipid vesicles (2 mg/ml) were prepared in buffer B by mixing PtdCho/PtdEtn/PA in 50:50:0, 45:45:10, 40:40:20 and 25:25:50, or 50:50:0, 49.5:49.5:1, 48.75:48.75:2.5 and 47.5:47.5:5 molar ratios. Vesicles were washed once in buffer B and 200 μg/ml of lipid vesicles were mixed with neutrophil cytosol (4×10^7 cell equivalent/ml) for 30 min at 4 °C. Where indicated, sufficient $CaCl_2$ was added to give a free Ca^{2+} concentration of 1 μM. Samples were centrifuged ($175{,}000 \times g$, 30 min) and the vesicles washed in buffer B prior to analysis using 10% SDS-PAGE gels. Gels were silver stained or incubated overnight with SYPRO® Ruby according to manufacturer's instructions.

2.6. In-gel protein digest and mass spectrometry analyses

The SYPRO® Ruby stained bands were excised and in-gel protein digest was performed on a MassPrep liquid handling station (Micromass Inc, Beverly, MA, USA) according to the manufacturer's specifications and using sequencing grade modi-

fied trypsin. Extracted peptides were lyophilized using a vacuum evaporator and resuspended in 3 μl of 0.1% trifluoroacetic acid (TFA) solution. The matrix used for MALDI analysis was α-cyano-4-hydroxycinnamic acid (20 mg/ml in 50% acetonitrile, 0.1% TFA). Equal volumes of peptides and matrix solution were mixed, spotted on a stainless steel MALDI sample plate and air-dried at RT. MALDI-TOF-MS spectra were acquired on a Voyager-DE PRO Biospectrometry workstation and analyzed using DataExplorer software version 4.0. The instrument was operated in the positive-ion reflector delayed-extraction mode. The ProFound program (http://prowl.rockefeller.edu/cgi-bin.ProFound.exe) was used to search the non-redundant NCBI protein database for matching peptide mass fingerprints. Search criteria allowed a maximum of 1 missed cleavage by trypsin, complete carboxamidomethylation of cysteine, partial methionine oxidation and mass deviation smaller than 60 ppm.

LC/MS/MS spectra were obtained by microcapillary reverse-phase chromatography coupled to a LCQ DecaXP quadrupole ion trap mass spectrometer with a nanospray interface. Samples were loaded onto a 75-μm internal diameter C18 column and eluted with a gradient of water–acetonitrile–0.1% formic acid at a flow rate of 500 nl/min. Resulting peptide spectra were interpreted using the SEQUEST algorithm [16] and searched against proteins in the NCBI non-redundant protein database.

2.7. Translocation assays

Neutrophils (10^7/ml) were pre-treated with 1 mM DFP (10–20 min) at RT and incubated at 37 °C for 5 min with ADA (0.1 U/ml) to prevent adenosine accumulation prior to stimulation with 100 nM fMLF or 100 nM PMA. Incubations were stopped by adding 5 volumes of cold (4 °C) KCl-Hepes relaxing buffer (buffer A: 100 mM KCl, 50 mM Hepes, 5 mM NaCl, 3.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM PMSF, 2.5 μg/ml of both aprotinin and leupeptin, pH 7.2). In experiments with propranolol, neutrophils were not treated with DFP. Where indicated, neutrophils were pre-treated with 1-butanol or 2-butanol for 1 min, 5 μM PP2 or PP3 for 10 min, propranolol for 5 min, 3 μM GFX for 5 min or with 0.1 μM or 1 μM FIPI for 60 min before stimulation. Cells were also pre-incubated with 10 μM CB prior to stimulation with fMLF. Cell suspensions were sonicated 20 s and centrifuged 7 min at 700 × *g*. Unbroken cells and nuclei were discarded and supernatants ultracentrifuged at 180,000 × *g* for 45 min. Membrane pellets were washed in buffer A and samples were assayed for protein content.

2.8. Electrophoresis and immunoblotting

The Bradford assay was used to determine protein concentration according to manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Proteins (25–50 μg) were separated on a 10% SDS-PAGE gel and transferred to Immobilon polyvinylidene difluoride (PVDF) membrane for 3 h at 500 mA. Immunoblotting were performed using the indicated Abs and revealed with either HRP-conjugated secondary anti-rabbit Ab (1/20,000) or anti-mouse Ab (1/20,000) and the western lightning chemiluminescence kit (Perkin Elmer, Waltham, MA, USA).

2.9. PLD activity

Neutrophils were pre-labeled with 1-O-[^{3}H]alkyl-2-lyso-phosphatidylcholine (2 μCi/10^7 cells, 90 min). Cells (8×10^6/ml) were pre-incubated at 37 °C for 5 min and treated with 10 μM CB for 5 min prior to stimulation with 100 nM fMLF for 10 min in the presence of 1% ethanol. The samples were processed as previously described to monitor the levels of [^{3}H]PEt formed [17].

2.10. Phosphorylation assay

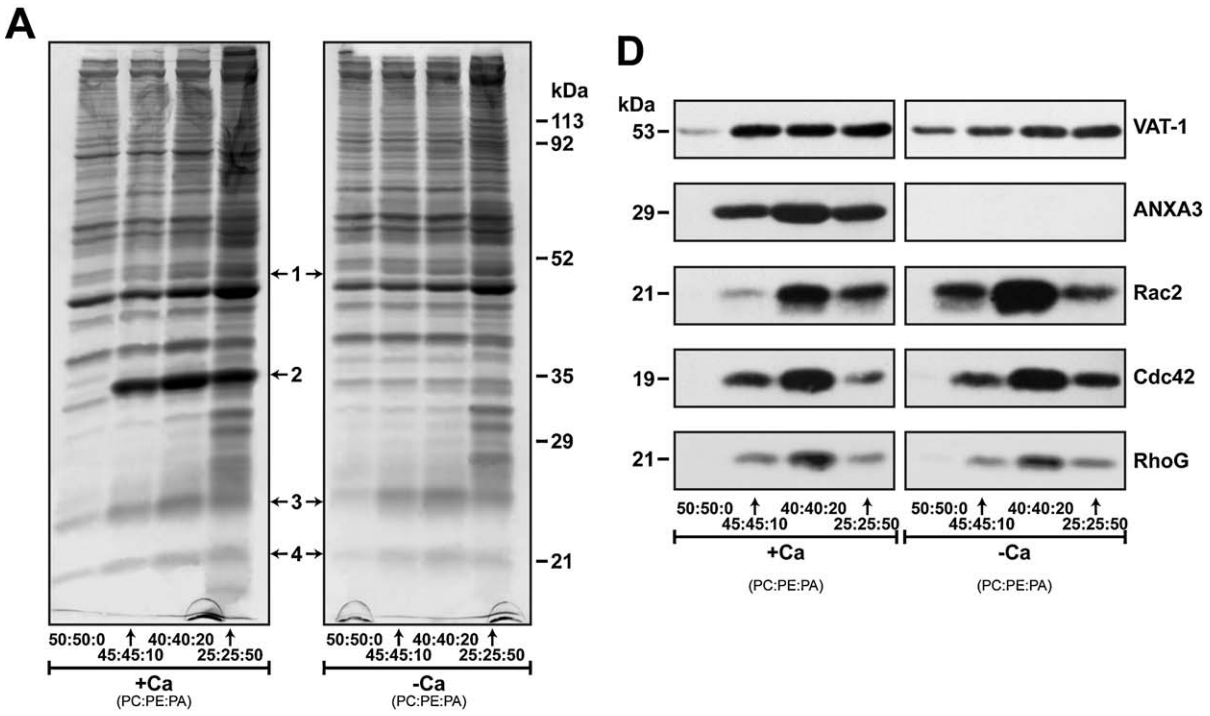
Neutrophils were incubated with [^{32}P] (1 mCi/10^7 cells) in HBSS for at least 90 min. After washing and warming at 37 °C for 5 min, cell suspensions (10^7 cells/ml) were stimulated with PMA or fMLF (100 nM) for the indicated times in the presence or absence of 3 μM GFX for 5 min. Where indicated, cell suspensions were incubated with 10 μM CB and ADA (0.1 U/ml) for 5 min prior to stimulation with fMLF. Cells were lysed and immunoprecipitation was performed overnight at 4 °C with the VAT-1 C-terminal Ab. The immune complexes were collected and proteins were separated using 10% SDS-PAGE gels. Gels were dried, exposed to a PhosphorImager screen, and analyzed in a PhosphorImager BAS-1800II (Fujifilm, Tokyo, Japan).

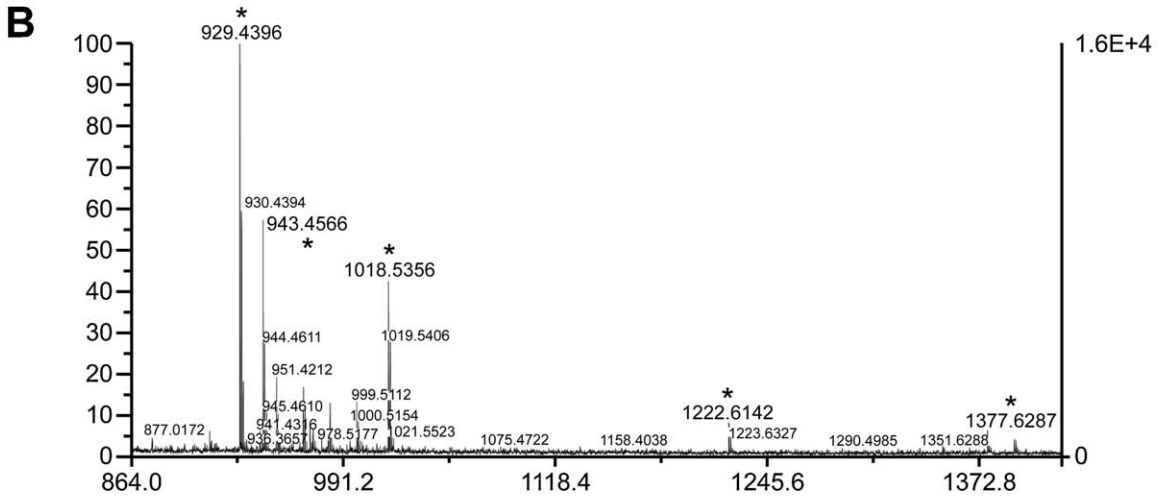
2.11. Subcellular fractionation

Neutrophil subcellular organelles were isolated following the method of Kjeldsen et al. with modification [18]. Cells (6.5×10^8) were treated with 1 mM DFP for 15 min at RT prior to stimulation with fMLF or DMSO for 1 min at 37 °C. Incubations were stopped by diluting the cells 5-fold with ice-cold HBSS. Samples were then centrifuged and resuspended in 10 ml ice-cold KCl-Hepes relaxation buffer (100 mM KCl, 50 mM Hepes, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DFP, 2.5 μg/ml aprotinin and 2.5 μg/ml leupeptin, pH 7.2). Neutrophils were pressurized (400 psi, 10 min) in a nitrogen bomb (Parr Instrument, Moline, IL, USA). Cavitates were centrifuged at 400 × *g* for 5 min to pellet unbroken cells. Supernatants were laid onto 3 × 4.5 ml Percoll step gradients (1.050; 1.090; 1.120 g/ml). After centrifugation (37,000 × *g*, 30 min), 18 fractions were collected (1 ml each), starting from the bottom of the tube. This procedure allows the separation of primary, secondary, and tertiary granules and plasma-membrane-enriched fractions. Each fraction was centrifuged (100,000 × *g*, 90 min) to pellet Percoll and samples were processed for immunoblot analyses using Abs against VAT-1, RhoG and marker proteins of various membrane compartments: MPO (primary granules), LF (secondary granules), MMP9 (tertiary granules), CD32a (plasma membrane).

2.12. Confocal microscopy

Neutrophils (7.5×10^5) were warmed at 37 °C for 5 min and incubated for a further 5 min with ADA (0.1 U/ml) and CB (10 μM) prior to stimulation with 100 nM fMLF for 90 s or incubated with an equal volume of DMSO. Incubations were stopped by diluting the cell suspensions with 500 μl ice cold HBSS. Cells were centrifuged (3500 rpm, 5 min at 4 °C) and resuspended in 4% paraformaldehyde (Laboratoire Mat, Québec, QC, Canada) for 20 min at RT. Fixed cells were cytospinned (1000 rpm, 10 min) onto glass coverslips coated with 1 μg/ml poly-L-lysine, permeabilized with 0.1% Triton X-100 for 15 min and blocked for 30 min with 10% FBS in PBS. After washing with PBS, cells were incubated at RT for 60 min with C-terminal VAT-1 Ab (dilution 1/250) or the pre-immune antiserum. After washing with PBS containing 0.01% Triton X-100 and 5% FBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody for 30 min. LF was detected by incubating cells at RT for 60 min with Texas Red-conjugated rabbit anti-human LF or with control Texas Red-conjugated rabbit IgG Ab at 1:100 dilution. Where indicated nuclei were labeled for 5 min with PI (500 nM). Samples were mounted using Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) and viewed under an Olympus IX-70 laser scanning confocal microscope (Olympus, Hamburg, Germany) using a 100× UPlanApo/1.35 NA oil immersion objective. Samples were scanned sequentially at each excitation wavelength to minimize crosstalk





C

gi|18379349|ref|NP_006364.2| vesicle amine transport protein 1; synaptic vesicle membrane protein VAT-1 homolog; membrane protein of cholinergic synaptic vesicles [Homo sapiens]

1	11 21	31	41	51	
MSDEREVAEA	ATGEDASSPP	PKTEAASDPQ	HPAASEGAAA	AAASPPLLRC	LVLTGFGGYD
61	71	81	91	101	111
KVKLQSRPAA	PPAPGPGQLT	LRLRACGLNF	ADLMARQGLY	DRLPPLPVTP	GMEGAGVVIA
121	131	142	151	161	171
VGEGVSDRKA	GDRVMVLNRS	GMWQEEVTVP	SVQTFLIPEA	MTFEEAAALL	VNYITAYMVL
181	191	201	211	221	231
FDFGNLQPGH	SVLVHMAAGG	VGMAAVQLCR	TVENVTVFGT	ASASKHEALK	ENGVTHPIDY
241	251	261	271	281	291
HTTDYVDEIK	KISPKGVDIV	MDPLGGSDTA	KGYNLLKPMG	KVVTYGMANL	LTGPKRNLMA
301	311	321	331	341	351
LARTWWNQFS	VTALQLLQAN	RAVCGFHLGY	LDGEVELVSG	VVARLLALYN	QGHIKPHIDS
361	371	381	391		231
VWPFEKVADA	MKQMQEKKNV	GKVLLVPGPE	KEN		

Fig. 1. Characterization of neutrophil PA-binding proteins.
Vesicles containing increasing concentrations of PA were mixed with neutrophil cytosol (4×10^7 cell eq/ml, 30 min, 4 °C) in the presence or the absence of a final free Ca^{2+} concentration of 1 μM. (A) SYPRO® Ruby stained protein pulled down with PA-enriched vesicles. (B) MALDI-TOF-MS spectrum of protein band 2. * Indicates the monoisotopic mass of the peptides that match the sequence of ANXA3. (C) Amino acid sequence of human VAT-1 (NCBI Reference Sequence NP_006364). The peptides identified by MALDI-TOF-MS are underlined. (D) Immunoblots of VAT-1, ANXA3, Rac2, Cdc42 and RhoG affinity purified with PA-containing vesicles. One experiment representative of three is presented in A and D.

between signals. Digital images were processed with Olympus Fluoview FV300 (version 4.3) acquisition software. Pearson's coefficient analysis was performed using the JACoP plug-in in Image J software (Rasband. W.S., U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/).

2.13. Statistical methods

Student's paired *t*-test was used for comparisons between experimental groups. Analyses were performed using GraphPad Prism version 5. A p-value <0.05 was considered significant.

3. Results

3.1. In human PMN cytosol, VAT-1, ANXA3, Rac2, Cdc42 and RhoG bind PA

Although PLD-derived PA has been implicated in neutrophil migration and degranulation [6,19,20], PA-binding proteins remain poorly characterized in leukocytes. Human neutrophil cytosols were therefore incubated with phospholipid vesicles containing increasing concentrations of PA in the presence or absence of calcium. Proteins pulled down were resolved using SDS-PAGE gels. SYPRO® Ruby (Fig. 1A) or silver (data not shown) staining of gels allowed us to focus on four protein bands that were specifically enhanced by incorporating PA into the phospholipid vesicles. These four bands were cut out and proteins were trypsin-digested. MALDI-TOF-mass spectrometry (MS) or liquid chromatography tandem-mass spectrometry (LC/MS/MS) analyses resulted in the generation of various spectra. A search in the non-redundant NCBI protein database for matching peptide mass fingerprints revealed 5 peptides specific for human ANXA3 (Fig. 1B) and 6 peptides for human VAT-1 (Fig. 1C). Up to eight putative PA-binding proteins were identified in human neutrophils (Table 1). Most of these proteins are known to modulate the organization of the actin cytoskeleton and cell shape such as Rho small GTPases Cdc42, RhoG, Rac2 and a subunit of the actin related protein 2/3 complex subunit 4 (ARPC4). Other identified proteins include the 26S protease regulatory subunit 7 and elongation factor-1-gamma (EF1G). The ability of VAT-1, ANXA3, Rac2, Cdc42 and RhoG to bind PA-containing vesicles in a calcium-dependent or -independent manner was determined by Western blotting (Fig. 1D). The binding of ANXA3 to PA-vesicles, but not that of VAT-1, Rac2, Cdc42 and RhoG, was calcium-dependent, which is in line with the general property of annexins that associate with membranes in a calcium-regulated manner and through interaction with negatively charged phospholipids [21]. Since various proteins known to bind to PA such as Annexin A4, and Raf-1 have also been reported to interact with PS [7,22], we incubated neutrophil cytosols with phospholipid vesicles containing physiologically relevant concentrations of either PA or PS in the presence of calcium in order to define the binding specificity of VAT-1, Rac2, Cdc42 and RhoG for anionic phospholipids. As shown in Fig. 2, Cdc42, RhoG and at a lesser extent Rac2 interacted with both anionic phospholipids with a preference for PA-enriched vesicles. PA increased the binding of Rho GTPases to vesicles in a concentration-dependent manner as estimated by Western blotting and with a threshold detection limit of ~1 nmol% (equivalent to a bulk concentration of 2.9 μM) of total phospholipids. In contrast, VAT-1 bound PA or PS-containing liposomes with the same affinity.

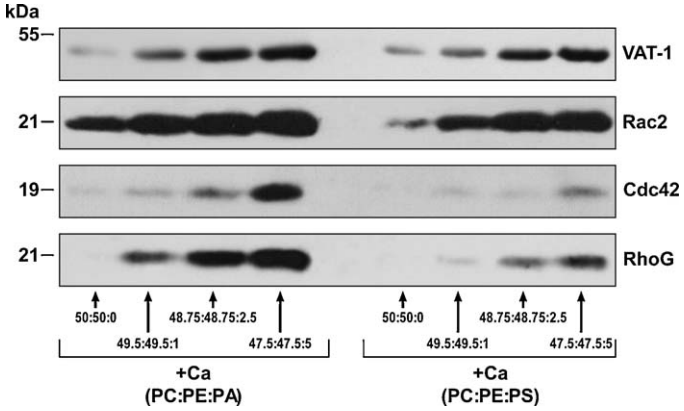


Fig. 2. Contrary to that of VAT-1, the binding of Cdc42 and RhoG is selective for PA. PA or PS vesicles (200 μg/ml) were mixed with neutrophil cytosol (4×10^7 cell eq/ml) for 30 min at 4 °C in the presence of a final free Ca^{2+} concentration of 1 μM. Samples were processed and probed with VAT-1, Rac2, Cdc42 and RhoG Abs as described in Section 2. One experiment representative of three is presented.

3.2. VAT-1 translocation to membrane is partially controlled by Src kinases

Src kinases play an important role in cell signaling by promoting phosphorylation on tyrosine residues and some studies have underlined their role in the activation of PLD [23,24]. To assess whether Src kinases could be involved in fMLF-stimulated PLD activity, neutrophils were pre-incubated with the specific Src kinase inhibitor PP2 [25], or the inactive analogue PP3. Neutrophils were also primed with CB and treated with ADA, to prevent accumulation of adenosine, for 5 min prior to stimulation to increase the amount of PEt produced by PLD [17]. As shown in Fig. 3A, increasing concentrations of PP2 but not PP3 decreased fMLF-induced PEt formation. PEt formation was reduced by 74% in cells treated with 10 μM of PP2. Increasing the concentration of PP2 up to 25 μM did not further reduce fMLF-mediated activation of PLD. We also verified whether other fMLF-induced responses

Table 1
Identification of PA-binding proteins from neutrophil lysates.

Protein	Accession number	Molecular weight (kDa)	MALDI-TOF-MS		LC/MS/MS	
			Peptides	Coverage	Peptides	Coverage
Vesicle Amine Transport Protein 1	NP_006364	41.9 (1)	n.d.	n.d.	6	24.2%
26S Protease Regulatory Subunit 7	P46472	48.9 (1)	n.d.	n.d.	5	12.5%
EF-1-gamma	Q9D8N0	50 (1)	n.d.	n.d.	1	3.0%
Annexin A3	XP_034350	35 (2)	5/18	18%	n.d.	n.d.
Small GTP binding Protein Rac2	NP_002863	21.4 (3)	4/39	20%	6	34.4%
Small GTP binding Protein Cdc42	NP_593536	21.3 (3)	n.d.	n.d.	3	14.1%
Small GTP binding Protein RhoG	XP_006153	21.2 (4)	3/12	20%	3	20.4%
ARP 2/3 Complex 20 kDa Subunit 4 (ARPC4)	BAB31059	19.6 (4)	n.d.	n.d.	3	16.1%

(·) SYPRO® Ruby stained bands analyzed by MALDI-TOF-MS and LC/MS/MS. Vesicles containing increasing concentrations of PA were mixed with neutrophil cytosol (4×10^7 cell eq/ml, 30 min, 4 °C). Samples were centrifuged (175,000 × *g*, 30 min) and the vesicles washed prior to 10% SDS-PAGE analysis. Proteins were then stained with Sypro® Ruby and prepared for MALDI-TOF-MS or LC/MS/MS analysis as described in Section 2. Non-redundant NCBI protein database was used to match peptides. n.d. = not detected.

such as transient increases in the levels of cytosolic calcium were also sensitive to inhibition by PP2 in neutrophils. Fig. 3B shows that PP2 and PP3 did not affect the fMLF-induced increase of intracellular free calcium levels. Taken together the results suggest that fMLF-mediated activation of PLD is dependent on Src kinase and fMLF-mediated calcium mobilization is not altered by a concentration of PP2 that inhibits the formation of PEt by more than 70%.

Since fMLF increases PLD activity resulting in a production of PA, through a mechanism involving Src kinases (Fig. 3A), the next series of experiments examined whether fMLF could affect the subcellular distribution of the PA-binding protein VAT-1 in neutrophils. VAT-1 is present in the cytosol and the crude membrane fraction as well (Fig. 3C). Following stimulation of the cell suspensions with fMLF the amount of VAT-1 associated with membranes increased, while the amount recovered in the cytosol decreased. Fig. 3C shows that PP2 (10 μM) but not PP3 reduced by almost 50% ($p = 0.0445$) fMLF-mediated increase in VAT-1 association with membranes suggesting that Src kinases regulate the subcellular localization of VAT-1 through a mechanism involving, at least in part, activation of PLD.

3.3. VAT-1 translocation to membrane is partially controlled by PLD and PKC

In order to further characterize the role of PLD-produced PA in VAT-1 or ANXA3 translocation to neutrophil membranes, cells were pre-treated with increasing concentrations of propranolol, an inhibitor of PA phosphatase (PAP), which prevents the production of diacylglycerol (DAG) from PA leading to accumulation in cells [26]. As illustrated in Fig. 4A, treatment with 10 μM propranolol increased fMLF-mediated VAT-1 translocation to membranes nearly twofold ($p = 0.0299$). Enhanced recruitment by fMLF of VAT-1 to membranes was observed for all concentrations of propranolol tested, however the stimulatory effect on VAT-1 translocation decreased as the concentration of propranolol increased. In the presence of 200 μM propranolol, the highest concentration tested, the amounts of VAT-1 recruited to membranes in response to fMLF was still superior to that induced by fMLF alone (Fig. 4A). We also monitored the effect of propranolol on fMLF-mediated recruitment of ANXA3 (Fig. 4B). At concentrations found to enhance VAT-1 translocation to membranes (50 μM), propranolol decreased fMLF-induced ANXA3 binding to membranes. The amount of ANXA3 recruited to membranes was decreased by ~29% ($p = 0.0333$). Because propranolol can affect other targets [27–29], we also used the ability of PLD to catalyze a transphosphatidylation reaction to reduce the level of PA in cell membranes. In these experiments cells were incubated with 1-butanol, a short-chain primary alcohol, which is used by PLD to produce PBut at the expense of PA, or 2-butanol, a secondary alcohol that is not used by PLD for transphosphatidylation reactions. As shown in Fig. 4C, 1-butanol, but not 2-butanol, reduced VAT-1 translocation to membranes. The fMLF-induced recruitment of VAT-1 was decreased by 56% ($p = 0.0202$) with 0.25% 1-butanol. Concerns regarding the efficacy and the specificity of 1-butanol to fully prevent PA production have grown since this alcohol could not entirely block PA production at the concentration used [30], and it may have other effects on cells [31]. As a third complementary approach to study the role of PLD-derived PA in the recruitment of VAT-1 to membranes, neutrophils were pre-incubated with FIPI, an analogue of halopemide which was recently described to be a selective inhibitor of PLD-1 and PLD-2 activity [32]. As illustrated in Fig. 4D, the lowest concentration of FIPI (0.1 μM) significantly reduced fMLF-mediated VAT-1 translocation ($p = 0.0197$), thereby confirming previous results obtained using 1-butanol and propranolol. Taken together, these data

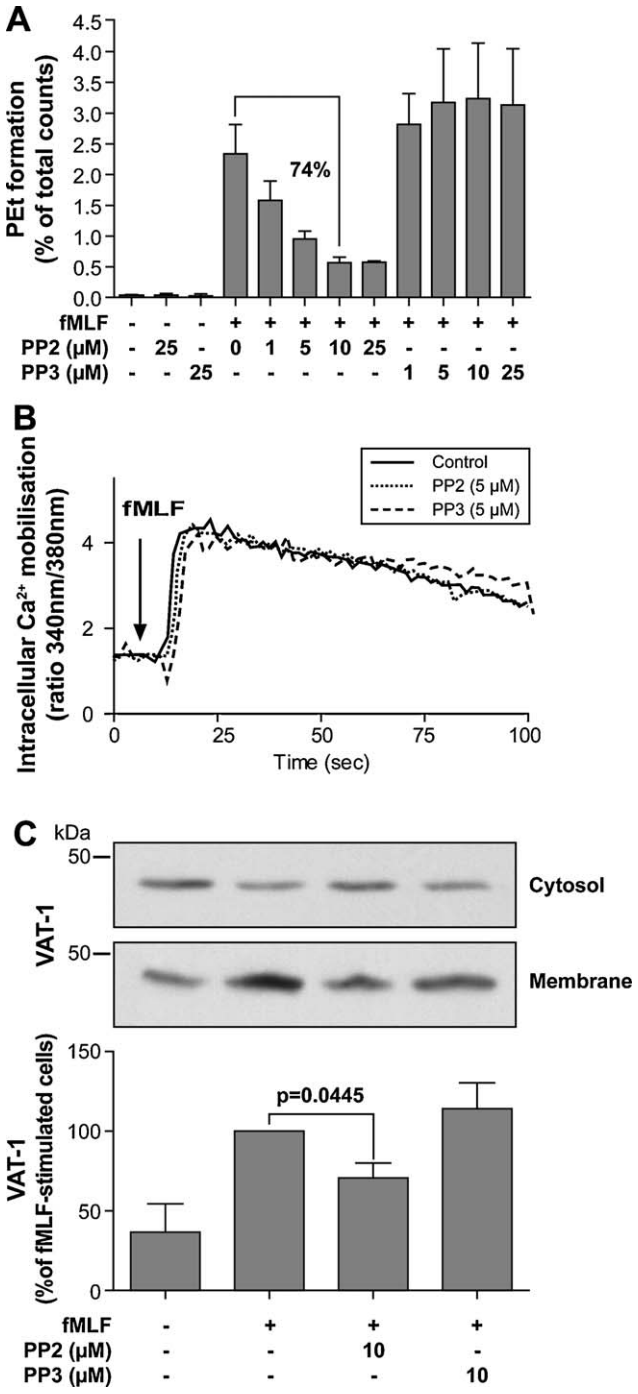


Fig. 3. Role of PA-derived PLD in fMLF-induced VAT-1 translocation to neutrophil membranes.

(A) Neutrophils labeled with 1-O-[^{3}H]alkyl-2-lyso-phosphatidylcholine were incubated with CB (10 μM) and ADA (0.1 IU/ml) for 5 min prior to stimulation with fMLF in the presence or absence of PP2 or PP3. [^{3}H]PEt was separated and quantified as described in Section 2. Results are expressed as mean ± SD of 2 independent experiments. (B) Neutrophils were incubated in the presence or absence of PP2 or PP3 for 10 min and stimulated with fMLF at the time indicated by the arrow. Cytosolic calcium concentration was monitored as described in Section 2. One experiment representative of 3 is presented. (C) Cell suspensions were pretreated with CB and ADA and stimulated with fMLF for 2.5 min with or without PP2 or PP3. The membrane and cytosolic fractions were analyzed by immunoblotting using VAT-1 Ab as described in Section 2. Histograms represent the quantification of protein translocation to membranes. Results are expressed as mean ± SD of 3 independent experiments.

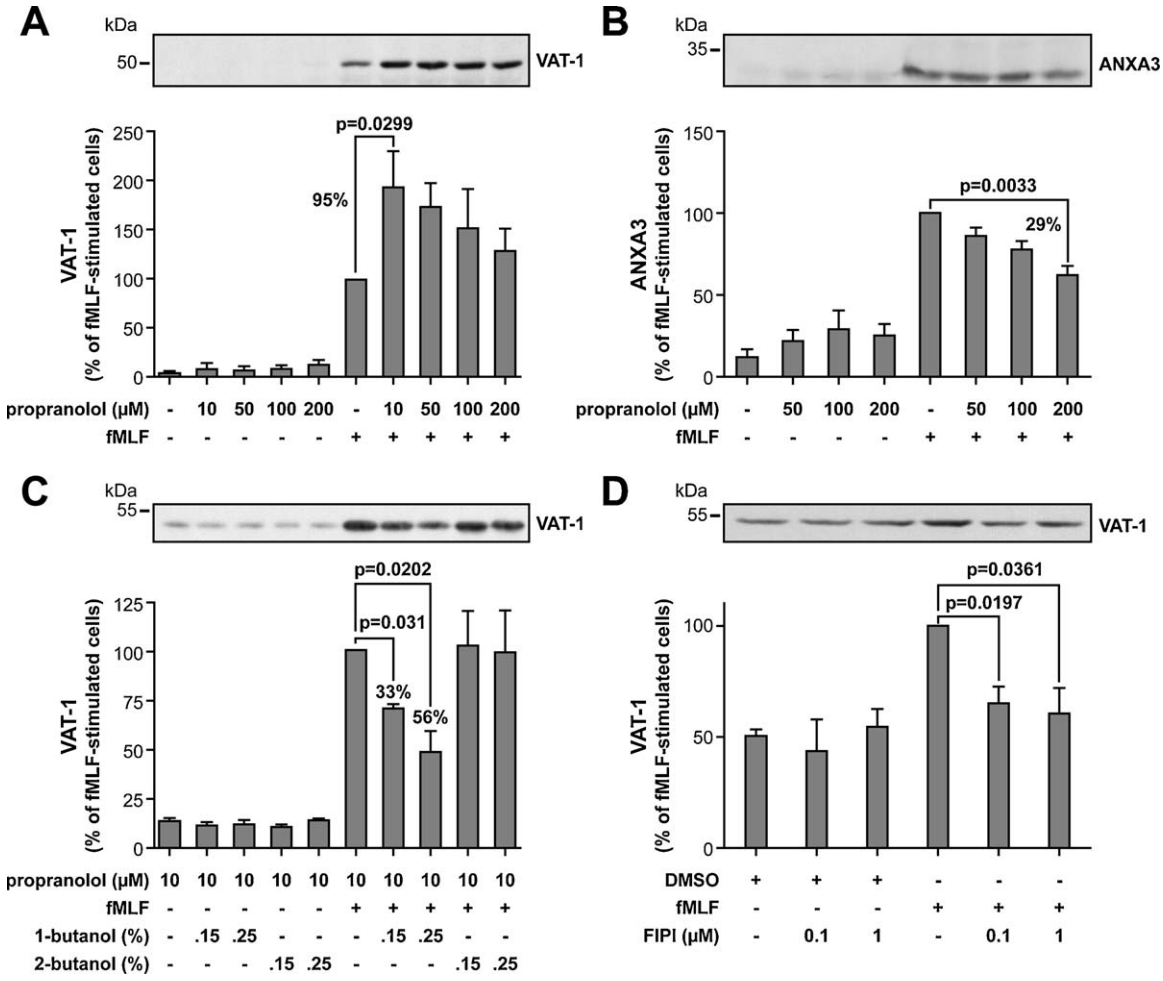


Fig. 4. VAT-1 and ANXA3 translocation to membrane is partially controlled by PLD.
Cell suspensions were pre-warmed at 37 °C and pretreated with CB and ADA. Where indicated the samples were pretreated with propranolol for 5 min (A, B), with propranolol for 5 min and then 1-butanol or 2-butanol for 1 min (C), or with FIPI for 60 min (D) prior to stimulation with fMLF for 1 min. The membrane fractions were assayed for protein content and proteins were separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane and probed using VAT-1 (A, C, D) or ANXA3 (B) Abs. Histograms represent the quantification of protein translocation to membranes. The results are expressed as mean ± SD of at least 3 independent experiments.

suggest that the binding of VAT-1 to neutrophil membranes upon fMLF stimulation requires the formation of PLD-derived PA.

To examine further the extent to which the PLD pathway regulates the subcellular distribution of VAT-1, neutrophils were also stimulated with concentrations of PMA reported previously to activate PLD-1 and/or PLD-2 in PKCα-dependent manner [1,3]. The amounts of VAT-1 associated with membrane fractions were increased 1.43 ± 0.68 fold and 2.46 ± 1.21 fold in response to stimulation with 100 nM PMA for 2.5 min and 10 min, respectively (Fig. 5A). On one hand, when neutrophils were pretreated with the non-selective inhibitor of PKC isoforms GFX [33], PMA-induced translocation of VAT-1 to membranes was completely abolished. On the other hand, inhibition of PLD activity with FIPI also entirely antagonized PMA-mediated recruitment of VAT-1 to membranes (Fig. 5B; $p = 0.008$), thereby suggesting the involvement of PKCs and PLD in the recruitment of VAT-1.

3.4. PMA- but not fMLF-induced VAT-1 phosphorylation was PKC dependent

Protein phosphorylation by protein kinases constitutes a major intracellular signaling event that affects the properties of various proteins including activity, stability and/or localization within cells. Though the enzymatic activity and the function of VAT-1 are not known, we examined whether VAT-1 was phosphorylated in response to stimulation with fMLF or PMA for 2.5 min and 10 min, respectively. As shown in Fig. 6A, PMA induced a strong phosphorylation of VAT-1 ($p < 0.0001$) whereas that induced by fMLF remained weak but significant ($p = 0.0014$). On one hand, VAT-1 phosphorylation induced by PMA was reduced by 57% when cell suspensions were preincubated with the PKC inhibitor GFX (Fig. 6B). On the other hand, GFX did not affect fMLF-induced VAT-1 phosphorylation (Fig. 6C). Although Src kinases were found to regulate fMLF-induced translocation of VAT-1 to membranes (Fig. 3C), VAT-1 was not phosphorylated on tyrosine as estimated by Western blotting with anti-phosphotyrosine Abs (data not shown). These data suggest that PMA-mediated phosphorylation of VAT-1 is PKC-dependent whereas that induced by fMLF uses another kinase pathway.

3.5. VAT-1 was localized to intracellular membrane compartments in neutrophils.

In the next series of experiments we examined the distribution of VAT-1 using the subcellular fractionation method on a three-layer Percoll gradient [34]. To assess the localization of neutrophil organelles in the gradient, 18 collected fractions were analyzed for the presence of myeloperoxidase (MPO, primary granules), LF (secondary granules), matrix metallo-proteinase 9 (MMP9, tertiary granules) and CD32a (plasma membrane). These fractions were

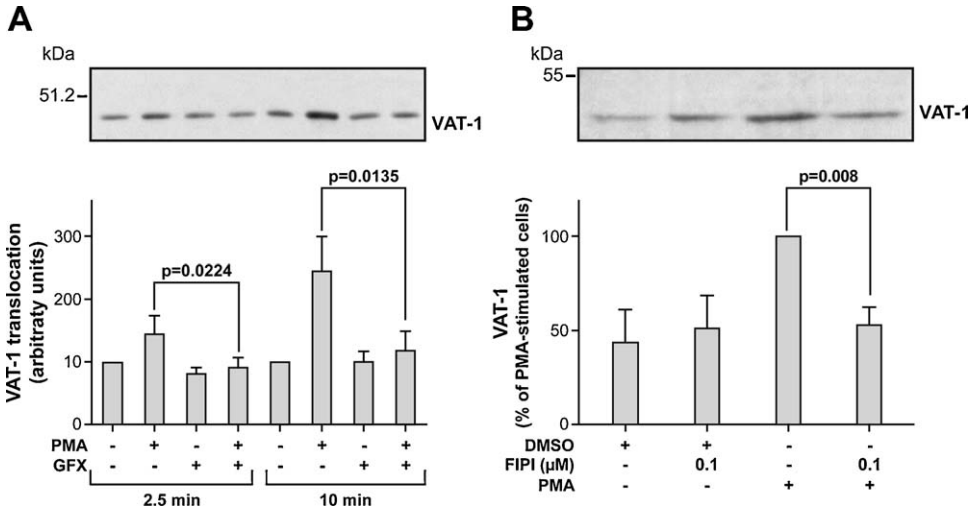


Fig. 5. VAT-1 translocation to membrane is mediated by PKC and PLD after PMA activation.
Cell suspensions were pre-warmed at 37 °C for 5 min and incubated with ADA prior to stimulation with PMA for the indicated times. Where indicated, cell suspensions were incubated with a PKC inhibitor (GFX) for 5 min (A) or a PLD inhibitor (FIPI) for 60 min (B) prior to stimulation with PMA. The membrane fractions were assayed for protein content and proteins were separated by 10% SDS-PAGE. Samples were transferred to a PVDF membrane and probed using VAT-1 antibody. Histograms represent the quantification of protein translocation to membranes. The results are expressed as mean ± SD of 5 independent experiments for A and 4 for B.

immunoblotted with VAT-1, RhoG and PLD-1 Abs. As illustrated in Fig. 7A, VAT-1 colocalized with primary (fractions 1–4), secondary (fractions 5–7), and tertiary (fractions 8–10) granules, and plasma membrane and secretory vesicles (fractions 11–13) protein markers. VAT-1 was also present in fractions 14–18 that were previously shown to contain the cytosolic marker lactate dehydrogenase (LDH) [35]. To further characterize the potential contribution of PLD enzymes we also assessed the subcellular distribution of PLD-1, which is the only PLD isoform detectable at the protein level in human neutrophils [36]. The distribution PLD-1 partially overlapped with VAT-1 in fractions corresponding to tertiary granules (fractions 8–10), secretory vesicles and plasma membrane (fractions 11–13) and the cytosolic fractions (14–18). Stimulation with fMLF redistributed VAT-1 in fractions 9–10 overlapping partially with the marker of tertiary granules MMP9 and fractions 11–12 positive for the plasma membrane marker CD32a (Fig. 7B). Stimulation with fMLP also relocalized PLD-1 in fractions 8–11 that partially overlap with the tertiary granules, secretory vesicles and plasma membrane markers. In contrast, the co-localization of RhoG with the plasma membrane marker CD32a was not altered following stimulation of the cells with fMLF.

Because secretory vesicles and plasma membranes were recovered in the same percoll gradient fractions, immunocytochemical staining of neutrophils followed by fluorescence microscopy was used to better visualize the localization of VAT-1. Fig. 8A shows a strong intracytoplasmic granular staining with accentuation in the cell periphery. The intracytoplasmic granular staining pattern of VAT-1 was conserved in fMLF-stimulated neutrophils. No well-defined staining of the plasma membrane was observed. Since VAT-1 may decorate various populations of vesicles we examined, using confocal microscopy, the co-localization of VAT-1 with LF in specific granules. Some LF positive granules appeared to lack labeling for VAT-1 and as shown in Fig. 8B, VAT-1 co-localized partially with LF in control and fMLF-activated neutrophils with a mean Pearson's coefficient of 0.740 and 0.786, respectively. As some proteins such as p47phox have a distinct distribution in neutrophils in response to soluble or insoluble stimulators [37], we assayed phagocytosis of IgG-opsonized polystyrene beads to further characterize the localization of VAT-1 in neutrophils. VAT-1 was not enriched at the phagocytic cup at the plasma membranes (arrow in Supplemental Data 1). We also noticed that VAT-1 was not recruited at the membrane of sealed phagosomes that surrounds fully internalized particles (asterisks in Supplemental Data 1). Taken together, these data suggest that VAT-1 is recruited to intracellular organelles other than secondary granules and phagosomes.

4. Discussion

Originally considered as the simplest membrane phospholipid and restricted to its role in the synthesis of membrane lipids and storage lipids, PA has increasingly been recognized to be a pleiotropic bioactive lipid involved in cell growth, proliferation, membrane trafficking and cytoskeleton reorganization [8]. In human neutrophils, the production of PA is mainly the consequence of PLD activity since the presence of DAG kinase inhibitor had no effect on the level of PA as monitored by mass measurements [38]. Although neutrophils constitute the first line of defense against invading pathogens and produce significant amounts of PA only a few studies have reported the effects of PA on cell activation or identified targets of this lipid. So far, only Dock2 [10], flavocytochrome B [39] tyrosine kinase Fgr [9] and NADPH oxidase [40] were reported to bind to and to be regulated by PA in neutrophils. The data reported here identify 8 new putative PA-binding proteins including EF1G, 26S protease regulatory subunit 7, ARPC4, ANXA3, Rac2, Cdc42, RhoG and VAT-1 in neutrophil cytosol. The binding of ANXA3, Rac2, Cdc42, RhoG and VAT-1 to PA-enriched vesicles was validated by immunoblotting. We then focused on VAT-1 in neutrophils. We report that VAT-1 localizes to the cytosol and to intracytoplasmic membrane compartments, and is recruited to membranes in a calcium-independent manner by PLD-derived PA. Furthermore, depending on the stimulus, VAT-1 was phosphorylated through signaling pathways involving PKC-dependent and independent pathways.

The coupling of PA-containing vesicles and mass spectrometry allowed us to identify various PA-binding partners from human neutrophil cytosol. Several of these proteins, including subunits of the ARP complex and of the proteasome, small GTPases, annexins, elongation factors and VAT-1 were also identified in a proteome analysis of acidic phospholipid-binding proteins in rat brain cytosol and membranes [13]. EF1G was one of these acidic phospholipid-binding proteins and EF1A1 was reported to bind to

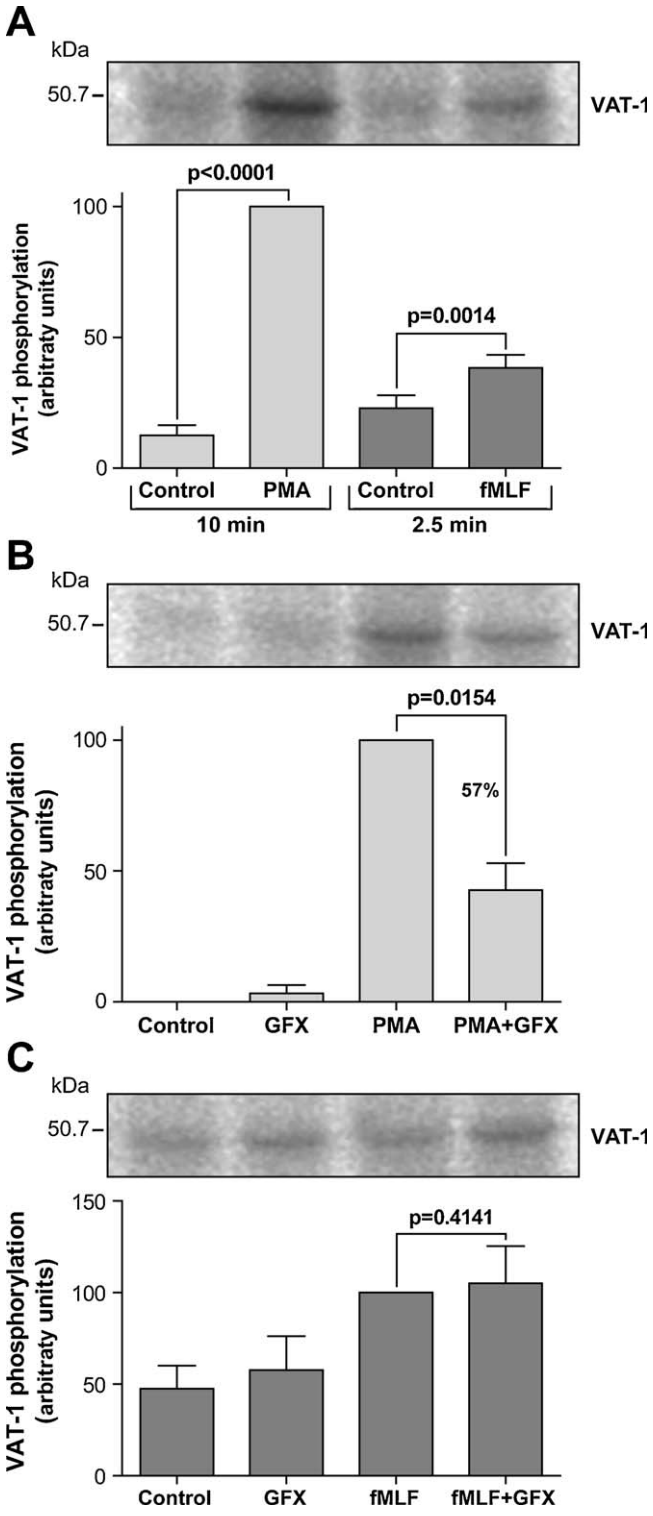


Fig. 6. PMA- but not fMLF-induced VAT-1 phosphorylation was PKC dependent. Neutrophils were incubated with 1 mCi [^{32}P] for 90 min (A) or 120 min (B, C). Cells suspensions (2×10^7 cells/ml) were pre-warmed at 37 °C for 5 min and pretreated in the presence or absence of GFX (3 μM) prior to stimulation with PMA or fMLF for 10 min and 2.5 min, respectively. Where indicated, cell suspensions were incubated with CB and ADA for 5 min prior to fMLF stimulation. VAT-1 was immunoprecipitated and the samples were analyzed by 10% SDS-PAGE. Histograms represent the quantification of VAT-1 phosphorylation. The results are expressed as mean ± SD of 4 independent experiments for (A) and of 3 for (B) and (C).

acidic liposomes [13]. Though the functional significance of elongation factor binding to negatively charged phospholipids is unclear, one possible function could be spatiotemporal regulation of protein translation [41]. Our data suggest that the 26S protease regulatory subunit 7 is a target of PA in neutrophils. Using atomic force microscopy the 20S proteasome subunit was reported to specifically bind membrane bilayers containing phosphatidylinositol but not those containing PtdCho or PA [42] and other proteasome protein subunits that precipitated with acidic phospholipids were also identified by Tsujita et al. [13]. Though proteasomal protein subunits were shown to bind distinct anionic phospholipids, none of these studies indicated whether the proteins interact directly with acidic liposomes and indirectly through interaction with acidic phospholipid-binding proteins.

The PA-binding proteins Cdc42, RhoG, Rac2 and ARPC4 can be grouped together as regulators of actin cytoskeleton dynamics and cell shape. In this regard, the interaction of PA with several regulators of the actin cytoskeleton is intriguing since this lipid has been reported to promote stress fiber formation [43,44]. Although Rho family members Rac1, Cdc42 and RhoA are known to activate PLD [45], we have shown here that several members of this family

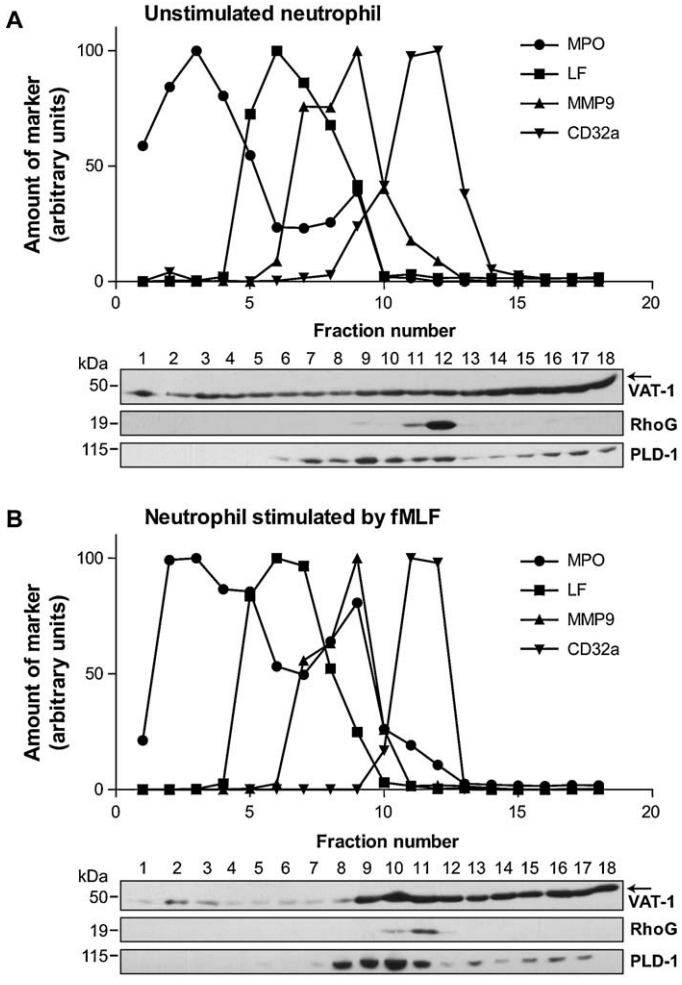


Fig. 7. Subcellular localization of VAT-1, PLD-1 and RhoG in human neutrophils. Neutrophils were stimulated or not with fMLF and postnuclear supernatants were fractioned on a Percoll gradient as described in Section 2. The gradient fractions were analyzed for MPO (primary granules), LF (secondary granules), MMP9 (tertiary granules), CD32a (plasma membrane), VAT-1, PLD-1 and RhoG by immunoblotting. The distribution of MPO, LF, MMP9, CD32a, VAT-1, PLD-1 and RhoG in unstimulated (control DMSO) and fMLF-stimulated neutrophils are shown in panels A and B, respectively. One experiment representative of four is presented.

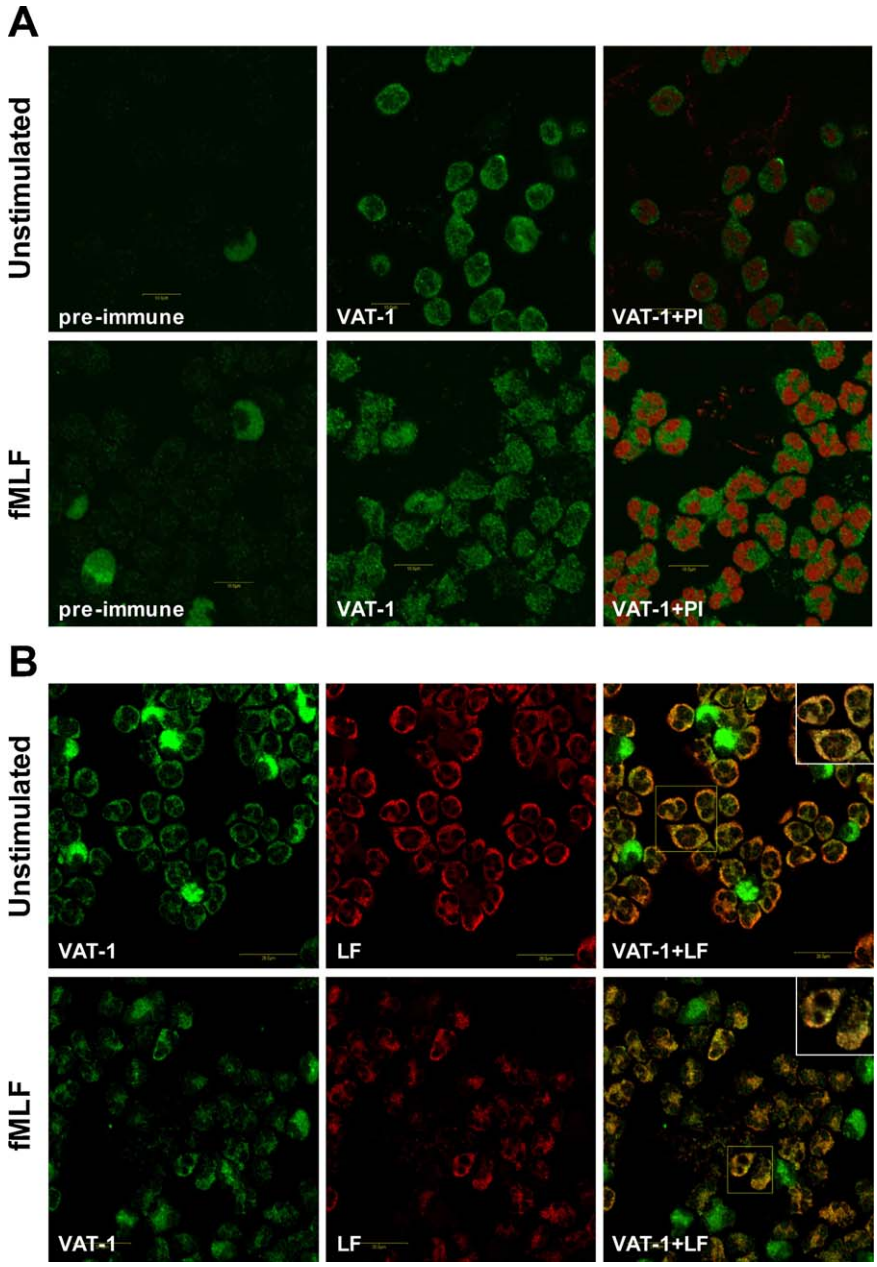


Fig. 8. Intracellular staining of VAT-1 in neutrophils.
(A) Unstimulated (control DMSO) and fMLF-activated neutrophils were cytospinned and subjected to immunofluorescence labeling using VAT-1 antibody and PI (nuclear staining) as described in Section 2. (B) Unstimulated (control DMSO) and fMLF-activated neutrophils were double-labeled with VAT-1 and lactoferrin (LF) Abs. Insets in B show a magnification of the boxed areas. One experiment representative of three is presented for panels A and B

could also interact with PLD-derived PA. The C-terminal polybasic (PB) motif is essential for Rho biological functions and targeting [46–48]. The PB motif of Rac1 but not of Rac2 interacts with anionic phospholipids including phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 3,4,5-trisphosphate and PA [49,50]. To assess phospholipid binding of Rho small GTPases several studies have used the GST-tagged PB motif or proteins [49,51]. However C-terminal isoprenylation may also contribute to membrane avidity and phospholipid recognition since Rac1 and Cdc42 as well were also shown to bind to PS when isoprenylated [52]. Our data indicate that endogenous Cdc42 and RhoG interacted more strongly with PA-containing liposomes when compared to PS. Although the amounts of PA present in cells are lower than those of PS, the concentrations of PA in cellular membranes change swiftly following stimulation. The data indicate that various anionic phospholipids, including PLD-derived PA, could contribute to a spatiotemporal recruitment and differential targeting of Rho GTPases to membrane microdomains [53]. The presence of the small GTPase RhoG in plasma membranes as well as in endoplasmic reticulum and Golgi membranes has been previously reported [54,55]. RhoG was also recruited to *Y. pseudotuberculosis*-containing phagosomes in transfected COS1 cells [56]. In neutrophils RhoG is found to colocalize with the plasma membrane marker CD32a and stimulation with fMLF does not affect its membrane compartmentalization.

Annexins form a large family of calcium and phospholipid binding proteins [57]. Previous work has shown that ANXA3 (also called lipocortin III) isolated from neutrophil cytosol interacts with PS-containing vesicles in a Ca^{2+}-dependent manner [58]. ANXA3

promotes Ca^{2+}-dependent aggregation of isolated specific granules [58] and translocates to phagosomal membranes in neutrophils [59]. Taken together, our data suggest that ANXA3 may be involved in membrane trafficking through binding to PLD-derived PA in Ca^{2+}-dependent manner in addition to PS. VAT-1 was originally described in the electric organ of Pacific electric ray *Torpedo californica* as a major component of synaptic vesicles [60]. VAT-1 from Torpedo has been predicted to be a 41-kDa integral membrane protein and shows highest sequence homology with members of the medium chain dehydrogenase superfamily [61]. Its C-terminal domain shares some homology with annexins and is capable of binding Ca^{2+} [62]. Murine breast cancer cell lines [63] and human glioma cells [64] have been reported to express VAT-1. We show here that VAT-1 is expressed by human neutrophils and has the capacity to bind PA- or PS-containing vesicles with similar affinity. Although there is no consensus PA-binding motif, clusters of basic amino acids (Lys or Arg), hydrophobic residues or lipid-binding pockets have been implicated in the binding of the tyrosine phosphatase SHP-1 [65], Raf [7], mTOR [66], and $p47^{phox}$ to PA [67]. The phospholipid-binding domain of VAT-1 remains to be characterized. However our findings suggest that the phospholipid-binding domain of VAT-1 may be different from that of Rho GTPases [51–53,68] since these molecules have a distinct sensitivity to PS.

Chemotactic factors such as fMLF are widely used to study degranulation, migration, and calcium fluxes in neutrophils. Activation of Src kinases by fMLF has been linked to regulation of neutrophil degranulation [69,70]. The respiratory burst and F-actin polymerization are reduced in neutrophils deficient in Src-family kinases [71] and Src kinases also regulate PLD signaling in various cell types [23,72]. Our study shows that the selective Src kinase inhibitor PP2 [25] reduces fMLF-induced PEt synthesis in a concentration-dependent manner but does not affect the transient increase in the levels of cytosolic calcium. Inhibition of PLD activity by PP2 also correlated with signification inhibition of fMLF-induced recruitment of VAT-1 to neutrophil membranes, thereby suggesting a functional link between PLD activation and the recruitment of VAT-1. The involvement of PLD-derived PA in relocalizing VAT-1 from cytosol to membranes was examined using three complementary approaches. First, propranolol, which has been shown to increase the amount of intracellular PA by inhibiting the PA phosphohydrolases [26,36], increased VAT-1 translocation to membranes. Although fMLF-induced VAT-1 translocation was more strongly enhanced by the lowest concentration of propranolol, all the concentrations of propranolol tested were found to increase the recruitment of VAT-1 when compared to cells stimulated with fMLF alone. However, possible non-specific effects of propranolol such as alteration of membrane fluidity, blockage of β-adrenergic receptors [27,28] or sequestration of anionic phospholipids including PS [29,73] can not be ruled out. PA is produced by PLD activation, but also by other pathways including diacylglycerol kinase (DGK) phosphorylating DAG, acyl transferase adding a fatty acid to lysoPA and enzymes of the *de novo* pathway from glycerol-3-phosphate to dihydroxyacetone-3-phosphate [8]. PLD has a propensity to catalyze a transphosphatidylation reaction in the presence of short-chain primary alcohols, a property that has been used to study the role of PLD-derived PA in cellular responses. We show that 1-butanol but not 2-butanol partially inhibited VAT-1 recruitment to cell membranes, thereby suggesting a role for PLD-derived PA in relocalizing cytosolic VAT-1 to membranes. However, increasing concerns have emerged regarding the efficacy of 1-butanol to fully prevent PA production, and the fact that PBut does not entirely function as an inert lipid [30,31]. As further evidence of a role of PLD, we demonstrated that the specific inhibitor of PLD activity FIPI inhibits almost entirely fMLF-mediated translocation of VAT-1 to membranes.

The regulation of PLD enzymes is complex and involves a large number of factors including PKC [3]. Activation of PLD by PKC involves direct phosphorylation events as well as phosphorylation-independent mechanisms through interaction with the N-terminal domain of PLD-1 [74]. In neutrophil-like cells activation of PLD by PKC activators such as PMA, but not fMLF, is inhibited by PKC inhibitors [75]. We show here that GFX, a competitive inhibitor preventing PKC activity exclusively via the ATP-binding site [33] as well as FIPI, a selective PLD inhibitor, entirely antagonized PMA-induced VAT-1 translocation. The effect of PMA on VAT-1 translocation may involve the activation of PLD by PKC or direct phosphorylation by PKC enzymes. Inhibitors of PKC inhibit PMA- but not fMLF-mediated activation of PLD in human granulocytes [75]. Though regulation of VAT-1 translocation by phosphorylation cannot be excluded, inhibition of VAT-1 recruitment by FIPI suggests a role for PLD-derived PA in cells stimulated with PMA.

Protein phosphorylation is a reversible and dynamic process, which plays a key role in regulation of cell activation. PMA and fMLF induce similar but not identical effects on protein phosphorylation in neutrophils [76,77]. Indeed a protein with an apparent molecular weight of 48 kDa was more heavily phosphorylated in PMA-treated cells [77]. The 48 kDa protein is possibly a subunit of NADPH oxidase that is phosphorylated and recruited to membranes in neutrophils stimulated with various agonists [78]. In this study, we identified another protein of ~48 kDa, VAT-1, whose phosphorylation level was more strongly induced by PMA than by fMLF. The difference in VAT-1 phosphorylation might reflect distinct signaling pathways used by these two agents since phoshorylation induced by PMA is PKC-dependent and that stimulated by fMLF is independent of PKC. It will be interesting to identify the phosphorylation sites and the kinases that phosphorylate VAT-1 in human neutrophils.

The functions of VAT-1 in vertebrates remain to be defined. The protein of the Pacific electric ray *Torpedo californica* [79], as well as the murine VAT-1 homolog [63], has ATPase activity. However using a nonradioactive ATPase assay, human VAT-1 affinity purified from cells over-expressing VAT-1 showed no ATPase activity (data not shown). A dehydrogenase/reductase activity has been suggested since VAT-1 has sequence homology with this enzyme family and the ability to bind nicotinic nucleotide compounds [80], but this remains unproven. Recent studies suggest a role for VAT-1 in human keratinocyte physiology [81] as well as in glioma invasion [64]. In cancer cells, VAT-1 is localized in the cytosol, mainly in the nuclear periphery region, and to discrete ruffle-like membrane structures [64]. To provide potential clues to VAT-1 function its distribution was examined in neutrophils [18]. VAT-1 seems to co-localize with distinct intracellular membrane compartments including primary granules, secondary granules and tertiary granules and stimulation with fMLF redistributes VAT-1 to fractions that overlap with tertiary granule and plasma membrane markers. Immunolabeling indicates that VAT-1 is concentrated in the cell periphery of unstimulated neutrophils and shows a more granular pattern following stimulation with fMLF. There was no clear labeling of the plasma membrane, and co-localization with LF and the phagocytosis assay ruled out the possibility that VAT-1 was recruited to secondary granules or phagosomes of activated neutrophils. Moreover PLD-1, which was previously reported to have a punctate staining in control and fMLF-stimulated human neutrophils by confocal microscopy [36], was recovered in the same fractions as VAT-1 thereby suggesting a functional link between the formation of PLD-derived PA and VAT-1 recruitment to membranes. Some slight differences of the localization of VAT-1, CD32a and LF were observed between confocal microscopy and cell fractionation. This discrepancy may be due to the fact that using fractionation the integrity of cells was broken and membranes originating from

different compartments (plasma membrane, Golgi, reticulum) co-localize with neutophil organelles in Percoll gradients, which may increase artifacts as suggested in a previous study [82].

In conclusion we report on the characterization of neutrophil proteins that bind anionic phospholipids with distinct affinities and calcium dependence. Many of these proteins are involved in the regulation of membrane traffic, actin cytoskeleton dynamics, transcription and protein translation. We identified for the first time VAT-1 as a neutrophil protein that binds anionic phospholipids. VAT-1 is localized in the cytosol and co-localizes with different intracellular membrane compartments including secretory granules. The data suggest that the recruitment of cytosolic VAT-1 to membranes involves signaling through PLD, PKC and Src family kinases.

Conflict of interest

The authors declare no conflict of interest or financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.09.014.

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