Annexin A3 plays a role in cytoplasmic calcium oscillation by extracellular calcium in the human promyelocytic leukaemia HL-60 cells differentiated by phorbol-12-myristate-13-acetate

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Abstract

The roles of annexin A3 (ANXA3) in macrophages are not fully understood. In contrast to C5a, we have demonstrated that C-terminal ribosomal protein S19 (RP S19)-tagged S-tagged C5a (S-tagged C5a/RP S19) raises an alternative cytoplasmic calcium oscillation by extracellular calcium during macrophage migration into apoptotic cells. We here differentiated human promyelocytic leukaemia HL-60 cells bearing with either control sense RNA and shRNA for ANXA3 mRNA or a vector cDNA with or without ANXA3 cDNA into macrophage-like cells by phorbol-12-myristate-13-acetate and found that a fluorescence ratio (340 nm/380 nm) upon the S-tagged C5a/RP S19-induced alternative cytoplasmic calcium oscillation by extracellular calcium was an equilateral association with a dose of ANXA3. Moreover, the ANXA3-dependent modification was partially reflected upon the S-tagged C5a-induced classical cytoplasmic calcium oscillation by both intracellular and extracellular calcium. ANXA3 seems to extend the C5aR-mediated cytoplasmic calcium oscillation by extracellular calcium at least in the HL-60 macrophage-like cells.

Keywords

Annexin A3 • C5a receptor • Calcium • Macrophages • RP S19

Abbreviations.

annexin A3; ANXA3, 4-bromoenol lactone; BEL, C5a receptor; C5aR, calcium-independent phospholipase A2; iPLA2, calcium-dependent cytoplasmic phospholipase A2; cPLA2, G protein-coupled receptor; GPCR, HL-60 macrophage-like cells; differentiated from human promyelocytic leukaemia HL-60 cells by the addition of phorbol-12-myristate-13-acetate, N-(p-aminocinnamoyl) anthranilic acid; ACA, ribosomal protein S19; RP S19.

Introduction

Annexins (ANXs) function not only as an inhibitor for calcium-dependent cytosolic phospholipase A2 (cPLA2) but also as an organizer between calcium signaling and lipid signaling during remodeling of phospholipid bilayer in cell migration (Bandorowicz-Pikula et al., 2012). ANXA2 expressed in osteoblasts of bone marrow interacts with stromal cell-derived factor for localization and homing of hematopoietic stem cells via CXCR4, belongs to Gi protein-coupled receptor (GiPCR) (Jung et al., 2011). A tetramer form of ANXA2 attracts human monocyte-derived macrophages via toll-like receptor 4 (Maratheftis et al., 2007). Conversely, ANXA3 is mainly expressed in macrophages (Le Cabec et al., 1992). However, a role of ANXA3 in macrophage migration is unclear.

The *C5a receptor (C5aR)* gene, which generates no mRNA splice variants, encodes the C5aR protein, which belongs to GiPCR (Gerard et al., 1993). In a case of C5a, phospholipase C β 2 (PLC β 2) is classically interacts with the G $\beta\gamma$ subunits in a calcium-dependent manner for making phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphates (IP3) and diacylglycerol (Cathcart, 2009). However, the source of calcium to interact with PLC β 2 is still unclear. After the binding of IP3 to the IP3R expressed on endoplasmic reticules intracellular calcium is released and calcium-released activation of the calcium channel expressed on the plasma membrane is opened for activation of protein kinase C (Mollapour et al., 2001). Both phosphatidylinositol 3-kinase signal and extracellular-signal-regulated kinase 1/2 (ERK1/2) signal are transmitted through small GTP binding protein Ras and mitogen-activated protein kinase (MAPK) kinase Raf. In the interval, cPLA2 activated by extracellular calcium through extracellular calcium through calcium-released activation of the calcium channel hydrolyses arachidonic acid for extension of cytoplasmic calcium oscillation by extracellular calcium. The above classical cytoplasmic calcium oscillation is crucial for the C5a-induced macrophage migration via the monocyte C5aR.

We have demonstrated that ribosomal protein S19 (RP S19) polymer produced by apoptotic cells functions either as the monocyte C5aR agonist for an induction of macrophage migration or as the neutrophil C5aR antagonist for an inhibition of neutrophil migration (Nishiura et al., 2011b). The diametrically opposite mechanisms are suggested to be one of phagocytic cell clearance systems in cell homeostasis (Lauber et al., 2004). To understand a nature of RP S19 polymer, we have found that L₁₃₁DR and the C-terminus (I₁₃₄AGQVAAANKKH₁₄₅) moiety of RP S19 display binding and migration inhibitory properties against the neutrophil C5aR (Shibuya et al., 2001; Shrestha et al., 2003). Therefore, an S-tagged C5a/RP S19 complex harbouring both the mutant Gly73Asp-C5a and the RP S19 C-terminus moiety results primarily in macrophage infiltration (Nishiura et al., 2010a; Oda et al., 2008).

By using S-tagged C5a/RP S19, we recently showed that the monocyte C5aR-mediated alternative cytoplasmic calcium oscillation by extracellular calcium was inhibited *via* the catalysis of the ADP-ribosylation of the G α subunit (pertussis toxin), a p38MAPK inhibitor (SB203580), a non-specific PLA₂ inhibitor (N-(p-Amylcinnamoyl)anthranilic Acid (ACA)), non-specific calcium and/or magnesium channel inhibitors (2-aminoethoxydiphenyl borate (2-APB) and MgCl₂) in the human promyelocytic leukaemia HL-60 cells differentiated by phorbol-12-myristate-13-acetate (the HL-60 macrophage-like cells) (Nishiura et al., 2011b). In this paper, we assessed a contribution of ANXA3 with cytoplasmic calcium oscillation by extracellular calcium during the C5aR-mediated macrophage migration.

Methods

Vectors and HL-60 Transformations

ANXA3 cDNA was prepared by RT-PCR from total mRNA template isolated from human hepatocellular liver carcinoma HepG2 cells. The ANXA3 cDNA were inserted into a pAcGFP1-C1 vector (clontech, CA, USA) and a CSII-EF-MCS vector (RIKEN, Tsukuba, Japan). For the ANXA3 mRNA knockdown, we prepared control sense RNA and shRNA oligoribonucleotides (Table 1). They were inserted into the pENTR4-H1 vector. H1-shcontrol and H1-shANXA3 cassettes were replaced with the RfA region in the pCS-RfA-EG vector by LR reaction (Invitrogen, CA, USA).

HL-60 cells were obtained from the RIKEN BioResource Center, Japan. To establish HL-60 transformed cells containing pAcGFP1-ANXA3 cDNA, HL-60 cells were suspended in DMEM at a density of 5×10^6 cells/mL and subjected to electroporation with 40 μ g of linearized plasmid DNA using a Gene Pulsar II (Bio-Rad, CA, USA) in a 4-mm gap cuvette at 200 V and 950 μ F. 24 positive clones were confirmed using 250 μ M geneticin and western blotting and the best clone was selected based on the cell growth rate (data not shown).

To establish HL-60 transformed cells bearing CSIII-ANXA3 cDNA, 293T cells (5×10^6 cells/mL) were maintained in DMEM medium containing 10% FBS in poly-lysine coated 10-cm tissue culture plates for 24 hrs after which fresh medium was added. A plasmid DNA (450 μ L) solution containing 17 μ g packaging plasmid and 10 μ g VSV-G- and Rev-expressing plasmids was mixed with 50 μ L 2.5 M CaCl_2 and 500 μ L 2x HEBS (1.5 mM Na_2HPO_4 , 280 mM NaCl, and 50 mM HEPES). The HIV-1-based lentivirus was recovered by ultracentrifugation and resuspended in 50 μ L HBSS medium. We mixed the lentivirus with 100 μ L HL-60 cells (1×10^6 cells/mL) at a multiplicity rate of infection of 10, and stable transfected clones were selected using 1 mM Zeocin. Successfully transformed cells were sometimes sorted using a BD FACSAriaTM Cell Sorter (Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cells were suspended in lysis buffer (1×10^6 cells/100 μ L) (7 M urea, 2 M thiourea, 2% Triton X-100, 18 mM dithiothreitol and 0.1% bromothymol blue) and applied to a 12% SDS polyacrylamide gel. The proteins were electrophoretically transferred from the SDS polyacrylamide gel to an Immobilon Transfer MembraneTM (Millipore, MA, USA) using a Semi Dry Electrobloetter (Sartorius, Goettingen, Germany) for 90 min at an electric current of 15 V. The membrane was treated with 4% Block AceTM (Dainippon, Kyoto, Japan) for 30 min at 22°C. The first reaction was performed with anti-p38MAPK rabbit IgG (200 ng/mL, Cell Signaling Technology®, Boston, USA) or anti-ANXA3 rabbit IgG (200 ng/mL, Proteintech Group, Inc., CA, USA) in PBS containing 0.03% Tween 20 for 1 hr at 22°C. After washing, the second reaction was performed with HRP-conjugated anti-rabbit IgG goat IgG (20 ng/mL, Santa Cruz Biotechnology, CA, USA) for 30 min at 22°C. The enhanced chemiluminescence reaction was performed using the ECL Plus Western Blotting Detection SystemTM (GE).

Calcium Imaging

Cells (2×10^6 cells/mL) were loaded with calcium-sensitive Fura 2-AM (1 μ M) in the calcium buffer (HBSS containing 20 mM HEPES and 3% BSA, pH. 7.4) for 30 min at 37°C (Dojindo, Kumamoto, Japan). Samples were placed directly into the cell suspension in cuvette after a 5-minute baseline recording. Recordings were made with an F-2500 calcium imaging system with FL Solutions (Hitachi, Tokyo, Japan) at intervals of 5 sec that calculated the ratio of fluorescent signals obtained at 37°C with excitation wavelengths at 340 and at 380 nm and with an emission wavelength at 510 nm.

Chemotaxis Assay

Cells (2×10^6 cells/mL) in the calcium buffer were prepared for the chamber assay. The assay was performed for 90 min using a Nucleopore filter with a pore size of 5 μ m (Falk et al., 1980). The total number of cells that migrated beyond the lower surface of the membrane was counted in five microscopic

high-power fields.

Statistical Analysis

The results of representative examinations were confirmed by multiple experiments at least with triplicate samples. Statistical significance was calculated by the non-parametric or parametric tests offered in the two way analysis of variance window, respectively. Values are expressed as mean $\pm$ SD. A p-value <0.05 was considered statistically significant and shown as $P<0.05$: * and $P<0.01$: **.

Results

To examine a role of ANXA3 in the C5aR-mediated cytoplasmic calcium oscillation through plasma membrane during the C5aR-mediated macrophage migration, we prepared the HL-60 macrophage-like cells with either control sense RNA (Control) or shRNA (Knockdown) for ANXA3 mRNA (Fig. 1A). ANXA3 protein expression in the Control HL-60 macrophage-like cells was reduced to approximately 10% following ANXA3 knockdown. To confirm a sequential order of ANXA3 in the calcium signalling, the HL-60 macrophage-like cells were collected at several time points after a stimulation with S-tagged C5a/RP S19 (10^{-8} M) (Fig. 1B). Phosphorylation of p38MAPK in the HL-60 macrophage-like cells was slightly blocked by ANXA3 knockdown, indicating a position of ANXA3 downstream of p38MAPK. The fluorescence ratio (340 nm/380 nm) was monitored in the knockdown HL-60 macrophage-like cells after a stimulation with either S-tagged C5a or S-tagged C5a/RP S19 (10^{-8} M) (Fig. 1C). A suppressive effect of ANXA3 knockdown upon the S-tagged C5a/RP S19-induced alternative cytoplasmic calcium oscillation by extracellular calcium was stronger than that upon the S-tagged C5a-induced classical cytoplasmic calcium oscillation by both intracellular and extracellular calcium in the HL-60 macrophage-like cells. The different effects of ANXA3 knockdown were reflected in macrophage migration (Fig. 1D). These data indicated a participation of ANXA3 in cytoplasmic calcium oscillation by extracellular calcium.

To confirm an effect of ANXA3 upon cytoplasmic calcium oscillation, we prepared the ANXA3 HL-60 cells with or without enhanced green fluorescent protein (EGFP)-fused protein cDNA. In the HL-60 macrophage-like cells, the EGFP-fused ANXA3 protein expressed in the cytoplasm localised to plasma membranes after stimulation with S-tagged C5a/RP S19 (10^{-8} M) (Fig. 2A). The effect of ANXA3 overexpression without EGFP on the S-tagged C5a/RP S19-induced alternative cytoplasmic calcium oscillation by extracellular calcium was greater than that observed on the S-tagged C5a-induced classical cytoplasmic calcium oscillation by both intracellular and extracellular calcium (10^{-8} M) (Fig. 2B). The C5aR ligand-specific differences were reflected in cell migration (Fig. 2C).

We suggested that extracellular calcium and/or magnesium channels are opened by calcium-independent PLA2 (iPLA2) and the opening is extended by calcium-dependent cPLA2 and ANXA3 via the S-tagged C5a/RP S19-guided p38MAPK pathway (Cathcart, 2009; Mishra et al., 2008). To determine a role of cPLA2 in the ANXA3-dependent alternative cytoplasmic calcium oscillation by extracellular calcium, we used the ANXA3 HL-60 macrophage-like cells. The ANXA3 HL-60 macrophage-like cells were pretreated with or without an iPLA₂ inhibitor (20 μ M 4-bromoenol lactone (BEL)) and collected at several time points after a stimulation with S-tagged C5a/RP S19 (10^{-8} M) (Fig. 3A). Phosphorylation of p38MAPK was not suppressed by BEL, indicating an activation of iPLA2 downstream of p38MAPK. Importantly, cytoplasmic calcium oscillation by extracellular calcium was blocked by BEL, while the opening time was shortened by a non-specific PLA2 inhibitor ACA (20 μ M) (Fig. 3B), indicating a presence of cPLA2 upstream of the ANXA3-dependent alternative cytoplasmic calcium oscillation by extracellular calcium. To further confirm the C5a/RP S19-induced alternative cytoplasmic calcium oscillation by extracellular calcium through extracellular calcium and/or magnesium channels, the ANXA3 HL-60 macrophage-like cells were pretreated with either control PBS or a calcium channel blocker (1 μ M ruthenium red (RR)), 20 μ M ACA, a calcium/magnesium channel blocker (50 μ M 2-APB) and a magnesium channel blocker (1 mM MgCl₂). The 10^{-8} M S-tagged C5a/RP S19-dependent alternative cytoplasmic calcium oscillation by extracellular calcium in the ANXA3 HL-60 macrophage-like cells was inhibited by either ACA, 2-APB or MgCl₂ (Fig. 3C). These inhibitory effects were reflected in cell migration (Fig. 3D). These above data indicated that ANXA3 at least in part contributes with the extension of cytoplasmic calcium oscillation by extracellular calcium through calcium and/or magnesium channels in the HL-60 macrophage-like cells.

Discussion

As well as ANXA1 (MMDB ID: 22037), ANXA3 (MMDB ID: 55212) contains 4 ANX repeats for binding to phospholipids in plasma membrane and vesicles (Fig. 2A). Trp₁₂, Lys₂₆ and Lys₂₉ in an N-terminus (M₁-P₄₄) before the first α helix (S₄₅-M₅₆) are crucial for a sensitivity of 8 pieces of calcium binding to ANXA1 (Bitto and Cho, 1999). Trp₅ in an N-terminus (M₁-P₂₀) before the first α helix (S₂₁-I₃₁) is conserved for a sensitivity of 5 pieces of calcium binding to ANXA3 (Hofmann et al., 2000). Conversely, we here found that the potential of ANXA3 to cytoplasmic calcium oscillation by extracellular calcium through the S-tagged C5a/RP S19-guided p38MAPK pathway is higher than that through the S-tagged C5a-guided ERK1/2 pathway (Fig. 2B). Probably, specific phosphorylation sites Thr¹¹⁸ and Ser¹⁴⁶ in ANXA3 by p38MAPK (Group-based Prediction System 2.0 software of the CUCKOO workgroup in ExPASy Proteomics Tools) are crucial for a conformational change to extend alternative cytoplasmic calcium oscillation by extracellular calcium, especially via calcium and/or magnesium channels, through an increment of membrane permeabilization.

In addition, we newly found a translocation of the EGFP-fused ANXA3 protein from cytoplasm to nucleus (Fig. 2A). There are interesting reports about a binding protein of ANXAs in nucleus. Y²³ phosphorylation of ANXA2 promotes proliferation and invasion of human breast cancer SK-BR-3 cells via a Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylation in the nucleus (Liu, 2007; Wang et al., 2012). ANXA4 modulates NF- κ B transcriptional activity, depending on an interaction with NF- κ B p50 subunit and an intracellular calcium ion level in human kidney cancer cell lines (Jeon et al., 2010). Our data may suggest a unique mechanism of ANXA3 in nucleus for modification of cell movement.

When the GDP form of the G α i2 subset is exchanged to the GTP form via the guanidine exchange factor activity of the monocyte C5aR, the G $\beta\gamma$ subset commonly interacts with PLC β 2 at the EF hand domain (I₁₄₁-K₁₇₆) in a calcium-dependent manner. In a case of P2Y (GqPCR) ligand ATP, a source of calcium supply is extracellular calcium through ligand-gated ion channels P2Xs (del Rey et al., 2006). No one has found a specific ligand-gated ion channel of the C5a ligands yet.

We recently showed that RP S19 polymer binding to the monocyte C5aR guides a G $\beta\gamma$ subset-dependent p38MAPK pathway at least in an intracellular calcium release-independent manner (Nishiura et al., 2010b). The p38MAPK signal is artificially produced by S-tagged C5a stimulation following simultaneous treatment with the phosphatidylinositol 3-kinase and PLC inhibitors (LY294002 and U73122, respectively) at half concentrations of IC₅₀. The RP S19 C-terminus interacts with the Gla domain of prothrombin at low calcium concentrations (Nishiura et al., 2011a). These data indicate that the RP S19 C-terminal K₁₄₃K₁₄₄ moiety acts as a competitor against calcium for the activation of PLC β 2 in macrophages. There is an interesting report that phospholipase D (PLD) downstream of the interaction of the G $\beta\gamma$ subsets with ADP-ribosylation factor exchange factors not only catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid and choline but also drives the p38MAPK pathway (Knizhnik et al., 2012; Song et al., 2011). We here found that the S-tagged C5a/RP S19-induced p38MAPK phosphorylation upstream of both alternative extracellular calcium oscillation and cell migration was inhibited by a PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) (Supplemental Fig. 1), indicating a participation of PLD in the G $\beta\gamma$ subsets-dependent p38MAPK pathway via the monocyte C5aR. We need further experiments to study a selection mechanism of the monocyte C5aR in a ligand specific manner.

It has been reported that a reciprocal activation between iPLA₂ and cPLA₂ at the front side of cells affects on a maximum potency of cell migration (Cathcart, 2009). We here confirmed that the iPLA₂ inhibitor (BEL) is more efficient than the non-specific PLA₂ inhibitor (ACA) for the alternative cytoplasmic calcium oscillation by extracellular calcium through extracellular calcium and/or magnesium channels including transient receptor potential melastatin 7 in the ANXA3 HL-60 macrophage-like cells (Figure 3 and Supplemental Figure 1-3). The ready state of iPLA₂ bound by calmodulin at hydrophobic anchor residues (V₆₉₁-F₇₀₉) is altered to the active state via the replacement of calcium from the EF hand domains (Harteneck, 2005). The ready state of transient receptor potential melastatin 7 is also maintained at least in part by the binding of calmodulin to the hydrophobic anchor residues of M₁-R₁₄ (Jenkins et al.,

2001; Mason et al., 2012). These data again indicated a presence of a specific ligand-gated ion channel of the C5aR ligands.

Conclusion

ANXA3 especially expressed in macrophages plays a role in an extension of the C5aR-mediated extracellular calcium entry.

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Figure legends

Fig. 1. Cytoplasmic calcium oscillation was suppressed by ANXA3 knockdown. A, HL-60 cells bearing either control sense RNA (Control) or shRNA (Knockdown) for ANXA3 mRNA were prepared and differentiated into macrophage-like cells. ANXA3 protein expression levels were assessed *via* western blot analysis (n=3). B, Phosphorylation of p38MAPK induced by S-tagged C5a/RP S19 (10^{-8} M) was assessed for the Control and Knockdown HL-60 macrophage-like cells by western blotting (n=3). C, The cytoplasmic calcium oscillation induced by either S-tagged C5a or S-tagged C5a/RP S19 (10^{-8} M) was measured in the Knockdown HL-60 macrophage-like cells (n=4). D, The chemotactic potential of the Control and Knockdown HL-60 macrophage-like cells against either S-tagged C5a or S-tagged C5a/RP S19 at several concentrations was measured in a 48-well chemotaxis chamber (n=6). P values of less than 0.05 were considered to indicate statistical significance (P<0.01: **).

Fig. 2. Cytoplasmic calcium oscillation by extracellular calcium was extended by ANXA3 overexpression. A, The localisation of EGFP-ANXA3 in the HL-60 macrophage-like cells after activation of S-tagged C5a/RP S19 (10^{-8} M) was assessed using an Olympus Fluoroview FV300 microscope (n=3, x3000). B, The cytoplasmic calcium oscillation induced by either S-tagged C5a or S-tagged C5a/RP S19 (10^{-8} M) was measured in the plasmid (Mock) and ANXA3 (ANXA3) overexpressing HL-60 macrophage-like cells (n=4). C, The chemotactic potential of the Mock and ANXA3 HL-60 macrophage-like cells against either S-tagged C5a or S-tagged C5a/RP S19 at several concentrations was measured in a 48-well chemotaxis chamber (n=6).

Fig. 3. Cytoplasmic calcium oscillation was inhibited by PLA₂. A, Phosphorylation of p38MAPK induced by S-tagged C5a/RP S19 (10^{-8} M) was modified using BEL in the ANXA3 HL-60 macrophage-like cells as assessed by western blot analysis (n=3). B, The cytoplasmic calcium oscillation induced by either S-tagged C5a or S-tagged C5a/RP S19 (10^{-8} M) was modified with either BEL or ACA in the ANXA3 HL-60 macrophage-like cells (n=4). C and D, The ANXA3 HL-60 macrophage-like cells were pretreated with control PBS, RR, ACA, 2-APB and MgCl₂ and measured cytoplasmic calcium oscillation and cell migration (n=4).

Figure 1

A

HL-60 macrophage-like cells

B

C

Knockdown HL-60 macrophage-like cells

D

HL-60 macrophage-like cells

Figure 2

A

C5a/RP S19 (-)

C5a/RP S19 (+)

ANXA3 HL-60 macrophage-like cells

B

C

Figure 3

A

B

C

D

Graphic abstract

Annexin A3 (ANXA3) extends cytoplasmic calcium oscillation by extracellular calcium (Green→Yellow→Blue) in human promyelocytic leukaemia HL-60 cells differentiated by phorbol-12-myristate-13-acetate.

Table 1. Oligoribonucleotides for ANXA3 mRNA

shRNA	sense	GATCTGGACATTGTGGACAGCATAAAATTCAAGAGATTTTATGC TGTCCACAATGTCCGCGCG
	antisense	CGCGCGGACATTGTGGACAGCATAAAATCTCTTGAATTTTATGC TGTCCACAATGTCCA
Control sense RNA	sense	GATCTGGACATTGTGGACAGCATAAAATTCAAGAGATTTTCGCG CG
	antisense	CGCGCGCTCTTGAATTTTATGCTGTCCACAATGTCCA