Microbial alkaloid staurosporine induces formation of nanometer-wide membrane tubular extensions (cytonemes, membrane tethers) in human neutrophils

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Key words: staurosporine, neutrophil, tubular or tubulovesicular extensions, membrane tethers, cytonemes, Salmonella enterica serovar typhimurium, actin cytoskeleton, cytochalasin D, protein kinase C, tyrosine protein kinase

Introduction

Long distance cellular adhesive interactions mediated by long tubular or tubulovesicular cellular protrusions—cytonemes, membrane tethers, nanotubes,—were firstly observed in various embryonic and blood cells and later were found in nerve and other cells. Study and measurement of these structures is strongly complicated by their small size, which is near the limit of resolution for optic microscopy. Recently, a number of long tubular and taper cellular protrusions varying twenty times in diameter (from 2,000 to 100 nm) are united as cytonemes and nanotubes.

We determine the neutrophil tubular or tubulovesicular extensions (cytonemes) as membrane tethers with strongly uniform diameter along the entire length. The diameter can vary in the range 150–240 nm. High rate of development (1 μm/min and more) and flexibility also characterize neutrophil cytonemes. In human neutrophil physical and chemical factors can cause formation of membrane tubular extensions. Pulling of long and thin membrane tethers from the cell bodies was observed upon neutrophil flowing over spread platelets or immobilized P-selectin at the physiological rate. Neutrophils attached to platelet P-selectin by P-selectin glycoprotein ligand-1 receptors located on the neutrophil microvillus tips. Following microvillus elongation under shear stress resulted in the membrane tethers formation. Similar membrane tethers can be pulled from the neutrophil bodies by a micropipette manipulation.

Membrane tubulovesicular extensions resembling neutrophil membrane tethers in size and behaviour appeared on the neutrophil cell bodies upon adhesion to fibronectin-coated substrata in Na+-free extracellular medium or in the presence of actin-disrupting agents cytochalasin D or B, an alkylating agent 4-bromophenacyl bromide, inhibitors of glucose metabolism and inhibitors of vacuolar type ATPases, chloride channel inhibitors. Nitric oxide (NO), the physiological regulator of leukocyte adhesion to endothelium, appears to be a natural causative factor for TVE formation. NO-induced neutrophil tubulovesicular extensions connected neutrophils to substrata and to the other cells, and bound and aggregated pathogenic bacteria over a distance of several cell diameters.

The mechanism of membrane tubular extensions formation remains to be elucidated. Cell membranes undergo continuous curvature changes required for formation of tubular and vesicular carriers for intracellular membrane trafficking, exocytosis and endocytosis. It is shown recently that proteins containing BAR or F-BAR domain possess the ability to induce membrane...
on single serine in cofilin can block this activity. In human neutrophils staurosporine specifically inhibits the constitutively active serine 3 cofilin kinase, thus promoting actin depolymerization.\(^{19,21}\) There is a close interplay between the mechanisms that control actin dynamics and those that mediate plasma membrane invagination and fission. Cofilin and cofilin kinase are shown to regulate actin filament population required for the dynamin-dependent apical vesicular carrier fission from Trans-Golgi network.\(^{22}\)

In the present work, we demonstrate that STS, the natural alkaloid isolated from the bacterium \textit{Streptomyces staurosporeus}, induces formation of nanometer-wide tubular extensions connecting human neutrophils in a network upon adhesion to fibronectin-coated substrata. STS possesses multiple biological activities ranging from antibiotic to antihypertensive and anti-cancer.\(^{23-25}\) One of the target points for STS is inhibition of protein kinases. STS prevents ATP binding to a kinase due to its stronger affinity to the ATP-binding site. To elucidate whether inhibition of protein kinases is a causative factor for development of neutrophil tubulovesicular extensions we studied the effect of protein kinase C and tyrosine kinase inhibitors of different structure on neutrophil spreading on fibronectin-coated substrata. We also compared STS-induced extensions with those that were induced by cytochalasin D and a NO donor, diethylamine NONOate in size and behaviour.

Neutrophils appear to be professional phagocytes. STS was shown to inhibit internalization (phagocytosis) of bacteria or opsonized erythrocytes by phagocytes and other cells.\(^{26-29}\) At that it does not reduce but often increases adherence of objects for phagocytosis to the cells.\(^{28,30}\) We suggest that staurosporine facilitates extracellular binding of bacteria due to formation of long membrane tubular extensions strongly widening the area of the neutrophil contact interactions. To test whether these extensions play a role in binding of bacteria, we have studied neutrophil interaction with serum-opsonized \textit{S. typhimurium} species in control conditions and in the presence of protein kinase C inhibitors H-7 and STS, and of protein kinase C activator phorbol 12-myristate 13-acetate (PMA).

**Figure 1.** Scanning electron microscopy images of human neutrophils attached to fibronectin-coated substrata in control conditions and in the presence of inhibitors of protein kinases, and diethylamine NONOate. Cells were attached to fibronectin-coated substrata during 20 min at 37°C in control conditions (A) and in the presence of: tyrosine kinase inhibitors, 100 µM genistein (B) and 100 µM tyrphostin AG 82 (C); protein kinase C inhibitors, 10 µM bisindolylmaleimide VII (D), staurosporine 200 nM (E and F) and 10 µM Ro 31-8220 (G); 1 mM diethylamine NONOate (NO) (H). All pictures represent typical images observed in three analogous experiments.
Human neutrophils have a round shape in suspension. Upon adhesion to fibronectin they attached and spread on fibronectin-coated substrata. The control cells plated at the density $10^6$ cells/ml did not contact each other and appeared with a smooth surface (Fig. 1A). Neither tyrosine kinase inhibitors genistein and tyrphostin AG 82 (Fig. 1B and C), nor protein kinase C inhibitors bisindolylmaleimide VII (Fig. 1D) and H-7 (data not shown) altered significantly the neutrophil morphology when compared to the control cells (Fig. 1A). Neutrophils plated to fibronectin in the presence of STS (Fig. 1E and F) or RO 31-8220, a protein kinase C inhibitor structurally related to STS, had ruffled surface and were interconnected into network by thin tubular extensions (Fig. 1G). STS-induced extensions (Fig. 1E and F) like NO-induced neutrophil extensions (Fig. 1H) had definitely tubular shape and reached 60–120 µm (several cell diameters) in length during 20 min (Fig. 1F) but have fewer diameter (Table 1). These data indicate that to induce membrane tubular extension formation STS and RO 31-8220 possess other activities in addition to inhibition of protein kinase C or tyrosine kinase.

We suggest that staurosporine facilitate cytoneme formation through depolymerization of actin cytoskeleton. Depolymerization of actin filaments by cytochalasin D (Cyt D) induced cytonemes formation in neutrophils as revealed by phase contrast microscopy. Cyt D-induced extensions are characterized by rapid appearance and destruction due to swelling and lysis, which complicated fixation of extension for the electron microscopy study. This could be ascribed to the ability of Cyt D to stimulate secretion of lysosomal hydrolases disrupting cytonemes. Cyt D-induced extensions were partially conserved in neutrophils fixed during 10 min after addition of Cyt D (Fig. 2A and B) and resembled in size and behaviour STS-induced extensions (Table 1, Fig. 1E and F). Cells fixed 20 min after exposure

| Object of measurements | $D_{\min}$ | $D_{\max}$ | $D + SEM$ |
|------------------------|------------|------------|-----------|
| Control: tubulovesicular extensions connecting neutrophils in the presence of staurosporine, 200 nM | 128 | 188 | 158 ± 10 |
| Tubulovesicular extensions connecting *S. typhimirium* to neutrophils in the presence of staurosporine, 200 nM | 155 | 246 | 197 ± 8 |
| Tubulovesicular extensions developed in the presence of Ro 31-8220, 10 µM | 80 | 195 | 135 ± 5 |
| Tubulovesicular extensions developed in the presence of diethylamine NONOate, 1 mM | 150 | 240 | 184 ± 10 |
| Tubulovesicular extension developed in the presence of cytochalasin D, 5 µg/ml | 115 | 217 | 153 ± 3 |

Human neutrophils were attached to fibronectin-coated substrata during 20 min in the presence of STS, Ro 31-8220 or diethylamine NONOate and for 10 min in the presence of cytochalasin D and fixed for electron microscopy; The cytoneme diameters were measured on scanning electron images; The data presented are the mean values of diameter ($D + SEM$) obtained from the measurements of diameters of 15–25 tubular cellular extensions; Data of three independent analogous experiments were summarized; *p < 0.001 as compared to the control value.

![Figure 2](image-url)
Destruction of Cyt D-induced cytonemes was accompanied by appearance of specific round membrane invaginations on the neutrophil surface (Fig. 2B–D). These invaginations could represent exits for cytonemes or locuses of compensatory endocytosis upon cytonemes extrusion. Similar invaginations we observed on the neutrophil surface after shedding of NO-induced cytonemes as a result of interaction with bacteria.

Assembly of neutrophil cytoskeleton undergoes strong reorganization upon adhesion to substrata and depends on substrata. Human neutrophils adherent to a polystyrene plastic surface are vigorously activated and exhibited an increase of cytoskeleton-associated actin (F-actin) and a decrease of monomeric (G-actin) concentration when compared to suspended cells before plating. In contrast, fibronectin-adherent cells manifest only priming response and exhibit a decrease of F-actin and a rapid rise in G-actin concentration. The peak F-actin depolymerization occurred in the first minutes of adhesion and is followed by partial F-actin remodelling. In our experiments neutrophils plated to fibronectin during 20 min in control conditions exhibited actin staining without profound actin filaments at less spread cell periphery (Fig. 3A and B), thus indicating adhesion-induced actin depolymerization. Cytochalasin D-treated cells exhibited diffusive staining of cytoplasm typical for G-actin (Fig. 3C and D). Staurosporine-treated cells (Fig. 3E and F) like cytochalasin-treated cells were diffusively stained with FITC-phalloidin around the whole cell body including tubular extensions. Staurosporine could induce tubular extensions formation by blocking of actin remodelling in adherent neutrophils. Staurosporine specifically inhibits the neutrophil serine 3 coflin protein kinase, thus keeping actin in the depolymerized state. The neutrophil serine 3 coflin kinase is constitutively active and insensitive to a variety of selective antagonists of protein kinases (H-7, HA1004, ML-7, KN-62) but is blocked by STS. Another target point for STS in neutrophil cytoskeleton is inhibition of phosphorylation of L-plastin, a leukocyte-specific actin-bundling protein, which is phosphorylated on serine residue in response to adhesion or phagocytosis. L-plastin is a single protein, which interact with 4-bromophenacyl bromide, a drug capable of inducing cytoneme formation in more than 90% of cells. L-plastin is also one of the major S-nitrosation targets for NO, another potent inducer of cytoneme formation.

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STS and Ro-31-8220, but not other protein kinases inhibitors (H-7, H-8, herbimycin A) can inhibit L-plastin phosphorylation.
Mechanism of staurosporine-induced tubular membrane extension formation in neutrophils remains to be elucidated. It is not excluded that staurosporine causes extensions formation affecting NO availability and synthesis. Staurosporine has been shown to activate inducible and constitutive nitric oxide synthase isoforms expression and to inhibit production of superoxide—a scavenger of NO.

To demonstrate that STS-induced tubular extensions represent membrane tethers with cytoplasm inside, we used fluorescent cytoplasm dye BCECF and fluorescent lipid BODIPY-labelled sulfatide. STS-induced tubulovesicular extensions of BCECF-loaded neutrophils contained fluorescent dye along with neutrophil cytoplasm (Fig. 4A). Fluorescent analogue of natural lipid, BODIPY-sulfatide, permeated in the plasma membrane of STS-treated neutrophils and in STS-induced tubular extensions thus indicating their membranous structure (Fig. 4B).

The most impressive structures were formed during interaction of STS-treated neutrophils with bacteria. We compared human neutrophil interactions with serum-opsonized S. typhimurium bacteria in control conditions and in the presence of protein kinase C activator PMA and protein kinase C inhibitors H-7 and STS. In control conditions, neutrophils plated to fibronectin-coated substrata mainly ingested added S. typhimurium species. The specific membrane ruffles were left on the cell surface (Fig. 5A, large arrows). Such ruffles were shown to indicate the sites of S. typhimurium entering into the cells as a result of phagocytosis. In the presence of PMA bacteria were ingested by neutrophils in a similar way, leaving ruffles on the cell surface (Fig. 5B, large arrows).
In the presence of protein kinase C inhibitors H-7, S. typhimurium species were attached to the neutrophil surface, while ruffles were practically absent (Fig. 5C). However, when bacteria were added to STS-treated cells, STS-induced tubular extensions of cells bound S. typhimurium species along with the cell surface (Fig. 5D–F). Interaction with bacteria resulted in a statistically significant increase in STS-induced extension diameters (Table 1) and appearance of multiple bulges along the extensions (Fig. 5D and E, arrows). The latter are supposed to transport neutrophil substances along the cytonemes. The interaction with bacteria caused shedding of cytonemes together with bound bacteria and bulges and formed a kind of adhesive for bacteria islands far from the cell surface (Fig. 5D and F, arrowheads).

We demonstrated that STS-induced extensions are membrane tubules with the average diameter 160 nm. They strongly differ from 15 nm-wide neutrophil “extracellular traps”—fibers consisted of granule proteins and chromatin without lipid membrane. These fibers are formed as a result of neutrophil cell death and can bind and kill bacteria extracellularly. STS-induced extensions also do not resemble long F-actin-containing filopodia of human neutrophils, which bind bacteria and keep them until bacteria undergo phagocytosis. STS-induced tubular membrane extensions creates a way for neutrophils to contact other cells and to capture bacteria over a distance from the cell surface. Transportation of neutrophil substances along cytonemes towards bound bacteria could deliver neutrophil bactericidal agents for destination without dilution and host tissues injury (Fig. 4). The binding of bacteria by cytonemes is not a prerequisite for phagocytosis of bacteria by neutrophils but is followed by shedding of tubular extensions together with bound bacteria. Following lysis of tubular extensions besides bound and aggregated bacteria could release neutrophil bactericides closely to bound pathogens. Such a mechanism could contribute to antibiotic activity of staurosporine.

Materials and Methods

Bicarbonate-free Hank’s solution, phorbol 12-myristate 13-acetate, staurosporine, H-7, bisindolylmaleimide VII, Ro31-8220 methanesulfonate salt, genistein, cytochalasin D, FITC labeled phalloidin were purchased from Sigma (Steinheim, Germany), tyrophostin AG 82 (A25) was from Alexis (Lausen, Switzerland). Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). Fibronectin was from Calbiochem (La Jolla, USA). Diethylyamine NONOate was from Cayman (Massy, France). Acetoxymethyl ester of 7-(2′,7′-bis(2carboxyethyl)-5,6)-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR). BODIPY-sulfatide, 3-O-sulpho-D-galactosyl-[β1-1-N-[7-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl) heptanylo]-D-erythro-sphingosine, was prepared by a standard procedure used for such compounds, details of the synthesis will be published elsewhere.

Blood of healthy volunteers with no pharmacological therapy in the 2 weeks preceding sampling was used for neutrophil preparation. Blood was taken via venous puncture as approved by Ministry of Public Health Service of Russian Federation. Blood experimental procedures were approved by the Institutional Ethics Committee of A.N. Belozersky Institute. Neutrophils were isolated from freshly drawn donor blood on a bilayer gradient of Ficoll-Paque (at densities 1.077 and 1.125 g/ml). Washed neutrophils were resuspended in bicarbonate-free Hank’s solution containing 10 mM HEPES, pH 7.35. Glass cover slips were incubated in Hank’s solution containing 5 µg/ml fibronectin for 2 h at room temperature and were thoroughly washed with phosphate-buffered saline. Neutrophils (10⁶ cells/ml) were plated on protein coated cover slips in corresponding buffer and incubated for 20 min at 37°C. All test substances were dissolved in Hank’s solution, DMSO or ethanol, and were added to the cells before plating. Corresponding amounts of DMSO or ethanol (not exceeding 5 µl/ml) were added to the control cells.

Salmonella enterica serovar Typhimurium cells of virulent strain C53 were a kind gift of Prof. F. Norel. Bacteria were grown in Luria-Bertranbroth and then washed twice using physiological solution with centrifugation at 2,000 g. Concentration of stock suspension was 2 x 10⁶ CFU/ml.

Bacteria were opsonized with fresh serum from the same donor whose blood was used for neutrophil preparation. Serum was isolated by centrifugation of clotted blood. For opsonization, bacteria (4 x 10⁹/ml) were incubated for 10 min in Dulbecco’s solution containing 10% serum. Then bacteria were washed by repeated sedimentation in Dulbecco’s medium. Neutrophils were plated onto fibronectin-coated slides during 15 min at 37°C, then bacteria were added (10:1 or 20:1 bacteria/cell) and cells were further incubated at 37°C for 5 min. After that cell were fixed for scanning electron microscopy.

Photomicroscope Opton III (Germany) was used for phase contrast and fluorescent microscopy of neutrophils. The pH-sensitive fluorescent dye BCECF and BODIPY-sulfatide were used as a cytoplasmic marker, and a lipid fluorescent dye, respectively. For fluorescent microscopy, neutrophils after preparation were incubated with 5 µM BCECF (20 min at 37°C) and then were used in experiments. BODIPY-labelled sulfatide (5 µg/ml) was added to the attached cells (incubation for 5 min) at the end of experiments.

For actin cytoskeleton staining neutrophils were plated to fibronectin-coated substrata during 20 min in the presence of test drugs, fixed in 4% paraformaldehyde in Hank’s-HEPES solution without Ca²⁺ and Mg²⁺ and containing 5 mM EDTA (pH 7.3), permeabilized during 10 min with 0.1% Triton X-100 and stained with FITC-phalloidin.

For scanning electron microscopy, cells were fixed for 30 min in 2.5% glutaraldehyde, postfixed for 15 min with 1% osmium tetroxide in 0.1 M cacodylate (pH 7.3), dehydrated in an acetone series, then critical-point-dried with liquid CO₂ as a transitional fluid in a Balzers apparatus, sputter-coated with gold-palladium and observed at 15 kV with a Camscan S-2 or JSM-6380 scanning electron microscope.

Tubular extension diameters were measured directly on highly magnified scanning electron images and calculated based on the respective bar’s value. The data expressed as mean ± standard deviation.
error (D ± SEM). Student’s test for unpaired observations was applied. Values of p less than 0.05 were regarded as significant.

Acknowledgements

This work was supported by Grants of Russian Foundation of Basic Research 09-04-00367, 07-04-00410, 09-04-00313.

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