Lysophosphatidic acid induced red blood cell aggregation in vitro


Reprint from J. Bioelectrochem. (2012) 87, 89-95.

Abstract
Under physiological conditions healthy RBCs do not adhere to each other. There are indications that RBCs display an intercellular adhesion under certain (pathophysiological) conditions. Therefore we investigated signalling steps starting with transmembrane calcium transport by means of calcium imaging. We found a lysophosphatidic acid (LPA) concentration dependent calcium influx with an EC$_{50}$ of 5 μM LPA. Downstream signaling was investigated by flow cytometry as well as by video-imaging comparing LPA induced with »pure« calcium mediated phosphatidyserine exposure and concluded the coexistence of two branches of the signaling pathway. Finally we performed force measurements with holographic optical tweezers (HOT): The intercellular adhesion of RBCs (aggregation) exceeds a force of 25 pN. These results support (i) earlier data of a RBC associated component in thrombotic events under certain pathophysiological conditions and (ii) the concept to use RBCs in studies of cellular adhesion behavior, especially in combination with HOT. The latter paves the way to use RBCs as model cells to investigate molecular regulation of cellular adhesion processes.

26.1 Introduction
Adhesion between cells is a vital property that is essential for multi-cell organism. This holds true all the way from primitive cell clusters to mammals that can be regarded as the most complex organisms known. Most organs of the human body form tissues that rely on cell to cell adhesion. The adhesion processes are complex and versatile ranging from direct cell–cell contacts, like occluding junctions, desmosomes or gap junctions, to processes involving extracellular matrix proteins. One of the few organs, where the constitutive cells instead of adhering to each other form a complex liquid, is the blood. The predominant cell type in the blood is the red blood cell (RBC). Although the normal physiological function of RBCs is devoid of intercellular adhesion, there are conceptual reviews proposing the active involvement of RBCs in aggregation processes$^{1,2}$. Already more than 30 years ago, Evan Evans studied intercellular adhesion of RBC on the level of individual cell using micropipettes$^3$. Such early studies were based either on hydrodynamic interaction forces or interaction mediated by macromolecules$^4$ – both of them being reversible. The nature of these effects is based on »physical adhesion« and is therefore different from cross bridging or binding associated »biological adhesion«, although the borderline in-between is blurred.

However, experimental evidence for an involvement of RBC aggregation in vivo was published recently$^5$. Our own previous work connected intercellular RBC adhesion to an increase in intracellular calcium$^6$. Therefore RBC adhesion is both a relevant (patho)physiological process and a model system to study particular aspects of the adhesion process.

Here we investigate several aspects of the RBC aggregation in greater detail ranging from the stimulation of the calcium increase to the cellular signaling cascade.
Material and methods

26.2 RBC preparation and fluorescence microscopy

For experiments, fresh blood from healthy donors was obtained by a fingertip needle prick or human venous blood was drawn from healthy donors. Heparin or EDTA was used as an anticoagulant. The obtained blood was used within one day. The cells were washed three times by centrifugation (2000 g, 3–5 min) in a HEPES buffered solution of physiological ionic strength containing the following (in mM): 145 NaCl, 7.5 KCl, 10 glucose and 10 HEPES, pH 7.4, at room temperature. The buffy coat and plasma were removed by aspiration. For Ca²⁺ imaging, RBCs were loaded with 4–5 μM Fluo-4 AM (Molecular Probes, Eugene, USA) from a 1 mM stock solution in dimethyl sulfoxide with 20% Pluronic (F-127, Molecular Probes). Loading was performed in 1 ml of solution of physiological ionic strength at an RBC hematocrit of approximately 1% for 45 min at 37 °C. The cells were washed by centrifugation once more and equilibrated for de-esterification for 15 min. LPA, prepared from a stock solution of 1 mM in distilled water, and the Ca²⁺ ionophore 4-bromo-A23187, prepared from a stock solution of 1 mM in ethanol, were obtained from Sigma-Aldrich (St. Louis, USA). To investigate phosphatidylserine (PS) exposure, cells were stained with annexin V-FITC (Molecular Probes). Annexin V-FITC was delivered in a unit size of 500 μl containing 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, and 0.1% bovine serum albumin. Five hundred microliters of annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) was added to 1 μl of washed, packed RBCs previously treated with 4-bromo-A23187. 5 μl of annexin V-FITC was then added, and the cells were mixed gently. The probes were incubated at room temperature in the dark for 15 min. Subsequently, the samples were washed once in annexin binding buffer by quick centrifugation (20 s, 12 000 g) to remove unbound annexin V-FITC and resuspended in 500 μl of the same buffer and placed on ice. RBCs showing PS exposed on their outer membrane leaflet were measured in the FL-1 channel using an argon laser at 488 nm of a fluorescence-activated flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, New Jersey). The setting of the negative fluorescent gate was obtained in the absence of the Ca²⁺ ionophore 4-bromo-A23187 (negative control). The annexin V-FITC positive RBCs can be calculated in percentage by comparing the number of positive and negative signal events with the control. CellQuest Pro (BD Biosciences) software was used for data acquisition and analysis. The experiment was repeated three times on blood from different donors. For each experiment, 30 000 events were counted. The data are presented as mean values ± SD. For statistical comparison unpaired, two