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The influence of PAMAM-OH dendrimers on the activity of human erythrocytes ATPases

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ABSTRACT

Dendrimers are a relatively new and still not fully examined group of polybranched polymers. In this study polyamidoamine dendrimers with hydroxyl surface groups (PAMAM-OH) of third, fourth and fifth generation (G3, G4 and G5) were examined for their ability to influence the activity of human erythrocyte plasma membrane adenosinetriphosphatases (ATPases). Plasma membrane ATPases are a group of enzymes related, among others, to the maintenance of ionic balance inside the cell. An inhibition of their activity may result in a disturbance of cell functioning. Two of examined dendrimers (G4 and G5) were found to inhibit the activity of Na^+/K^+ ATPase and Ca^{2+} ATPase by 20–30%. The observed effect was diminished when higher concentrations of dendrimers were used. The experiment with the use of pyrene as fluorescent probe sensitive to the changes in microenvironment's polarity revealed that it was an effect of dendrimers' self-aggregation. Additional studies showed that PAMAM-OH dendrimers were able to decrease the fluidity of human erythrocytes plasma membrane. Obtained results suggest that change in plasma membrane fluidity was not caused by the dendrimer–lipid interaction, but dendrimer–protein interaction. Different pattern of influence of dendrimers on ATPases activity and erythrocyte membrane fluidity suggests that observed change in ATPases activity is not a result of dendrimer–lipid interaction, but may be related to direct interaction between dendrimers and ATPases.

1. Introduction

Dendrimers are a relatively new class of highly branched polymers. The first syntheses of dendrimers were reported in late 70s and early 80s of a previous century [1,2]. Important attributes of dendrimers are their high monodispersity, regular, poly-branched three dimensional organization and the presence of many terminal groups [3]. There are different types of dendrimers, depending on the building monomers, for example: PAMAM (polyamidoamine) or PPI (polypropyleneimine) dendrimers. The size of molecules and number of terminal groups depend on the generation of the dendrimer [4]. Since their first synthesis, a lot of studies related to properties of dendrimers have been performed. Dendrimers were found to be very useful in the field of pharmacy and medicine. For example they can be used as solubility enhancers for hydrophobic molecules [5] or as a part of a drug-delivery system [6]. Cationic dendrimers turned out to be good non-viral vehicles for gene delivery, that can be utilized for therapeutic applications [7–9]. There were also trials performed in order to assess the possibility of application of a dendrimer based molecule as a contrast agent in MR imaging [10,11].

The main factor that limits the possibility of the applications of dendrimers in medicine is their toxicity. Toxic activity is mainly related to cationic dendrimers. Cationic dendrimers possess surface groups that bear positive charge or groups (such as amine groups) that can be protonated under physiological conditions [12,13]. Interactions between positively charged surface of dendrimers and negatively charged lipid membranes lead to toxic effects of dendrimers [14]. This interaction may lead to nanoscale hole formation in lipid bilayers [15]. As a result of interaction between cationic dendrimers and erythrocytes, hemolysis may be observed [16]. Moreover, cationic dendrimers reveal cytotoxic activity against mammalian cells [17]. In order to reduce above-mentioned toxic effects, modifications of dendrimers' surface may be introduced. Such modifications include for example substitution of surface amine groups with: PEG [18], acetyl group [19] or saccharide moiety [20]. In the case of PAMAM dendrimers one of the possible modification is the replacement of surface amine groups for hydroxyl groups. This modification leads to the loss of surface charge. Such modified PAMAM dendrimers (called PAMAM-OH) have been shown to possess

Abbreviations: ATPase, adenosinetriphosphatase; CH, cholesterol; DLS, dynamic light scattering; DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetraacetic acid disodium salt dihydrate; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; G3, G4, G5, third, fourth and fifth generation of dendrimer; PAMAM, polyamidoamine dendrimer; PAMAM-OH, polyamidoamine dendrimer with hydroxyl surface groups; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; PPI, polypropyleneimine dendrimers; SM, sphingomyelin; TMA-DPH, N,N,N-trimethyl-4-(6-phenyl-1,3,5,-hexatrien-1-yl)phenylammonium p-toluenesulfonate; Tris, tris (hydroxymethyl)aminomethane

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significantly reduced ability to interact with lipid membranes [21], as well as reduced toxicity against mammalian cells [22,23]. Moreover, this modification of PAMAM dendrimers considerably reduced their capability of induction of hemolysis in comparison with unmodified PAMAM dendrimers [24]. Because of the presence of hydroxyl groups on the surface, dendrimer molecule can participate in many interactions due to the possibility of hydrogen bond formation. Hydrogen bonds are relatively weak interactions, when compared to covalent bonds, nevertheless they are very important type of interactions, widely found in biological systems [25]. Large number of hydroxyl groups on the surface of PAMAM-OH dendrimers may lead to strong interactions with components of biological systems due to formation of many hydrogen bonds by one dendrimer molecule. Such interactions may occur on the surface of cells. As an effect, a disturbance of function of membrane proteins may be observed.

An important group of membrane proteins is related to the transport of ions across the cell membrane. Among them two ATPases play crucial role in maintenance of intracellular ion concentration. The first of them is the Na^+,K^+-ATPase, that transports Na^+ ions outside the cell and K^+ ions into the cell [26]. The second is Ca^{2+}-ATPase that transports Ca^{2+} outside the cell [27]. Both above-mentioned ATPases require Mg^{2+} ions for their activity. Another membrane ATPases, which activity is dependent on the presence of Mg^{2+} ions, but which are not related to ion transport, are the enzymes responsible for asymmetrical distribution of lipids between the inner and the outer leaflet of plasma membrane. Those enzymes are called flippase and floppase and are responsible for at least a part of Mg^{2+} dependent basal ATPase activity [28–31].

Despite of reduced toxic effect and lowered hemolytic potential, interaction of PAMAM-OH with membrane ATPases may result in abnormality of cell functioning. The incorrectness of membrane ATPases activity may be the cause of very severe effects. For example in a case of Na^+,K^+-ATPase it was shown that the decrease of the activity of this enzyme may result in the apoptotic death of cell. If the decrease of above-mentioned enzyme activity is not high enough to trigger apoptotic death, still the affected cell is more susceptible to apoptosis [32,33].

The aim of this study was the evaluation of the effect of PAMAM-OH dendrimers on the activity of Mg^{2+} dependent erythrocyte membrane ATPases. In order to establish whether the observed influence of dendrimers on ATPases activity was indirect and related to interaction between dendrimers and lipid components of cell membrane, additional studies related to influence of dendrimers on lipid membranes fluidity were also performed. Studying interaction between dendrimers and ATPases led to an interesting observation. In some cases disturbance of ATPase activity was not proportional to the increased concentration of dendrimers. In order to explain this observation several experiments related to self-aggregation of dendrimers were performed. This article is the first, to our knowledge, to investigate the influence of dendrimers on the activity of erythrocyte membrane ATPases.

2. Materials and methods

2.1. Materials

Sodium chloride, potassium chloride, calcium chloride, magnesium chloride hexahydrate, potassium phosphate monobasic, copper (II) sulphate(VI), sodium carbonate, sodium citrate monohydrate, malachite green oxalate salt, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), Folin–Ciocalteau reagent, pyrene, hydrochloric acid, sulphuric acid, methanol, chloroform and tetrahydrofuran (THF) were purchased from POCh (Poland). Bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), adenosine 5′-triphosphate disodium salt hydrate (ATP), ammonium heptamolybdate tetrahydrate, poly(vinyl alcohol) (PVA), trichloroacetic acid (TCA), ouabain octahydrate, 1,6-diphenyl-1,3,5-hexatriene (DPH), *N,N,N*-trimethyl-4-(6-phenyl-1,3,5,-hexatrien-1-yl)phenylammonium *p*-toluenesulfonate (TMA-DPH), cholesterol (CH), L-α-phosphatidylcholine from egg yolk (PC), L-α-phosphatidylethanolamine from bovine brain (PE), L-α-phosphatidyl-L-serine from bovine brain (PS), sphingomyelin from bovine brain (SM) and PAMAM-OH dendrimers (generations 3, 4 and 5) were purchased from Sigma-Aldrich. PAMAM-OH dendrimers were obtained in the form of a solution in methanol. Methanol from dendrimer solutions was evaporated prior to preparation of PAMAM-OH water solutions. Water used for preparation of all solutions was obtained from Mill-Q system (Millipore).

The buffer used in all experiments, except for ATPase activity assay, contained 100 mM Tris–HCl buffer (pH 7.4), 85 mM NaCl, 10 mM $MgCl_2$, and 10 mM KCl. The buffer was chosen to resemble the environment of ATPase activity assay.

2.2. Preparation of erythrocyte ghosts

Human blood from healthy adult donors was obtained from a local blood bank. Erythrocyte ghosts were obtained using previously described methods [34,35] with some modifications. Erythrocytes were cooled to 4 °C and centrifuged at 400*g* for 10 min. Next the buffy coat was removed, and erythrocytes were washed three times with one volume and three times with ten volumes of isotonic sodium chloride solution and centrifuged at 700*g* for 5 min. The buffer used for hemolysis and following washing was 20 mM Tris–HCl buffer, pH 7.4 containing 1 mM EDTA and 0.5 mM PMSF (as proteolysis inhibitor). Erythrocytes were hemolysed with ten volumes of ice-cold buffer and centrifuged at 19,000*g* for 20 min at 0 °C. Next, supernatant was discarded and lysed erythrocytes were washed under the same conditions until complete removal of hemoglobin. Erythrocyte ghosts were suspended in small amount of hemolytic buffer. The protein concentration was determined using the method of Lowry [36] with bovine serum albumin as a standard. Erythrocyte ghosts were kept frozen at −20 °C and used within a week from the preparation.

2.3. ATPase activity assay

Activity of erythrocyte ghosts membrane ATPases was measured according to the method described previously [37] with some modifications. Enzymes activities were determined in the medium containing 100 mM Tris–HCl buffer (pH 7.4), 85 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA and 1 mM ATP. Reaction was started by the addition of ATP containing solution to the solution containing erythrocyte ghosts (protein concentration of 0.3 mg/mL) and PAMAM-OH dendrimers in a concentration range between 0.01 mg/mL and 1 mg/mL. Samples were incubated for 1 hour at 37 °C. Reaction was stopped by the addition of equal volume of ice-cold 0.6 M trichloric acid. Samples were centrifuged at 19,000*g* for 5 min and the supernatant was used for the determination of the amount of liberated inorganic phosphates. Activity of Na^+/K^+-ATPase was determined from the difference in the formation of inorganic phosphates in the absence and presence of 0.1 mM ouabain. Activity of Ca^{2+}-ATPase was determined from the difference in the formation of inorganic phosphates in the presence and absence of 0.2 mM $CaCl_2$. Activity of ATPase in the absence of Ca^{2+} ions and the presence of ouabain was determined as the basal Mg^{2+}-ATPase and calculated from the difference in the formation of inorganic phosphates in the presence and the absence of Mg^{2+} and K^+ ions.

The amounts of liberated inorganic phosphates were determined according to the improved malachite green assay [38], adapted for the use in 96-well plates. Supernatant samples of 40 μL were placed in 96-well plates. Next 160 μL of water was added, followed by the addition