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The effect of polyamidoamine dendrimers on human erythrocyte membrane acetylcholinesterase activity

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Abstract

Polyamidoamine (PAMAM) dendrimers impact on activity of acetylcholinesterase was studied. It has been shown that dendrimers induce a biphasic effect: depending on their concentrations they increase or decrease enzyme activity. It may be due to two types of interactions: direct—between dendrimers and the enzyme; indirect—via a modification of the physical state of membrane phospholipids affecting the acetylcholinesterase.

Keywords: Acetylcholinesterase; Erythrocyte membrane; Erythrocyte ghosts; Michaelis–Menten constant; Dendrimer

1. Introduction

Dendrimers are a new class of polymers which have attracted much interest since their first synthesis in the mid-1980s due to their unique nanoscopic architecture [1]. Unlike linear polymers, they have well-defined structure. Dendrimers consist of a central core and branched monomers. The cyclic manner in which they are built results in a globular shape and a large number of end groups on the surface. The more layers of branched units are attached, the higher generation of dendrimer is obtained. Polyamidoamine (PAMAM) dendrimers are based on an ethylenediamine core and branched units are built from both methyl acrylate and ethylenediamine.

In current studies, we used the fourth generation (G4) of two types of polyamidoamine dendrimers, PAMAM G4 and PAMAM-OH G4 (Fig. 1). PAMAM G4 dendrimers possess 64 amino groups on a surface whereas PAMAM-OH G4 dendrimers have the same number of hydroxyl groups at chain-ends. Molecular weight for PAMAM G4 and PAMAM-OH G4 equals 14,215 and 14,279 Da, respectively. Their diameters are similar and equal to approximately 40 Å.

The specific structure makes dendrimers suitable for a variety of biomedical applications. Among them, the use of dendrimers as carrier molecules for drugs has been of great interest. Drug molecules can either be attached to dendrimers' end groups, or encapsulated in the macromolecule interior [2,3]. Both strategies are very promising in a targeted anti-tumour therapy. To pursue studies on dendrimers in therapeutic applications, more information about their biological properties is needed.

We have previously shown that dendrimers interact with serum albumin and change its conformation [4]. There are articles that postulate haematotoxic and cytotoxic actions of some types of dendrimers [5,6]; however, the detailed molecular mechanism of these actions is still unknown. Thus, this study was undertaken to reveal more information about dendrimers impact on an enzyme—acetylcholinesterase (AChE, EC 3.1.1.7.) and to answer a question how dendrimers affect its function. Moreover, changes in AChE activity are a good indicator of membrane alterations.

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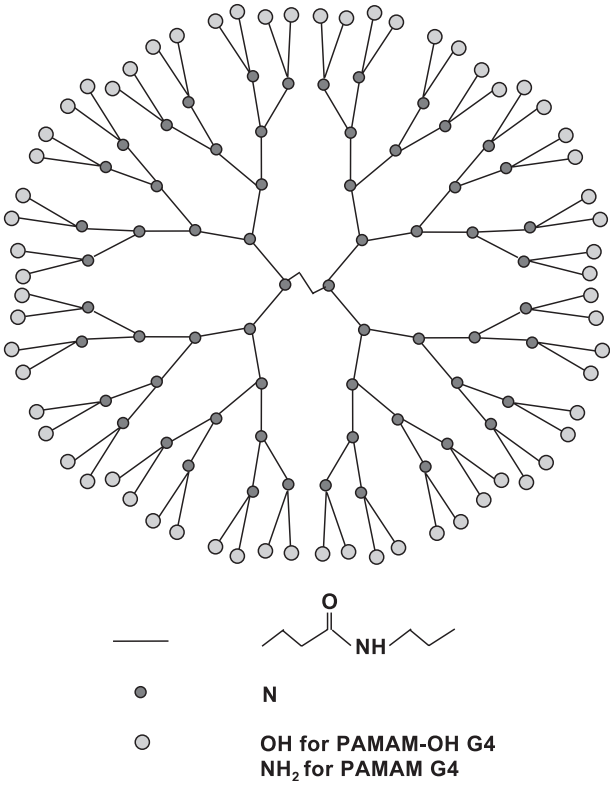

Fig. 1. Scheme of a polyamidoamine dendrimer.

AChE is a very efficient protein catalyst that hydrolyses its physiological substrate acetylcholine at one of the highest known catalytic rates [7]. It is a key enzyme in cholinergic neurotransmission and signal transduction. Located at neuromuscular junctions, it is responsible for preventing re-excitation after the stimulated cell has recovered from first action potential [8]. Irreversible inhibition of AChE induces a constant excitation of the parasympathetic nervous system and muscle tissues which leads to death [9]. AChE has also been found on the surface of mammalian erythrocytes. Its physiological role is still unknown. Erythrocyte AChE is a hydrophobic integral membrane enzyme anchored in the outer monolayer via carboxy-terminal binding domain. AChE may exist as globular monomers, dimers and oligomers [10]. Dimeric AChE is the predominant species present in the native erythrocyte membrane [11]. The dimer is built up of two identical subunits which have molecular weights of approximately 73,000 Da. The monomers are covalently linked together by disulfide bonds.

2. Experimental

2.1. Materials

5,5′ -dithiobis (2-nitrobenzoic acid) (Ellman reagent) and acetylthiocholine iodide were purchased from Sigma (USA). PAMAM and PAMAM-OH dendrimers (both G4) were obtained from Aldrich (UK). All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

2.2. Preparation of erythrocyte ghosts

Blood from healthy donors was purchased from the Central Blood Bank in Lodz. Blood was taken into 3% sodium citrate. Erythrocytes were separated from plasma and leukocytes by centrifugation for 10 min at 4 °C at 600×*g* and washed three times with ice-cold phosphate-buffered saline (PBS: 0.15 mol/l NaCl, 1.9 mmol/l NaH_2PO_4, 8.1 Na_2HPO_4 mmol/l, pH 7.4). The erythrocyte ghosts were prepared from washed cells according to the method of Dodge et al. [12] with some modifications. The erythrocytes were haemolysed with 20 volumes of 10 mmol/l phosphate buffer, pH 7.8, containing 0.1 mmol/l EDTA and 0.1 mmol/l PMSF (phenylmethylsulphonyl fluoride) as proteolytic inhibitors and centrifuged for 20 min at 4 °C at 20,000×*g*. Then the ghosts were resuspended in ice-cold 5 mmol/l phosphate buffer, pH 7.4, and centrifuged again. The process was continued until the ghosts were free of residual haemoglobin. The protein content in the erythrocyte ghosts was determined by the method of Lowry et al. [13], using bovine serum albumin as a standard.

2.3. Acetylcholinesterase activity estimation

AChE activity in isolated erythrocyte membranes was assayed by the method of Ellman et al. [14] in which acetylthiocholine is used as the substrate and the product, thiocholine, reacts with Ellman reagent to form a yellow anion 5-thio-2-nitrobenzoic acid. The formation of this product is an indicator of AChE activity.

Erythrocyte ghosts suspension was diluted with 10 mmol/l phosphate buffer, pH 8.0, to 0.03 mg/ml protein concentration. To 1 ml sample of diluted ghosts Ellman reagent was added (its final concentration in the sample was 100 μmol/l). Then dendrimers in concentration range from 25 to 150 μmol/l were added. The kinetics of acetylthiocholine iodide hydrolysis was recorded spectrophotometrically (Pharmacia LKB-Biochrom 4060, UK) at room temperature and the rate of the reaction was calculated from the equation:

$$v = \frac{\text{O.D.}^{412} \cdot F}{13,600 \cdot 1000} \left[\frac{\text{mol acetylthiocholine}}{\text{min·mg of protein}} \right],$$

where O.D.^{412} is an increase of absorbance at 412 nm for 1 min, and F is a dilution coefficient in respect to the protein concentration.

Two parameters were calculated from Lineweaver–Burke graph: V_{max}, which is the maximal rate of the enzymatic reaction and the Michaelis–Menten constant (K_m), which corresponds to the concentration of the

substrate at which the reaction rate equals to the half of its maximal value.

All results are expressed as a mean value±S.D. of six experiments. Statistical significance was assessed using Student–Fisher test.

3. Results and discussion

In the presence of both types of dendrimers, PAMAM G4 and PAMAM-OH G4 we observed a biphasic effect on the activity of membrane-bound AChE (Figs. 2 and 3). Lower dendrimer concentrations caused a statistically significant increase of the enzyme activity. For higher dendrimer concentrations, we observed an inhibition of AChE. The efficiency of the inhibition was similar for both types of dendrimers; however, the maximum of activation occurred at a different range of dendrimer concentration −25 μmol/l for PAMAM-OH G4 and 100 μmol/l for PAMAM G4.

The biphasic influence of dendrimers on AChE activity may be a result of an alteration in protein structure but may also reflect the structural changes in the whole membrane. It is believed that the measurement of AChE activity gives valuable information about the structural changes in the membrane under actions of various factors (e.g., laser irradiation, oxidative stress). AChE properties depend on, e.g., membrane fluidity. It has been shown that there is some correlation between membrane rigidity and the value of the Michaelis–Menten constant (K_m). The K_m constant corresponds to the affinity of the enzyme to the substrate. The greater the membrane rigidity, the smaller the K_m [15]. Similar biphasic alteration of AChE activity was observed upon addition of tetracaine, which was strongly connected with changes in fluidity of membrane lipid hydrocarbon chains and charged lipid head groups [16]. It has been earlier reported that polyamidoamine dendrimers impact on human red blood cell morphology and membrane integrity [17], change rotational mobility of fatty acid chains [18], and disrupt anionic lipid vesicles [19]. On the other hand, there are proofs that dendrimers interact with proteins and

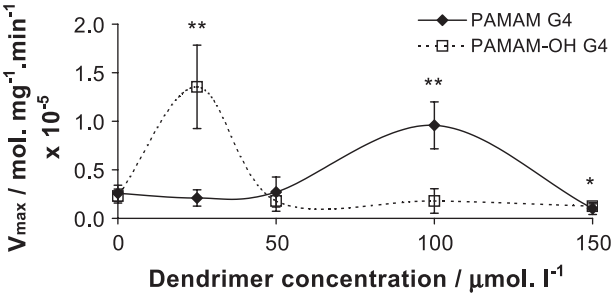

Fig. 2. The effect of dendrimers on the maximal rate of the enzymatic reaction. Results are expressed as means±S.D. of six experiments. Statistical significance was assessed using Student–Fischer test, *P<0.05, **P<0.01.

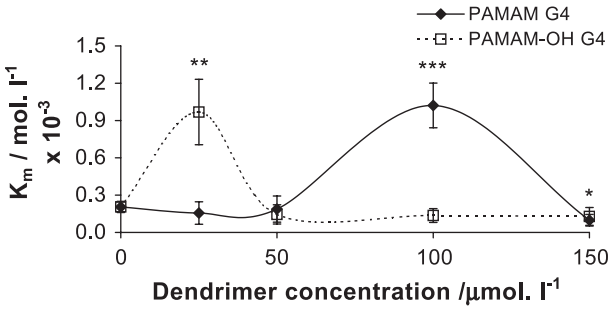

Fig. 3. The effect of dendrimers on the Michaelis–Menten constant. Results are expressed as means±S.D. of six experiments. Statistical significance was assessed using Student–Fischer test, *P<0.05, **P<0.01, ***P<0.001.

change their conformation, e.g., they create a layer on the surface of serum albumin [4]. Especially, the inhibition of AChE activity observed for higher dendrimer concentration may be a result of direct interactions between dendrimers and the enzyme. The type of the inhibition was determined from the Lineweaver–Burke plot. After addition of dendrimers, the value of the Michaelis–Menten constant K_m decreased similarly to the value of V_{max}. The equivalent change in K_m and V_{max} indicates an uncompetitive inhibition [20]. X-ray diffraction data have revealed that the active site of AChE is at the bottom of a deep and narrow gorge [21]. Although the base of the gorge near the active site is negatively charged [22,23], we did not observe differences between the AChE behaviour upon cationic PAMAM G4 and neutral PAMAM-OH G4. This could be due to large dimensions of dendrimer molecules that do not enable them to reach the active site.

To sum up, changes of AChE activity upon addition of dendrimers are a consequence of both direct interactions between dendrimers and the enzyme and indirect via membrane condition modifications (Fig. 4). It is highly possible that two effects play an equally significant role.

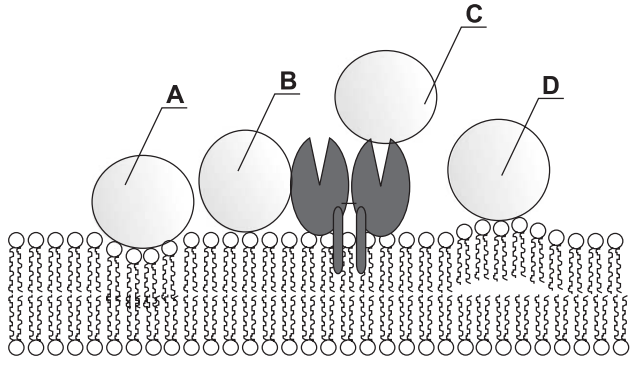

Fig. 4. Scheme of dendrimer interactions with an enzyme and a membrane. (A) Partial dendrimer incorporation into a lipid bilayer; (B) simultaneous dendrimer interactions with an enzyme and a lipid bilayer; (C) dendrimer interactions with an enzyme; (D) pulling out the outer monolayer by a dendrimer molecule.

Acknowledgements

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