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Structural effects of Zn^{2+} on cell membranes and molecular models

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ABSTRACT

Zinc is an essential element for nutrition as well as for the proper development and function of brain cells, and its traces are present in a wide range of foods. It is a constituent of many enzyme systems and is an integral part of insulin and of the active site of intracellular enzymes. However, excessive accumulation of zinc or its release from the binding sites may become detrimental for neurons. With the aim to better understand the molecular mechanisms of the interaction of zinc ions with cell membranes, it was incubated with intact human erythrocytes, isolated unsealed human erythrocyte membranes (IUM), cholinergic murine neuroblastoma cells, and molecular models of cell membranes. These consisted in bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), phospholipid classes present in the outer and inner monolayers of most plasmatic cell membranes, particularly that of human erythrocytes, respectively. The capacity of zinc ions to perturb the bilayer structures of DMPC and DMPE was assessed by X-ray diffraction, DMPC large unilamellar vesicles (LUV) and IUM were studied by fluorescence spectroscopy, intact human erythrocytes were observed with scanning electron microscopy (SEM), and neuroblastoma cell morphology was observed under inverted microscope. This study presents evidence that 0.1 mM Zn and higher concentrations affect cell membrane and molecular models.

1. Introduction

It is widely recognized that marine ecosystem can become contaminated by trace metals from numerous and diverse sources. However, anthropogenic activities such as mining and industrial ore and metal processing still remain the main cause of the increased amount of heavy metals which have been dumped into oceans [1]. They occur in all compartments in the marine environment with a tendency to be accumulated in organisms from different tropic levels of the marine webs, thus toxic trace metals become a potential hazard for man and mammals [2,3]. It has been reported that zinc concentration in the human consuming bivalve mussel *Perumytilus purpuratus* present in the Southern coast of Chile is as high as 233 μg/g [2]. Among the first-raw transition metals, zinc is only second to iron in terms of abundance and importance in chemical, structural and regulatory roles in biological systems [4]. Zinc is an essential element of nutrition and traces are present in a wide range of foods. It is a constituent of many enzyme systems and is an integral part of insulin and of the active site of intracellular enzymes concerned with RNA synthesis, proteolytic cleavage, dehydrogenation reactions, hydration of CO_2 and many other activities [5,6]. It is also present in Alzheimer's disease amyloid plaques [7]. Zinc binds to negatively-charged groups such as carboxylates and thiolates by electrostatic interactions, and to neutral residues such as carbonyls and imidazoles via orientation-dependent charge–dipolar interactions [4]. It has previously been indicated that several agents induce leakage of ions and low molecular weight metabolites through membrane lesions in cells. However, leakage is known to be inhibited by divalent cations such as Zn^{2+} [8]. On the other hand, high concentrations of Zn^{2+} ions are released during synaptic transmission, being shown to bind to β-amyloid peptide and affect the peptide's interactions with lipid bilayers [9]. Zinc uptake into cholinergic neuroblastoma cells mediated by M1 muscarinic receptors was found to alter a transcriptional regulation of their zinc finger genes [10]. However, in pathological conditions, excessive accumulation of zinc or its release from the binding sites may become detrimental for neurons [11]. These effects of Zn^{2+} ions have prompted us to study the structural effects that this ion induces to cell membranes.

In the course of *in vitro* systems search for the toxicity screening of chemicals of biological relevance, different cellular models have been applied to examine their adverse effects. The cell membrane is a diffusion barrier which protects the cell interior. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with chemical species. In order to better

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understand the molecular mechanisms of the interaction of Zn^{2+} with cell membranes, molecular models of the erythrocyte membranes have been utilized. Erythrocytes were chosen because, although less specialized than many other cell membranes, they carry on enough functions in common with them (such as active and passive transport, and the production of ionic and electric gradients) to be considered representative of the plasma membrane in general. The molecular models consisted of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of the human erythrocyte membrane, respectively [12,13]. The capacity of Zn^{2+} to perturb the multibilayer structures of DMPC and DMPE was evaluated by X-ray diffraction. In an attempt to further elucidate the effect of Zn^{2+} ions on cell membranes, the current work also examined their influence on the morphology of intact human erythrocytes, while isolated unsealed human erythrocyte membranes (IUM) and DMPC unilamellar vesicles (LUV) were studied by fluorescence spectroscopy. These systems and techniques have been used in our laboratories in order to determine the interaction with and the membrane-perturbing effects of other metal cations [14–17]. In this work we also investigated Zn^{2+} uptake into murine cholinergic neuroblastoma cells and its effect on their morphology, assessed by inverted microscopy.

2. Materials and methods

2.1. X-ray diffraction studies of phospholipid multilayers

The capacity of Zn^{2+} to perturb the structures of DMPC and DMPE multilayers was determined by X-ray diffraction. Synthetic DMPC (lot 140PC-224, MW 677.9), DMPE (lot 140PE-54, MW 635.9) from Avanti Polar Lipids (ALA, USA), $ZnCl_2$ from Merck (Germany) were used without further purification. About 2 mg of each phospholipid was introduced into 2 mm diameter special glass capillaries (Glas-Technik and Konstruktion, Berlin, Germany), which were then filled with 150 μl of (a) distilled water and (b) aqueous solutions of $ZnCl_2$ in a range of concentrations (10 μM up to 1 mM). The specimens were incubated for 1 h at 30 °C and 60 °C with DMPC and DMPE, respectively, centrifuged for 10 min at 2000 rpm and X-ray diffracted with Ni-filtered CuKα from a Bruker Kristalloflex 760 (Karlsruhe, Germany). Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. The relative reflection intensities were obtained in an MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 18 ± 1 °C, which is below the main phase transition temperature of both DMPC and DMPE. Higher temperatures would have induced transitions to the more fluid liquid crystalline phases making harder the detection of structural changes induced by Zn. Each experiment was repeated three times, and additional experiments were carried out when there were doubts.

2.2. Fluorescence measurements of DMPC large unilamellar vesicles and isolated unsealed human erythrocyte membranes

The influence of Zn^{2+} on the physical properties of DMPC unilamellar vesicles (LUV) and isolated unsealed human erythrocyte membranes (IUM) was examined by fluorescence spectroscopy using DPH and laurdan (Molecular Probe, Eugene, OR, USA) fluorescent probes. DPH is widely used as a probe for the hydrophobic regions of the phospholipid bilayers because of its favorable spectral properties. Its steady state fluorescence anisotropy measurements were used to investigate the structural properties as it provides a measure of the hindered rotational diffusion of the fluorophor, restricted within a certain region such as the cone, due to the lipid acyl chain packing order. On the other hand, its time resolved fluorescence decay data provide information related to the polarity of its environment. Laurdan, an amphiphilic probe, has a high sensitivity of excitation and emission spectra to the physical state of membranes. With the fluorescent moiety within a shallow position in the bilayer, laurdan fluorescence spectral shifts provide information on the polarity and molecular dynamic properties at the level of the phospholipid polar headgroups. The quantification of the laurdan fluorescence shift was effected using the generalized polarization (GP) concept [18].

DMPC large unilamellar vesicles (LUV) suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspensions (final lipid concentration 0.4 mM) through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Costar Corp., MA, USA) under nitrogen pressure at 10 °C above the lipid phase transition temperature. Human erythrocyte membranes (IUM) were prepared from blood obtained from healthy male volunteers according to the method of Dodge et al. [19]. DPH and laurdan were incorporated into DMPC LUV and IUM suspensions by addition of small aliquots of concentrated solutions of the probes in dimethyl sulfoxide and ethanol, respectively, in order to obtain a proportion of the probe to lipids of *ca.* 0.1 mol%. After the probe addition, the samples were incubated at 37 °C for 1 h. Steady state and time resolved fluorescence measurements were performed on a K2 multifrequency phase shift and modulation spectrofluorometer (ISS Inc., Champaign, IL, USA) interfaced to computers. Software from ISS was used for data collection and analysis. For both probes, the exciting light was from an ISS Light Emitting Diode (LED) emitting at 370 nm, which can be modulated up to 600 MHz during time resolved measurements. DMPC LUV suspensions were measured at 18 °C and 37 °C, and IUM suspensions at 37 °C using 5 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circulator (Cole-Parmer, Chicago, IL, USA) and monitored before and after each measurement using an Omega digital thermometer (Omega Engineering Inc., Stanford, CT, USA). Anisotropy measurements were made in the L configuration using Glan–Thompson prism polarizers (ISS) in both exciting and emitting beams. The emission was measured using a WG-420 Schott high-pass filter (Schott WG-420, Mainz, Germany) with negligible fluorescence. DPH fluorescence anisotropy (r) was calculated according to the definition: $r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$, where $I_{||}$ and $I_{\perp}$ are the corresponding vertical and horizontal emission fluorescence intensities with respect to the vertically polarized excitation light [20]. Laurdan fluorescence spectral shifts were quantitatively evaluated using the GP concept (see above) which is defined by the expression $GP = (I_b - I_r)/(I_b + I_r)$, where I_b and I_r are the emission intensities at the blue and red edges of the emission spectrum, respectively. These intensities have been measured at the emission wavelengths of 440 and 490 nm, which correspond to the emission maxima of laurdan in the gel and liquid crystalline phases, respectively [21]. For fluorescence decay determination, the exciting light was polarized parallel to the vertical laboratory axis, and the emission was viewed through a Glan–Thompson polarizer oriented at 54.7° [22]. Phase and modulation values were obtained as previously described [23,24] at 10 modulation frequencies in the interval of 5–40 MHz for DPH. Dimethyl-POPOP in ethanol (τ = 1.45 ns) was used as a reference of intensity decay. Time resolved fluorescence data was analyzed using the Globals Unlimited software package (Laboratory for Fluorescence Dynamics, University of California at Irvine, Irvine, CA). The DPH phase and modulation data were analyzed either by assuming a sum of discrete exponential [25] or continuous distribution models which assumed Lorentzian or Gaussian distributions [23,24]. $ZnCl_2$ was incorporated in IUM