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Acetylsalicylic acid (aspirin) and salicylic acid interaction with the human erythrocyte membrane bilayer induce *in vitro* changes in the morphology of erythrocytes

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ABSTRACT

Despite the well-documented information, there are insufficient reports concerning the effects of salicylate compounds on the structure and functions of cell membranes, particularly those of human erythrocytes. With the aim to better understand the molecular mechanisms of the interaction of acetylsalicylic acid (ASA) and salicylic acid (SA) with cell membranes, human erythrocyte membranes and molecular models were utilized. These 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. The capacity of ASA and SA to perturb the multibilayer structures of DMPC and DMPE was evaluated by X-ray diffraction while DMPC unilamellar vesicles (LUV) were studied by fluorescence spectroscopy. Moreover, we took advantage of the capability of differential scanning calorimetry (DSC) to detect the changes in the thermotropic phase behavior of lipid bilayers resulting from ASA and SA interaction with PC and PE molecules. In an attempt to further elucidate their effects on cell membranes, the present work also examined their influence on the morphology of intact human erythrocytes by means of defocusing and scanning electron microscopy, while isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy. Results indicated that both salicylates interact with human erythrocytes and their molecular models in a concentration-dependent manner perturbing their bilayer structures.

Introduction

Acetylsalicylic acid (ASA)[1] is one of the most widely used medications, amply used as an analgesic to relieve minor aches and pains, as an antipyretic to reduce fever, and as an anti-inflammatory medication. These medicinal properties, particularly fever relief, have been known since ancient times [1]. It also has an antiplatelet effect by inhibiting the production of thromboxane. Much of this is believed to be on account of decreased production of prostaglandins and thromboxanes, which is due to its irreversible inactivation of the cyclooxygenase (COX) enzyme required for prostaglandin and thromboxane synthesis [2]. Although a major clinical use of aspirin (ASA) therapy is to improve the survival of patients with myocardial infarction and unstable angina, which is usually attributed to the antiplatelet action of aspirin, involvement of salicylates in other mechanisms of action cannot be excluded. Accordingly, aspirin has recently been shown to protect endothelial cells against oxidative stresses, possibly by promoting the synthesis of ferritin [3]. The main effects of orally ingested ASA, especially in higher doses, are gastrointestinal ulcers, stomach bleeding, and tinnitus [4]. On the other hand, salicylic acid (SA) is known for its ability to ease aches and pains and reduce fevers. However, it is so irritating to mucosa that it can only be used externally [4]. Although toxic in large quantities, SA is used as a food preservative, bactericide and antiseptic. Increasing numbers of reports assert that salicylate side effects including gastric intestinal toxicity are actually due to nonspecific interactions with biomembranes [5].

Despite the extensive and well documented information about both salicylates, the reports on their effects on cell membranes, particularly on those of the human erythrocyte are amazingly scanty. Cell membrane is a diffusion barrier which protects the interior of

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[1] *Abbreviations used:* ASA, acetylsalicylic acid; SA, salicylic acid; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; SEM, scanning electron microscopy; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; *r*, fluorescence anisotropy; GP, generalized polarization; DPH, 1,6-diphenyl-1,3,5-hexatriene; laurdan, 6-dodecanoyl-2-dimethylaminonaphtalene; DM, defocusing microscopy; RBC, red blood cell suspension.

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the cell. Therefore, its structure and functions are susceptible to alterations as a consequence of interactions with chemical species. With the aim to better understand the molecular mechanisms of the interaction of ASA and SA with cell membranes, human erythrocyte membranes and molecular models of red cell membranes were utilized. Human erythrocytes were chosen since with one membrane only and no internal organelles they constitute an ideal cell system for studying basic drug–biomembrane interactions [6]. On the other hand, although less specialized than many other cell membranes, they carry out 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 of cell membranes consisted of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) bilayers, representative of phospholipid classes located in the outer and inner monolayers of cell membranes, particularly of the human erythrocyte, respectively [7,8]. The capacity of ASA and SA to perturb the bilayer structures of DMPC and DMPE was evaluated by X-ray diffraction and differential scanning calorimetry (DSC), DMPC large unilamellar vesicles (LUV) and isolated unsealed human erythrocyte membranes (IUM) were studied by fluorescence spectroscopy, and intact human erythrocytes were observed by scanning electron (SEM) and defocusing (DM) microscopy. These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other drugs, particularly of anti-inflammatories such as diclofenac [9], naproxen [10,11], ibuprofen [12], and mefenamic acid [13].

Materials and methods

X-ray diffraction studies of DMPC and DMPE multilayers

The capacity of ASA and SA to perturb the structures of DMPC and DMPE multilayers was evaluated by X-ray diffraction. Synthetic DMPC (lot 140PC-246, MW 677.9) and DMPE (lot 140PE-60, MW 635.9) from Avanti Polar Lipids (AL, USA), acetylsalicylic acid and salicylic acid (99.5%, BP, Ph. Eur, USP) from Merck, Germany were used without further purification. 2 mg of each phospholipid were introduced into Eppendorf tubes which were then filled with 150 μl of (a) ASA aqueous solutions in a range of concentrations (0.02–0.5 mM) for DMPC experiments, (b) 0.1–5 mM for DMPE experiments, (c) 0.1–1.0 mM SA for DMPC experiments, and (d) 0.5–10 mM SA for DMPE experiments. The specimens were shaken, incubated for 30 min at 30 °C and 60 °C with DMPC and DMPE, respectively and centrifuged for 10 min at 2500 rpm. Samples were then transferred to 1.8 mm diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) and X-ray diffracted utilizing Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray system. Specimen-to-detector 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 °C ± 1 °C, which is below the main phase transition temperature of both DMPC (23.3 °C) and DMPE (50.0 °C). Higher temperatures would have induced transitions onto more fluid phases making the detection of structural changes harder. Each experiment was performed in triplicate and in case of doubts, additional experiments were carried out.

Differential scanning calorimetry (DSC) studies of phospholipid model systems

Appropriate amounts of lipids (DMPC or DMPE) dissolved in chloroform were gently evaporated to dryness under a stream of gaseous nitrogen until a thin film on the wall of the glass test tube was formed. To remove the remnants of moisture, the samples were subsequently exposed to vacuum for 1 h and then dry lipid films were suspended in buffer (1 mM EDTA/10 mM Hepes/50 mM KCl, pH 7.0). ASA or SA was added in the concentration range of 0.02–5.0 mM. The multilamellar liposomes (MLV) were prepared by vortexing the samples at the temperature above gel-to-liquid crystalline phase transition of the pure lipid (about 30 °C for DMPC and 60 °C for DMPE). DSC experiments were performed using a NANO DSC Series III System with Platinum Capillary Cell (TA Instruments). The calorimeter was equipped with the original data acquisition and analysis software. In order to avoid bubble formation during heating mode the samples were degassed prior to being loaded by pulling a vacuum of 0.3–0.5 atm on the solution for a period of 10–15 min. Then the sample cell was filled with about 400 μl of MLV suspension and an equal volume of buffer was used as a reference. The cells were sealed and thermally equilibrated for about 10 min below starting temperature of the run. All measurements were made on samples under 3-bar pressure. Data were collected in the range of 5–40 °C (DMPC) and 30–70 °C (DMPE) at the scan rate 1 °C min^{-1} both for heating and cooling. Scans of buffer as a sample and a reference were also performed to collect the apparatus baseline. For the check of the reproducibility each sample was prepared and recorded at least three times. Each data set was analysed for thermodynamic parameters with the software package supplied by TA Instruments.

Fluorescence measurements of large unilamellar vesicles (LUV) and of isolated unsealed human erythrocyte membranes (IUM)

The influence of ASA and SA on the physical properties of DMPC LUV and 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. Their steady-state fluorescence anisotropy measurements were used to investigate the structural properties of DMPC LUV and IUM as it provides a measure of the rotational diffusion of the fluorophor, restricted within a certain region such as a cone due to the lipid acyl chain packing order. Laurdan, an amphiphilic probe, has high excitation sensitivity and emission spectra to the physical state of membranes. With the fluorescent moiety within a shallow position in the bilayer, laurdan provides information about the polarity and/or molecular dynamics at the level of the phospholipid glycerol backbone. The quantification of the laurdan fluorescence spectral shift was effected by means of the general polarization (GP) concept [14]. DMPC 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. Erythrocytes were separated from heparinized venous blood samples obtained from normal casual donors by centrifugation and washing procedures. IUM were prepared by lysis, according to Dodge et al. [15]. DPH and laurdan were incorporated into LUV and IUM by addition of 2 μl/ml aliquots of 0.5 mM solutions of the probe in dimethylformamide and ethanol, respectively, in order to obtain final analytical concentrations of 1×10^{-3} mM, and incubated them at 37 °C for 45 min. Fluorescence spectra and anisotropy measurements were performed in a phase shift and modulation K_2 steady-state and time resolved spectrofluorometer (ISS, Inc., Champaign, IL, USA) interfaced to computer. Software from ISS was used for both data collection and analysis. LUV suspensions measurements were carried out at 18 °C and 37 °C, and IUM measurements were made at 37 °C using 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external bath circu-