Biophysical Chemistry 142 (2009) 27–33

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Effect of acetylsalicylic acid on the current–voltage characteristics of planar lipid membranes

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 27 February 2009
Accepted 1 March 2009
Available online 12 March 2009

Keywords:
Acetylsalicylic acid (ASA)
Planar lipid membranes
Cholesterol
Transmembrane transport
Passive diffusion

ABSTRACT

Monitoring of influence of acetylsalicylic acid (ASA) on lipid bilayer conductance may contribute to better understanding of molecular mechanisms underlying passage of ASA into cells. This paper presents effects of increasing sweeping potential on stability of egg yolk phosphatidylcholine planar bilayer lipid membranes (BLM) without or with cholesterol incubated in the presence of ASA. We demonstrated that current flow through bilayer membranes generated fluctuating pores in their structure. Presence of cholesterol in the membrane caused an increase in the value of the breakdown potential, thus confirming that cholesterol had a stabilizing effect on BLM. Otherwise, ASA significantly reduced these values regardless of cholesterol concentration. Overall, by destabilizing the lipid bilayer, ASA contributed to the formation of metastable single pores, which facilitated ASA diffusion through a bilayer. Our data point out that ASA transport across the lipid bilayer takes place predominantly via the process of passive diffusion. In conclusion, the effects of ASA on lipid bilayer stability may contribute to drug transport through membrane lipid bilayers.

1. Introduction

Acetylsalicylic acid (ASA, aspirin), the most commonly used antiplatelet drug, has been used for years for prevention of primary and secondary cardiovascular complications. It inhibits the synthesis of thromboxane A_2 in blood platelets by irreversible acetylation of cyclooxygenase, the cellular enzyme catalyzing the formation of an unstable endoperoxide intermediate prostaglandin H_2. In blood platelets, it acetylates the serine-530 residue close to the active site of cyclooxygenase (COX) [1], which is thought to constitute the molecular mechanism underlying the non-enzymatic inhibition of the cyclization of arachidonic acid [2,3]. However, to inhibit the activity of COX, ASA has to penetrate the platelet membrane lipid bilayer, getting into the cell through the mechanism yet not fully elucidated.

The optimization of a successful and effective ASA-dependent therapy has become a challenge largely due to the so-called "aspirin-resistance," to a major extent encountered in special groups of patients at risk for cerebro- and cardiovascular complications [4]. Recently, we have demonstrated an association between reduced platelet sensitivity to ASA (Aspirin®) and a higher plasma cholesterol concentration [5]. Accordingly, it has been hypothesized that elevated cholesterol causes alterations in the platelet membrane lipid profile [6], and hence, it may significantly affect platelet function by changing the dynamic properties of cell membrane [7]. However, the question of whether and how ASA molecules may be freely diffused or be transported across the membrane lipid bilayer has not been resolved so far.

In the present study, we aimed at verifying the hypothesis that ASA, due to its interaction with lipid membranes, is able to alter the current–voltage characteristics of a lipid bilayer, which may further affect the incidence of the pore formation, and consequently facilitate the transmembrane passage of the drug. The possible effect of bilayer lipid composition on the rate of such diffusion has also been studied. In order to resolve the question, we monitored (a) the current–voltage characteristics as the function of lipid bilayer stability, and (b) pH changes, as due to the penetration of weak organic acid (acetylsalicylate) through the bilayer of planar lipid membranes composed of phosphatidylcholine and containing the increasing fractions of cholesterol.

2. Materials and methods

2.1. Chemicals

L-α-phosphatidylcholine (*from egg yolk*) and 3-2*sn*-phosphatidylethanolamine (*from bovine brain*) were purchased from Fluka GmbH (Buchs, Switzerland). All other chemicals, including cholesterol,

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doi:10.1016/j.bpc.2009.03.003

acetylsalicylic acid (ASA), and salicylic acid (SA), were from Sigma (St. Louis, MO, USA). Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, IA, USA).

2.2. Formation of planar lipid membranes

Planar lipid membranes (black lipid membranes, BLMs) were formed at 22 °C in a Teflon chamber with two compartments (*cis* and *trans*), separated by a diaphragm with the orifice for bilayer formation (1.5 mm in diameter), using the technique previously described by Mueller et al. [8]. Briefly, the bilayer was obtained by bubbling the lipid solution toward the orifice, and the potential across was maintained at −60 mV. Applying very short impulses of a given voltage accelerated the formation of the membranes.

The lipid bilayers used as model membranes in this study were composed of lipids naturally present in biomembranes: phosphatidylcholine (100% phosphatidylcholine considered as a control) and cholesterol, mixed at varying proportions (90–70/10–30%), with small portions (up to 5%) of phosphatidylethanolamine added to some samples in order to verify its effect on bilayer stability, formation time, and lifespan. Firstly, we prepared a chloroform mixture of egg yolk phosphatidylcholine with 0, 10, 20, or 30 mol% cholesterol, evaporated off chloroform under nitrogen, and the lipid film (total lipids 1 mg) was dissolved in nonane (C_9H_{20}) to give the solution of 25 mg/mL. Both compartments of the Teflon chamber were filled with electrolyte (PBS, pH 7.4), and lipid bilayers were formed on the orifice of the chamber's diaphragm by applying a drop of previously prepared lipid solution. Membrane formation was monitored using a video camera and by recording an increased capacitance between compartment electrodes. The *V–A* characteristics were monitored during 30 min following BLM stabilization at RT. The values of the 'breakdown voltage' (the moment of BLM rupture) and the 'pre-rupture voltage' (the moment of formation of metastable single pores, *msp*), as well as the time interval between the application of a given voltage and the onset of the rupture, were evaluated based on current–voltage characteristics using the relevant plots [9,10] (Fig. 1).

2.3. System for the study of current–voltage characteristics of planar lipid membranes

A customized multifunctional BLM system, in which two chambers of a Teflon cell, each containing a platinized electrode, are separated by a septum with the orifice of 1.5 mm, was used to study the current–voltage (*V–A*) characteristics up to the picoampere level, as well as to monitor the kinetics of electric conductivity of planar BLMs [9,10]. The device allows measuring of the penetration of acid/alkaline ions (like SA or ASA) through a lipid bilayer, which is stabilized by the constant electric field of −30 mV/−20 mV. Since it is possible to modulate both voltage and current (amperage), the device also ensures the possibility of analyzing the resistance of the lipid bilayer, which appears linear as long as the bilayer remains intact, while the formation of holes and pores in the bilayer results in the appearance of non-linear regions. In addition, the fluctuations in lipid composition of a bilayer may affect *V–A* characteristics, i.e., the formation of holes or pores is strongly influenced by varying cholesterol concentration [9,10]. The formation of the BLM over the orifice was monitored on the plot of the current versus a sweeping membrane potential, as well as by means of a microvideo system, which enabled observation of the membrane formation in a real-time mode on a PC monitor and saving of the video files. For each series of measurements, the calibration curve has been recorded, which further enabled estimation of the bilayer resistance, R_m.

The data analysis of all the acquired signals — current–voltage characteristics, pH and video signals — was performed by means of the in-house software [9,10]. In the used BLM system, there were two principal components of current flowing through the lipid bilayer: the charging current, I_C, and the ohmic current, I_R. These can be determined as follows:

$$I_C = \frac{dU}{dt} = C_m A \tag{1}$$

$$I_R = \frac{U}{R_m} \tag{2}$$

$$I = I_R + I_C = \frac{U}{R_m} + C_m A_t \tag{3}$$

where C_m and R_m represent, respectively, membrane capacitance and membrane resistance, A_t is the scan rate in mV s^{-1}, and the capacitance charging current, I_c, is constant [11–13]. As shown in Eq. (3), increasing the scanning voltage results in an increased current through the resistor. When scan rate A_t is constant, with fixed values of C_m and R_m, the current I is linearly related to the sweeping potential U. Hence, the slope reflects R_m, while C_m may be determined by measuring of the $I = f(U)$ response [11–13].

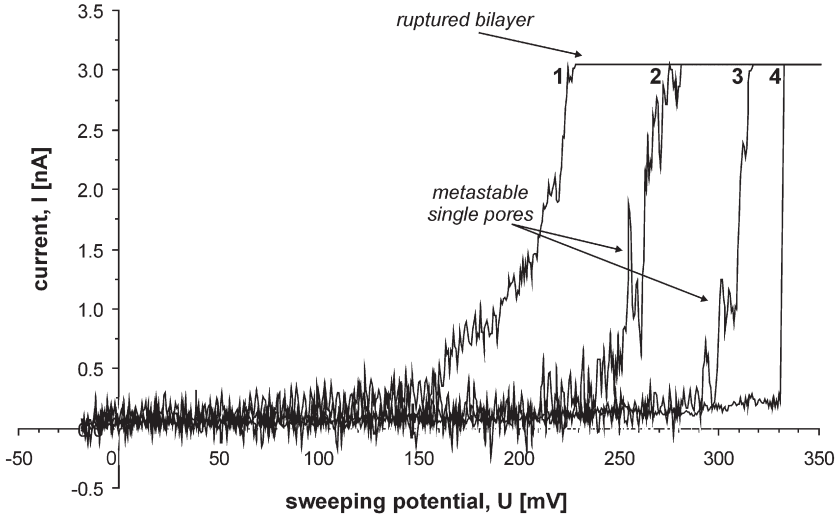

Fig. 1. Current–voltage characteristics of planar lipid membranes with varying lipid composition. The dependence of current (I) on the sweeping potential (U) for BLMs composed of egg yolk phosphatidylcholine (PCh) and the increasing portions of cholesterol (CH): (1) 100% PCh; (2) 90% PCh, 10% CH; (3) 80% PCh, 20% CH; (4) 70% PCh, 30% CH, in PBS, pH 7.4, 20 °C. The formation of metastable single pores ('spikes') and bilayer rupture shown by arrows. The range of the sweeping potential was from −60 mV to 650 mV. The recording time was 20 s. For experimental details, see Sections 2.2 and 2.3.

Using this device, we also monitored the transport of ASA and SA through planar membranes with varying lipid composition. The passage of weak acids (ASA, SA) results in the local pH alterations in the source/target chamber and unequivocally changes the value of the 'breakdown voltage' and the 'pre-rupture voltage'.

2.4. Monitoring of the ASA/SA transmembrane diffusion using planar lipid membranes

The above-described BLM system was used to monitor the transmembrane diffusion of ASA (and/or SA) and to measure the effect of cholesterol content on ASA/SA passage. Following the 30 min stabilization of the formed BLMs formed over the orifice in a chamber filled with PBS, ASA solution (ethanol stock, final concentration 1 mmol/l) or pure electrolyte (blank) was added respectively to *cis* or *trans* compartments. The diffusion was allowed to proceed at room temperature with a potential held at −20 mV for up to 15 min. The collected samples of buffer taken off each compartment were immediately frozen at −20 °C, within 2–3 h deeply frozen at −70 °C, and thus stored until analyzed.

2.5. HPLC analysis of ASA and SA concentrations

As far as we considered the possible hydrolysis of ASA in either compartment in the course of the experiment, our analysis comprises the determinations of both ASA and SA in aliquots of buffer from either compartment of a Teflon chamber. The analyses of ASA and SA concentrations in the collected samples were performed with HPLC technique, using a Waters 590 HPLC pump, equipped with the Rheodyne type injector with a 20 µl sample loop, the Waters 2487 UV–Vis detector (240 nm), and the Column Thermostat Jetstream II Plus. Chromatographic measurements were performed with the column Luna 5 µ C18 (2) 250×4.6 mm of Phenomenex (Torrance, CA, USA) using a mobile isocratic phase of water/methanol 30/70 v/v acidified to pH 2.5 with phosphoric acid. The flow rate was set at 0.5 ml/min and the column temperature to 25 °C.

ASA and SA stock solutions (1 mol/l) were prepared in 96% ethanol freshly on the day of analysis, and diluted with PBS, pH 7.4, to the required concentrations. The concentration of ethanol was maintained the same in all samples regardless of the actual solute concentration. Prior to the analysis, the aliquots of ASA or SA stock solutions were diluted with a saline, vortexed vigorously, and transferred into test tubes to perform HPLC analysis. The working solutions of ASA and SA for calibration curves were prepared within the concentration range of 1–100 µmol/l. The analysis was performed in the samples containing both ASA and SA mixed at a molar ratio of 0.005–200. All measurements were performed within the range of linear dependence between ASA or SA concentration and HPLC detection (see Section 3.2). The Bland–Altman repeatability coefficient was 13.8% and 12.3% for ASA and SA, respectively, and the accuracy was respectively 2.0 and 0.8% within the concentration range of 0.05–1 mol/l and 12–16% for lower concentrations (0.005–0.05 mol/l). The values recorded for the mixtures of ASA and SA corresponded very well to those of calibration standard curves (R^2>0.999, p<0.01 for both ASA and SA; Bland–Altman agreement as given by the averaged differences: 11.0 µmol/l and 7.2 µmol/l for ASA and SA, respectively).

2.6. Statistical analysis

The results are expressed as arithmetic mean ± standard error of mean (SEM) or median (*Me*) and interquartile range (IQR: from lower quartile, Q1, to upper quartile, Q3). The normality of data distribution was verified with Shapiro–Wilk's test. Two inference tests were used depending on data normality. Data that showed right-skewed distribution were log-transformed prior to further analysis. The differences were determined with either the Student *t* test or various models of one-way and two-way ANOVA on either raw or transformed data. The Mann–Whitney *U* test was used for nonparametric data. In all two-sample inference tests, we employed the Bonferroni correction for multiple testing. The analysis of outliers was performed based on the residual analysis and the estimates of Cook's distances and 2.5 sigma deviation. Pearson's linear correlations were used to assess simple associations. Multiple non-linear regression was employed for the analysis of current–voltage characteristics [14,15]. We used the Rosenbrock's and quasi-Newton's estimation method for resolving of the segment regression equation of $F(x)=a_0+a_1*x+a_2*(x-p)$ for the condition when $(U>p)$. The equation describes the relationship between the sweeping potential $(x=U)$ and the current $(F(x)=I)$, where p means the cutoff point. The used loss function was given by the least squares method and the case selection was for $F(x)=I<3.046$ nA. The value of the potential on a curve relevant to the cutoff point was interpreted as the potential, at which the formation of *msp* would start. The potential, for which the highest value (boundary) of a current was recorded, was regarded as the breakdown (rupture) potential.

3. Results and discussion

3.1. Effects of lipid composition and acetylsalicylic acid on lipid bilayer stability

The planar lipid membranes had a rather short formation time (below 2 min), showed the formation of metastable single pores for pre-rupture voltages at +258 (+218; +282) mV, and ruptured at the voltage of +293 (+248; +314) mV, strongly depending on their lipid composition (cholesterol concentration). The resistance (R_m) of intact BLMs composed of merely phosphatidylcholine was 17.4 (14.7; 20.7)*10^9 Ω cm^{-2}. The addition of cholesterol up to the concentration of 20 mol% stabilized BLMs, which corresponded to increased R_m, whereas further enrichment of the membranes with cholesterol (over 25 mol%) resulted in the reduced R_m values. In the presence of ASA, R_m became increased; the significant rise, however, was noted only for the membranes containing 20 mol% cholesterol (Fig. 2). In addition, the incidence of the formation of metastable single pores, observed in 44% of cases (n=34), was dependent on lipid composition and modulated by ASA. The destabilizing effect of ASA was the strongest in membranes

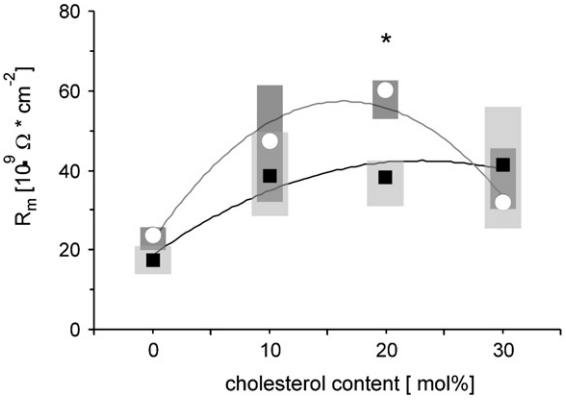

Fig. 2. Effect of ASA on the resistance of planar lipid membranes at various cholesterol concentrations. Data presented as median (symbols) and interquartile range (boxes) (n=6–8) for planar lipid membranes composed of phosphatidylcholine (70–100 mol%) and cholesterol (0–30 mol%) in the absence (solid squares, light grey boxes) and presence of ASA (open circles, dark grey boxes). R_m values calculated using the slopes of calibration curves and the BLM orifice diameter of 1.5 mm. The range of the sweeping potential was from −60 mV to 650 mV. For further experimental details, see Sections 2.2 and 2.3. Significance of differences between varying cholesterol contents were estimated with the Kruskal–Wallis test and the all pairwise multiple comparisons Conover–Inman test: No ASA: $R_{m\ chol\ 0}$ *vs.* $R_{m\ chol\ 10\ mol\%}$, p<0.05. ASA: $R_{m\ chol\ 0}$ *vs.* $R_{m\ chol\ 10\ mol\%}$, p<0.035; $R_{m\ chol\ 0}$ *vs.* $R_{m\ chol\ 20\ mol\%}$, p<0.003. The significant difference between samples incubated in the absence or presence of ASA, estimated with the use of Mann–Whitney *U* test, is marked with the asterisk, p<0.03.

composed of pure phosphatidylcholine, where the incidence of pore formation raised to almost 100% (Fisher's exact test $p<0.04$). The enrichment of phosphatidylcholine membranes in cholesterol stabilized the bilayer and lowered the incidence of the pores formation at lower cholesterol content. This effect vanished, however, when membranes were overloaded with cholesterol, which facilitated bilayer rupture (Fig. 3). Considering the incidence of the formation of metastable single pores, the enrichment of BLMs in cholesterol resulted in overall stabilizing of the lipid bilayer, which was particularly distinct for ASA (*NS* in the absence and $p<0.035$ in the presence of ASA by chi^2 test of independence; Kendall's $\tau_\beta=0.251$, $p=0.063$, in the absence, and $\tau_\beta=0.393$, $p<0.002$, in the presence of ASA).

The cholesterol content prevented lipid membranes against rupture up to 20 mol%: both the pre-rupture (formation of metastable single pores) and rupture (breakdown) voltage was significantly higher for BLMs with 10 mol% and 20 mol% compared to pure PC membranes. This effect became reverted above the cholesterol content of 20 mol% concentration (Figs. 4 and 5). The presence of ASA resulted in significant reductions in both the pre-rupture and rupture voltage at all but the highest cholesterol concentration (Figs. 4 and 5).

Membrane lipid bilayer stability seems crucial in the formation of transmembrane aqueous pores [16]. As far as this phenomenon occurs under the influence of the electric field, it is known as electroporation. During this process, membranes exhibit a sharp transition to a higher conductance, which indicates the formation of membrane pores (or defects). The phenomenon of pore formation has been extensively studied with respect to numerous biomedical applications, including loading cells with molecules for drug delivery purposes or transporting molecules into and out of cells for therapeutic purposes [17]. According to the electroporation theory, BLM is considered a system in a metastable state, containing a fluctuating population of pores [18]. In the present study, we used that system to explore the effects of ASA on the pore formation and on the current–voltage characteristics of model BLMs composed of phosphatidylcholinea and cholesterol. Our current study is one of numerous drug research studies on the planar lipid bilayer, but it is the first one for studying the interactions of ASA with BLMs and considering the possible mechanism(s) of transport of ASA across a lipid bilayer.

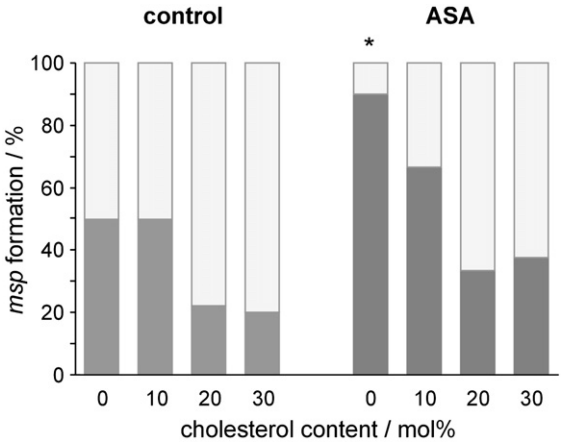

Fig. 3. Effect of ASA on the incidence of the formation of metastable single pores (*msp*) in planar lipid membranes at various cholesterol concentrations. Data presented as normalized proportions of the events (the formation of *msp*, dark grey) ($n=8$–10) for planar lipid membranes composed of phosphatidylcholine (70–100 mol%) and cholesterol (0–30 mol%) in the absence (control) and presence of 1 mmol/l ASA. For experimental details, see Sections 2.2 and 2.3. Significance of differences between varying cholesterol contents were estimated with χ^2 test for trend and the exact Fisher's test: No ASA: χ^2 test for trend and inter-group comparisons: beyond significance. ASA: χ^2 test for trend $p<0.01$; $r_{\text{chol 0}}$ *vs.* $r_{\text{chol 20 mol\%}}$, $p_{1\alpha}<0.02$; $r_{\text{chol 0}}$ *vs.* $r_{\text{chol 30 mol\%}}$, $p_{1\alpha}<0.03$. $^*p_{1\alpha}<0.04$, in the absence versus in the presence of ASA, by the exact Fisher's test.

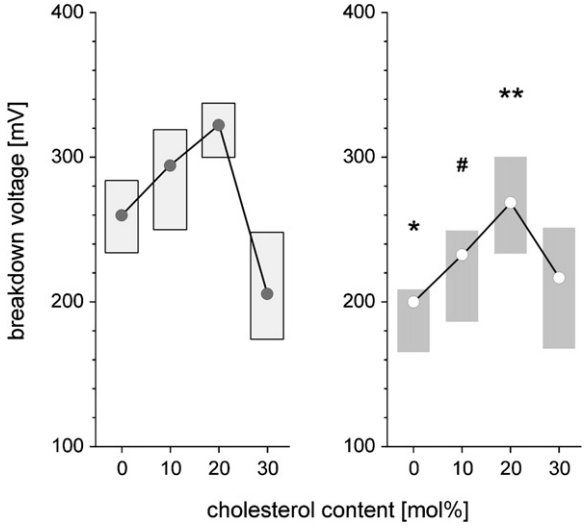

Fig. 4. Effect of ASA on the value of the sweeping potential (U) needed for rupture of planar lipid membranes ('breakdown potential') at various cholesterol concentrations. Data presented as median (circles) and interquartile range (boxes) ($n=6$–8) for planar lipid membranes composed of phosphatidylcholine (70–100 mol%) and cholesterol (0–30 mol%) in the absence (light grey) and presence of ASA (dark grey). For experimental details, see Sections 2.2 and 2.3. Significance of differences between varying cholesterol contents were estimated with the Kruskal–Wallis test and the all pairwise multiple comparisons Conover–Inman test: No ASA: $U_{\text{rupture chol 0}}$ *vs.* $U_{\text{rupture chol 10 mol\%}}$, $p<0.015$; $U_{\text{rupture chol 10 mol\%}}$ *vs.* $U_{\text{rupture chol 30 mol\%}}$, $p<0.04$; $U_{\text{rupture chol 20 mol\%}}$ *vs.* $U_{\text{rupture chol 30 mol\%}}$, $p<0.002$. ASA: $U_{\text{rupture chol 0}}$ *vs.* $U_{\text{rupture chol 10 mol\%}}$, $p<0.015$; $U_{\text{rupture chol 10 mol\%}}$ *vs.* $U_{\text{rupture chol 30 mol\%}}$, $p<0.03$; $U_{\text{rupture chol 20 mol\%}}$ *vs.* $U_{\text{rupture chol 30 mol\%}}$, $p<0.04$. The significant differences between samples incubated in the absence or presence of ASA, estimated with the use of Mann–Whitney U test, are marked with the symbols: $^*p<0.02$, $^{**}p<0.01$, $^{\#}p<0.002$.

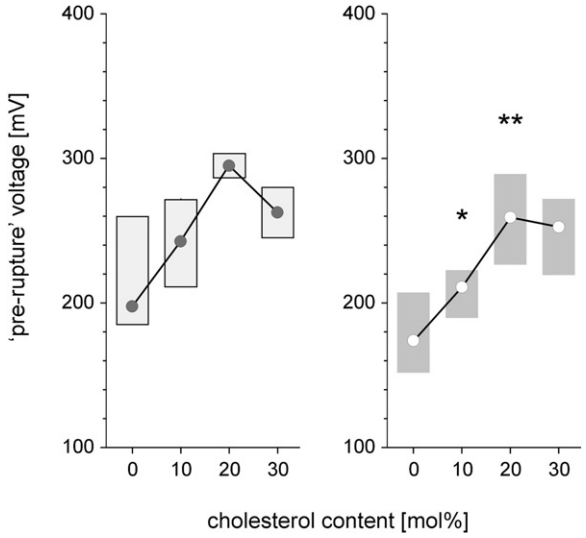

Fig. 5. Effect of ASA on the value of the sweeping potential (U) needed for the formation of metastable single pores (*msp*) in planar lipid membranes at various cholesterol concentrations Data presented as median (circles) and interquartile range (boxes) ($n=6$–8) for planar lipid membranes composed of phosphatidylcholine (70–100 mol%) and cholesterol (0–30 mol%) in the absence (light grey) and presence of ASA (dark grey). For experimental details, see Sections 2.2 and 2.3. Significance of differences between varying cholesterol contents were estimated with Kruskal–Wallis test and the all pairwise multiple comparisons Conover–Inman test: No ASA: $U_{msp\ \text{chol 0}}$ *vs.* $U_{msp\ \text{chol 20 mol\%}}$, $p<0.0001$; $U_{msp\ \text{chol 0}}$ *vs.* $U_{msp\ \text{chol 30 mol\%}}$, $p<0.015$; $U_{msp\ \text{chol 10 mol\%}}$ *vs.* $U_{msp\ \text{chol 20 mol\%}}$, $p<0.001$; $U_{msp\ \text{chol 10 mol\%}}$ *vs.* $U_{msp\ \text{chol 30 mol\%}}$, $p=0.06$. ASA: $U_{msp\ \text{chol 0}}$ *vs.* $U_{msp\ \text{chol 20 mol\%}}$, $p<0.003$; $U_{msp\ \text{chol 0}}$ *vs.* $U_{msp\ \text{chol 30 mol\%}}$, $p<0.003$; $U_{msp\ \text{chol 10 mol\%}}$ *vs.* $U_{msp\ \text{chol 20 mol\%}}$, $p<0.05$; $U_{msp\ \text{chol 10 mol\%}}$ *vs.* $U_{msp\ \text{chol 30 mol\%}}$, $p<0.05$. The significant differences between samples incubated in the absence or presence of ASA, estimated with the use of Mann–Whitney U test, are marked with the symbols: $^*p=0.054$, $^{**}p<0.01$.

The effects of cholesterol on the phosphatidylcholine BLM resistance and capacitance have been earlier extensively studied by Naumowicz et al. [11,19]. Our present results confirmed the earlier observations in showing that cholesterol stabilized and strengthened the lipid bilayer. Lipid bilayers containing cholesterol may also exhibit phase separation into cholesterol-rich (liquid-ordered) and cholesterol-poor (liquid disordered) domains with the increase of cholesterol content [11,19–24], although the molecular organization of such cholesterol-containing bilayers is still a matter of debate, as is the significance of the structures like lipid rafts and hexagonal superlattices [25–29]. With the increasing fraction of cholesterol, the BLM became more stable at the beginning, and the threshold voltage of bilayer breakdown (rupture) was initially increased. The resistance (R_m) of the lipid bilayer increased along with the increasing cholesterol content up to 20 mol%; these dependences, however, were not linear, which could be caused by the formation of some bonds in the membrane [19,22,25,27]. Within the stabilizing concentrations of membrane cholesterol, the lipid bilayer remained more resistant to pore formation than pure PCh membranes, which is in line with findings by other authors [30]. In the presence of ASA, the value of membrane resistance was additionally raised compared to control membranes and such a tendency appeared significant within the stabilizing range of cholesterol concentrations, i.e., up to the cholesterol content of 20 mol%. However, while the addition of cholesterol to pure phosphatidylcholine BLMs reduced the incidence of pore formation, ASA accelerated the creation of metastable single pores and facilitated membrane rupture. Under conditions of our experiment, the BLMs composed of pure phosphatidylcholine are in the gel state, and their enrichment in cholesterol has a stabilizing effect due to clustering of the sterol within the phosphatidylcholine lipid core [11–13,18,21,23,24,30–34]. ASA is known to spontaneously decompose (hydrolyze) into acetate and SA, although this process is relatively slow and takes 5–6 h [35]. Xiang and Anderson [36] studied the permeability of acetic acid through the dipalmitoylphosphatidylcholine/cholesterol, dihexadecylphosphatidylcholine/cholesterol, or dimyristoylphosphatidylcholine/cholesterol vesicles using an NMR and found that the addition of cholesterol significantly reduced permeability of vesicles to acetic acid in both ordered and disordered liquid crystalline phases. The similar effect was observed for SA. McLaughlin [37] showed that SA adsorbs on neutral phosphatidylcholine or phosphatidylserine bilayers at pH 7, producing negative surface potentials. Song et al. [38] studied the salicylate effects on the dipalmitoylphosphatidylcholine bilayer by molecular dynamics. They found that SA significantly decreased the area per head group of lipid molecules and increased the tail order of lipid bilayer. The most important is that salicylate significantly increased the electrostatic potential at the water–lipid interface and significantly affected dipole moment fluctuations of the bilayer molecules [38]. All these findings are in support of our present findings, as they can explain both the increased resistance of the bilayer upon addition of ASA and the increased formation of holes in the lipid matrix. The significant increase of dipole motions in alternating current can produce the microperturbations of the bilayer, and the penetration of SA molecules in such micropores can increase the probability of formation of metastable single pores. Such interactions are likely to change the conductivity of the unmodified lipid bilayers under conditions of moderate electric fields, as driven by the formation of the voltage-induced metastable single pores, with lifetimes of about 3 ms [18,32] and a theoretical pore radius of 0.6 nm [31], as described by the integrated and modified Nernst–Planck equation [31,32]. At pre-rupture (pre-breakdown) voltage, these metastable single pores may become stabilized and form great pores (single ion channels) [32], thus providing a potential possibility of perpetuated diffusion of acetylsalicylic acid through the bilayer. Importantly, these effects can be pronounced in the case of ASA. Gutknecht [39] studied the passive transport of SA and ASA through dipalmitoylphosphatidylcholine lipid bilayers and found that the alterations caused by salicylic and acetylsalicylic acids differed significantly: the conductance of the lipid bilayer caused by ASA was at 50-fold less than that caused by SA. The author interpreted these results by showing the differences in the structures of SA and ASA. In salicylate, the location of hydroxyl adjacent to the carboxyl group permits the formation of an internal hydrogen bond [40] that delocalizes the negative charge. In ASA, the internal hydrogen bond is absent [39] and, hence, such a charge displacement is not likely to occur.

At relatively large ASA concentrations, the agent may also considerably alter the equilibrium between LOH^- and LH^+ forms of PCh formed upon the interaction of ASA with the bilayer [41]. Thus, ASA is likely to affect the delicate balance of forces presented between the polar heads of phospholipids, which include electrostatic repulsive and attractive interactions. It has been suggested that the stronger lateral repulsion between molecules of some lipids may contribute to pore formation [42]. This implies that, whereas cholesterol reduces the repulsive interactions between phospholipid polar heads, ASA may act opposite, facilitating the interactions between the positive charge of the PCh head and the negative charge of the adjacent PCh molecule, which finally results in easier formation of pores in the bilayer [43].

The above-discussed data can be also supporting for our outcomes on ASA transmembrane diffusion.

3.2. Effects of lipid bilayer cholesterol on ASA transmembrane diffusion

In order to validate the above reasoning, we monitored the movements of ASA through BLMs built up of pure phosphatidylcholine and cholesterol. The aliquots of buffer taken from both compartments contained both acetylsalicylic as well as small proportions of salicylic acid, due to a hydrolysis. Therefore, the rate of ASA transport through the membrane lipid bilayer was calculated taking into account a possible slow spontaneous hydrolysis of ASA. The transport of ASA was negligibly slow: while its initial concentration in the source compartment was 1 mmol/l, the concentration of ASA in the target compartment following the 15 min diffusion did not exceed 9 μmol/l, which means that merely up to one percent of the initial amount of ASA in the source compartment migrated through a bilayer. Our present findings also show that the transport of ASA was dependent on the cholesterol/phosphatidylcholine ratio in a bilayer: while the fastest at lower cholesterol content (10 mol%), it fell down to below 0.1% of initial value from 20 mol% (Fig. 6).

Overall, although we revealed that the transmembrane passage of ASA remained in a reciprocal relation to the content of cholesterol in

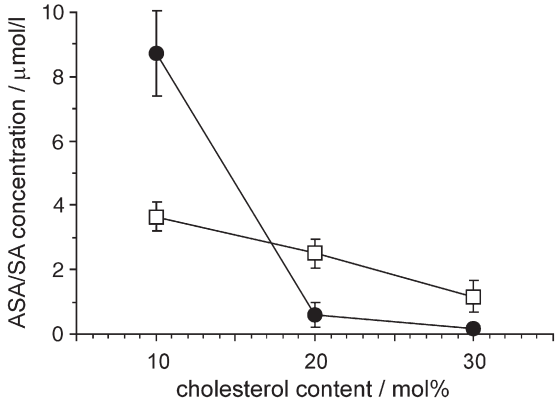

Fig. 6. Effect of cholesterol content on acetylsalicylic and salicylic acid transport through planar lipid membranes. Data presented as mean ± SEM ($n = 4–12$) represent the concentrations of acetylsalicylic (solid circles) and salicylic acid (open squares) in the target compartment (B) of the chamber in the course of passive diffusion through planar lipid membranes composed of phosphatidylcholine (70–100 mol%) and cholesterol (0–30 mol%). Significance of differences, estimated with the Kruskal–Wallis test and the all pairwise multiple comparisons Conover–Inman test for independent measurements, was: ASA: $p < 0.0001$ $\mu_{10} \neq \mu_{20} = \mu_{30}$; salicylic acid: $p < 0.001$ $\mu_{10} \neq \mu_{20} \neq \mu_{30}$. For experimental details, see Sections 2.4 and 2.5.

BLMs, i.e., the higher content of BLM cholesterol, the lower rate of ASA movement through BLM, the absolute values of the rate, at which ASA penetrated BLM, was extremely slow. Based on our present data and the literature reports [36–38,44] data, we can conclude that the transport takes place via the process of passive diffusion. The small amounts of ASA penetrating the membrane may be also interpreted in view of the arguments on the ASA-mediated changes in a conductance of lipid membranes, presented above. McLaughlin and Dilger [44] have investigated the transport of weak acids through the bilayer and showed that this transport is a passive one, including the transport of both protons (H^+) and anions (HA_2^-). According to their report, the H^+ transport is significant at pH close to pK_A of a weak acid. At neutral pH, far from pK_A, the transport is very slow and is determined by anionic dimers HA_2^-. The dimers HA_2^- enter the bilayer, penetrate it, being transported from the *cis* to *trans* side of bilayer due to flip-flop motions, and finally leave the bilayer. These outcomes are in a good agreement with our data. pK_A of ASA is 3.5 and we observed a very slow passive transport of ASA through a bilayer. Of course, the additional diffusion though metastable single pores may also take place but is not determining/dominating in the course of ASA diffusion. Worth mentioning is that the action of ASA anions, which are considered as structure-breakers for the lipid bilayer, may be also related to their chaotropic/kosmotropic features toward the biological membranes [45–48].

These observations are in line with our earlier findings showing that high cholesterol is an important factor determining a reduced response of blood platelets to ASA and its differentiated acetylating potential toward platelet proteins. It still remains a matter of debate what may be potential mechanisms of such association and whether they concern the transport of ASA through the cell membrane lipid bilayer. However, the altered membrane lipid status, and particularly enhanced content of platelet membrane cholesterol, appear to retard ASA penetration across platelet membranes and to lower ASA potency to acetylate its intraplatelet target, COX-1 [6]. It seems also consistent with the outcomes of numerous studies on natural and model membranes. The enrichment of membranes with cholesterol favors the interactions of cholesterol with membrane phospholipids, which may result in the increased membrane cohesion, consequently leading to reduced passive permeability of the membrane lipid bilayer to small molecules [33,36,49,50].

Our results, showing the slow transport of ASA through the phosphatidylcholine/ cholesterol bilayer, point out to the possible existence of an active transporter for ASA in blood platelets. Such proton/monocarboxyl hydrate co-transporter (MCT1), which recognizes and transports some monocarboxylic acids, was found in the small intestine [51,52]. As far as blood platelets have a greater number of various transporters on their surface plasma membranes, it seems quite likely that one of them may act as a co-transporter for ASA.

Acknowledgements

The research was supported by the Polish State Committee for Scientific Research grants PBZ-KBN-101/T09/2003/9 and 2P05A14529, Medical University of Lodz fund 502-16-240, and NATO project CBP.NUKR.CLG 981884. The abovementioned sponsors had no role(s) in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

We appreciate the technical assistance from Pawel Ziemba, M.Sc.

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