Interaction of ibuprofen with eukaryotic membrane lipids

Henning Lygre, Grete Moe and Holm Holmsen

Department of Odontology—Dental Pharmacology, Department of Biochemistry and Molecular Biology, University of Bergen, Bergen, Norway

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With the versatility of the molecular mechanism of amphiphilic drugs there is the possibility that ibuprofen could interact with eukaryotic model membrane lipids. Using the Langmuir technique, we first determined the pressure/molecular area isotherms of glycerophospholipids monolayers at 37°C, and, second, using differential scanning calorimetry (DSC), phase transition parameters in liposomes of the same lipids. Ibuprofen interacted in a concentration-independent manner with monolayers of saturated phosphatidylcholines (PC, i.e. markers of the outer membrane leaflet of eukaryotic cells). Ibuprofen was found to interact with liposomes of saturated and unsaturated phosphatidylcholines and -serines (PS, i.e. markers of the inner membrane leaflet of eukaryotic cells), and saturated ethanolamines (PE, i.e. markers of the inner membrane leaflet of eukaryotic cells). A lowering of the lipid melting temperature (T_m) and a change of enthalpy (ΔH) of the gel to liquid-crystalline phase transitions of liposomes were detected. □ *Calorimetry; cell membrane; ibuprofen; lipids*

Henning Lygre, Department of Odontology—Dental Pharmacology, University of Bergen, Armauer Hansens Hus, NO-5021, Bergen, Norway. Tel. +47 55 97 46 74, fax. +47 55 97 46 05, e-mail. henning.lygre@odont.uib.no

One of the basic tenets of pharmacology is that drug molecules have to exert a chemical influence on one or more constituents of cells in order to produce pharmacological responses. Drug molecules must get so close to cellular molecules that their function is altered. Most drugs produce their effects by binding, in the first instance, to protein molecules. However, it is believed that carbohydrates, lipids, and other macromolecules can be targets for drugs (1). The interaction of drugs with biological membranes is of major importance for their pharmacokinetic and pharmacodynamic properties.

Non-steroidal anti-inflammatory drugs (NSAIDs) possess anti-inflammatory, analgesic, and antipyretic activities. It has been demonstrated that the molecular basis for NSAIDs is through inhibition of prostaglandin H synthase (cyclooxygenase: PGHS) activity, causing interdiction of the production of pro-inflammatory prostaglandins (2). However, recent reports indicate that NSAIDs may activate and bind to peroxisome proliferator-activated receptor (PPAR) α and γ isoforms (3, 4). Ibuprofen is a NSAID which is a colorless, amphiphilic, crystalline stable solid with molecular weight 206.3 (Fig. 1), and relatively insoluble in water, but readily soluble in most organic solvents. The thermogram of pure ibuprofen shows a single endothermic peak at 71.7 ± 0.1°C, due to the melting of the drug (5). The amphiphilic compound ibuprofen is a frequently used analgesic in dentistry (6).

It has previously been demonstrated that amphiphilic drugs partition into cellular membranes to different extents (7, 8), thus changing the organization of the membrane. Differential scanning calorimetry (DSC) studies have revealed that amphiphilic drugs may decrease the main transition temperature and increase the transition interval of the lipid bilayer (9), indicating a lowering of the phase transition's cooperativity of the membrane. Anesthetics have a similar mechanism of action via a change in the lateral pressure profile in membranes, where they induce an increase in the lateral pressure near the interface. Accordingly, anesthetics influence the dynamic ordering processes of the membrane by accumulating at the interfacial regions of coexisting domains of gel and fluid lipids (10). The composition of the phospholipids in cellular membranes has also been shown to be an important factor for the interdigitation of the amphiphilic drug chlorpromazine with the acyl chains of lipids (11). Hence, the molecular mechanism of amphiphilic drugs appears to be versatile.

Biological membranes are complex, but well-organized, multi-molecular assemblies composed of a lipid bilayer and a variety of proteins. Eukaryotic cells have numerous membrane systems, the best characterized being the endoplasmatic reticulum, Golgi membranes, plasma, mitochondrial, lysosomal, and nuclear membranes (12). The major classes of lipids in biological membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin, diphosphatidylglycerol (DPG) and phosphatidylinositol (PI). In eukaryotic membranes the glycerol-based phospholipids predominate, particularly PC, PE, PS, and PI. Glycerolipids make up the essential milieu of cellular

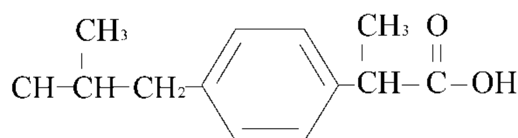

Fig. 1. Structure formula of ibuprofen.

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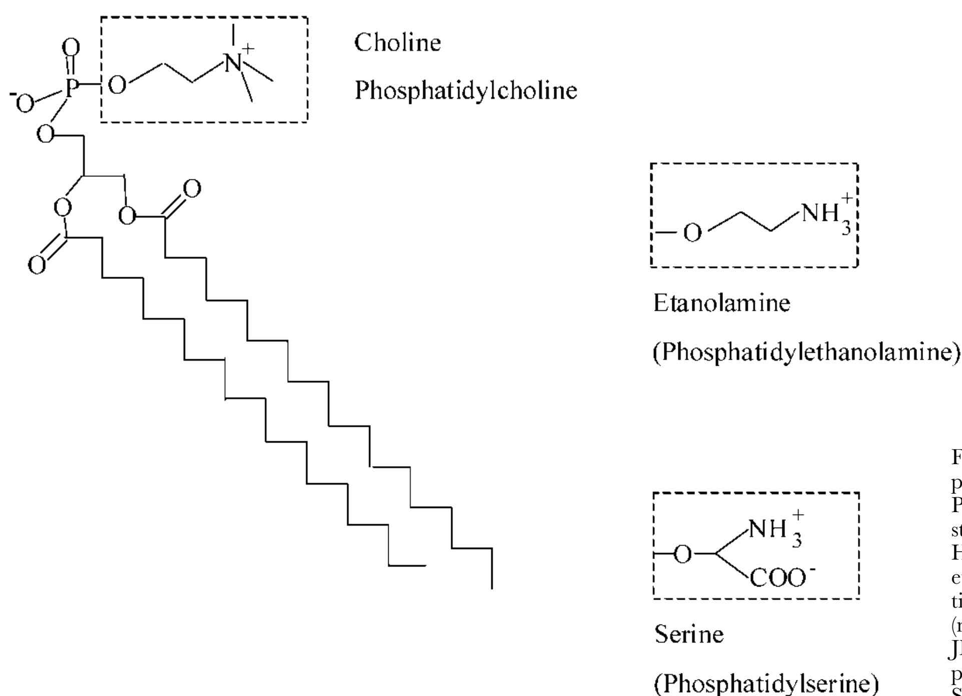

Fig. 2. The structure of the phospholipid molecule dipalmitoyl-PC (DPPC) in the liquid crystalline state represented schematically. Head groups for phosphatidylethanolamine (DPPE) and phosphatidylserine (DPPS) are also shown (modified from Vance DE, Vance JE. Biochemistry of lipids, lipoproteins and membranes. Elsevier Science B.V.; 1996. p. 4).

membranes, and act as a barrier for the entry of compounds into cells (12). The most abundant constituents of cellular membranes are proteins and glycerophospholipids.

A phospholipid molecule consists of a polar head group and two non-polar, hydrophobic fatty acyl chains (Fig. 2). In eukaryotic plasma membranes the glycerol-based phospholipids are predominant and unevenly distributed between the outer and inner leaflets, including phosphatidylchloline (PC, outer), phosphatidylethanolamine (PE, inner), phosphatidylserine (PS, inner), phosphatidylinositol (PI), and cardiolipin. In glycerophospholipids there is usually a saturated acid esterified at the *sn*-1 position of the glycerol backbone and an unsaturated fatty acid at the *sn*-2 position. The fluidity of membranes depends on the nature of the acyl chain region comprising the hydrophobic domain of most membrane lipids. Most lipid species in isolation can undergo a transition from a viscous gel (frozen) state to the fluid (melted) as the temperature is increased. The local fluidity, as dictated by the gel or liquid-crystalline nature of membrane lipids, may regulate membrane-mediated processes. Transitions between the gel and liquid-crystalline phases can be monitored by a variety of techniques, including nuclear magnetic resonance (NMR), electron spin resonance, and fluorescence. DSC, which measures the heat absorbed (or released) by a sample as it undergoes an endothermic (or exothermic) phase transition, is particularly useful.

With the aim of studying interactions between a given effector and cell membranes, several approaches are possible, i.e. living cells or cellular membranes. Natural membranes are complex entities with a great variety of lipids and proteins. If one is interested in specific aspects of a given biological phenomenon occurring at membrane level, the best choice is to use membrane models (13). A lipid monolayer of glycerophospholipids spread at the air/water interface has been shown to represent a simple model of a single membrane leaflet of cells (14).

The aim of the present study was to reveal potential molecular interactions between the amphiphilic drug ibuprofen and different kinds of glycerophospholipids found in eukaryotic cells. Model membranes made of lipids were used in two ways—as monolayers and as liposomes (i.e. concentric bilayers).

Materials and methods

Chemicals

In biological systems, fatty acids usually contain an even number of carbon atoms, i.e. between 14 and 24. The 16- and 18-carbon fatty acids are the most common.

1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine (POPS, 16:0/18:1) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 16:0/16:0) were purchased from Sigma (St. Louis, Mo., USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, 16:0/16:0) were purchased from Avanti Polar Lipids Inc. (Alabaster, Ala., USA). Racemic (RS mixture) ibuprofen ($C_{13}H_{18}O_2$) [2-(4-isobutylphenyl)-propionic acid] was purchased from Sigma-Aldrich Norway AS (Oslo, Norway).

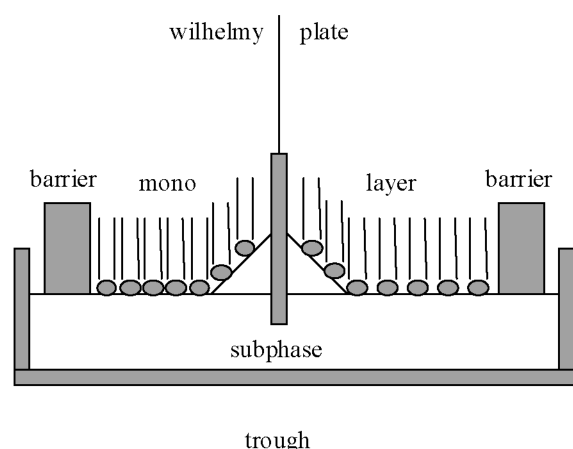

Fig. 3. Schematic representation of the device for the monolayer technique.

Monolayer technique

In 1917, Irving Langmuir (15) introduced the experimental and theoretical modern concepts on monolayers. The film state of a given substance spread on an aqueous sub-phase is characterized by the main parameters temperature T, surface pressure π, surface area and number of molecules, these last two parameters being expressed as area per molecule, A. Therefore, at constant T, an equation of state of the film has the general form:

$$\pi = \pi(A)_T$$

The surface pressure area (π-A) isotherm of a monolayer constitutes the essential characterization of the properties of the film. The trough and the barriers are often made of Teflon (Fig. 3). The whole device rests on an anti-vibration plate placed inside a thermostat box (13). Our experiments were performed using an M 1000 Minitrough (75 × 364 × 5 mm) made of Teflon (KSV Instruments, Helsinki, Finland). All experiments were performed at 37°C with two movable barriers, constant speed (5 mm/min) on a Ringer solution (0.15 M NaCl, 5.6 mM KCl, 1.7 mM CaCl, pH 7.4). The surface pressure was measured using the Wilhelmy plate detector. The lipids were dissolved in chloroform (1 mg/mL) applied in droplets on either side of the Wilhelmy plate using a Hamilton pipette, and the solvent allowed to evaporate before compression of the monolayer. Control samples of 20 μL were used. Samples containing lipids and ibuprofen were mixed 1:2 prior to application. Ringer solution was used as a sub-phase solution. Samples of 40 μL were used. All experiments were repeated five to seven times. The median value was chosen for statistics.

Liposome preparation

Liposomes were made of glycerophospholipids dissolved in chloroform. Different concentrations of ibuprofen were added, and the mixtures were lyophilized and allowed to dry overnight. Samples of dry powder were suspended in 1.5 ml Ringer solution to give a 4 mM lipid suspension, which contains multilamellar liposomes, and sonicated for 4 min. Unilamellar vesicles were obtained by freeze-thawing seven times.

Differential scanning calorimetry

DSC measures the heat absorbed (or released) by a sample as it undergoes an endothermic (or exothermic) phase transition. Three parameters of interest in such traces are (Table 1): (i) the area under the transition peak, which is proportional to the enthalpy of the transition (ΔH); (ii) the width of the transition, which gives a measure of the 'cooperativity' of the transition ($T_{c1/2}$); and (iii) the transition temperature (T_m) itself. The enthalpy of the transition reflects the energy required to melt the acyl chains. Cooperativity reflects the number of molecules that indergo transition simultaneously.

Measurements were performed with liposomes suspended in Ringer solution with a Microcal VP-DSC differential scanning calorimeter (MicroCal LLC, Northampton, Mass., USA) with cell volumes of 0.5 mL at the indicated scan rates. Ringer solution was used as the reference. All samples were degassed and the calorimetric

Table 1. Differential scanning calorimetry values of transition temperature (T_m), half-width ($T_{c1/2}$), and enthalphy change (ΔH) for the liposome studied. DPPC 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. DPPE 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine. POPS 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine

Glycerophospholipid	μM Ibuprofen	Tm (°C)	Tc1/2 (°C)	ΔH (kJ/mol)
DPPC	0	41.78	0.499	12.79
	1	41.79	0.499	10.62
	25	41.85	0.503	9.23
	50	41.72	0.624	10.13
	100	41.55	0.874	10.27
DPPE	0	67.52	0.626	8.4
	1	67.46	0.627	6.34
	25	67.38	0.627	6.78
POPS	0	15.76	2.63	0.34
	1	13.53	2.26	0.38
	100	16.16	3.38	0.61

cells were kept under an excess pressure of 30 psi to prevent degassing during the scan. A scanning rate of 1.5°C/min was used for all samples. The original scans were processed by subtraction of the buffer baseline and further correction by defining a progress baseline from the pre- to the post-transition regions using the Origin TM software (MicroCal LLC) provided with the instrument.

Statistics

The median, the non-parametric measure of central tendency, was used.

Results

Monolayer studies

DPPC and POPS were used for the monolayer technique. Ibuprofen interacted with monolayers of DPPC in a concentration-independent manner, and increased the mean molecular area (MMA) of the membrane lipids significantly for concentrations up to 25 μM. At higher concentrations (50 and 100 μM) ibuprofen caused, surprisingly, a slight decrease in MMA (Fig. 4). Ibuprofen interacted with POPS in a concentration-independent manner. The molecular area of the membrane lipids was evidently increased for low concentrations of ibuprofen i.e. 1 μM (Fig. 5).

DSC studies

Ibuprofen had effects on the thermograms (Figs 6–8) affecting the transition temperatures (T_m), transition interval ($T_{c1/2}$), and the corresponding enthalpies (ΔH) (Table 1) of the phosholipid liposomes studied.

A typical thermogram of pure 16:0/16:0-PC (DPPC) is given in Fig. 6 (control) showing a main transition at 41.78°C and a pretransition at 36°C. Incorporation of

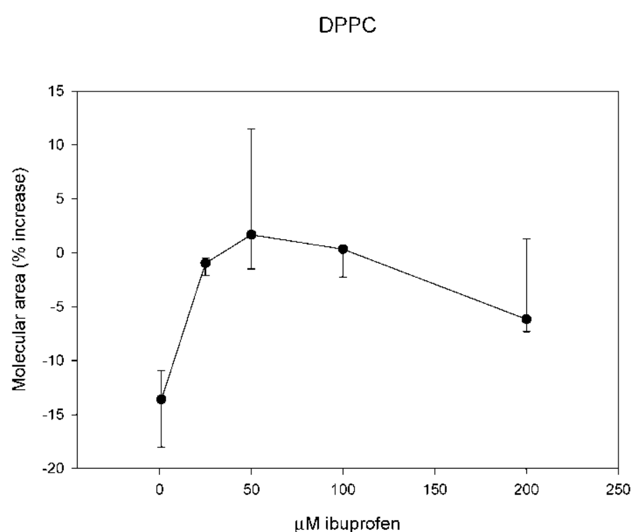

Fig. 4. The effect of ibuprofen on the DPPC monolayer. Results are presented as the difference in molecular area at a surface tension of 30 mM/m between control and added samples, and different concentrations of ibuprofen. Minimum and maximum values are indicated.

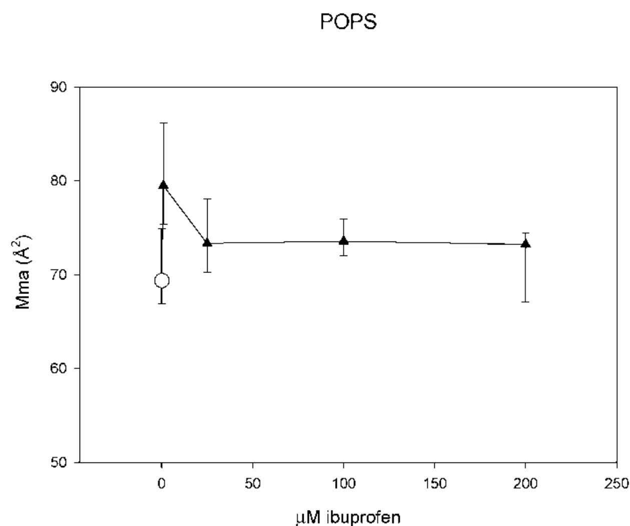

Fig. 5. The effect of ibuprofen on POPS monolayers. Results are presented as the difference in molecular area at a surface tension of 30 mM/m between control and added samples, and different concentrations of ibuprofen. Minimum and maximum values are indicated. Circle indicates control value.

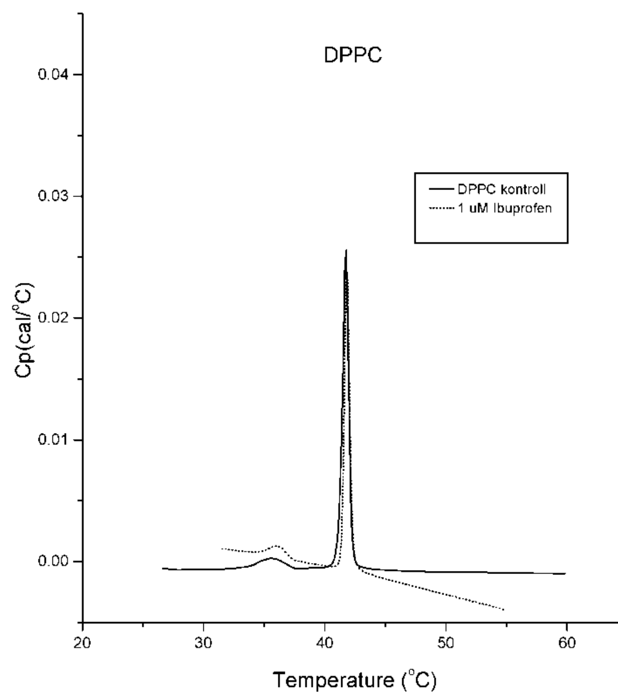

Fig. 6. Differential scanning calorimetry thermograms obtained from aqueous liposome suspensions of DPPC with added ibuprofen 1 μM.

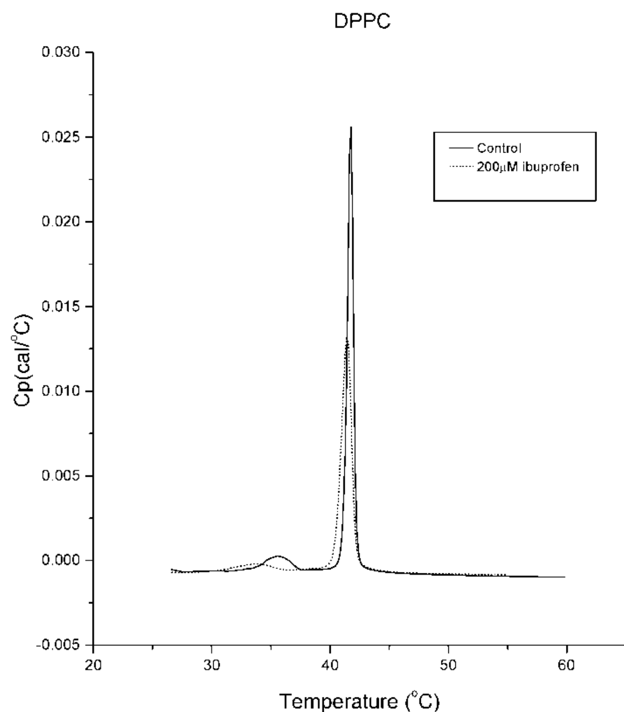

Fig. 7. Differential scanning calorimetry thermograms obtained from aqueous liposome suspensions of DPPC with added ibuprofen 200 μM.

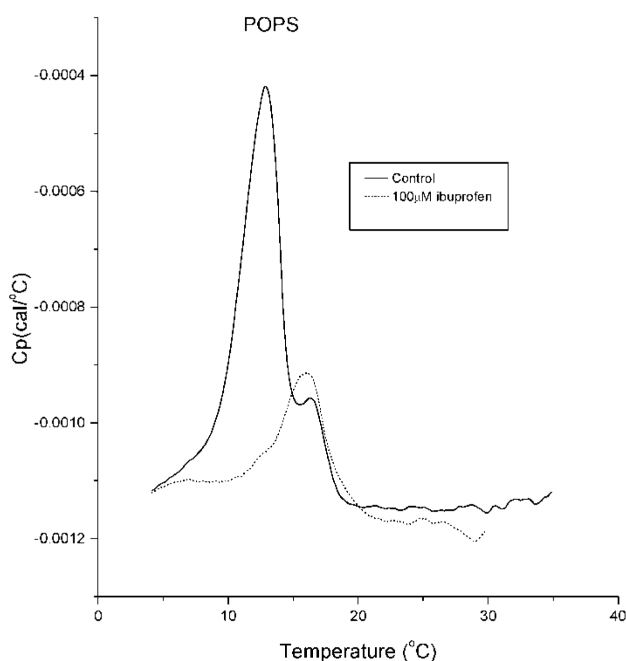

Fig. 8. Differential scanning calorimetry thermograms obtained from aqueous liposome suspensions of POPS with added ibuprofen 100 μM.

ibuprofen up to 200 μM in the liposomes caused very little change in T_m. However, a reduction of ΔH was revealed for all applied concentrations from 1 to 200 μM (Table 1). $T_{c1/2}$ increased for concentrations from 25 to 200 μM (Fig. 7, Table 1).

Keeping the two acyl groups saturated and changing the head group from phosphocholine to phosphoethanolamine (eliminating three N-methyl groups), as in DPPE, resulted in higher T_m, pretransition temperature and ΔH (Table 1). When ibuprofen was added it lowered the ΔH in small (1 μM) and medium concentrations (25 μM) (Table 1), but did not alter T_m appreciably.

Changing the head group to phosphoserine gave a thermogram with two transition temperatures, one large at 12.7°C and a smaller one at 15.7°C. These were due to two ionizing forms of POPS, one with protonated serine carboxyl at the higher temperature and the other with deprotonated negatively charged serine at the lower temperature. The presence of ibuprofen caused almost complete disappearance of the T_m for the protonated form and increased the T_m and enthalpy for the charged form (Fig. 8, Table 1).

The addition of ibuprofen alters the physicochemical characteristics of the liposomes, although whether these are due to domain formations and/or changes in phospholipid packing cannot be probed with thermograms alone. These effects were dependent on the head group, as well as on the length of the saturated and unsaturated acyls we studied.

Discussion

Cellular membranes are complex systems. Their functions reach far beyond the physical separation between the cell and its surroundings, or the delineation of the cell's different compartments. Interactions between the cell and its environment are governed by processes taking place at the plasma membrane. Lipids are diverse in their forms and show great variations among different membranes. The apparent specialization of lipids in tissues, plasma membranes, and organelles might have a role in the biological functioning of different cells, i.e. drug delivery to a cell may occur via different processes at the plasma membrane of different cells. Elucidation of molecular interactions at membranes is therefore a major challenge.

It is well known that water dispersions of amphiphilic molecules may undergo different phase transitions when temperature or their composition is varied. The occurrence of phase transitions in monolayers of amphiphile molecules, at the air/water interface and in bilayer lipid membranes, has also been reported. Interactions through lipid components in the cell membrane may result in disturbance of signal transduction mechanisms. The potency of ibuprofen to associate with and to alter the physical properties of phospholipid membranes has been demonstrated in our study.

By using the monolayer technique, it is possible to

explore a potential relationship between the surface pressure and the apparent molecular area of membrane lipids (Figs 4, 5). PC membranes have been used to model the eukaryotic outer membrane cell leaflet, and PS and PE used as models of the inner membrane leaflet. A saliva-like Ringer solution was used as a sub-phase. The acyl chains of fatty acids were altered, in regard to chain length and degree of desaturation, to determine the importance of the acyl chains for possible interactions. Unsaturated and saturated acyl chains were present in the *sn*-2-position and *sn*-1-position, respectively, just as in naturally occurring phospholipids. Adding a compound at low concentration in a monolayer is assumed to affect the surface pressure exclusively by its interaction with lipid molecules (16). Accordingly, interactions of ibuprofen and membrane lipids may be demonstrated as an altered relationship between surface pressure and molecular area (Figs 4, 5).

When the fatty acyl chains of lipid molecules in bilayer membranes exist in an ordered, rigid state, all of the C-C bonds have a trans-conformation, whereas in the disordered state some are in the gauche conformation. The transition from the rigid (all trans) to the fluid (partly gauche) state occurs fairly abruptly as the temperature is raised above T_m, the melting temperature (Table 1). The small peak (the pretransition) (Figs 6, 7) appears to result from a change in tilt of fatty acyl chains with respect to the bilayer plane, and represents a small endothermic reorganization in the packing of the gel-state lipid molecules prior to melting (12). The major peaks (Figs 6–8) arise from a phase transition in which crystalline fatty acyl chains become disordered because of the introduction of kinks. PC and PS undergo a phase transition when heated, as detected here by DSC, which measures the rate of uptake of heat on warming a sample (Figs 6–8). It has long been known that the melting profiles are influenced by secondary components, e.g. proteins, cholesterol, or anesthetics (17).

We used DSC in order to characterize the influence of ibuprofen on the thermotropic properties of the phospholipids. Adding a foreign molecule to a phospholipid system would normally be expected to change the transition temperature of the phospholipid if both molecules are miscible (18). For DPPC we observed a broadening of the transition peak and a shift of the T_m to lower temperatures when ibuprofen was added (Fig. 4). A reduction in ΔH was revealed for all applied concentrations of ibuprofen (1 to 200 μM) (Table 1). According to Goldman & Isenberg (19) this is indicative of partial insertion of the foreign compound into the hydrophobic region of the lipid membrane. Hence, ibuprofen perturbs the cooperative behavior of the phospholipids. This could be explained by the establishment of a molecular interaction between the phospholipid acyl chains and the aromatic rings and sec-butyl group of the ibuprofen molecule. These findings are in accordance with the effects from abietic acid and triclosan on DPPC (18, 20). Abietic acid is a major compound of the oleoresin synthesized by many conifers, and constitutes a major class of environmental toxic compounds with potential health hazards to animal, human, and plant life. Triclosan exhibits pharmacological effects towards prokaryotic and eukarotic membranes.

For POPS we observed an increase of ΔH, and almost disappearance of the charged form of the lipid, when ibuprofen was added in high concentrations, i.e. 100 μM. The appearance of only the protonated form with ibuprofen suggests that the drug only interferes with this form. Anthony et al. (21) have shown by densitometry that the enthalpy change (the excess heat), ΔH, and the relative volume change (the excess volume), ΔV, are proportional functions in the lipid melting transition range. This may influence the elastic properties of membranes, i.e. the area compressibility and the membrane bending modulus (22).

Conclusions

The main result of this study is that ibuprofen can perturb the packing of phospholipid monolayers using lipid components that are known to exist in eukaryotic cells. The perturbing effects of ibuprofen on membrane structures suggest that this molecule would alter membrane functions, affecting not only lipids, but also indirectly the proteins of the membrane, the function of which is highly dependent on membrane structure. Hence, research is necessary to gain insight into the mechanisms by which the amphiphilic drug ibuprofen exerts effects at the molecular level.

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