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EFFECT OF ASPIRIN ON CONFORMATION AND DYNAMICS OF MEMBRANE PROTEINS IN PLATELETS AND ERYTHROCYTES

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Abstract—The effect of the chemical modifications induced by aspirin (acetylsalicylic acid), acetyl chloride or salicylate on platelet membranes and erythrocyte ghosts has been investigated by means of fluorescence quenching and ESR spectroscopy in relation to our earlier findings of acetylation-induced reduction of platelet and erythrocyte membrane lipid fluidity. Only aspirin was found to induce disorders in the lipid–protein matrix and membrane protein conformation. The apparent distance separating the membrane tryptophan and bound 1-anilino-8-naphthalenesulphonate (ANS) molecules was decreased after aspirin action in both platelet and erythrocyte membranes. This resulted in a significant increase in the maximum energy transfer efficiency. The decrease in the ratio of the amplitudes of low-field peaks of weakly to strongly immobilized fractions of maleimide spin label (4-maleimido-2,2,6-6-tetramethylpiperidine-1-oxyl) and the rise in the relative rotational correlation time of iodoacetamide spin label [4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl] indicate that aspirin effectively immobilizes membrane proteins in the plane of the lipid bilayer, whereas neither acetyl chloride or salicylate gave rise to detectable effects. We conclude that aspirin-induced alterations in membrane protein structure induce a reorganization of lipid assembly as well as rearrangements in the membrane protein pattern, and consequently alterations in lipid–protein interactions. Thus, the interaction of aspirin with platelet and erythrocyte membranes may induce local conformational changes in membranes, which are discussed in connection with impairment of platelet function. A new mode of protein chemical modification by aspirin is suggested which involves the generation of reactive salicylic residue during the fast degradation of aspirin under physiological conditions.

More than a decade ago aspirin (acetylsalicylic acid) was recognized to inhibit normal platelet function. The mechanism of this action of aspirin [1–3] is to impair platelet activation and α-granule secretion by irreversible inhibition of platelet cyclo-oxygenase. Since the introduction of the concept that aspirin may be helpful in preventing the initiation of thrombus formation its efficacy in the prevention of acute myocardial infarction has been thoroughly explored [1, 4]. A number of studies have been completed which suggest that there may be a beneficial effect of aspirin in primary and secondary prevention. In contrast to coexisting risk factors, control of which has been established as beneficial, aspirin is not free of undesirable side-effects [for review see 5]. It was suggested that the failure of aspirin to offer significant protection in many clinical trials may be due to the presence of a salvage pathway in platelets provided by the mechanism of membrane modulation [6]. Regardless of the revealed undesirable effects aspirin is still regarded as an important drug in the treatment of numerous disorders. Its several mechanisms of action include inhibition of prostaglandin production by acetylation of prostaglandin synthetase [2].

Previous experiments showed that the reduction in membrane fluidity due to aspirin was independent of its acetylating effect on platelet cyclo-oxygenase. Treatment of both platelet and erythrocyte membranes with aspirin caused a rise in 1,6-diphenylhexatriene-1,3,5 fluorescence polarization, thus indicating a reduction in membrane lipid fluidity. Further, the ingestion of aspirin also caused a reduction in the membrane fluidity of platelets under *in vivo* conditions [7]. These studies have furnished support for the protective role of aspirin against non-enzymatic glycosylation in diabetic subjects [8–10], but have also suggested that the rigidifying effect of aspirin on membrane fluidity may have resulted from occupation of the sites available for glycation and not the presence of glucose moieties *per se* at these sites. The occupation of these sites as a result of acetylation with other agents, like acetyl chloride, produced a similar reduction in membrane lipid fluidity [7].

To explore further the possible mechanisms of the altered dynamics of blood cell membranes we employed ESR and fluorescence techniques in an attempt to monitor the changes in the structure and

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conformation of platelet and erythrocyte membrane proteins as induced by aspirin, acetyl chloride or salicylate.

MATERIALS AND METHODS

Chemicals. Phenylmethanesulphonylfluoride (PMSF*) was from Merck (Darmstadt, Germany). 1-Anilino-8-naphthalenesulphonate (ANS), maleimido-TEMPO (4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl, MSL), iodoacetamido-TEMPO [4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl,ISL]and acetyl chloride were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Aspirin was from Bayer AG (Germany); sodium salicylate was from Aldrich (Germany). All other chemicals were of A.R. grade from POCh (Gliwice, Poland). Water used for solution preparation and glassware washing was passed through a Millipore water purification unit (Millipore, U.K).

Preparation of suspensions of intact platelets and of platelet and erythrocyte membranes. Blood was obtained from individuals under the guidelines of the Helsinki Declaration for human research and the studies were approved by the committee on the Ethics of Research in Human Experimentation at the Medical School of Lódź. None of the volunteer subjects were known to have taken aspirin or any other drug that is known to affect platelet function within the previous 10 days.

Blood (100 mL) was collected into a plastic syringe from a forearm vein through an 18 gauge needle into a plastic syringe containing acid-citrate-dextrose solution [11].

For preparation of platelet membranes, platelet-rich plasma was centrifuged at 1000 *g* for 15 min and the platelet pellet was resuspended in ice-cold phosphate-buffered saline (PBS) at pH 7.4 containing PMSF (0.35 mg/mL), sodium azide (0.13 mg/mL) and $EDTANa_2$ (0.37 mg/mL). Platelet membranes were prepared by a modification [12] of the method of Fox *et al.* [13]. The platelets were sonicated at 0° and centrifuged at 7000 *g* for 20 min at 4° to remove any intact platelets and granules. The supernatant fluid was then centrifuged at 90,000 *g* for 60 min to obtain a particulate membrane fraction. The membrane pellet was resuspended in ice-cold PBS (1–2 mL) containing PMSF, sodium azide and EDTA.

Red blood cells washed four times with PBS pH 7.4 were subjected to moderate haemolysis in Tris–HCl/$EDTANa_2$ buffer pH 7.0 as described previously [14]. The isolated erythrocyte membranes were resuspended in ice-cold PBS with PMSF, sodium azide and $EDTANa_2$. The protein content of the platelet and erythrocyte membrane suspensions was measured [15] and adjusted to 1 mg/mL. The selected samples of platelet and erythrocyte membranes pretreated with aspirin, acetyl chloride or salicylate were subjected to lipid extraction according to Vickers and Rathbone [16]. The phospholipid content of the liposomes made up in PBS pH 7.4 from the extracted membrane lipids and in the whole membranes was assayed according to Ames [17].

Incubation of platelet and erythrocyte membranes with aspirin, salicylate and acetyl chloride. The suspensions of platelet or erythrocyte membranes (final protein concentration of 800 μg/mL) were treated for 1 hr at 37° with or without aspirin, acetyl chloride or salicylate at a final concentration of 0.55 mmol/L. After treatment, the membranes were washed three times with PBS containing PMSF, sodium azide and EDTA, and then subjected to spin labelling.

ESR and fluorescence measurements. Platelet and erythrocyte membranes were labelled by the introduction of 2 μL of ethanol solution of 100 mmol/L MSL per 1 mL of membrane suspension (approx. 3 mg/mL protein), and the labelled samples were then incubated for 1 hr. The incubation with ISL (2 μL of 100 mmol/L ISL solution per 1 mL of membrane suspension, approx. 3 mg/mL protein) was performed overnight. The final ethanol concentration did not exceed 0.05% (v/v). The unbound spin label was removed by four subsequent washings with PBS. It was checked that no ESR signal could be detected in the supernatant after the last wash. In all the ESR spectra, the ordinate was represented as the amplitude of an ESR signal and expressed in arbitrary units. ESR scans were routinely recorded as the first derivatives of absorption spectra. The estimated ratios were calculated from the ESR graphs taking into account the relevant amplitudes measured as the heights of the respective peaks (expressed in metric units). ESR measurements were performed at the ambient temperature (23 ± 1°) in a Brüker SX-300E spectrometer.

The binding of magnesium ANS (5 mmol/L in PBS) with platelet and erythrocyte membrane lipids was analysed in the system containing membrane samples or liposomes corresponding to 150 μg/mL protein or 5 μg/mL phospholipid phosphorus, respectively, and increasing concentrations of ANS (10–80 μmol/L) [18]. The excitation (2.5 nm slit) and emission (5 nm slit) wavelengths for ANS were 360 and 470 nm, respectively. Tryptophan fluorescence was excitated at 295 nm and recorded at 333 nm. All fluorescence measurements were performed at a temperature of 23 ± 1° using a Perkin–Elmer LS-5B spectrofluorometer.

Statistical analysis. Means ± SD are given. The normal distribution of data was confirmed using the Shapiro–Wilk's test. Analysis of variance and Tukey's test for multiple comparisons were employed to assess the significance of differences among groups. A linear model of regression was performed to assess the significance of ANS emission wavelength shift [19].

RESULTS

In order to check the contribution to the fluorescence of ANS of the proteins and lipids of erythrocyte and platelet membranes the fluorescence signals originating from the whole membranes and the liposomes made up of the lipids extracted from

* Abbreviations: ANS, 1-anilino-8-naphthalenesulphonate; ISL, iodoacetamide spin label; MSL, maleimide spin label; PMSF, phenylmethylsulphonylfluoride; PBS, phosphate-buffered saline.

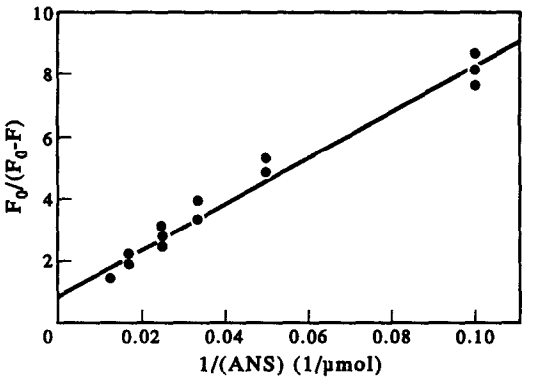

Fig. 1. Double-reciprocal plot of fluorescence intensity vs ANS concentration. F_0 and F denote the tryptophan fluorescence in the absence or presence of ANS, respectively.

these membranes were recorded. Prior to the extraction of lipids the membranes were pretreated with aspirin and related compounds. The averaged ratios of the fluorescence of ANS-labelled liposomes to the fluorescence of ANS embedded in the relevant intact membrane preparations for platelet and erythrocyte membranes were 93.1 ± 8.7% (N = 6) and 96.7 ± 11.2% (N = 8), respectively (the differences with respect to the theoretical mean of 100% were insignificant by the Student's t-test). As the slopes of the curves of the ANS fluoresence intensity vs ANS concentration were insignificant ($P < 0.15$ and $P < 0.28$, respectively, for platelet and erythrocyte membrane-derived liposomes), we concluded that the concentration of label did not significantly affect the mechanism of ANS incorporation into the membrane lipid pockets in either the platelet or the erythrocyte membranes. These results show that only a negligible fraction of the label binds to membrane proteins, and consequently the major fraction of membrane-bound ANS can be attributed to membrane lipids. Accordingly, the ANS fluorescence signal arising from the ANS-labelled membranes may be attributed to the ANS molecules bound predominantly in the membrane lecithin-sphingomyelin and lecithin-cholesterol pockets [20].

The double-reciprocal plot of the decrease in the fluorescence intensity of the tryptophan vs ANS concentration is presented in Fig. 1. The intercept of the ordinate gives the reciprocal maximum transfer efficiency which corresponds to a state of complete occupation of all ANS binding sites in the membrane [21]. The apparent interchromophore separation, R', which is the distance separating the membrane tryptophan and bound ANS molecules was determined from transfer efficiency (E) values according to the formula [21]:

$$R' = R_0(1 - E)^{1/6}$$

where R_0 is the distance at which the coefficient E equals 0.5. Since the value of R_0 is not given in the literature for platelet membranes, for the purpose of this study the ratios of R'_s (treated sample) to R'_0 (control) $R_r = (R'_s/R'_0)$ were estimated instead for both platelet and erythrocyte membrane preparations.

The average values of the maximum transfer efficiency and the apparent interchromophore separation calculated for platelet membranes are presented in Table 1, and those obtained for erythrocyte ghosts are given in Table 2. For the membranes of both types of blood cell the coefficients

Table 1. Energy transfer efficiency (E) and relative apparent interchromophore separation (R_r) between membrane tryptophan residues and ANS molecules, ISL rotational correlation time (τ_c), and immobilization of MSL (expressed as h_w/h_s ratio) in platelet membranes incubated with aspirin, salicylate and acetyl chloride

	Control μ_0	Salicylate μ_1	Aspirin μ_2	Acetyl chloride μ_3
Energy transfer efficiency	0.881 ± 0.061	0.864 ± 0.082	0.981 ± 0.070	0.873 ± 0.051
$\alpha_T < 0.015$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.01$				
Relative interchromophore distance ($R_r = R'_s/R'_0$)	1.00	1.022 ± 0.093	0.736 ± 0.057	1.011 ± 0.085
$\alpha_T < 0.003$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.001$				
ISL rotational correlation time (τ_c) (nsec)	11.21 ± 0.67	11.61 ± 0.91	13.83 ± 1.03	11.09 ± 1.07
$\alpha_T < 0.001$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.01$				
MSL h_w/h_s ratio	6.08 ± 0.43	5.99 ± 0.72	4.01 ± 0.39	6.12 ± 0.60
$\alpha_T < 0.001$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.01$				

Values are means ± SD; N = 8.

The significance of differences is given as calculated by analysis of variance (α_{ANOVA}) and by Tukey's test (α_T).

Table 2. Energy transfer efficiency (E) and relative apparent interchromophore separation (R_r) between membrane tryptophan residues and 1-anilino-8-naphthalenesulphonate (ANS) molecules, ISL rotational correlation time (τ_c), and immobilization of MSL (expressed as h_w/h_s ratio) in erythrocyte ghosts incubated with aspirin, salicylate and acetyl chloride

	Control μ_0	Salicylate μ_1	Aspirin μ_2	Acetyl chloride μ_3
Energy transfer efficiency	0.829 ± 0.094	0.851 ± 0.103	0.963 ± 0.052	0.860 ± 0.077
$\alpha_T < 0.005$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.001$				
Relative interchromophore distance ($R_s = R'_s/R'_0$)	1.00	0.977 ± 0.104	0.774 ± 0.037	0.967 ± 0.076
$\alpha_T < 0.001$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.001$				
ISL rotational correlation time (τ_c) (nsec)	11.44 ± 0.88	11.05 ± 0.86	14.19 ± 1.44	10.81 ± 0.91
$\alpha_T < 0.001$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.001$				
MSL h_w/h_s ratio	5.95 ± 0.55	6.03 ± 0.62	3.70 ± 0.56	5.88 ± 0.63
$\alpha_T < 0.0001$		$\mu_0 = \mu_1 = \mu_3 \neq \mu_2$		
$\alpha_{ANOVA} < 0.001$				

Values are means ± SD; N = 11.

The significance of differences is given as calculated by analysis of variance (α_{ANOVA}) and by Tukey's test (α_T).

of the maximum transfer efficiency were significantly higher only in the case when membranes were treated with aspirin. Correspondingly, the apparent interchromophore separation, R, was significantly diminished in aspirin-treated membranes of platelets and red blood cells when compared with control values. In the treated preparations there was no significant shift of the emission maximum of tryptophan residues and only a small *ca.* 3 nm shift in the emission maximum of ANS when the ANS concentration was increased from 10 to 80 μmol/L (from 464 to 467 nm and from 465 to 468 nm in platelet and erythrocyte membranes, respectively). As estimated by the means of the Student's t-test for comparing two slopes, the blueshift was approximately the same in the two cell types. In the absence of ANS, fluorescence was emitted by tryptophan residues with a maximum at 332 nm (λ_{exc} = 295 nm). When adding increasing amounts of ANS the tryptophyl fluorescence band was quenched and a second peak appeared with a maximum at 467–468 nm. These data indicate the changes concerning ANS localization in the membrane lipid bilayer, interactions of ANS with membrane proteins and consequently the possible alterations in lipid–protein organization, which occur in platelet and erythrocyte membranes upon the action of aspirin.

In order to monitor the possible alterations in membrane protein conformation two protein spin labels, MSL and ISL, were employed. Both the spin labels are reactive toward thiol groups in proteins: whereas ISL anchors preferentially to the surface of labelled protein, MSL can easily penetrate protein molecules and bind also to the intrinsic -SH groups [22]. As shown in Fig. 2 the spectrum of MSL attached to erythrocyte membranes consists of two dominant classes of spin label. The broad anisotropic spectrum arose from the subpopulation of label molecules embedded into a strongly immobilized membrane environment, the narrower one represents

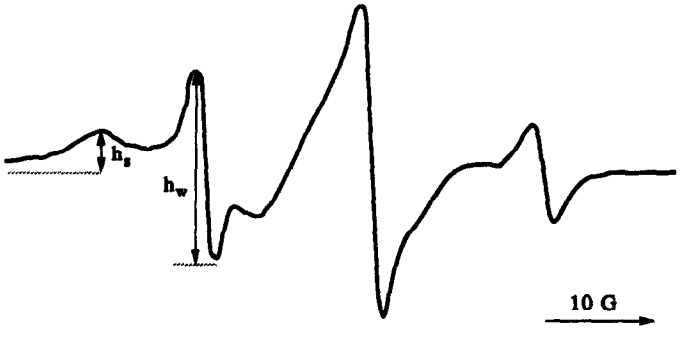

Fig. 2. ESR spectrum of MSL attached to erythrocyte membrane ghosts; h_w and h_s correspond to weakly and strongly immobilized membrane MSL residues.

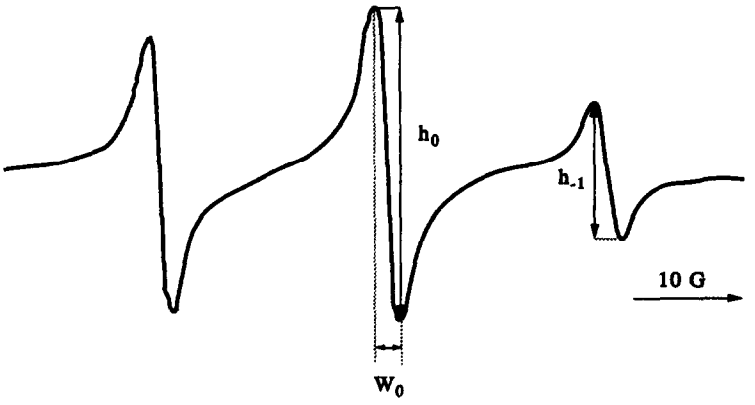

Fig. 3. ESR spectrum of ISL attached to -SH groups of erythrocyte membrane proteins; W_0, h_0 and h_{-1} are midfield line width, mid-field line height and high-field line height, respectively.

the subpopulation of weakly immobilized or more mobile MSL residues. The ratio of the amplitudes of low-field peaks of weakly (h_w) to strongly (h_s) immobilized fractions of MSL is regarded as an indicator of the physical state of membrane proteins and, moreover, a sensitive measure of the conformational state of the proteins [22]. This ratio was significantly reduced in both platelet and erythrocyte membranes after incubation with aspirin (Tables 1 and 2), thus suggesting that the thiol groups in membrane proteins may be much less mobile. This observation was confirmed by the relevant increase in the values of the relative rotational correlation time (τ_c) of ISL in membranes treated with aspirin. Relative rotational correlation times τ_c were calculated from the heights and widths of spin label hyperfine lines according to the equation [23]:

$$\tau_c = 6.5 \times 10^{-10} \times W_0[(h_0/h_{-1})^{1/2} - 1]$$

where W_0, h_0 and h_{-1} are mid-field line width, mid-field line height and high-field line height, respectively (Fig. 3). The higher the value of τ_c, the slower the rotational mobility of ISL, implying the stronger immobilization of protein moieties. The values of τ_c estimated for the samples incubated with aspirin were statistically different from each other (Tables 1 and 2), indicating considerable changes in the surroundings of surface thiol groups in platelet and erythrocyte membrane proteins. Treatment with either acetyl chloride or salicylate had no effect similar to that which occurred following incubation with aspirin. Both the ESR parameters of MSL or ISL and the parameters estimated from the quenching of membrane protein tryptophan remained the same, indicating that neither acetyl chloride nor salicylate were effective in changing membrane protein conformation.

DISCUSSION

There appear to be at least two independent ways by which aspirin could influence platelet function. As revealed recently, it can reduce membrane fluidity [7], which has been associated with hypersensitivity of platelets to agonists [12, 24], and it can block the cyclo-oxygenase enzyme involved in the arachidonate pathway. The rigidizing effect of aspirin and acetyl chloride on membrane lipid fluidity indicated that occupation of the sites potentially available for glycation with acetyl groups might have occurred. The effect was confirmed in the *in vivo* monitoring study, in which the ingestion of aspirin induced a similar reduction in membrane lipid fluidity, and declined in about 1 week. It was established in several studies that the acetylation of plasma albumin after ingestion of aspirin occurs at a lysine residue and therefore aspirin is thought to compete with glucose for these sites [8, 10, 25]. Some recent studies have shown that glycation itself is able to induce well-marked alterations in the membrane dynamics of erythrocytes from diabetic subjects and these changes resemble very much those induced by aspirin [26]. As we have found, in both platelet and erythrocyte membranes incubated with aspirin the maximum energy transfer efficiency of the molecules is high; consequently, the calculated distance between them is low. In membranes subjected to aspirin action ANS molecules may bind to membrane more efficiently, and subsequently occupy the sites which are further away from the membrane proteins, namely membrane lipids. Thus, the data indicate that in the samples chemically modified with aspirin the membrane tryptophan residues are exposed to the solvent molecules to a greater extent and/or that the quenchable fraction of membrane tryptophan is greater than in control samples.

The spectrum of ANS is characterized by a small shift towards longer wavelengths, which can be accounted for by an increase in the polarity of the environment, thus suggesting diminished contact with hydrophobic phospholipid fatty acyl chains. This would imply that membrane-bound ANS molecules may move towards the external environment to a greater extent in aspirin-treated membranes, which supposedly results in more efficient energy transfer between ANS and membrane protein tryptophan residues. These tryptophan

residues might originally be screened from ANS by phospholipid molecules. Since treatment with acetylsalicylic acid seems to affect the conformation and structure of the integral proteins of platelet and erythrocyte membranes, the order in the bilayer lipids is likely to be influenced also as the proteins embedded in the lipid bilayer become less immobilized and more relaxed. Consequently, some of the tryptophan residues may become exposed or, at least, less hindered by membrane phospholipid molecules. Such an exposure of selected membrane tryptophan residues might be responsible for the diminished interchromophore distance and the enhanced energy transfer efficiency in membranes upon incubation with aspirin.

In addition to the previously reported reduction in membrane lipid fluidity, the conformation of membrane proteins undisputably seems to be considerably altered due to aspirin action. MSL employed in this study revealed a significantly increased immobilization of membrane proteins in aspirin-treated platelet and erythrocyte membranes. It is not surprising that the altered MSL spectrum was accompanied by a corresponding increase in the relative rotational correlation time, τ_c, of the ISL. Although the two spin labels have essentially different preferences when labelling protein -SH groups, the resultant changes are compatible. Evidently, the surroundings of the surface thiol groups of membrane proteins in control and modified membranes are not very similar: the values of τ_c in the modified membranes are 25 and 23% greater than those of control membranes for the MSL and ISL, respectively. Likewise, after the increase in protein rigidity in the membranes treated with aspirin, the intrinsic thiol groups become less mobile, as revealed by the decrease in the MSL h_w/h_s parameter. This retardation in the motion of protein molecules has been interpreted to reflect aggregation or "clustering" of the protein in the plane of the membrane [27] and is likely to underlie the significant increase in the interaction of membrane protein tryptophan and ANS molecules.

Based on the above, it seems reasonable to conclude that the well-marked alterations in membrane protein exposure and conformation corroborate the lowered lipid fluidity reported in our previous study [7]. Whether and how far membrane protein mobility is influenced by the acetylation-induced fluctuations in lipid fluidity certainly remains to be established. However, it seems reasonably certain that two coexisting mechanisms occur upon the action of aspirin. On one hand, the aspirin-induced alterations in the structure and conformation of membrane proteins may result in the reorganization of membrane lipid assemblies and as such underlie the rigidizing effect of aspirin on membrane lipid fluidity [7]. In consequence, the changes in the dynamics of the lipid bilayer and the lowered lipid fluidity may render some membrane integral proteins more exposed to the external environment [28]. If so, one should expect any alterations in the dynamics of bilayer lipids to be the possible cause of the observed changes in the physico-chemical properties of platelet and erythrocyte membrane proteins given their modification with aspirin. These alterations occurred after incubation with aspirin at concentrations relevant to its physiological concentrations in the blood (range 0.2–0.6 mmol/L) of subjects taking the drug.

Others have reported that acetylating agents like aspirin and other compounds containing a reactive acetyl group affect cell membrane structure and function. Aspirin, like the reversible inhibitor of cyclo-oxygenase, indomethacin, was found to induce pronounced alterations in the structure of rat liver microsomes [29] and acetylation–deacetylation processes were found to be involved in membrane function, possibly in ion and/or transmitter channels [30, 31]. The data reported by Mehta *et al.* [32] indicated that aspirin decreased platelet α2-receptor affinity for agonists as well as antagonists and that these effects of aspirin were independent of circulatory or dynamic intraplatelet changes. Further, it has been postulated that the progressive nature of the inhibition of platelet function with low aspirin concentrations might be due to either slow aspirin transport across the platelet membrane or delayed interaction with cyclo-oxygenase. The data reported by Parker and Gralnick [33] indicate that platelets in which the cyclo-oxygenase pathway is blocked by the action of aspirin can increase surface expression of platelet-von Willebrandt factor as a consequence of platelet shape change. The authors speculated that this process might have exposed platelet-von Willebrandt factor bound to the membrane complex of glycoproteins IIb and IIIa, or possibly glycoprotein Ia, within the surface-connected canalicular system.

The results presented here are only partly compatible with those reported earlier and concerning acetylation-induced reduction in membrane lipid fluidity [7]. It does not seem surprising that sodium salicylate, a naturally produced neutral catabolite of aspirin, does not affect membrane protein dynamics nor lowers membrane lipid fluidity. On the other hand we did not observe a significant effect of another acetylating agent, acetyl chloride, suggesting that different chemical mechanisms underlie the reduction in membrane phospholipid mobility and the dynamic properties of membrane proteins. Whereas the process of acetylation most likely accounts for the reduced membrane lipid fluidity, membrane proteins may be modified not only by the attachment of the reactive acetyl residues but also by salicylic residues generated *in situ* when aspirin is decomposed under physiological pH. The two fragments of the aspirin molecule are of different sizes, and there exists the possibility that the attachment of small acetyl residues to protein amino groups does not cause major alterations in protein conformation and consequently in membrane protein dynamics. The larger salicylic residues appear to be a steric hindrance, which influences protein structure and conformation. In the cyclo-oxygenase enzyme, both the small acetyl groups and the larger salicylic residues are likely to inhibit irreversibly the activity of the enzyme when bound at its active site. Though hitherto only the process of non-enzymatic acetylation has been considered to be a potential mechanism of aspirin action, we suggest that the attachment of salicylic residues is more important in

impairing the cyclo-oxygenase function in affected platelets.

The results of the present study as well as our earlier findings suggest that the occurrence of aspirin-induced alterations in membrane protein conformation and the corresponding fluctuations of lipid fluidity argue variations in the structure of membrane proteins following *in vitro* incubation with aspirin. In conclusion, it should be emphasized that the altered dynamics of membrane proteins in platelet and red blood cells by aspirin might cause the undesirable side-effects accompanying use of acetylsalicylic acid in numerous clinical trials, and thus contribute to the reduce efficiency of this antiinflammatory drug in antithrombotic therapy [1–4].

REFERENCES

1. Chesebro JH and Fuster V, Dynamic thrombosis and thrombolysis—role of antithrombins. *Circulation* **83**: 1815–1817, 1991.
2. Dusting GJ and MacDonald PS, Prostacyclin and vascular function—implications for hypertension and atherosclerosis. *Pharmacol Ther* **48**: 323–344, 1990.
3. Wilson KM, Siebert DM, Duncan EM, Somogyi AA, Lloyd JV and Bochner F, Effect of aspirin infusions on platelet function in humans. *Clin Sci* **79**: 37–42, 1990.
4. Theroux P, Antiplatelet and antithrombotic therapy in unstable angina. *Am J Cardiol* **68**: B92–B98, 1991.
5. Lekstrom JA and Bell WRTI, Aspirin in the prevention of thrombosis. *Medicine (Baltimore)* **70**: 161–178, 1990.
6. Rao GH, Escolar G and White JG, Epinephrine reverses the inhibitory influence of aspirin on platelet-vessel wall interactions. *Thromb Res* **44**: 65–74, 1986.
7. Winocour PD, Watała C, Perry DW and Kinlough-Rathbone RL, Decreased platelet membrane fluidity due to glycation or acetylation of membrane proteins. *Thromb Haemost* **68**: 577–582, 1992.
8. Ceriello A, Dello Russo P, Curcio F, Giugliano D and D'Onoforio F, Acetylsalicylic acid and lysine inhibit protein glycosylation in vitro. A preliminary report. *Diabete Metab* **10**: 128–129, 1984.
9. Ceriello A, Giugliano D, Quatraro A, Donzella C, Dipalo G and Lefebvre PJ, Vitamin-E reduction of protein glycosylation in diabetes—new prospect for prevention of diabetic complications. *Diabetes Care* **14**: 68–72, 1991.
10. Hun-Opfer C and Mata-Segreda JF, Non-enzymatic acetylation of proteins by aspirin as protection against the secondary complications of diabetes mellitus. *Acta Physiol Pharmacol Latinoam* **36**: 313–316, 1986.
11. Aster RH and Jandl JH, Platelet sequestration in man. I. Methods. *J Clin Invest* **43**: 239, 1964.
12. Winocour PD, Watała C and Kinlough-Rathbone RL, Membrane fluidity is related to the extent of glycation of proteins, but not to alterations in the cholesterol to phospholipid molar ratio in isolated platelet membranes from diabetic and control subjects. *Thromb Haemost* **67**: 567–571, 1992.
13. Fox JEB, Say AK and Haslam RJ, Subcellular distribution of the different platelet proteins phosphorylated on exposure of intact platelets to ionophore A23187 or to prostaglandin E_1. Possible role of a membrane phosphopolypeptide in the regulation of calcium ion transport. *Biochem J* **184**: 651–657, 1979.
14. Marchesi VT and Palade GE, The localization of Mg-Na-K-activated adenosine triphosphatase on red blood cell membranes. *J Cell Biol* **35**: 385–404, 1967.
15. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Vickers JD and Rathbone MP, The effect of membrane cholesterol depletion upon erythrocyte membrane-bound proteins. *Can J Biochem* **57**: 1144–1152, 1979.
17. Ames BN, Assay of inorganic phosphate, total phosphate and phosphates. *Methods Enzymol* **8**: 115–118, 1966.
18. Watała C and Jóźwiak Z, 1-Anilino-8-naphthalenesulphonate binding parameters in red cell membranes. Does diabetes mellitus affect cell membrane dynamics? *J Clin Chem Clin Biochem* **27**: 839–845, 1989.
19. Zar J, *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, NJ, 1984.
20. Slavik J, Anilinonaphthalenesulphonate as a probe of membrane composition and function. *Biochim Biophys Acta* **694**: 1–25, 1982.
21. Audley UP and Chakrabarti B, Interaction of 8-anilino-1-naphthalenesulfonate with rod outer segment membrane. *Biochemistry* **20**: 1687–1698, 1981.
22. Berliner LJ, The spin label approach to labeling membrane protein sulfhydryl groups. *Ann NY Acad Sci* **414**: 153–161, 1983.
23. Keith A, Bulfield G and Snipes W, Spin labeled *Neurospora* mitochondria. *Biophys J* **10**: 618–629, 1970.
24. Tandon NN, Harmon JT and Jamieson GA, Influence of membrane fluidity on platelet activation in cholesterol-modified and hypercholesterolemic platelets. In: *Lipid Domains and the Relationship to Membrane Function* (Eds. Aloia RC, Curtain CC and Gordon LM), pp. 83–99. Alan R. Liss, New York, 1988.
25. Hawkins D, Pinckard RN, Crawford IP and Farr RS, Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid. *J Clin Invest* **48**: 536–542, 1969.
26. Watała C, Hyperglycaemia alters the physico-chemical properties of proteins in erythrocyte membranes of diabetic patients. *Int J Biochem* **24**: 1755–1761, 1992.
27. Clague MJ and Cherry RJ, A comparative study of band 3 aggregation in erythrocyte membranes by melittin and other cationic agents. *Biochim Biophys Acta* **980**: 93–99, 1989.
28. Shinitzky M, Membrane fluidity and cellular functions. In: *Physiology of Membrane Fluidity* (Ed. Shinitzky M), Vol. 1, pp. 1–43. CRC Press, Boca Raton, FL, 1984.
29. Russanov EM, Dimitrova DE, Ivancheva EA and Kirkova MDTI, I The effects of aspirin, indomethacin and their copper complexes on phospholipase activity and on lipid peroxidation in rat liver microsomes. *Acta Physiol Pharmacol Bulg* **12**: 36–43, 1986.
30. Berl S, Clarke DD, Colon AD and Nunez R, Is acetylation of protein involved in membrane function? *J Neural Transm* **19**: 105–119, 1983.
31. Darveau RP, Hancock RE and Benz R, Chemical modification of the anion selectivity of the PhoE porin from the *Escherichia coli* outer membrane. *Biochim Biophys Acta* **774**: 67–74, 1984.
32. Mehta JL, Mehta P and Lawson D, Alterations in platelet alpha 2-adrenoceptors by aspirin. *Agents Actions* **24**: 196–203, 1988.
33. Parker RI and Gralnick HR, Effect of aspirin on platelet-von Willebrand factor surface expression on thrombin and ADP-stimulated platelets. *Blood* **74**: 2016–2021, 1989.