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PHASE SEPARATION IN PHOSPHATIDYLCHOLINE BILAYERS AS A PREDICTOR OF INHIBITION OF BLOOD PLATELET AGGREGATION BY AMANTADINES

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Summary

The ability of eleven amantadine derivatives to induce phase separation in dipalmitoyl phosphatidylcholine bilayers was studied by differential scanning calorimetry. The relative potency varied with the shape and size of the hydrocarbon cage. These agents also markedly inhibited blood platelet aggregation. The relative potencies of these compounds to induce phase separation showed a significant correlation ($r = 0.70$) with their platelet inhibitory activity suggesting that their pharmacologic action may be at the level of the platelet membrane. The effective concentration of the parent component amantadine is similar to its pharmacologic concentration suggesting its use as an anti-platelet drug.

Introduction

Arterial thrombosis occurs in response to a disruption of vascular endothelium which results in the exposure of subendothelial components of the vessel wall to blood platelets [1]. The conclusion that blood platelets are involved in thrombotic vascular disease follows from the observations that: (a) platelets show enhanced sensitivity to aggregating agents in type II hyperlipoproteinemia [2], myocardial infarction and stroke [3,4], coronary atherosclerosis [5], and diabetes [6]; (b) platelet microaggregates circulate in peripheral vascular disease and transient cerebrovascular ischemia [7]; and (c) platelet survival de-

Abbreviations: HHW′, half-height width; ID_{50}, concentration of the amantadine derivative that inhibits platelet aggregation by 50%.

creases in coronary disease [8], hyperlipoproteinemia, and cerebrovascular disease [9]. A variety of pharmacologic agents have been employed to modify platelet behavior in vivo [10] and in vitro [11]. Some drugs like aspirin and non-steroidal anti-inflammatory agents appear to act by inhibiting platelet cyclo-oxygenase [12], an enzyme required for the synthesis of prostaglandin cyclic endoperoxides [13,14] and thromboxane A2 [14]. However, the actions of several lipid-soluble drugs, including antihistamines (e.g. cyproheptadine [15]), hypolipemic agents (e.g. clofibrate [16] and halofenate [17]), and agents active on the central nervous system (e.g. chlorpromazine [18]), appear to be localized at the platelet membrane. However, direct evidence for membrane modification by these drugs has not been reported.

The initial adhesion of blood platelets to the vessel wall and the aggregation of platelets to each other result in the formation of a platelet thrombus which later is reinforced by fibrin [19]. Consequently, inhibitors of platelet aggregation are of considerable interest as they offer the potential for treating or preventing arterial thrombosis. We now wish to present results which suggest that differential scanning calorimetry of doped lipid bilayers can be employed to predict the relative potencies of platelet inhibiting drugs whose action is localized at the platelet membrane.

Methods and Results

Amantadine [1], a lipophilic quasi-spherical compound, which has been shown to be effective in preventing influenza A2 [20] and ameliorating the symptoms of Parkinson's disease [21], enters all cell membranes including those of the nervous system [20]. The structural formulae of the eleven amantadine derivatives whose hydrochloride salts were employed in this study are given in Figs. 1a, 1b and 1c.

Platelets for the aggregation studies were prepared from venous blood drawn from fasting blood donors who had abstained from all medication for at least 2 weeks prior to venesection. Venous blood was collected through siliconized needles into plastic syringes and then anticoagulated with sodium citrate (final concentration 0.013 M). Platelet-rich plasma with a platelet count of 350 000 ± 50 000 was prepared by centrifugation of the samples at 170 g for 10 min at 23° C. The remaining blood was centrifuged at 12 000 × *g* for 4 min at 23° C to

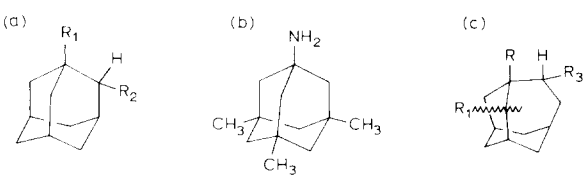


Fig. 1. (a) Structure of seven amantadine derivatives: (1) $R_1 = NH_2$, $R_2 = H$; (2) $R_1 = NHCH_3$, $R_2 = H$; (3) $R_1 = NHC_2H_5$, $R_2 = H$; (4) $R_1 =$ NH-cyclic-C_6H_{11}, $R_2 = H$; (5) $R_1 = CH_2NH_2$, $R_2 = H$; (6) $R_1 = CH(CH_3)NH_2$, $R_2 = H$; (7) $R_1 = H$, $R_2 = NH_2$. (b) Structure of trimethylamantadine [8]. (c) Structure of three homoamantadine derivatives: (9) $R_1 = NH_2$, $R_2 = R_3 = H$; (10) $R_2 = NH_2$, $R_1 = R_3 = H$; (11) $R_3 = NH_2$, $R_1 = R_1 = R_2 = H$.

produce platelet-poor plasma which had less than 1000 platelets per μl. Platelet aggregation was studied with an aggregometer according to a modification [3] of the method of Born [22]. As aggregation occurs, the amount of light scattering decreases and more light is transmitted. The extent of aggregation was determined by the percent difference in light transmission between platelet-rich and platelet-poor plasma after 15 min of stirring at 1200 rev./min and 37°C. The sensitivity of the sample to ADP (Sigma Chemical Co., St. Louis, Mo.) was determined at the lowest concentration of the agent which would produce a "second wave" response [2]. This threshold concentration was found to show a high correlation (r = 0.95) with the threshold determined by the use of serotonin release [23]. It was found in preliminary studies that if the final concentration of methanol exceeded 0.5 M then platelet aggregation was inhibited. Therefore no more than 5 μl of methanol containing the amantadine derivative was added to 500 μl of platelet-rich plasma (final concentration of methanol 0.25 M) before being incubated at 37°C for 10 min without stirring. The threshold concentration of ADP was then added and the percent aggregation of platelets for a given concentration of the amantadine derivative was compared to the percent aggregation of platelets with the solvent, methanol (see Fig. 2). By carrying out analogous experiments for a number of concentrations of the drug, the concentration of the amantadine derivative that inhibited platelet aggregation by 50% (i.e. ID_{50}) could readily be determined (see Fig. 3).

Unsonicated multilamellar liposomes containing DL-1,2-dipalmitoyl phosphatidylcholine (Sigma Chemical Co.) and amantadine derivatives were prepared for the differential scanning calorimetry experiments according to the procedures employed for similar studies with other variously substituted amantadines [24]. A known volume of a methanolic solution of an amantadine derivative was evaporated to dryness in a small test tube. An aliquot of liposomes was tranferred onto the film of amantadine residue and the mixture was vigorously

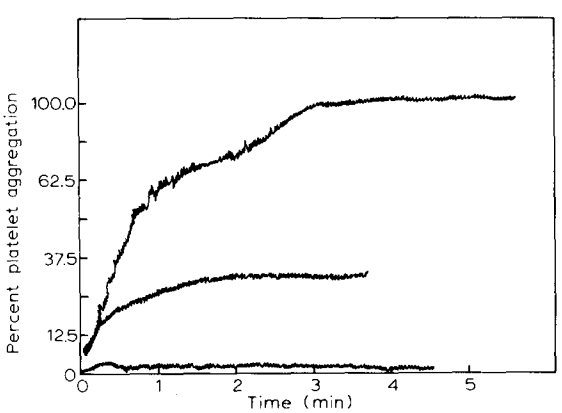
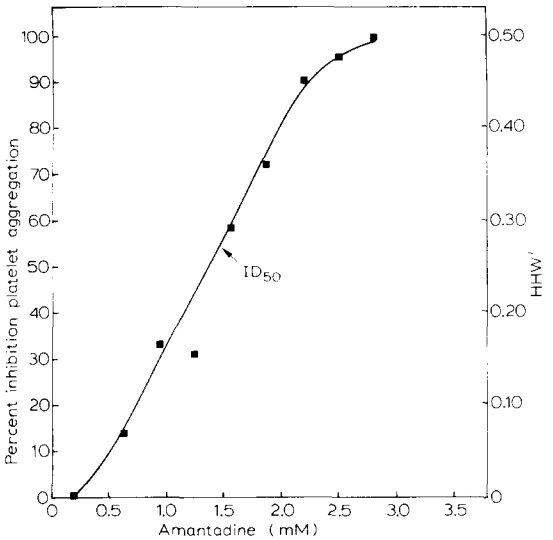


Fig. 2. Platelet-rich plasma (500 μl) was incubated 10 min at 37°C with (top) 2.25 μl of a methanol solution of amantadine (structure 1) (final concentration of structure 1, 2.79 mM), or with (middle) 1.3 μl of this solution (final concn. of structure 1, 161 mM), or with (lower) 3 μl of methanol (no drug). Platelet aggregation was performed as described in the text.



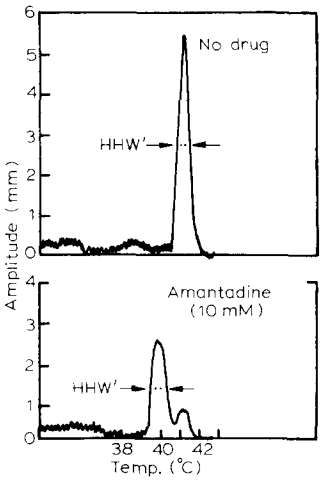


Fig. 3. By means of the experimental procedure employed in Fig. 2, the percent inhibition of platelet aggregation induced by various concentrations of structure 1 was determined and thus the concentration of structure 1 required to inhibit platelet aggregation by 50% (ID_{50}) was obtained.

Fig. 4. Differential scanning calorimetry profiles of pure dipalmitoyl phosphatidylcholine liposomes (70 mM) and of such liposomes doped with structure 1 (10.67 mM). The half-height widths (HHW') are noted.

shaken with two 3-mm glass beads on a Vortex mixer under a nitrogen atmosphere at 45—50°C for 5 min. The sample was then allowed to stand for 24—48 h before being employed for the differential scanning calorimetry experiment. For comparison, some samples were prepared by premixing the lipid and amantadine derivative in a solvent and then generating the liposomes from a mixed film. The differential scanning calorimetry profiles of the liposomes prepared by either method were identical.

All differential scanning calorimetry samples were 75 mM in dipalmitoyl phosphatidylcholine, 50 mM in KCl, 5 mM in Tris buffer, and were prepared at pH 7.4. The samples were analyzed using a Perkin-Elmer DSC-1B calorimeter operating at a sensitivity of 1 mcal and a scanning rate of 1.25°C/min towards increasing temperature. The phase transition profile for pure dipalmitoyl phosphatidylcholine liposomes shows a sharp symmetrical transition commencing (T_c) at 41.0°C which is essentially complete in 1.6°C (Fig. 4). It was found for all of the cage amines in this study that liposomes doped with them showed another sharp transition at a lower temperature. As the concentration of the amantadine was increased, the area of the parent peak decreased and the area of the new peak increased. Thus, as the concentration of the amantadine derivative is increased, T_c decreases and the combined half-height width (HHW') of the two transitions increases (see Fig. 5). None of the amantadine derivatives had a phase transition by itself in the temperature regions studied. In order to compare the relative potencies of the amantadine derivatives, we have defined an arbitrary constant, HHW'_{100}, which is the concentration of drug at which the HHW' of the phase transition profile of pure dipalmitoyl phosphatidylcholine

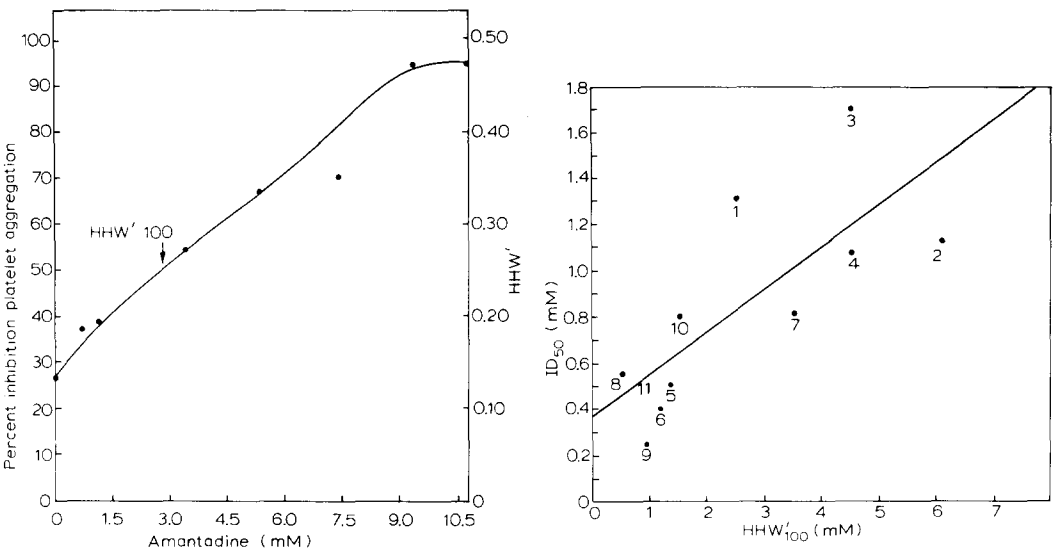


Fig. 5. The concentration-dependent HHW′ curve for structure 1 from which the concentration at which HHW′ of pure dipalmitoyl phosphatidylcholine liposomes doubles (HHW'_{100}) was determined.

Fig. 6. Plot of ID_{50} vs. HHW'_{100} for eleven amantadine derivatives. The equation of the line obtained by linear regression analysis of the data is $y = 0.38 + 0.18x$ with $r = 0.70$ and $P < 0.02$.

liposomes has been increased by 100%. This parameter offers certain advantages over measurement of either integrated peak areas or T_c. While at low solute concentrations the total peak area of the transition is fairly constant, it is not possible to resolve the individual peaks. On the other hand, at moderately high solute concentration the onset or midpoint of the transition cannot be adequately defined and/or accurately measured. By contrast, HHW′ can be easily determined over a wide concentration range and it can be employed in the analysis of the several types of transition profiles that are induced by a variety of solutes [25].

In Fig. 6 the HHW'_{100} values obtained from the differential scanning calorimetry curves for the 11 amantadine derivatives examined are plotted against the ID_{50} values for these compounds from the platelet aggregation studies. The correlation coefficient (r) is 0.70 ($P < 0.02$).

Discussion

The differential scanning calorimetry results obtained for the amantadine derivatives are consistent with our earlier observations that the position and orientation of a sclute within the lipid bilayer are critical factors in determining its relative potency [24]. For a given substituent, the shape and size of the hydrocarbon cage are exceedingly important [24]. This point is well illustrated by the trimethyl-substituted amantadine (Fig. 1 [8]) and the homoamantadines (Fig. 1 [9—11]) which are all significantly more potent than the parent amantadine (Fig. 1 [1]). The results further suggest that in the presence of amantadine derivatives an additive induced phase separation occurs in the bilayer in which

parent and modified phases independently coexist. Since these compounds generate in the bilayer a phase with a lower transition temperature, the fluidity of the doped bilayer only increases in certain regions.

There is a significant relationship between the relative potencies of various amantadines in inhibiting platelet aggregation in vitro and the abilities of these compounds in inducing phase separation in dipalmitoyl phosphatidylcholine bilayers. It has recently been shown that cholesterol incorporation into blood platelets increases the microviscosity of the platelet membrane [26], increases the sensitivity of the platelets to ADP and epinephrine [23] and alters platelet cyclic AMP metabolism [27]. It may follow here that a drug-induced increase in the fluidity of the membrane and/or the formation of border domains between gel and liquid crystalline regions in the membrane lead to decreased platelet aggregation. However, it remains to be determined if drugs which alter the organization of the bilayer in a qualitatively different fashion [24,28] produce similar results. Since the clinical plasma concentration of amantadine is 0.5 mM [29] and comparable to the ID_{50} concentration, and since it is virtually non-toxic [20], these results suggest that amantadine should be considered as an anti-platelet agent.

Acknowledgements

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References

1 French, J.E., (1971) Semin. Hematol. 8, 84
2 Carvalho, A.C., Colman, R.W. and Lees, R.S. (1974) New Eng. J. Med. 290, 434
3 Sano, T., Boxer, M.G.J., Boxer, L.A. and Yokoyama, M. (1971) Thromb. Diath. Haemorrh., 25, 524
4 Goldenfarb, P.B., Cathey, M.H., Zucker, S., Wilber, P. and Corrigan, Jr., J.J. (1971) Circulation 43, 538
5 Frishman, W.H., Weksler, B., Christodoulou, J.P., Smithen, C. and Killip, T. (1974) Circulation 50, 887
6 Sagel, J., Colwell, J.A., Crook, L. and Laimins, M. (1975) Ann. Int. Med. 82, 733
7 Wu, K.K. and Hoak, J.C. (1974) Lancet 2, 924
8 Gilbert, J.B. and Mustard, J.F. (1963) J. Atherscler. Res. 3, 623
9 Murphy, E.A., Robinson, G.A., Roswell, A.C. and Mustard, J.F. (1967) Blood 30, 26
10 Genton, E., Hirsch, J., Gent, M. and Harker, L. (1975) New Eng. J. Med. 293, 1174
11 Weiss, H.J. (1972) Hemostasis Thromb. 1, 199
12 Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3400
13 Hamberg, M., Svensson, J., Wakabayaski, T. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 345
14 Hamberg, M., Svensson, J. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3824
15 Goldman, B., Aledort, L.M., Puszkin, E. and Burrows, L. (1971) Circulation 44, Supp. 2, 68
16 Carvalho, A.C., Colman, R.W. and Lees, R.S. (1974) Circulation 50, 570
17 Colman, R.W., Bennett, J.S., Sheridan, J.F., Cooper, R.A. and Shattil, S.J., (1976) J. Lab. Clin. Med. 88, 282
18 Mills, D.C.B. and Roberts, G.C.K. (1967) Nature 213, 35
19 Weiss, H.J. (1975) New Eng. J. Med. 293, 531
20 Parkes, D. (1974) Adv. Drug Res. 8, 11
21 Barbeau, A., Mars, H., Botez, M.I. and Joubert, M. (1971) Can. Med. Assoc. J. 105, 42

22 Born, G.V.R. (1962) Nature 194, 927
23 Shattil, S.J., Anaya-Galindo, R., Bennett, J., Colman, R.W. and Cooper, R.A. (1975) J. Clin. Invest. 55, 636
24 Jain, M.K., Wu, N.Y.-M., Morgan, Jr., T.K., Briggs, M.S. and Murray, Jr., R.K. (1976) Chem. Phys. Lipids 17, 71
25 Jain, M.K. and Wu, N.Y.-M. (1977) J. Membrane Biol., in the press
26 Shattil, S.J. and Cooper, R.A. (1976) Biochemistry 15, 4832
27 Sinha, A.K., Shattil, S.J. and Colman, R.W. (1977) J. Biol. Chem., in the press
28 Jain, M.K., Wu, N.Y.-M and Wray, L.V. (1975) Nature 255, 494
29 Biandrate, P., Tognoni, G., Belvedere, G., Frigerio, A., Rizzo, M. and Morselli, Pl.L. (1972) J. Chromatogr. 74, 31