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Trifluoperazine inhibits Sendai virus-induced hemolysis

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Sendai virus-induced hemolysis, a manifestation of virus-red cell fusion, is inhibited by exposure of the virus to 50 μM and higher concentrations of trifluoperazine. Trifluoperazine does not disrupt the virus, since trifluoperazine-treated virus with no hemolytic activity sediments slightly faster than untreated virus on sucrose density gradients and contains viral proteins in proportions characteristic of untreated virus. Trifluoperazine affects the fusion protein to a greater extent than the hemagglutinin, since trifluoperazine-treated virus with no hemolytic activity is as active or nearly as active in agglutinating red cells. The partition coefficient of trifluoperazine between the virus membrane and buffer is lower at 4°C than, but the same at 37°C, as that between the red cell membrane and buffer. Nevertheless, virus-independent red cell lysis and inactivation of virus-mediated hemolysis occur when the red cell and viral membranes, respectively, contain similar concentrations of trifluoperazine. Furthermore, 13–28% more trifluoperazine is necessary to achieve either effect at 4°C or at 25°C than at 37°C. Changes in the surface activity of trifluoperazine do not explain these results, insofar as the critical micellar concentration of (0.75 mM) and maximal reduction in surface tension by (40 dyn/cm) trifluoperazine are the same at 25°C and 37°C. The fluorescence of viral tryptophan decreases by approx. 25% when viral hemolysis is inactivated by trifluoperazine, by trypsin treatment or by heating at 100°C for 5 min.

Introduction

The study to be described grew out of an ongoing search in this laboratory [21] for inhibitors of Sendai virus-mediated fusion, the actions of which might help to clarify the molecular mechanism of this as yet obscure process [37]. Sendai virus-mediated fusion reflects the concerted action of the two glycoproteins in the viral membrane. The hemagglutinin, HN, binds to target cell receptors [25,29], whereupon the fusion protein, F, causes fusion of the target membrane with the viral membrane [12,30]. Because fusion may require the interaction of a hydrophobic site on F protein [14] with the target membrane [27], inhibitors known to bind specifically to hydrophobic sites on various proteins were screened for their ability to inhibit Sendai virus-induced hemolysis. Hence, as reported here, trifluoperazine (TFP) and chlorpromazine were found to inhibit Sendai virus hemolysis, which is a convenient indicator of Sendai virus fusion, provided that late-harvest-type virus is used [13].

In an earlier report, the phenothiazine tranquilizers had been demonstrated to inhibit virus-dependent, cell-cell fusion by acting on target cells of Sendai and other paramyxoviruses [26]. In contrast, it is shown here that trifluoperazine can also

Abbreviations: TFP, trifluoperazine; HAU, hemagglutination units; Mops-buffered saline, 0.15 M NaCl/0.01 M 4-morpholinepropanesulfonic acid (Mops) (pH 7); histidine-buffered saline, 0.15 M NaCl/0.01 M histidine (pH 6); phosphate-buffered saline, 0.15 M NaCl/0.01 M sodium phosphate (pH 7); SDS, sodium dodecyl sulfate.

inhibit Sendai virus hemolysis by interacting with the virus, rather than its target cell. Although Sendai virus inactivation requires TFP concentrations an order of magnitude higher than those acting on calcium-binding proteins, such as troponin C [18] and calmodulin [23], the direct inactivation of Sendai virus by trifluoperazine is of interest, since this loss of viral hemolytic activity is not due to a dissolution of Sendai virus, as mediated by detergents, and appears to result from inactivation of the viral fusion protein, F, rather than the hemagglutinin, HN. In addition, study of this phenomenon has revealed (1) a more ordered viral membrane structure at low than at high temperatures and (2) decreased fluorescence of viral tryptophan correlated with inactivation of viral hemolysis by TFP, trypsin or heat treatment.

Materials and Methods

Materials

Virus. Sendai virus (Z strain, obtained from Dr. F.J. Martin) was grown in 10–11-day, embryonated chicken eggs and harvested after 72 h. After purification by differential centrifugation, the concentrated virus was stored in small aliquots at −20°C. Two different virus harvests were used for these experiments – one with a protein concentration of 3.3 mg/ml and a hemagglutination titer of 20500 HAU/ml and another with a protein concentration of 8 mg/ml and a hemagglutination titer of 81800 HAU/ml. Protein was measured according to a modification of the method of Lowry et al. [22].

Red cells and red cell ghosts. Blood was drawn from a healthy human donor and stored at 4°C for no longer than 1 week before use. For hemagglutination or hemolysis assays, the red cells were washed free of serum and white cells in more than 10 vol. of 0.15 M NaCl/0.01 M MOPS (pH 7) (Mops-buffered saline) during three or four low-speed centrifugations at 5000 rpm in a Sorvall RC-2B centrifuge for 5 min at 4°C. Red cell ghosts were prepared by a modification of the procedure of Dodge et al. [9]. The phospholipid content of ghost membranes was determined by phosphate analysis, according to a modification of the procedure of Bartlett [3].

Phenothiazines. Trifluoperazine dihydrochloride and chlorpromazine hydrochloride were obtained from Sigma (St. Louis, MO). Stock solutions were made by dissolving the drugs in 0.15 M NaCl/0.01 M histidine (pH 6) (histidine-buffered saline) and adjusting the pH to 6 with 10 M NaOH and the concentration to 50 mM with histidine-buffered saline. Solutions of TFP were checked spectrophotometrically at 254 nm and taken to have a molar extinction coefficient of 30110 $M^{-1} \cdot cm^{-1}$ [8]. Trifluoperazine, chromatographed on silica gel thin-layer plates with the solvents of Gariepy and Hodges [11], gave a single spot under ultraviolet illumination. The pH values of all dilutions of TFP stock solutions were checked to ensure that these values were within a tenth of a unit of pH 6 and well below pH 7.5, at which point unprotonated TFP will precipitate [11].

Methods

Viral and TFP-induced hemolysis. Sendai virus was preincubated with serial dilutions of trifluoperazine in 11 μl of histidine-buffered saline for 30 min at various temperatures prior to cooling and addition of cold Mops-buffered saline and 9 μl of 20% (v/v) red cells. The final assay mixture of 200 μl was incubated for 10 min at 4°C and for 30 min at 37°C prior to centrifugation of the red cells and spectrophotometric reading of the supernatant at 540 nm. TFP-induced hemolysis was measured in the same way, except for the omission of Sendai virus and the incubation of the final assay mixture for 30 min at various temperatures, rather than at 37°C only.

Direct determination of TFP uptake by a centrifugation method. Aliquots of Sendai virus or red cell ghosts were incubated in duplicate for 60 min at different temperatures in 0.2 ml of various dilutions of TFP and centrifuged for 15 min at 15000 rpm in a Sorvall centrifuge at the temperature of incubation. Centrifuged samples were kept at the temperature of incubation while supernatant aliquots were removed for dilution in water and spectrophotometric reading at 254 nm. No protein could be detected in the supernatant after centrifugation.

The partition coefficient, K_p, is defined by the following equation:

$$K_p = \frac{N_m / V_m}{N_b / V_b} \quad (1)$$

where N is the number of moles of TFP and V is the volume in cm^3 of the viral or red cell membrane (m), buffer (b) or both (t). To determine the partition coefficient, K_p, and the uptake of TFP by the viral or red cell membrane, Eqn. 1 was re-arranged [19] to give:

$$V_b/N_b = (K_p/N_t)V_m + V_b/N_t \quad (2)$$

V_b/N_b was plotted against V_m and linear-regression analysis gave a straight line, the slope of which, K_p/N_t, was solved for K_p.

The volume of the membrane was taken to be the lipid compartment of the membrane under study, which was calculated for the virus to be $0.39 \cdot 10^{-3}$ cm^3 per mg of viral protein, assuming that the density of lipid is 1.0 g/cm^3 [10], that lipid is 28% of the total weight of Sendai virus [4] and that protein is about 72% of the total weight of SV5 and Newcastle Disease virus, representative paramyxoviruses [7]. Similarly, the volume of the red cell membrane was calculated to be $5 \cdot 10^{-13}$ cm^3 per cell, based on the lipid content of a red cell ghost being $5 \cdot 10^{-13}$ g [35], the density of lipid being 1.0 g/cm^3 [10] and 1 ml of red cells containing $5 \cdot 10^9$ cells. Red cells were counted with a hemacytometer. The volume of the ghost membrane was taken to be 1.5-times the phospholipid volume, which specified an amount of lipid consistent with the lipid/protein ratio of red cells [35].

Indirect determination of TFP uptake by its effects on membrane functions. The method of Lieber et al. [19] for estimating chlorpromazine uptake by red cells was adapted to estimating TFP uptake by Sendai virus and by red cells. Eqn. 1, which defines K_p, is rearranged to give:

$$V_b/V_m = [K_p/(N_m/V_m)](N_t/V_m) - K_p \quad (3)$$

where N_t is the number of moles of TFP which causes inactivation of half of the Sendai virus or lysis of half of the red cells exposed to inhibitor and which is found to vary with the buffer/membrane ratio, V_b/V_m. Plotting V_b/V_m vs. N_t/V_m gave a straight line defined by linear-regression analysis, the y intercept of which is $(-K_p)$. Knowing K_p allows calculation of the viral or red cell uptake of TFP for any V_m.

Sucrose gradient centrifugation of Sendai virus. Aliquots of Sendai virus were resuspended in histidine-buffered saline. Samples with or without TFP at concentrations giving viral protein/TFP ratios similar to those in the hemolysis assays were incubated for 30 min at 37°C, then layered onto 20–60% (w/v) sucrose in histidine-buffered saline + 0.02% NaN_3 and centrifuged in a SW-50.1 rotor at 45 000 rpm for 100 min at room temperature. Addition of TFP to sucrose gradients used for analysis of TFP-treated Sendai virus did not affect the results. Gradients were pumped through a fluorometer cell to record tryptophan fluorescence (excited at 290 nm and emitted at 340 nm) before 12-drop fractions were collected for assay of viral hemolysis and polyacrylamide gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis was performed according to a modification of the Laemmli procedure [16]. The separation gel consisted of 8% polyacrylamide, made from 40% acrylamide + 1.5% bisacrylamide, in 0.375 M Tris (pH 8.8) and 0.1% SDS. The stacking gel consisted of 3% polyacrylamide, made from 40% acrylamide + 1.5% bisacrylamide, in 0.125 M Tris (pH 6.8) and 0.1% SDS. Samples directly from the sucrose gradients or not centrifuged were adjusted to contain 0.32 M Tris (pH 6.8)/10% SDS/25% β-mercaptoethanol/0.005% pyronin Y, heated at 100°C for 5 min, layered onto gels and electrophoresed for 3.5 h at 64 V (8 V/cm of gel). Gels were fixed and stained with 0.05% Coomassie blue in 25% isopropanol/10% acetic acid and destained with 0.005% Coomassie blue in 10% isopropanol/10% acetic acid and with 10% acetic acid.

Surface tension. The surface tension of TFP in histidine-buffered saline at 25 and at 37°C was determined by the Wilhelmy plate method [2,21]. The temperatures of tested solutions were controlled by a circulating water-bath and directly measured with a thermister probe.

Hemagglutination. Viral hemagglutination was assayed in plastic microtiter plates by the Salk pattern method [21]. Untreated or TFP-treated Sendai virus was titered in 25 μl of Mops-buffered saline prior to addition of 25 μl of 1% washed red cells in Mops-buffered saline and agitation of the plates. Plates were left overnight at 4°C.

Fluorescence of viral tryptophan and trifluopera-

zine. Virus and/or trifluoperazine were incubated at 37°C for 30 min in histidine-buffered saline under conditions of virus inactivation. Trypsin treatment consisted of incubating 66 μg of Sendai virus protein with 12 μg of trypsin (Type I, Sigma) in 0.01 M sodium phosphate (pH 7) for 20 min at 37°C, followed by dilution with 0.15 M NaCl/0.01 M sodium phosphate (pH 7) containing 24 μg of turkey egg white trypsin inhibitor (Sigma) and centrifugation for 1 h at 15000 rpm prior to dilution. Samples containing virus and/or TFP were diluted 1 : 100 with appropriate buffers and excited at 290 nm or at 340 nm and the emission recorded from 310 to 550 nm or from 350 to 550 nm, respectively. Slits were 10 nm wide.

Results

Temperature-dependent inhibition of viral hemolysis by trifluoperazine

Sendai virus was preincubated with serial dilutions of trifluoperazine in 0.15 M NaCl/0.01 M histidine (pH 6) for 30 min at 4, 25 and 37°C prior to dilution and addition of 20% (v/v) red cells and incubation in 0.15 M NaCl/0.01 M Mops-histidine buffer (pH 7) for 10 min at 4°C and for 30 min at 37°C. Fig. 1A shows that as the TFP concentration was raised, hemolysis first decreased, then increased. The decrease in hemolysis reflects inactivation of viral hemolysis, whereas the increase in hemolysis reflects the well-known [31], directly hemolytic effect of TFP itself. That the increase in hemolysis in Fig. 1A does represent the hemolytic activity of TFP itself was demonstrated by repeating the experiment of Fig. 1A in the absence of virus, as shown in Fig. 1B. It should be noted that the TFP concentrations to which the red cells were exposed in the experiment of Fig. 1A were 1/20th the initial TFP concentrations given in Fig. 1A and to which the virus was exposed. The A_{540} of samples containing the highest concentrations of TFP in both Fig. 1A and B includes contributions from TFP itself, which formed a visible precipitate on dilution with pH 7 buffer. Results similar to those in Fig. 1A and B were obtained with chlorpromazine (not shown), except that somewhat higher concentrations of chlorpromazine were necessary for the degree of viral inhibition given by TFP.

A striking feature of the TFP inactivation of viral hemolysis in Fig. 1A is its temperature dependence, i.e., the higher the temperature of virus-TFP preincubation, the lower the minimally inhibitory concentration of TFP. In contrast, red cell lysis by TFP appears less temperature-dependent, i.e., the minimally hemolytic concentration of TFP is nearly the same at 4, 25 and 37°C. The decrease in hemolysis caused by TFP in Fig. 1A is attributed to an effect of TFP on the virus, and not on the red cells, because viral hemolysis was not inhibited when the virus was added to red cells and TFP, which had been diluted to 1/20th of the preincubation concentrations of TFP, i.e., no preincubation of virus and TFP (not shown).

Characteristics of TFP inactivation of Sendai virus hemolysis

Three aspects of the nature of TFP inactivation of Sendai virus were addressed: (1) whether TFP disrupts Sendai virus, (2) whether TFP affects viral hemagglutination, as well as viral hemolysis, and (3) whether the surface activity of TFP is temperature-dependent. Fig. 2 (top panel) shows that Sendai virus incubated in 0.98 mM TFP at 37°C for 30 min (unbroken line) sedimented slightly faster than untreated virus (dashed line) during centrifugation through sucrose gradients containing 0.098 mM TFP, at which concentration TFP did not inactivate viral hemolysis. Fig. 2 (middle panel) also indicates that TFP-treated virus displayed no hemolytic activity (unbroken line) while untreated virus remained hemolytic (dashed line) after sucrose gradient centrifugation. Nevertheless, as shown in Fig. 2 (bottom panel), the centrifuged, TFP-treated virus subjected to SDS-polyacrylamide gel electrophoresis ('T') contained all viral proteins in the same proportions as untreated virus.

Viral hemagglutination by TFP-treated and untreated Sendai virus was assayed to determine whether the activity of the hemagglutinin, HN, was affected by TFP during inactivation of viral hemolysis. The hemagglutination titer was $40.9 \cdot 10^3$ HAU/ml for the TFP-treated virus and $81.8 \cdot 10^3$ HAU/ml for the untreated virus, whereas undiluted, TFP-treated virus had no demonstrable hemolytic activity while a 1/64 dilution of untreated virus gave 37.5% of maximal viral hemoly-

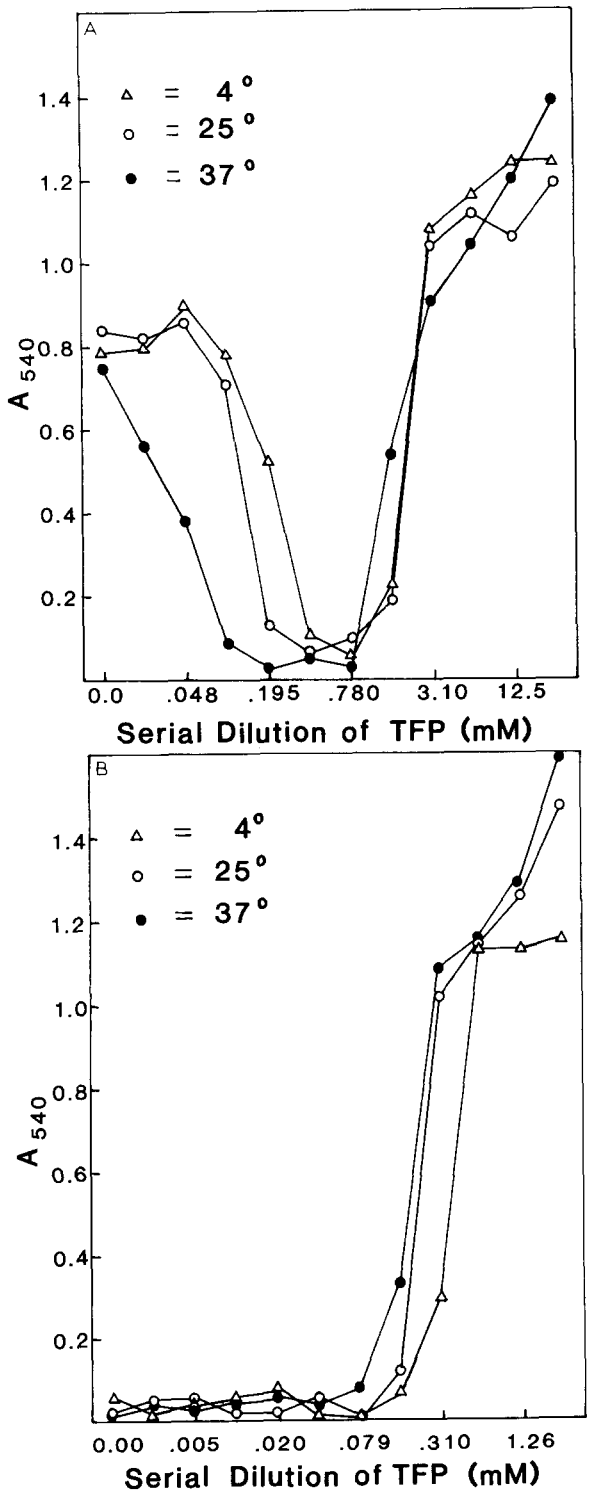

Fig. 1. Temperature-dependent inhibition of viral hemolysis by TFP. (A) Sendai virus (1 μg protein) was preincubated in 11 μl of serial dilutions of trifluoperazine in 0.15 M NaCl/0.01 M histidine (pH 6) for 30 min at 4, 25 and 37°C prior to dilution with cold 0.15 M NaCl/0.01 M Mops (pH 7) and addition of 9 μl of 20% (v/v) red cells. The 200 μl assay mixture was incubated for 10 min at 4°C and for 30 min at 37°C and centrifuged. The supernatants were read at 540 nm. (B) 11 μl of serial dilutions of trifluoperazine in 0.15 M NaCl/0.01 M histidine (pH 6) were mixed with 180 μl of cold 0.15 M NaCl/0.01 M Mops (pH 7)/9 μl 20% (v/v) red cells, incubated for 10 min at 4°C and for 30 min at 4, 25 or 37°C and centrifuged. The supernatants were read at 540 nm.

sis. Thus, TFP reduced the hemolytic activity of Sendai virus much more than its hemagglutination activity.

Finally, the surface activity of TFP was measured as a function of concentration at 25 and at 37°C to determine whether the temperature dependence of TFP-inactivation of viral hemolysis could be due to an increase in the surface activity of TFP with temperature. As measured by the Wilhelmy plate method, the critical micellar concentration of TFP was 0.75 mM and the maximum surface tension of TFP in 0.15 M NaCl/0.01 M histidine (pH 6) was 40 dyn/cm at both 25 and 37°C (Fig. 3). The histidine-buffered saline buffer had the same surface tension as water, i.e., about 70 dyn/cm.

Measurement of TFP uptake by Sendai virus

The temperature-dependent inactivation of Sendai virus by TFP could reflect either a temperature-dependent sensitivity of the virus to TFP and/or a temperature-dependent uptake of TFP by the virus. These possibilities were tested by determining (1) the partition coefficient of TFP between viral membranes and buffer as a function of TFP concentration and temperature and (2) the concentrations of TFP required for inactivation of viral hemolysis at different temperatures. Calculations based on these data would indicate whether inactivation at lower temperatures were precluded by insufficient TFP uptake at the lower temperatures, relative to the TFP uptake sufficient to inactivate at 37°C. First, TFP uptake was estimated directly, according to a centrifugation method for separating viral membrane from buffer [19]. The partition coefficients thus determined are plotted as solid symbols in Fig. 4 against the concentration of TFP, N_t/V_b, at which they had been obtained.

TFP uptake by Sendai virus was also measured

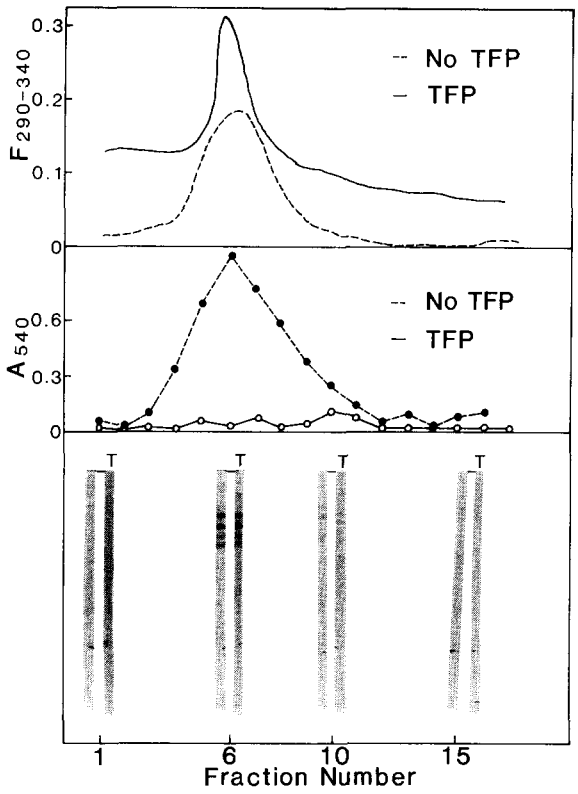

Fig. 2. Sendai virus (330 μg protein) was incubated with or without 10^{-7} mol of TFP in 0.1 ml of 0.15 M NaCl/0.01 M histidine (pH 6) for 30 min at 37°C. Samples were layered onto gradients of 20–60% (w/v) sucrose in the incubation buffer plus 0.02% NaN_3 and centrifuged in an SW-50.1 rotor at 45000 rpm for 100 min at room temperature. Upper panel: Centrifuged gradients were pumped through a fluorometer flow cell and the tryptophan fluorescence (excitation at 290 nm and emission at 340 nm) was recorded for untreated (dashed line) and TFP-treated (unbroken line) virus. Middle panel: 12-drop fractions were collected from each gradient containing TFP-treated (unbroken line) or untreated (dashed line) virus and 25 or 50 μl of each fraction incubated with 15 μl of 20% (v/v) red cells/160 μl Mops-buffered saline for 10 min at 4°C and for 30 min at 37°C. After centrifugation, supernatants were read at 540 nm. Lower panel: The remaining portions of selected fractions from gradients containing TFP-treated ('T') or untreated virus were layered onto gels described in Materials and Methods and subjected to SDS-polyacrylamide gel electrophoresis.

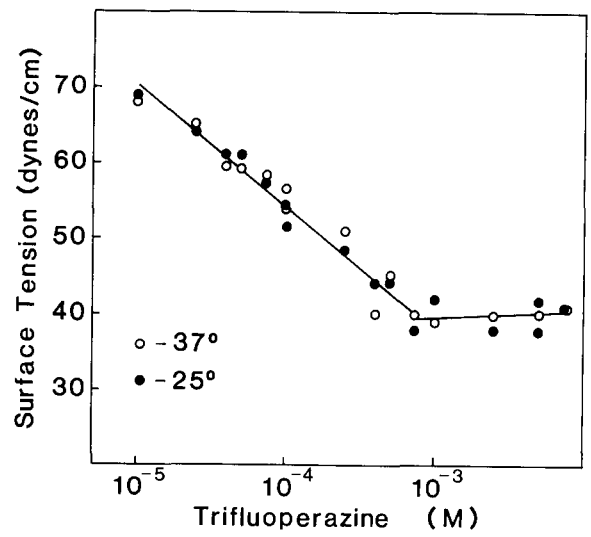

Fig. 3. Surface activity of trifluoperazine at 25 and at 37°C. The surface tension of various concentrations of trifluoperazine in 0.15 M NaCl/0.01 M histidine (pH 6) was measured by the Wilhelmy plate method [2,21].

under conditions of viral hemolysis inactivation at 4, 25 and 37°C, according to the indirect method of Lieber et al. [19]. With this method, drug uptake by the membrane is indicated by a manifestation of its presence in the membrane such as hemolysis or inactivation of viral hemolysis, rather than by direct measurement of the drug. Increasing concentrations of TFP were applied at four increasing ratios of virus/buffer for 75 min at 4, 25 and 37°C. Aliquots of the TFP-treated virus were then assayed for hemolytic activity in the presence of excess red cells to determine the TFP concentrations which had inhibited half of the hemolytic activity of the virus at a particular virus/buffer ratio. The TFP concentrations inactivating half of the Sendai virus assayed were plotted (not shown) as N_t/V_m against the relevant ratio of buffer to virus, V_b/V_m, to obtain an intercept, $(-K_p)$, the negative of the partition coefficient of TFP between the virus membrane and the buffer. The partition coefficients between the virus and the buffer at 4, at 25 and at 37°C are plotted vs. the TFP concentration at which they were obtained as open symbols in Fig. 4. Each of the indirectly determined partition coefficients (open symbols) is within 10% of the straight line defined by linear-regression analysis of the directly determined partition coefficients (solid symbols). As found by Lieber et al. [19], therefore, use of the membrane action of a lipid-soluble drug to measure drug uptake by the membrane gave the same partition coefficient of the drug as its direct measurement.

Three aspects of Fig. 4 are noteworthy: (1) The partition coefficient of TFP between the virus

membrane and buffer is temperature-dependent, as emphasized by the inset plot of the Y intercepts or maximum partition coefficients of the larger plot vs. temperature. (2) The partition coefficient decreases with increasing phenothiazine concentrations, as reported by others [6,19,36]. (3) When the partition coefficients defined by the straight lines in Fig. 4 are used to calculate the uptake of TFP by Sendai virus, it is clear that the amounts of TFP taken up at 25 and at 4°C are enough to inactivate the virus at 37°C, although not enough to inactivate the virus at 25 and at 4°C. For example, when its membrane contains $0.39 \cdot 10^{-3}$ mol TFP/cm^3 lipid at 37°C in the presence of 0.17 mM TFP, the virus loses half of its hemolytic activity, but when it contains as many moles of TFP/cm^3 lipid in the presence of 0.22 mM TFP at 25°C or in the presence of 0.29 mM TFP at 4°C, the virus retains all of its hemolytic activity. In other words, even when the TFP concentration in the virus membrane at 25 and at 4°C is the same as that which can reduce viral activity by 50% at 37°C, viral hemolytic activity is unaffected at 25 and at 4°C. Reduction of the viral hemolytic activity by 50% does not occur at 25°C until the TFP concentration in the viral membrane is $0.44 \cdot 10^{-3}$ mol TFP/cm^3 lipid or at 4°C until the TFP concentration in the viral membrane is $0.5 \cdot 10^{-3}$ mol TFP/cm^3 lipid. Thus, 13 and 28% more TFP must enter the viral membrane at 25 and at 4°C,

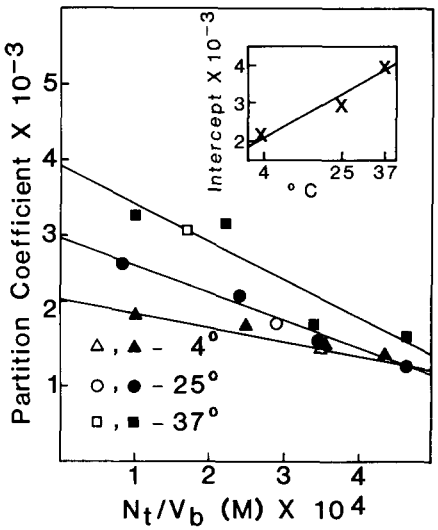

Fig. 4. Partition coefficient, K_p, of TFP between virus membranes and buffer plotted as a function of TFP concentration, N_t/V_b, at 4, 25 and 37°C. The open symbols represent values of K_p determined by indirectly measuring TFP by assaying viral hemolysis, and the solid symbols represent values of K_p determined by directly measuring TFP spectrophotometrically. For direct measurement of TFP partition, $(25–100) \cdot 10^{-6}$ cm^3 of viral lipid were incubated for 60 min at various temperatures in 200 μl of 0.15 M NaCl/0.01 M histidine (pH 6) containing 0.1–0.5 mM TFP. For indirect measurement of TFP partition, $2.5 \cdot 10^{-6}$, $5.0 \cdot 10^{-6}$, $7.5 \cdot 10^{-6}$ and $10.0 \cdot 10^{-6}$ cm^3 of viral lipid were incubated at various temperatures for 75 min in 24 μl of 0.15 M NaCl/0.01 M histidine (pH 6) containing various concentrations of TFP. 5 μl of TFP-treated virus was then diluted with 40 μl of 10% red cells/195 μl of Mops-buffered saline, incubated for 10 min at 4°C and for 45 min at 37°C. The intercepts or maximal partition coefficients from the larger plot are re-plotted against temperature in the inset to indicate the significant dependence on temperature of the partition coefficient of TFP between the viral membrane and buffer.

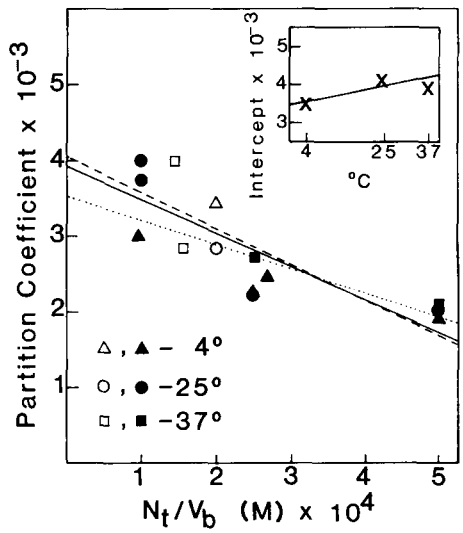

Fig. 5. Partition coefficient, K_p, of TFP between red cell membranes and buffer plotted as a function of TFP concentration, N_t/V_b, at 4, 25 and 37°C. The open symbols represent K_p values determined indirectly by measuring TFP-induced hemolysis, whereas the solid symbols represent K_p values determined directly by measuring TFP spectrophotometrically. For TFP-induced hemolysis, $(2.5–5.0) \cdot 10^{-6}$ cm^3 of red cell lipid were incubated in 40 μl of 0.15 M NaCl/0.01 M histidine (pH 6) for 70 min, and for direct measurement of K_p, $(16.3–97.8) \cdot 10^{-6}$ cm^3 of red cell ghost lipid were incubated in 200 μl of 0.15 M NaCl/0.01 M histidine (pH 6) for 60 min. Lines representing data at the same temperature of incubation are dotted for 4°C, dashed for 25°C and unbroken for 37°C. The intercepts or maximal partition coefficients given in the larger plot are re-plotted against temperature in the inset to indicate the relatively slight dependence on temperature of the partition coefficient of TFP between the red cell membrane and the buffer.

respectively, than at 37°C to inactivate the same amount of Sendai virus.

TFP-induced hemolysis as a function of temperature

Uptake of TFP by red cells and TFP-induced hemolysis were measured as a function of temperature to determine whether uptake of TFP by viral membranes and TFP inactivation of viral hemolysis were virus-specific in their temperature dependence. The partition coefficients of TFP between red cells and buffer, determined by both direct and indirect methods as a function of TFP concentration and temperature and plotted against N_t/V_b in Fig. 5, are more variable than partition coefficients of TFP between virus and buffer in Fig. 4. Nevertheless, the y intercepts or maximal partition coefficients of TFP between red cells and buffer in the inset of Fig. 5 are clearly less temperature-dependent than the y intercepts or maximal partition coefficients of TFP between virus and buffer in the inset of Fig. 4. Thus, TFP uptake by Sendai virus is significantly more temperature-dependent than TFP uptake by red cells.

On the other hand, virus and red cells are similar, insofar as more TFP is necessary at lower temperatures to cause both the inactivation of viral hemolysis and the lysis of red cells. When half of the red cells are lysed at 4, 25 and 37°C, the uptake of TFP by red cell membranes, calculated from the partition coefficients in Fig. 5, are $0.5 \cdot 10^{-3}$, $0.53 \cdot 10^{-3}$ and $0.42 \cdot 10^{-3}$ mol TFP/cm^3, respectively. Thus, not much different from the inactivation of virus, 21 and 16% more TFP must enter the red cell membrane at 25 and at 4°C, respectively, than at 37°C to lyse the same number of red cells.

Fluorescence of viral tryptophan and TFP

To probe the proximity of TFP to the viral proteins, TFP-treated Sendai virus and appropriate control samples were monitored for fluorescence energy transfer from tryptophans in viral proteins to TFP. Samples were excited at 290 nm, the excitation maximum of tryptophan, while their emission was scanned from 310 to 550 nm so as to include the emission maximum of tryptophan at 340 nm, the excitation maximum of TFP at 340 nm and the emission maximum of TFP at 480 nm. The scan in Fig. 6 of untreated virus, diluted 1 : 100 with 0.01 M Mops (pH 7) (A) features a single peak due to tryptophan. In contrast, the scan of virus exposed to and completely inactivated by 0.75 mM TFP, then diluted 1 : 100 with 0.01 M Mops (pH 7) (D) features a peak of tryptophan fluorescence about 3/4 that of untreated virus and a second, barely visible peak in the region of TFP emission probably due to direct excitation of TFP itself, rather than secondary excitation by tryptophan emission. Scans B and C

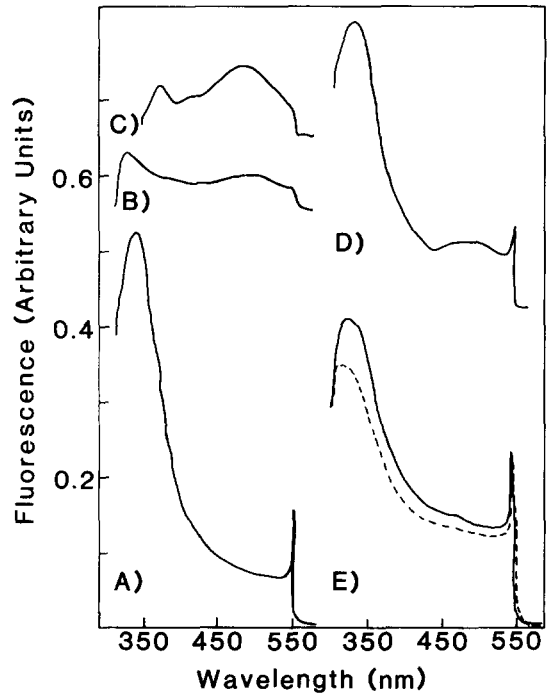

Fig. 6. Fluorescence of viral tryptophans and TFP. (A) Untreated Sendai virus (115 μg protein) in histidine-buffered saline diluted 1 : 100 in 10 mM Mops (pH 7) and excited at 290 nm, the excitation maximum of viral tryptophan. (B) 0.75 mM TFP in histidine-buffered saline incubated at 37°C for 30 min, diluted 1 : 100 in 10 mM Mops and excited at 290 nm. (C) Same as (B) but excited at 340 nm, the excitation maximum of TFP. (D) Sendai virus (115 μg protein)/49 nmol of TFP (0.75 mM) were incubated in histidine-buffered saline at 37°C for 30 min, diluted 1 : 100 in 10 mM Mops (pH 7) and excited at 290 nm. (E) Sendai virus (60 μg of viral protein) was incubated with (dashed line) or without (unbroken line) 12.6 μg of trypsin in 200 μl of 0.01 M sodium phosphate (pH 7) for 20 min at 37°C. 1.8 ml of cold 0.15 M NaCl/0.01 M sodium phosphate (pH 7) (phosphate-buffered saline) containing 24 μg of turkey egg white trypsin inhibitor was added and the samples centrifuged for 1 h at 15000 rpm, resuspended in 0.2 ml of phosphate-buffered saline, diluted 1 : 100 in phosphate-buffered saline and excited at 290 nm.

were taken of 0.0075 mM TFP in 0.01 M Mops (pH 7) – i.e., the concentration of TFP in the TFP-treated virus samples (D) – excited at 290 nm and at 340 nm to estimate the TFP emission due to excitation of tryptophan or to direct excitation of TFP, respectively. Mixing of TFP with purely lipid vesicles had no effect on the TFP fluorescence (not shown), so the fluorescence of TFP excited at 340 nm in (C) minus that of TFP excited at the maximum for viral tryptophan in (B) is a reasonable estimate of the maximum TFP fluorescence possible in the event of energy transfer from viral tryptophan to TFP. Scans of Sendai virus not exposed to TFP but heated for 5 min at 100°C to abolish its hemolytic activity (not shown) evidenced a loss of viral tryptophan fluorescence comparable to the scan of Sendai virus inactivated by TFP (D).

The reduction in viral tryptophan fluorescence associated with inactivation by TFP treatment (D) or by heating (not shown) may reflect quenching of F protein tryptophans, three of which reside in the extracellular surface fragment of F protein [5]. Specific removal of the extracellular fragment of F protein and consequent inactivation of hemolytic activity by trypsin treatment, according to the procedure of Shimizu and Ishida [33], also results in a comparable reduction of viral tryptophan fluorescence (Fig. 6E, dashed line). Thus far, the data allow the possibility that the tryptophans, which are quenched after heating and TFP treatment, are the same tryptophans which are removed by trypsin digestion.

Discussion

The biological effects of phenothiazines vary qualitatively as a function of their concentration: (1) from nM to 100 nM levels, at which these drugs are neuroleptic and therapeutically useful [32], (2) through μM to 50 μM levels, at which these drugs bind to and/or perturb proteins such as troponin C [18] and calmodulin [23], as well as inhibit hemolysis and block nerve conduction [31], and (3) above 50 μM to near mM levels, at which these drugs are hemolytic [31], act on membrane-bound enzymes through their phospholipid environment [28] and, as reported here, inactivate the hemolytic activity of Sendai virus. Since TFP attained concentrations of $(0.39–0.5)\cdot 10^{-3}$ mol TFP/cm^3 of lipid in the viral membrane – levels which produce holes in red cell [19] and retroviral membranes [38] – the original bilayer arrangement of viral lipids was probably perturbed. Nevertheless, Sendai virus proteins remained associated in proportions characteristic of the native virus and with material sedimenting slightly faster than native virus (Fig. 2).

Given a gross membrane perturbation by TFP, therefore, it is not surprising that the viral fusion protein apparently undergoes an irreversible, conformational or organizational change to a non-fusogenic state on exposure to TFP. The much greater effect of TFP on viral hemolysis, than on viral hemagglutination points to a selective inhibition of the fusion protein and not the hemagglutinin. A similar denaturation may occur on heating to 100°C for 10 min (e.g., Ref. 15) and on exposure to fluid-phase fatty acids [21], such that Sendai virus becomes non-hemolytic. The approx. 25% decrease in tryptophan fluorescence of TFP-treated Sendai virus (Fig. 6D) could be evidence for a conformational change in F protein or for the proximity of TFP to F protein tryptophans. That trypsin, which selectively inactivates viral hemolysis by removing about 2/3 of F protein [33], effects a comparable decrease in viral tryptophan fluorescence (Fig. 6E) allows, but does not prove, the possibility that TFP directly or indirectly causes the fluorescence of F protein tryptophans to be quenched.

Comparison of viral and red cell membranes with respect to their uptake of and perturbation by TFP as a function of temperature reveals two noteworthy facts:

(1) Viral uptake of TFP is more temperature-dependent than red cell uptake of TFP. Specifically, the partition coefficient of TFP between red cells and buffer is 1.06-times at 37°C, 1.2-times at 25°C and 1.69-times at 4°C that of TFP between virus and buffer. In other words, the partition coefficients are the same at 37°C but that for Sendai virus is depressed compared to that for red cells at 4°C. Two kinds of structural changes may account for less uptake of phenothiazines by viral membranes at lower temperatures: (a) Ordering of lipid packing. Chlorpromazine partitions 2–3-times more readily into liquid-crystal-phase than into

gel-phase phospholipid vesicles [36] and more than twice as readily into fluid-phase phospholipid vesicles as into phospholipid vesicles containing 20 mol% cholesterol [20]. That the partition coefficients of TFP into virus membrane display greater temperature dependence than those of TFP into red cell membrane may indicate the existence of more regions in viral membranes of more tightly packed lipid, which become progressively less tightly packed from 4 to 37°C and take up more TFP as a result. (b) Ordering of protein packing. Another well-documented, temperature-dependent characteristic of Sendai virus is its lysis of artificial lipid vesicles [24,34], as well as red cells [17], which has recently been correlated with a temperature-dependent increase in the rotational mobility of F protein labeled with eosin and detected by fluorescence spectroscopy [17]. Because the mobility of diphenylhexatriene in viral membranes did not increase correspondingly, the increased mobility of F protein with temperature appeared not to reflect increased fluidity of the hydrophobic region of the bilayer [17]. Since F protein activity rises sharply above 30°C [17,24,34], whereas the partition coefficient of TFP increased more linearly with temperature, however, ordering of lipid – rather than protein – packing may be more relevant to the effect of temperature on TFP uptake. It is premature to discount proteins as agents of temperature-dependent changes in the structure of the viral membrane, however, since trypsin treatment which selectively inactivated F protein was found to enhance the unusually restricted movement of the phospholipid headgroups in the Sendai virus membrane [1].

(2) Although viral uptake of TFP is more temperature-dependent than red cell uptake of TFP, the occurrence of both inactivation of viral hemolysis and lysis of red cells require the presence in both types of membranes of more TFP at lower temperatures. Not only are the amounts of TFP associated with both lysed, red cell and inactivated, viral membranes at 37°C similar (i.e., $0.42 \cdot 10^{-3}$ and $0.39 \cdot 10^{-3}$ mol TFP/cm^3 lipid, respectively), but the increments in TFP uptake necessary to produce these effects at lower temperatures are also similar. Thus, the need for more TFP to inactivate Sendai virus at lower temperatures does not reflect a specifically viral characteristic, in contrast with the temperature-dependent, viral uptake of TFP. It should be pointed out that since the surface tension and the critical micellar concentration of TFP are the same at 25 and at 37°C (Fig. 3), neither the temperature dependence of TFP uptake by Sendai virus nor the temperature dependence of TFP effects on both virus and red cells can be explained by temperature-dependent changes in the surface activity of TFP. As stated at the outset of this Discussion, inactivation of viral hemolysis by TFP is definitely in that class of phenothiazine effects which includes red cell lysis, although the mechanism of inactivation of Sendai virus by trifluoperazine remains to be determined.

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