Proteolysis of ankyrin and of band 3 protein in chemically induced cell fusion

Ca²⁺ is not mandatory for fusion

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1. Human erythrocytes were fused by incubation with 0.5–2 mM-chlorpromazine hydrochloride at pH 6.8–7.6. 2. Fusogenic preparations of chlorpromazine were cloudy suspensions of microdroplets, and below pH 6.8 chlorpromazine gave clear solutions that were inactive. 3. Unlike control cells, the lateral mobility of the intramembranous particles of the PF-fracture face of chlorpromazine-treated cells was relatively unrestricted, since the particles were partly clustered at 37°C and they exhibited extensive cold-induced clustering. 4. Ca²⁺ stimulated fusion, but fusion was only very weakly inhibited by EGTA (10 mM) and by *N*-ethylmaleimide (50 mM); pretreatment of the cells with Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one) (7.5 mM) markedly inhibited fusion. 5. Changes in the membrane proteins of erythrocytes fused by chlorpromazine, before and after treatment with chymotrypsin to remove band 3 protein, were investigated. 6. The several observations made indicate that the Ca²⁺-insensitive component of fusion is associated with degradation of ankyrin (band 2.1 protein) to band 2.3–2.6 proteins and to smaller polypeptides by a serine proteinase that is inhibited by Tos-Lys-CH₂Cl, and that the component of fusion inhibited by EGTA and *N*-ethylmaleimide is associated with degradation of band 3 protein to band 4.5 protein by a Ca²⁺-activated cysteine proteinase. 7. Proteolysis of ankyrin appeared to be sufficient to permit the chlorpromazine-induced fusion of human erythrocytes, but fusion occurred more rapidly when band 3 protein was also degraded in the presence of Ca²⁺. 8. Since other cells have structures comparable with the spectrin–actin skeleton of the erythrocyte membrane, the observations reported may be relevant to the initiation of naturally occurring fusion reactions in biomembranes. 9. It is also suggested that, should polypeptides with fusogenic properties be produced from integral and skeletal membrane proteins by endogenous proteolysis, their formation would provide a general mechanism for the fusion of lipid bilayers in biomembrane fusion reactions.

Abbreviations used: Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

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In this laboratory and elsewhere considerable attention has been paid to changes in the organization of the lipid components of membranes that occur during membrane fusion (for review see Lucy, 1982). However, as far as the control of biomembrane fusion reactions is concerned, an understanding of changes in membrane structure that precede the fusion of lipid bilayers is probably at least as if not more important. To this end, we have investigated the behaviour of membrane proteins in the chemically induced fusion of erythrocytes

and, on the basis of a previous study on human erythrocytes fused by oleoylglycerol, it was suggested that proteolytic activity that results in an increased freedom of movement of integral membrane proteins could be an important early feature of membrane fusion (Quirk *et al.*, 1978). Such a proteinase-mediated movement could facilitate membrane fusion in areas of lipid bilayer free from protein, in accordance with the model that we originally proposed from this laboratory (Ahkong *et al.*, 1975). Subsequent work on rat erythrocytes fused by benzyl alcohol implicated the activity of a Ca^{2+} -activated cysteine proteinase in the fusion process, and it was then proposed that this proteinase acts to degrade spectrin-binding proteins that attach band 3 protein to the membrane cytoskeleton (Ahkong *et al.*, 1980).

In the present paper, work is reported on the fusion of human erythrocytes induced by chlorpromazine. This compound has not previously been investigated as a chemical fusogen, and it is of particular interest because the fusion induced occurs at a high incidence in the presence of EGTA, thereby contrasting with the emphasis that other workers have placed on the importance of Ca^{2+} in the fusion of lipid bilayers in model systems (Papahadjopoulos *et al.*, 1979) and that we have given previously in chemically induced cell fusion to the activation of cellular proteinases by the entry of exogenous Ca^{2+} (Blow *et al.*, 1979; Ahkong *et al.*, 1980). The present observations thus clarify our previous finding that the fusion of human erythrocytes by oleoylglycerol was not completely inhibited by EGTA (Quirk *et al.*, 1978), as it now appears that at least two proteinases are involved in the chemically induced fusion of these cells, only one of which is stimulated by Ca^{2+} and is inhibited by EGTA and by *N*-ethylmaleimide.

With regard to the more general significance of the observations described, recent developments have indicated that the erythrocyte membrane may serve as a valid model for more complex membranes. The present work may therefore be generally relevant to the early stages of biomembrane fusion reactions. By analogy with the fusogenic proteins of certain viruses, polypeptides formed by the endogenous proteolysis of membrane proteins may, if they contain appropriate hydrophobic sequences, also provide a general means of inducing the fusion of the lipid bilayers in such reactions.

Materials and methods

Erythrocytes

Blood from human donors was mixed with cold citrate anti-coagulant (De Gowin *et al.*, 1949) and used for experiments on cell fusion on the same

day, or stored at 4°C for up to 1 week when used for the preparation of spectrin or inside-out vesicles. The blood was washed three times in a salt solution containing NaCl (116 mM), glucose (1 mg/ml), penicillin G (120 µg/ml) and streptomycin sulphate (100 µg/ml), buffered at pH 7.4 with sodium cacodylate (10 mM). The buffy coat was carefully removed with each washing and the erythrocytes were resuspended in 4 vol. of the buffered salt solution, which additionally contained dextran (80 mg/ml) (Ahkong *et al.*, 1973). They were stored at 4°C until used. Examination of the cells by optical microscopy showed that there was virtually no contamination by leucocytes.

Reagents

All reagent chemicals used were commercially obtained and were of analytical grade. Dextran (average M_r 60000–90000; clinical grade), Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl, Tos-Arg-OMe, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, chlorpromazine hydrochloride, histamine dihydrochloride, EGTA, DL-dithiothreitol, α -chymotrypsin (Tos-Lys-CH₂Cl-treated) and α -chymotrypsin (inactivated by di-isopropyl phosphorofluoridate) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Sodium cacodylate, acrylamide (specially pure for electrophoresis), sodium dodecyl sulphate (specially pure) and Coomassie Brilliant Blue R250 were from BDH Chemicals (Poole, Dorset, U.K.). Glutaraldehyde (Gurr) and Alcian Blue 8GX were supplied by Hopkin and Williams (Chadwell Heath, Essex, U.K.).

Incubation of erythrocytes with chlorpromazine

Chlorpromazine hydrochloride was dissolved to a concentration of 15 mM in the salt/dextran solution and the pH re-adjusted to 7.4. At this pH chlorpromazine formed a white milky suspension. Erythrocytes (approx. 3×10^8 cells/ml) in the salt/dextran medium were pre-incubated with or without various proteinase inhibitors or thiol-specific reagents for 15 min at 37°C. CaCl_2 , MgCl_2 , EGTA or EDTA was then added to a final concentration of 10 mM (or as otherwise indicated) and the incubation was continued for a further 5 min period. Chlorpromazine was subsequently added (final concentration 2 mM or as otherwise indicated) and the progress of the incubation was followed by removing samples for phase-contrast microscopy.

Stock solutions of the proteinase inhibitors Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl and phenylmethanesulphonyl fluoride were prepared in methanol at 0.1 M. Stock solutions of Tos-Arg-OMe (20 mM), *N*-ethylmaleimide (50 mM) and histamine (200 mM) were prepared in the salt/dextran medium and the pH was adjusted to pH 7.4. In some experiments,

erythrocytes were pre-incubated in the salt/dextran medium with inhibitor, methanol or *N*-ethylmaleimide at 37°C for various time periods and were then washed twice in the salt/dextran medium. The cells were then resuspended in the same medium and incubated as above. Cells incubated with chlorpromazine were fixed as for electron microscopy (see below), and counted by using phase-contrast microscopy. The fusion index (expressed as a percentage) was determined as described previously (Quirk *et al.*, 1978).

Treatment of erythrocytes with α -chymotrypsin

Washed human erythrocytes were resuspended in buffered salt solution without added dextran, pH 7.4, and incubated with α -chymotrypsin (200 μ g/ml final concentration) for 1 h at 37°C. The cells were washed twice, resuspended to 50% haematocrit, and the chymotrypsin reaction was inhibited by treatment with Tos-Phe-CH₂Cl (100 μ g/ml) for 5 min at 37°C (Siegel *et al.*, 1980). After two further washes, the erythrocytes were resuspended in salt/dextran medium, pH 7.4, and maintained at 4°C until used within approx. 1 h.

Freeze-fracture electron microscopy

Incubated erythrocytes were treated in one of two ways. First, samples (1 vol.) were fixed at the temperature indicated for 30 min in a solution of glutaraldehyde (2%, v/v) in 0.1 M-sodium cacodylate/HCl buffer, pH 7.4. Glycerol (30%) was added and the samples were centrifuged in a Beckman micro-centrifuge. Pellets were mounted between two Balzers gold discs sandwiched together and frozen in liquid Freon 22. Secondly, very small (approx. 10 μ l) samples of unfixed erythrocytes were placed on thin copper supports maintained at the temperature indicated in the Results section and equilibrated at this temperature for 10 min. A gold 400-mesh grid was then placed on to the specimen and a second copper support was placed over this to form a sandwich, which was then frozen in liquid Freon 22. This method gives very high freezing rates (Gulik-Krzywicki & Costello, 1978). No differences were found in the observations made by the two methods. Freeze-fracturing was performed as described previously (Lang & Brown, 1981). Replicas were examined in an AEI EM6B electron microscope.

Analyses of membrane proteins

Erythrocyte 'ghosts' were prepared by the hypotonic lysis method of Hanahan & Ekholm (1974) with the modification reported previously (Ahkong *et al.*, 1980). Protein was determined by the method of Lowry *et al.* (1951). Samples for gel electrophoresis were solubilized in a modified Laemmli's sample buffer (Ahkong *et al.*, 1980); 20–40 μ l,

containing 40–50 μ g of proteins, was loaded per lane. Electrophoresis was performed in slab gels (5 or 7%, w/v, acrylamide in the separating gel; 2.5% acrylamide in the stacking gel), with the buffer system of Laemmli (1970). The proteins were detected by staining with Coomassie Blue, essentially as described by Fairbanks *et al.* (1971).

Purification of spectrin and its binding to spectrin-depleted vesicles

Packed erythrocyte 'ghosts' (5–6 ml) were resuspended in 5–10 vol. of extraction buffer (0.3 M-sodium phosphate buffer, pH 8.0, containing 0.1 M-EDTA and 0.1 M-phenylmethanesulphonyl fluoride) and incubated at 37°C for 30 min. The extraction and purification of spectrin were then carried out, essentially as described by Litman *et al.* (1980), at 0–4°C. At the end of this procedure, pooled peak fractions from Sepharose chromatography that contained highly purified spectrin (band 1 and 2 proteins) were concentrated by ultrafiltration to final protein concentrations of 1–2 mg/ml, and were then stored at 0–4°C for up to 2 weeks.

Inside-out spectrin-depleted vesicles were prepared by the procedure described by Steck (1974), and binding studies were then performed by mixing the vesicles or resealed 'ghosts' (600 μ g of membrane protein) with spectrin (0–200 μ g) in a total volume of 1 ml of buffer containing 20 mM-KCl, 0.5 mM-NaN₃ and 0.7 mM-sodium phosphate buffer, pH 6.6, followed by incubation for 90 min at 0°C. Membrane-bound spectrin was separated from free spectrin by centrifugation at 20000 *g* for 40 min, and the supernatants were removed for the determination of their protein concentrations. The protein composition of the membrane pellets was then determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Results

Effects of pH

The optimum pH for the fusion of human erythrocytes by chlorpromazine was 7.4, and the fusion did not occur below pH 6.8. If the pH was higher than 7.6, the cells were lysed rather than fused. Experiments were performed over the range pH 6.0–8.0 in steps of 0.2 pH unit, and we found that chlorpromazine hydrochloride forms a milky white dispersion in the salt/dextran medium used at pH values greater than 6.8. The dispersed material appeared as spherical microdroplets (diameter approx. 1 μ m) when viewed by phase-contrast optical microscopy and in negatively stained preparations in the electron microscope (Fig. 1a). As with the fusion of human erythrocytes by microdroplets of oleoylglycerol (Quirk *et al.*, 1978), the

Fig. 1. Morphological behaviour of human erythrocytes on treatment with chlorpromazine

(a) An electron micrograph of a suspension of chlorpromazine (15 mM) in salt/dextran medium, pH 7.4, negatively stained with 1% ammonium molybdate as described in the Materials and methods section. Magnification $\times 60000$. (b) Human erythrocytes in salt/dextran medium, pH 7.4, containing 10 mM- CaCl_2 after 5 min incubation at 37°C in the presence of 2 mM-chlorpromazine hydrochloride. Microdroplets of chlorpromazine are present on the cell surfaces (arrow), and some clumping of the cells is apparent. (c) Cells incubated as in (b) but after 30 min with 2 mM-chlorpromazine. The cells appear swollen, and some multicellular bodies are visible (arrows). (d) Cells incubated for 30 min in the presence of 2 mM-chlorpromazine as in (c) but with 10 mM-EGTA replacing 10 mM- CaCl_2 . Cell doublets (arrow) are seen. Phase-contrast microscopy was used for (b)–(d); magnification $\times 800$.

microdroplets of chlorpromazine were frequently observed adhering to the surfaces of the erythrocytes (Fig. 1b).

Morphology and time course of cell fusion: role of Ca^{2+}

Human erythrocytes incubated at 37°C in the presence of 10 mM- Ca^{2+} and chlorpromazine (2 mM at pH 7.4) rapidly became spherical and then aggregated. The first cells to fuse, usually within 7 min, were dumb-bell-shaped doublets (Fig. 1c), as in the fusion of these cells by oleoylglycerol (Quirk *et al.*, 1978). On further incubation, tri- and multicellular bodies formed, some of which appeared to be very swollen (Fig. 1d). After 60 min of incubation, 50–80% of the cells had fused [mean fusion index, 64.7 ± 10.1 (mean $\pm$ s.d. in ten experiments)] (Table 1). By that time about 5–10% of the cells had

lysed and the rest were single cells. Fusion was also induced by lower concentrations of chlorpromazine (0.4–2 mM), but it was much slower when the concentration was less than 1 mM.

Unlike the virtually complete inhibition by EGTA of the fusion of rat erythrocytes by benzyl alcohol (Akhong *et al.*, 1980), fusion was only partially inhibited when EGTA or EDTA was present during the incubation of human erythrocytes with chlorpromazine. Thus after 60 min of incubation with 2 mM-chlorpromazine in the presence of 10 mM-EGTA, the mean fusion index was still 30.6 ± 9.6 (mean $\pm$ s.d. in nine experiments) (Table 1). Under these conditions, most of the fused erythrocytes were doublets, and large multicellular bodies were very rarely seen. Mg^{2+} did not stimulate fusion to the extents observed with added Ca^{2+} .

Table 1. *Fusion indices of human erythrocytes treated with chlorpromazine*

Human erythrocytes were incubated, under the conditions shown in the Table, as described in the Materials and methods section. The concentration of chlorpromazine hydrochloride used was 2 mM in all experiments, except for those with α -chymotrypsin-treated cells, when 1 mM-chlorpromazine was used. The fusion indices shown are mean values $\pm$ S.D. for the numbers of independent experiments shown in parentheses.

Pretreatment	Addition on incubation	Incubation time (min) after addition of chlorpromazine	Fusion index
None	10 mM-CaCl ₂	30	34.7 $\pm$ 6 (8)
None	10 mM-EGTA	30	24.6 $\pm$ 3.9 (8)
None	10 mM-CaCl ₂	60	64.7 $\pm$ 10.1 (10)
None	10 mM-EGTA	60	30.6 $\pm$ 9.6 (9)
1 mM-Tos-Lys-CH ₂ Cl	10 mM-CaCl ₂ + 1 mM-Tos-Lys-CH ₂ Cl	30	24.1 $\pm$ 2.7 (4)
1 mM-Tos-Lys-CH ₂ Cl	10 mM-EGTA + 1 mM-Tos-Lys-CH ₂ Cl	30	23.4 $\pm$ 2.3 (4)
7.5 mM-Tos-Lys-CH ₂ Cl	10 mM-CaCl ₂	30	9.2 $\pm$ 1.3 (4)
7.5 mM-Tos-Lys-CH ₂ Cl	10 mM-EGTA	30	8.8 $\pm$ 1.4 (4)
50 mM-N-Ethylmaleimide	10 mM-CaCl ₂	30	30.8 $\pm$ 4.3 (5)
50 mM-N-Ethylmaleimide	10 mM-EGTA	30	24.9 $\pm$ 3.4 (5)
α -Chymotrypsin	10 mM-CaCl ₂	30	75.0 $\pm$ 5.9 (5)
α -Chymotrypsin	10 mM-EGTA	30	73.6 $\pm$ 6.3 (5)

Dextran was not essential for fusion, but in its absence cells treated with chlorpromazine were extensively lysed and fewer cells were fused.

Perturbation of membrane structure by chlorpromazine

By freeze-fracture, small vesicles were observed adjacent to the plasma membranes of the treated cells. These may have been formed by invagination of the membrane around microdroplets of chlorpromazine, and they probably corresponded to the invaginations observed by Ruutu & Collan (1972) in thin sections of chlorpromazine-treated human erythrocytes. Other vesicles were clearly associated with the line of fusion between two cells, and these were apparently produced by multiple fusions between adjacent cells (Fig. 2a).

The appearance of the intramembranous particles of the P-fracture faces of untreated and control cells was consistent with observations made by other workers (Weinstein, 1974; see also Quirk *et al.*, 1978). However, by contrast with the normal immobility of the intramembranous particles of human erythrocytes (Elgsaeter *et al.*, 1976) that results from the binding of a proportion of the molecules of band 3 protein to the membrane skeleton (reviewed by Gratzer, 1981), the intramembranous particles of erythrocytes treated with chlorpromazine in the presence of EGTA were aggregated even when the cells were fixed at 37°C (Fig. 2b). More extensive aggregation (cold-induced clustering) was seen when the treated cells were subsequently incubated for 10 min at 0°C and then fixed at 0°C (Fig. 2c). Both at 37°C (Fig. 2d) and at 0°C (Fig. 2e) the intramembranous particles of cells treated with chlorpromazine in the presence of Ca²⁺ were, by comparison, even more aggregated.

Similar observations were made when the treated cells were rapidly frozen, without chemical fixation or addition of glycerol, as described in the Materials and methods section.

Inhibition of fusion by protein reagents

Pre-incubation with a low concentration (1 mM) of Tos-Lys-CH₂Cl was partially inhibitory. It diminished fusion in the presence of 10 mM-Ca²⁺ to 69% of the control value, but it had no noticeable effect on fusion in the presence of EGTA (Table 1). Higher concentrations of Tos-Lys-CH₂Cl (3–7.5 mM) caused a greater inhibition, and fusion indices of less than 10 were then observed after 30 min with chlorpromazine in the presence of either Ca²⁺ or EGTA. When cells were pretreated with 7.5 mM-Tos-Lys-CH₂Cl and then washed before incubation, fusion was still strongly inhibited (Table 1).

Incubation of rat erythrocytes with 1 mM-N-ethylmaleimide virtually prevents their fusion by benzyl alcohol (Ahkong *et al.*, 1980). However, with human erythrocytes and chlorpromazine, 5 mM-N-ethylmaleimide did not affect fusion in the presence of Ca²⁺ or of EGTA. When the cells were pretreated with 50 mM-N-ethylmaleimide and then washed before incubation with chlorpromazine, there was some inhibition in the presence of Ca²⁺ (fusion index 89% of the control value), but there was still no effect on the fusion induced by chlorpromazine in the presence of EGTA (Table 1). These observations indicate that the fusion of human erythrocytes by chlorpromazine that occurs in the presence of EGTA, unlike the benzyl alcohol-induced fusion of erythrocytes (which is prevented by EGTA), does not involve a (Ca²⁺-activated) cysteine proteinase. Conversely, the fact

Fig. 2. *Ultrastructure of human erythrocytes treated with chlorpromazine*

(a)–(e) Electron micrographs of freeze-fractured preparations of human erythrocytes prepared as described in the Materials and methods section. (a) A multicellular body formed after 30 min of incubation in salt/dextran medium, pH 7.4, in the presence of 2 mM-chlorpromazine and 10 mM-EGTA. The fracture has exposed part of the cytoplasm to reveal intracellular vesicles, some of which lie in the region where membrane fusion appears to have occurred (arrows). Magnification $\times 10000$. (b)–(e) PF-fracture faces of membranes from erythrocytes treated as described below. Magnification $\times 75000$. (b) Cells were treated as in (a), and then maintained at 37°C for 10 min before being fixed at 37°C. (c) Cells were treated as in (a), and then maintained at 0°C for 10 min and fixed at 0°C. Aggregation of the intramembranous particles is apparent. (d) Cells were treated as in (a), but with 10 mM- Ca^{2+} replacing 10 mM-EGTA, and they were then maintained at 37°C for 10 min and fixed at 37°C. Extensive aggregation of intramembranous particles and a decrease in their number are apparent. (e) Cells were treated as in (d), but maintained at 0°C for 10 min and then fixed at 0°C. A very extensive aggregation of the intramembranous particles has occurred, and protuberances free from intramembranous particles are visible (arrows).

that Tos-Lys- CH_2Cl markedly inhibited the fusion of human erythrocytes by chlorpromazine when EGTA was present suggests that a serine protein-

ase is concerned in fusion occurring in the presence of EGTA.

The serine proteinase inhibitor phenylmethane-

sulphonyl fluoride (1 mM) had no effect on the fusion of human erythrocytes by chlorpromazine, probably because the inhibitor fails to enter the treated cells (cf. Siegel *et al.*, 1980). Neither Tos-Arg-OMe (2 mM), a substrate analogue of serine proteinases, nor Tos-Phe-CH₂Cl was inhibitory. Histamine (20 mM), a substrate for the Ca²⁺-activated transglutaminase in human erythrocytes (Lorand *et al.*, 1978), slightly stimulated the fusion of human erythrocytes by chlorpromazine.

Binding of spectrin by chlorpromazine-treated membranes

To investigate possible relationships of chlorpromazine-induced cell fusion to the degradation of spectrin-binding proteins, the spectrin-binding properties of inside-out vesicles prepared from cells treated in the presence of 10 mM-Ca²⁺ were studied. Although 2 mM-chlorpromazine was used for most of the present work (Table 1), it was necessary to use 1 mM-chlorpromazine for these experiments because the membranes of cells treated with the higher concentration of fusogen did not endocytose extensively in the vesiculation buffer. When spectrin (200 µg) was added to 600 µg of inside-out vesicles from control or chlorpromazine-treated cells, the quantity of spectrin bound was 94 ± 8 µg (mean $\pm$ S.D.) (i.e. 156 µg of spectrin/mg of membrane protein) and 68 ± 12 µg (mean $\pm$ S.D.) (113 µg of spectrin/mg of membrane protein) respectively in three experiments. These observations indicate that the treatment of human erythrocytes with chlorpromazine leads to a breakdown of spectrin-binding proteins.

Changes in membrane proteins: proteinase activity

When the cells were fused by incubation at 37°C with 2 mM-chlorpromazine for 15 min in the presence of 10 mM-Ca²⁺, band 2.1 protein was decreased, and there was an increased staining of band 2.3–2.6 proteins (compare Figs. 3a and 3c). After 30 min with 2 mM-chlorpromazine and 10 mM-Ca²⁺ there was less band 2.3 protein, and two or three sharp bands were present in the region normally occupied by the diffuse band 3 region (Fig. 3b). These observations are consistent with the fusion of cells by chlorpromazine being associated with the degradation of the spectrin-binding protein ankyrin (band 2.1 protein) successively to a series of smaller proteins by one or more endogenous proteinases (Siegel *et al.*, 1980). Band 1, 2 and 4.1 proteins were decreased with chlorpromazine and 10 mM-Ca²⁺ after 30 min (Fig. 3b), and this was probably due to the transglutaminase activity that is discussed below. The loss of band 3 protein observed under these conditions was, however, unlikely to have been due solely to transglutaminase activity, as it was accompanied by an

Fig. 3. Electrophoretic patterns of 'ghosts' prepared from human erythrocytes

Erythrocytes were incubated and 'ghosts' then prepared and subjected to electrophoresis as described in the Materials and methods section. (a) Membranes from cells incubated at 37°C in salt/dextran medium, pH 7.4, with 10 mM-Ca²⁺ for 5 min, followed by incubation for a further 15 min in the presence of 2 mM-chlorpromazine. (b) Membranes from cells treated as in (a), but after 30 min of incubation with 2 mM-chlorpromazine in the presence of 10 mM-CaCl₂. (c) Membranes from cells incubated in a control experiment at 37°C in salt/dextran medium, pH 7.4, with 10 mM-EGTA for 35 min. (d) Membranes from cells treated as in (a), but with 10 mM-EGTA instead of 10 mM-CaCl₂, and then incubated for 15 min in the presence of 2 mM-chlorpromazine. (e) Membranes from cells treated as in (a), but with 10 mM-EGTA instead of 10 mM-CaCl₂, and then incubated for 30 min in the presence of 2 mM-chlorpromazine. (f) Membranes from cells that were pre-treated with 7.5 mM-Tos-Lys-CH₂Cl for 15 min at 37°C, washed twice in the salt/dextran medium, and then further incubated for 30 min with 2 mM-chlorpromazine and 10 mM-CaCl₂. The insets at the top of each lane are from electrophoretic separations performed in 5%-acrylamide gels, for comparison with the complete electrophoretic profiles that are shown for 7%-acrylamide gels.

increased staining in the region of band 4.5, which was indicative of the proteolytic degradation of band 3 protein.

Erythrocytes incubated at 37°C for 15 min with 2 mM-chlorpromazine in the presence of 10 mM-EGTA also showed an increase in material migrat-

ing in the region between bands 2.1 and 2.6 (Fig. 3*d*). It is to be noted, however, that band 3 was not noticeably decreased after 30 min with chlorpromazine when 10 mM-EGTA was present, nor was band 4.5 enhanced (Fig. 3*e*), by comparison with cells treated with chlorpromazine and 10 mM- Ca^{2+} (Fig. 3*b*). *N*-Ethylmaleimide also largely prevented the loss of band 3 protein (results not shown).

The marked inhibition of chlorpromazine-induced cell fusion by pretreatment with 7.5 mM-Tos-Lys- CH_2Cl (Table 1) was paralleled in a 30 min incubation with Ca^{2+} and chlorpromazine by diminished losses of band 2.1 and 3 proteins and by smaller increases in the staining of bands 2.3, 2.6 and 4.5 (Fig. 3*f*). Tos-Lys- CH_2Cl , which is known to inhibit cysteine proteinases (Whitaker & Perez-Villasenor, 1968), as well as serine proteinases, therefore appeared to inhibit proteolysis of band 3 protein and of ankyrin (band 2.1 protein).

These several observations indicate that the component of chlorpromazine-induced cell fusion of human erythrocytes that is inhibited by EGTA and *N*-ethylmaleimide is associated with the degradation of band 3 protein to band 4.5 protein by a Ca^{2+} -activated cysteine proteinase. Conversely, the Ca^{2+} -insensitive component of fusion appears to be associated with degradation of ankyrin to band 2.3–2.6 proteins by a serine proteinase that is inhibited by Tos-Lys- CH_2Cl .

Chymotrypsin-treated erythrocytes

To monitor further the appearance of new bands in the band 3 region produced by proteolysis of ankyrin, band 3 protein was cleaved by pretreating the erythrocytes with α -chymotrypsin: the enzyme was then inhibited with Tos-Phe- CH_2Cl . As described by Siegel *et al.* (1980), this procedure degraded band 3 protein and produced a fragment with an M_r of approx. 60 000 that appeared in the band 4.5 region (compare Fig. 4*b* with Fig. 4*a*). When the chymotrypsin-treated cells were fused by incubation with 1 mM-chlorpromazine in the presence of 10 mM-EGTA, some five or six new bands were formed in the region originally occupied by band 3 (compare Fig. 4*d* with Fig. 4*b*). Similar new bands were produced when α -chymotrypsin-treated cells were fused with chlorpromazine in the presence of 1 mM- and 5 mM- Ca^{2+} (compare Figs. 4*e* and 4*f* with Fig. 4*c*).

It is noteworthy that, whereas incubation of untreated erythrocytes with 2 mM-chlorpromazine for 30 min gave fusion indices of 34.7% and 24.6% respectively in the presence of Ca^{2+} and of EGTA (Table 1), the chymotrypsin-treated cells gave corresponding indices of 75.0% and 73.6% when incubated with only 1 mM-chlorpromazine for 30 min. Exogenous proteolysis of band 3 protein

Fig. 4. Electrophoretic patterns of 'ghosts' prepared from human erythrocytes pretreated with α -chymotrypsin. Erythrocytes were incubated and 'ghosts' then prepared and subjected to electrophoresis as described in the Materials and methods section. (a) Membranes from untreated erythrocytes. (b)–(g) Membranes from cells that were pretreated with α -chymotrypsin as described in the Materials and methods section, and that were then incubated in salt/dextran medium, pH 7.4, at 37°C as follows: (b) with 10 mM-EGTA for 35 min; (c) with 10 mM- CaCl_2 for 35 min; (d) with 10 mM-EGTA for 5 min followed by a further 30 min with 1 mM-chlorpromazine; (e) with 1 mM- CaCl_2 for 5 min followed by a further 30 min with 1 mM-chlorpromazine; (f) with 5 mM- CaCl_2 for 5 min followed by a further 30 min with 1 mM-chlorpromazine; (g) with 10 mM- CaCl_2 for 5 min followed by a further 30 min with 1 mM-chlorpromazine.

thus greatly enhanced fusion, and it also abolished the partial inhibition by EGTA of fusion. This latter observation supports the idea that the component of chlorpromazine-induced cell fusion that is inhibitable by EGTA is associated with the endogenous proteolysis of band 3 protein to band 4.5 protein by a Ca^{2+} -activated (cysteine) proteinase.

Transglutaminase activity

When the concentration of Ca^{2+} was increased to 10 mM for the fusion by 2 mM-chlorpromazine of chymotrypsin-treated erythrocytes, there was a loss of band 1, 2, 4.1 and 4.2 proteins (Fig. 4*g*), together with the appearance of large- M_r material

that did not enter the gel (results not shown). Similar observations were made on the fusion of cells that had not be pretreated with chymotrypsin, which were consistent with the activation of erythrocyte transglutaminase (Lorand *et al.*, 1978) by entry of Ca^{2+} into the cytoplasm. This suggests that chlorpromazine, like the fusogens studied previously by Blow *et al.* (1979), increases membrane permeability to Ca^{2+} . Histamine (20mM) prevented the changes in the gel patterns attributable to transglutaminase activity in the present work, including the disappearance of band 4.1 protein, which is the most sensitive indicator of the action of this enzyme (Lorand *et al.*, 1978), but it did not prevent the changes that are attributable to proteinase activity. Since cell fusion was not inhibited by histamine, it is concluded that transglutaminase activity is not involved in cell fusion induced by chlorpromazine.

Discussion

Chlorpromazine hydrochloride is a cationic amphipath whose interaction with erythrocyte membranes has been widely studied, and in our experiments the concentration of chlorpromazine used (2mM) was close to the critical micelle concentration of 4mM at 0.1 *I* reported by Scholtan (1955). Cell fusion was observed, however, only at pH values greater than 6.8, when microdroplets of chlorpromazine, which adhered to the cell surfaces, were easily seen. Nevertheless, it is problematic whether fusion was actually induced by the microdroplets or by smaller micelles. Several studies have shown that chlorpromazine readily binds to erythrocyte membranes (Kwant & Seeman, 1969; Seeman & Kwant, 1969; Tenforde *et al.*, 1978), and Sheetz & Singer (1974) have proposed that the stomatocytogenic effect of chlorpromazine is caused by its intercalation into the cytoplasmic half of the phospholipid bilayer, which results in an expansion of the inner monolayer with respect to the outer. The quantity of chlorpromazine that actually partitions into membranes is currently, however, a matter of some controversy (Conrad & Singer, 1981; Gains & Dawson, 1982).

The behaviour of the intramembranous particles in the present work differed from that seen when human erythrocytes were fused with oleoylglycerol (Quirk *et al.*, 1978). Earlier, cold-induced clustering was not observed (possibly because of the presence of low-melting oleoylglycerol in the membrane), but the random distribution of the intramembranous particles nevertheless implied that band 3 protein was at least partly free to move in the treated cells. The present findings, made with both fixed and unfixed preparations, clearly show that the intramembranous particles of cells treated

with chlorpromazine have an increased lateral mobility, which is most marked when chlorpromazine is used with Ca^{2+} . [Similar changes have been seen by Gazitt *et al.* (1977) in erythrocytes treated with 0.24–0.48mM-chlorpromazine, although no fusion was reported.] Our observations therefore support the suggestion of Ahkong *et al.* (1975), and of Volsky & Loyter (1978), that membrane fusion occurs in protein-free areas of lipid bilayer after the aggregation or lateral movement of integral membrane proteins.

It is apparent that the increased lateral mobility of the intramembranous particles resulted from the activation by chlorpromazine of endogenous proteinases that act to degrade band 2.1 protein (ankyrin) and band 3 protein. It also appears that proteolysis of ankyrin is sufficient to permit fusion to occur in this system, but fusion is more rapid when band 3 protein is also degraded. Our work suggests that proteolysis of ankyrin to band 2.3–2.6 proteins and to proteins migrating in the band 3 region is achieved by a serine proteinase that is inhibited by Tos-Lys- CH_2Cl , whereas the breakdown of band 3 protein to band 4.5 protein is due to a Ca^{2+} -activated cysteine proteinase. The latter enzyme may be similar to that involved in the fusion of rat erythrocytes induced by benzyl alcohol (Ahkong *et al.*, 1980), and in each case the enzyme may be the Ca^{2+} -dependent cysteine proteinase, with a pH optimum between 7.0 and 7.5, termed calpain I (Murachi *et al.*, 1981). It is clear, however, that Ca^{2+} -activated cysteine proteinase activity is much more important in the fusion of rat erythrocytes by benzyl alcohol than it is with human erythrocytes fused by chlorpromazine, since only with the former is fusion effectively prevented by EGTA or *N*-ethylmaleimide. This may partly be a reflection of differing effects on the membranes of the two chemical fusogens. It is nevertheless noteworthy that the content of calpastatin, an endogenous inhibitor of calpain I, is extremely small in rat erythrocytes, whereas in human erythrocytes there is much more inhibitor than enzyme (Murachi *et al.*, 1981).

With regard to the degradation of ankyrin to band 2.3–2.6 proteins and to polypeptides in the band 3 region, it is relevant that Siegel *et al.* (1980) have reported that the endogenous proteolysis of band 2.1 protein in human erythrocyte-'ghost' membranes occurs through two pathways, one of which is a cascade of cleavages that produces band 3' protein and is inhibited by phenylmethanesulphonyl fluoride. In previous work, the fusion of human erythrocytes by oleoylglycerol gave rise to a new sharp band corresponding to band 3' protein (Quirk *et al.*, 1978). By contrast, two bands were clearly visible in the band 3 region of chymotrypsin-treated erythrocytes fused by chlorproma-

zine (Figs. 4d–4f). This difference between the two systems may be related to the fact that Tos-Phe-CH₂Cl, but not Tos-Lys-CH₂Cl, inhibited oleoylglycerol-induced fusion, whereas the reverse observations were made with chlorpromazine. These findings indicate that more than one serine proteinase can mediate cell fusion, and that different fusogenic chemicals enhance the activity of different proteinases.

Numerous biomembrane fusion reactions are known to be initiated by an increase in the intracellular concentration of Ca²⁺, and much consideration has been given to Ca²⁺ in some theoretical models for membrane-fusion reactions (Poste & Allison, 1973; Papahadjopoulos *et al.*, 1979). Furthermore, in our early studies, the chemically induced fusion of hen erythrocytes was prevented by EGTA (Ahkong *et al.*, 1973), and on the basis of related work it was later proposed that an increase in the concentration of intracellular Ca²⁺ initiates or facilitates events leading to fusion (Blow *et al.*, 1979). Until now, however, it has not been clear why human erythrocytes treated with oleoylglycerol in the presence of EGTA fused to an extent of 25% of that occurring in the presence of Ca²⁺ (Quirk *et al.*, 1978), and it seemed possible that this was some kind of experimental artifact. The present observations clearly show that chemically induced cell fusion can occur without Ca²⁺, and they also indicate how this occurs. Whereas we formerly regarded the entry of exogenous Ca²⁺ as mediating the fusion of erythrocytes by chemicals, it is apparent that this is not generally so: at least two proteinases are involved in the fusion of human erythrocytes by membrane-active compounds, only one of which is activated by Ca²⁺. Our present observations also contrast with the emphasis that has been placed by Papahadjopoulos *et al.* (1979) on the role of Ca²⁺ in the fusion of lipid bilayers as a model for the actions of this cation in biomembrane fusion reactions.

Not all chemically induced cell fusion seems to involve a proteolytic breakdown of the membrane skeleton. Fusion of human erythrocytes induced by poly(ethylene glycol), which has been reported not to involve proteolysis of membrane proteins (Schindler *et al.*, 1980; Smith & Palek, 1982), probably causes ultrastructural changes in the membrane skeleton by the dehydrating action of its concentrated solutions (Blow *et al.*, 1978; Aldwinckle *et al.*, 1982), and membrane-active fusogens such as chlorpromazine, oleoylglycerol and benzyl alcohol seem more appropriate as models for the unknown chemical mediators of biomembrane-fusion reactions that occur *in vivo* than does 35–50% poly(ethylene glycol). We previously suggested that the proteolytic degradation of membrane proteins may therefore be of general signifi-

cance in biomembrane-fusion reactions (Ahkong *et al.*, 1978), and, since several recent investigations have indicated that other cells seem to have structures that are comparable with the spectrin-actin skeleton of the erythrocyte membrane (Haest, 1982; Baines, 1983), the findings reported in the present paper may well be relevant to the initiation of naturally occurring fusion reactions, including exocytosis and endocytosis in biomembranes. It has, for example, been reported that the Ca²⁺-dependent fusion of cultured myoblasts into multinucleated myotubes appears to require the activity of a neutral metalloendoproteinase at the time of fusion, although the relationship between the requirement for Ca²⁺ and the activity of the enzyme is at present unknown (Couch & Strittmatter, 1983).

Finally, it is noteworthy that the addition of chlorpromazine to phospholipid vesicles has been reported to produce non-bilayer phases rather than large liposomes (Verkleij *et al.*, 1982). This may indicate that regions of lipid bilayer in erythrocyte membranes undergoing fusion (probably free from integral proteins) are additionally perturbed in chlorpromazine-treated cells by comparison with similarly treated liposomes. Conceivably, the products of the proteolysis of integral or skeletal membrane proteins could have membrane-perturbing properties and, by analogy with the behaviour of the F protein of Sendai virus (Hsu *et al.*, 1981), such polypeptide fragments might penetrate the lipid bilayer to mediate cell fusion. The production *in situ* of fusogenic polypeptides by endogenous proteolysis may, indeed, be a general mechanism for the fusion of lipid bilayers in biomembrane-fusion reactions, some aspects of which are imitated by viruses.

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