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MEMBRANE EXPANSION OF THE ERYTHROCYTE BY BOTH THE NEUTRAL AND IONIZED FORMS OF CHLORPROMAZINE

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SUMMARY

1. A charged form of chlorpromazine, chlorpromazine methochloride, protected erythrocytes from hypotonic hemolysis. It had about 1/75th the potency of chlorpromazine HCl.

2. The anti-hemolytic effect of chlorpromazine·HCl was potentiated at higher pH's when erythrocytes, originally suspended in pH 7, were hypotonically hemolyzed in solutions of different extracellular pH's. In these experiments the pH was different on each side of the membrane.

3. No such pH dependency of chlorpromazine potency was seen when the pH was the same on both sides of the membrane. Intact cells were hemolyzed in hypotonic solutions of different pH. A Coulter counter and computer was used for measuring the mean cell volume of these fresh erythrocyte ghosts. The intracellular pH of the spherical ghosts was assumed to be equal to the extracellular pH. It was found that chlorpromazine·HCl increased the ghost mean cell volume to about the same extent over the pH range 5.9–9.8. $5 \cdot 10^{-6}$ M chlorpromazine·HCl increased the mean cell volume by 5.2 μ^3 and the membrane area by about 3.2 μ^2 over a wide pH range.

4. It is concluded that both the free base and the charged form of chlorpromazine contributed to its membrane action of expansion and anesthesia; both forms of the drug apparently expand the erythrocyte membrane by about an equal amount.

5. It is concluded that the charged form of chlorpromazine affects the erythrocyte membrane from both the internal and external aspects of the membrane. This is identical to the situation which obtains in nerve fibers where both sides of the membrane are sensitive to quaternary anesthetics.

INTRODUCTION

There have been many claims in the literature that the uncharged form or free base, B, is the active form in producing local anesthesia[1–22]. This conclusion is based on the general observation that the potency of the local anesthetics is enhanced at higher extracellular pH's. There is also much evidence that the charged form of the anesthetic may be active[23,24]. ARIENS *et al.*[25] have recalculated the data of SKOU[17,18] and concluded that BH^+ was active. They assumed, however, that the intracellular pH did not change when the extracellular pH was altered; this assumption does not hold for other cells[26,27]. Quaternary anesthetics are also active[18,19,30].

Inspection of the results of both DETTBARN[31] and RITCHIE *et al.*[32–35] indicates that possibly both amine species, charged and neutral, contribute to the total anesthetic activity. RITCHIE AND GREENGARD[32], while obtaining complete anesthetic block at pH 7 of C fibers which had been pre-exposed to chlorpromazine, found that the C potential recovered by 50–75 % between pH 8 and 9 and that there was no further degree of recovery at higher pH's up to 10.5 (see Fig. 2 of ref. 32); Fig. 3 of their paper also shows that in general only 50 % of the fibers ever recovered at pH 10.5. The chlorpromazine pK_a is either 8.1 (ref. 36), 6.8 (ref. 37) or 9.3 (ref. 38). At pH 10.5, therefore, the amount of ionized chlorpromazine trapped in the region between the neurolemma and the Schwann sheath would be very small. The results of RITCHIE AND GREENGARD could, therefore, be explained by postulating that both forms of the anesthetic are active, and that possibly the cationic form is about twice as active as the neutral form.

The erythrocyte is more suitable for studies to resolve some of these problems than the nerve cell because the plasma membrane is directly exposed (unlike the 80 %-covered node of Ranvier[38,40]) and the internal pH can be varied according to the techniques described in this paper. It is known that anesthetics have an anti-hemolytic and membrane-expanding effect on erythrocyte membranes (refs. 1–4, 26, 42–45). These effects occur at anesthetic concentrations which are almost identical to those which anesthetize nerve fibers.

METHODS

Preparation of erythrocyte stock suspensions

All experiments were done on human erythrocytes. A sample of venous blood was drawn from a fasting volunteer, heparinized at 50 units/ml blood, centrifuged at 1500 × *g* for 12 min, and the plasma and buffy coat removed. The cells were resuspended and diluted in 154 mM NaCl, 10 mM sodium phosphate buffer (pH 7.0), to make a stock cell concentration of about $4 \cdot 10^8$ cells/ml. For the Coulter counter experiments the stock suspension of erythrocytes was prepared using unbuffered 154 mM NaCl and the cell concentration was $1.8 \cdot 10^6$ cells/ml.

Anti-hemolytic effect of chlorpromazine·HCl and chlorpromazine methochloride

The anti-hemolytic effect or erythrocyte-protecting effect of chlorpromazine on hypotonic hemolysis was studied by adding 0.1 ml of the stock cell suspension ($4 \cdot 10^8$ cells/ml) to 1.5 ml of chlorpromazine·HCl in 66.6 mM NaCl (pH 7). The exact degree of hemolysis was measured by centrifuging the cells 5 min after the onset of hemolysis and measuring the amount of hemoglobin in the supernatant by means of a spectrophotometer at a wavelength of 543 mμ.

Preincubation experiments with chlorpromazine methochloride

The effect of pre-exposing the cells to chlorpromazine methochloride before testing the membrane-protecting action of this drug was studied as follows: Aliquots of 0.1 ml ($4 \cdot 10^8$ cells/ml) were added to 1.5 ml of chlorpromazine methochloride in 154 mM NaCl (pH 7). The suspension was mixed and then left for different periods of time. The cells were later centrifuged and resuspended in a chlorpromazine methochloride solution of the same concentration but now in 66.6 mM NaCl (pH 7).

Extracellular pH variations of erythrocytes

The effect of altering the extracellular pH on the anti-hemolytic action of chlorpromazine was studied as follows: Aliquots of 0.1 ml of the stock erythrocyte suspension ($4 \cdot 10^8$ cells/ml) were added to 1.5 ml of chlorpromazine in either 88 mM NaCl (pH 5), 77.8 mM NaCl (pH 6), 66.6 mM NaCl (pH 7) or 57.8 mM NaCl (pH 8); the amount of hemolysis under these conditions in the absence of chlorpromazine was 60%.

Under the conditions of these experiments the intracellular and extracellular pH were not equal because the erythrocytes in the stock suspension were suspended in pH 7 solution.

The effect of pH on chlorpromazine-induced membrane expansion

The effect of pH on the chlorpromazine-induced membrane expansion of erythrocyte ghost membranes was studied by the sealed-ghost-expansion method of SEEMAN *et al.*[3], as described in an accompanying paper. Aliquots of 0.5 ml from the stock suspension of intact erythrocytes ($1.8 \cdot 10^6$ cells/ml) were added to Coulter counter vials containing 10 ml of a solution of NaCl and 10 mM sodium phosphate buffer (pH 5.7–10) such that the total ideal osmolarity was between 65 and 120 mosM. The exact osmolarity does not matter as long as all cells are converted into ghosts and enough NaCl is present to keep the conductivity of the solution high[3]. It was assumed that during the ensuing period of hypotonic hemolysis the intracellular pH equilibrated with the extracellular pH through the transiently existing holes in the membrane[41]. The pH values of the hypotonic solutions were adjusted by adding different proportions of 10 mM Na_2HPO_4, NaH_2PO_4 and small amounts of 1 M NaOH. The addition of erythrocytes to these solutions did not alter the pH, since the erythrocyte stock suspension solution was unbuffered and the amount of erythrocyte protein and hemoglobin added was negligible.

About 1.5 h after the onset of hemolysis the ghost membranes had sealed[3,41] and were spherical in shape[3] (as indicated by phase-contrast appearance). Aliquots of chlorpromazine were added to the sealed spherical ghosts, the contents mixed and the vials left again for 2 h to allow the cells to pass through the transient period of irregularly shaped cells[3]. After 2 h the ghosts were again spherical, and the mean cell volume was measured by means of a Coulter counter and mean cell volume computer[3].

MATERIALS

Pure samples of chlorpromazine·HCl and chlorpromazine methochloride were kindly provided by Smith Kline and French, Philadelphia.

RESULTS

Erythrocyte protection by tertiary and quaternary chlorpromazine

The results in Fig. 1 show that both chlorpromazine·HCl and chlorpromazine methochloride at low concentrations reduced hypotonic hemolysis. The concentration of chlorpromazine·HCl inhibiting erythrocyte osmotic hemolysis by 50% was usually between $2 \cdot 10^{-6}$ and $5 \cdot 10^{-6}$ M. The quaternary was about 75-fold less effective.

Fig. 2 shows that pretreating the intact cells with chlorpromazine methochloride led to a greater anti-hemolytic effect.

At higher concentrations both compounds were directly hemolytic, an effect which may be explained by the potent surface activity of these particular compounds and phenothiazine derivatives in general[1,46–52].

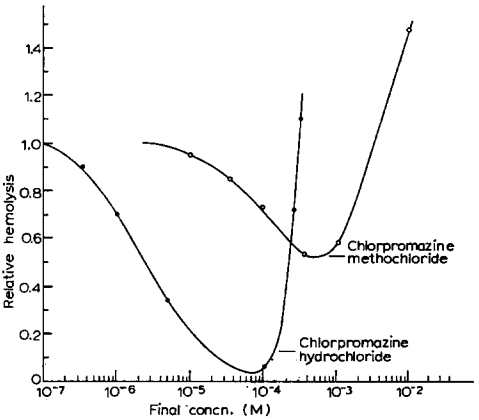

Fig. 1. Effect of chlorpromazine·HCl and chlorpromazine methochloride concentrations on hypotonic hemolysis of intact erythrocytes. In the absence of any drug, the relative hemolysis of 1.0 indicates an absolute hemolysis of about 60%. The quaternary chlorpromazine is about 1/75th less effective in protecting the cells. Low concentrations of both substances protect the erythrocytes, while high concentrations of both these surface-active tranquilizers are directly lytic to the membranes.

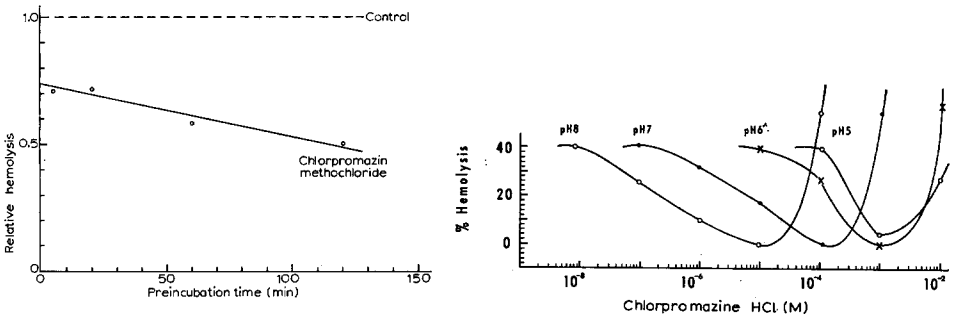

Fig. 2. Intact erythrocytes were pretreated with chlorpromazine methochloride in isotonic solution for varying periods of time. The cells were then placed into hypotonic solution containing the same concentration of the drug. The protection of the erythrocytes was enhanced by longer pretreatment with the drug. A relative hemolysis of 1.0 represents an absolute hemolysis of 60%.

Fig. 3. Effect of extracellular pH on erythrocyte stabilization by chlorpromazine·HCl. An increase in extracellular pH potentiated the protective effect of chlorpromazine if the intracellular pH was kept approx. 7. Intact erythrocytes, suspended and equilibrated in pH 7, were added to chlorpromazine solutions of different pH.

Extracellular pH variations on erythrocytes

The results in Fig. 3 indicate that the apparent anti-hemolytic potency of chlorpromazine·HCl is greatly enhanced when the extracellular pH is increased. At pH 5, for example, the protective concentration is of the order of $5 \cdot 10^{-4}$ M, while

at pH 8 it is in the range of 10^{-7} M. The direct lytic potency also increases at higher extracellular pH.

The effect of pH on chlorpromazine-induced membrane expansion

Using the sealed-ghost-expansion method[3], the intact erythrocytes were hypotonically hemolyzed in solutions of different pH's (5.9–10.5), and the ghosts were allowed to seal before the chlorpromazine·HCl was added.

TABLE I

CHLORPROMAZINE EXPANSION OF GHOST VOLUME IS INDEPENDENT OF pH

The effect of pH on chlorpromazine-induced expansion of the ghost volume. Intact erythrocytes were hemolyzed in hypotonic solutions of varying pH; the pH was considered, therefore, to be the same on both sides of the ghost membrane. Chlorpromazine was added and 2 h later the volume of the expanded ghost was measured. The same volume increment of around 5.2 μ^3 occurred over a wide pH range for the chlorpromazine concentration of $5 \cdot 10^{-6}$ M showing that the expansion was independent of pH. The volumes of the control ghosts varied from week to week and between individuals. Above pH 9 the ghosts started to fragment, and above pH 10.5 the cells were greatly disrupted.

Extracellular and intracellular pH	*Volume of control ghost (μ_3) (mean ± S.D.)*	*Increment in volume caused by $5 \cdot 10^{-6}$ M chlorpromazine·HCl (μ^3) (mean ± S.D.)*
5.9	152.3 ± 1.10	4.9 ± 1.01
6.3	154.2 ± 0.90	6.9 ± 1.10
6.8	151.0 ± 1.80	7.2 ± 1.70
6.96	130.3 ± 0.50	3.6 ± 0.40
7.0	139.3 ± 1.89	6.5 ± 0.61
7.0	145.1 ± 0.55	2.7 ± 0.93
7.3	146.6 ± 1.00	5.3 ± 0.90
7.5	142.98 ± 0.30	4.8 ± 0.20
7.6	146.4 ± 0.80	4.3 ± 0.70
7.8	149.4 ± 0.70	3.5 ± 0.80
8.4	144.34 ± 0.40	5.2 ± 0.30
8.6	136.5 ± 1.00	5.7 ± 1.20
9.0	112.7 ± 1.27	5.7 ± 0.81
9.8	100.7 ± 0.42	6.9 ± 1.14
10.5	Cells disrupted at this pH	

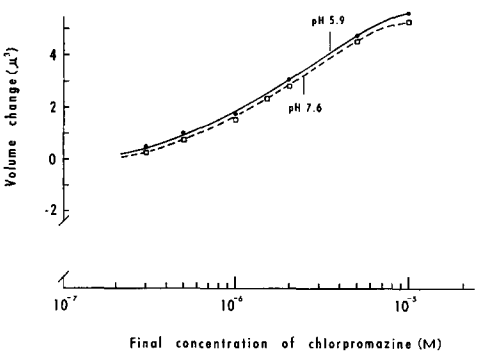

Fig. 4. Effect of pH on membrane area expansion by chlorpromazine. The expansion of the erythrocyte ghost spherical volume and area is independent of the pH. Intact erythrocytes were hemolyzed in solutions of different pH's. The ghost, therefore, has the same pH inside as outside. Chlorpromazine was then added and the cell volumes monitored later, according to the sealed-ghost-expansion method.

The effect of pH on the chlorpromazine-induced expansion of the sealed ghost membrane is shown in Table I and in Figs. 4 and 5. These results directly indicate that both the B and BH^+ forms of chlorpromazine are about equal in eliciting membrane area expansion of the erythrocyte ghost. The results in Fig. 4, for example, show the increase in the spherical volume in cubic microns brought about by chlorpromazine at low concentrations and at two different pH values, 5.9 and 7.6.

The data in Table I and Fig. 5 show the increases in ghost volume brought about by $5 \cdot 10^{-6}$ M chlorpromazine·HCl at different pH values. The mean increase in ghost cell volume was about 5.2 μ^3. For a spherical ghost of 146 μ^3 in volume and 134 μ^2 in area, this would represent an increment in membrane area of around 3.2 μ^2 or 2.4 %.

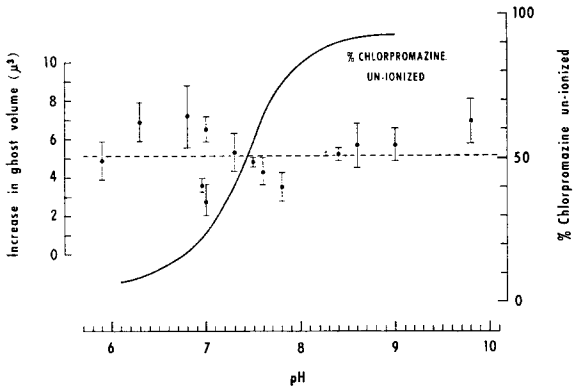

Fig. 5. A comparison, at different pH values, of the concentration of un-ionized chlorpromazine molecules and the chlorpromazine-induced expansion of the ghost. The drug-induced increase in the cell volume is about 5.2 μ^3 (or 3.2 μ^2) over a wide pH range for $5 \cdot 10^{-6}$ M chlorpromazine. This effect does not parallel the concentration of chlorpromazine base (see text). The fact that the drug effect is roughly constant at difference pH values suggests that both ionized and un-ionized forms of chlorpromazine are approximately equally effective in acting on the membrane.

This finding agrees with earlier work[3,4] which demonstrated that the ghost membrane area expanded by 1.5–3 % at concentrations of drugs which inhibited hypotonic hemolysis by 50 %. Higher concentrations of chlorpromazine (which are required for anesthesia of the squid giant axon[53], for example) expand the membrane even more (see Fig. 4).

Fig. 5 also shows the fractional amount of un-ionized chlorpromazine at different pH values, using a value of 7.4 as the apparent pK_a for chlorpromazine. Although the exact value is in dispute (see INTRODUCTION), the choice of other pK_a values does not change the interpretation of these results, which indicate that both forms of chlorpromazine are active.

DISCUSSION

The results indicate that (a) the charged form of chlorpromazine can exert anti-hemolytic and membrane-expanding effects, and (b) the charged form is approximately equal in membrane-expanding potency to the uncharged form of chlorpromazine.

As mentioned in the INTRODUCTION, most workers have found that altering the pH either reduces or potentiates the anesthetic potency of tertiary amines. It has also been shown[51] that the penetration of chlorpromazine into phospholipid films (especially lecithin) spread at an air–water interface is reduced at lower pH values. In all the biological preparations, however, the pH on both sides of the membrane is not the same and there are many interpretational difficulties because of this[25]. These problems are readily appreciated when one compares the results in Fig. 3 with those in Figs. 4 and 5.

There is a possibility that the intracellular pH of the ghost started to change after the membrane was sealed and that it was not the same as the extracellular pH at the time when the drug was added. This is unlikely for two reasons. The experiments were carried out at room temperature and this would tend to reduce enzymatic activity. Secondly, it is known that at room temperature, while the erythrocyte ghost membrane holes seal and become impermeable to large molecules such as ferritin[41], the ghost membrane is still rather leaky to cations such as K^+ (refs. 55, 56).

In earlier work on this subject[26] it was concluded that some of the anti-hemolytic effect of chlorpromazine could be attributed to the charged or ionized form acting on the inside of the membrane. NARAHASHI AND FRAZIER[57] have recently found that quaternary local anesthetics also effectively block squid giant axon action potentials when applied to the inside of the cell. In the present results, Figs. 1 and 2, the action of the quaternary is presumably on the exterior aspect of the membrane, since it is known that charged compounds permeate erythrocytes rather slowly[58]. As mentioned in the INTRODUCTION, quaternary compounds also anesthetize when applied externally to the nerve membrane[18,28–30]. The erythrocyte membrane and the nerve cell membrane are, therefore, pharmacologically similar in the limited sense that they are both affected by charged anesthetic compounds acting on both sides of the plasmalemma.

Interpreting the results of Fig. 5 as showing that the neutral and charged forms of chlorpromazine are roughly equal in membrane-expanding potency is compatible with the results of both DETTBARN[31] and RITCHIE AND GREENGARD[32]. A recent paper[59] confirms that both forms of the amine anesthetics are active.

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