BBA 75359

THE DISPLACEMENT OF MEMBRANE CALCIUM BY A LOCAL ANESTHETIC (CHLORPROMAZINE)

W. O. KWANT AND PHILIP SEEMAN

Department of Pharmacology, University of Toronto, Toronto 5 (Canada)

(Received July 2nd, 1969)

SUMMARY

1. The effect of chlorpromazine on the membrane concentration of Ca^{2+} was examined. Erythrocyte ghosts, which were free of hemoglobin and Ca^{2+}, were mixed with $^{45}Ca^{2+}$ in the presence of different concentrations of Ca^{2+} and chlorpromazine. The amount of Ca^{2+} adsorbed to the membrane was determined from the amount of $^{45}Ca^{2+}$ remaining in the supernatant.

2. It was found that [^{3}H]sorbitol rapidly equilibrated over the entire water space of the ghost suspension. [^{14}C]Inulin, however, completely permeated only about 80 % of the ghosts. The water space for $^{45}Ca^{2+}$ was found to be similar to that for inulin; this was obtained by saturating the membrane sites for Ca^{2+} (which occurred between 10 and 50 mM Ca^{2+}), measuring the radioactivity in the supernatant, and comparing it with the control value.

3. The membrane concentration of Ca^{2+} was determined as a function of the extracellular Ca^{2+} concentration. At free concentrations of Ca^{2+} below 1 mM the adsorption isotherm indicated one set of binding sites with a saturation capacity of 0.081 mole Ca^{2+} per l of solid (and 30 % hydrated) membrane, and with an affinity constant of 1940 l membrane solid per mole Ca^{2+}.

4. The addition of saponin (2 mg/mg dry ghost) greatly increased the inulin space and the adsorption of Ca^{2+} to the membranes. Rehemolysis of the ghosts also increased the binding of Ca^{2+} to the membranes. Saponin and rehemolysis presumably exposed intracellular sites by disrupting the membrane.

5. Chlorpromazine, at free concentrations of 1–10 μM (or total concentrations of 0.1–1 mM) competitively displaced the membrane-bound Ca^{2+}. The maximum amount of membrane Ca^{2+} displaced when the membrane was saturated with chlorpromazine (0.066 mole chlorpromazine per l solid membrane) was 0.021 mole Ca^{2+} per l membrane solid. It was concluded that 1 Ca^{2+} was displaced from the membrane by about 2 chlorpromazine molecules.

INTRODUCTION

This study describes the adsorption of Ca^{2+} to erythrocyte membranes and shows that a local anesthetic (chlorpromazine) effectively competes with and displaces the Ca^{2+} from these cell membranes. The work was carried out for the following reasons.

(1) It has been known since the early work of LOCKE[1] in 1894 that Ca^{2+} is essential for the maintenance of the electrical excitability of cell membranes[2–19]; low Ca^{2+} concentrations lead to depolarization, spontaneous electrical discharges and ultimately block membrane excitability. Local anesthetics act on the cell membrane[20] and prevent the production of action potentials[21–24]. Since membrane excitation requires Ca^{2+} and since local anesthetics prevent this excitation, the thought has developed that anesthetics might compete with Ca^{2+} and might block Na^+ channels which are normally operative in the action potential[22,25,26]. This idea is supported by the fact that external Ca^{2+} competes with the local anesthetics and reverses the membrane stabilization brought about by them[18,27–33]. It was of interest, therefore, to examine whether local anesthetics could compete with, and displace, Ca^{2+} from cell membranes. Although there are a number of studies demonstrating that local anesthetics can displace Ca^{2+} from lipid molecules[34–45], there is no information on whether or not these compounds can actually displace the Ca^{2+} associated with biological membranes.

(2) Membrane-associated Ca^{2+} is also required for excitation–contraction coupling[46–48] and stimulus–secretion coupling[49–52]. Local anesthetics interfere with this coupling and it is of interest to know whether this occurs as a result of the displacement of membrane Ca^{2+}.

(3) It is known that local anesthetics expand erythrocyte membranes at the same concentrations which anesthetize nerve fibers[53–60]. This occurs with all anesthetics except tetrodotoxin. One of the possible mechanisms of this membrane-expanding effect is that the anesthetic may displace membrane-bound Ca^{2+} which normally serves to keep the membrane in a condensed state[59].

METHODS

(1) *Preparation of the stock ghost suspension*

Human blood, stored in acid–citrate–dextrose medium, was obtained from the Red Cross Blood Transfusion Service of Toronto.

As has been described previously[61,62], the blood was centrifuged, the plasma and buffy coat removed, and the erythrocytes washed 3 times in 154 mM NaCl in 6.25 mM sodium phosphate buffer, pH 7.4. Erythrocyte ghosts were then prepared using the method of DODGE *et al.*[63]. All the washing and hemolysing solutions contained 1 mM EDTA (sodium salt). The ideal osmolarity of the hemolysing solutions was 20 mosM. As has been shown by HARRISON AND LONG[64], EDTA effectively removes the Ca^{2+} bound to these membranes.

The resulting ghosts were then washed 3 times in 15 mM Tris–HCl buffer (pH 7.0) and finally concentrated by centrifugation at 36900 $\times g$ for 40 min. The concentration of ghosts in the stock ghost suspension, as determined by the dry weight, was usually between 1 and 2 % (g dry membrane per 100 ml ghost suspension). The dry weight of each preparation was obtained by drying an aliquot of the stock ghost suspension at 80° for 16–20 h; the dry weight of the buffer salts was subtracted from the total dry weight to give the dry weight of the ghost membranes.

(2) *Determination of $^{45}Ca^{2+}$ activity*

$^{45}CaCl_2$ was obtained from Atomic Energy of Canada Ltd. and was diluted with distilled water to a solution of 8.08 mM. The specific activity was 12 mC/mmole.

For experiments this solution was diluted 1:100 in 15 mM Tris–HCl buffer (pH 7.0) to give a stock solution of 80.8 μM.

Aliquots of 0.1 ml of this stock solution or from the experimental supernatants (see below) were mixed with 10 ml of liquid scintillator described by BRAY[65].

For single label experiments $^{45}Ca^{2+}$ was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 3380) using an amplification of 9.5 % with the lower and upper windows set at 20 and 800 units, respectively. The high tension voltage was set at 1467 V.

It was observed that the apparent disintegration rate (disint./min) of the $^{45}Ca^{2+}$ in the presence of liquid scintillator was not constant and diminished more rapidly than the half life of 165 days (for $^{45}Ca^{2+}$) could predict.

For example, 8 h after the addition of liquid scintillator, the disint./min of the sample was 97 % of the original value, while after 6 days, approx. 83 % of the original activity was left. If this loss would have been due to decay, it should have occurred only after 44 days.

Since in the first 20 h the apparent drop in disintegration rate was independent of the amount of Ca^{2+} present, all samples were counted within this period. The necessary corrections were determined by counting the first set of vials at the beginning and at the end of each series. Since the loss in disintegration rate appeared to be linear with time, a correction was made for the absolute time when the vial was counted. This correction never exceeded 3 % except in one instance where a 4 % correction was applied. We have recently been informed that this effect results from the adsorption of $^{45}Ca^{2+}$ to the glass vial and is prevented by the addition of EDTA to the liquid scintillation medium.

A quench curve was determined using acetone as the quenching agent. The quench correction was made according to the standard procedure and was usually smaller than 2 %.

(3) *Determination of the accessible water space*

For reasons explained in the results the accessible water space for Ca^{2+} was assumed to be the same as that for inulin. To determine this space, a 0.2-ml aliquot of ^{14}C- or ^{3}H-labeled inulin dissolved in Tris–HCl buffer ([*carboxyl*-^{14}C]inulin, 2.03 mC/g, Mallinckrodt Nuclear, 25 mg/100 ml buffer, or [*methoxy*-^{3}H]inulin, 89.2 mC/g, New England Nuclear, 3.62 mg/100 ml buffer) was added to a 0.5-ml aliquot of the stock ghost suspension to which 0.2-ml aliquots of varying Ca^{2+} concentrations had been added. The 10 mm × 75 mm pyrex test tubes were then centrifuged in rubber jackets at 36900 × g for 20 min and the radioactivity in the supernatant was determined.

If all the water in the test tube was accessible to inulin the stock inulin should have been diluted 2/9, as was found in control tubes containing 0.5 ml of buffer replacing the stock ghost suspension. The dilution was less than this factor 2/9, and the volume of water accessible to inulin (V_W) was calculated from:

$$V_W = \frac{(\text{disint./min per ml})_{\text{stock}} \times 0.2 \text{ ml/tube}}{(\text{disint./min per ml})_{\text{supernatant}}} \quad (1)$$

where (disint./min per ml) and the subscripts are the disintegrations found per ml of stock or supernatant. The accessible water space was found to be independent of

the Ca^{2+} concentration, in the range of concentrations used for these experiments (0.05–1 mM). In some experiments, two radioactive labels were used simultaneously to determine both the accessible water space, with [^{3}H]inulin, and the Ca^{2+} adsorption using $^{45}CaCl_2$. To count both isotopes simultaneously, the liquid scintillation spectrometer was set up in such a way that no counts due to ^{3}H were present in Channel II, used to count $^{45}Ca^{2+}$. (Channel I for ^{3}H: amplification 81 % and window settings of 40 and 440; Channel II for $^{45}Ca^{2+}$: amplification 9.5 % and window settings 110 and 800.) An external standard was used to determine the quench curve for ^{3}H in its own channel (Channel I) and for $^{45}Ca^{2+}$ in both Channels I and II. To separate the counts in Channel I, the following formula was used:

$$\text{Disint./min } {}^3\text{H} = \left(\frac{\text{counts/min}_\text{I} - \dfrac{\text{counts/min}_\text{II} \times \text{Eff } {}^{45}\text{Ca}_\text{I}^{2+}}{\text{Eff}{}^{45}\text{Ca}_\text{II}^{2+}}}{\text{Eff } {}^3\text{H}_\text{I}} \right) \quad (2)$$

where disint./min ^{3}H is the disintegration rate of ^{3}H in the scintillation vial, counts/min$_\text{I}$ and counts/min$_\text{II}$ are the counts/min in Channel I and II respectively, and Eff $^{45}Ca_I^{2+}$, Eff $^{45}Ca_{II}^{2+}$ and Eff ^3H_I are the efficiencies of $^{45}Ca^{2+}$ and ^{3}H in the channel indicated by the subscript.

(4) *Determination of the adsorption of Ca^{2+} to the membrane and the influence of chlorpromazine on this adsorption*

Aliquots of ghosts (0.5 ml) were mixed with 0.2-ml aliquots of $^{45}CaCl_2$ solutions of varying concentrations. These $^{45}CaCl_2$ solutions were prepared by mixing the radioactive stock solution with equal parts of $CaCl_2$ solutions of different concentration. After mixing, and after 10 min incubation at room temperature (21°), a second 0.2-ml aliquot, containing chlorpromazine and [^{3}H]inulin, was added. In some experiments, [^{3}H]sorbitol or saponin was added.

All the substances added were dissolved in 15 mM Tris–HCl buffer, pH 7.0. The contents were again mixed and incubated for 10 min at 21°. The ghosts were centrifuged at 36900 × *g* for 20 min at 21° in a temperature-controlled centrifuge. The radioactivity in 0.1-ml samples of the supernatant was determined in the liquid scintillation spectrometer as described in METHODS, Sections 2 and 3.

The membrane concentration of Ca^{2+}, Ca^{2+}_{mem}, in moles Ca^{2+} per l of hydrated solid membrane was obtained as follows:

$$Ca^{2+}_{mem} = \frac{Ca^{2+}_{dry\ mem} \times 1000 \times 1.17}{1.00/0.70} \quad (3)$$

where $Ca^{2+}_{dry\ mem}$ is the amount of Ca^{2+} bound per g of dry membrane, where the amount of water in the hydrated membrane is taken as 30 % of the total weight of the hydrated solid membrane[66] and where the density of the wet membrane is taken as 1.17 (refs. 67, 68).

$Ca^{2+}_{dry\ mem}$ was obtained as follows:

$$Ca^{2+}_{dry\ mem} = \frac{(Ca^{2+}_{stock} \times 0.0002 - Ca^{2+}_{sup} \times V_W)\ 0.0009}{0.5 \times \text{dry weight}} \text{ moles/g dry membrane} \quad (4)$$

where Ca^{2+}_{stock} is the Ca^{2+} molarity in the 0.2-ml aliquot of the added $CaCl_2$ solution, where Ca^{2+}_{sup} is the Ca^{2+} molarity found in the supernatant, where V_W is the accessible water space as determined by labeled inulin, and where dry weight is the dry weight of the added ghost suspension in g dry membranes per ml ghost suspension. The final volume in each tube was 0.9 ml, 0.5 ml of which had been added from the ghost suspension. Ca^{2+}_{sup} was measured as follows:

$$Ca^{2+}_{sup} = \frac{Ca^{2+}_{stock} \times (\text{disint./min per ml})_{sup}}{(\text{disint./min per ml})_{stock}} \quad (5)$$

where Ca^{2+}_{stock} and Ca^{2+}_{sup} are the Ca^{2+} concentrations in the stock solution and in the supernatant, respectively, and where $(\text{disint./min per ml})_{stock}$ and $(\text{disint./min per ml})_{sup}$ are the disintegration rates found per ml of stock and supernatant, respectively.

Control experiments in which the ghosts were replaced by buffer showed better than 99 % recovery of the radioactive labels.

(5) *Influence of rehemolysis on Ca^{2+} adsorption*

The stock ghost suspension was mixed 1:1 with a 6 % NaCl (w/v) solution in 15 mM Tris–HCl buffer, pH 7.0. The ghosts were left overnight at 4° to allow the NaCl to enter the ghosts.

On the following day the ghosts were centrifuged at 36900 × *g* for 40 min and these concentrated cells were used for the rehemolysis experiments. Aliquots of 0.2 ml of $^{45}Ca^{2+}$ and [^{3}H]inulin in 15 mM Tris–HCl buffer were mixed with 0.5 ml of the NaCl-loaded ghosts in 10 mm × 75 mm tubes. The resulting drop in tonicity should have caused rehemolysis. After waiting 10 min for resealing, an aliquot of 0.2 ml of 3 % NaCl (w/v) was added.

For control experiments the NaCl-loaded cells were mixed with 0.2 ml Tris–HCl buffer only and, after resealing, the $^{45}Ca^{2+}$ and [^{3}H]inulin were added together in the 0.2-ml aliquot of 3 % NaCl. The tubes were centrifuged and the supernatants were analysed for $^{45}Ca^{2+}$ and ^{3}H. Inulin spaces and Ca^{2+} adsorption were determined as described above.

RESULTS

It was found that inulin and Ca^{2+} did not equilibrate over the entire water space of the final ghost mixtures; sorbitol did. These results are shown in Fig. 1.

The data in Fig. 1 were obtained by mixing ghosts with $^{45}Ca^{2+}$, with [^{3}H]sorbitol, and with [^{14}C]inulin in the presence of different concentration of Ca^{2+}. The ordinate shows the isotope concentration in the control tube without any ghosts. Ratio values less than 1.0 indicate that the membranes have adsorbed or concentrated the isotope. Ratio values which are more than 1.0, however, indicate that the isotope has not fully equilibrated across the membranes and has, therefore, been excluded to a certain extent from the entire water space.

The ratio for [^{3}H]sorbitol was exactly 1.0 at all extracellular Ca^{2+} concentrations ranging from 0.1 to 100 mM. Sorbitol, therefore, was not adsorbed and was not excluded, but was distributed over the entire water available.

The inulin ratio at low concentrations of Ca^{2+} was 1.06 in the experiment shown

in Fig. 1. In other experiments it was found that the inulin ratio was the same over a wide range of Ca^{2+} concentrations of up to 20 mM Ca^{2+} at least.

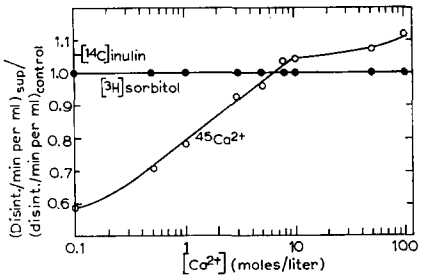

Fig. 1. Showing that erythrocyte ghosts are completely permeable to sorbitol but not to inulin, and that the membranes strongly adsorb Ca^{2+}. Radioactive sorbitol, inulin and Ca^{2+} were mixed with ghosts and the radioactivity in the supernatant was measured. The ordinate plots the disint./min of the supernatant relative to the control where there were no cells. The ordinate value for inulin was 1.06.

The ratio for $^{45}Ca^{2+}$ was considerably less than 1.0 at low concentrations of Ca^{2+}, and this reflects the fact that the membranes adsorbed the Ca^{2+}. At higher concentrations of Ca^{2+} the uptake of Ca^{2+} by the membranes became saturated; the $^{45}Ca^{2+}$ ratio, therefore, increased and exceeded 1.0 at Ca^{2+} concentrations above 8 mM. In the 10–50-mM concentration range, where the membrane sites for Ca^{2+} were virtually all occupied (see later results) the Ca^{2+} ratio was 1.06, in good agreement with the value for inulin. Inulin and Ca^{2+}, therefore, distributed over a similar water space in the ghost suspension, but this space was less than the total volume of water in the tube, as monitored by [^{3}H]sorbitol.

The increase in the water space available to Ca^{2+} in the presence of membrane 'holes'

(A) Saponin 'holes'

Electron microscopic studies employing ferritin and colloidal gold have shown that saponin makes permanent 'holes' in the erythrocyte membrane[69,70]. It has also been found in this laboratory (M. CHAU AND P. SEEMAN, unpublished observations) that a saponin concentration of 2 mg/mg of erythrocyte ghost increases the water space accessible to inulin; the [^{14}C]inulin ratio drops from 1.25 to 1.05 (*cf.* Fig. 1).

The addition of 2 mg saponin per mg ghost caused a fall in the concentration of Ca^{2+} in the supernatant of from 50 (without saponin) down to 15 μM. Although this finding supports the idea of Ca^{2+} being partly excluded when no saponin is present, there is also a possibility that the chemical alteration of the membrane opens up new Ca^{2+} adsorption sites and that these new sites account for the complete drop of Ca^{2+} in the supernatant.

(B) Transient 'holes' during hemolysis

It was found that when ghosts underwent rehemolysis, the water space accessible to inulin increased. It is known that during osmotic hemolysis the erythrocyte membrane develops 'holes' which rapidly seal[69,71].

Ghosts were loaded with NaCl and subjected to rehemolysis in the presence of [^{3}H]inulin, as outlined in METHODS, Section 5. After rehemolysis the space inaccessible to inulin decreased to 7.5% from the control space of 12.3%, a relative drop of 39%. In another experiment with ghosts of a different preparation and

suspended at different concentration, rehemolysis decreased the inulin-inaccessible space to 13.8 % compared to the control value of 17.4 % indicating a relative opening of 23 % of the originally closed space. Although the absolute magnitude of the inulin-inaccessible space varied for different preparations (apparently depending on the time interval after ghost preparation), rehemolysis invariably opened about a third of the closed ghosts to inulin.

While rehemolysis in 3 % NaCl (see METHODS) was effective in making the intracellular water available, it was found that this high concentration of NaCl competed with Ca^{2+} for adsorption to the membrane. This effect has been described by GENT *et al.*[72]. For example, as shown in Fig. 1, the Ca^{2+} concentration in the supernatant falls by about 40 % at 0.1 mM Ca^{2+} when only 15 mM Tris–HCl buffer was present. In the presence of 3 % NaCl, however, the drop in supernatant concentration at 80 μM was only 0.7 % without rehemolysis (see METHODS, Section 5) and 7.6 % with rehemolysis. Rehemolysis, therefore, opened the cells, but the NaCl reduced the binding of Ca^{2+} to the membrane.

The adsorption of Ca^{2+} to erythrocyte ghost membranes

Because of the agreement between the water spaces for inulin and Ca^{2+} at high Ca^{2+} concentrations (where all the membrane sites for Ca^{2+} were occupied), the water spaces accessible to inulin and to Ca^{2+} were assumed to be equal at all lower Ca^{2+} concentrations.

The membrane concentration of Ca^{2+} was determined as a function of the extracellular Ca^{2+} concentration (see METHODS, Section 4). A reciprocal plot of the Ca^{2+} concentration in the hydrated membrane *versus* the free Ca^{2+} concentration in the supernatant is shown in Fig. 2.

At free concentrations below 1 mM one set of binding sites was present. Using Formula 6 (see refs. 61 and 73 for further references), the membrane concentration of Ca^{2+} (Ca^{2+}_{mem}) at saturation of the binding sites was 0.081 M ($Ca^{2+\,max}_{mem}$) and the affinity constant (K) was 1940 l/mole. The free energy of adsorption was −6.8 kcal/mole.

$$\frac{1}{Ca^{2+}_{mem}} = \frac{1}{Ca^{2+\,max}_{mem}} + \frac{1}{K\,Ca^{2+\,max}_{mem}}\left(\frac{1}{Ca^{2+}_{sup}}\right) \qquad (6)$$

The displacement of membrane Ca^{2+} by chlorpromazine

It was found that chlorpromazine displaced the Ca^{2+} which had adsorbed to the membranes. This displacement occurred at low chlorpromazine concentrations of the order of 1–10 μM (free concentrations) which are known to be membrane-stabilizing to nerve cells and to erythrocytes[74,75].

The results showing the displacement of membrane Ca^{2+} by chlorpromazine are presented in Fig. 3. The data demonstrate the competition of Ca^{2+} and chlorpromazine for the same sites on the membranes. The adsorption isotherms for Ca^{2+} in Fig. 3 show that as the chlorpromazine concentration was increased there was no change in $Ca^{2+\,max}_{mem}$, the number of Ca^{2+} sites of the membranes.

It was possible to show that 1 Ca^{2+} was displaced by approx. 2 chlorpromazine molecules. This was done as follows. The membrane concentration of Ca^{2+} was plotted as a function of the reciprocal of the total concentration of chlorpromazine

in the test tube (see Fig. 4). From extensive studies done earlier[61] it is known that all the membrane sites for chlorpromazine are occupied by the drug when it is present in extremely high concentration. In Fig. 4, therefore, extrapolation of the Ca^{2+}_{mem} to where $1/chlorpromazine_{total}$ became zero yielded the minimum Ca^{2+}_{mem} concentration still present when the chlorpromazine uptake was maximal. The difference

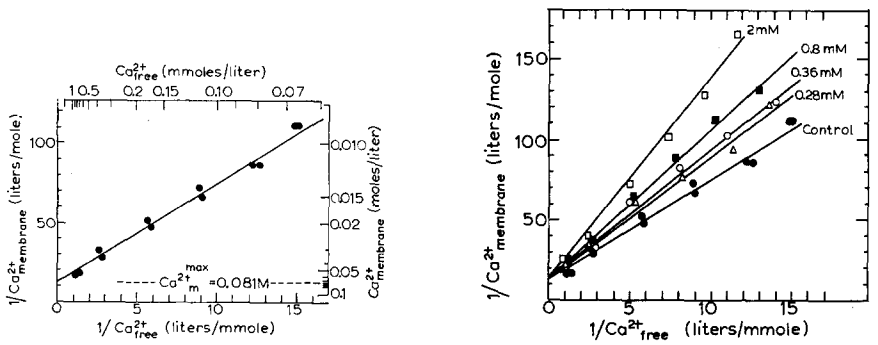

Fig. 2. The adsorption of Ca^{2+} to erythrocyte ghost membranes. The ordinate shows the amount of Ca^{2+} adsorbed (or its reciprocal) expressed as moles per l of solid (hydrated) membrane. The membrane concentration of Ca^{2+} is 0.081 M when the isotherm is extrapolated to an infinitely high free concentration of Ca^{2+}.

Fig. 3. The displacement of membrane Ca^{2+} by chlorpromazine. The ordinate represents the reciprocal of the membrane concentration of Ca^{2+}, in l of solid (hydrated) membrane per mole Ca^{2+}. The concentrations of chlorpromazine indicated in the figure represent the total concentration of the drug in the tube; the free concentration of chlorpromazine in the supernatant is less than 1/10th of the total concentration.

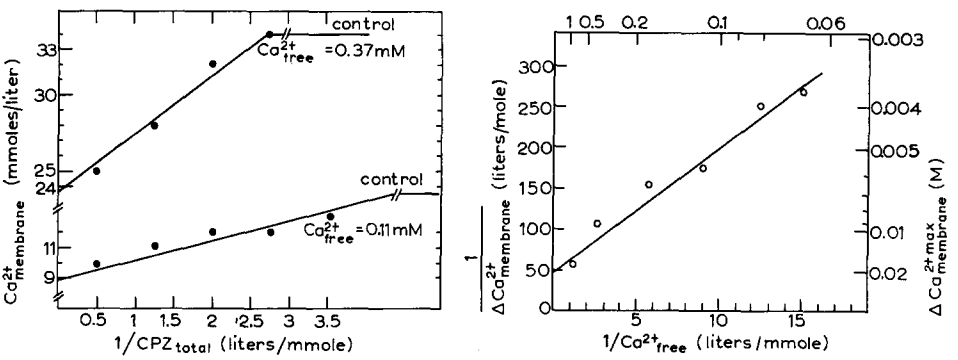

Fig. 4. The membrane concentration of Ca^{2+} at different total concentrations of chlorpromazine (CPZ). This figure shows that the amount of chlorpromazine-induced displacement of Ca^{2+} increased at higher concentrations of free Ca^{2+}. The maximum amount of Ca^{2+} displaced represented by Ca^{2+max}_{mem} (*cf.* Fig. 5), is the difference between Ca^{2+}_{mem} in the absence of chlorpromazine (control) and the Ca^{2+}_{mem} in the presence of a maximum amount of membrane chlorpromazine (which occurs when $1/chlorpromazine_{total} = 0$).

Fig. 5. The maximum amount of membrane-bound Ca^{2+} which is displaced by maximum amounts of membrane-bound chlorpromazine is plotted on the right ordinate as a function of the amount of free Ca^{2+}. This maximum amount of membrane-bound Ca^{2+} which is displaced is about 0.02 mole Ca^{2+} per l of solid (hydrated) membrane; the membrane-bound amount of chlorpromazine under these conditions is 0.066 M.

ΔCa^{2+}_{mem} between this minimum Ca^{2+}_{mem} and the control Ca^{2+}_{mem} represented the maximum amount of drug-induced displacement that occurred at this Ca^{2+}_{free}. This extrapolation was made for all the six Ca^{2+} concentrations tested. As the free concentration of Ca^{2+} increased, more Ca^{2+}_{mem} could be displaced by the drug. The drug-induced displacement, ΔCa^{2+}_{mem}, was hence graphed as a function of the Ca^{2+}_{free}, using the reciprocal plot shown in Fig. 5.

Since the reciprocal plot (in Fig. 5) was linear, it was possible to determine the maximum amount of the drug-induced displacement (Ca^{2+max}_{mem}) which occurred at the highest possible concentration of free Ca^{2+}. Extrapolating to zero for $1/Ca^{2+}_{free}$, the maximum amount of membrane Ca^{2+} that was displaced by chlorpromazine at saturation was 0.021 mole Ca^{2+} per l of solid membrane.

It has been shown previously that the membrane concentration of chlorpromazine at saturation is 0.066 mole drug per l solid membrane. 1 Ca^{2+}, therefore, is removed by about 3 molecules of chlorpromazine. In the present system, however, not all the possible binding sites for Ca^{2+} were occupied since it was shown that some of the ghosts were closed or impermeable to Ca^{2+}. More binding of Ca^{2+} would have resulted in more drug-induced displacement. The maximum error in these data would have occurred if all the ghosts were impermeable to Ca^{2+}. Opening up these cells would double the surface area available for Ca^{2+} adsorption and drug-induced displacement. It seems reasonable to assume, therefore, that the absolute displacement could at most have amounted to twice that found. This would result in a displacement of 1 Ca^{2+} by 1.5 molecules of chlorpromazine. It may be concluded, therefore, that 1 Ca^{2+} is displaced from the membrane by about 2 chlorpromazine molecules (1.5–3.1).

DISCUSSION

The results have shown (1) that the water space of ghosts which was accessible to inulin and to Ca^{2+} was less than the total water content, (2) that saponin or rehemolysis increased this water space, (3) that the membrane concentration of Ca^{2+} was 0.081 M at saturation, and (4) that chlorpromazine in membrane-stabilizing concentrations displaced membrane-associated Ca^{2+}.

The membrane concentration of Ca^{2+}

The concentration of Ca^{2+} in the membrane when all the sites were occupied was about 0.08 M. This value agrees approximately with that of GENT *et al.*[72] who found about 0.05 mole of Ca^{2+} per kg of dry ghost membrane at an equal ionic strength. GENT *et al.*[72] assumed that all the erythrocyte ghosts were open since freezing and thawing did not increase the amount of Ca^{2+} adsorption. While the ghosts used in the present study were not entirely open to inulin, it was calculated that the majority of the cells were open. The packed cell volume of the main stock suspension of ghosts had a value of at least 90 %. The addition of 0.5 ml ghosts and 0.4 ml buffer with or without other solutes, should lead to an inulin space of at least 50 % if all the cells were closed. The experimentally determined inulin space varied, but averaged around 85 % in these experiments. The volume of the ghosts which was unavailable to inulin was, therefore, of the order of one-fifth of the total intracellular ghost volume.

The displacement of membrane Ca^{2+} by local anesthetics

The fact that chlorpromazine, a potent local anesthetic, displaces Ca^{2+} from the plasmalemma supports the idea that local anesthetics may act in this manner to block nerve conduction[35–42]. It has already been mentioned (see INTRODUCTION) that erythrocytes and nerve membranes are stabilized at the same concentrations by the same drugs (except tetrodotoxin); this fact justifies the extrapolation of the present pharmacological findings to nerve membranes.

It is not surprising that very low free concentrations of chlorpromazine in the supernatant (of the order of 10 μM) should compete with Ca^{2+} concentrations about 100-fold higher. This is because the concentration of chlorpromazine in the membrane is very high, of the order of 4000 to 10000 times higher than that in the supernatant[61,62].

The present results showing chlorpromazine-induced displacement of plasma membrane Ca^{2+} should be distinguished from the effect of chlorpromazine on subcellular membranes. BALZER *et al.*[77] found that chlorpromazine did not affect the Ca^{2+}-storing capacity of sarcoplasmic reticulum; they did find, however, that the rate of Ca^{2+} exchange was reduced by the drug. In the present experiments with cell membranes, it was found that chlorpromazine did reduce the membrane capacity of Ca^{2+}. Since the procedure of the present experiments was to adsorb Ca^{2+} first and then to desorb it with the drug, the rate of Ca^{2+} exchange did not enter into the calculations; the supernatant concentrations were treated as if they were in a final equilibrium state. It was assumed that the concentration of Ca^{2+} in the extracellular space was the same as that in the intracellular space which was accessible to inulin.

CARVALHO[78], on the other hand, using the same preparation as BALZER *et al.*[77], did find a local anesthetic-induced reduction in the Ca^{2+} bound to the rabbit skeletal muscle sarcoplasmic reticulum.

The displacement of Ca^{2+} from lipid molecules by drugs[37–45] is not restricted to local anesthetics. Amino acids, cholinergic compounds and many other drugs are effective in displacing the lipid-associated Ca^{2+}. It remains to be determined, therefore, whether the displacement of Ca^{2+} from the plasmalemma is restricted to anesthetics or not. Current studies are in progress.

Changes in membrane properties that may occur as a result of the anesthetic-induced displacement of membrane Ca^{2+}

The following changes in membrane physiology might be expected to occur as a result of the chlorpromazine-induced displacement of the cell membrane Ca^{2+}.

(1) Occupation of the Ca^{2+} sites on the membrane by the local anesthetic may impede the translocation rates (both passive and active) of various ions, including Ca^{2+} itself[79,53,22].

(2) The Ca^{2+} displacement may cause the cell membrane to expand in area. Membrane expansion is known to occur in the presence of local anesthetics[53–62].

(3) The viscosity of the membrane may change[80]. It has been found that local anesthetics 'fluidize' the membrane[60,81].

(4) The Ca^{2+}-ATPase (Ca^{2+}-activated ATPase) of the cell membrane may be inhibited. This enzyme has been considered to play a contractile role in the cell membrane[82].

(5) Membrane Ca^{2+} would not now be available for excitation–contraction

coupling and stimulus–secretion coupling (see INTRODUCTION). This would include the inhibition of nerve action potentials which apparently requires membrane Ca^{2+}.

Chemical nature of the binding sites of Ca^{2+}

Previous work indicated that chlorpromazine attached to the membrane partially by means of a hydrophobic bond, and that the binding sites could be lipid or protein[61]. HARRISON AND LONG[64] have recently shown that essentially all the Ca^{2+} of the erythrocyte is associated with the membrane. These authors have also found that the membrane Ca^{2+} could not be extracted with chloroform–methanol. The Ca^{2+} of the membrane appear to be associated, therefore, with either the membrane protein or triphosphoinositol (which is present in the membrane at the same molarity as Ca^{2+}; triphosphoinositide is not extracted by chloroform–methanol; see ref. 64 for a discussion on this matter). It appears possible, therefore, that chlorpromazine and Ca^{2+} may be competing for membrane protein sites.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Janet Forstner, Dr. J. Manery-Fisher, Mrs. T. Ambus and Mrs. E. Dryden for many interesting discussions and their kind help and cooperation, to Professor H. L. Booij and Dr. J. Van Steveninck for their assistance in arranging W. Kwant's stay in Canada, and to the Medical Research Council of Canada for their support (Grant MA-2951).

REFERENCES

1 F. S. LOCKE, *Centr. Physiol.*, 8 (1894) 166.
2 F. BRINK, *Pharmacol. Rev.*, 6 (1954) 243.
3 P. FATT AND B. KATZ, *J. Physiol.*, 120 (1953) 171.
4 B. FRANKENHAEUSER AND A. L. HODGKIN, *J. Physiol.*, 137 (1957) 218.
5 B. FRANKENHAEUSER, *J. Physiol.*, 137 (1957) 245.
6 N. ISHIK AND M. SATO, *Japan. J. Physiol.*, 7 (1957) 51.
7 J. C. DALTON, *J. Gen. Physiol.* 41 (1958) 529.
8 J. C. DALTON, *J. Gen. Physiol.*, 42 (1959) 971.
9 W. J. ADELMAN AND J. ADAMS, *J. Gen. Physiol.*, 45 (1959) 655.
10 T. NARAHASHI AND T. YAMASAKI, *J. Physiol.*, 151 (1960) 75.
11 W. J. ADELMAN AND J. W. MOORE, *J. Gen. Physiol.*, 45 (1961) 93.
12 F. J. JULIAN, J. W. MOORE AND D. E. GOLDMAN, *J. Gen. Physiol.*, 45 (1962) 1217.
13 E. A. LIBERMAN, L. M. TSOFINA AND M. N. VAINTAVAIG, *Biophysics USSR (Engl. Transl.)*, 6 (1961) 29.
14 K. KOKETSU AND K. NODA, *J. Cellular Comp. Physiol.*, 59 (1962) 323.
15 T. NARAHASHI, *J. Cellular Comp. Physiol.*, 64 (1964) 73.
16 S. HAGIWARA, S. CHICHIBU AND K. NAKA, *J. Gen. Physiol.*, 48 (1964) 163.
17 T. NARAHASHI, *Comp. Biochem. Physiol.*, 19 (1966) 759.
18 M. P. BLAUSTEIN AND D. E. GOLDMAN, *J. Gen. Physiol.*, 49 (1966) 1043.
19 I. TASAKI, A. WATANABE AND L. LERMAN, *Am. J. Physiol.*, 213 (1967) 1465.
20 T. NARAHASHI, N. C. ANDERSON AND J. W. MOORE, *Proc. 10th Biophys. Soc.*, 147 (1966).
21 R. E. TAYLOR *Am. J. Physiol.*, 196 (1959) 1071.
22 A. M. SHANES, *Pharmacol. Rev.*, 10 (1958) 59.
23 B. HILLE, *Nature*, 210 (1966) 1220.
24 B. HILLE, *J. Gen. Physiol.*, 50 (1967) 1287.
25 A. L. HODGKIN, *Biol. Rev.*, 26 (1951) 339.
26 J. M. TOBIAS, *J. Cellular Comp. Physiol.*, 52 (1958) 89.
27 J. ACEVES AND X. MACHINE, *J. Pharmacol. Exptl. Therap.*, 140 (1963) 138.
28 M. B. FEINSTEIN, *J. Pharmacol. Exptl. Therap.*, 152 (1966) 516.
29 M. B. FEINSTEIN AND M. PAIMRE, *Nature*, 214 (1967) 151.

30 R. P. RUBIN, M. B. FEINSTEIN, S. D. JAANUS AND M. PAIMRE, *J. Pharmacol. Exptl. Therap.*, 155 (1967) 463.
31 W. W. DOUGLAS AND T. KANNO, *Brit. J. Pharmacol. Chemotherap.*, 30 (1967) 612.
32 R. P. RUBIN AND E. MIELE, *Arch. Pharmak. Exptl. Pathol.*, 260 (1968) 298.
33 J. M. FRANKENHEIM AND S. SHIBATA, *J. Pharmacol. Exptl. Therap.*, 163 (1968) 17.
34 M. B. FEINSTEIN, *J. Gen. Physiol.*, 48 (1964) 357.
35 M. P. BLAUSTEIN, *Biochim. Biophys. Acta*, 135 (1967) 653.
36 S. EHRENPREIS, *Biochim. Biophys. Acta*, 44 (1960) 561.
37 S. EHRENPREIS, *Nature*, 201 (1964) 887.
38 M. B. FEINSTEIN AND M. PAIMRE, *Biochim. Biophys. Acta*, 115 (1966) 33.
39 M. P. BLAUSTEIN AND D. E. GOLDMAN, *Science*, 153 (1966) 429.
40 W. G. NAYLER, *J. Pharmacol. Exptl. Therap.*, 153 (1966) 479.
41 D. E. GOLDMAN AND M. P. BLAUSTEIN, *Ann. N.Y. Acad. Sci.*, 137 (1966) 967.
42 H. HAUSER AND R. M. C. DAWSON, *Biochem. J.*, 109 (1968) 909.
43 G. A. ROGENESS, L. G. KRUGMAN AND L. G. ABOOD, *Biochim. Biophys. Acta*, 125 (1966) 319.
44 F. CARPENEDO, M. FERRARI AND M. FURLANUT, *J. Pharm. Pharmacol.*, 20 (1968) 733.
45 S. J. MULE, *Biochem. Pharmacol.*, 18 (1969) 339.
46 A. SANDOW, *Pharmacol. Rev.*, 17 (1965) 265.
47 E. BOZLER, *Am. J. Physiol.*, 216 (1969) 671.
48 C. CAPUTO AND M. GIMENEZ, *J. Gen. Physiol.*, 50 (1967) 2177.
49 W. W. DOUGLAS, *Brit. J. Pharmacol.*, 34 (1968) 451.
50 B. KATZ AND R. MILEDI, *J. Physiol.*, 195 (1968) 481.
51 S. M. KIRPEKAR AND Y. MISU, *J. Physiol.*, 188 (1967) 219.
52 A. ISHIDA, *Japan. J. Physiol.*, 18 (1968) 471.
53 P. SEEMAN, *Intern. Rev. Neurobiol.*, 9 (1966) 145.
54 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1632.
55 P. SEEMAN AND J. WEINSTEIN, *Biochem. Pharmacol.*, 15 (1966) 1737.
56 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1753.
57 P. SEEMAN, *Membrane Stabilization and Expansion by Drugs*, in E. DEUTSCH, E. GERLACH AND K. MOSER, *Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes*, Georg Thieme Verlag, Stuttgart, 1968, p. 384.
58 P. SEEMAN, W. O. KWANT, T. SAUKS AND W. ARGENT, *Biochim. Biophys. Acta*, 183 (1969) 490.
59 P. SEEMAN, W. O. KWANT AND T. SAUKS, *Biochim. Biophys. Acta*, 183 (1969) 499.
60 J. METCALFE, P. SEEMAN AND A. S. V. BURGEN, *Mol. Pharmacol.*, 4 (1968) 87.
61 W. O. KWANT AND P. SEEMAN, *Biochim. Biophys Acta*, 183 (1969) 530
62 W. O. KWANT, S. ROTH AND P. SEEMAN, *Federation Proc.*, 28 (1969) 614.
63 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
64 D. G. HARRISON AND C. LONG, *J. Physiol.*, 199 (1968) 367.
65 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
66 J. CLIFFORD, B. A. PETHICA AND E. G. SMITH, in L. BOLIS AND B. A. PETHICA, *Membrane Models and the Formation of Biological Membranes*, North Holland, Amsterdam, 1968, p. 19.
67 M. BARCLAY, R. K. BARCLAY, E. S. ESSNER, V. P. SKIPSKI AND O. TEREBUS-KEKISH, *Science*, 156 (1967) 665.
68 C. D. MITCHELL AND D. J. HANAHAN, *Biochemistry*, 5 (1966) 51.
69 P. SEEMAN, *J. Cell Biol.*, 32 (1967) 55.
70 R. BAKER, *Federation Proc.*, 26 (1967) 1785.
71 M. DURRUTI-CUBRIA, E. SEIFEN AND H-L. SCHMIDT, *Z. Physiol. Chem.*, 348 (1967) 1043.
72 W. L. GENT, J. R. TROUNCE AND M. WALSER, *Arch. Biochem. Biophys.*, 105 (1964) 582.
73 H. SCHNEIDER, *Biochim. Biophys. Acta*, 163 (1968) 451.
74 P. ROSENBERG AND E. BARTELS, *J. Pharmacol. Exptl. Therap.*, 155 (1967) 532.
75 W. O. KWANT AND J. VAN STEVENINCK, *Biochem. Pharmacol.*, 17 (1968) 2215.
76 H. J. SCHATZMANN AND F. F. VINCENZI, *J. Physiol.*, 201 (1969) 369.
77 H. BALZER, M. MAKINOSE AND W. HASSELBACH, *Arch. Exptl. Pathol. Pharmakol.* 260 (1968) 444.
78 A. P. CARVALHO, *J. Gen. Physiol.*, 52 (1968) 622.
79 A. S. KUPERMAN, B. T. ALTURA AND J. A. CHEZAR, *Nature*, 217 (1968) 673.
80 D. W. DEAMER AND D. G. CORNWELL, *Biochim. Biophys. Acta*, 116 (1966) 555.
81 J. METCALFE AND A. S. V. BURGEN, *Nature*, 220 (1968) 587.
82 P. WINS AND E. SCHOFFENIELS, *Arch. Intern. Physiol. Biochem.*, 26 (1969) 812.