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CHLORAL HYDRATE INHIBITION *IN VITRO* OF ATPase IN MEMBRANE OF RAT ERYTHROCYTES AND IN MICROSOMES OF DOG KIDNEY EXTERNAL MEDULLA

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Abstract—The study of the general anesthetic chloral hydrate and its effects on rat erythrocyte membranes and dog kidney microsomes showed that ATPases were reversibly inhibited in every case. The inhibition was cooperative in the cases of $(Mg^{2+} + Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase of rat erythrocyte membrane, while Ca^{2+}-ATPase and $(Mg^{2+} + Ca^{2+})$-ATPase were non-cooperative. The chloral hydrate concentrations necessary to diminish the activity of the enzyme to half of the V_{max} (I_{50}) were 6 mM for Ca^{2+}-ATPase from erythrocyte membranes and 82 mM for Mg^{2+}-ATPase from intact external kidney medulla microsomes. When Ca^{2+}-ATPase was studied in the absence of Mg^{2+} in these microsomes, the affinity for Ca^{2+} was very low, but the enzyme was sensitive to chloral hydrate.

It has been postulated that depressant drugs, like local anesthetics that block peripheral nerve conduction, affect the activity of $(Na^+ + K^+)$-ATPase of microsomal membrane from bovine cerebral cortex [1]. Other investigators found that local anesthetics such as tetracaine inhibit $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase in erythrocyte membrane in humans [2].

In this investigation, we studied the general anesthetic chloral hydrate (2,2,2-trichloro-1,1-ethanediol) which is widely used in veterinary work. We thought that it would be of interest to establish the mechanisms by which this drug exerts its effects at the biochemical level.

MATERIALS AND METHODS

White albino rats weighting 200–300 g were used for the experiments. Blood was obtained by cardiac puncture after ether anesthesia, utilizing heparin as the anticoagulant. All the following steps for the preparation of erythrocyte membrane were carried out at 0–5°. The erythrocytes were separated by centrifugation and washed three times with a 155 mM Tris–HCl buffer (pH 8.0) (buffer A).

The white cells were eliminated during the washings. The erythrocyte pellet was hemolyzed for 20 min in 20 vol. of a buffer composed of 15 mM Tris–HCl, 1 mM ethyleneglycolbis (amino-ethylether) tetra-acetate (EGTA) (pH 8.0) (buffer B). The suspension was centrifuged at 27,000 *g* for 20 min, and the pellet was washed three times with buffer B or until total elimination of hemoglobin. The pellet was finally suspended in buffer A and centrifuged at 27,000 *g* for 20 min; the membranes so obtained were resuspended in a volume of buffer A equal to the original volume of the blood red cells and were frozen in liquid nitrogen for not more than 7 days before use.

The membrane protein concentration was 2.5 to 3.5 mg/ml. The microsomal fraction (intact microsomes) from external medulla of dog kidney was prepared according to the method of Jørgensen [3].

The microsomes were resuspended in sucrose solution containing 0.25 M sucrose and 0.03 M histidine (pH 7.2) to a final concentration of protein of 11–16 mg/ml. This suspension was fractionated and kept in liquid nitrogen for no longer than a month.

A $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.1.6.3) preparation purified from dog kidney (grade IV, Sigma Chemical Co., St. Louis, MO) [4] was used in some experiments.

ATPase determination. The enzyme assay mixture was similar to that described by Bloj *et al.* [5]. For $(Mg^{2+} + Na^+ + K^+)$-ATPase determination, the medium contained 25 mM Tris–HCl buffer (pH 8.0), 2 mM Mg^{2+}, 80 mM Na^+, 33 mM K^+, 1 mM ATP and 75–100 μg of membrane protein from erythrocytes and 10–25 μg of microsomal protein, in a final volume of 2.0 ml. Mg^{2+}-ATPase was determined without Na^+ and K^+; the protein concentration was doubled compared to that used for $(Mg^{2+} + Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was calculated as the difference between the $(Mg^{2+} + Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase. $(Mg^{2+} + Ca^{2+})$-ATPase was determined in the presence of 0.2 mM Ca^{2+} and 2 mM Mg^{2+}, whereas Ca^{2+}-ATPase was calculated as the difference between the $(Mg^{2+} + Ca^{2+})$-ATPase and the Mg^{2+}-ATPase, except for microsomal Ca^{2+}-ATPase which was determined in the absence of Mg^{2+}.

A microsomal fraction of external medulla of dog kidney (11.4 mg protein/ml) was washed with a buffer containing 0.25 M sucrose, 0.03 M histidine,

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0.01 M EDTA (pH 7.2) in a proportion of 1:30 (v/v) and centrifuged at 48,000 *g* for 20 min, the pellet was washed twice with 25 ml of the same buffer without EDTA, centrifuging each time at 48,000 *g* for 20 min. The pellet was finally resuspended to 4.9 mg protein/ml. This fraction was used to determine the ATPase activity with different concentrations of Ca^{2+} in the absence of Mg^{2+}.

The assay mixture was incubated at 37° for 60 min in every case. The reaction was stopped by adding 0.1 ml of 7% SDS (laurylsulfate) [6] (final concentration 0.33%).

Phosphate was determined by the Fiske–Subbarow method [7]. Enzyme activity was expressed as relative activity (extinction at 660 nm) or specific activity. The method of Lowry *et al.* [8] was used for protein determination, utilizing bovine seroalbumin as standard.

The values of *n* (Hill coefficients) were calculated using the following equation [9, 10]:

$$\log (v/V - v) = \log K_i - n \log (I)$$

where *V* is the velocity in the absence of the inhibitor, *v* the velocity with the inhibitor, *n* the Hill coefficient, and K_i the constant of the Hill equation. I_{50} represents the concentration of inhibitor that reduced the V_{max} to 50% and was calculated from the graphs of Hill.

Chloral hydrate does not interfere with P_i determination at a concentration up to 100 mM of the inhibitor.

RESULTS

Enzyme activity versus enzyme concentration and time of incubation. Figure 1 shows the $(Mg^{2+} + Na^+ + K^+)$-ATPase activity of rat erythrocyte membranes expressed as extinction at 660 nm versus micrograms of membrane protein. The control exhibited linear activity up to a concentration of 180 μg protein. This figure shows that chloral hydrate (CH) inhibited the enzyme by 46 and 66% when the concentration of the anesthetic was 40 and 70 mM respectively. These percentages of inhibition were constant for all the enzyme concentrations utilized. Figure 2 represents the $(Mg^{2+} + Na^+ + K^+)$-ATPase activity with the anesthetic against incubation time. A CH concentration of 30 mM produced a 35% inhibition that was constant throughout the time of incubation.

The enzyme activity was linear for the control during the 60 min that the experiment lasted. There was only a change in the slope in the presence of CH when compared to the control.

Reversibility of $(Mg^{2+} + Na^+ + K^+)$*-ATPase activity by CH in erythrocyte membranes by washing.* $(Mg^{2+} + Na^+ + K^+)$-ATPase activity decreased to 34% of the controls in the presence of 70 mM CH. The enzyme activity was restored to normal values when the membranes preincubated with the inhibitor were subjected to several washings with buffers A and B (Table 1).

Effect of CH concentration on ATPases. The inhibitory effects of CH on the $(Mg^{2+} + Na^+ + K^+)$-ATPase of erythrocyte membranes prompted us to investigate the effects of the anesthetic on other ATPase activities present in the erythrocyte membrane. Fig. 3A shows the inhibitory effect of CH on ATPase activities. $(Na^+ + K^+)$-ATPase was more sensitive to CH than was Mg^{2+}-ATPase, according to the I_{50} (Table 2). The curves were sigmoidal suggesting a cooperative behavior of the enzyme activities, and Hill coefficients confirmed this (Fig. 3B and Table 2) since the values of *n* were above 2 in every case. With concentrations of CH of less than 5 mM, the original ATPase activity was 100% when compared to the controls (Fig. 3A).

The effects of different concentrations of CH on $(Mg^{2+} + Ca^{2+})$- and Ca^{2+}-ATPase activities of erythrocyte membranes were more pronounced than the activities with Mg^{2+} and $(Na^{2+} + K^+)$ as shown in Fig. 4. These activities were more sensitive than those shown in Fig. 3A. The I_{50} values were 18 and

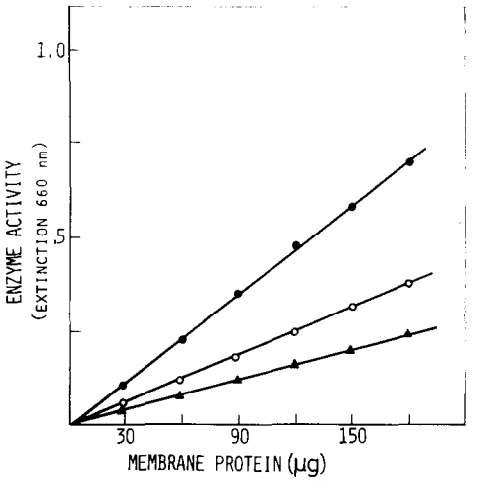

Fig. 1. $(Mg^{2+} + Na^+ + K^+)$-ATPase activity of rat erythrocyte membranes in relation to enzyme concentration, with and without chloral hydrate. Key: (●) control; (○) 40 mM chloral hydrate; and (▲) 70 mM chloral hydrate. (Time of incubation: 60 min.)

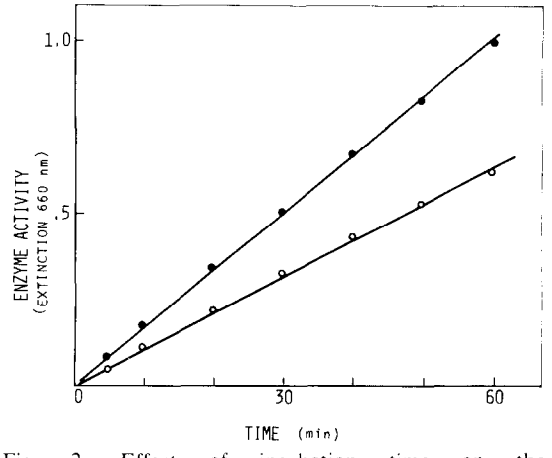

Fig. 2. Effect of incubation time on the $(Mg^{2+} + Na + K^+)$-ATPase activity of rat erythrocyte membranes, with and without chloral hydrate. Key: (●) control; and (○) 30 mM chloral hydrate.

Table 1. Reversibility of chloral hydrate inhibition by washing*

$(Mg^{2+} + Na^+ + K^+)$-ATPase of erythrocyte membrane	Specific activity (μmoles P_i/mg protein/hr)	Percent activity compared to control
Control	2.95	
Preincubated	1.00	34
Preincubated and washed	3.06	103

* A suspension aliquot of rat erythrocyte ghosts (1.25 mg protein) was preincubated at 37° for 5 min in the presence of 70 mM CH, 15.5 mM Tris–HCl (pH 8.0), 1 mM $ATPNa_2$, 80 mM Na^+, 32 mM K^+ and 2 mM Mg^{2+}. The control was subjected to the same treatment omitting CH. Both preparations were centrifuged, and the resulting pellets were washed twice with buffer B and once with buffer A. After the last washing the pellet was resuspended in the same buffer, and the $(Mg^{2+} + Na^+ + K^+)$-ATPase activity was determined. The results are the average of two independent experiments.

6 mM respectively (Table 2). Ca^{2+}-ATPase activity was the most sensitive to CH; the activity fell rapidly and was abolished completely when the concentration was 30 mM (Fig. 4). The *n* values for these activities were −1.1 and −1.0 (Table 2), which would indicate that CH is not an allosteric effector for $(Mg^{2+} + Ca^{2+})$- and Ca^{2+}-ATPase from rat erythrocyte membranes. $(Mg^{2+} + Ca^{2+})$-ATPase activity of erythrocyte membranes was more sensitive to CH than Mg^{2+}-ATPase, possibly because Ca^{2+} may act as a sensitizing ion.

CH concentration effects on ATPase activities from microsomes of the external medulla of dog kidney. Figure 5 shows the effects of CH on $(Mg^{2+} + Na^+ + K^+)$-, Mg^{2+}- and $(Na^+ + K^+)$-ATPase activities from dog kidney microsomal fractions obtained by the method of Jørgensen. The most important characteristic is that the curves are not sigmoidal. The percentage of the enzyme activity was less than 100% even at the lowest concentration of CH; similar curves were obtained for inhibition of the purified enzyme by CH (Sigma, grade IV) (not shown).

The *n* values for the three activities appeared to be close to −1.0 for both intact and purified microsomal enzymes; these values were lower than those obtained from erythrocytes. The I_{50} values (Table 2) show that intact and purified microsomes had an $(Na^{2+} + K^+)$-ATPase that appeared to be more sensitive to CH than the corresponding activity of erythrocyte membrane; on the other hand, the Mg^{2+}-ATPase activities were less sensitive in microsomes than in erythrocyte membranes. The 100% inhibition of Mg^{2+}-ATPase and $(Mg^{2+} + Na^+ + K^+)$-ATPase of intact or purified microsomes was attained with 300 mM CH, which fact justifies our application of the Hill equation to calculate the *n* values.

Activation of ATPase by Ca^{2+} in microsomes of external medulla of dog kidney. Figure 6 shows the Ca^{2+} activation of ATPase in intact microsomes in

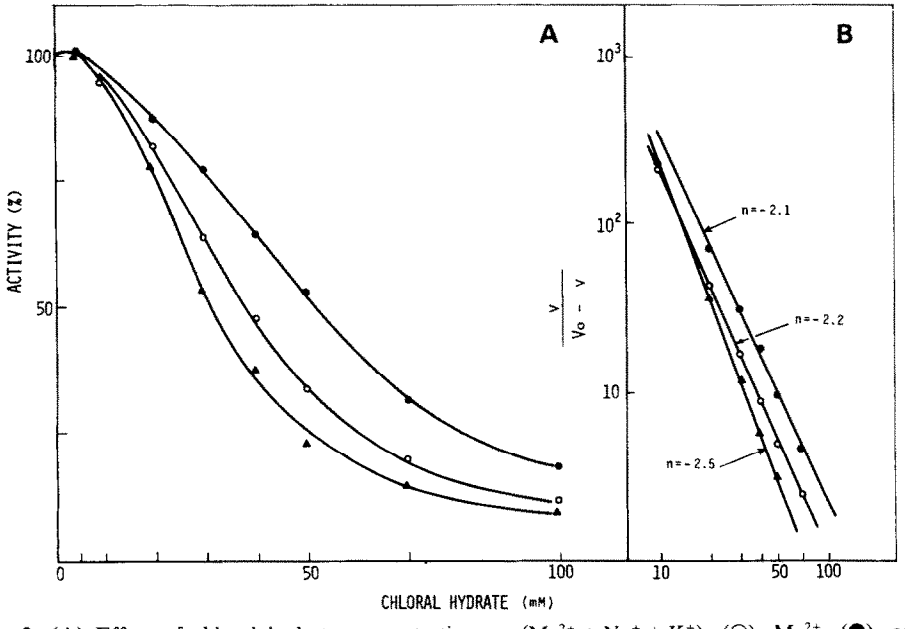

Fig. 3. (A) Effect of chloral hydrate concentration on $(Mg^{2+} + Na^+ + K^+)$- (○), Mg^{2+}- (●), and $(Na^+ + K^+)$- (▲) ATPase activities of rat erythrocyte membranes. (B) *n* Values (Hill coefficients) for the three ATPase activities.

Table 2. n Values (Hill coefficients) and I_{50} for chloral hydrate inhibition on ATPase activities from different sources

	Enzyme source					
	Rat erythrocyte membrane		Dog kidney external medulla microsomes			
			Purified (Sigma, grade IV)		Intact	
	n	I_{50} (mM)	n	I_{50} (mM)	n	I_{50} (mM)
$(Mg^{2+} + Na + K^-)$ATPase	−2.2	39	−1.34	21	−1.37	39
Mg^{2+}-ATPase	−2.1	50	−1.11	62	−1.13	82
$(Na^- + K^-)$-ATPase	−2.5	35	−1.70	14	−1.70	17
$(Mg^{2+} + Ca^{2+})$-ATPase	−1.1	18				
Ca^{2+}-ATPase	−1.0	6			−1.34*	62*

* n Values and I_{50} for Ca^{2+}-ATPase were independent of added Mg^{2+} in dog kidney microsomes (determined at the optimal Ca^{2+} concentration of 2.5 mM).

the absence of Mg^{2+}. The optimum concentration of Ca^{2-} was 2.5 mM; higher concentrations were slightly inhibitory. We called this ATPase activity: Ca^{2-}-ATPase independent of added Mg^{2-}. It has also been found in the plasma membrane of skeletal muscle [11], cardiac muscle [12] and vascular smooth muscle [13]. When the CH effect was determined in the presence of 2.5 mM Ca^{2+} (optimum concentration), the activity showed an n of −1.34 and an I_{50} of 62 mM (Table 2).

DISCUSSION

$(Mg^{2-} + Na^- + K^+)$-ATPase from rat erythrocyte membrane was inhibited by CH (Figs. 1 and 2). Figure 2 shows that, when the activity was determined at different concentrations of CH (40 and 70 mM), the lines of the graph met at the origin, thus indicating that the inhibition was reversible [14]. The reversibility of the reaction was confirmed since the membranes of rat erythrocytes preincubated with the inhibitor and washed several times regained 100% of the enzyme activity (Table 1).

On the other hand, the ATPase activities associated with erythrocyte membranes such as Mg^{2-}- and $(Na^{2-} + K^+)$-ATPases showed sigmoidal curves (Fig. 3A) with n values above 2 (Fig. 3B and Table 2). Although we did not use purified enzyme preparations, these results may indicate that more than one molecule of the inhibitor interacts with the enzyme molecule or that it is influenced by the medium surrounding it. Supporting this fact are results obtained with inhibitors of membrane ATPase, where cooperative behavior was observed when subjected to the action of an effector [15, 16]. $(Mg^{2+} + Ca^{2+})$-ATPase and Ca^{2+}-ATPase activities in erythrocyte membrane exhibited hyperbolic curves of inhibition (Fig. 4) with n values of −1.1 and −1.0 respectively; this would suggest non-cooperative behavior between the enzyme and the inhibitor. Similar results were obtained with Mg^{2-}- and $(Na^+ + K^-)$-ATPase activities of intact micro-

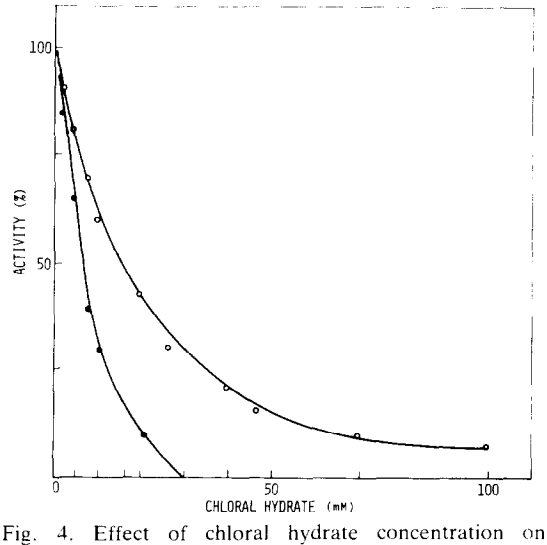

Fig. 4. Effect of chloral hydrate concentration on $(Mg^{2-} + Ca^{2-})$- (○) and Ca^{2-}- (●) ATPase activities of rat erythrocyte membranes.

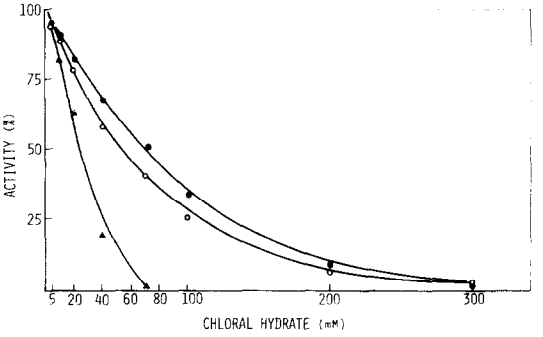

Fig. 5. Effect of chloral hydrate concentration on $(Mg^{2-} + Na^- + K^-)$- (○), Mg^{2+}- (●), and $(Na^- + K^+)$ (▲) ATPase activities of dog kidney external medulla intact microsomes.

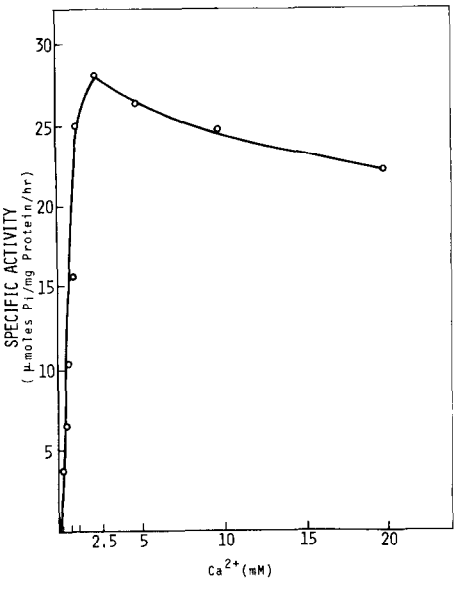

Fig. 6. Effect of Ca^{2+} concentration on dog kidney microsome ATPase.

somes and microsomal purified fractions (Sigma, grade IV) of dog kidney, which also exhibited non-sigmoidal curves with very similar n values that were much lower than for similar preparations from erythrocytes (Table 2).

Ca^{2+}-ATPase activity was the most sensitive to CH in erythrocyte membranes, probably because of a sensitizing activity of Ca^{2+}, while Mg^{2+}-ATPase was the least sensitive (Table 1). Preliminary work in our laboratory also indicated that CH blocked −SH groups that are essential for the enzyme activity [17], but additional work is necessary to elucidate the real mechanism of choral hydrate action.

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