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INHIBITION OF ATPASE ACTIVITY IN HUMAN RED CELL MEMBRANES BY TETRACAINE*

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Abstract—This study describes the inhibition by tetracaine of two transport-related enzymes in human red cell membranes: NaK-ATPase and Ca-ATPase. Tetracaine (2·5 mM) inhibited both enzymes between pH 7·0 and 7·8; the degree of inhibition increased as the pH increased. Inhibition of NaK-ATPase increased sharply between pH 7·4 and 7·8, whereas inhibition of Ca-ATPase increased gradually. Concentration-effect studies were conducted at tetracaine concentrations between 0·25 and 2·5 mM at pH 7·8. NaK-ATPase was significantly inhibited by 1·0 mM tetracaine and inhibition increased sharply with tetracaine concentration. Ca-ATPase was significantly inhibited by 0·5 mM tetracaine, but inhibition was essentially linear with tetracaine concentration. In the presence of 2·5 mM tetracaine the $\kappa_{0.5}$ for activation of NaK-ATPase by Na^+ and K^+ was increased 3- and 5-fold respectively. The V_{max} was also decreased. In the case of Ca-ATPase, tetracaine did not alter the $\kappa_{0.5}$ for activation by Ca^{2+}, but decreased V_{max}. These results demonstrate that NaK-ATPase is more sensitive than Ca-ATPase to inhibition by tetracaine and suggest that the non-ionized form of the local anesthetic is primarily responsible for the inhibition of both enzymes.

Local anesthetics block excitability in nerve and other tissues by acting on the plasma membrane to prevent the passive inward movement of Na^+, which initiates the action potential [1]. This effect alone can explain anesthesia. Nevertheless, local anesthetics could also modify other membrane functions which are required for the maintenance of the excitable state. For example, an inhibition of the active transport of Na^+ and K^+, or of Ca^{2+}, could contribute to the duration of action or efficacy of local anesthetics. There are conflicting reports in the literature concerning the effect of local anesthetics on active transport [2–5], and the question of whether there is or is not significant inhibition appears unresolved. We therefore pursued this problem with the aim of finding conditions under which inhibition might be demonstrated.

In the work described here we studied the effect of tetracaine on two transport-related enzymes in human red cell membranes: NaK-ATPase† and Ca-ATPase. The former enzyme mediates the active transport of Na^+ and K^+ [6], and the latter mediates the active efflux of Ca^{2+} [7]. The red cell was chosen for study because it contains a single homogeneous membrane and is frequently used as a model system to evaluate drug–membrane interactions [8].

EXPERIMENTAL

Outdated human blood was obtained from the blood bank, and membranes were prepared as previously described [9]. This involved lysis and repeated washing in 1 mM Tris–EDTA (pH 7·8). The protein content of each preparation was measured by the method of Lowry *et al.* [10].

The following conditions were common to all experiments: ATP, 2 mM; Mg^{2+}, 2 mM; and Tris–HCl, 63 mM (pH 7·0, 7·4 or 7·8 at 38°). The reaction volume was 2 ml. The conditions for assay of the three ATPase activities are as follows—(1) Mg-ATPase: Na^+, 60 mM; Tris–EGTA, 0·25 mM; and ouabain, 0·5 mM; (2) NaK-ATPase: Na^+, 120 mM; K^+, 15 mM; and Tris–EGTA, 0·25 mM; (3) Ca-ATPase: Na^+, 60 mM; Ca^{2+}, 0·2 mM; and ouabain, 0·5 mM. All cations were added as the Cl^- salt. Variations from this standard protocol are described in the text.

Assays were carried out at 38°. ATPase activity was determined by measuring the inorganic phosphate (Pi) produced, using a modification of the method of Fiske and SubbaRow [11], as described by Bond and Green [9]. When tetracaine was present, 300 mg Dowex-50 cation-exchange resin was added after the reaction was stopped by the addition of 1·2 M perchloric acid. This step was required to remove tetracaine, which interferes with Pi determinations.

Activity is expressed either as μmoles Pi/mg of protein/hr or as per cent of control activity. In all cases NaK-ATPase or Ca-ATPase activity was calculated from the total activity by subtracting the Mg-ATPase activity measured concurrently. Experimental data represent the average of two or more experiments performed in duplicate. Statistical comparisons between mean values were performed with Student's *t*-test.

RESULTS

Time-course and pH dependence of inhibition. In preliminary experiments we found that 2·5 mM tetracaine inhibited both ATPase activities at each pH.

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† Abbreviations: NaK-ATPase, $(Na^+ + K^+ + Mg^{2+})$-ATP phosphohydrolase (EC 3.6.1.3); Ca-ATPase, $(Ca^{2+} + Mg^{2+})$-ATP phosphohydrolase (EC 3.6.1.3); EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; $\kappa_{0.5}$, concentration of ligand required to half maximally activate the enzyme; V_{max}, maximum velocity.

Table 1. ATPase activities of human red cell membrane in the absence, and presence of tetracaine

	ATPase activity* (μmoles Pi/mg protein/hr)	Per cent activity with 2·5 mM tetracaine†
Mg-ATPase		
pH 7·0	0·14 ± 0·01 (5)	82·8 ± 2·6
pH 7·4	0·15 ± 0·01 (5)	81·0 ± 4·1
pH 7·8	0·15 ± 0·01 (5)	88·2 ± 4·2
NaK-ATPase		
pH 7·0	0·37 ± 0·05 (4)	75·7 ± 4·5
pH 7·4	0·37 ± 0·03 (6)	64·8 ± 1·9
pH 7·8	0·35 ± 0·04 (7)	11·4 ± 2·6
Ca-ATPase		
pH 7·0	0·91 ± 0·07 (5)	86·4 ± 3·6
pH 7·4	0·95 ± 0·06 (5)	70·2 ± 2·5
pH 7·8	0·74 ± 0·04 (7)	53·3 ± 3·2

* Values are expressed as mean ± S.E. Numbers in parentheses refer to the number of duplicate assays.

† Values are expressed as per cent ± S.E.

The onset of inhibition was rapid, and inhibition did not increase with time during a typical incubation period. These enzymes are therefore stable in the presence of tetracaine, and activity measured after a fixed incubation time in subsequent experiments is an accurate reflection of rate.

We examined the pH dependence of inhibition by tetracaine in order to determine whether the ionized or non-ionized form of the drug is the more effective inhibitor. Tetracaine (p*K*a = 8·24) undergoes substantial changes in state of ionization as pH is varied within the physiologic range. We selected pH 7·0, 7·4 and 7·8 for study, where the per cent of the drug in the non-ionized form is 6, 13 and 27 per cent respectively.

Table 1 compares the specific activities of the ATPases under study and shows the influence of pH on inhibition by 2·5 mM tetracaine. Mg-ATPase was inhibited slightly, but this was independent of pH. Inhibition of NaK-ATPase and Ca-ATPase was more pronounced and increased as the pH increased. At pH 7·8, a marked difference in the sensitivity of the two enzymes to tetracaine became apparent. These results indicate that the non-ionized form of tetracaine is responsible for the inhibition of both enzymes or is at least more effective than the ionized form.

Inhibition as a function of tetracaine concentration. Figure 1 shows the concentration dependence of inhibition by tetracaine at pH 7·8. In these experiments, at constant pH, the concentrations of ionized and non-ionized tetracaine increased proportionately. NaK-ATPase was not significantly inhibited by tetracaine at concentrations below 1 mM. Beyond this point, inhibition increased sharply with increasing concentration. Ca-ATPase was significantly inhibited by 0·5 mM tetracaine ($P < 0·01$), but in contrast to NaK-ATPase, inhibition was linear with concentration over the entire range.

A comparison of the results of Table 1 and Fig. 1 reveals two similarities. As pH was increased at a fixed tetracaine concentration (Table 1) or as tetracaine concentration was increased at a fixed pH (Fig. 1), inhibition of NaK-ATPase increased abruptly, whereas inhibition of Ca-ATPase increased gradually. The experimental variable which increased in parallel with inhibition in both experiments was the concentration of non-ionized tetracaine.

Effect of tetracaine on the kinetics of activation of NaK-ATPase and Ca-ATPase by cations. Figure 2 shows the effect of 2·5 mM tetracaine on the kinetics of activation of NaK-ATPase by K^+ at a saturating concentration of Na^+. Tetracaine increased the $K_{0.5}$ for K^+ by 5-fold and decreased the maximum velocity (V_{max}) about 30 per cent. We consistently found that K^+ became inhibitory at concentrations greater than 10 mM in the absence of tetracaine, but not in its

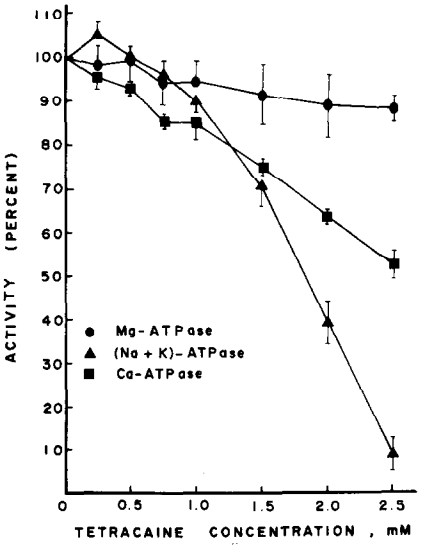

Fig. 1. ATPase activities as a function of tetracaine concentration. Mg-ATPase (●——●); NaK-ATPase (▲——▲); Ca-ATPase (■——■). The pH was 7·8 throughout. Other assay conditions are described in Experimental. Activity is expressed as a per cent of the activity without tetracaine present. Mean values were obtained from four to seven experiments. Vertical bars represent S.E.M.

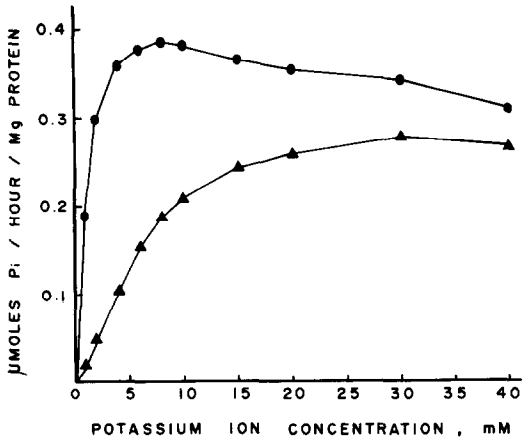

Fig. 2. NaK-ATPase activity as a function of K^+ concentration with and without tetracaine. No tetracaine (●——●); 2·5 mM tetracaine (▲——▲). The pH was 7·4. Other conditions are described in Experimental. The data represent the average of three experiments run in duplicate.

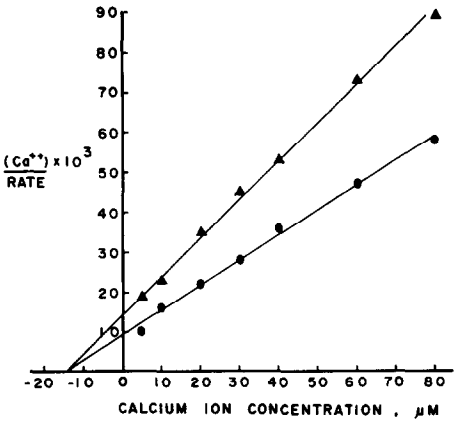

Fig. 4. Ca-ATPase activity as a function of Ca^{2+} concentration with and without tetracaine. No tetracaine (●——●); 2·5 mM tetracaine (▲——▲). Data are expressed as a modified Lineweaver–Burk plot. The pH was 7·4. Other conditions are described in Experimental. Values are the average of four experiments run in duplicate.

presence; for this reason, the two curves converged at higher K^+ concentrations.

Curves for activation of this enzyme by both Na^+ and K^+ are sigmoid, suggesting cooperativity in the binding of these cations [12]. It appeared from the results of Fig. 2 that the curve for activation by K^+ might be more sigmoid with tetracaine than without, and that tetracaine increased the $K_{0.5}$ for K^+, at least in part, by increasing the cooperativity for binding. In order to test this point, we did additional experiments at lower K^+ concentrations and constructed Hill plots from the data. The slopes of the lines with and without tetracaine were identical; the Hill coefficient, a measure of sigmoidicity, was 1·6 for both.

Figure 3 shows that tetracaine also increased the $K_{0.5}$ for Na^+ by about 3-fold. It is clear that tetracaine interfered with Na^+ binding at low Na^+ concentrations.

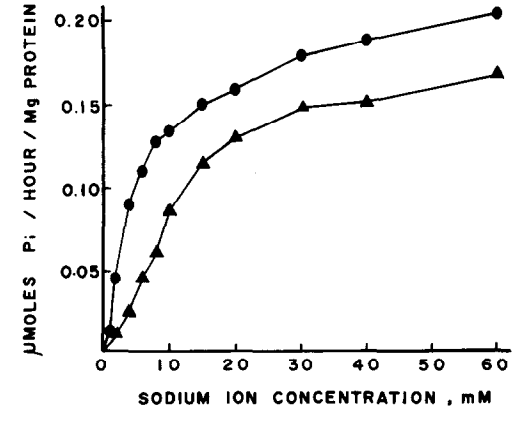

Fig. 3. NaK-ATPase activity as a function of Na^+ concentration with and without tetracaine. No tetracaine (●——●); 2·5 mM tetracaine (▲——▲). The K^+ concentration was 20 mM.

Activation of Ca-ATPase by Ca^{2+} was studied in the same way (Fig. 4). Tetracaine had no effect on the $K_{0.5}$ for Ca^{2+}, but decreased the V_{max} by 30 per cent.

DISCUSSION

Inhibition of NaK-ATPase and Ca-ATPase by tetracaine increased with increasing pH. Therefore, it appears that the non-ionized drug is more inhibitory than the ionized. However, the ionized form appeared to produce some inhibition of NaK-ATPase. For example, at pH 7·0 (Table 1), the concentrations of non-ionized and ionized tetracaine were 0·15 and 2·35 mM, respectively, and NaK-ATPase activity was 76 per cent of control. A similar concentration of non-ionized tetracaine was achieved with 0·5 mM tetracaine at pH 7·8 (Fig. 1), but no inhibition was evident. Thus the inhibition at pH 7·0 must be due exclusively to the ionized form. Since 90 per cent inhibition was achieved by the combination of 0·68 mM non-ionized and 1·82 mM ionized tetracaine (2·5 mM tetracaine at pH 7·8), it is clear that the non-ionized form of the drug is more inhibitory than the ionized. Similar considerations show that inhibition of Ca-ATPase can be attributed almost exclusively to the non-ionized form of tetracaine at each pH.

The non-ionized, lipid-soluble form of tetracaine can distribute freely into the membrane, whereas the ionized form is largely confined to the interface by polar interactions with the charged heads of membrane phospholipids and with water [13]. Both NaK- and Ca-ATPase are lipoproteins and both are inactivated when phospholipids are removed or enzymatically modified [14, 15]. Phospholipids are believed to supply a structural frame-work necessary to maintain these enzymes in functionally active conformations [14], and tetracaine could inhibit by altering or disrupting the interaction between lipid and protein.

Inhibition of NaK-ATPase appeared to be a sigmoid function of tetracaine concentration over the

range studied (Fig. 1). This suggests that more than one molecule of tetracaine is required to inhibit one active site of NaK-ATPase and that the interaction of tetracaine with the enzyme might be cooperative.

Tetracaine altered the interaction of NaK-ATPase with Na^+ and K^+, increasing the $\kappa_{0.5}$ for both cations (Figs. 2 and 3). Inhibition was thus most pronounced when either cation was present at a subsaturating concentration. In the case of K^+, this was not associated with an increased cooperativity of binding, because tetracaine did not change the Hill coefficient for activation. A number of inhibitors of this enzyme increase the $\kappa_{0.5}$ for K^+, and most of these are lipid soluble [2, 16, 17]. Effects on the $\kappa_{0.5}$ for Na^+ have not generally been considered, but results which have been reported are variable. Organic solvents decreased the $\kappa_{0.5}$ for Na^+ [16] but ethanol had no effect [17]. Therefore, it appears that there are differences in the mechanism of inhibition by lipid-soluble agents.

It is unlikely that tetracaine or other drugs increase the $\kappa_{0.5}$ for Na^+ or K^+ by directly competing with these cations at their respective binding sites. This effect may be the result of a conformational change which alters the affinity of the enzyme for these cations. In this way one could explain a decrease as well as an increase in $\kappa_{0.5}$.

Tetracaine did not alter the $\kappa_{0.5}$ for activation of Ca-ATPase by Ca^{2+}. This contrasts with the competitive interaction between Ca^{2+} and local anesthetics at sites where these drugs block Na^+ conductance in excitable tissues [18, 19]. The cationic form of the local anesthetic appears to be required for blockade, however [20]. Johnson and Inesi [21] reported that Ca-ATPase in sarcoplasmic reticulum was unaffected by 0·8 mM tetracaine at pH 6·8. This concentration of tetracaine, at pH 7·0, also had little effect on Ca-ATPase in red cells (Table 1).

Previous workers [2–5] have considered the effect of local anesthetics on NaK-ATPase and on transport, but with somewhat conflicting results. Judah and Ahmed [2] reported that dibucaine significantly inhibited both the active uptake of K^+ by liver slices and NaK-ATPase in liver microsomes. Inhibition by dibucaine was not reduced by increasing the K^+ concentration. This result is in contrast to our findings with tetracaine, where inhibition was largely overcome by K^+. Judah and Ahmed [2] found that procaine had no effect on either transport or ATPase activity. In preliminary studies we also found that procaine (2·5 mM) had no effect on NaK- or Ca-ATPase. Procaine ($pKa = 8·95$) is almost completely ionized at physiological pH and its partition coefficient is about two orders of magnitude lower than that for tetracaine [22]. This could account for the negative result.

Andersen and Gravenstein [3] and Andersen [4] reported that the active transport of Na^+ and K^+ in red cells was reduced by five local anesthetics. Although the fluxes of both cations were altered, attempts to distinguish between effects on active and passive movements were not completely successful.

Askari and Rao [5] studied the effects of several drugs on ouabain-sensitive Na^+ efflux from reconstituted red cell ghosts at pH 7·4. Procaine (1–10 mM) had no effect. The authors state that the effects of lidocaine and tetracaine were similar to those obtained with procaine. These findings appear to conflict with the results of the present study. However, differences in experimental conditions could, in part, explain the discrepancy. For example, Askari and Rao [5] incubated reconstituted ghosts in a Na^+-free medium with 6 mM K^+. In the absence of competing extracellular Na^+, this concentration of K^+ is completely saturating [23], and inhibition by tetracaine might be largely overcome.

In summary, we have shown that the activity of two transport-related enzymes in human red cell membranes is significantly inhibited by the local anesthetic tetracaine. This finding suggests that active cation transport in intact cells might also be inhibited by local anesthetics in concentrations reached in clinical situations. The inhibition of active cation transport systems could play a role in the pharmacologic as well as toxic effects of local anesthetics.

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