Biochemical Pharmacology. Vol. 26, pp. 2241–2245. Pergamon Press, 1977. Printed in Great Britain.

IRREVERSIBLE INACTIVATION OF HUMAN RED CELL ATPASE ACTIVITY BY TETRACAINE*

GUY H. BOND and PATRICIA M. HUDGINS
Department of Physiology Kirksville College of Osteopathic Medicine, Kirksville, MO 63501, U.S.A.

(*Received* 22 *November* 1976; *accepted* 17 *March* 1977)

Abstract—Red cell NaK- and Ca-ATPase activities are irreversibly inactivated when membranes are exposed to tetracaine under appropriate conditions. NaK-ATPase was inactivated upon exposure of membranes to tetracaine at 20° in the absence of ligands. Ca-ATPase was stable at this temperature, but inactivation could be demonstrated at 47°. The rate of inactivation of both enzymes increased with tetracaine concentration over a similar range. Inactivation also increased with pH, suggesting a correlation between the non-ionized form of the local anesthetic and inactivation of both enzymes. All ligands of NaK-ATPase with the exception of ATP afforded marked protection against inactivation by tetracaine. The degree of protection was a hyperbolic function of ligand concentration in the exposure medium. The concentration of each ligand required for half-maximal protection ($K_{0.5}$) was approximately 1 mM. These results demonstrate that the binding of some ligands to NaK-ATPase stabilizes that enzyme in conformational states which are resistant to attack by tetracaine. Ca-ATPase was more resistant to inactivation by tetracaines, and ligand effects were not evident.

The local anesthetic, tetracaine, reversibly inhibits NaK-ATPase and Ca-ATPase in human red cell membranes under standard assay conditions, i.e. in the presence of MgATP and the required cations [1]. This report describes a second, related effect of tetracaine; that is, both enzymes can be irreversibly inactivated when membranes are exposed to tetracaine under appropriate conditions. There are a number of similarities between the kinetics of inhibition and inactivation, suggesting that the two events are related with regard to mechanism.

Each ligand of NaK-ATPase, with the exception of ATP, affords marked protection against inactivation of this enzyme by tetracaine. Ca-ATPase is more resistant to inactivation and protective effects of ligands are not evident.

A number of agents, or treatments, can irreversibly inactivate these enzymes, and individual ligands or combinations of ligands frequently alter the rate of inactivation. In the case of NaK-ATPase, Robinson [2–5] has accumulated evidence which supports the view that ligands modify inactivation by binding to their respective sites on the enzyme to yield conformational states with altered susceptibilities to inactivation.

This study was undertaken in order to define the conditions which modulate drug-enzyme interactions to produce these two distinctly different functional alterations, i.e. inhibition and inactivation. The study of inactivation offers the advantage over inhibition studies in that drug-enzyme interactions can be detected without the restriction that all ligands necessary for activity need be present, and the influence of individual ligands can be tested separately.

* This investigation was supported by USPHS NIH grant AM 19162.

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.

EXPERIMENTAL

Preparation of membranes. Membranes were prepared from freshly outdated human blood as described previously [6]. Protein was measured by the method of Lowry *et al.* [7].

ATPase assay. The following conditions were common for the assay of all activities: 63 mM Tris–HCl (pH 7.2), 2 mM Mg^{2+} and 2 mM Na_2 ATP. The reaction vol. was 2 ml and the temperature was 38°. For assay of Mg-ATPase the medium also contained 60 mM Na^+, 0.25 mM Tris–EGTA and 0.25 mM ouabain. For NaK-ATPase: 120 mM Na^+, 10 mM K^+ and 0.25 mM Tris–EGTA. For Ca-ATPase: 60 mM Na^+, 0.25 mM ouabain and 0.2 mM Ca^{2+}. In all experiments, NaK- and Ca-ATPase activities were calculated as the difference between the total ATPase activity measured under the respective assay conditions, and the Mg-ATPase measured concurrently.

ATPase activity was measured in terms of inorganic phosphate (Pi) production according to a modification of the method of Fiske and SubbaRow [8]. Other details have been described previously [6]. When tetracaine was present, 300 mg of Dowex-50 cation exchange resin was added to each tube after stopping the reaction with 1.2 M perchloric acid. This step removed tetracaine, which interferes with Pi measurement.

Inactivation experiments. Both enzymes were inactivated by exposing membranes to tetracaine in a medium containing 50 mM Tris–HCl (pH 7.8) and 0.25 mM EGTA. For inactivation of NaK-ATPase the temperature was 20° and for Ca-ATPase it was 47°. At 47° there was a significant inactivation of Ca-ATPase even without tetracaine. This was minimized

by EGTA, which chelates traces of contaminant Ca^{2+}, which in turn increases the rate of thermal inactivation of this enzyme [9].

In most experiments, membranes were added to the medium containing 2.5 mM tetracaine and 1 ml aliquots were removed immediately and after 40 min of exposure for measurement of ATPase activity remaining under the standard assay conditions described above. This gave a tetracaine concentration during assay of 1.25 mM, which inhibited ATPase slightly (<15 per cent). Nevertheless, subtracting the activity of the 40 min sample from the activity of the zero-time sample gave an absolute measure of the amount of inactivation during exposure.

To study the influence of ligands on inactivation, these were included in the exposure medium at various concentrations. To facilitate comparisons between experiments, the protection against inactivation afforded by a ligand was normalized in the following way, and is expressed as relative protection:

$$\text{Relative protection} = \left[1 - \frac{\text{Activity lost (ligand present)}}{\text{Activity lost (ligand absent)}}\right].$$

In this way the protection afforded by a ligand is expressed on a scale of zero to one. A value of zero represents no effect, and a value of one represents full protection.

RESULTS

In preliminary experiments we established that NaK-ATPase and Ca-ATPase activities were stable for up to one hour when membranes were exposed at 20° to the pre-incubation medium described in Experimental. Addition of tetracaine to this medium, at 20°, caused a progressive, dose-dependent inactivation of NaK-ATPase (Fig. 1 A). The activity which disappeared during exposure under these conditions could not be restored by washing the membranes free of tetracaine before assay. The enzyme was thus irreversibly inactivated during exposure to tetracaine. In addition, the activity which remained was linear with time during assay, showing that the enzyme had not become unstable following exposure to tetracaine.

Ca-ATPase was unaffected by exposure at 20° to 2.5 mM tetracaine, the highest concentration tested with either enzyme. When the temperature was increased to 47° during exposure, however, a progressive, dose-dependent inactivation of Ca-ATPase was also evident (Fig. 1 B). If these data are corrected for the thermal inactivation in the absence of tetracaine, the activity lost at 40 min due to 1.25 mM, 2.0 mM and 2.5 mM tetracaine was 13, 28 and 44 per cent, respectively.

We examined inactivation of NaK-ATPase by 2.5 mM tetracaine at pH 7.0, 7.4 and 7.8 in order to alter the proportions of ionized and non-ionized tetracaine during exposure. At these three pH's the percentages of tetracaine (pK_a = 8.24) in the non-ionized form are 6, 13 and 27 per cent, respectively. The activity remaining after 40 min of exposure to tetracaine was 91, 63 and 27 per cent, respectively (n = 3 at each pH). On the basis of this limited series of experiments there appears to be a correlation between the amount of inactivation and the concentration of non-ionized tetracaine. In other experiments a similar pH-dependence for inactivation of Ca-ATPase was found.

Tetracaine reversibly inhibits NaK-ATPase under assay conditions, but does not inactivate the enzyme [1]. It is clear, therefore, that one or more ligands of the enzyme must prevent inactivation during assay. We first tested activating monovalent cations, and found that addition of either Na^+, K^+

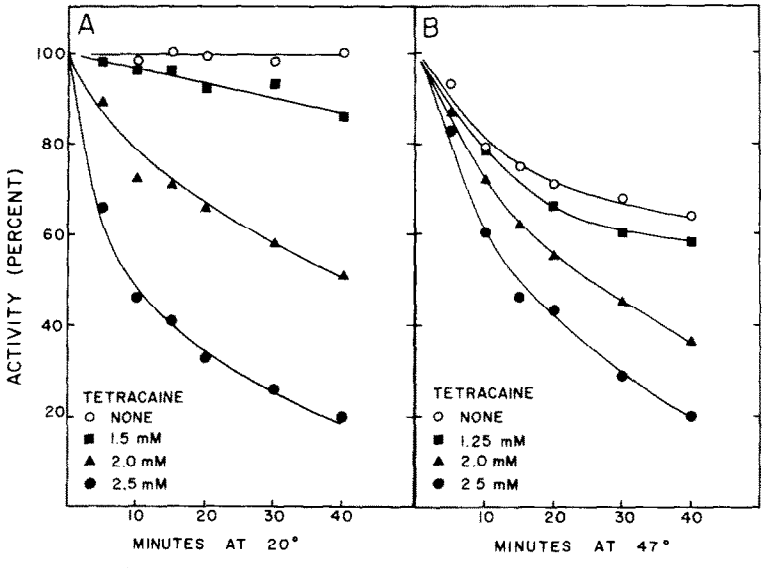

Fig. 1. Time course and concentration-dependence of ATPase inactivation during exposure of membranes to tetracaine. Panel A: NaK-ATPase activity remaining after exposure to tetracaine at 20°. Panel B: Ca-ATPase activity remaining after exposure to tetracaine at 47°. At timed intervals, aliquots of the exposure media were removed for assay of residual ATPase activity as described in Experimental. In both panels, activity is expressed relative to the activity at zero time. Each curve represents the average of 2–7 experiments run in duplicate.

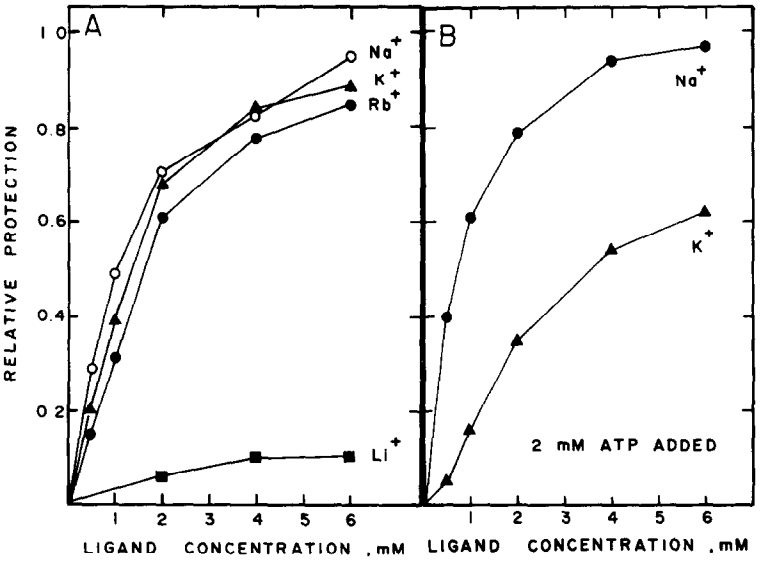

Fig. 2. Relative protection afforded by monovalent cations against inactivation of NaK-ATPase during exposure to 2.5 mM tetracaine for 40 min. Relative protection was calculated as described in Experimental. Panel A: monovalent cations were added to the exposure medium at the indicated concentrations. Panel B: Na^+ or K^+ were added to the exposure medium along with 2 mM Tris–ATP. Curves represent the average of 2–8 experiments run in duplicate.

or Rb^+ to the exposure medium, in low concentrations, effectively protected against inactivation (Fig. 2 A). An estimate of the concentration of each ligand giving half maximal protection ($K_{0.5}$) was made by linear regression analysis of Woolf plots of the same data. The $K_{0.5}$ values obtained for Na^+, K^+ and Rb^+ were 1.5 mM, 1.2 mM and 1.4 mM, respectively. In all cases, complete protection from inactivation was afforded by each cation at saturating concentrations (maximum relative protection = 1.0). Only slight protection was afforded by Li^+ over the same concentration range (Fig. 2 A).

Mg^{2+} and MgATP also protected NaK-ATPase from inactivation (Fig. 3). The $K_{0.5}$ for Mg^{2+} was 1.0 mM and for MgATP it was 1.4 mM. In both cases, complete protection was afforded by saturating concentrations of these ligands. The concentration of MgATP present during exposure to tetracaine was slightly lower than indicated because of dissociation of MgATP to yield free Mg^{2+} and ATP. However, free Mg^{2+} at any concentration of MgATP can account for only a small fraction of the protection afforded by MgATP. Taking a dissociation constant for MgATP of 32 μm [10], the free Mg^{2+} concentration in the presence of 2 mM MgATP would be 0.24 mM.

In contrast to other ligands, ATP protected poorly. The relative protection by 1.0 mM ATP was only 0.1 ± 0.02 (mean $\pm$ S.E., $n = 10$). Thus ATP appeared to bind to the enzyme, but the resulting complex was nearly as susceptible as the free enzyme to inactivation. The interaction between ATP and the enzyme was more evident when protection by either Na^+ of K^+ was studied in the presence of 2 mM ATP (Fig. 2 B). The $K_{0.5}$ for protection by Na^+ was unaffected by ATP, but that for K^+ was increased from 1.2 mM to 3.6 mM. Thus the binding of ATP to the enzyme apparently reduced the affinity for K^+.

Since inactivation of Ca-ATPase by tetracaine could be demonstrated only at temperatures well above 38°, there was no reason to conclude that one or more ligands of the enzyme would necessarily prevent inactivation. We nevertheless tested this possibility, and examined various ligands separately, but found no evidence of protection in any case. Among the ligands studied were Na^+ and K^+, either of which can activate the enzyme up to 1.6-fold [6, 11]. Ca^{2+} increases the rate of thermal inactivation in the absence of tetracaine [9]. When tetracaine and Ca^{2+} were both present, their effects appeared additive.

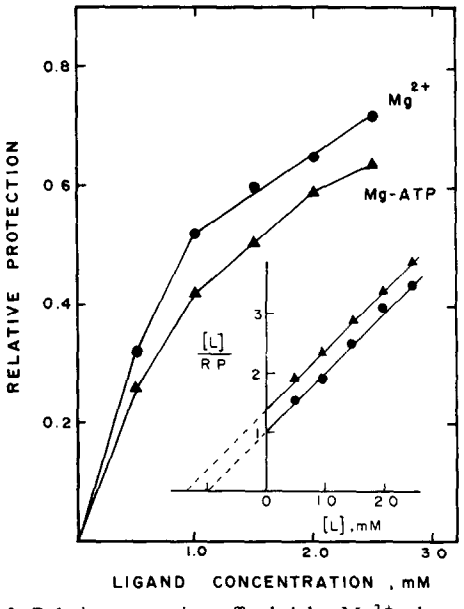

Fig. 3. Relative protection afforded by Mg^{2+} alone and equimolar Mg^{2+} and Trix–ATP against inactivation of NaK-ATPase during exposure to 2.5 mM tetracaine for 40 min. Inset shows a Woolf plot of the same data. Curves represent the average of 2–4 experiments run in duplicate.

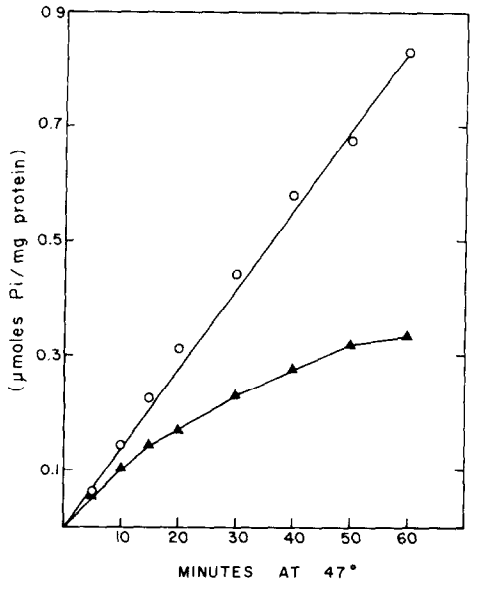

Fig. 4. Ca-ATPase activity at 47° in the absence (○) and presence (▲) of 2.5 mM tetracaine. Aliquots of the complete standard assay medium were removed at timed intervals for P_i determinations.

Figure 4 shows an experiment in which P_i release was measured as a function of time at 47° with and without tetracaine. In the control curve P_i release was linear with time for up to one hour, indicating that the enzyme was stable at this temperature in the presence of all ligands required for activity. In the presence of tetracaine, however, P_i release declined progressively with time and essentially ceased after about one hour. Thus inactivation by tetracaine was evident even when the enzyme was actively hydrolyzing ATP.

DISCUSSION

The inactivation of NaK- and Ca-ATPase by tetracaine is similar in several respects to the inhibiton of these enzymes described previously [1]. For example: (1) the pH-dependence of inactivation and inhibition was similar. (2) Ca-ATPase was less sensitive than NaK-ATPase to inactivation and inhibition. (3) Inactivation and inhibition of both enzymes occurred over a similar range of tetracaine concentrations. (4) Inactivation of NaK-ATPase was prevented by low concentrations of either Na^+ or K^+, and inhibition could be partially overcome by increasing the concentration of either cation at a fixed concentration of the other. (5) Ca^{2+} did not protect Ca-ATPase from either inactivation or inhibition by tetracaine. On the basis of these similarities it appears that inactivation and inhibition represent extreme consequences of a common mechanism.

A number of agents irreversibly inactivate NaK-ATPase, and the rate of inactivation is altered by ligands of the enzyme. For example: Na^+ protects against inactivation by dicyclohexylcarbodiimide [12]; ATP protects against inactivation by *N*-ethylmaleimide [13] or by photooxidation in the presence of methylene blue [2]; and both Mg^{2+} and K^+ are required for inactivation by Be^{2+} [14] or F^- [15]. Thus specific enzyme-ligand complexes have discrete susceptibilities to inactivation by particular agents in comparison to the free enzyme. Furthermore, the sites which ligands occupy to alter inactivation appear to be the sites which they occupy to elicit NaK-ATPase activity.

Robinson [2–5] has applied this principle to measure dissociation constants for Na^+, K^+, Mg^{2+} and MgATP (or ATP). The values which we obtained for cation concentrations giving half-maximal protection against inactivation by tetracaine are close to the dissociation constants which Robinson reported. It thus appears that protection against inactivation by tetracaine, as by other agents, results from the formation of specific complexes involving active sites of NaK-ATPase. This conclusion is strengthened by the relative failure of Li^+ to protect against inactivation by tetracaine. Li^+ has a low affinity for the K^+-site on the enzyme, as demonstrated both kinetically and in studies dealing with inactivation [4].

The protection afforded by MgATP and the relative ineffectiveness of ATP requires comment. The K_m for phosphorylation of NaK-ATPase by MgATP in the presence of Na^+ is about 0.5 μM [16], and ATP is also bound at this site with a similar dissociation constant [17]. In our experiments, the $K_{0.5}$ for protection by MgATP was about 1.4 mM, and this is at least an order of magnitude too high to reflect binding at the substrate site. There is convincing evidence, however, for a second low affinity site for MgATP or ATP. The dissociation constant of this site is 0.5 mM for both ligands, as determined both kinetically and from the protection afforded by either ligand against inactivation by photooxidation in the presence of methylene blue [2, 5]. It seems likely that MgATP protected against inactivation by tetracaine by forming a complex with this low affinity site. Apparently ATP also bound at this site because ATP was able to influence the ability of K^+ to protect the enzyme. This result is consistent with the finding of Post *et al.* [18] that ATP binding at this site increases activity by displacing K^+ from a stable inactive complex with the dephosphoenzyme. The inability of ATP to protect can best be explained if we assume that ATP and MgATP yield different enzyme conformations with different susceptabilities to inactivation.

Among the agents which inactivate NaK-ATPase, tetracaine is unusual in that most ligands, individually, afford protection. With other inactivating agents, ligand effects are highly selective, suggesting that different ligands yield distinctly different conformational states which apparently differ in the extent to which sensitive groups are exposed to attack. Judging from our results with tetracaine, on the other hand, it is necessary to conclude that various ligand-induced conformations also have a common feature, not evident in the free enzyme, which confers resistance to inactivation. Inactivation of Ca-ATPase by tetracaine could be demonstrated only at temperatures at the limit of thermal stability of the enzyme. Although ligand-dependent conformational changes can be demonstrated with this enzyme [9], they do not prevent attack by tetracaine.

In a limited series of experiments we found that the local anesthetic dibucaine was more potent than tetracaine as an inhibitor of NaK-ATPase. Dibucaine

also inactivated the enzyme and ligand protection could be demonstrated, although the effects of ligands were not tested individually. Etidocaine did not significantly inhibit NaK-ATPase at concentrations as high as 5 mM, nor did it cause inactivation. There is, therefore, a positive correlation between inhibition and inactivation, and the results described in this paper are not unique to tetracaine.

REFERENCES

1. G. H. Bond and P. M. Hudgins, *Biochem. Pharmac.* **25,** 267 (1976).
2. J. D. Robinson, *Nature, Lond.* **233,** 419 (1971).
3. J. D. Robinson, *Ann. N.Y. Acad. Sci.* **242,** 185 (1974).
4. J. D. Robinson, *Biochim. biophys. Acta* **384,** 250 (1975).
5. J. D. Robinson, *Biochim. biophys. Acta* **429,** 1006, (1976).
6. G. H. Bond and J. W. Green, *Biochim. biophys. Acta* **241,** 393 (1971).
7. O. H. Lowry, N. J. Rosebrough, N. J. Farr and R. J. Randall, *J. biol. Chem.* **139,** 265 (1951).
8. C. H. Fiske and Y. SubbaRow, *J. biol. Chem.* **66,** 375 (1925).
9. G. H. Bond, *Biochim. biophys. Acta* **288,** 423 (1972).
10. J. D. Robinson, *FEBS Lett.* **47,** 352 (1974).
11. H. J. Schatzmann and G. L. Rossi, *Biochim. biophys Acta* **241,** 379 (1971).
12. W. Schoner, *Angew. Chem. Int. Ed. Engl.* **10,** 882 (1971).
13. D. C. Tosteson, *Ann. N.Y. Acad. Sci.* **137,** 577 (1966).
13. D. C. Tosteson, *Ann. N.Y. Acad. Sci.* **137,** 577 (1966).
14. G. Toda, *J. Biochem. Tokyo* **64,** 464 (1968).
15. L. J. Opit, H. Potter, J. S. Charnock, *Biochim. biophys. Acta* **120,** 159 (1966).
16. R. L. Post, A. K. Sen and A. S. Rosenthal, *J. biol. Chem.* **240,** 1437 (1965).
17. C. Hegyvary and R. L. Post, *J. biol. Chem.* **246,** 5240 (1971).
18. R. L. Post, C. Hegyvary and S. Kume, *J. biol. Chem.* **247,** 6350 (1972).