Biochemical Pharmacology, Vol. 25, pp. 237–239. Pergamon Press, 1976. Printed in Great Britain.

COMMENTARY

SODIUM–POTASSIUM ADENOSINE TRIPHOSPHATASE—A RECEPTOR FOR DIGITALIS?

ARNOLD SCHWARTZ
Department of Cell Biophysics and Division of Myocardial Biology, Baylor College of Medicine and The Methodist Hospital, Texas Medical Center, Houston, Texas 77025, U.S.A.

The active glycoside ingredients of the flowering plant *Digitalis purpurea* and its derivatives have not only produced incalculable benefits to mankind in terms of clinical medicine, but also it has encouraged countless scientists through the years to carry out provocative and inventive experiments yielding information on a number of biological systems.

The purpose of this commentary is to reiterate the importance of one such enzymatic system, to describe to those who do not work in this interesting area recent developments, and to discuss the interesting controversy that has developed concerning the possibility that Na^+, K^+-ATPase may represent a component of a digitalis receptor.

Skou[1] was the first to recognize that Na^+, K^+-ATPase associated with the membrane may represent the enzymatic expression of the sodium–potassium pump. Skou isolated this enzyme in relatively crude form from a homogenate derived from crab nerve and localized the activity in a microsomal (small membrane) fraction of the preparation. He later showed that ouabain (Strophanthin G) was able to specifically inhibit the sodium- and potassium-stimulated portion of the enzyme activity. Skou[2] clearly indicated a number of important criteria that had to be fulfilled in order for the Na^+, K^+-ATPase to be "elected" as the chemical counterpart of the sodium–potassium pump. To the best of this author's knowledge, the criteria have been fulfilled[3]. The investigations of countless investigators (notable among them are Post, Albers and Skou) have led to the following series of reactions which probably describe the nature and complexity of this enzyme system:

$$E_1 + ATP \overset{Mg,Na}{\rightleftharpoons} E_1P + ADP \quad (1)$$

$$E_1P \overset{Mg}{\rightleftharpoons} E_2P \quad (2)$$

$$E_2P + H_2O \overset{K^+}{\rightleftharpoons} E_2 + Pi \quad (3)$$

$$E_2 \rightarrow E_1 \quad (4)$$

If the enzyme preparation is treated with *N*-ethylmaleimide or oligomycin, the enzymatic preparation becomes resistant to potassium and sensitive to ADP, i.e. a sodium-dependent ADP–ATP exchange reaction can be demonstrated. The native enzyme preparation is sensitive to potassium and relatively resistant to ADP. Accordingly, it seems appropriate to refer to the initial phosphorylation event as being catalyzed by a kinase-type reaction and the phosphatase reaction as being catalyzed by a potassium-dependent enzymatic component. Indeed, if the latter reaction occurs on the outside portion of the membrane and the former reaction occurs on the inside portion of the membrane, a convenient "chemical" mechanism might exist for the transport of the sodium and potassium. It is not known whether the sodium and potassium are transported in a sequential manner, as the above series of equations would suggest, or whether they are transported in a simultaneous manner, as some recent evidence would suggest[4]. It seems clear from extensive investigations that this enzymatic system is an allosteric type that is intimately associated with the cell membrane. Ligand-specific sites include magnesium, ATP, sodium, potassium, possibly ouabain-binding and calcium. The locations of these sites are not clear. It seems reasonable to suppose that potassium sites are located on the outside portion of the membrane and that the sodium sites are located on the inside. The stoichiometry (Na:K: ~ P) is probably 3:2:1, at least in intact systems.

The specificity of the inhibitory action of those cardiac glycosides that exert a positive inotropic action on the heart is well known[3]. The mechanism of inhibition of Na^+, K^+-ATPase is fascinating and has attracted the attention of a number of investigators, not only because it represents an interesting tool to dissect the mechanism of action of the Na^+, K^+-ATPase, but also from the point of view of pharmacology. It seems clear that digitalis glycosides inhibit the Na^+, K^+-ATPase by first binding to a specific site and/or region which produces a conformational change that leads to an inhibition of enzyme activity. My colleague, Hideo Matsui, and I found some years ago that sodium and potassium appeared to modulate the interaction of ouabain with isolated Na^+, K^+-ATPase, as shown in Fig. 1. In order to prove this relationship, we aquired some ^{3}H-digoxin and found that the saturable binding occurred only to the Na^+, K^+-ATPase (Fig. 2). This binding seems to be a pseudo irreversible type. We have recently shown that sodium increases and potassium decreases the rate constant of digitalis binding to Na^+, K^+-ATPase[5] (Fig. 1). George Lindenmayer and I showed some years ago that, in the presence of magnesium and inorganic phosphate, but in the absence of ATP, ouabain bound quite well to Na^+,

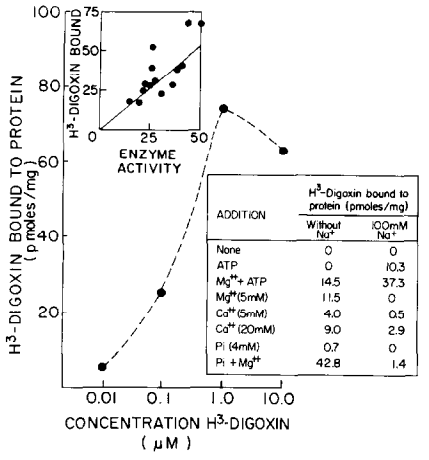

Fig. 1. Concentration and specific binding of ^{3}H-digoxin to a Na^+, K^+-adenosine triphosphatase. The enzyme was prepared from fresh calf heart and used within 1 week after isolation. The binding was carried out in tubes containing: 50 mM Tris-HCl (pH 7·4), 2 mM Tris adenosine triphosphatase, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, and ^{3}H-digoxin at indicated concentrations, with and without 10^{-4} M unlabeled ouabain; the tubes were shaken in a water bath at 37° for 3 min. The binding reaction is complete in about 1 min. The inset (top left) represents specific binding of 10^{-6} M ^{3}H-digoxin as a function of specific activity of various preparations measured as the amount of inorganic phosphate released/mg of protein/hr in a system identical to the binding experiment, except that KCl (10 mM) was added. ^{3}H-digoxin binding is expressed as pmoles/mg of protein. The hydrolysis of adenosine triphosphate under these conditions is completely inhibited by 10^{-4} M ouabain. These results are typical of many experiments. The inset (bottom right) shows the effect of Na^+ on specific ^{3}H-digoxin binding supported by various cations, adenosine triphosphates and anions. The cations, except when otherwise indicated, were employed in a 5 mM concentration; Tris salt of ATP in a 2 mM concentration; the anions in a 2–4 mM concentration. The conditions were the same, with specific activity of the cardiac enzyme varying between 26 and 40. The results were compiled from many experiments.

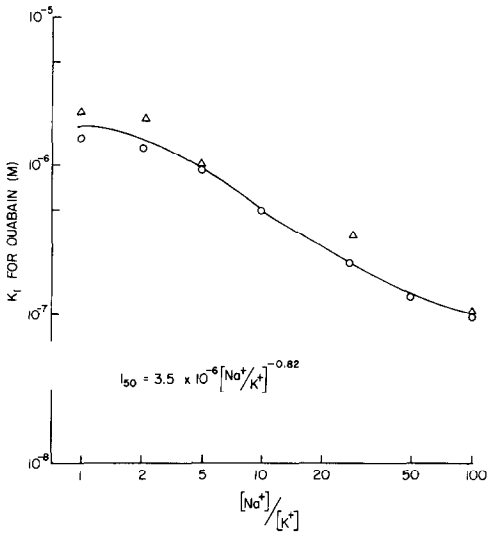

Fig. 2. Relationship between half-maximal inhibitory concentration of ouabain on Na^+, K^+-ATPase and the ratio of Na^+/K^+. The K_i obtained was plotted against Na^+/K^+ ratios on a full logarithmic scale. The symbols, ○ and △, represent different enzyme preparations.

K^+-ATPase. Moreover, a ouabain-treated enzyme was able to incorporate inorganic phosphate into a phosphoenzyme that was indistinguishable from E–P produced by reacting the enzyme with ATP^{32} in the presence of sodium. This and other evidence is consistent with the idea that Na^+, K^+-ATPase is a conformationally sensitive enzyme with multiple sites.

It appears logical to assume that digitalis interacts with a site on an intact system located on the external surface and that this somehow affects activity. The site could be associated with a potassium-sensitive region[4]. We have shown that at least the isolated enzyme system behaves in many ways like the intact system, in that the stoichiometry of ligand-induced activity seems to be 3:2:1, with two nonequivalent potassium sites and three equivalent sodium sites, presumably on the outside and inside of the membrane system, respectively, for each ATP hydrolyzed[4]. In order to study the kinetics of the enzyme activity as well as the possible role of digitalis and calcium, it was necessary to purify the enzyme. A number of investigators have been working actively in this field[3]. We succeeded in purifying the enzyme from canine kidney medulla[6]. It seems accepted that the enzyme system consists of at least two protein components, one involved in the phosphorylation reaction and the other, a glycoprotein, whose function is not known. It is also quite clear that phospholipid components are required for enzymatic activity. It is less clear what phospholipid components, if any, are necessary for digitalis action, and indeed it is not yet clear if there is any specificity with respect to lipid material for enzymatic activity. If the Na^+, K^+-ATPase is an allosteric enzyme, then it should be possible to prepare antibodies to specific conformations. An antibody to the Na^+, K^+-ATPase has been isolated and reported by a number of investigators[3]. Recently, we prepared a globulin fraction, after injecting a purified, high activity Na^+, K^+-ATPase from canine renal medulla into rabbits. This fraction was further purified by reacting with a purified Na^+, K^+-ATPase, and then two components of the antibody were isolated by reacting the purified Na^+, K^+-ATPase antibody with an ouabain enzyme. A fraction was recovered in the supernatant that did not bind to the ouabain-treated enzyme. This fraction was labeled "anti-Digitalis Receptor" because it prevented digitalis binding but did not prevent ATPase activity. The fraction that bound to the Na^+, K^+-ATPase, which was subsequently eluted from the enzyme, was labeled "anti-CAT" because it inhibited the catalytic component of the enzyme but did not affect ouabain binding. Further work using those two components is in progress, but these preliminary observations are consistent with the postulation that Na^+, K^+-ATPase possesses specific digitalis-reacting sites.

The characteristics of ouabain binding and inhibition of the Na^+, K^+-ATPase encourage thoughts about the pharmacological importance of this enzyme system. It is well known that digitalis

produces an increased calcium entry into the cell, and it is equally well known that digitalis does not seem to specifically affect any other enzymatic system thus far studied. Does the purified Na^+, K^+-ATPase have anything to do with the calcium? Calcium inhibits the Na^+, K^+-ATPase in the presence of sodium and potassium [3]. It seems logical to assume that calcium binds to the enzyme, but it has been difficult to demonstrate because of the lack of purity of the enzyme. With the advent of relatively purified preparations, this has now become possible. Investigations in this laboratory have very recently produced some preliminary evidence that is encouraging in this direction. Using a highly purified Na^+, K^+-ATPase from sheep kidney medulla, binding sites associated with the phospholipid component for calcium have been demonstrated. Ouabain produced a significant decrease in affinity for calcium in the absence of sodium and increased affinity in the presence of sodium. Recent experiments utilizing stopped-flow spectrophotometric analysis have indicated that the rates of binding of calcium are significantly altered when the enzyme is treated with ouabain. No other ATPase associated with membrane thus far examined is affected by ouabain. It appears that ouabain, when bound to the enzyme, may modulate the affinity of an energy-independent calcium-binding site. This phenomenon may be due to altered microarchitectural changes in the enzyme. We suggest that cardiac glycosides increase an extracellular-bound pool of calcium by an action on the Na^+, K^+-ATPase which is responsible for the well known increase of contractility produced by digitalis [7].

The above evidence seems to be consistent with the definition of a pharmacological receptor of a drug. Our recent studies *in vivo* corroborate the postulation made originally by Repke [8] that this enzyme is indeed the receptor for digitalis. Using a species that is highly sensitive to digitalis, the cat, hearts were suspended according to the Langendorff procedure, and ouabain was infused until a positive inotropic effect was obtained, with no evidence of toxicity. The enzyme was isolated from the hearts and found to be inhibited. When the drug was washed out, contractility returned to normal and enzyme activity returned to normal. Re-infusion of another concentration of another dose of ouabain again caused an increase in inotropic action and with a concomitant decrease in enzymatic activity. The use of ^{3}H-ouabain yielded results quite consistent with the hypothesis that Na^+, K^+-ATPase is the pharmacological receptor for digitalis. We feel that these results fit the general suggestion made by Langer, Reuter, Baker and other investigators that a sodium–calcium exchange system existing in the membrane may be affected by the cardiac glycosides in such a way as to produce an increase in sodium. This increase, when presented to the carrier, augments the rate of transport across the membrane so that the sodium–carrier complex can release sodium, pick up calcium and bring it to the interior of the cell.

I hasten to add the disclaimer that these experiments in no way prove that Na^+, K^+-ATPase is a receptor for digitalis. They merely, I hope, suggest the direction of future studies in order to explain fully the mechanism of action of Na^+, K^+-ATPase, the exact molecular changes that occur when digitalis interacts with this enzyme system and, of great importance, to differentiate between an arrhythmogenic action of digitalis and its positive inotropic effect.

Acknowledgement—I wish to acknowledge my sincerest appreciation to my colleagues, Drs. Julius C. Allen, George E. Lindenmayer, Hideo Matsui, Lois K. Lane, Barry Van Winkle, Mark L. Entman and André Gervais. Without their valuable advice and collaboration this work could not have been carried out.

REFERENCES

1. J. C. Skou, *Biochim. biophys. Acta* **23**, 394 (1957).
2. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
3. A. Schwartz, G. E. Lindenmayer and J. C. Allen, *Pharmac. Rev.* **27**, 3 (1975).
4. G. E. Lindenmayer, H. K. Thompson, Jr. and A. Schwartz, *J. Physiol., Lond.* **236**, 1 (1974).
5. G. E. Lindenmayer and A. Schwartz, *J. biol. Chem.* **248**, 1291 (1973).
6. L. K. Lane, J. H. Copenhaver, G. E. Lindenmayer and A. Schwartz, *J. biol. Chem.* **248**, 7197 (1973).
7. A. Gervais, L. K. Lane and G. E. Lindenmayer, *Fedn. Proc.* **34**, 715 (1975).
8. K. Repke, in *New Aspects of Cardiac Glycosides* (Ed. B. Uvnas), p. 47. MacMillan, New York (1963).