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RAPIDLY REVERSIBLE INHIBITION OF FROG MUSCLE SODIUM PUMP CAUSED BY CARDIOTONIC STEROIDS WITH MODIFIED LACTONE RINGS

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SUMMARY

We have measured the reversibility of the inhibition of the Na^+ pump caused by several cardiotonic steroids (ouabain, dihydrouabain, strophanthidin, AY-22241 and digitoxigenin). Either saturation of the lactone ring, as in dihydrouabain, or a modification of the position of attachment of the ring, as in AY-22241, caused a marked increase in the rate of recovery of the pump after washing out the inhibitors. These results indicate that the lactone plays a central role in stabilizing the inhibitor–receptor complex.

INTRODUCTION

In many cells cardiotonic steroids cause an inhibition of the Na^+–K^+ pump which is only very slowly reversed by washing repeatedly with inhibitor-free solutions [1–5]. This slow reversibility has been attributed to a stable binding of the glycosides to a membrane site [2, 3, 5]. The stability of the binding is not uniform in all organs and species; among the most notable extremes in this variability are the tissues of the rat, where a very loose binding between the glycoside and the membrane site has been proposed [3, 5].

In a recent communication, it was shown [6] that a new semisynthetic glucoside (AY-22241) produced rapidly reversible alterations of the electrical activity of dog Purkinje Fibres. This glycoside differs only from the natural cardenolides in the position of attachment of the lactone ring to the steroid nucleus [7]. This observation suggested that it would be of interest to test the effects of alterations of the lactone ring on the reversibility of inhibition of the sodium pump. If minor structural changes produce drastic modifications in action, they may offer clues to the nature of the inhibitor–receptor interactions. Furthermore, the availability of reversible inhibitors of the sodium pump may be of great use in certain experimental conditions.

In this study we have compared the reversibility of the inhibition caused by ouabain, strophanthidin, digitoxigenin and two cardiotonic steroids with minor mod-

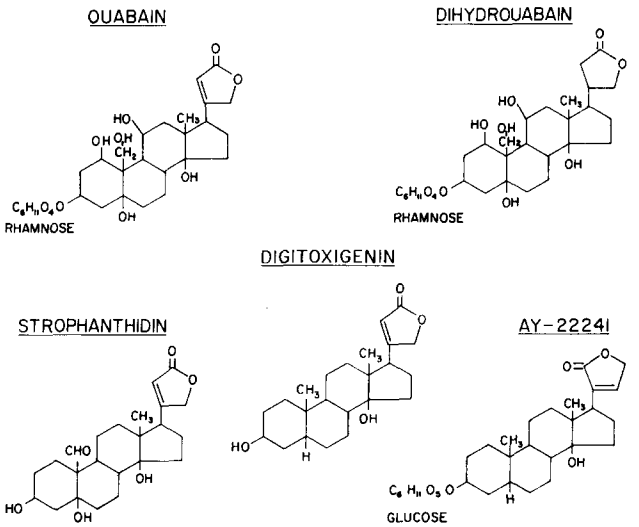

Fig. 1. The structure of the cardiotonic steroids used in this study. Digitoxigenin has the simplest structure. On the right side the steroids with modified lactones are shown.

ifications of their lactone ring: AY-22241 and dihydrouabain (see Fig. 1) on the Na^+ efflux of frog muscle.

Paired sartorius muscles dissected from the same frog (*Rana pipiens*) were used to provide control and test muscles. The techniques used to measure the efflux of labelled sodium have been described several times [8, 9]. An apparatus that transfers the muscle automatically from container to container was used [10]. The Ringer's solution used had the following composition (in mmoles/l): NaCl, 115; KCl, 2.5; $CaCl_2$, 2.0; Tris–Cl buffer, 3.0; pH 7.4. Values are given as averages $\pm$S.E. The cardiotonic steroids were initially dissolved in 100% ethanol in concentrations such that the final alcohol concentration in the efflux solutions never exceeded 0.05% (v/v).

In Fig. 2, the effects produced on Na^+ efflux when concentrations of ouabain, dihydrouabain and strophanthidin that had maximum inhibitory effects were used, are illustrated. The effects of the three cardiotonic steroids were of similar magnitude. An average inhibition of sodium efflux of $47\pm3.6\%$ was found for all the experiments ($n = 28$) in which maximally inhibitory concentrations of these substances were used. This figure is in agreement with previous measurements which have shown that only about 45% of the sodium efflux from frog skeletal muscle is inhibited by cardiotonic steroids [8, 9, 11, 12]. It is generally accepted that this ouabain-sensitive fraction of efflux corresponds to the activity of the Na^+–K^+ dependent ATPase.

Although the maximum inhibition produced by all the three cardiotonic steroids was of a similar magnitude, they were not equally effective. The concentrations producing about half maximum inhibition were: for ouabain, $1 \cdot 10^{-7}$ M; for strophanthidin, $1 \cdot 10^{-6}$ M and for dihydrouabain, $8 \cdot 10^{-7}$ M. These values are similar to those previously reported for ouabain and strophanthidin [1, 9].

The most striking difference between the effects of the different steroids was observed when the muscles were transferred into inhibitor-free solutions. Two rep-

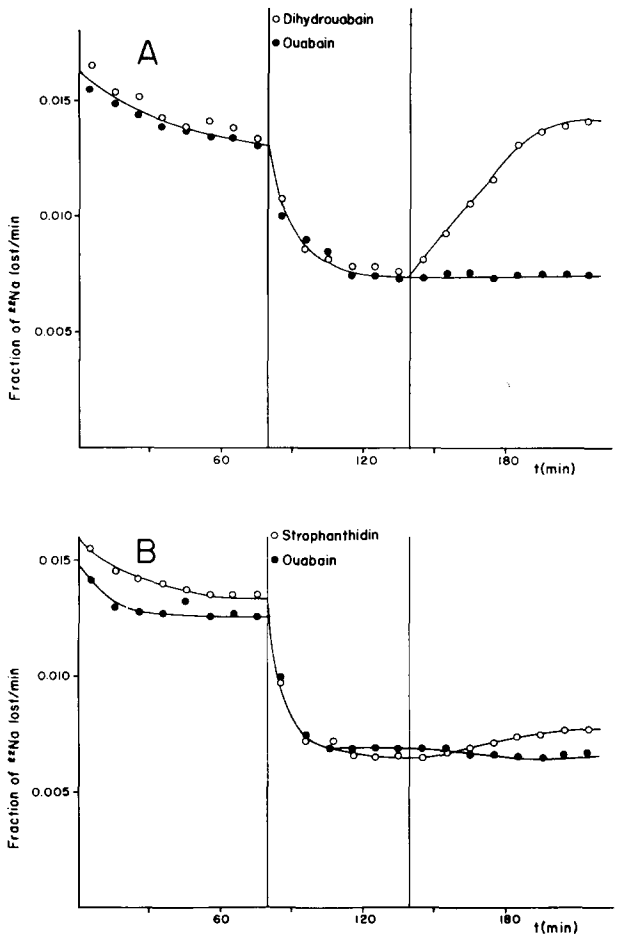

Fig. 2. Comparison of the effects of ouabain, dihydrouabain and strophanthidin. The muscles were exposed to the steroids only during the period between the vertical lines. A, ouabain ($5 \cdot 10^{-6}$ M) and dihydrouabain ($1 \cdot 10^{-5}$ M). B, ouabain ($5 \cdot 10^{-6}$ M) and strophanthidin ($3 \cdot 10^{-5}$ M). Temperature 25 °C.

resentative experiments are illustrated in Fig. 2. 80 min after washing in ouabain-free solution, the efflux remained at essentially the same level as when the inhibitor was present, while the efflux from dihydrouabain-treated muscles (Fig. 2A) increased rapidly after washing in inhibitor-free solutions, reaching levels close to those observed during the control period. In eight muscles that were treated with $1 \cdot 10^{-5}$ M dihydrouabain, the time necessary to recover half of the efflux inhibited by the glycoside averaged 28 ± 2.6 min. When strophanthidin was used (Fig. 2B), washing into inhibitor-free solutions did not result in rapid recovery. There was, however, a slow and consistent tendency for the efflux to increase. In eight muscles, recovery was followed for 80 min in strophanthidin-free solutions. At the end of this period, the average recovery was $11 \pm 0.2\%$ of the inhibited efflux. A slow recovery from strophanthidin inhibition has already been observed previously [1].

In two muscles, Na^+ efflux was followed for 3 h into dihydrouabain-containing solutions. No spontaneous tendency towards recovery was observed while the muscles were in the glycoside-containing solutions. In one experiment, ouabain was added after a muscle had recovered from inhibition with dihydrouabain; ouabain caused its usual inhibitory effects.

Fig. 3 shows one of four experiments in which the effects of AY-22241 and strophanthidin were compared. The concentration of AY-22241 used was $5 \cdot 10^{-6}$ M, because we were unable to dissolve greater amounts of this compound without increasing the alcohol concentration in the Ringer's solution. This concentration of AY-22241 produced an inhibition of Na^+ efflux that ranged between 53 and 65% of the effect produced by $3 \cdot 10^{-5}$ M strophanthidin in the control muscle. However, as in the experiments with dihydrouabain, the effects of AY-22241 were readily reversed after washing with inhibitor-free solutions (Fig. 3).

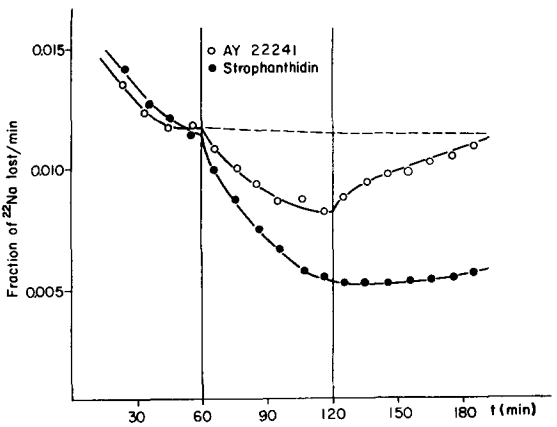

Fig. 3. Comparison of the effects of strophanthidin ($3 \cdot 10^{-5}$ M) and AY-22241 ($5 \cdot 10^{-6}$ M). The muscles were exposed to the steroids only during the period indicated between the vertical lines. Temperature 25 °C.

In two experiments, digitoxigenin ($3 \cdot 10^{-5}$ M) produced an inhibition of a similar magnitude to $3 \cdot 10^{-5}$ M strophanthidin. The rate of recovery after washing digitoxigenin was similar to that observed for strophanthidin.

The results described here show that saturation of the lactone ring, or modification of its position of attachment to the steroid nucleus, markedly increase the rate of recovery of the sodium pump after washing out the inhibitors.

It is reasonable to assume that the differences in speed of recovery between the different steroids studied here result from differences in the stability of binding between inhibitor and its receptor, rather than from metabolic inactivation of the inhibitors, since inactivation takes place mainly in organs like the liver and occurs at a slow rate [13].

From measurements of the effects of numerous cardiotonic steroids on the ATPase activity, it has been concluded [14] that the binding between the steroid and the enzyme can be described in terms of three components: (1) Component A is the site that interacts either with the 3-β-hydroxyl group of the sterol, or in the case of

the glycoside derivatives, with the alcohol groups attached to the 3-β-hydroxyl group. (2) Component B interacts mainly with the 14-hydroxyl group of the inhibitor. For this discussion we also include as parts of the B component, the interactions with other substituents in the carbons of the cyclopentanophenanthrene ring. We are thinking particularly of substituents in carbons 5 and 19, since a 5-β-hydroxyl group and the presence of an aldehyde or a hydroxyl methyl group at C-19 result in more potent compounds than those having methyl groups in C-19 and C-5. (3) Component C interacts with the lactone ring at C-17.

Provided our assumptions are correct, our results imply that the site of attachment of the lactone ring to the enzyme (Component C) is probably the most important in stabilizing the steroid–enzyme complex. Although it is known that the effects of the aglycones are more readily reversed than those of their respective glycosides, the speed of recovery from inhibition with strophanthidin was considerably longer than that observed with the steroids with modified lactones. The few experiments with digitoxigenin provide additional support to this conclusion. Furthermore, the finding that a modification in the position of attachment of the lactone ring is sufficient to increase reversibility, suggests that in addition to binding forces, resulting from the double bond and the carbonyl oxygen, there is a requirement of an adequate spatial orientation of the lactone ring with respect to the enzyme site. In line with this conclusion is the finding that compounds with inversion at C-17, i.e., the 17α-cardenolides, completely lack cardiotoxic activity [15].

Although our experiments are restricted to a relatively small number of cardiotonic steroids, it is known that a systematic pattern of effects is found when structure–activity relationships among cardiac steroids are considered i.e., among the cardenolides, the monosides are the most active, rings A/B joined *trans* give higher activities than when joined in a *cis* configuration; saturation of the lactone reduces activity etc. [2, 15–18]. Also, our experiments were limited only to the frog muscle sodium pump. However, since observations in this system are at least qualitatively similar to findings in other cells and cell free Na^+–K^+ ATPase preparations [1–4, 16–18], we feel that they provide a useful approach in clarifying the glycoside–receptor interactions.

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