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Interaction of ouabain and cassaine with $Na^+ + K^+ -$ ATPase and its relationship to their inotropic actions

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INTRODUCTION

Sodium - potassium - activated ATPase (ATP phosphohydrolase E.C. 3.6.1.3) is a membrane-bound enzyme which is thought to be closely related to, if not identical with, the sodium pump activity of cell membranes. *In vivo*, this enzyme pumps sodium out of and potassium into the cell and it has therefore, during its transport cycle, access to both sides of the cell membrane (Post *et al.*, 1969). The transport function of $Na^+ + K^+$-ATPase is required for volume control in the cell, but it also subserves many other cellular functions (Post, 1968).

The normal reaction cycle of the sodium pump of red cell membranes is shown in Fig. 12.1. Three sodium ions are pumped out of the cell and two potassium ions are pumped into the cell with the concomitant hydrolysis inside the cell of one ATP molecule to ADP and inorganic phosphate (P_i). This reaction requires Mg^{2+} inside the cell and is specifically inhibited, from outside the cell only, by low concentrations of cardiac glycosides such as ouabain (Post, 1968).

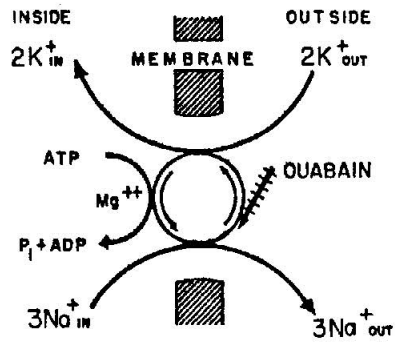

Figure 12.1 Stoicheiometry and localisation of the sodium and potassium pump in human erythrocytes. Reprinted by permission from Post (1968)

If cells are broken and their plasma membrane fragments isolated, this pump activity appears as a magnesium-dependent, sodium- and potassium-stimulated ATPase whose activity is specifically inhibited by cardiac glycosides such as ouabain. If these membrane fragments are incubated with Mg^{2+}, [γ-^{32}P] ATP and 100 mM potassium ion, a slowly accumulating and stable phosphorylation of the membranes is observed (Fig. 12.2). If sodium ion is substituted for potassium ion, there is an immediate increase of about tenfold in the level of labelling of these membranes, followed by a slow increase, as before (Post, Sen and Rosenthal, 1965).

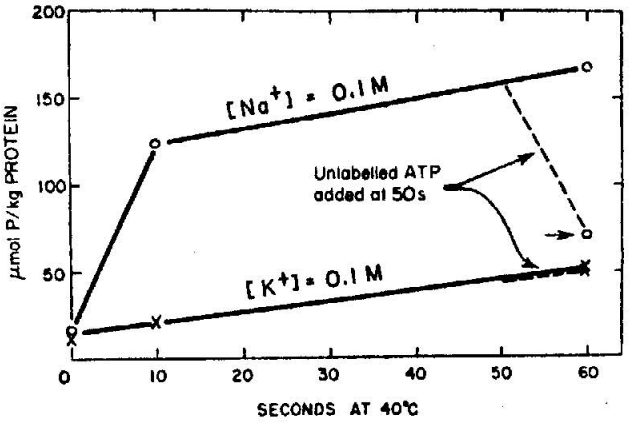

Figure 12.2 Onset and turnover of Na^+-dependent labelling of kidney membranes. The initial volume for each experiment was 1.1 ml. The reaction was started with 0.5 μmol of Mg-[γ-^{33}P] ATP in 0.1 ml. It was stopped with acid, and radioactivity was measured in the washed precipitate. The values at zero time were determined by adding acid before [γ-^{32}P] ATP. For two points, unlabelled MgATP, 5 μmol in 0.1 ml, was added at 50 s. This reduced the specific activity of the [γ-^{32}P] ATP to one-eleventh of the initial value. The labelling shown by the *unmarked arrow* was calculated by adding one-eleventh of the increment in labelling at 60 s due to sodium ion to the labelling at 60 s in the presence of potassium ion. Reprinted by permission from Post *et al.* (1965)

At 0°C the Na^+-stimulated ^{32}P labelling of these membranes turns over relatively slowly, as may be demonstrated by adding unlabelled ATP to the system (Fig. 12.2). It is thus possible to directly observe the action of K^+ on it, as shown in Fig. 12.3. In this experiment, the phospho-enzyme was first formed in the presence of Na^+ and Mg^{2+} and [γ-^{32}P] ATP, then isolated by the addition of excess cold EDTA, which blocks the Mg^{2+}-requiring phosphorylation reaction. Under these conditions the sodium-stimulated ^{32}P labelling of the membrane decays slowly with a half-life of about 8 s. If, however, a small amount of K^+ is added to the system, the rate at which the phospho-enzyme breaks down is greatly accelerated (Fig. 12.3), suggesting a potassium-accelerated dephosphorylation

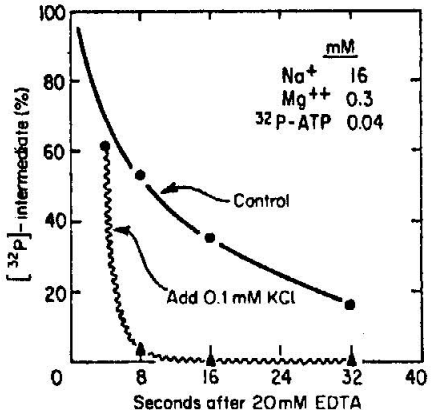

Figure 12.3 Dephosphorylation of the phosphorylated intermediate by K^+. The temperature was 0°C. Reprinted by permission from Post (1968)

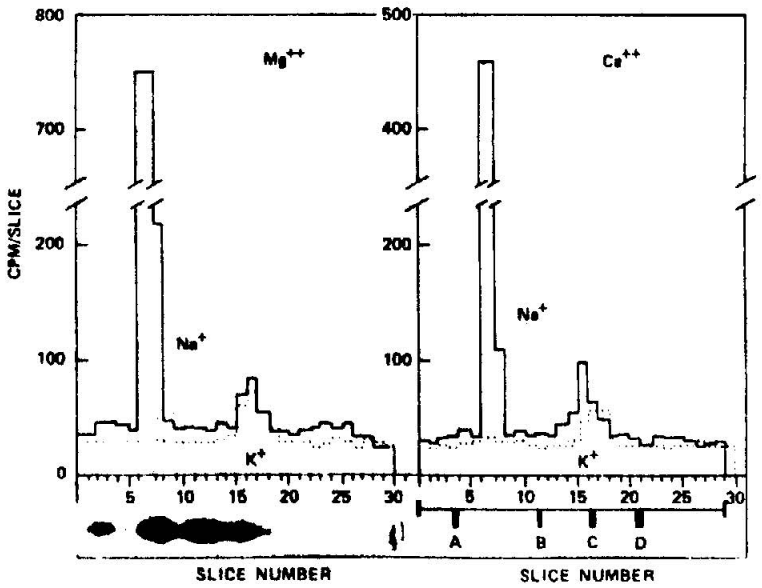

Figure 12.4 Gel electrophoresis of rat brain ATPase phosphorylated in the presence of Mg^{2+} or Ca^{2+}. Rat brain ATPase was phosphorylated by [γ-^{32}P] ATP in the presence of 100 mM Na^+ or 100 mM K^+ and either 1 mM Mg^{2+} or 1 mM Ca^{2+}. Phosphorylation was for 5 s at 0°C, and denaturation, washing and electrophoresis were performed as described in Tobin *et al.* (1975). Mg^{2+}-stimulated labelling was 500.6 pmol ^{32}P/mg protein, while Ca^{2+}-stimulated labelling was 247 pmol ^{32}P/mg protein. The solid lines show radioactivity observed in each slice when labelling was performed in the presence of Na^+, the dotted lines labelling in the presence of K^+. The lower panels show the staining patterns of the ATPase preparations (right-hand panel) or migration distances of thyroglobulin (A), ovalbumin (B), catalase (C) or lysozyme (D) standards. Reprinted by permission from Tobin *et al.* (1975)

(Post, 1968). Thus the experiments of Figs. 12.2 and 12.3 present evidence that the reaction cycle of $Na^+ + K^+$-ATPase involves a Na^+-stimulated phosphorylation of these membranes and a K^+-accelerated hydrolysis of the phospho-enzyme complex.

If membrane preparations phosphorylated in the presence of Na^+, Mg^{2+} and [γ-^{32}P] ATP are solubilised in sodium dodecyl sulphate (SDS) and run on acrylamide gels at pH 2.4, the material whose phosphorylation is stimulated by Na^+ runs as a single band with an apparent molecular weight of about 95 000 (Tobin, Akera and Brodie 1975) (Fig. 12.4). This phosphorylated material has been identified as a β-aspartyl phosphate group (Post and Orcutt, 1973). Thus the Na^+-stimulated phosphorylation of the plasma membrane observed in the earlier experiments appears to be associated with the phosphorylation of aspartic acid residues in membrane polypeptides of about 95 000 molecular weight. This reaction cycle is summarised in Fig. 12.5.

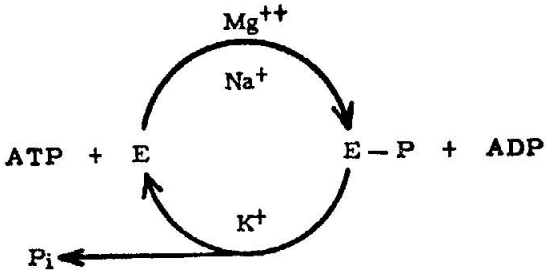

Figure 12.5 A partial reaction sequence for formation and breakdown of a phosphorylated intermediate in ($Na^+ + K^+$)-ATPase. Reprinted by permission from Post (1968)

THE EFFECTS OF OUABAIN

As pointed out earlier, both the sodium pump and the sodium-potassium-ATPase activity of broken membranes are very specifically inhibited by cardiac glycosides such as ouabain. The availability in the mid-1960s of [^{3}H]-ouabain made it possible to study the interaction of ouabain with this enzyme in detail. The right-hand panel of Fig. 12.6 shows an experiment in which guinea-pig kidney $Na^+ + K^+$-ATPase was incubated with micromolar concentrations of [^{3}H]-ouabain in the presence of Na^+, Mg^{2+} and various nucleotide triphosphates. In the presence of Na^+, Mg^{2+} and [^{3}H]-ouabain no significant binding of [^{3}H]-ouabain to these membrane preparations was observed. At indicated zero time, however, the nucleotides were added and binding commenced. In the presence of low concentrations of ATP and CTP binding increased rapidly, peaked at about two minutes, and then declined slowly. Nucleotides other than ATP and CTP were less effective in supporting [^{3}H]-ouabain binding, though ADP also supported a significant level of [^{3}H]-ouabain binding. The left-hand panel of Fig. 12.6 shows

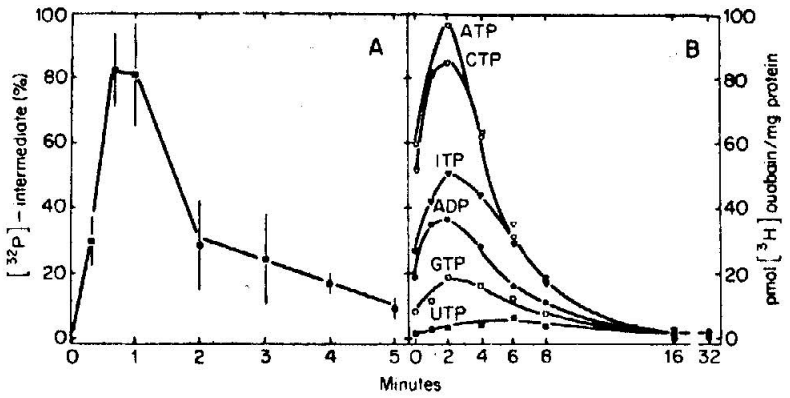

Figure 12.6 Time course of [γ-^{32}P] ATP-dependent labelling and nucleotide-supported [^{3}H]-ouabain binding to $Na^+ + K^+$-ATPase. (A) Guinea-pig kidney enzymes were incubated with 100 mM Na^+ and 5 mM Mg^{2+} at 37°C. At indicated zero time 25 μM [γ-^{32}P] ATP was added to start the labelling reaction. The reaction was stopped with trichloroacetic acid. (■–■) shows the amount of ^{32}P-labelling of the enzyme at the indicated time points. Labelling is expressed as a percentage of peak labelling for each of four different experiments which averaged 122 pmol ^{32}P/mg protein. (B) At indicated zero time the binding reaction was started by the addition of 50 μM of each of the indicated nucleotides and stopped by centrifugation at the indicated time points. The symbols show the binding of [^{3}H]-ouabain supported by each of the nucleotides with no deductions made for background labelling. Reprinted by permission from Tobin *et al.* (1974)

that under these conditions the phosphorylation of these membranes by [γ-^{32}P] ATP is also transient, consistent with the concept that the [^{3}H]-ouabain binding observed in Fig. 12.6 requires prior phosphorylation of the enzyme (Tobin *et al.*, 1974).

Though the experiment of Fig. 12.6 is suggestive, there are a number of problems with the conclusion that [^{3}H]-ouabain binding in the presence of Na^+, Mg^{2+} and ATP requires phosphorylation of the enzyme by ATP. In the first place, with the exception of ATP, all of the nucleotides used in the experiment of Fig. 12.6 are very poor substrates for the overall $Na^+ + K^+$-ATPase activity. Secondly, it seems rather unlikely that the ADP-stimulated binding observed in Fig. 12.6 can be explained on the basis of a direct phosphorylation from ADP. Therefore, in an effort to resolve these discrepancies we studied the action of a non-phosphorylating analogue of ATP, β-γ-methylene ATP, on the binding of [^{3}H]-ouabain to guinea-pig kidney $Na^+ + K^+$-ATPase.

β, γ-Methylene ATP is an analogue of ATP in which the terminal bridge oxygen of the nucleotide molecule has been replaced by a methylene group. This analogue is structurally similar to ATP and binds to the enzyme but is not hydrolysed by it, presumably because the terminal phosphonate group cannot

be transferred to the enzyme. Use of this analogue should therefore allow one to distinguish between effects due to the binding of ATP (which would be mimicked by β-γ-methylene ATP) and those due to both binding and phosphorylation as shown by authentic ATP.

The experiment of Fig. 12.7 was designed to reveal any tendency of β-γ-methylene ATP to inhibit [^{3}H]-ouabain binding to $Na^+ + K^+$-ATPase. Na^+ and Mg^{2+} were required in the system to stimulate phosphorylation by ATP. To stimulate [^{3}H]-ouabain binding to the enzyme in the presence of Na^+ and Mg^{2+}, 1 mM inorganic phosphate was included in the system (Tobin and Sen, 1970). Under these conditions [^{3}H]-ouabain binding equilibrated at about 40 per cent of the level observed in the presence of Na^+, Mg^{2+} and ATP. At 15 min this binding was challenged by the addition of either ATP or β-γ-methylene ATP. As shown in Fig. 12.7, ATP produced a prompt increase in the equilibrium level of [^{3}H]-ouabain binding, while β, γ-methylene ATP produced an equally prompt decline to zero in the equilibrium level of [^{3}H]-ouabain binding. The experiment shows that the β, γ-methylene analogue of ATP is strongly inhibitory to P_i-suppor-

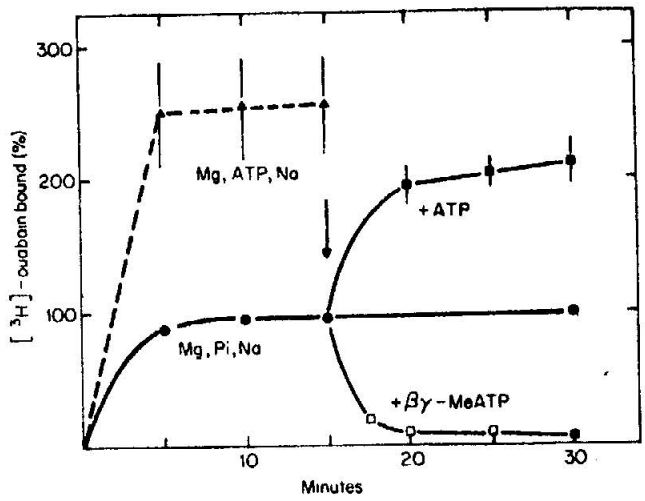

Figure 12.7 Inhibition of the equilibrium level of [^{3}H]-ouabain binding by β, γ-methylene-ATP. Guinea-pig kidney enzymes were incubated with 5×10^{-7} M [^{3}H]-ouabain at 37°C. The binding reaction was started by the addition of 4 mM Mg^{2+} at zero time and stopped at the indicated times. (●–●) shows binding in the presence of 4 mM Mg^{2+}, 1 mM P., and 60 mM Na^+. After 15 min, binding in the presence of Na^+, Mg^{2+} and P_i was challenged by the addition of 3 mM ATP (■–■) or 3 mM β, γ-methylene-ATP (□–□). (▲–▲) shows [^{3}H]-ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{2+} and 5 mM ATP and (♦) shows binding in the presence of 4 mM Mg^{2+}, 1 mM P_i and 2.5×10^{-4} M unlabelled ouabain. Binding in the presence of Na^+, Mg^{2+} and P_i at 30 min was arbitrarily taken as 100 per cent. Maximal binding (4 mM Mg^{2+}, 1 mM P_i without Na^+) averaged 94.2 pmol [^{3}H]-ouabain/mg protein. Reprinted by permission from Tobin *et al.* (1973)

ted [^{3}H]-ouabain binding. Broadly similar observations have been made with α,β-methylene ADP and β,γ-imido ATP. These experiments are thus consistent with the idea that in the presence of Na^+ and ATP phosphorylation of this enzyme is required to support [^{3}H]-ouabain binding to these membranes (Tobin *et al.*, 1974).

Most of the experiments and experimental designs for the [^{3}H]-ouabain binding experiments presented up to this point were based on the premise that the interaction of [^{3}H]-ouabain with the cardiotonic steroid binding site of $Na^+ + K^+$-ATPase was readily reversible. However, reports from other laboratories suggested that [^{3}H]-ouabain binding, in the hands of other workers, was essentially irreversible. This led us to examine the factors which determined the rate at which [^{3}H]-ouabain dissociated from its binding sites on this enzyme. The first variable that we investigated was temperature, as shown in Fig. 12.8. It turned out that [^{3}H]-ouabain binding to guinea-pig kidney enzyme is highly temperature-dependent, binding being relatively labile ($t_{\frac{1}{2}}$ = 3 min) at 37°C, but very stable ($t_{\frac{1}{2}}$ = 9 h) at 0°C (Tobin and Sen, 1970). This finding has turned out to be of considerable practical importance, since it allows one to stabilise or labilise the enzyme-ouabain complex simply by varying the temperature; this has become an important manoeuvre in studies on the $Na^+ + K^+$-ATPase-ouabain interaction.

Another important variable which affects the rate of dissociation of the ouabain-enzyme complex is the species and tissue from which the enzyme is prepared.

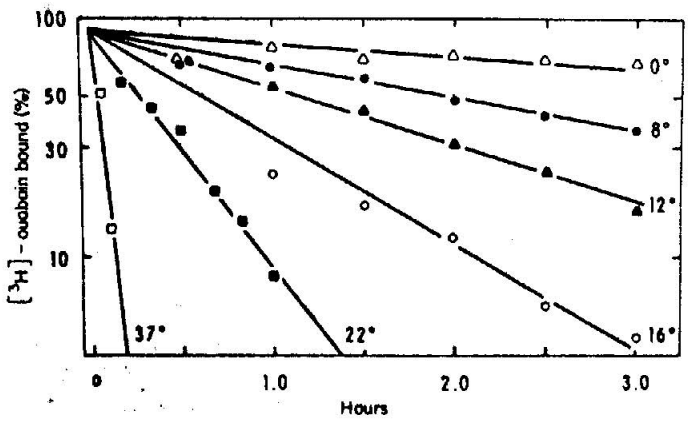

Figure 12.8 Stability of enzyme–ouabain complex at different temperatures. Enzyme, 4 mM $MgCl_2$, 1 mM H_3PO_4 and 2.5×10^{-7} M [^{3}H]-ouabain were incubated for 20 min at 37°C. The tubes were then cooled to the indicated temperature and 10 mM EDTA and 5×10^{-4} M unlabelled ouabain added. The reaction was stopped by centrifugation at the indicated time points. At 22 and 37°C only unlabelled ouabain was added. At 37°C the reaction was stopped by rapidly freezing (20 s) the tubes in an acetone–dry ice mixture. After thawing at 0°C, the tubes were centrifuged as before. The pH of the system did not change (7.4 ± 0.1 pH units) with temperature. Reprinted by permission from Tobin and Sen (1970)

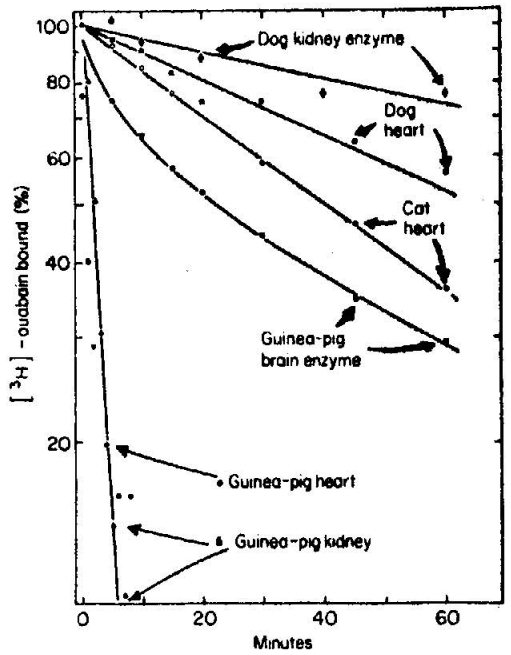

Figure 12.9 Dissociation of enzyme–ouabain complex in different tissues and species: [^{3}H]-ouabain–enzyme was formed and its dissociation followed as described by Tobin *et al.* (1972b). The symbols indicate the percentage of the binding at zero time in a given tissue remaining at the times indicated. The enzymes prepared from different tissues were as follows: ▲, guinea-pig kidney; ●, guinea-pig heart; ○, guinea-pig brain; ■, dog heart; ◆, dog kidney; ○, cat heart. Reprinted by permission from Tobin *et al.* (1972b)

Figure 12.9 shows an experiment in which we compared the rates of dissociation of [^{3}H]-ouabain from $Na^+ + K^+$-ATPase preparations from a number of tissues at 37°C. The experiment shows that [^{3}H]-ouabain dissociates rapidly and at approximately similar rates from guinea-pig heart and kidney enzymes, but more slowly from dog heart and kidney $Na^+ + K^+$-ATPases (Tobin, Henderson and Sen, 1972b; Tobin and Brody, 1972).

The observation that the rate of dissociation of [^{3}H]-ouabain from cardiac $Na^+ + K^+$-ATPase obtained from different species is variable and slow raised an interesting possibility concerning the rate of loss of ouabain-induced inotropy in certain species. The most straightforward model which one can assume for $Na^+ + K^+$-ATPase as the cardiotonic receptor for cardiac glycosides would be a simple occupancy model. If this were the case, the rates of dissociation of the ouabain–enzyme complex observed in Fig. 12.9 should be rate-limiting for the offset of ouabain-dependent inotropy in perfused hearts. From a survey of the literature

it was readily apparent that ouabain-induced inotropy in guinea-pig hearts decayed at just about the same rate as [^{3}H]-ouabain binding to guinea-pig heart ATPase in the experiment of Fig. 12.9. Therefore the test of the simplest possible receptor model for the cardiotonic action of ouabain involving $Na^+ + K^+$-ATPase in perfused hearts was to determine whether the rates of dissociation observed in Fig. 12.9 are comparable with the rates of offset of inotropic responses due to ouabain.

Because Langendorff perfusion experiments are done under specific temperature and ionic conditions, we repeated the experiments of Fig. 12.9 under conditions applicable to the perfusion experiments. The data of Fig. 12.10 show that when conditions appropriate to perfusion experiments were used (temperature 27°C; binding supported by Na^+, Mg^{2+} and ATP; 5 mM K^+ present in the system; Akera *et al.*, 1973), the rate at which [^{3}H]-ouabain dissociated from these ATPase preparations was somewhat slower than previously. However, clear

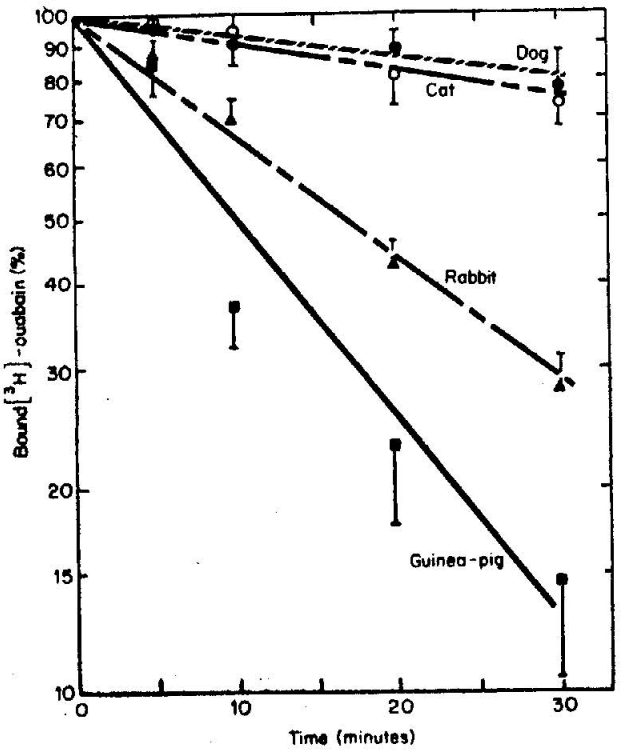

Figure 12.10 Dissociation of [^{3}H]-ouabain from cardiac $Na^+ + K^+$-ATPase *in vitro* at 27°C. Bound [^{3}H]-ouabain was expressed as the percentage of that immediately after the addition of unlabelled ouabain in each enzyme preparation. ●, dog; ○, cat; ▲, rabbit; and ■ guinea-pig. Each point represents the mean of five experiments. Vertical lines indicate SEM. Reprinted by permission from Akera *et al.* (1973)

separations in rates of dissociation from the ATPases chosen were observable, allowing comparison with inotropy decay rates (Akera *et al.*, 1973).

Figure 12.11 shows the rates at which inotropy due to ouabain was lost in perfused guinea-pig, rabbit, kitten and puppy hearts. Inotropy was lost rapidly in the guinea-pig and rabbit hearts, consistent with the rapid dissociation of ouabain from $Na^+ + K^+$- ATPase in these tissues. Inotropy was lost relatively slowly, however, in the perfused puppy and kitten hearts, consistent with the relatively slow dissociation of [^{3}H]-ouabain from its binding sites on $Na^+ + K^+$-ATPase in these tissues. This relationship is underscored in Fig. 12.12, where the half-times for dissociation and loss of inotropy are compared. The experiment shows a good correlation between the rates of loss of inotropy and the rates of dissociation of [^{3}H]-ouabain from $Na^+ + K^+$-ATPase over the range accessible with currently

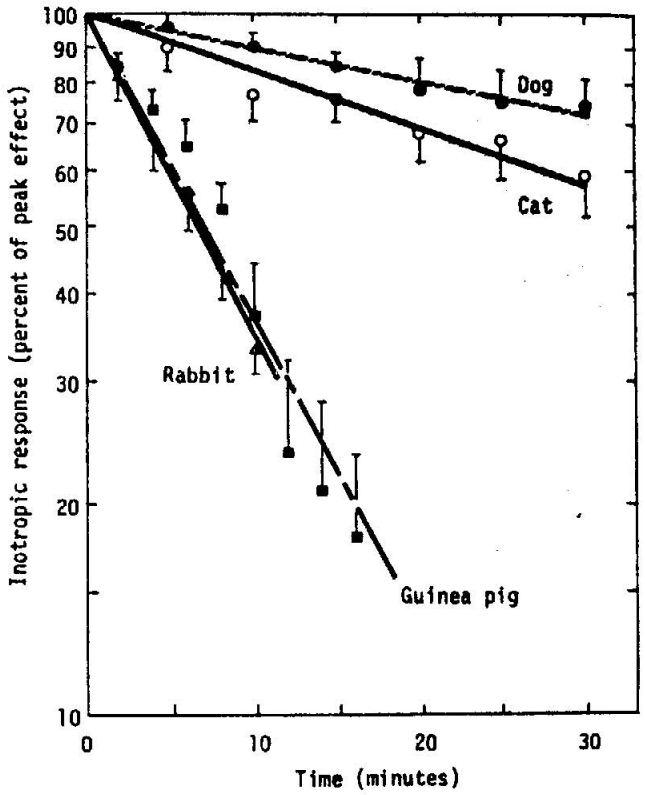

Figure 12.11 Washout of positive inotropic effect of ouabain in Langendorff preparations at 27°C. The maximal positive inotropic effect was arbitrarily set at 100 per cent. ● Dogs; ○ cats; ▲ rabbits; and ■ guinea-pigs. Each point represents the mean of four experiments. Vertical lines indicate SEM. Reprinted by permission from Akera *et al.* (1973)

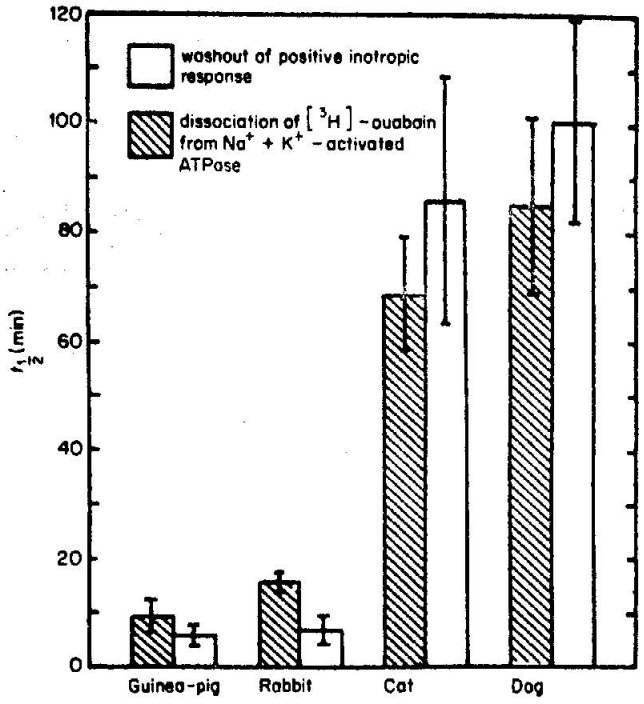

Figure 12.12 Half-times of the inotropic effect of ouabain and the dissociation of [^{3}H]-ouabain from cardiac ($Na^+ + K^+$)-activated ATPase at 27°C. Half-time was calculated from Fig. 12.10 and 12.11 by fitting a linear regression line to the data obtained in each experiment. Mean of four (inotropic data) and five (dissociation data) experiments in each species, respectively. Vertical lines indicate SEM. Reprinted by permission from Akera *et al.* (1973)

available techniques. The experiment is consistent with the concept that $Na^+ + K^+$-ATPase is the cardiotonic receptor for cardiac glycosides, that occupancy of the receptor is closely linked to the inotropic effect and that the rate at which glycosides dissociate from this receptor is rate-limiting for offset of the inotropic effect of these drugs (Akera *et al.*, 1973).

Other studies in this area have shown that the time course of both onset and offset of ouabain-induced inotropy in guinea-pig heart tissue is associated with the binding and dissociation of ouabain from cardiac $Na^+ + K^+$-ATPase. Concomitant with these increases and decreases in ouabain binding, the ability of cardiac slices to take up ^{86}Rb is also lost and then recovered. These experiments and others (Ku *et al.*, 1974a; Ku *et al.*, 1975, 1976; and Post *et al.*, 1969) comprise a substantial body of evidence which suggests that inhibition of $Na^+ + K^+$-ATPase by cardiac glycosides is closely linked to development of their positive inotropic effects (Akera and Brody, 1975).

THE EFFECTS OF CASSAINE

Comparative studies on the cardiac glycosides suggest the existence of several structural requirements for their cardiotonic actions. These requirements are generally considered to be a cyclopentanoperhydrophenanthrene nucleus with A/B *cis*, B/C *trans* and C/D *cis* fusion of the four-ring structure, a C_{14}-OH group and an unsaturated lactone ring in the β configuration on C17 (Kahn, VanAtta and Johnson, 1963). The erythrophleum alkaloids, however, share many of the pharmacological actions of the cardiac glycosides while satisfying none of these structural requirements (Fig. 12.13). In particular, the erythrophleum group of alkaloids are cardiotonic and cardiotoxic like the cardiac glycosides and are also specific inhibitors of monovalent cation transport and of $Na^+ + K^+$-ATPase (Bonting *et al.*, 1964; Peters, Rabin and Wasserman, 1974).

To further investigate the hypothesis that inhibition of $Na^+ + K^+$-ATPase can account for the positive inotropic effects of various groups of drugs we examined the interactions of cassaine with $Na^+ + K^+$-ATPase in some detail.

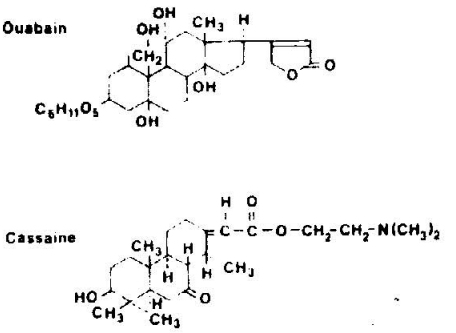

Figure 12.13 Structural formulae of ouabain and cassaine

These studies commenced with the working hypothesis that, because of the structural differences outlined, the interaction of cassaine with $Na^+ + K^+$-ATPase would be distinguishable from that of the cardiac glycosides. It was further hoped that such differences in interaction at the enzymatic level would be reflected in differences in the cardiotonic effects of cassaine. Thus, it might be possible to correlate events at the enzymatic level with those occurring at the level of the perfused heart, and these studies might provide further evidence in support of the possibility that $Na^+ + K^+$-ATPase is the positive inotropic receptor for the cardiac glycoside and erythrophleum alkaloid group of drugs.

Figure 12.14 shows inhibition of rat brain $Na^+ + K^+$-ATPase by cassaine and ouabain. In this experiment cassaine had about one-fourth of the apparent affinity of ouabain for this enzyme preparation. Further, the dotted line in Fig. 12.14 shows that preincubation of the enzyme with cassaine in the absence of K^+ does not increase the apparent affinity of cassaine for this enzyme. This

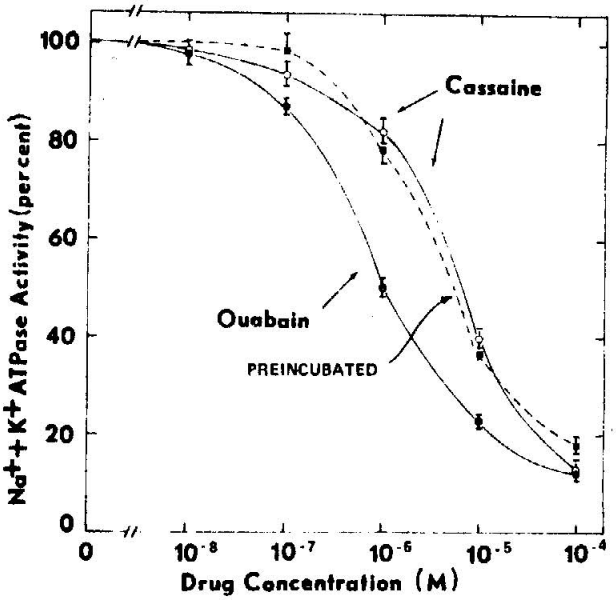

Figure 12.14 Inhibition of rat brain $Na^+ + K^+$-ATPase by ouabain and cassaine. About 30 μg of enzyme was incubated with the indicated concentrations of cassaine or ouabain in the presence of 100 mM Na^+, 15 mM K^+, 5 mM Mg^{2+} and 50 mM Tris HCl buffer, pH 7.6 at 37°C. The ATPase reaction was started by the addition of 5 mM ATP and stopped 10 min later. The solid circles (●–●) show inhibition in the presence of the indicated concentrations of ouabain, the open circles (○–○) inhibition in the presence of indicated concentrations of cassaine. The solid squares (■–■) show inhibition when the enzyme was preincubated with the indicated concentrations of cassaine, $Na^+ + K^{2+}$ and ATP for 10 min and the ATPase reaction started by adding K^+. $Na^+ + K^+$-ATPase activity is expressed as a percentage of that observed in the absence of ouabain which averaged 185.5 ± 9.0 μmol P_i/mg protein/h. Reprinted by permission from Tobin *et al.* (1975)

is in marked contrast to what is observed with ouabain, where preincubation can increase the apparent I_{50} for ouabain on this enzyme up to tenfold. In the case of ouabain this effect depends on the stability of the enzyme - ouabain complex (Akera, 1971), so one interpretation of this effect is that the cassaine - enzyme complex is considerably less stable than the ouabain - enzyme complex.

Figure 12.15 shows a direct test of the ability of cassaine to displace [^{3}H] - ouabain from its binding sites on $Na^+ + K^+$ - ATPase. In this experiment rat brain $Na^+ + K^+$ - ATPase was incubated with low concentrations of [^{3}H] - ouabain of high specific activity in the presence of Na^+, Mg^{2+} and ATP. At the time points indicated by the arrows cassaine was added either before, with or after the [^{3}H] - ouabain. The experiment shows that these low concentrations of cassaine do decrease the binding of [^{3}H] - ouabain to this enzyme and that the amount of

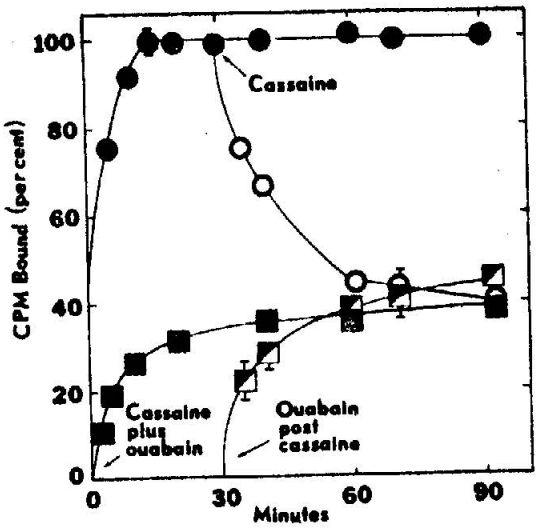

Figure 12.15 Effect of cassaine on [^{3}H]-ouabain binding to $Na^+ + K^+$-ATPase. Rat brain enzyme was incubated at 37°C in the presence of 100 mM Na^+, 0.2 mM Mg^{2+} and 5 mM ATP. The solid circles show the radioactivity bound when tracer amounts (0.3×10^{-9} M) of [^{3}H]-ouabain were added to the binding system. The solid squares show radioactivity bound when the same concentrations of cassaine and [^{3}H]-ouabain were added together. The half squares (◪) show radioctivity bound when the [^{3}H]-ouabain was added 30 min after the cassaine. The points are the mean ± SEM of four separate experiments with radioactivity bound expressed as a percentage of that bound at 90 min in the presence of Na^+, Mg^{2+} and ATP, which averaged 11.8 ± 1.0 pmol [^{3}H]-ouabain/mg protein. Reprinted by permission from Tobin *et al.* (1975)

this decrease was essentially independent of the sequence of addition of the ligands. The experiment suggests that the interactions of low concentrations of cassaine with this enzyme are fully reversible and is consistent with the idea that cassaine and ouabain may compete for the same binding sites on this enzyme.

While the data of Fig. 12.15 may suggest a direct displacement of [^{3}H]-ouabain from its binding sites on this enzyme by cassaine, other ligands of this enzyme such as Na^+ and ATP (and the ATP analogues) can readily displace [^{3}H]-ouabain from its binding sites on this enzyme by what appear to be indirect or allosteric interactions (Tobin and Sen, 1970; Tobin, Banerjee and Sen, 1970, Tobin *et al.*, 1974). We therefore next investigated the actions of cassaine on the intermediate steps of the $Na^+ + K^+$-ATPase reaction to choose between these possibilities. The rationale behind this approach was that if cassaine produced its effects by binding at the ouabain-binding site of $Na^+ + K^+$-ATPase, one might expect its actions on the intermediate steps of the reaction cycle of this enzyme to be similar to those of ouabain. Because radiolabelled cassaine was not available

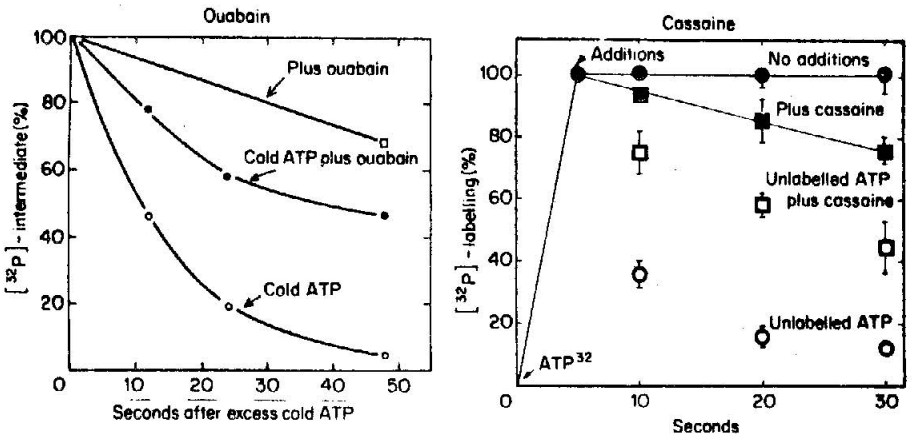

Figure 12.16 In the experiment shown in the left-hand panel guinea-pig kidney $Na^+ + K^+$-ATPase was phosphorylated from [γ-^{32}P] ATP with Mg^{2+} and Na^+. Once formed, E-P was isolated at indicated zero time by the addition of unlabelled ATP (open circles), or unlabelled ATP plus 2.5×10^{-4} M ouabain (solid circles). The open square shows the level of E-P when ouabain alone was added to the system at zero time. Reprinted by permission from Sen *et al.* (1969).

The right-hand panel shows the actions of cassaine on E-P formed similarly in rat brain ATPase. The solid circles show the steady state levels of E-P, the solid squares levels of E-P in the presence of 1×10^{-4} M cassaine. The open circles show spontaneous turnover of the phosphorylated intermediate when it was isolated by the addition of unlabelled ATP, while the open squares show its turnover in the presence of unlabelled ATP and 1×10^{-4} M cassaine. Reprinted by permission from Tobin *et al.* (1975)

to us, we used an indirect method following ^{32}P labelling of this enzyme from [γ-^{32}P] ATP as described by Sen, Tobin and Post (1969).

Figure 12.16 compares the reactivity of ouabain and cassaine with the phosphorylated intermediate of Na^+K^+-ATPase. The left-hand panel shows the reaction of guinea-pig kidney phosphoenzyme with ouabain at 0°C. In this experiment the phosphorylated intermediate was first formed in the presence of Na^+, Mg^{2+} and [γ-^{32}P] ATP. Then, at indicated zero time, the ^{32}P phosphoenzyme was isolated by the addition of excess unlabelled ATP and the spontaneous turnover of E-P exposed. When ouabain was added to the system with the unlabelled ATP, the turnover of the phosphoenzyme was slowed. However, if ouabain alone was added to the system, the amount of E-P present also declined, in apparent contradiction to the ability of ouabain to stabilise E-P. Other experiments have shown that this action is due to the generation of a dephosphoenzyme resistant to rephosphorylation (Sen *et al.*, 1969). Thus this experiment shows that ouabain interacts readily with the phosphoenzyme, slows its turnover and then gives rise to a form of the enzyme which is not readily rephosphorylated. Since ouabain

binding is virtually irreversible at 0°C, this resistance to rephosphorylation results in essentially complete inhibition of turnover (Sen *et al.*, 1969).

The right-hand panel of Fig. 12.16 shows that essentially the same results were observed with cassaine. Cassaine alone reduces the steady state level of E-P in much the same manner as ouabain. The spontaneous turnover of the phosphoenzyme was also slowed by cassaine. The experiment shows that the qualitative actions of cassaine and ouabain on the spontaneous turnover and steady state levels of the phosphoenzyme are similar.

Figure 12.17 shows a further parallelism in the actions of ouabain and cassaine on the phosphorylated intermediate of $Na^+ + K^+$-ATPase. In the left-hand panel of Fig. 12.17 $Na^+ + K^+$-ATPase was phosphorylated in the presence of Na^+, Mg^{2+}, [γ-^{22}P] ATP and ouabain. Phosphorylation was essentially complete at 3 s and then the level of the phosphoenzyme slowly declined owing to the ouabain present. When the ouabain-treated phosphoenzyme was challenged with K^+, the dephosphorylation obtained was only partial, showing that in the presence of ouabain a K^+-resistant phosphoenzyme accumulates. This action of ouabain on E-P is typical of the cardiac glycosides (Sen *et al.*, 1969; Post *et al.*, 1969). The

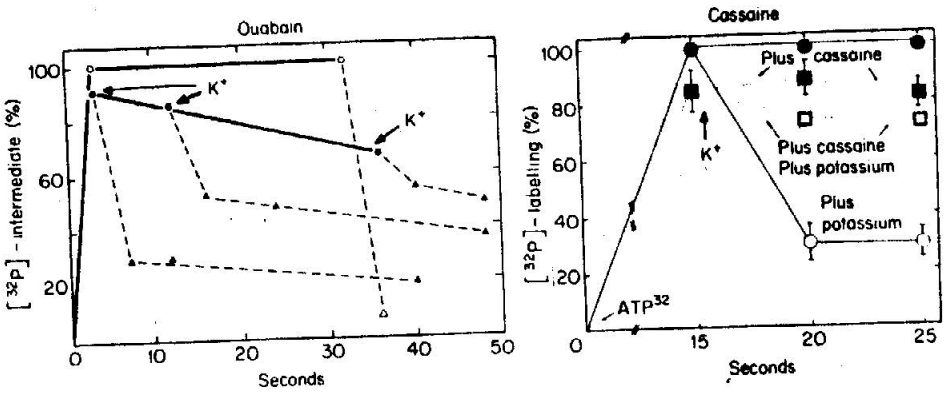

Figure 12.17 Resistance of ouabain or cassaine-treated phospho-enzymes to dephosphorylation by K^+.

The left-hand panel shows the effect of ouabain on the K^+-sensitivity of E-P. The solid circles show levels of E-P when this enzyme was phosphorylated from [γ-^{32}P] ATP, Mg^{2+} and Na^+ in the presence of 2.5×10^{-4} M ouabain. At the time points indicated by the arrows 15 mM K^+ was added to the system, and the solid triangles show the subsequent levels of E-P observed. The open circles and open triangle show steady state levels of E-P and the action of K^+ in the absence of added ouabain. Reprinted by permission from Sen *et al.* (1969).

The right-hand panel shows a similar experiment with cassaine. The solid circles show the steady state level of E-P when in the presence of Na^+, Mg^{2+} and ATP, the open circles the levels of E-P after addition of K^+ at 15 s. The solid squares show steady state levels of E-P when cassaine (1×10^{-4} M) was added to the system 5 s after the addition of [γ-^{32}P] ATP. The open squares show the effect of K^+ (added at 15 s) on the cassaine-treated phospho-enzyme. Reprinted by permission from Tobin *et al.* (1975)

right-hand panel of Fig. 12.17 shows that qualitatively similar results are obtained with cassaine. When the enzyme was phosphorylated in the presence of cassaine, the steady state level of the phosphoenzyme was reduced, much as was observed with ouabain. If this cassaine-reacted phosphoenzyme was then challenged with K^+, it was found to be largely resistant to K^+, in contrast to the native phosphoenzyme. The experiments show that with respect to potassium ion the actions of both ouabain and cassaine on the phosphorylated intermediate of $Na^+ + K^+$-ATPase are similar.

From the similarity of the cassaine- and ouabain-induced patterns of reactivity of the phosphoenzyme, it is probably reasonable to conclude that cassaine produced its effects by interacting with and stabilising a configuration of this enzyme similar to that stabilised by ouabain. However, if cassaine stabilised a configuration of this enzyme similar to that stabilised by ouabain, it can then antagonise [^{3}H]-ouabain binding (Fig. 12.15) only by interacting directly at the cardiotonic

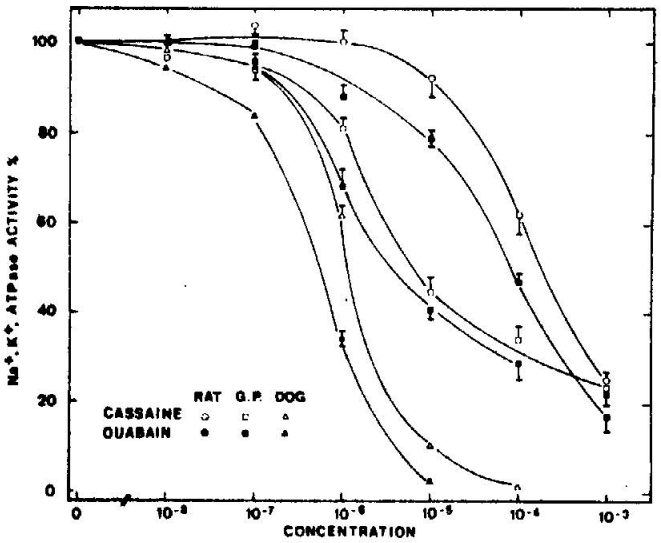

Figure 12.18 Inhibition of dog, guinea-pig (G.P.) and rat heart ATPases by cassaine or ouabain. Rat and guinea-pig heart $Na^+ + K^+$-ATPases were prepared as described by Akera *et al.* (1969), while the dog heart ATPase was prepared as described by Pitts, Lane and Schwartz (1973). Their $Na^+ + K^+$-ATPase activity was then assayed under standard conditions (100 mM Na^+, 15 mM K^+) in the presence of the indicated concentration of cassaine and ouabain. The solid triangles, squares and circles show inhibition of the dog, guinea-pig and rat enzyme preparations of ouabain. The open triangles, squares and circles show inhibition of these enzymes by the indicated concentrations of cassaine. All points are the mean ± SEM of determinations on four different enzymes except for the dog heart preparation, where only the points at 1×10^{-6} M are from four individual determinations. Reprinted by permission from Tobin *et al.* (1975)

steroid binding sites of $Na^+ + K^+$-ATPase. If cassaine does interact directly with the cardiotonic steroid binding sites of $Na^+ + K^+$-ATPase, one might then expect species-dependent differences in the sensitivity of different $Na^+ + K^+$-ATPase to cassaine, since the glycoside binding sites on $Na^+ + K^+$-ATPase are the only binding sites on this enzyme which show substantial species-dependent differences in ligand affinity (Tobin *et al.*, 1972b; Tobin and Brody, 1972; Akera *et al.*, 1969). Testing this hypothesis, Fig. 12.18 shows that similar species-dependent differences in sensitivity to cassaine are observed in dog, guinea-pig and rat heart ATPases. These experiments are therefore consistent with the idea that cassaine produces its inhibition of $Na^+ + K^+$-ATPase by interacting at the cardiotonic steroid binding site of $Na^+ + K^+$-ATPase.

At this point the data suggest that cassaine interacts with $Na^+ + K^+$-ATPase at the cardiotonic steroid binding site of this enzyme and the interaction appears to be qualitatively quite similar to that of ouabain. The only major quantitative

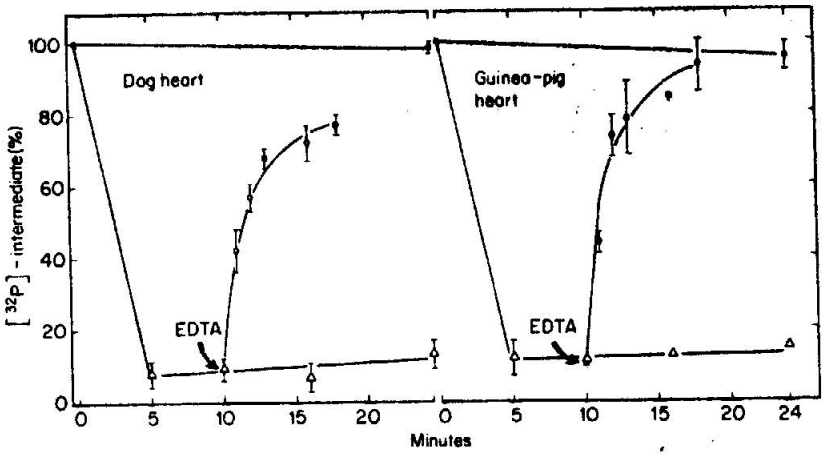

Figure 12.19 Rates of recovery of phosphorylation in cassaine-inhibited guinea-pig and dog heart enzymes. The left-hand panel shows the results of experiments with dog heart enzymes, the right-hand panel experiments with guinea-pig heart enzymes. About 150 μg of each enzyme was incubated with 100 mM Na^+ and 0.2 mM Mg^{2+} at 37°C. The experiment was started at indicated zero time by the addition of 1.0 mM UTP, with or without 1×10^{-4} M cassaine in the system. The solid circles show the labelling (after 4 s) from [γ-^{32}P] ATP added with 10 mM EDTA and 12 mM Mg^{2+} at the indicated times after UTP alone. The open triangles show the labelling observed in the presence of cassaine plus UTP at the indicated times. The open circles show the labelling observed in the presence of cassaine plus UTP when the 10 mM EDTA was added at 10 min; labelling with Mg^{2+} and [γ-^{32}P]-ATP was for 4 s, and labelling in the presence of 100 mM K^+ was subtracted as background labelling. The experiments with the dog heart enzyme are shown as the mean ± SEM of four individual experiments, those with the guinea-pig heart as the means ± SEM of three experiments. Maximal K^+-sensitive ^{32}P incorporation was 159 ± 26 pmol ^{32}p/mg protein in the guinea-pig heart enzymes. Reprinted by permission from Tobin *et al.* (1975)

difference suggested by these experiments is the implication of Fig. 12.16 that the enzyme - cassaine complex is less stable than the enzyme - ouabain complex. We therefore decided to investigate the rates of dissociation of the cassaine - enzyme complex in an attempt to identify a quantitative difference between the interactions of cassaine and ouabain with cardiac $Na^+ + K^+$-ATPase.

We estimated the rate of dissociation of cassaine from $Na^+ + K^+$-ATPase indirectly (Fig. 12.19) by the method used to demonstrate dissociation of strophanthidin bromacetate from this enzyme (Tobin *et al.*, 1973). In this method cassaine was bound to the enzyme in the presence of Na^+, Mg^{2+} and UTP. UTP was used instead of ATP because it phosphorylates $Na^+ + K^+$-ATPase, but does not interfere significantly with the subsequent phosphorylation of this enzyme from ATP under appropriate conditions (Tobin *et al.*, 1973; Tobin *et al.*, 1972a). In this way cassaine was allowed to bind to this enzyme in the presence of Na^+ and UTP and the cassaine - enzyme complex was then isolated by the addition of EDTA. Under these conditions the ability of the enzyme to accept a phosphate group from [γ-^{32}P] ATP after exposure to cassaine was recovered relatively rapidly and completely in the guinea-pig enzyme; more slowly and less completely in the dog heart enzyme (Fig. 12.19). The data suggest that cassaine dissociates rapidly from the guinea-pig enzyme in the presence of Na^+, UTP and excess EDTA and somewhat more slowly from dog heart ATPase.

The half-lives of the enzyme–cassaine estimated from the data of Fig. 12.19 are much shorter than the 10 and 95 min half-lives reported for the ouabain - enzyme complexes at 27°C in these species (Fig. 12.10). If the basis of the cardiotonic actions of cassaine is indeed its reversible interaction with the cardiotonic steroid binding site of $Na^+ + K^+$-ATPase, then one would expect the rates of offset of the positive inotropic responses due to cassaine to be (a) much faster than the rates of offset of the positive inotropic actions of ouabain, and (b) faster in the guinea-pig heart than in the dog heart. Figure 12.20 shows that the rates of guinea-pig heart than in the dog heart. Figure 12.20 shows that the rates of offset of the ouabain-induced positive inotropic effects in dog and guinea-pig hearts are about 10 and 55 min, respectively, in good agreement with data previously reported by Akera *et al.* (1973). However, if cassaine was substituted for ouabain as the inotropic agent, the half-lives of the inotropic effects were much shorter, about 2.5 min in the guinea-pig and 9.5 min in the dog heart preparation. These much faster offsets of cassaine-dependent inotropy are consistent with the lower apparent affinity of cassaine for cardiac $Na^+ + K^+$-ATPase preparations in Fig. 12.14 and the rapid recovery of guinea-pig and dog heart enzymes from inhibition by cassaine shown in Fig. 12.19.

In conclusion, it appears that the only quantitative difference between the interaction of cassaine and ouabain with $Na^+ + K^+$-ATPase is the faster dissociation rate of cassaine from the cardiotonic steroid binding site. This difference at the enzymatic level is reflected at the tissue level in the offset of cassaine-induced inotropy being four to five times faster than the offset of ouabain-induced inotropy. These observations make it very likely that the offset rates

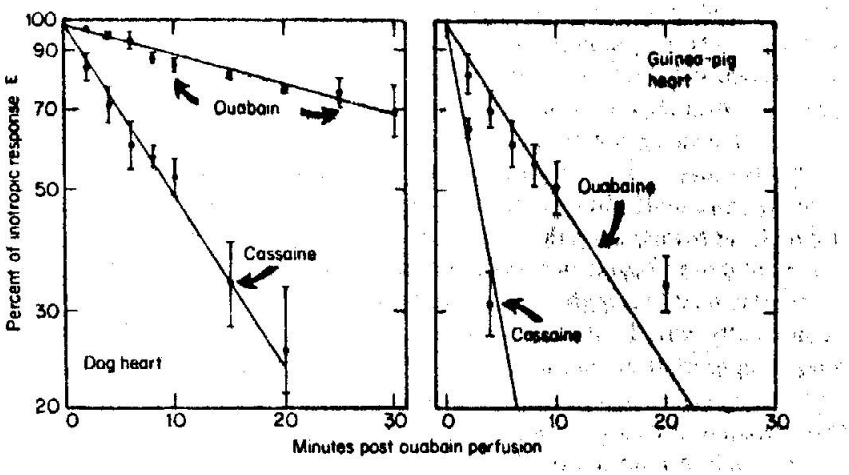

Figure 12.20 Rates of offset of ouabain- and cassaine-induced inotropic effects in dog and guinea-pig hearts. The left-hand panel shows experiments on puppy hearts, the right-hand panel experiments on guinea-pig hearts. Langendorff preparations of these hearts were perfused with Krebs–Henseleit solution containing 6×10^{-7} M drug (puppy hearts) or 1.2×10^{-6} M drug (guinea-pig hearts). After 20 min of drug perfusion the hearts were switched to drug-free solution and the rates of loss of the inotropic response measured. The guinea-pig heart data are the means ± SEM of five separate experiments, with ouabain producing a 35 ± 6.0 percent increase in cardiac contractile force and cassaine a 51 ± 3.0 per cent increase. The puppy heart experiments are shown as the means of four experiments, with ouabain producing a 34 ± 4.6 per cent inotropic response and cassaine a 23.0 ± 5.0 per cent inotropic response. Reprinted by permission from Tobin *et al.* (1975)

observed with ouabain are in fact determined by the rates of dissociation of ouabain from the $Na^+ + K^+$-ATPase and not due to diffusion barriers. If the rates of offset observed with ouabain were due to diffusion barriers, one would expect essentially similar rates with cassaine rather than the much faster dissociation rates observed. Thus the rapid offset rates of cassaine-induced inotropy correlate well with what is known about the interaction of cassaine with the $Na^+ + K^+$-ATPase (Peters *et al.*, 1974) and are in good agreement with the concept that the rate of dissociation of these drugs from the cardiotonic steroid binding site of $Na^+ + K^+$-ATPase is rate-limiting for the offset of their pharmacological effects in perfused hearts (Akera *et al.*, 1973).

ACKNOWLEDGEMENTS

Published as paper No. 76-4 with the permission of the Dean and Director and from the Equine Drug Research Program, Department of Veterinary Science, University of Kentucky, Lexington, Ky. 40506. This work was supported by grants from the Michigan Heart Association, Grant HL 16055 and HL 16052-01

from the National Institutes of Health, and General Research Support Grant NIH-RR-05623-04 to the College of Veterinary Medicine, Michigan State University, from the National Institutes of Health, and Grant BMS 74-18512 from the National Science Foundation. The technical help of Mrs Annie Han is gratefully acknowledged.

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