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INHIBITION OF THE SODIUM PUMP IN GUINEA-PIG VENTRICULAR MUSCLE BY DIHYDRO-OUABAIN: EFFECTS OF EXTERNAL POTASSIUM AND SODIUM

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SUMMARY

1. The inhibition of the electrogenic pump current in quiescent guinea-pig ventricular muscle by dihydro-ouabain (DHO) was studied with the three-micro-electrode voltage-clamp technique described previously (Daut, 1982*c*). From dose–response curves of the drug-induced current change (I_D) the equilibrium dissociation constant of the binding of DHO to the Na–K pump (K_D) and the electrogenic pump current flowing in the steady state (I_p) were inferred (Daut & Rüdel, 1982*b*).

2. The external K concentration ($[K]_o$) was varied between 2 and 4·5 mM (substituted by Na). K_D was found to increase with increasing $[K]_o$. A plot of log K_D *versus* log $[K]_o$ gave a straight line with a slope of about 1·5.

3. The time constants of the onset (τ_{on}) and decay (τ_{off}) of I_D are supposed to represent the chemical kinetics of binding and unbinding of the drug (Daut & Rüdel, 1981, 1982*b*). τ_{on} was found to be inversely related to $[K]_o$ whereas τ_{off} was found to be independent of $[K]_o$.

4. I_p was found to be independent of $[K]_o$. This was interpreted to indicate that in the steady state I_p is mainly determined by the passive influx of Na into the cell, which may be relatively insensitive to small changes in $[K]_o$.

5. The effects of $[K]_o$ on the drug-induced current change are consistent with competitive inhibition of the binding of DHO to the Na–K pump. It is suggested that K ions and cardiac glycosides compete for extracellular binding sites on the same conformation of the Na–K pump.

6. The external Na concentration ($[Na]_o$) was varied between 147 and 49 mM (substituted by choline or Tris). Reduction of $[Na]_o$ produced a proportional decrease of I_p. This may be a consequence of the accompanying reduction of passive Na influx and the resulting decrease in intracellular Na activity (a^i_{Na}).

7. Reduction of $[Na]_o$ markedly increased K_D. This effect may be mediated by competition between Na and K at the K-loading sites of the pump and/or by separate modulatory Na-binding sites.

8. It is concluded that the well known effects of external Na and K on the positive inotropic action of cardiac glycosides can be fully accounted for by the marked changes in the apparent binding affinity of the drug reported here.

INTRODUCTION

The positive inotropic effect of cardiotonic steroids on cardiac muscle is reduced by external K ions (Reiter, Stickel & Weber, 1966) and enhanced by external Na ions (Reiter, 1963). The molecular mechanisms underlying these effects are not yet clear. On one hand it has been suggested that these effects may be due to a change in intracellular sodium activity (a^{i}_{Na}) following the change in $[Na]_o$ or $[K]_o$ (Reiter, 1981). This interpretation was based on the notion that excitation–contraction coupling in cardiac muscle and the positive inotropic action of cardiac glycosides may be linked to a^{i}_{Na} through the Na–Ca exhange system (see Mullins, 1981). On the other hand the binding of ouabain to the Na–K pump was found to be inhibited by extracellular K and promoted by extracellular Na in squid axon (Baker & Manil, 1968; Baker & Willis, 1972*b*), erythrocytes (Schatzmann, 1953, 1965; Glynn, 1957; Gardner & Conlon, 1972; Sachs, 1974; Hobbs & Dunham, 1978; Furukawa, Bioezikian & Loeb, 1980) and other mammalian cells (Baker & Willis, 1970, 1972*a*). Similarly, ouabain binding to isolated Na–K-ATPase preparations was found to be modified by Na and K ions (Schwartz, Matsui & Laughter, 1968; Schwartz, Lindenmayer & Allen, 1975; Akera, Temma, Wiest & Brody, 1978). It has therefore been suggested that such effects may also modulate the action of cardiac glycosides in intact cardiac muscle (Akera *et al.* 1978; Akera, Hirai & Oka, 1979).

The aim of the experiments reported here was to find out to what extent a change in binding affinity can account for the effects of Na and K on the positive inotropic action of cardiac glycosides. Using the voltage-clamp technique described previously (Daut, 1982*c*) the inhibition of the electrogenic pump current in guinea-pig ventricular muscle by a fast-acting cardiac glycoside, dihydro-ouabain (DHO), was measured at various external Na and K concentrations. During short-lasting ($\sim$ 1 min) application of DHO the magnitude of this 'drug-induced current change' (I_D) appears to depend on the number of DHO molecules bound to the Na–K pump (Daut & Rüdel, 1981, 1982*b*). This measurement of binding is indirect, but it has the advantage that only specific binding to the intact cell, associated with inhibition of the Na–K pump, is recorded.

By evaluating the concentration-dependence of I_D with a Scatchard-type plot the two factors which may be modulated by $[Na]_o$ and $[K]_o$ were determined simultaneously: (i) the density of the electrogenic pump current flowing in the steady state, which reflects the turnover rate of individual pump molecules, and (ii) the apparent binding affinity of DHO. The simultaneous measurement of these two parameters is useful because it is not known whether they are interdependent (see Bodeman & Hoffman, 1976) or whether they may be influenced differently (and independently) by the change in $[Na]_o$ or $[K]_o$ and by the resulting change in a^{i}_{Na}.

The results presented in this paper suggest that a change in the binding affinity of the drug may play an important role in the modulation of the therapeutic and toxic effects of cardiac glycosides by external Na and K ions. A preliminary communication of this work has been published (Daut, 1982*a*).

METHODS

Voltage-clamp experiments were carried out with cylindrical strands of guinea-pig ventricular muscle (diameter 90–250 μm; length 700–1400 μm) using the technique described previously (Daut, 1982*c*; Daut & Rüdel, 1982*b*). Voltage-clamp pulses (500–1000 ms duration) to the resting potential were applied at intervals of 5–10 s. The preparations were superfused with different concentrations of dihydro-ouabain (DHO) for 0·5–2 min. Each application of the drug was followed by a wash-out period of at least 10 min. The results reported here are based on a relatively small number of experiments ($n = 14$) in which dose–response curves of the effects of DHO on the transmembrane current were recorded both in the modified Tyrode solution described below and in a solution containing a different concentration of K or Na. However, similar results were obtained in a large number of partially successful experiments. The modified Tyrode solution contained (mM): NaCl, 117; KCl, 3; $CaCl_2$, 2; $MgSO_4$, 1; Na pyruvate, 2; $NaHCO_3$, 26·2; NaH_2PO_4, 1·67; glucose, 10. The K concentration was varied by substituting NaCl for KCl in equimolar amounts. The Na concentration was varied by substituting isotonic choline Cl or Tris/Tris HCl for NaCl. The solutions were gassed with 95% O_2 and 5% CO_2 (pH 7·4). The temperature was 35–37 °C and was held constant within 0·3 °C during an individual experiment.

RESULTS

The effects of external K on the drug-induced current change

The effect of DHO on membrane potential and transmembrane currents in resting guinea-pig ventricular muscle were studied as described previously (Daut, 1982*c*; Daut & Rüdel, 1982*b*). Fig. 1 shows the depolarization produced in a cylindrical preparation of 180 μm diameter by superfusion with various concentrations of DHO (lower trace of each panel), at two different levels of external K. Voltage-clamp pulses (900 ms duration) to the resting potential were applied at intervals of 9 s. The change of the clamp current recorded during application and wash-out of the drug is shown in the upper trace of each panel. This 'drug-induced current change' (I_D) was suggested to represent the electrogenic pump current which is blocked by DHO and to be proportional to the number of drug molecules bound to the Na–K pump (Daut & Rüdel, 1981, 1982*b*).

When the K potassium concentration of the bathing Tyrode solution was increased from 3 to 4·5 mM a smaller I_D was observed with the same concentration of DHO (panels *B* and *D*). However, by increasing the drug concentration (*D*) in the presence of 4·5 mM-K current changes similiar to those measured in 3 mM-K could be produced (compare panels *A* and *D*, *B* and *E*, *C* and *F*). The results obtained in this experiment were evaluated using the modified Scatchard plot shown in Fig. 2. The data were fitted with straight lines by linear regression (correlation coefficient $< -0·95$). It was found that the point of intersection with the abscissa was shifted very little by the change in $[K]_o$, whereas the slope of the two lines differed by a factor of about two. Very similar results were obtained in four further experiments in which it was possible to obtain complete dose–response curves of I_D at two different external K concentrations (3 mM and 2 or 4·5 mM) in the same preparation. The data always well approximated by a straight line. This suggests that not only with 3 mM-K but also with other external K concentrations the measured I_D may be proportional to the number of DHO molecules bound.

This interpretation is based on the following assumptions: (1) The binding of one DHO molecule to an extracellular binding site leads to the blockage of one Na–K-ATPase molecule, (2) the concentration of the drug is large compared to the

concentration of the receptor, (3) the population of pump molecules is homogeneous, i.e. the average turnover rate is the same (apart from an unimodal statistical scatter) for all pump sites, and (4) the change in a^{i}_{Na} is slow compared to the equilibration of the drug with the receptor. The validity of these assumptions has been discussed previously (Daut & Rüdel, 1982*b*). The last assumption (4) is the most crucial one, and in some experiments intracellular Na accumulation did in fact cause a small decrease of I_D during prolonged drug application and a small 'after-hyperpolarization'

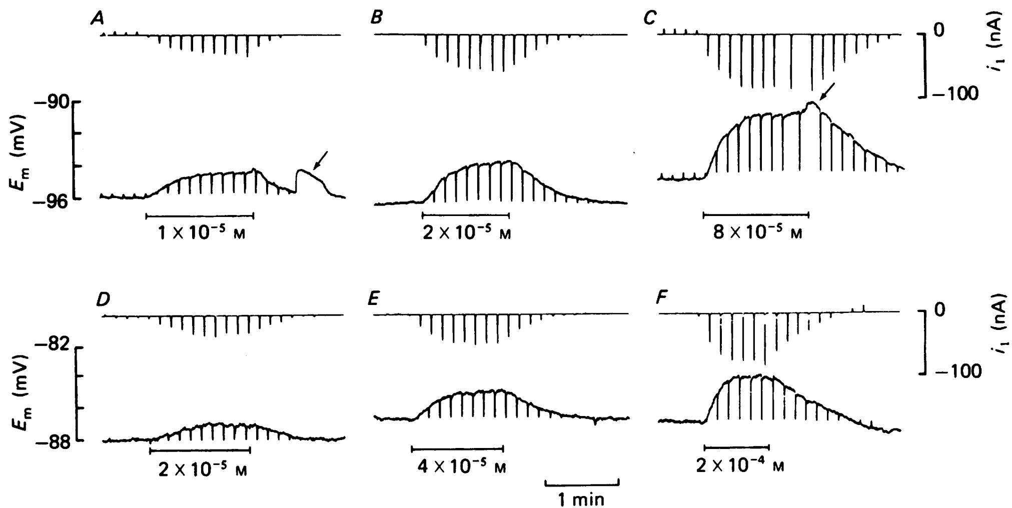

Fig. 1. The drug-induced current change measured in 3 mM-K (panels *A*, *B*, *C*) and 4·5 mM-K (panels *D*, *E*, *F*) in the same preparation. The duration of the voltage-clamp pulses was 900 ms. i_1 (upper traces) denotes the voltage-clamp current measured at the input of the current pump. In panels *A* and *D* there were small artifacts during the wash-out of the drug (arrows) caused by air bubbles near the reference electrode.

(see Daut, 1982*d*) during the wash-out of DHO. To minimize these artifacts the wash-out of the drug was usually started as soon as the current change had reached a plateau.

The magnitude of intracellular Na accumulation was estimated from the time integral of I_D using the parameters determined previously (Daut, 1982*d*; Daut & Rüdel, 1982*a*, *b*). The expected error in the current measurement was then calculated from the relative increase in a^{i}_{Na} and the fraction of pump molecules blocked by DHO (r), since only the fraction of pump molecules not blocked ($1-r$) can be stimulated by the rise in a^{i}_{Na}. The value of r was obtained from the ratio between I_D and the current change extrapolated for saturating drug concentrations, I_p (see below). It was found that under the present experimental conditions the error in the determination of the steady-state binding of DHO should be always less than 10%. The error should also be rather concentration-independent, because with high drug concentrations the number of unoccupied pump sites is smaller and binding equilibrium is reached faster, which allows the duration of drug application to be reduced. Therefore it appears that the conclusions drawn from the measurements reported here are not affected by the small but unavoidable distortion of the current records produced by intracellular Na accumulation.

The intersection of the straight lines in Fig. 2 with the abscissa represents the current change extrapolated for saturating drug concentrations, i.e. the total electrogenic pump current (I_p). This remained remarkably constant when $[K]_o$ was changed from 3 mM to 2 or 4·5 mM. The difference in the value of I_p obtained at two different external K concentrations in the same preparation was always less than 10%. Such differences are thought to be within the limits of experimental error. Moreover, the small changes in I_p recorded in successive dose–response curves were

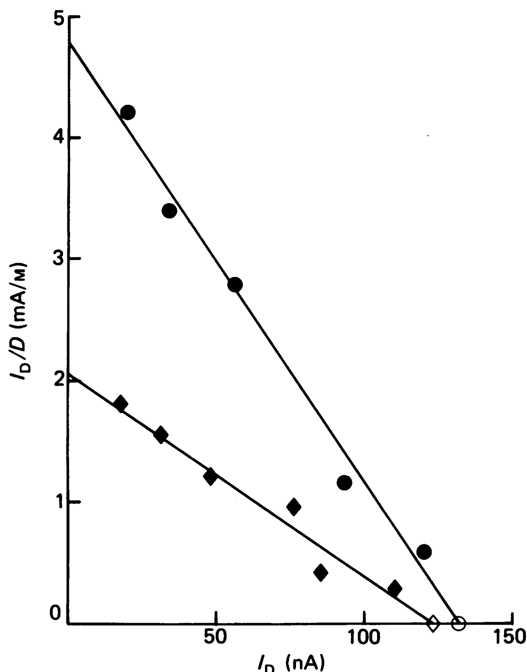

Fig. 2. Scatchard-type plot of the steady-state current changes (I_D) measured with various concentrations (D) of DHO. I_D corresponds to the amount of drug bound to the Na–K pump (abscissa); I_D/D corresponds to the ratio of bound to free drug concentration (ordinate); ●, 3 mM-external K; ◆, 4·5 mM-external K. The intersection of the linear regression lines with the abscissa (I_p) is indicated by open symbols. The data are from the same experiment as the records shown in Fig. 1.

not correlated with the direction in which $[K]_o$ was changed. These findings suggest that a change of $[K]_o$ between 2 and 4·5 mM does not appreciably change the electrogenic pump current flowing in quiescent guinea-pig ventricular muscle.

The slope of the straight lines obtained in the Scatchard plot equals $-1/K_D$, K_D being the equilibrium dissociation constant of the binding of DHO to the Na–K pump. It was found that K_D increased by a factor of nearly 2 when $[K]_o$ was changed from 2 to 3 mM or from 3 to 4·5 mM. Since the experiments shown in Fig. 1 and 2 required more than 2 h of stable recording a faster method was used to get a larger statistical sample of the function relating K_D to $[K]_o$. The method was simply to superfuse a preparation cumulatively with two convenient concentrations of DHO.

Fig. 3 shows a typical experiment in which K_D was determined in this way, first in 4·5 mM-K (*A*), and 10 min later in 3 mM-K (*B*). In both runs the preparation was

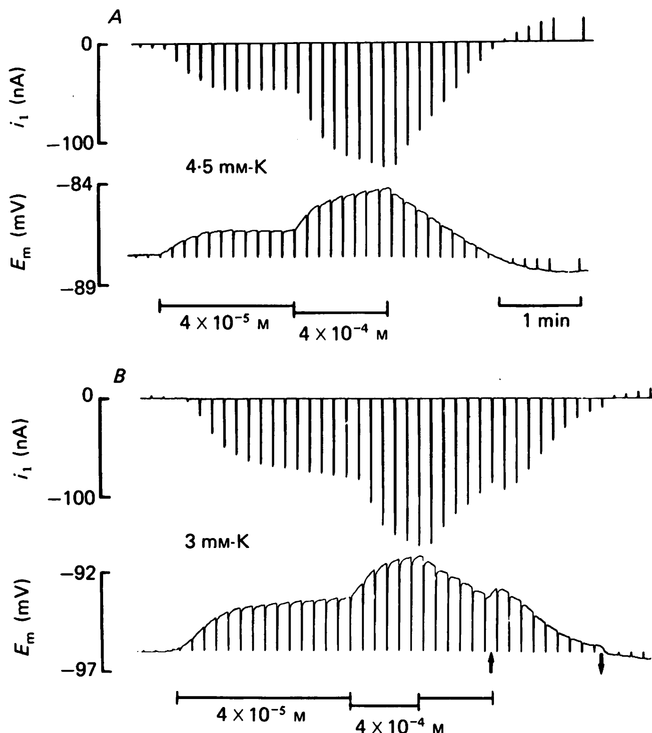

Fig. 3. The drug-induced current change measured during cumulative application of 4×10^{-5} and 4×10^{-4} M-DHO. *A*, in 4·5 mM-K. *B*, in 3 mM-K. A small, reversible potential shift of the reference electrode ($\simeq 400\ \mu V$) is indicated by arrows.

superfused first with 4×10^{-5} M and then with 4×10^{-4} M-DHO. It can be seen that the ratio of the currents induced by the two drug concentrations was larger in 4·5 mM-K. K_D was calculated from the steady-state pump currents (I_{D_1} and I_{D_2}) measured with the two different DHO concentrations (D_1 and D_2). Since the concentration dependence of I_D is given by the equation

$$I_D = I_p \frac{D}{D+K_D} \tag{1}$$

(Daut & Rüdel, 1982*b*) it follows that

$$K_D = \frac{I_{D_1} - I_{D_2}}{I_{D_1}/D_1 - I_{D_2}/D_2}. \tag{2}$$

Thus the equilibrium dissociation constant at a particular external K concentration can be calculated from a single record as shown in Fig 3. The numerical value of K_D determined with this method is more sensitive to small errors in the measurement, but on the other hand all slow changes of the state of the preparation are avoided. The values of K_D determined with this method were in good agreement with the values determined from the slope of the Scatchard plots. They are plotted in Fig. 5, together with the data obtained by a third method, which is described below.

The influence of external K on the time course of the drug-induced current change

The finding that the total electrogenic pump current was independent of $[K]_o$ whereas K_D was strongly dependent on $[K]_o$ is suggestive of competitive inhibition by $[K]_o$ of the binding of DHO to the Na–K pump. For competitive inhibition one would expect the binding rate constant of the drug to decrease with increasing $[K]_o$ whereas the rate constant of unbinding should be independent of $[K]_o$.

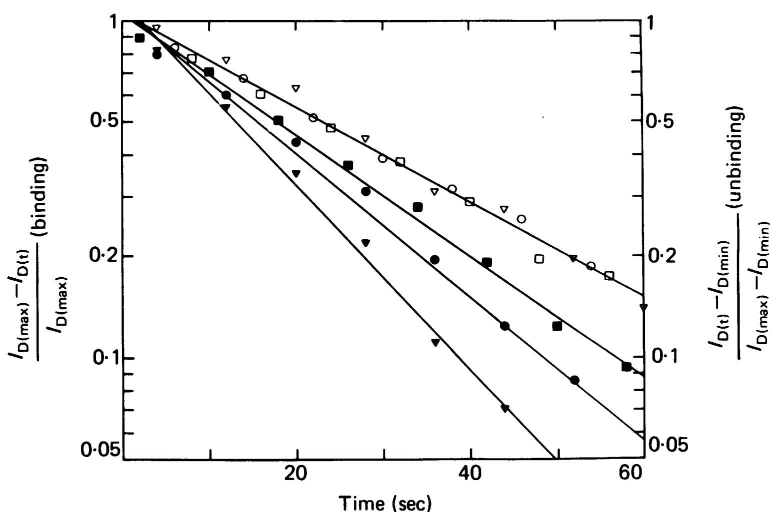

Fig. 4. Normalized, semi-logarithmic plot of the time course of the drug-induced change measured with 5×10^{-5} M-DHO. The filled symbols denote the onset of the current change, the open symbols denote the decay. ▼, 2 mM; ●, 3 mM; ■, 4 mM-external K. For calculating the linear regression lines, some points recorded at later times were also included. The correlation co-efficients were $< -0{\cdot}99$.

Fig. 4 shows that this was indeed the case. In this experiment the time course of the current change ($I_{D(t)}$) with 5×10^{-5} M-DHO was recorded in 2 (▼), 3 (●) and 4 (■) mM-K and plotted on a semi-logarithmic scale. The onset of $I_{D(t)}$ was normalized with respect to the I_D recorded in the steady state ($I_{D(max)}$) during application of the drug, so the ordinate gives $[(I_{D(max)} - I_{D(t)})/I_{D(max)}]$. The data of the three drug applications were fitted with three straight lines obtained by linear regression. The time constants (τ_{on}) given by these lines were 15·9 s (2 mM-K), 20·1 s (3 mM-K) and 24·2 s (4 mM-K).

For the $I_{D(t)}$ recorded during the wash-out of DHO the current measured during the peak of the after-hyperpolarization ($I_{D(min)}$) was taken as the reference level (see Daut & Rüdel, 1982*b*; Daut, 1982*d*). The currents recorded during the wash-out ($I_{D(t)} - I_{D(min)}$) were normalized with respect to the maximal current change recorded during the wash-out ($I_{D(max)} - I_{D(min)}$), so the ordinate gives $[(I_{D(t)} - I_{D(min)})/(I_{D(max)} - I_{D(min)})]$. Plotted in this way the three wash-out curves virtually coincide (open symbols). The continuous line was fitted to the lumped data of all three wash-out curves. The time constant of the decay of I_D (τ_{off}) was 30·7 s. Since

$1/\tau_{on} = (1+D/K_D)(-k_2t)$ and $1/\tau_{off} = -k_2t$ (see Daut & Rüdel, 1982*b*) K_D can be calculated from the equation

$$K_D = \frac{D}{\frac{\tau_{off}}{\tau_{on}} - 1}. \qquad (3)$$

Thus the analysis of the time course of the drug-induced current change represents another method of determining the function relating K_D to $[K]_o$. In thin preparations

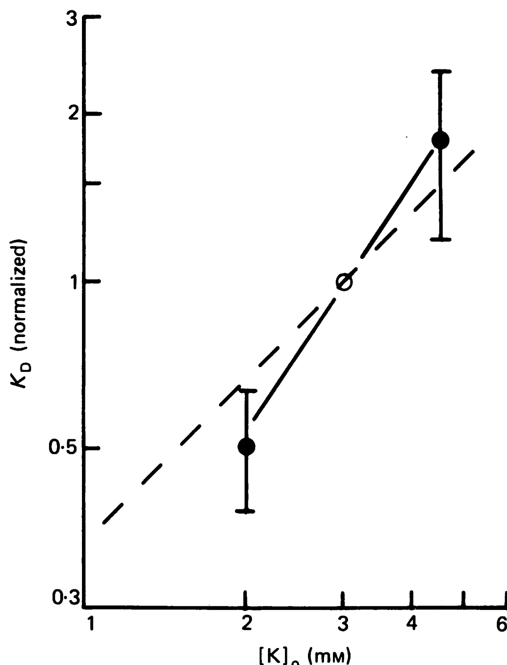

Fig. 5. Normalized, double-logarithmic plot of the equilibrium dissociation constant of DHO (K_D) against $[K]_o$. In each experiment a value of K_D was obtained in 3 mM-K (= 1) and in 2 or 4 mM-K. The filled circles indicate the mean of five experiments each, the vertical bars denote the standard deviation. The continuous line was drawn by eye. The dashed line has a slope of 1.

(diameter < 150 μm), where the diffusional delays are very small, the values of K_D calculated from equation (3) were in good agreement with the values calculated by the two other methods described above.

The data obtained with the three methods on the relative change of K_D produced by a change in $[K]_o$ are shown as a log–log plot in Fig. 5. The vertical bars indicate the standard deviation. The slope of the line drawn through the experimental points was 1·5. The possible implications of this finding for the molecular mechanism of the interaction between K and cardiac glycosides are discussed below.

The effects of external Na on the drug-induced current change

The replacement of external Na ions by other cations (choline or Tris) also had very pronounced effects on the change in voltage-clamp current measured during application of DHO. Fig. 6 shows records from an experiment in which dose–response

curves of I_D were obtained at three different external Na concentrations, 147 mM (*A* and *B*), 98 mM (*C* and *D*), and 49 mM (*E* and *F*). It can be seen that the I_D recorded with the same concentration of DHO was drastically reduced after lowering $[Na]_o$ (compare panels *B* and *C*, or *D* and *E*).

In contrast to the results obtained when increasing $[K]_o$ this reduction of I_D could not be overridden by increasing the concentration of DHO (compare panels *B* and *F*). The Scatchard plot of the results obtained in this preparation is shown in Fig. 7. The data could be fitted with straight lines which suggests that the one-to-one

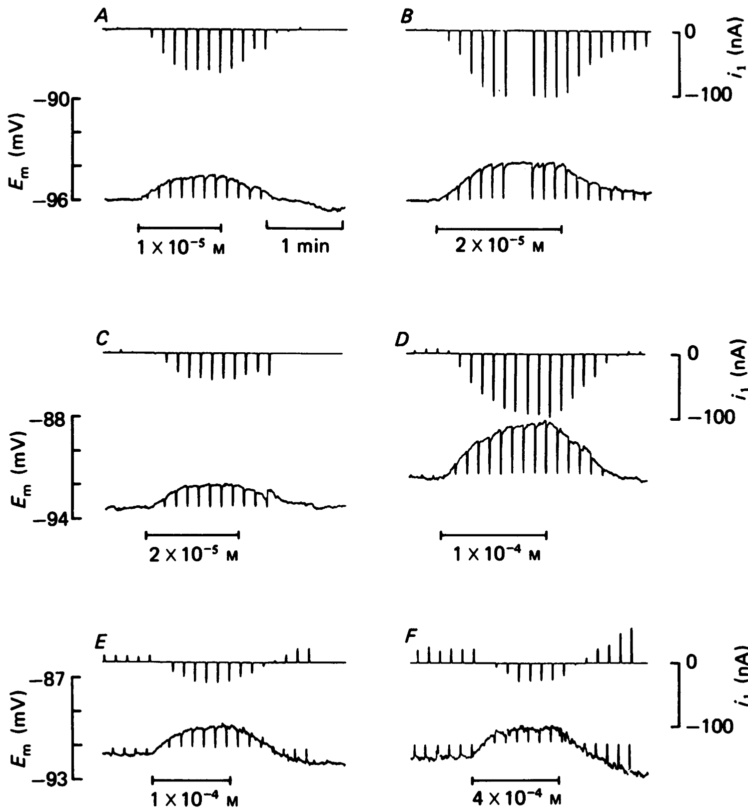

Fig. 6. The drug-induced current change measured in 147 mM (*A* and *B*), 98 mM (*C* and *D*) and 49 mM (*E* and *F*) external Na in the same preparation.

stoichiometry of the binding of the drug to the Na–K pump is not changed by reducing $[Na]_o$. The total electrogenic pump current flowing in the steady state at each value of $[Na]_o$ is given by the intersection of the straight lines with the abscissa (open symbols), and it can be seen that I_p was reduced after lowering $[Na]_o$. The slope of the straight lines was markedly reduced in low $[Na]_o$, i.e. the value of K_D was increased.

In Fig. 8 the effects of $[Na]_o$ on I_p and K_D observed in four preparations are summarized. Each point represents the relative magnitude of I_p or K_D measured with lowered external Na as compared with the value of I_p or K_D measured at normal $[Na]_o$ in the same preparation. The success rate of these experiments was rather low

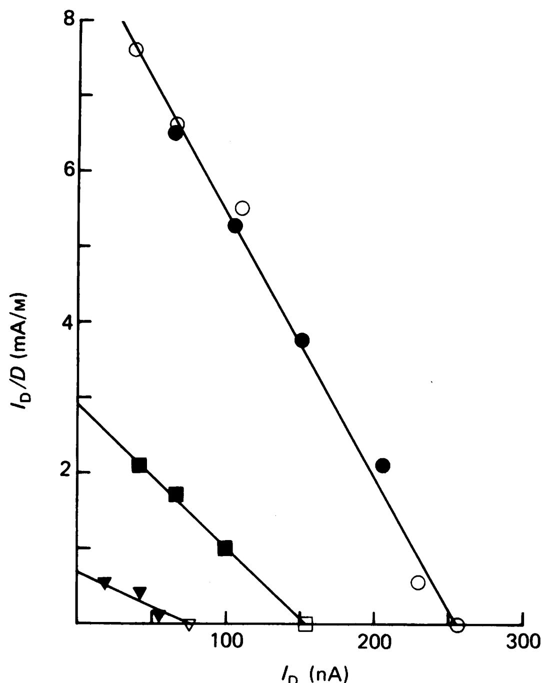

Fig. 7. Scatchard-type plot of the I_D measured in 147 mM (●), 98 mM (□), 49 mM (▼) and 147 mM (○) external Na (in that sequence). The intersections of the linear regression lines with the abscissa are indicated by open symbols.

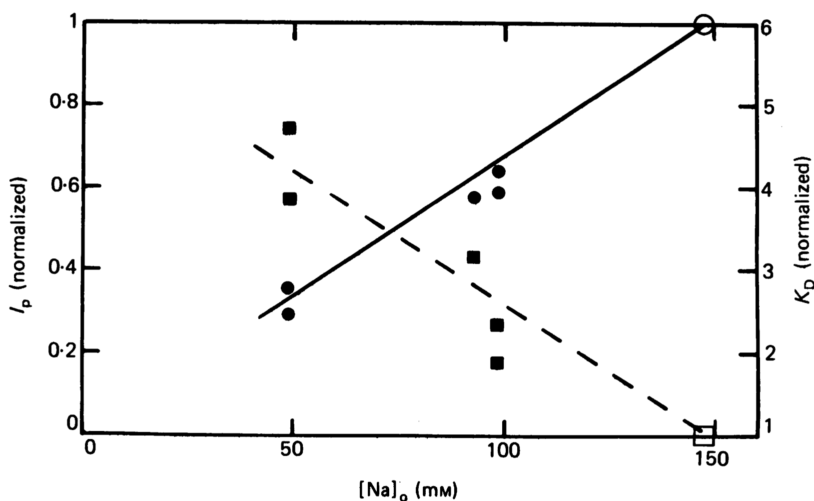

Fig. 8. The relative change of I_p (●, left-hand ordinate) and K_D (■, right-hand ordinate) recorded after a change of $[Na]_o$. The values recorded in normal $[Na]_o$ were taken as 1 (open symbols). The data were obtained from four different preparations using Scatchard-type plots as shown in Fig. 7. When $[Na]_o$ was reduced to less than $\frac{1}{3}$, I_D was very small and K_D appeared to be very large, but it is difficult to give precise figures because of the low signal-to-noise ratio of I_D in very low $[Na]_o$. The straight lines through the experimental points were drawn by eye.

because the decreased signal-to-noise ratio with lowered $[\mathrm{Na}]_o$ required extremely stable recording conditions for at least two hours. However, several partially successful experiments were carried out, the results of which are in accord with the data shown in Fig. 8.

The magnitude of I_p was found to be roughly proportional to $[\mathrm{Na}]_o$ (●). This finding is consistent with the results obtained in cardiac Purkinje fibres. It may be attributable to a linear relation between a^i_{Na} and $[\mathrm{Na}]_o$ (Ellis, 1977; Ellis & Deitmer, 1978) and between a^i_{Na} and I_p (Gadsby, 1980; Eisner Lederer & Vaughan-Jones, 1981*a*). The value of K_D derived from the Scatchard-type plots increased when $[\mathrm{Na}]_o$ was decreased (■, Fig. 9), i.e. the presence of external Na was found to promote the binding of DHO. This is in agreement with results obtained with different methods in various other preparations (see Introduction). The possible mechanisms underlying these effects are discussed below.

DISCUSSION

The effects of external K on the electrogenic pump current

The electrogenic pump current was found to be essentially unchanged when $[\mathrm{K}]_o$ was varied between 2 and 4·5 mM. This might appear to suggest that the activating effect of $[\mathrm{K}]_o$ on the Na–K pump is saturated at 2 mM-K. However, this interpretation is considered unlikely for the following reasons: (1) measurements with Na-selective micro-electrodes in guinea-pig ventricular muscle showed that changes in $[\mathrm{K}]_o$ in that range produced changes in a^i_{Na} in the opposite direction (J. Daut, unpublished; see also Ellis, 1977). (2) In erythrocytes the value of $K_{0\cdot5}$, the K concentration giving half-maximal activation of the Na–K pump, was found to be between 1 and 2 mM in the presence of external Na (Sachs & Welt, 1967; Garrahan & Glynn, 1967). The estimates given for $K_{0\cdot5}$ in mammalian cardiac tissues vary over a wide range (sheep Purkinje bundle: 10 mM, Deitmer & Ellis, 1978; 6 mM, Eisner & Lederer, 1980; 4 mM or < 1 mM, Eisner, Lederer & Vaughan-Jones, 1981*b*; guinea-pig atrium: 1·5 mM, Glitsch, Grabowski & Thielen, 1978; canine Purkinje fibre: 1 mM, Gadsby, 1980; rabbit atrio-ventricular node: 5–6 mM, Kurachi, Noma & Irisawa, 1981). The reason for this divergence may be that K depletion in the extracellular space of sheep Purkinje fibres during re-activation of the Na–K pump leads to an over-estimate of $K_{0\cdot5}$ (see Eisner *et al.* 1981*b*). But even if the lowest values of $K_{0\cdot5}$ are correct one would still expect some activation of the Na–K pump to occur in the range 2–4·5 mM-K (Gadsby, 1980).

A more plausible interpretation of the present results is that the magnitude of the electrogenic pump current may be mainly determined by the passive sodium influx (Cohen, Daut & Noble, 1976; Gadsby, 1980), because in the steady state at the resting potential Na extrusion via the Na–K pump equals the passive Na influx (Daut & Rüdel, 1982*b*). Thus activation of the Na–K pump by elevation of external K may produce a compensatory decrease in a^i_{Na} (and vice versa) rather than a change in pump current. The Na permeability of the cell membrane appears to be relatively insensitive to changes in $[\mathrm{K}]_o$ (Gadsby, 1980; Eisner *et al.* 1981*b*). Therefore variation of $[\mathrm{K}]_o$ may leave the passive Na influx essentially unchanged (if the concomitant change in E_m is not too large). This may explain the invariance of I_p measured after

changing $[K]_o$ from 3 mM to 2 or 4·5 mM, regardless of the possible activating effect of K ions on the Na–K pump. This hypothesis is also in accord with the results obtained by Bosteels & Carmeliet (1972) in cow Purkinje fibres. They found that increasing external K produced only a transient increase in ^{22}Na efflux (for 4–5 min), whereas the rate constant of ^{22}Na efflux measured in the steady state was relatively insensitive to variation of $[K]_o$ between 0·54 and 16·2 mM.

It may be worth noting that analogous considerations apply for the effects of low concentrations of cardiac glycosides. After partial inhibition of the Na–K pump the adaptation of a^i_{Na} to a new level takes only a few minutes in cardiac tissues (Eisner *et al.* 1981*b*; Daut, 1982*b*, *d*). Therefore the ionic fluxes measured at later times (e.g. the Na efflux and the K or Rb influx) may be quite insensitive to low concentrations of cardiac glycosides. Any changes in membrane properties measured under these conditions may be secondary consequences of intracellular Na or extracellular K accumulation (Cohen *et al.* 1976).

The effects of external K on the binding of DHO

It has been suggested in a previous paper (Daut & Rüdel, 1982*b*) that the drug-induced current change may be proportional to the number of DHO molecules bound to the Na–K pump and that the time course of the current change may reflect the chemical kinetics of the binding and unbinding of the drug. This hypothesis is confirmed by the present results, in particular by the absence of curvature in the Scatchard plots obtained in 2 and 4·5 mM-K (Fig. 2) and by the good agreement of the values of K_D calculated from the steady-state current changes (Fig. 2) and from the time constants of the onset and decay of I_D (Fig. 4).

In the concentration range explored here the interaction between external K ions and the binding of DHO may be adequately described by competitive inhibition. This interpretation is based on the following effects of $[K]_o$ on I_D: (1) the extrapolated binding of DHO at infinite concentrations (which corresponds to I_p) was essentially unchanged when $[K]_o$ was varied in the range 2–4·5 mM (Fig. 2). (2) K_D increased with increasing $[K]_o$ (Fig. 5). (3) The time constant of the onset of I_D (binding) increased with increasing $[K]_o$ (Fig. 4). (4) The time constant of the decay of I_D (unbinding) was independent of $[K]_o$ (Fig. 4).

The term competitive inhibition is used in a wider sense here, it should only be taken to indicate that DHO and K ions bind to the same conformer of the Na–K-ATPase and that the binding site is no longer available for association with the drug when it is occupied by K. This does not necessarily imply that K and DHO bind to the same or overlapping binding sites. It could also indicate that association of K with the K-loading site, from which it is transported inwardly, induces a conformational change which greatly reduces the binding affinity of the enzyme for DHO.

The present results are qualitatively consistent with the effects of external K on ouabain binding in intact erythrocytes (Glynn, 1957, 1964; Furukawa *et al.* 1980) and in isolated Na–K ATPase preparations (see Schwartz *et al.* 1975; Akera *et al.* 1978). With large concentrations of ouabain, however, deviations from simple competitive inhibition have been described (Glynn, 1957; Schwartz *et al.* 1975).

In erythrocytes the slope of the relation between log K_D and log $[K]_o$ was found

to be linear with a slope of 1 for values of $[K]_o$ between 1 and 5 mM (Furukawa *et al.* 1980). As Furukawa *et al.* pointed out, this is expected for one-to-one competition of K and ouabain for a common binding site, provided that $K_{0.5}$ is small compared to $[K]_o$. In the present experiments the function relating log K_D to log $[K]_o$ was also linear, but it had a slope of about 1·5 (Fig. 5). Although there was some scatter in the data the results do not appear to be compatible with a slope of 1, and thus with simple competitive inhibition. One possible explanation is that two K ions have to be bound to equivalent sites on the Na–K pump to prevent the binding of DHO. Since $K_{0.5}$ is probably not much smaller than $[K]_o$ (see above) such a mechanism could account for a slope between 1 and 2 in Fig. 5.

However, recent work on the relations between conformational changes and ion movements suggests that such an analysis may be misleading. Fig. 9 shows a simplified version of the reaction scheme proposed by Karlish, Yates & Glynn (1978), in which the binding of cardiac glycosides has been included. Both cardiac glycosides and K ions are assumed to bind most readily to the E_2 . P form of the Na–K-ATPase at binding sites facing the external surface of the membrane (Schwartz *et al.* 1968; Albers, Koval & Siegel, 1968; Hansen, 1979). The fractional life-span of this conformational state during the pump cycle is assumed to be relatively short under physiological conditions (Karlish, 1979; Glynn & Richards, 1982). It may be increased by intracellular Na which promotes phosphorylation (Blostein & Chu, 1977) and may be decreased by extracellular K which promotes dephosphorylation (Blostein & Chu, 1977; Blostein, Pershadsingh, Drapeau & Chu, 1979). The apparent rate constant of binding of DHO (but not of unbinding) is therefore expected to depend on external K and internal Na, as can be seen from Fig. 9.

In the present experiments a decrease in $[K]_o$ and the accompanying increase in a^i_{Na} should increase the fractional life-span of the E_2 . P-form of the enzyme despite the fact that the over-all turnover rate of the Na–K pump (I_p) remained constant. Thus a decrease of $[K]_o$ should increase the apparent binding affinity of DHO more than one would predict for simple competition for a constant binding site. This suggests that the slope of 1·5 in the relation between log $[K]_o$ and log K_D (Fig. 5) can be explained on the basis of competitive inhibition of cardiac glycoside binding by external K ions at the E_2 . P-form of the Na–K pump (reactions *b* and *i*), and that the availability of this conformation may be influenced by external K (and cogeners) and internal Na (reactions *c* and *g*). This interpretation of the present results is in line with the observation that the order of efficacy with which K cogeners (i) promote dephosphorylation of the E_2 . P-form of the Na–K-ATPase (Hegyvary & Post, 1971; Post, Hegyvary & Kume, 1972), (ii) affect ouabain binding (Baker & Willis, 1972*a*; Schönfeld, Schön, Menke & Repke, 1972; Erdmann & Schoner, 1973; Hobbs & Dunham, 1978), and (iii) activate the sodium pump (Whittam & Ager, 1964; Rang & Ritchie, 1968; Eisner & Lederer, 1979, 1980; Kurachi, Noma & Irisawa, 1981) is the same, namely $Rb \simeq K < Cs \simeq NH_4 < Li$.

The effects of external Na on the electrogenic pump current and on the binding of DHO

The electrogenic pump current measured in the steady state was found to be roughly porportional to the extracellular Na concentration (Fig. 8). The reason for this may be a fall in intracellular Na activity following the reduction of $[Na]_o$ and

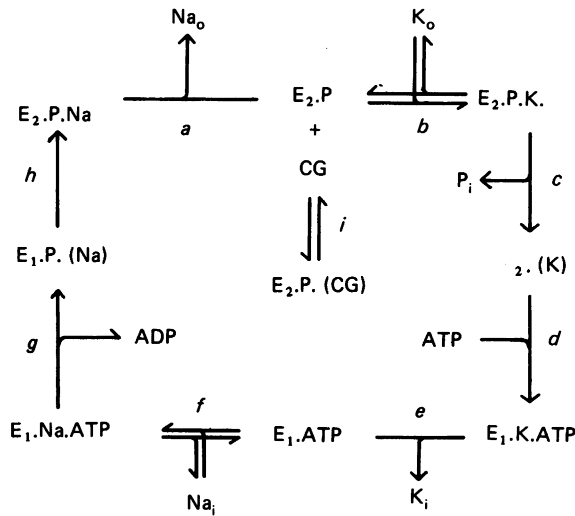

Fig. 9. Simplified version of the reaction scheme of the Na–K pump proposed by Karlish *et al.* (1978; see also Glynn, Karlish & Yates, 1979). Only the forward running mode of the Na–K pump is shown and two intermediary steps have been omitted. For simplicity only the binding of cardiac glycosides (CG) to the uncombined E_2. P-form of the Na–K-ATPase has been included. The main point is to visualize two aspects of the interaction between Na, K and cardiac glycosides. (1) Cardiac glycosides may compete with K ions (and K-cogeners) for the E_2. P-conformation of the Na–K-ATPase. Since the binding and unbinding of K is probably extremely fast (Glynn & Richards, 1982) the apparent binding affinity of cardiac glycosides may depend on the equilibrium dissociation constant of reaction *b*. (2) Na_i and K_o may influence the fractional life-span of the E_2. P conformation by shifting the equilibrium between the E_1 and E_2 forms of the enzyme; this would also modify the apparent binding affinity of cardiac glycosides. Similar models of cardiac glycoside binding have been suggested previously (Barnett, 1970; Lindenmayer & Schwartz, 1973; Inagaki, Lindenmayer & Schwartz, 1974; Sachs, 1974; Kropp & Sachs, 1977; Hobbs & Dunham, 1978). The main difference between the various schemes concerns the form of the phosphoenzyme to which cardiac glycosides are assumed to bind. Lindenmayer & Schwartz (1973) proposed that the affinity of cardiac glycosides may be determined by competition between Na and K at the K-loading site. This implies that cardiac glycosides can bind to the K-loading site when it is combined with Na (Inagaki *et al.* 1974; Schwartz *et al.* 1975). Kropp & Sachs (1977) made the assumption that cardiac glycosides are bound with equal affinity to all E_2. P forms free of K (with or without Na). Hobbs & Dunham (1978) assumed that cardiac glycosides bind mainly to the E_2. P. Na and E_2. P. K forms of the Na–K pump, because the fractional life-time of the uncombined phosphoenzyme (E_2. P) might be extremely small. They hypothesized that the apparent binding affinity of cardiac glycosides is mainly determined by the species of alkali metal ions bound to both the K-loading sites and the Na-discharge sites. The results of Furukawa *et al.* (1980) are compatible with simple one-to-one competition between ouabain and K for a common binding site, the fractional life-span of which is constant.

a proportionality between a^i_{Na} and I_p, as found in Purkinje bundles (Ellis, 1977; Eisner *et al.* 1981*a*). Such a mechanism would imply that the replacement of $[Na]_o$ by impermeant cations does not influence Na permeability and that the resulting fall in I_p is ultimately a consequence of a corresponding reduction in passive Na influx.

The equilibrium dissociation constant of the binding of DHO was found to be

inversely related to $[Na]_o$ (Fig. 8). This is consistent with some results obtained in cardiac muscle with different methods. By measuring the contractility of isolated perfused guinea-pig hearts under various ionic conditions Akera *et al.* (1979) obtained indirect evidence that the binding of ouabain to its receptor requires the presence of external Na. Dutta & Marks (1969) found that lowering external Na reduced the rate of uptake of [^{3}H] ouabain in guinea-pig hearts. This may also be related to the effect of $[Na]_o$ on ouabain binding to extracellular sites (cf. Akera, Olgaard, Temma & Brody, 1977). A reduction of the binding of [^{3}H]digoxin to dog heart during Na depletion *in vivo* has also been reported (Harrison & Wakim, 1969).

On the whole the properties of the electrogenic Na pump in mammalian cardiac muscle inferred from the present experiments appear to be similar to the properties of the Na–K-ATPase in erythrocytes and other preparations (see Introduction). This suggests that there are at least four possible molecular mechanisms by which the effects of $[Na]_o$ on the binding of DHO may be mediated. (1) The decrease of a^i_{Na} following the reduction of $[Na]_o$ may reduce the amount of phosphorylated enzyme present at any time (Blostein & Chu, 1977; Akera *et al.* 1977) and thus reduce the apparent binding affinity of cardiac glycosides as described above (Fig. 9). (2) External Na may compete with K at the K-loading site and thus counteract the inhibitory effects of external K on the binding of cardiac glycosides (Beaugé & Adragna, 1971; Lindenmayer & Schwartz, 1973; Hobbs & Dunham, 1978; see also Sachs, 1977). (3) External Na may act as an allosteric modifier at a binding site with relatively low affinity (Gardner & Conlon, 1972; Hobbs & Dunham, 1978; Furukawa *et al.* 1980). (4) The modulatory extracellular Na binding site may be the site from which Na is discharged after translocation (Hobbs & Dunham, 1978). This would imply that cardiac glycosides bind preferentially to the $E_2 . P . Na$ conformation of the Na–K pump. Of course the four hypothetical mechanisms mentioned are not mutally exclusive, and it is quite likely that at least two of these contribute to the relation between $[Na]_o$ and K_D shown in Fig. 8.

The modulation of the therapeutic and toxic effects of cardiac glycosides by extracellular K and Na

The concentrations of DHO and ouabain producing a half-maximal positive inotropic effect in guinea-pig ventricular muscle were found to be $1{\cdot}3 \times 10^{-5}$ M and $3{\cdot}3 \times 10^{-7}$ M, respectively, at a stimulation frequency of 1 Hz in Tyrode solution containing 4·7 mM-K (Reiter, 1967; Ebner & Reiter, 1977). From the present results (Fig. 5) and earlier work (Daut & Rüdel, 1982*b*) it appears that equilibrium dissociation constants of DHO and ouabain in 4·5 mM-K should be about 8×10^{-5} M and $2{\cdot}7 \times 10^{-6}$ M, respectively, in quiescent guinea-pig ventricular muscle. Recently, Marban & Tsien (1982) obtained some evidence that the binding of cardiac glycosides may in fact be independent of stimulation frequency and that the frequency dependence of the positive inotropic action of cardiac glycosides may be related to a transient after-effect of stimulation (see also Ebner & Reiter, 1977). Thus it may be that the values of K_D given above also apply for a stimulation frequency of 1 Hz. If this is so one would expect a half-maximal positive inotropic effect to be associated with a blockage of about 10–20 % of the pump molecules (eqn. (1)).

An antagonism between K ions and the therapeutic and toxic effects of cardioactive

steroids has been observed *in vivo* (Marcus, Nimmo, Kapadia & Goldsmith, 1971; Goldman, Coltart, Schweizer, Snidow & Harrison, 1975) and *in vitro* (Müller, 1963; Reiter, *et al.* 1966; Reiter, 1981). In a careful study of the effects of acetylstrophanthidin on isometric tension in cat papillary muscle Lee, Zelis & Mason (1977) found that the dose–response curve was shifted to the right by increasing $[K]_o$ from 3·5 to 7 mM, whereas the maximal increase in peak tension was unchanged. The rate of development of the positive inotropic effect of digoxin on cat papillary muscle was found to be inversely related to $[K]_o$ (Prindle, Skelton, Epstein & Marcus, 1971). These findings can be fully accounted for by the effect of external K on the binding of the drug described here. Thus the well known influence of K ions on the positive inotropic effect of cardiac glycosides may be largely attributable to a change in the apparent binding affinity. Nevertheless, it cannot be excluded that additional indirect effects of external K, e.g. on a^i_{Na} or on membrane potential (Reiter, 1981), may also play a role in modulating the effects of cardiotonic steroids.

It is well known that the effects of cardiac glycosides on mammalian ventricular muscle are diminished after lowering external Na (Reiter, 1963; Caprio & Farah, 1967; Akera *et al.* 1977). The experimental studies relevant to the mechanism of this Na dependence have been reviewed recently by Reiter (1981). In keeping with the notion that the positive inotropic action of cardiac glycosides may be related to a rise in intracellular Na (Wilbrandt, 1958; Glynn, 1964; Repke, 1964; Langer, 1968), he concluded that the reduction of the positive inotropic effect of cardiac glycosides in low $[Na]_o$ may be primarily due to the influence of the concomitant fall in a^i_{Na} on excitation–contraction coupling. Similar conclusions were reached in two recent studies of the sodium dependence of the effects of cardiac glycosides in cat ventricular muscle (Wiggins & Bentolila, 1980) and guinea-pig atrium (Linden & Brooker, 1980).

In contrast, Akera *et al.* (1977) proposed that it is the effect of a^i_{Na} on the binding affinity of cardiac glycosides which modifies the action of cardiac glycosides. The present experiments also suggest that in low $[Na]_o$ there is a marked reduction of the binding of cardiac glycosides to the Na–K pump. From the slope of the relation between K_D and $[Na]_o$ (Fig. 8) it appears that the change in binding affinity may in fact be the major factor determining the sodium dependence of the positive inotropic action of cardiac glycosides. However, it is not yet clear whether this effect is mediated by intracellular or extracellular Na binding sites or both.

Finally, the present results may help to clarify the mechanisms of the therapeutic and toxic actions of digitalis. The close resemblance of the effects of $[Na]_o$ and $[K]_o$ on the positive inotropic effect of DHO on one hand and on the binding of the drug inferred from the inhibition of the pump current on the other hand supports the idea that in guinea-pig ventricular muscle the positive inotropic effect of DHO may be mediated by inhibition of the Na–K pump (see also Akera *et al.* 1979; Lee, Kang, Sokol & Lee, 1980; Reiter, 1981; Daut, 1982*d*).

The toxic effects of cardiac glycosides are almost certainly caused by inhibition of the Na–K pump (Lee & Klaus, 1971). It is well known that the sensitivity of the heart to the toxic effects of digitalis is decreased in elevated $[K]_o$ (Vassalle, Greenspan & Hoffman, 1963). Conversely, the development of spontaneous arrhythmic activity in canine Purkinje fibres after application of digitalis was found to be hastened after increasing $[Na]_o$ and delayed after decreasing $[Na]_o$ (Lin & Vassalle, 1978). Therefore

it appears likely that the toxic effects of cardiac glycosides observed clinically may be precipitated not only by decreased plasma K but also by elevated plasma Na concentration, because in either case the fraction of pump molecules blocked by a particular dose of digitalis may be increased.

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