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THE INTERACTIONS OF POTASSIUM, SODIUM AND STROPHANTHIDIN DURING ACTIVE TRANSPORT OF SODIUM IONS IN FROG MUSCLE CELLS

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SUMMARY

1. After the addition of $1 \cdot 10^{-5}$ M strophanthidin to a Na^+-free medium, about 15 min are required for the K^+-stimulated Na^+ efflux from muscle fibers to diminish. The strophanthidin-sensitive Na^+-stimulated Na^+ efflux is diminished immediately after the addition of $1 \cdot 10^{-5}$ M strophanthidin.

2. The half-time of inhibition by $5 \cdot 10^{-7}$ M strophanthidin is 30 min in Na^+-free, 5 mM K^+ Ringer solution, 16 min in 80 mM Na^+, 5 mM K^+ Ringer solution and 9 min in K^+-free, 105 mM Na^+ Ringer solution.

3. The quantitative findings suggest that strophanthidin molecules react with two different membrane-located catalytic particles that are interconvertible and in equilibrium with one another. The equilibrium is sensitive to external K^+ and Na^+.

4. K^+-activated Na^+ extrusion in Na^+-free media follows Michaelis–Menten kinetics in the absence of strophanthidin and in the presence of submaximally inhibiting concentrations of strophanthidin.

5. The maximal stimulation of strophanthidin-sensitive Na^+ loss by K^+ is depressed by strophanthidin. The non-competitive kinetics observed indicate that K^+ and strophanthidin molecules react at different membrane sites. Also, the kinetics indicate that one strophanthidin molecule is bound per one functional catalytic site.

6. In the presence of external Na^+, activation of Na^+ extrusion by external K^+ follows kinetics with a marked sigmoidal character. The kinetics, under these conditions, remain sigmoidal when fixed submaximal concentrations of strophanthidin are present in the medium. A synergistic action of external Na^+ and strophanthidin is apparent.

7. The activation energy of K^+-activated Na^+ extrusion in muscle fibers is 22 kcal/mole and that for strophanthidin-sensitive Na^+-activated Na^+ efflux is 10.2 kcal/mole.

8. Experimental findings are interpreted on the basis of an allosteric model in which strophanthidin molecules react with two different forms of a membrane transport enzyme.

INTRODUCTION

Cardioactive steroids have been shown to act similarly on active Na^+–K^+ transport across cell membranes and on the activity of membrane (Na^+–K^+)-ATPase[1-3]. In both intact cells transporting cations and membrane-bound (Na^+–K^+)-ATPase systems, raising the K^+ concentration in the medium overcomes some of the inhibition occasioned by the presence of submaximally-inhibiting concentrations of cardioactive steroids[2-7]. The interaction between K^+ in the medium and cardioactive steroid inhibitors is non-competitive[2,5-7]. The degree of inhibition obtained with cardioactive steroids depends upon both the Na^+ and K^+ concentrations in the medium[5,6] The time required to attain a steady-state inhibition by cardioactive drugs also depends upon the ionic composition of the medium. In the squid giant axon, the half-time for inhibition by submaximal ouabain concentrations is shorter for Na^+-activated Na^+ efflux in a K^+-free medium than for K^+-activated Na^+ pumping[8].

The purpose of the present work was to study the influence of external Na^+ and K^+ on the time dependence and degree of inhibition obtained when cardioactive steroids act on the Na^+ pump in skeletal muscle cell membranes.

MATERIALS AND METHODS

Sartorius muscles from freshly sacrificed frogs, *Rana pipiens*, were used exclusively as experimental material. The detailed procedures for dissection, mounting and handling of muscles, cation analyses, and radioactive flux determination were as previously described[9,10]. Prior to the measurement of Na^+ efflux by means of $^{22}Na^+$, muscles were enriched with Na^+ as previously described[11]. Strophanthidin-sensitive Na^+ efflux was determined by previously applied methods[11].

Standard K^+-free Ringer solution was of the composition: NaCl, 120 mM, $CaCl_2$, 2 mM, Tris, 1 mM neutralized to a pH of 7.35. Solutions containing K^+ were prepared by adding KCl to the standard solution from an isosmotic KCl solution. The Na^+ concentration of Ringer solution was varied by mixing the standard solution with isosmotic Tris Ringer solution containing 2 mM $CaCl_2$. Strophanthidin obtained from Sigma Chemical Company was added to solutions at the time of use in flux experiments.

Experiments were carried out in a water bath regulated at 20.0 ± 0.1 °C.

RESULTS

Time dependence of strophanthidin inhibition

The time required to reach a steady state of sub-maximal inhibition with strophanthidin was found to depend upon the ionic composition of the medium. Fig. 1 shows that, in a Na^+-free medium with 5 mM K^+ present, the inhibition obtained with $5 \cdot 10^{-7}$ M strophanthidin requires about 50 min to reach a steady state. With the same drug concentration in K^+-free, 105 mM Na^+ Ringer solution, only 20 min were required to reach a steady state of inhibition. The time required to reach a steady-state inhibition in the presence of both external Na^+ and K^+ fell between these two values. The times required to reach 50% of the total steady-state in-

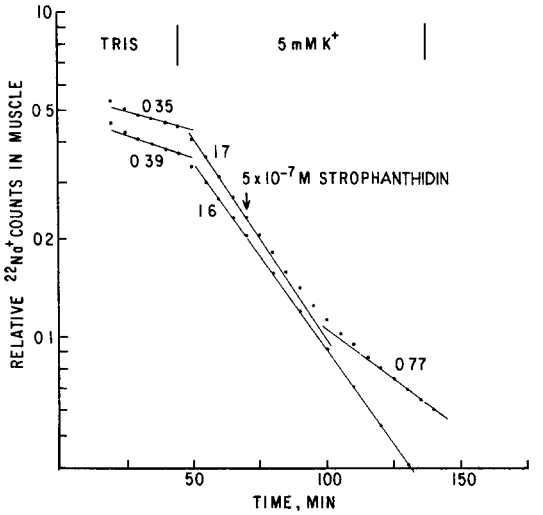

Fig. 1 The time course of the inhibition by strophanthidin in Na^+-free, 5 mM K^+ Ringer solution. The arrow indicates the time of adding strophanthidin The lower line represents a control experiment with no strophanthidin added The numbers on each curve refer to rate constants in h^{-1} units

TABLE I

HALF-TIME OF INHIBITIONS IN DIFFERENT IONIC ENVIRONMENTS

Ionic compositions		*Half-time (min)*
$[K^+]_0$ (*mM*)	$[Na^+]_0$ (*mM*)	
5	0	30 ± 4
5	80	16 ± 3
0	105	9 ± 1

hibition with $5 \cdot 10^{-7}$ M strophanthidin (half-time of inhibition) are shown in Table I for different ionic environments. Each value of the half-time is the average of 3 to 4 experimental results. The data suggest that the rate of binding of strophanthidin to muscle membrane sites at which inhibition occurs is increased by external Na^+ and decreased by external K^+. Experimental results indicate that the full steady-state inhibition occurs with no detectable delay when $1 \cdot 10^{-5}$ M strophanthidin is added to a K^+-free, 105 mM Na^+ Ringer solution in which muscles are extruding $^{22}Na^+$. When the same experiment is performed in a Na^+-free medium containing 5 mM K^+, about 15 min are required to obtain full inhibition.

The delay in the achievement of full inhibition with $5 \cdot 10^{-7}$ M strophanthidin cannot be due to a slow change in ion binding at the pump-activation sites. This was shown by performing some experiments in which the drug was present in the medium throughout the entire experiment with K^+ being added to the medium after the muscle membranes were well equilibrated with the drug. Fig. 1 shows that K^+ acts within a few minutes at the external activation sites. If external K^+ acts

similarly in the presence of $5 \cdot 10^{-7}$ M strophanthidin, this would argue against there being a slow equilibration of activation sites with external K^+ in the presence of the drug. A typical experiment is shown in Fig. 2. K^+ activates without detectable delay in the presence of the drug as well as in the absence of the drug. The results of many experiments of this type under a variety of experimental conditions indicate that the rate constants for Na^+ loss obtained when K^+ is added in the presence of the drug agree within experimental error with those measured by the method of adding the drug during the experiment with K^+ already present in the medium.

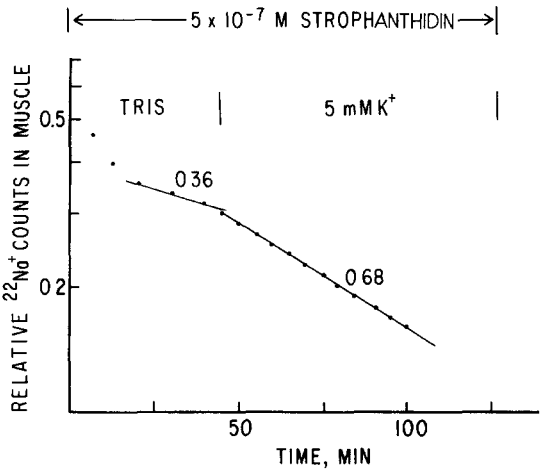

Fig. 2 The strophanthidin-sensitive Na^+ efflux rate constant shown by the change of the slopes of the Na^+ efflux rate constant upon the addition of K^+ with strophanthidin already present. The numbers above the curves refer to rate constants in h^{-1} units

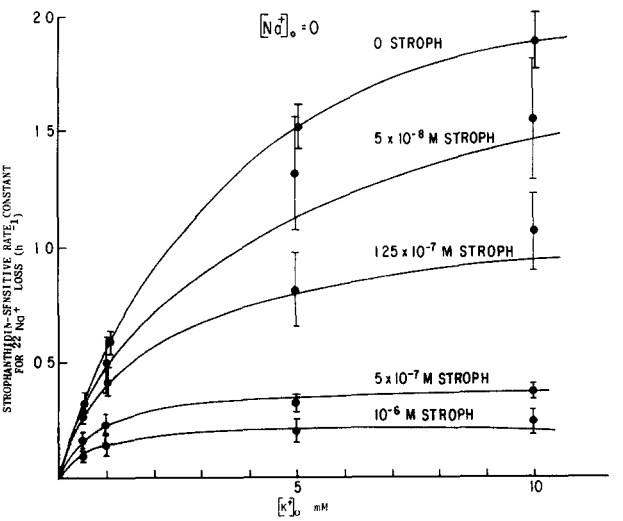

Fig 3. The strophanthidin-sensitive rate constant for Na^+ loss *versus* the external K^+ concentrations at four strophanthidin concentrations in Na^+-free Ringer solution Each point represents the average of six experimental values $\pm$ 1 S E

The kinetics of strophanthidin inhibition

The strophanthidin-sensitive rate constant for Na^+ loss from muscles to Na^+-free Ringer solution is plotted against the external K^+ concentration in Fig. 3 for different fixed strophanthidin concentrations. In the absence of the drug, the rate constant for Na^+ loss under these conditions has been shown to obey the Michaelis–Menten equation[11]. The hyperbolic shape of the curves is not changed by the drug. Increasing concentrations of strophanthidin progressively lower the maximal velocity of N^+ extrusion and also lower the Michaelis constant, K_m.

In the absence of external K^+, part of the unidirectional Na^+ efflux from muscle fibers is abolished by cardioactive steroids and probably represents an Na^+–Na^+

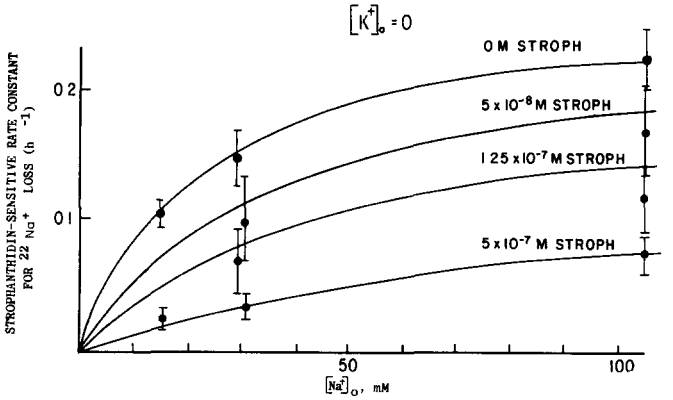

Fig 4 The strophanthidin-sensitive rate constant for Na^+ loss *versus* the external Na^+ concentrations at three strophanthidin concentrations in K^+-free Ringer solution. Each point represents the average of six experimental values ± 1 S.E.

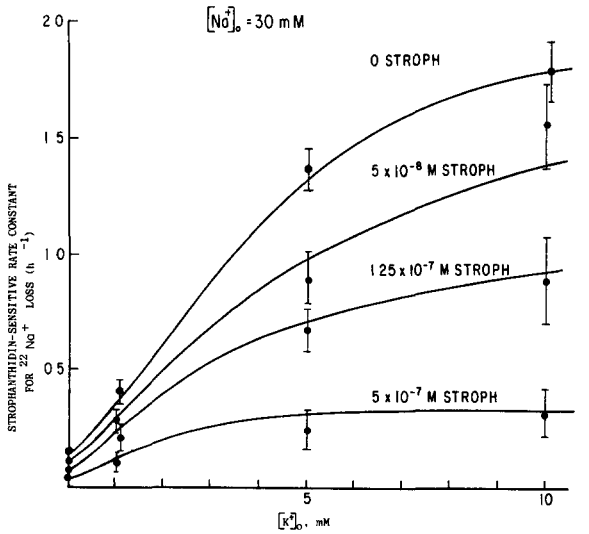

Fig. 5 The strophanthidin-sensitive rate constant for Na^+ loss *versus* the external K^+ concentrations at three strophanthidin concentrations in 30 mM Na^+ Ringer solution. Each point represents the average of six experimental values ± 1 S.E.

exchange[11,12]. The effect of varying concentrations of strophanthidin on this process was studied in K^+-free media as a function of the external Na^+ concentration. The results are shown in Fig. 4 where the strophanthidin-sensitive portion of the rate constant for Na^+ loss is plotted *versus* $[Na^+]_o$. Increasing concentrations of strophanthidin progressively depress the maximal rate of Na^+ extrusion and raise the K_m values.

K^+ activation curves were also determined in the presence of various external Na^+ concentrations and submaximally inhibiting concentrations of strophanthidin. The results with $[Na^+]_o = 30$, 80 and 120 mM are shown in Figs 5, 6 and 7, respectively.

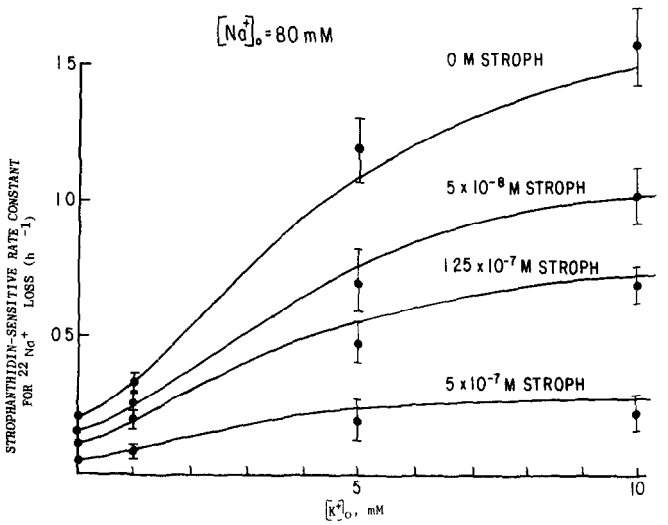

Fig. 6 The strophanthidin-sensitive rate constant for Na^+ loss *versus* the external K^+ concentrations at three strophanthidin concentrations in 80 mM Na^+ Ringer solution Each point represents the average of six experimental values $\pm$ 1 S.E.

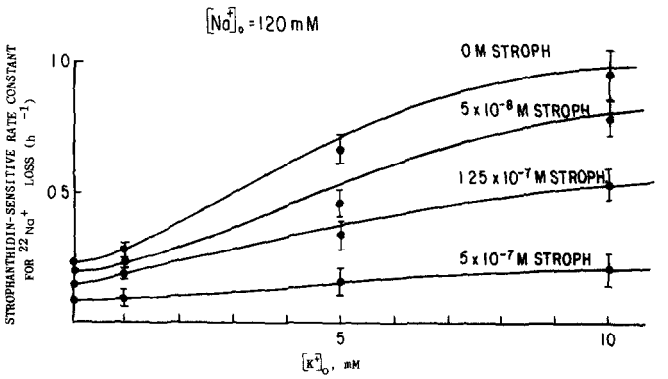

Fig. 7. The strophanthidin-sensitive rate constant for Na^+ loss *versus* the external K^+ concentrations at three strophanthidin concentrations in 120 mM Na^+ Ringer solution. Each point represents the average of six experimental values $\pm$ 1 S E

Theoretical treatment of kinetics

In the absence of external Na^+, an externally directed particle that catalyzes Na^+ transport is assumed to react with external K^+ and with strophanthidin molecules. The non-competitive kinetics observed indicate that K^+ and strophanthidin molecules react at different sites. We assume that the following reactions occur:

$$E + K^+ \underset{k_{11}}{\overset{k_1}{\rightleftharpoons}} EK^+ \tag{1}$$

$$E + D \underset{k_{22}}{\overset{k_2}{\rightleftharpoons}} ED \tag{2}$$

$$ED + K^+ \underset{k_{33}}{\overset{k_3}{\rightleftharpoons}} EK^+D \tag{3}$$

$$EK^+ + D \underset{k_{44}}{\overset{k_4}{\rightleftharpoons}} EK^+D \tag{4}$$

and

$$E_0 = E + EK^+ + ED + EK^+D \tag{5}$$

where E is the catalytic particle with unoccupied catalytic sites, EK^+ is the transport enzyme–K^+ complex, ED is the enzyme–strophanthidin complex and EK^+D is the enzyme–K^+–strophanthidin complex. E_0 is the total amount of enzyme present at the outer membrane surface. Assuming that only EK^+ is catalytically active in Na^+ transport, the rate of Na^+ extrusion will be proportional to $[EK^+]$. Solving Eqn. 1–5 yields the following equation for the rate of Na^+ extrusion:

$$v_K = \frac{V_K[K^+]_0}{[K^+]_0 + \frac{k_{11}}{k_1} + [D]\left\{\frac{k_4}{k_{44}}[K^+]_0 + \left(\frac{k_2}{k_{22}}\right)\left(\frac{k_{11}}{k_1}\right)\right\}} \tag{6}$$

where v_K is the K^+-activated strophanthidin-sensitive rate constant for Na^+ loss and V_K is the maximal K^+-activated rate constant for Na^+ loss. The curves in Fig. 3 are calculated from Eqn 6 using the following constants determined from the experimental data:

$$V = 2.5\ h^{-1}$$

$$\frac{k_1}{k_{11}} = 0.303\ mM^{-1}$$

$$\frac{k_2}{k_{22}} = 8 \cdot 10^2\ mM^{-1}$$

$$\frac{k_3}{k_{33}} = 4.17\ mM^{-1}$$

$$\frac{k_4}{k_{44}} = 1.1 \cdot 10^4\ mM^{-1}$$

The Na^+-activated strophanthidin-sensitive Na^+ efflux observed in the absence of external K^+ is treated in a similar manner by considering Reactions 1–5 to involve Na^+ instead of K^+. Solution of these equations subject to the assumption that only the form ENa^+ is active in promoting strophanthidin-sensitive Na^+ efflux yields the following equation for the rate of Na^+ loss

$$v_{Na} = \frac{V_{Na}[Na^+]_0}{[Na^+]_0 + \frac{k'_{11}}{k'_1} + [D]\left\{\frac{k'_4}{k'_{44}}[Na^+]_0 + \left(\frac{k'_2}{k'_{22}}\right)\left(\frac{k'_{11}}{k'_1}\right)\right\}} \quad (7)$$

where v_{Na} refers to the strophanthidin-sensitive Na^+-activated Na^+ efflux and V_{Na} is the maximal value of the same parameter The constants determined from the experimental data are·

$$V_{Na} = 0.269\ h^{-1}$$

$$\frac{k'_1}{k'_{11}} = 0.046\ mM^{-1}$$

$$\frac{k'_2}{k'_{22}} = 1.51 \cdot 10^4\ mM^{-1}$$

$$\frac{k'_3}{k'_{33}} = 0.005\ mM^{-1}$$

$$\frac{k'_4}{k'_{44}} = 0.164 \cdot 10^4\ mM^{-1}$$

The curves in Fig. 4 are plots of Eqn 7 with the constants given.

In the presence of both external Na^+ and K^+, more complicated kinetics are suggested due to the sigmoidal nature of the activation curves. The data in Figs 5, 6 and 7 are satisfactorily fit by an allosteric model based upon two conformations of the membrane enzyme. Details of the model are considered in the Appendix.

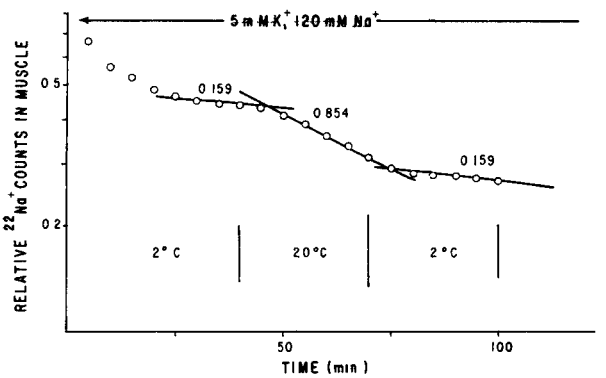

Fig 8 The Na^+ efflux rate constant increase upon changing the temperature of the Ringer solution from 2 to 20 °C, and its decrease to the same value upon changing the temperature back to 2 from 20 °C The numbers above the curves refer to rate constants in h^{-1} units

The activation energies of K^+- and Na^+-activated Na^+ pumping

The reversibility of the Na^+ efflux with respect to temperature in the range of 2 to 20 °C is shown in Fig. 8.

The strophanthidin-sensitive Na^+ efflux can be stimulated by external Na^+ alone or K^+ alone. The strophanthidin-sensitive Na^+ efflux rate constant for the Na^+–Na^+ exchange and the Na^+–K^+ coupling are obtained at various temperatures. Their relationships are shown in Fig. 9. According to the Arrhenius rate equation, one can calculate that the activation enthalpy of the Na^+ pump is 22 kcal/mole in the Na^+–K^+ coupling form and 10.2 kcal/mole in the Na^+–Na^+ exchange form. The two activation energies imply that the mechanism of Na^+–K^+ coupling is probably different from that of the Na^+–Na^+ exchange.

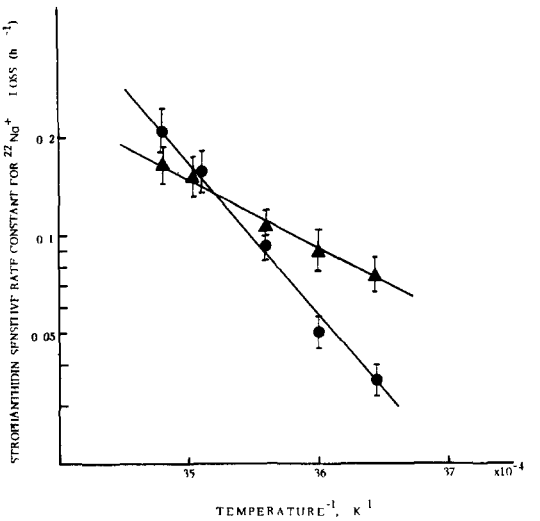

Fig 9 The Na^+ efflux rate constant plotted *versus* the logarithm of the reciprocal absolute temperature ▲, data collected in K^+-free, 120 mM Na^+ Ringer solution ●, data collected in 1 mM K^+, Na^+-free Ringer solution Each point is the average of three experimental values ± 1 S.E.

DISCUSSION

The reaction of strophanthidin with an inhibitory site on the outward-directed free membrane-enzyme, E (Reaction 2), exhibits different rate constants depending upon whether the external medium is K^+-free or Na^+-free. This indicates that the enzymatic particle, E, can exist in two forms, E^{Na} and E^{K}. The affinity of the form E^{Na} for strophanthidin is about 20 times greater than that of the E^{K} form as measured by kinetic analysis of steady-state inhibition curves Also, the half-time for development of inhibition by $5 \cdot 10^{-7}$ M strophanthidin is 3 times longer in Na^+-free media than in normal Na^+ Ringer solution. A similar finding has been reported for squid giant axons by Baker and Manil[8] who observed ouabain to act 10 times slower in Na^+-free media than in normal seawater. It seems evident that cardioactive steroids combine with a part of the transport mechanism that has an affinity for Na^+. The binding of Na^+ increases the subsequent rate of binding of cardioactive steroids.

In addition to acting as competitive inhibitors of activation by K^+ at the external activation sites[11,13], Na^+ in the medium acts to produce a form of the transport enzyme having a higher affinity for cardiotonic drugs, according to the present interpretation. Skou and Hilberg[14] have suggested that the transport system exists in both Na^+–Na^+ and Na^+–K^+ forms depending upon the Na^+ and K^+ concentrations in the solution. The work on red cells by Garrahan and Glynn[15] and on muscle by Keynes and Steinhardt[12] has shown that the addition of K^+ to a K^+-free medium converts some fraction of the Na^+-Na^+ exchange into a coupled Na^+-K^+ exchange. It is of interest that the activation energies for the two kinds of Na^+ pump operation measured in the present work differ by a factor of about 2. The kinetic studies on ionic activation and strophanthidin inhibition as well as the study of temperature coefficients suggest the possible application of an allosteric-type model. All of the kinetic data can be satisfactorily fit by assuming that the two forms, E^{Na} and E^K, are in equilibrium in the presence of both external Na^+ and K^+ and that high $[Na^+]_0$ favors the Na^+ form and high $[K^+]_0$ favors the K^+ form. A zero concentration of either species Na^+ or K^+ implies that the reaction proceeds completely to the opposite form. To fit all the data for all $[K^+]_0$, $[Na^+]_0$ and strophanthidin concentrations employed, it is necessary to postulate a 4-site model One of the sites is competitively occupied by K^+ or Na^+ while 3 of the sites are non-competitively occupied or unoccupied by Na^+. In this model, with the present data, it is not possible to distinguish ionic activation from ionic-induced conformation changes. If ionic activation corresponds to the postulated change in conformation, then only 4 sites would be required to fit the data. If ionic activation is a separate process, more sites would be required in the model. Strophanthidin binds at a different site as the observed kinetics were of the non-competitive type, agreeing with previous work on Na^+ transport in muscle[7] and on (Na^+–K^+)-ATPase[5,6]. External K^+ does, however, alter the affinity of the membrane for strophanthidin According to our interpretation, this action is the result of K^+ shifting the equilibrium in favor of a form, E^K, which has a lower affinity for the drug at low $[K^+]_0$ This finding is similar to that of Matsui and Schwartz[6] who observed that increasing $[K^+]$ at constant $[Na^+]$ caused an increase in K_i (decrease in affinity) for ouabain.

APPENDIX: ALLOSTERIC MODELLING

Spatially separated sets of conformation determining sites and of catalytic activity inducing sites of membrane (Na^+–K^+)-ATPase are postulated. For Na^+-loaded muscles, a hypothesis is proposed that the conformational sites of the enzyme are only determined by external Na^+ and K^+. Either 1 Na^+ occupying one specific site competing with K^+ or 3 Na^+ occupying three other non-competing sites is sufficient to change the enzyme to the form which only operates on the Na^+–Na^+ exchange. The enzyme form in Tris is not defined. All other conditions give the K^+ form which only operates on the Na^+–K^+ coupling. This empirical hypothesis is illustrated graphically in Fig. 10.

The probability of the enzyme being in the Na^+ form is

$$f(Na) = \frac{[Na^+]_0}{[Na^+]_0 + A[K^+]_0} + \left(1 - \frac{[Na^+]_0}{[Na^+]_0 + A[K^+]_0}\right) \cdot b \cdot [Na^+]_0^3 \qquad (1)$$

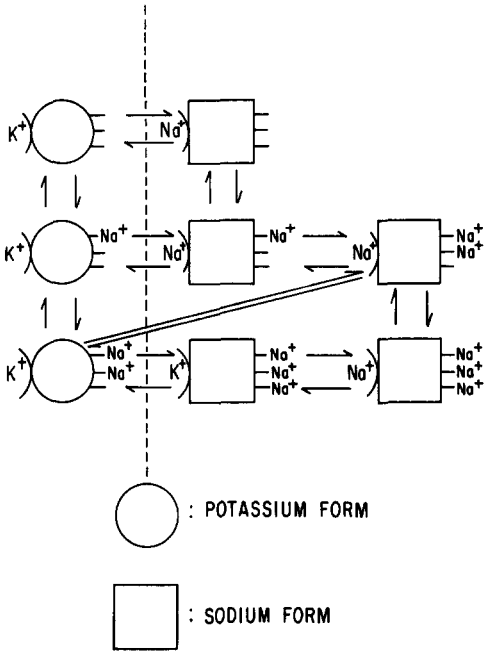

Fig 10. A graphical illustration of the K^+ enzyme form and the Na^+ enzyme form determined by the interactions between the conformation sites on the enzyme and K^+ and Na^+. Either 1 Na^+ bound to the competitive site or 3 Na^+ bound to the non-competitive sites suffices to produce the Na^+ form

When $[K^+]_0 = 0$, $[Na^+]_0 \neq 0$, $f(Na) = 1$ and when $[Na^+]_0 = 0$, $[K^+]_0 \neq 0$, $f(Na) = 0$. For $[K^+]_0$ and $[Na^+]_0$ both equal to zero, f(Na) is not defined. The probability of the enzyme being in the potassium form is

$$f(K) = 1 - f(Na) \tag{2}$$

The total strophanthidin-sensitive rate constant is

$$v_T = f(Na) \cdot v_{Na} + f(K) \cdot v_K \tag{3}$$

The constants A and b in Eqn 1 are determined by fitting the experimental data into Eqn 3. v_K and v_{Na} are given by Eqns 6 and 7,

$$A = 41,\ b = 2.15 \cdot 10^{-7}\ mM^{-3} \tag{4}$$

The theoretically calculated curves based on this hypothesis and the experimentally measured values are shown in Figs 5, 6 and 7.

ACKNOWLEDGEMENTS

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