J. Physiol. (1983), **334**, *pp.* 103–131
With 17 text-figures
Printed in Great Britain

THE EFFECTS OF LOW CONCENTRATIONS OF CARDIOTONIC STEROIDS ON MEMBRANE CURRENTS AND TENSION IN SHEEP PURKINJE FIBRES

BY G. HART*, D. NOBLE AND Y. SHIMONI
From the University Laboratory of Physiology, Parks Road, Oxford OX1 3PT

(*Received 9 February 1982*)

SUMMARY

1. Simultaneous measurements of voltage-clamp currents and tension were made in shortened sheep Purkinje fibres exposed to various concentrations of strophanthidin, ouabain and digoxin.

2. In 5·4 mM-K moderate doses (mean $2{\cdot}4 \times 10^{-7}$ M) of the drugs produced an inward shift of the current–voltage relationship at very negative potentials, consistent with an increase in cleft K concentration (Cohen, Daut & Noble, 1976*b*), which was always accompanied by an increase in tension. This change, which has been attributed to Na–K pump inhibition, was often better correlated with an increase in voltage-dependent tonic tension than in twitch tension.

3. Exposure to dihydro-ouabain gave a monotonic increase in tension but a delayed increase in inward current. This suggests (cf. Lee, Kang, Sokol & Lee, 1980) that minor changes in pump activity may not always change the current–voltage relationship.

4. Low concentrations of strophanthidin (5×10^{-9} to 5×10^{-7} M) produced an outward current shift at very negative potentials, this change becoming smaller with a more rapid onset and reversing on increasing the dose. This change is attributed to pump stimulation.

5. The outward current shift was often associated with a *negative* inotropic effect, which always reversed either spontaneously or on removal of the drug.

6. The alternative response at a narrower dose range (1×10^{-8} to 2×10^{-7} M) was an increase in twitch (not tonic) tension, termed the low-dose positive inotropic effect.

7. After a low concentration of cardiotonic steroid had given an early negative inotropic effect the bulk Ca concentration was reduced and the drug re-applied. The low-dose *positive* inotropic mechanism was then observed.

8. Outward current shifts and negative inotropy were also obtained with low concentrations of the clinically used glycosides digoxin and ouabain.

9. Low concentrations of strophanthidin applied to externally stimulated sheep ventricular trabeculae produced negative inotropy with lengthening of the action potential duration. Positive inotropy and action potential shortening occurred with higher doses.

10. A computer model of ionic currents and distributions in Purkinje fibres satisfactorily reproduced the changes in membrane currents and ionic gradients

* Present address: Department of Cardiology, The General Infirmary, Leeds LS1 3EX.

observed with cardiotonic steroids. The only perturbations capable of explaining our results were Na pump stimulation and inhibition.

11. It is concluded that cardiotonic steroids possess two inotropic mechanisms. The first is a low-dose positive inotropic mechanism causally unrelated to changes in sodium pump activity and possibly a direct release of a membrane-associated calcium fraction. Should this mechanism be unavailable then net pump stimulation at low doses will produce negative inotropy. The second mechanism is the well known Na-lag process.

INTRODUCTION

The mechanism by which cardiac glycosides alter the force of the heartbeat remains a controversial issue. Several groups have failed to establish a correlation between the degree of inhibition of the membrane Na–K-ATPase ('sodium pump') and increased force of contraction (reviewed by Noble, 1980, who also discusses the results of groups showing good correlation). Moreover, evidence is accumulating to show that at low concentrations, cardiac glycosides may in fact *increase* transmembrane sodium and potassium gradients in many tissues, including cardiac muscle. Cohen, Daut & Noble (1976*b*) studied the actions of 5×10^{-8} to 5×10^{-7} M-ouabain on short sheep-Purkinje fibres, using a two-micro-electrode voltage-clamp technique in the presence of 5·4 mM- or 8 mM-K. They found that these glycoside levels shifted the membrane current–voltage relationship in the *outward* direction, in contrast to higher concentrations, which produced an increase in inward current at potentials in the pacemaker range (-50 to -100 mV). Similarly the apparent reversal potential for the pacemaker current, 'E_{K_2}', became more negative with low and more positive with higher glycoside levels. These changes could be reproduced by decreasing and increasing, respectively, the bulk extracellular potassium concentration, $[K]_b$. They concluded that low concentrations of the glycosides may produce stimulation instead of inhibition of the sodium pump, thereby changing membrane currents by lowering the potassium concentration in the intercellular cleft spaces.

Other groups have also shown that providing K_b is not too low, glycosides may apparently stimulate the sodium pump. For example Ellis (1977) and Deitmer & Ellis (1978*b*) have observed a fall in intracellular Na activity, a^i_{Na}, in quiescent sheep-Purkinje fibres. Godfraind & Ghysel-Burton (1979) found an increase in K and a decrease in Na content in stimulated guinea-pig atria. Peters, Raben & Wassermann (1974) obtained an increase in activity of partially purified Na–K-ATPase from guinea-pig atria on exposure to low concentrations of ouabain. Ghysel-Burton & Godfraind (1980) have shown that raising $[K]_b$ increases the glycoside concentration at which an increase in K content is obtained.

The problem posed by these studies is that, as shown by Blood & Noble (1978), low concentrations of glycoside can induce a substantial increase in contractile strength, which suggests that at least some of the inotropic action may not be causally linked to sodium pump inhibition. We have therefore made simultaneous measurements of tension and voltage-clamp currents in short sheep Purkinje fibres in order to correlate changes in force of contraction with changes in K gradient. We have been able to confirm that, in a given fibre, a low concentration of cardiotonic steroid may increase

twitch tension while giving rise to an outward shift of membrane current. An unexpected finding during the course of our work was that in some fibres low doses produced a *negative* inotropic effect, correlated with net pump stimulation. Our results necessitate the invoking of two inotropic mechanisms for the glycosides: one related to net changes in sodium pump activity, the other a low-dose mechanism which is possibly a direct effect on a membrane-associated calcium store. A preliminary report has been presented to The Physiological Society (Earm, Hart, Noble & Shimoni, 1980).

METHODS

Preparations

Sheep hearts were obtained from the slaughterhouse immediately after exsanguination of the animal and carried to the laboratory in either oxygenated normal Tyrode solution at room temperature or, for later experiments, unoxygenated heparinized cardioplegic solution maintained at 2–4 °C. Similar experimental results were obtained with both transporting solutions, but the yield of healthy fibres was improved after initial rapid cooling and cold transportation. Free-running Purkinje fibres were dissected from both ventricles and immersed in oxygenated normal Tyrode solution at room temperature. After initial recovery of resting potential (approximately $\frac{1}{2}$ h) each fibre was shortened and one end fixed to the bath with an entomological pin. Another stainless steel pin, bent in the form of a hook, was inserted into the connective tissue at the other end of the fibre for later attachment to the tension transducer. The dimensions of the muscular core of the fibres varied between 1·3 and 2·0 mm in length and 0·05 and 0·15 mm in diameter.

Experimental set-up

The experimental set-up consisted of a rapid-flow system designed to provide a bath exchange time of approximately 5 s through a bath volume of 0·2–0·3 ml (Eisner & Lederer, 1979). Solutions were fed into the bath by a LKB Multi-perpex pump via a heat-exchanger provided by a Peltier element which was controlled by a feed-back circuit maintaining the bath temperature constant to within 0·2 °C for the course of an experiment. All experiments were performed at 35–36·5 °C. Tension was measured by an Akers transducer (element 803E; amplifier circuit as in Fig. 1 of Eisner & Lederer, 1979). Conventional glass micropipettes were filled with 3 M-KCl and bevelled from a tip resistance of 15–20 MΩ to 3–8 MΩ. The current-passing electrode (usually filled with 2M-K citrate, tip resistance 10–15 MΩ) was inserted close to the centre of the fibre; the voltage-measuring electrode was inserted at roughly one third of the distance from the centre to the end of the fibre. A conventional voltage-clamp circuit was used (Cohen, Daut & Noble, 1976*a*). Membrane voltage and current were monitored during each depolarizing step using a Tektronix 7313 dual-beam storage oscilloscope. A Devices four-channel pen-chart recorder provided a continuous record of voltage, current, tension and temperature. Recordings of these were also made on a Hewlett-Packard 3968A FM tape recorder.

Solutions

Normal Tyrode solution contained: NaCl, 140 mM; KCl, 5·4 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 10 mM. This solution was buffered with Tris-HCl (gassed with 100 % O_2) or 12 mM-$NaHCO_3$, 0·4 mM-NaH_2PO_4 (gassed with 95 % O_2, 5 % CO_2), pH 7·4 ± 0·1 at 36 °C. Cardioplegic solution contained: NaCl, 140 mM; KCl, 20 mM; $CaCl_2$, 2 mM; $MgCl_2$, 16 mM; $NaHCO_3$, 1·5 mM; procaine hydrochloride, 1·2 mM. Stock solutions of cardiotonic steroids were made in ethanol to the following concentrations and stored at 4 °C; strophanthidin (Sigma), 10^{-2}M; ouabain (Sigma), 10^{-2}M; dihydro-ouabain (Hommel), 10^{-2}M; digoxin (Sigma), 10^{-3}M. These were added to the Tyrode solution in the appropriate dilutions.

Experimental protocol

Our usual approach was to hold the membrane of the fibre at a potential between −65 and −85 mV, at which the holding current was near zero. A depolarizing pulse of around +50 mV was given for 1–2 s to elicit a twitch, followed a few seconds later by a hyperpolarizing pulse of around

−10 mV lasting 2–5 s. These pulses were repeated every 15 or 20 s and tension and currents were allowed to stabilize. A full current–voltage relationship was then obtained, which would take approximately 2 min. Phasic tension was allowed to return to steady state before a drug was applied. A change in cleft K concentration resulting from the action of a cardiotonic steroid produced a change in the current required to hold or hyperpolarize the membrane at the set potentials; at the peak of such a change, or before wash-off, a further current–voltage relationship was taken. The drug most commonly used was the aglycone strophanthidin, because its effects are more readily reversible than those of ouabain. In all respects the results obtained with strophanthidin are identical to those found with the glycosides. The results described below were obtained from thirty-eight Purkinje fibres and seventy-one applications of cardiotonic steroid.

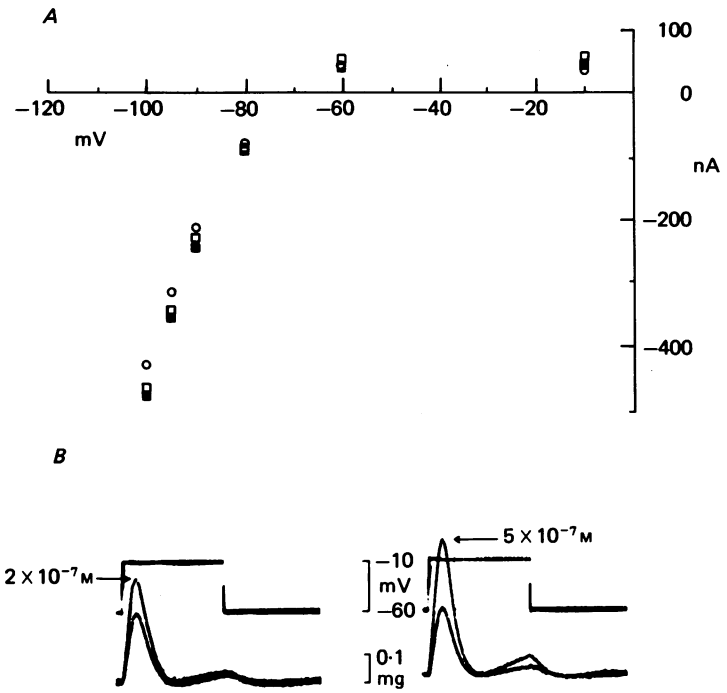

Fig. 1. Effects of moderate concentrations of strophanthidin. *A*, current–voltage relationship. ○, control; □, 18 min after 2×10^{-7} M-strophanthidin; ■, 15 min after 5×10^{-7} M-strophanthidin. *B*, tension elicited by a 1 s voltage step from a holding potential of −60 mV to −10 mV obtained at the same time as the current–voltage relations were recorded. Lower twitch tension amplitudes in each case represent control. Cycle time 15 s. Very much larger increases in inward current and tension were seen after more prolonged exposure or higher dose, but such effects were not usually fully reversible.

RESULTS

Current change in the direction of sodium pump inhibition

Fig. 1 illustrates the effects on currents and tension of exposure to moderate doses of strophanthidin. Part *A* shows the instantaneous current–voltage relationships of the fibre 18 min after exposure to 2×10^{-7}M-strophanthidin and 15 min after 5×10^{-7}M-strophanthidin; in each case there is an inward shift in current which is

fully reversible. The phasic component of tension in response to the depolarizing pulse increased (Fig. 1*B*). Note that with the higher glycoside concentration there is also an increase in voltage-dependent tonic tension.

An inward shift of the current–voltage relation was invariably accompanied by an increase in twitch tension. It was usual that an increase in voltage-dependent tonic tension was produced by a steroid concentration higher than that needed to increase the twitch tension alone. However, the onset of the increase in tonic tension and its

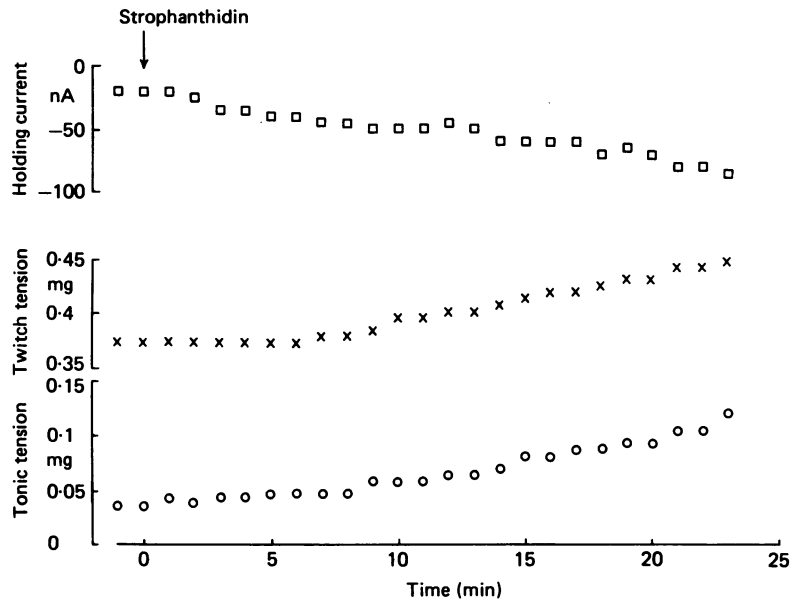

Fig. 2. Time course of increases in inward current and tension after 5×10^{-8} M-strophanthidin. For the sake of clarity points are plotted at 1 min intervals only. Holding potential −70 mV. □, holding current; ×, twitch tension; ○, tonic tension. Tonic tension was measured at the end of a 1 s depolarization to −10 mV. Cycle time 15 s. Vertical arrow in this and subsequent illustrations indicates the onset of perfusion with the stated concentration of cardiotonic steroid. Note that the increase in tonic tension begins earlier and is proportionately greater than the increase in twitch.

rise were sometimes better correlated with the onset and degree of inward current shift than the corresponding changes in twitch tension. Not uncommonly twitch tension would begin to rise several minutes *before* any shift in membrane current. Reasons for this delay will be discussed later. An increase in tonic tension, when seen, always occurred at the time of or slightly after an overt inward current change.

The percentage increase in tonic tension produced by a given glycoside dose was often considerably greater than the accompanying increase in twitch tension. This is illustrated in Fig. 2, which shows that after 23 min of exposure tonic tension at the end of a 1 s depolarizing clamp pulse had risen by over 300 %, while twitch tension, although rising by a similar total amount, had increased by only around 25 %. Note that the rise in tonic tension precedes by a few minutes the increase in the phasic

component. It appears that changes in voltage-dependent tonic tension are more closely linked with the development of a net inward current shift than are changes in the phasic component.

Positive inotropy without current change

In some fibres application of low doses of strophanthidin produced a positive inotropic effect with no shift in the current–voltage relation. The fibre from which Fig. 3 is taken showed a 30 % increase in twitch tension over 23 min, with no change in membrane currents. Note the small initial fall in tension – this will be discussed in more detail later.

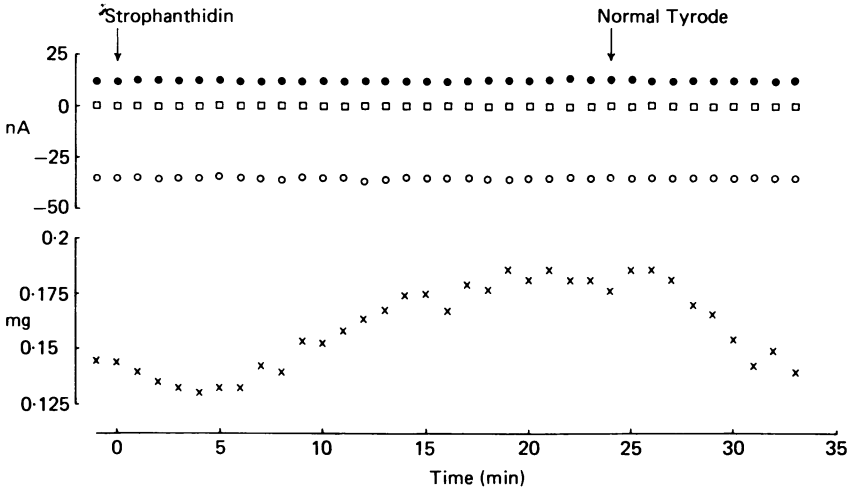

Fig. 3. Effects of 1×10^{-7} M-strophanthidin. Membrane currents: □, at −70 mV (holding potential); ●, at −20 mV; ○, at −90 mV. ×, twitch tension in response to a 1 s depolarization to −20 mV. Cycle time 15 s. Note lack of change of currents during an early negative followed by a positive inotropic response.

Two general interpretations of the lack of change of membrane current may be considered. First, that the drug at this dose had no net effect on sodium pump activity, and produced the rise in tension by an unrelated mechanism: evidence to support the possibility that such a mechanism may operate will be presented later. Secondly, that the sodium pump was inhibited, but not enough to give rise to a change in membrane current. Fibres with a relatively large extracellular cleft space would, for example, need a greater degree of pump inhibition to produce a given rise in cleft potassium concentration than fibres with narrower cleft spaces. We shall show later (see the section Computer Model) that this is a plausible suggestion from a quantitative point of view.

In order to test whether sodium pump inhibition may occur in the absence of a change in the current–voltage relationship, we used the glycoside dihydro-ouabain (DHO). This drug is without inotropic effect below about 10^{-7} M, above which it produces solely inhibition of the sodium pump (Ghysel-Burton & Godfraind, 1977).

Lee, Kang, Sokol & Lee (1980), using sheep Purkinje fibres, found a unique relationship between the increases in a^i_{Na} and contractile tension on exposure to DHO. It follows that positive inotropy resulting from this drug should be linked to sodium pump inhibition, however measured. Fig. 4 shows that twitch tension increases monotonically on application of DHO. There is no consistent increase in inward current, however, until 38 min after the tension has begun to rise, during which time, according to Lee *et al.* (1980), a^i_{Na} is constantly rising. This result is similar to the occasions on which an inhibitory dose of strophanthidin also produced a positive inotropic effect, to be followed many minutes later by an inward shift of the holding current. It may be concluded, then, that no current change after exposure to

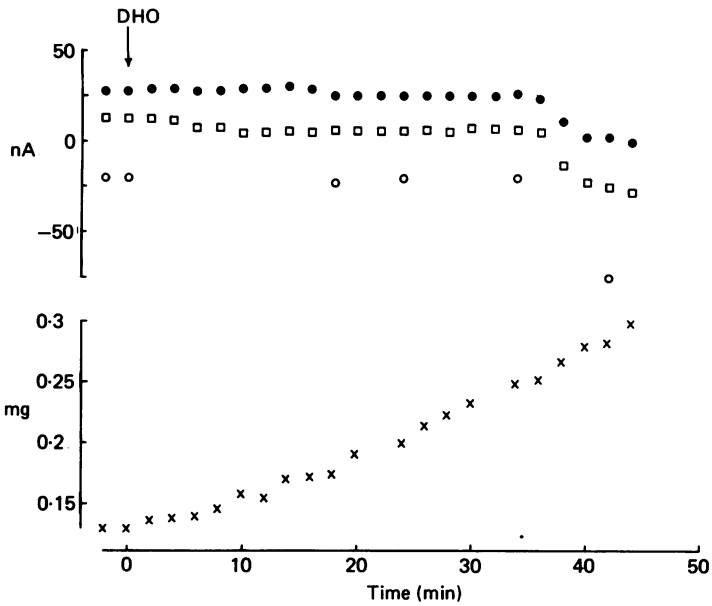

Fig. 4. Effects of 5×10^{-7} M-dihydro-ouabain (DHO). Experiment performed in 8 mM-K_b. Membrane currents: ●, at −74 mV (holding potential): □, at −84 mV; ○, at −94 mV. ×, twitch tension in response to a 1 s depolarization to −19 mV. The breaks in the tension record were due to the taking of current–voltage relationships. Cycle time 15 s. Although twitch tension increases from the beginning there is no consistent inward change of membrane currents (this is particularly significant at −94 mV) until 38 min after the drug was applied.

cardiotonic steroid does not necessarily denote that sodium pump activity has remained unchanged, and thus that positive inotropy in these fibres is necessarily dissociated from net pump inhibition. The corollary, though, is emphasized: that when membrane current does change, a significant degree of sodium pump inhibition or stimulation has already taken place.

Current change in the outward direction

During twenty-nine of the seventy-one exposures to cardiotonic steroid we found an outward shift of membrane current at negative potentials. This current change

may be mimicked by lowering $[K]_b$ and was interpreted by Cohen *et al.* (1976*b*) as evidence for net stimulation of the sodium pump by low glycoside concentrations. Net outward current shifts resulted, on average, from lower drug concentrations than those required for inhibition. For eleven exposures producing current shift in the inward direction alone, the mean concentration of cardiotonic steroid was $2{\cdot}36 \times 10^{-7}$ M, whereas the mean concentration was $5{\cdot}5 \times 10^{-8}$ M in thirteen exposures where only outward current shift was observed. The effects of different concentrations on the direction of change are shown in Fig. 5. At a concentration of 5×10^{-8} M,

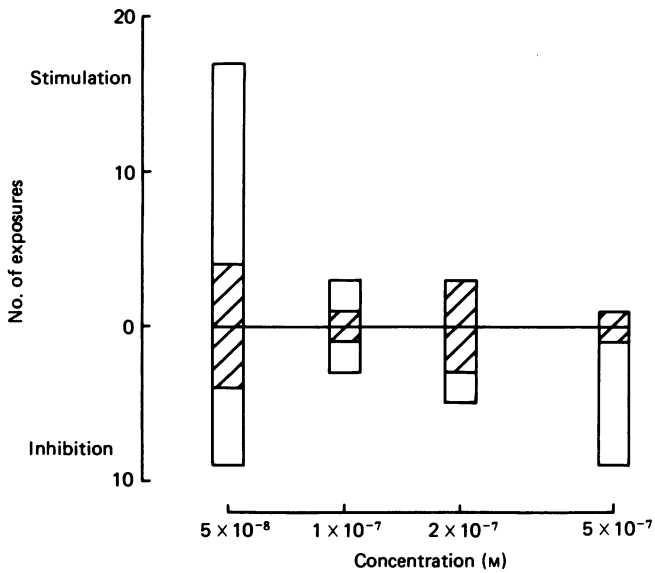

Fig. 5. Effects of different concentrations of strophanthidin and ouabain on the presumed direction of change of net pump activity in 5·4 mM-K, 2 mM-Ca. Exposures producing both stimulating and inhibitory changes were counted twice and are represented by the hatched areas. Fibres showing no current change but negative inotropy (see later) were classified as showing stimulation. Fibres showing no current change but delayed positive inotropy were classified as showing inhibition. The important feature is the relative frequency of each change in pump activity at a given dose, since the total number of exposures at each dose was different.

effects that we interpret to indicate pump stimulation occurred approximately twice as frequently as those interpreted as pump inhibition. At 1×10^{-7} M they occurred with equal frequency. The dominant effect at 2×10^{-7} M was described as inhibition, while one result indicating stimulation occurred at 5×10^{-7} M.

An example of outward current shift is shown in Fig. 6*A*. This instantaneous current–voltage diagram was taken 4–6 min after exposure to strophanthidin. Fig. 6*B* shows samples of these current responses to hyperpolarizing voltage steps before, during and after strophanthidin. The apparent reversal potential, 'E_{K_2}', moves from -95 mV to around -100 mV after strophanthidin.

It may be noted at this point that a new interpretation of the pacemaker current in Purkinje fibres has emerged since the work of Cohen *et al.* (1976*a*, *b*) (see DiFrancesco, 1981*a*, *b*), in which

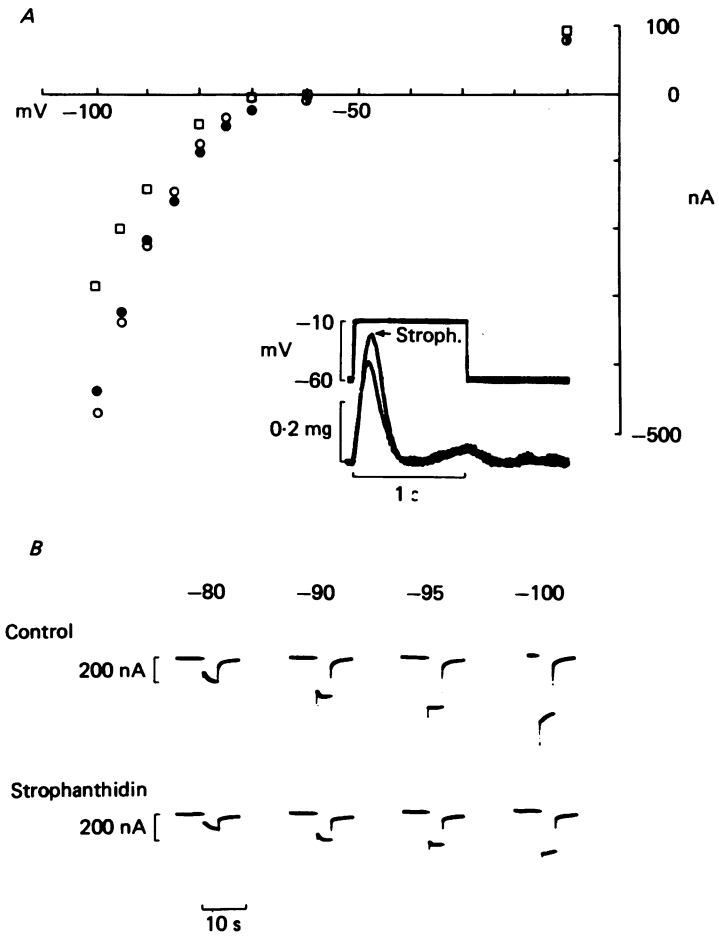

Fig. 6. Positive inotropic effect with net outward current shift after 5×10^{-8} M-strophanthidin. *A*, current–voltage diagram ●, before; □, 4–6 min after exposure to strophanthidin; ○, after wash-off. Inset shows the increase in twitch tension at 4 min after drug application. Lower twitch record is control. *B*, examples of current records used to construct the current–voltage diagram. Upper set, before exposure; lower set, 4–6 min after strophanthidin. Currents are in response to hyperpolarizations to the voltages shown at the top of the records from a holding potential of −60 mV. Cycle time 15 s.

the pacemaker current is found to be an inward current and the apparent reversal is caused by the 'overlap' of a decaying outward current due to cleft K depletion. We shall show later (see Discussion) that this reinterpretation does not affect the conclusions to be drawn from experiments of the kind described in this paper.

The outward current shift shown in Fig. 6 persisted until wash-off at 15 min after the onset of drug exposure, when the current–voltage relationship returned to control. Not uncommonly, though, such an outward shift was transient, reaching a peak 3–8 min after the onset of drug perfusion and returning by 15–25 min. In line with the results of Deitmer & Ellis (1978*b*) the outward shift tended to be more transient

with higher concentrations of cardiotonic steroid. Nevertheless it must not be presumed that outward current change which reverses spontaneously necessarily implies that net sodium pump activity, or a^{i}_{Na}, has returned to former levels (see the section on Computer Model).

Outward current change and positive inotropic effect

The inset of Fig. 6*A* shows that in this fibre twitch tension increased. This occurred at the time of the outward current shift. This was transient for, despite the persistent current change, the twitch had returned to base line 8 min after the strophanthidin was applied. The onset of this early positive inotropic effect was always correlated

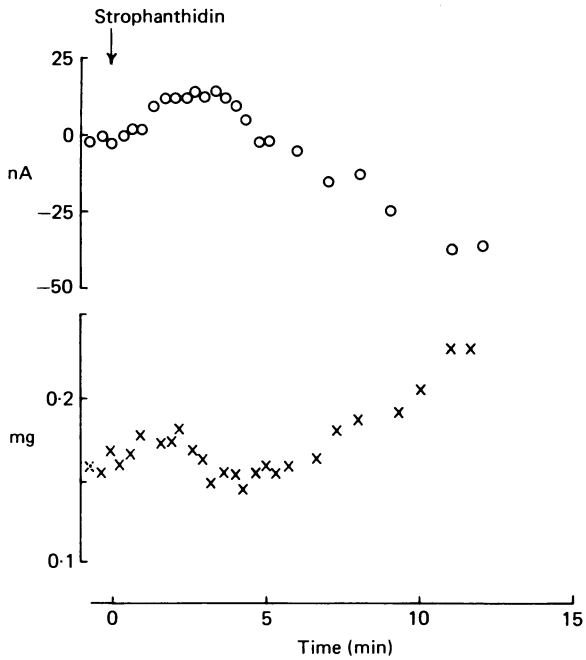

Fig. 7. Two inotropic mechanisms on exposure to 5×10^{-8} M-strophanthidin. ○, membrane current at holding potential of −70 mV. ×, twitch tension in response to a 1 s depolarization to −10 mV. Cycle time 20 s. The smaller tension rise associated with the outward current change gives way to a second, larger inotropic response with a net inward current shift.

with the onset of an outward current shift. Nevertheless, we sometimes observed that the twitch tension remained elevated when the current–voltage diagram had returned to control. The magnitude of the peak rise in twitch tension varied between 7·7 and 72·7 % with a mean of 29 % (n = 13).

This early rise in tension must be distinguished from the slower, often larger positive inotropic effect which is associated with higher glycoside concentrations. It was commonly possible to discern these separate mechanisms in one fibre, particularly if more than one glycoside application were feasible (see later). Fig. 7 illustrates the

changes in tension and holding current in such a fibre. There is an early positive inotropic effect associated with an outward current shift; by 5–6 min tension and current have returned to base line. There follows a second, larger tension rise at the same time as an overt inward current shift. Clearly from this Figure it is necessary to invoke more than one inotropic mechanism: the early positive inotropic effect *cannot* have been a secondary consequence of the process producing an inward current shift.

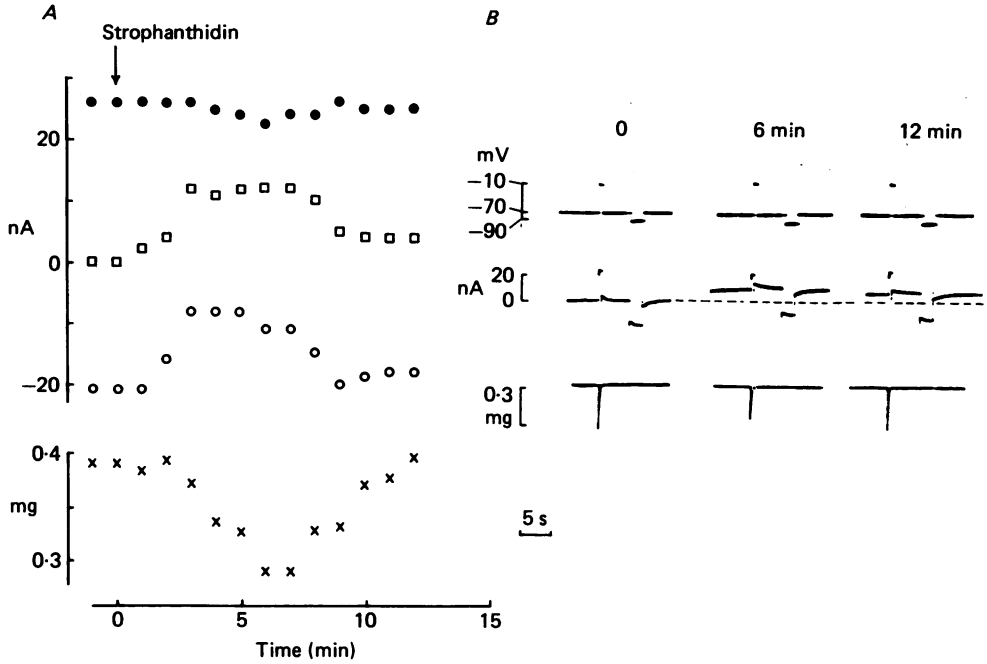

Fig. 8. Negative inotropy with outward current change on exposure to 5×10^{-8} M-strophanthidin. *A*, instantaneous membrane currents: □, at −70 mV (holding potential); ●, at −10 mV; ○, at −90 mV. ×, twitch tension in response to a 1 s depolarization to −10 mV. Cycle time 15 s. *B*, examples of records of membrane voltage (upper traces), current (centre) and tension (lower) at 0, 6 and 12 min after the onset of strophanthidin perfusion. Note that the twitch is shown here as a *downard* deflexion.

A further point of distinction between the two inotropic responses is that the increase in twitch tension associated with an outward shift of membrane currents was never accompanied by an increase in voltage-dependent tonic tension. This suggests that the low-dose positive inotropic mechanism may not depend on a rise in background cytosolic calcium concentration.

Negative inotropic effect

We have described above a positive inotropic effect temporally associated with the onset of net outward current shift, which occurred in thirteen cases of cardiotonic steroid application producing this direction of shift. In four of the twenty-nine fibres

in which an outward current shift was seen there was no change in twitch tension. A surprising finding in twelve of the applications in which an outward current change occurred was the association of a *negative inotropic effect*. Fig. 8*A*, as an example, shows membrane currents at the holding potential (−70 mV) and on clamping to −10 and −90 mV; an outward current shift after application of strophanthidin reaches a plateau at 3–7 min and later reverses. Twitch tension, shown below, decreases by 24% during this period. Fig. 8*B* shows examples of the voltage, current and tension records at the onset, peak and end of this period.

This reduction in twitch tension was sometimes transient, lasting 3–20 min, or sustained until the glycoside was removed. It must be stressed that negative inotropic responses were regarded as acceptable only if they were reversible, either spontaneously or on wash-off of the drug. This rules out the possibility that the negative inotropic responses we have described were due to deterioration of the fibres. The magnitude of the fall in twitch tension varied between 7·9 and 35%, with a mean of 17·2% ($n = 20$). In a further fibre we observed a fall in tension of 80·7% which reversed spontaneously after several minutes and was associated with a marked outward shift in current. Although this negative inotropic effect was atypically large we can find no reason to believe it was artifactual.

Unlike the early positive inotropic effect which in 2 mM-Ca^{2+} was always associated with an outward current shift, in eight fibres we studied a negative inotropic effect was seen without a change in the membrane current–voltage relationship. An example is the early negative inotropic effect shown in Fig. 3. This is probably linked with the observation that the negative inotropic effect was produced on average by slightly lower concentrations of cardiotonic steroid than the early positive effect. The mean concentration during seven exposures where the tension response was an early positive inotropic effect only was $5{\cdot}9 \times 10^{-8}$ M, compared with an average dose of $5{\cdot}0 \times 10^{-8}$ M in nine fibres with a negative tension change alone.

The effects of different doses of cardiotonic steroid in one fibre

So far we have described changes resulting from exposure to a single dose of cardiotonic steroid. In order to define better the concentration ranges at which effects occur and to confirm the pattern of our interpretation, it is necessary to look at different doses in a given fibre. Clearly this was not possible in most cases because of the difficulty of maintaining stable electrode impalements for long periods in a contracting fibre. The main effects of multiple exposures in thirteen fibres are listed in Table 1.

Dose range for net pump stimulation

From the work of Deitmer & Ellis (1978*b*) and Godfraind & Ghysel-Burton (1979) it can be expected that above a threshold of approximately 1×10^{-9} M, increasing concentration of cardiotonic steroid will produce a smaller and more transient ion gradient increase. Indeed, the results in guinea-pig atrium suggest the presence of a 'watershed' concentration at which no net change in ion gradients will be seen. It is therefore of interest to determine whether the negative inotropic effect is smaller and more transient at higher doses. In Fig. 9 are plotted the effects on twitch tension of two concentrations of strophanthidin; this illustration is from fibre B in Table 1 and

TABLE 1. Effects of multiple exposures on currents and tension. Experiments performed using 2 mM-Ca_b, 5·4 mM-K_b unless otherwise noted

Experiment	Steroid	Concentration (M)	Direction of current change	Change in twitch tension
A	Ouabain	2×10^{-8}	No change	No change
		5×10^{-8}	Outward (transient)	Increase (transient)
		1×10^{-7}	No change	No change
		5×10^{-7}	No change	Increase
B	Strophanthidin	5×10^{-8}	Outward	Decrease (transient)
		1×10^{-7}	No change	Decrease then increase
		2×10^{-7}	Inward (delayed)	Decrease then increase
C	Strophanthidin (K_b = 8 mM)	5×10^{-8}	Outward	Decrease (transient)
		5×10^{-7}	Inward	Increase
D	Strophanthidin	5×10^{-8}	Outward	Increase (transient)
		1×10^{-7}	Outward	Increase
		2×10^{-7}	Inward (delayed)	Decrease then increase
		5×10^{-7}	Inward	Increase
E	Ouabain	5×10^{-8}	No change	Decrease (transient)
		1×10^{-7}	Inward	Increase
F	Strophanthidin	5×10^{-8}	No change	Decrease then increase
		5×10^{-7}	Inward	Increase
G	Strophanthidin	5×10^{-8}	Outward	Decrease (transient)
		5×10^{-7}	Inward (delayed)	Increase
H	Strophanthidin	5×10^{-8}	No change	Decrease (transient)
		5×10^{-7}	Inward (delayed)	Increase
I	Strophanthidin	5×10^{-8}	Outward (transient)	Increase (transient) then increase
		5×10^{-8}	No change	Decrease then increase
		3×10^{-7}	No change	Increase
J	Strophanthidin	2×10^{-8}	No change	No change
		5×10^{-8}	Outward then inward	Increase (transient) then increase
K	Strophanthidin	5×10^{-8}	Outward (transient)	Increase (transient)
		1×10^{-7}	Outward	Increase
		2×10^{-7}	No change	Increase
		5×10^{-7}	Outward then inward	Decrease then increase
L	Strophanthidin (K_b = 8 mM)	5×10^{-9}	Outward	Decrease
		1×10^{-8}	Outward (transient)	No change
M	Strophanthidin (K_b = 8 mM)	1×10^{-8}	Outward (transient)	Increase (transient)
		5×10^{-8}	Outward	No change
		5×10^{-7}	Outward (transient)	Increase (delayed)

the response to 5×10^{-8} M has been illustrated in Fig. 8. The higher dose produced no early change in the current–voltage relationship (not shown in Figure), and a smaller, faster negative inotropic effect before net inward current change and positive inotropy occurs. The lower dose, by contrast, produces only the negative inotropic effect correlated with outward current change, as shown in Fig. 8.

Dose range for the low-dose positive inotropic mechanism

The dose range for the low-dose positive inotropic mechanism appears to lie between about 1×10^{-8} and 2×10^{-7}M. This is narrower than that for outward current shifts (and the negative inotropic effect). The concentration threshold can be seen in fibres A, D, J and K (Table 1).

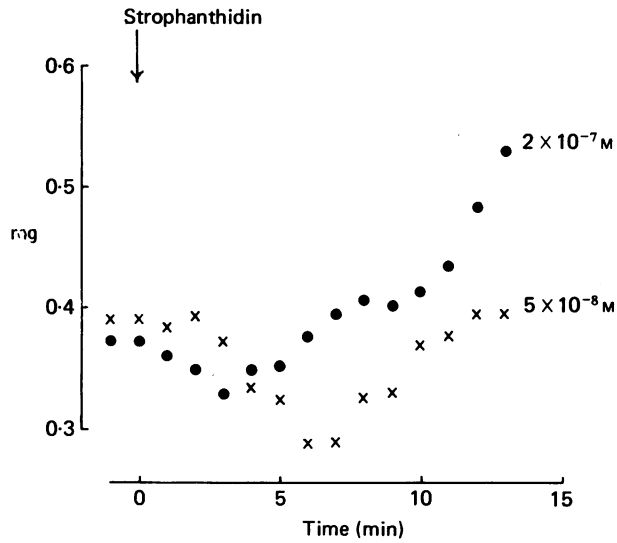

Fig. 9. Effects on twitch tension of two different concentrations of strophanthidin: ×, 5×10^{-8} M; ●, 2×10^{-7} M. The negative inotropic effect is smaller and more transient with the higher dose.

When the concentration range for the low-dose positive mechanism has been exceeded – or this inotropic mechanism has been saturated – then if an outward current change still occurs it may be accompanied by *negative* inotropy. This is illustrated by fibres D and K in Table 1, where on increasing the concentration low-dose positive inotropy gives way to negative inotropy during the transient outward current shift with the higher steroid doses.

The existence of an upper concentration limit to the low-dose positive inotropic mechanism, beyond which negative inotropy may still occur, suggests that over a narrow dose range no net tension change may result from the opposing interaction of these mechanisms. This was very occasionally seen, and is illustrated in fibres A and M of Table 1. In fibre M, which showed low-dose positive inotropy, 5×10^{-8} M-strophanthidin produced an outward current shift but resulted in no tension change. An additional explanation for the lack of effect of 1×10^{-7} M-ouabain in fibre A may,

of course, be that there was no net change at all in pump activity. When this occurs at a dose higher than that required for the low-dose positive inotropic effect, there will be no response in either current or tension, even though both will change in response to concentrations higher or lower than this intermediate area.

The effects of reduced extracellular calcium on the inotropic response

For reasons given earlier, it is not possible causally to connect the low-dose positive inotropic mechanism with a change in the state of the membrane sodium pump. Cardiotonic steroids might, by another mechanism, increase the intracellular calcium

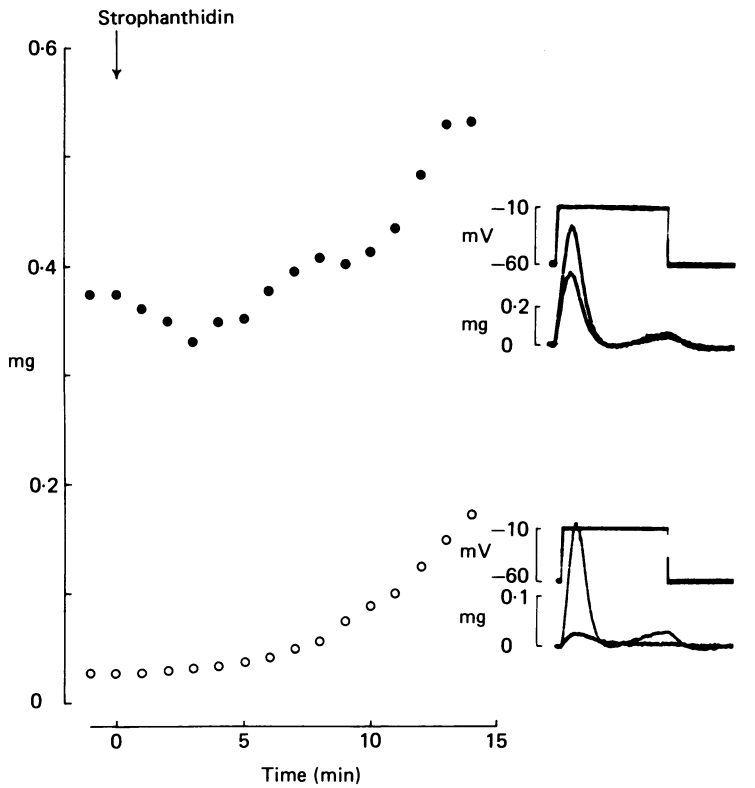

Fig. 10. Effects of reducing extracellular calcium on the twitch tension response to 2×10^{-7} M-strophanthidin. ●, 2 mM-Ca_b, ○, 0·5 mM–Ca_b. Insets show examples of the tension responses to the 1 s voltage steps illustrated, taken at 15 min after onset of drug perfusion. The lower amplitude twitch in each case represents the control. Note that the tension scale is different for the two inset records. The transient negative inotropic effect is absent in the lower calcium concentration and the low-dose positive inotropic mechanism is revealed.

transient on depolarization. Carrier, Lüllmann, Neubauer & Peters (1974) have shown that ouabain may increase the size of a rapidly exchanging membrane calcium fraction, and that the size of this store decreases with the age of the preparation. They further found that the size of this store, along with contractile force, increases much more with ouabain in aged than in freshly dissected fibres.

We therefore investigated the effects of lowering extracellular calcium on the inotropic response, and Fig. 10 illustrates one of four pairs of exposures in three fibres showing the same result. In the upper part of the Figure it can be seen that in 2 mM-Ca^{2+} this dose of strophanthidin produced first a negative and then a positive inotropic effect. The membrane currents did not change until the tension had risen appreciably, when they became more inward. After lowering extracellular calcium to 0·5 mM the same dose this time gave no early fall in tension, but a positive inotropic effect, followed after 8 min by a steeper rise in tension. This rise is proportionately

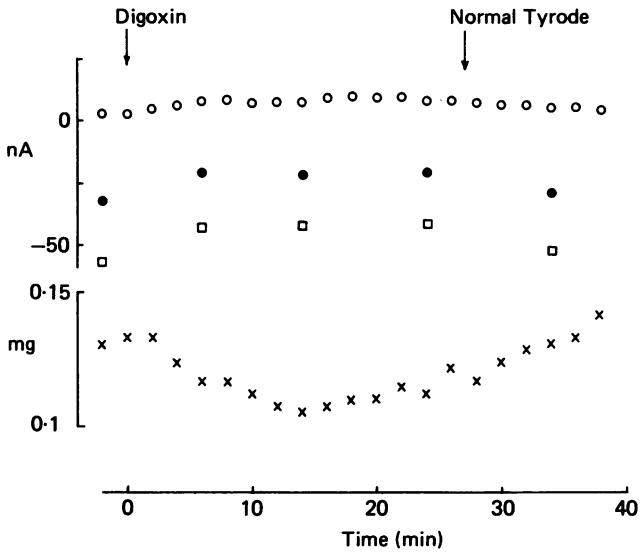

Fig. 11. Negative inotropic effect of $1{\cdot}2 \times 10^{-7}$ M-digoxin. Experiment performed in 8 mM-K_b. Membrane currents: ○, at −84 mV; ●, at −94 mV; □, at −99 mV. ×, twitch tension in response to a 1 s depolarizing step to −19 mV. Cycle time 15 s. For the sake of clarity values are plotted at 2 min intervals. There is a 21 % reduction in twitch tension associated with an outward shift in the current–voltage relationship. Tension change is spontaneously reversing by the time of wash-off.

very much greater than that given by the drug in 2 mM–Ca^{2+}. This result has also been found with 5×10^{-8} M-ouabain and 1×10^{-7} M-strophanthidin. It is possible that in 2 mM-Ca^{2+} the low-dose mechanism was saturated, becoming available on lowering background calcium concentration.

In another fibre an early positive inotropic effect was observed (associated with net outward current change) in *both* 2 mM- and 0·2 mM-Ca^{2+}; this later developed into a further tension rise with net inhibition. The early increase in tension was very much greater in the lower calcium concentration. It may be concluded that fibres with a lower level of resting calcium are much more likely to show a *positive* inotropic response to low doses of cardiotonic steroid.

Effects of ouabain and digoxin

The effects of strophanthidin described above have also been found with ouabain and digoxin. Two fibres treated with ouabain have been included in Table 1. Digoxin,

the most commonly prescribed glycoside in clinical practice, has also given negative inotropic responses. Fig. 11 illustrates the tension and current changes in a fibre in response to $1{\cdot}2 \times 10^{-7}$ M-digoxin.

Inotropic response in sheep ventricular trabeculae

The reason that Purkinje fibres were used in the above study and not working myocardium is that in the former preparation it is very much easier to achieve good control of membrane voltage while at the same time measuring twitch tension. Nevertheless we were interested to find out particularly whether the negative

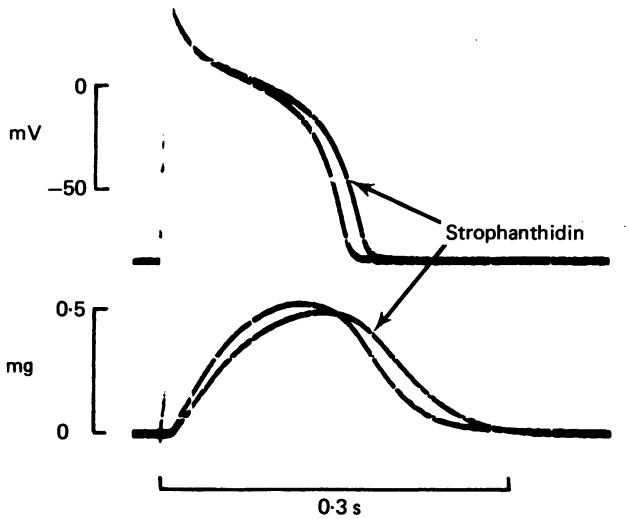

Fig. 12. Action potentials (upper record) and twitch tension (lower record) from a sheep ventricular trabeculum driven at 0·2 Hz, before and 2 min after 5×10^{-8} M-strophanthidin. The aglycone produced a lengthening of the action potential and a 9% fall in twitch tension. Both effects had reversed spontaneously by 10 min after the onset of the exposure.

inotropic response could be obtained in ventricular muscle. We therefore isolated from the endocardial surface free-running ventricular trabeculae having roughly the same overall dimensions as the Purkinje fibres and connected them in the bath to the tension transducer. Action potentials elicited by external stimulation were recorded by a single KCl micro-electrode. Fig. 12 is an example showing the action potential and twitch before and 2 min after 5×10^{-8} M-strophanthidin. The aglycone has produced a reduction in twitch tension with a *lengthening* of the action potential. Both returned to control values after 10 min of perfusion. Higher doses produced an increase in tension associated with a *shortening* of the action potential duration. Shortening is the effect usually seen on the action potential with glycoside (Edmands, Greenspan & Fisch, 1967; Ito, Hollander, Marks & Dutta, 1970), although its interpretation is complex (Daut, 1980). The most obvious explanation of the lengthening of the action potential in association with the reduction in tension is, by analogy with the Purkinje fibre, pump stimulation giving rise to a reduction in cleft [K], but this is not the only possible explanation. It is clear, though, from these results

that low doses of strophanthidin can produce the opposite effects on tension and action potential in the ventricle to those produced by higher doses. There is therefore no reason to believe that our results apply only to Purkinje fibres.

Computer model

In this section we present results based on using DiFrancesco & Noble's (1980, 1981, 1982) model of Purkinje fibre electrical activity to reproduce the ionic current changes observed in our experiments and in those of Cohen *et al.* (1976*b*).

The equations are almost identical with those given by DiFrancesco & Noble (1982) except that:

(i) Instead of a linear dependence of sodium pump activity on extracellular K, a non-linear relation was used. This allows for the fact that the sodium pump is half-maximally activated at about 1 mM-K_b (Gadsby, 1980) and that activation follows a simple Michaelis – Menten-type relation. For simplicity we have assumed first-order kinetics for the binding and activation.

(ii) Intracellular sodium concentration, $[Na]_i$, was computed by solving the equation

$$\frac{d[Na]_i}{dt} = \frac{\Sigma i_{Na}}{FV} - \frac{3i_p}{FV}, \tag{1}$$

where Σi_{na} is the sum of the various sodium currents (fast sodium current, background sodium current and the sodium components of i_{si} and i_f) and i_p is the Na-K exchange pump current.

Since $[Na]_i$ is included as a variable it is also necessary to take account of sodium activation of the pump. This was also assumed to be determined by a simple first-order binding with a K_m set to 20 mM. In fact the K_m for internal sodium activation may be even higher than this (Deitmer & Ellis, 1978*b*). Our calculations do not critically depend on this parameter since, even with a K_m of 20 mM, the pump activity is virtually linearly dependent on $[Na]_i$ in the range of values relevant to our purpose. This is the result obtained experimentally by Eisner, Lederer & Vaughan-Jones (1981).

Thus the total pump current is then given by:

$$i_p = \left(\frac{[K]_c}{K_{M,K} + [K]_c}\right)\left(\frac{[Na]_i}{K_{M,Na} + [Na]_i}\right)\bar{i}_p \tag{2}$$

where $K_{M,K} = 1$ mM, $K_{M,Na} = 20$ mM and $\bar{i}_p$ is the maximum current when the pump is fully activated. A value of 300 nA for $\bar{i}_p$ gives a reasonable value (about 20 nA) for the resting sodium pump current. This corresponds to the value obtained experimentally (Eisner *et al.* 1981).

For all the computations the current changes are sufficiently slow to allow the gating variables to be at their steady-state value with no loss of accuracy and greatly reduced computation time. $[K]_b$ was set to 5·4 mM as this was the value chosen for most of the experiments. A value of 5% for the restricted extracellular cleft space volume best reproduced changes in pump activity. The program was then run to find a zero-current potential appropriate to these values and the steady-state values of

$[K]_c$, $[Na]_i$ and $[Ca]_i$ incorporated. The 'membrane' was then clamped at this potential (−70·85 mV) and after 6 min pump activity suddenly changed to a new value.

Fig. 13 shows the results of a 14 min period of stimulation of pump activity by different degrees. The immediate effect is an outward current change, a fall in $[K]_c$ and a more gradual fall in $[Na]_i$. This change takes about 10 min to be complete, which

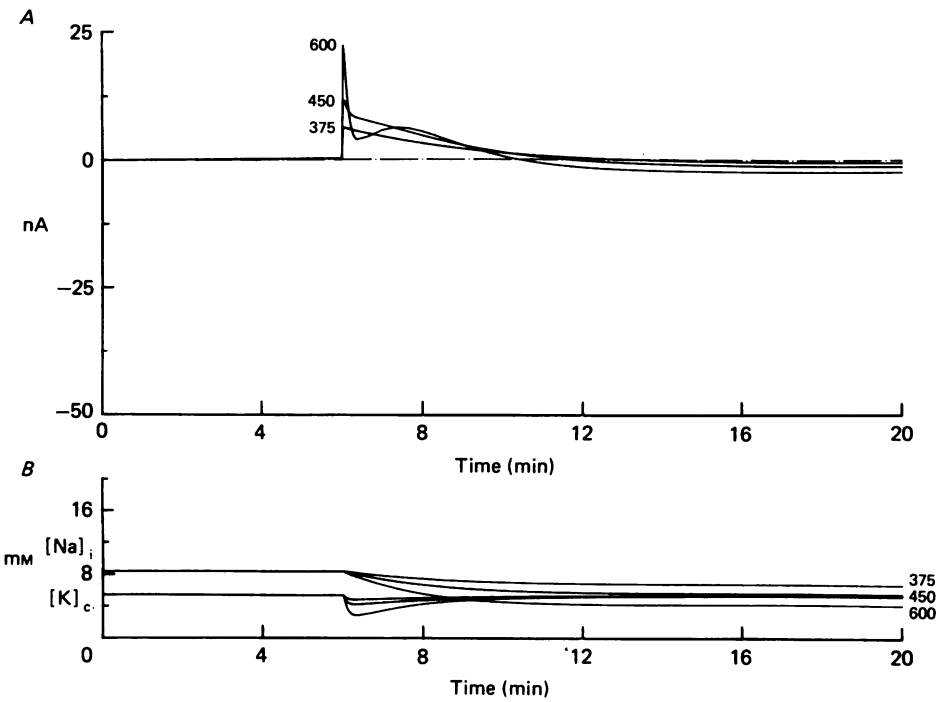

Fig. 13. Sodium pump stimulation. Changes in membrane current (*A*), $[Na]_i$ (*B*, upper records) and $[K]_c$ (*B*, lower records) in response to step increases in maximum pump current from 300 nA to 375 nA, 450 nA and 600 nA at 6 min. The inflexion in the current record after a doubling of pump activity is due to the effect of the reduced $[K]_c$ on the instantaneous rectifier. It is noteworthy that the currents eventually become net inward, while $[Na]_i$ remains low. This is again a result of the 'holding' potential being in the region of the cross-over of the current–voltage curves with different $[K]_b$ values and is an exception to the rule that inward current shift at negative potentials signifies pump inhibition. It is, however, uncommon for this effect to be seen overtly after glycoside administration but it is illustrated in Fig. 16 of Cohen *et al.* (1976*b*). The numbers on the right of the curves in *B* correspond to the curves for $[Na]_i$. $[K]_c$ at 20 min has become virtually the same for all three changes in pump activity and is slightly lower than the $[Na]_i$ reached with an $\bar{i}_p$ of 450 nA. This and the remaining Figures were drawn using a Hewlett-Packard 7220A terminal plotter.

corresponds well to the measured time for $[Na]_i$ changes following low cardiotonic steroid doses. The fall in $[Na]_i$ produced by a 25 % increase in $\bar{i}_p$ is similar to the fall in a^i_{Na} seen with $1{\cdot}5 \times 10^{-8}$ M-strophanthidin (Deitmer & Ellis, 1978*b*).

Current–voltage diagrams at 2 and 14 min after increasing pump activity are shown in Fig. 14*A* and *B* respectively. Fig. 14*A* closely mimics the change seen in Fig. 6.

From these results it can be seen that a *transient* electrical change similar to that frequently observed in our experiments would be expected to occur even if the pump stimulation were maintained. This is due to a reduction in pump activity consequent on a steady, lower level of $[Na]_i$. A more permanent or longer-lasting electrical change will occur only if either the rate of change of $[Na]_i$ is much reduced (as might happen in a much larger preparation) or factors other than the sodium pump are important

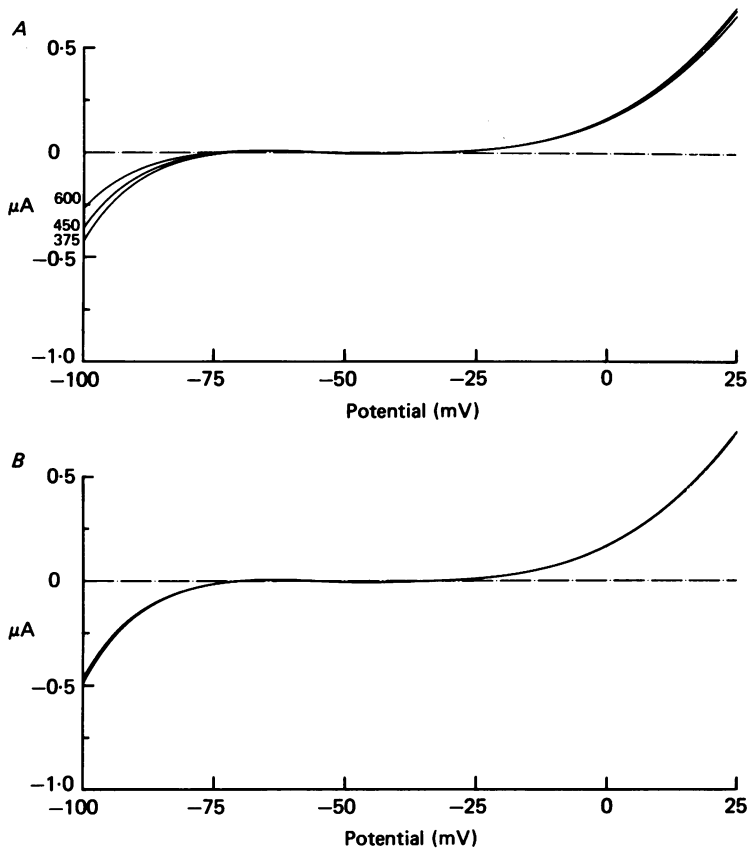

Fig. 14. Current–voltage relationships after sodium pump stimulation. *A*, 2 min after step increases in maximum pump current from 300 nA to the values shown by the corresponding curves (nA). *B*, 14 min afterwards. The current–voltage diagrams have returned very close to control.

in determining the steady level of $[Na]_i$ (Deitmer & Ellis, 1978*a*). It could be, therefore, that the difference between a preparation showing a maintained electrical response to pump stimulation and one showing a transient response lies in the relative importance of the sodium pump and other factors in determining the steady level of $[Na]_i$. Nevertheless the commonest cause of a transient outward current shift (and transient negative inotropy) at cardiotonic steroid concentrations which are other than very low is probably the development of net pump inhibition, as a later and slower effect of cardiotonic steroids.

No other simple perturbation of the model was found to be capable of reproducing the above results. An increased K conductance (which would account for the increased outward current at the holding potential) would produce an *inward* current shift negative to E_K. In fact the outward shift is larger at more negative and smaller at more positive levels of membrane potential (e.g. Fig. 6; E_K in 5·4 mM-K is −81 mV). A decrease in inward background current, which would also reproduce the outward current shift and a fall in $[Na]_i$, would not account for the negative shift in the potential (formerly called E_{K_2}) at which i_f and depletion currents balance to produce a zero current change with time. A reduced inward background current would slowly reduce pump activity (by reducing $[Na]_i$) and so lead to K accumulation, with an inward shift of the current–voltage relationship.

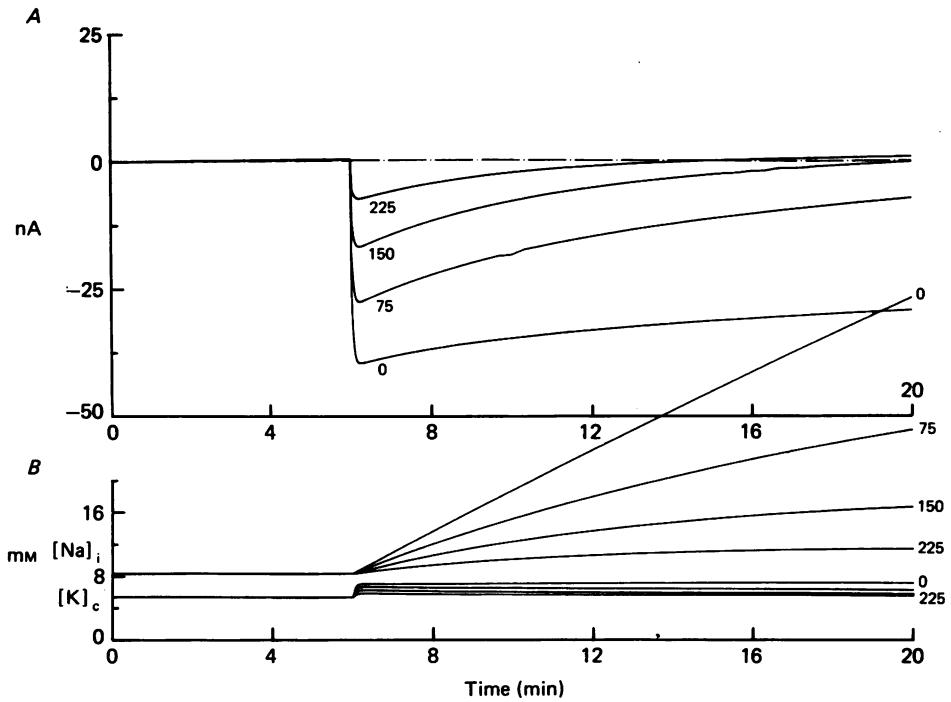

Fig. 15. Sodium pump inhibition. Changes in membrane current (*A*), $[Na]_i$ (*B*, upper records) and $[K]_c$ (*B*, lower records) in response to step reductions in maximum pump current from 300 nA to 225 nA, 150 nA, 75 nA and 0 nA at 6 min.

It should be noted that the model not only reproduces the changes in current–voltage relations but also the shifts in 'E_{K_2}' observed both in our experiments and in those of Cohen *et al.* (1976*b*). The reasons for this are that, despite the radical reinterpretation of i_{K_2}, the apparent reversal potential still accurately senses the K^+ gradient. The quantitative reasons for this are fully discussed by DiFrancesco & Noble (1982). It is because of this reinterpretation that we have used the instantaneous rather than the steady-state current–voltage relations, but the conclusions from the work of Cohen *et al.* (1976*b*) remain unchanged.

Fig. 15*A* shows the currents obtained from step reductions in $\bar{i}_p$ from 300 nA to the values shown. At 2 and 14 min after the reduction in $\bar{i}_p$ current–voltage relationships were obtained from the model and are shown in Fig. 16*A* and *B* respectively. It is interesting to note that, experimentally, whereas the outward

current change due to stimulation may be transient, the inward current change due to inhibition is usually maintained. This asymmetry is well reproduced by the model. A comparison of Figs. 14*B* and 16*B* shows that the current–voltage relationships 14 min after stimulation have all returned to virtually the same curve, in contrast to the persistent inward shift after inhibition.

Finally, we have also computed the effects of *gradual* changes in pump activity. Evidence suggests that cardiotonic steroid binding occurs over many minutes

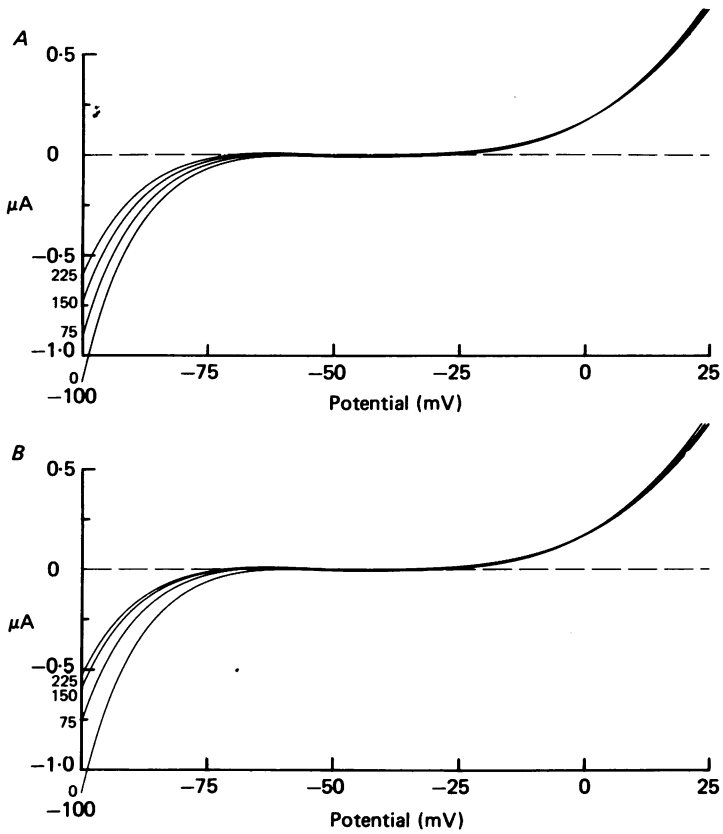

Fig. 16. Current–voltage relationships after sodium pump inhibition. *A*, 2 min after step reductions in maximum pump current from 300 nA to the values shown by the corresponding curves. These relations were obtained at 8 min in Fig. 15. *B*, 14 min afterwards (at 20 min in Fig 15). Note that the curves have become less inward at negative potentials than at 2 min after the onset of inhibition.

(Bentfeld, Lüllmann, Peters & Proppe, 1977) and Fig. 17 illustrates the effects of changing $\bar{i}_p$ in a gradual manner from 300 nA at 6 min to the values shown at 20 min. It can be seen that very little current change occurs with moderate degrees of change in pump activity, despite substantial changes in $[Na]_i$. The current change after pump stimulation is transient and returns towards the base line value as $[Na]_i$ stabilizes at a new, lower level (cf. Fig. 8*A*). The current changes in the inhibitory direction

do not, however, show this spontaneous reversal, but for greater degrees of inhibition increase more rapidly as more pump sites are blocked. This is similar to the current response illustrated in Fig. 4 and explains those instances of pump inhibition where a rise in tension occurred to be followed minutes later by a delayed and rapidly increasing inward current shift. These computations are also important in explaining those experiments in which a rise in tension was seen with no net current change. (e.g. Fig. 3).

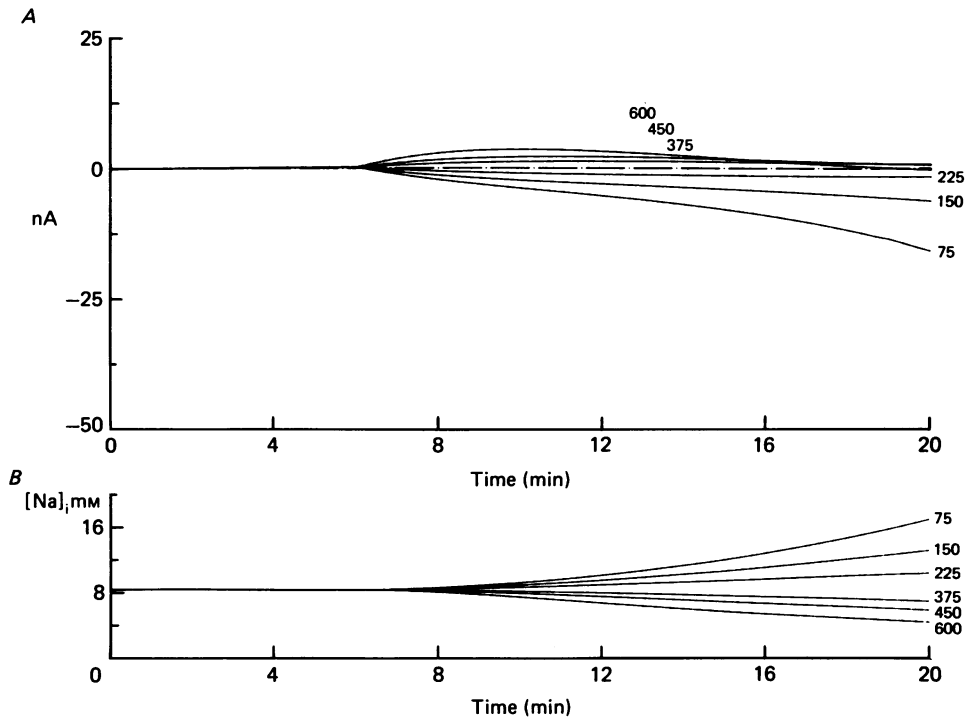

Fig. 17. Slow changes in pump activity. The value of $\bar{i}_p$ was changed gradually from 300 nA at 6 min to reach 600 nA, 450 nA, 375 nA, 225 nA, 150 nA and 75 nA by 20 min. *A*, membrane currents. The order of curves in the stimulatory direction is given by the numbers which correspond to the peaks of outward current shift. *B*, $[Na]_i$. Current change in the stimulatory direction is transient, whereas in the inhibitory direction it is larger and sustained.

There are thus four main reasons for seeing no overt current change in the face of a positive inotropic response to cardiotonic steroids:

(i) The changes in pump activity are sufficiently gradual for measurable current change to be delayed.

(ii) The preparation has a relatively large cleft space volume.

(iii) The potential at which the current is measured is in the cross-over region and the current change is below threshold for clear identification.

(iv) The glycoside dose is at the 'watershed' concentration between stimulation and inhibition, the rise in tension being due to the low-dose positive inotropic effect.

The conclusion to be drawn from these results is that all the important features of the experimental results are accurately reproduced by the model. These features include:

1. The fact that the main current change following pump stimulation or inhibition is at very negative potentials. Positive to the resting potential the changes are small and may even be in the opposite direction.

2. The transient nature of the outward current shifts attributed to pump stimulation contrasted with the more permanent nature of the inward current shifts produced by pump inhibition.

3. The fact that small gradual changes may produce little or no overt change in net ionic current.

DISCUSSION

The experiments which we have described are among the first studies of cardiotonic steroid action where measurements of pump activity and tension were undertaken together during the course of a single drug exposure. The results are quite complex and a large number of experiments (seventy-one) was necessary to introduce some degree of order. We shall first summarize a scheme for the various effects we have observed and then discuss the individual components.

The 'established' action of cardiotonic steroids, which may occur at most concentrations and is invariably seen at and above 5×10^{-7} M, is to inhibit the membrane sodium pump. This is accompanied by an increase in twitch and often in voltage-dependent tonic tension. At low cardiotonic steroid levels and in the presence of a sufficiently high $[K]_b$ the majority of fibres show an outward current change that we interpret as due to pump stimulation. If the dose is low enough this may be sustained; at higher doses it is often followed by inhibition. Pump stimulation may be accompanied by a decrease in twitch tension which is probably a direct result of the fall in $[Na]_i$. Alternatively there may be an increase in twitch tension from a causally unrelated mechanism. This general scheme encompasses all the results we have seen. We shall now discuss each aspect in turn.

Sodium pump inhibition

Our results confirm that it is usually necessary to apply more than 2×10^{-7} M cardiotonic steroid to obtain pump inhibition. However, pump inhibition is not always confined to high or even moderate levels of cardiotonic steroid. We have even observed (one experiment) an inward current shift and positive inotropy at 1×10^{-8} M-strophanthidin in 8 mM-K_b. A key to the interpretation of these results is that the fibres showing low-dose inhibition as the only current response all had measurable voltage-dependent *tonic* tension in response to a depolarizing clamp pulse *before* the drug was applied. This suggests a higher resting $[Ca]_i$ and $[Na]_i$. Less reserve pump activity would be available to give rise to stimulation. It is possible that these were fibres which were Ca-loaded from the trauma of dissection or because of deterioration in pump activity with the metabolic decline of ageing. They often showed the development of transient inward currents and after-contractions (Kass, Lederer, Tsien & Weingart, 1978; Kass, Tsien & Weingart, 1978) early during the cardiotonic steroid exposure, which again suggests that these fibres were Ca-loaded.

On the other hand the presence of voltage-dependent tonic tension before cardiotonic steroid exposure did not always indicate that stimulation would not be seen. Fig. 6 is an example where outward current shift occurred in such a fibre and shows that stimulation is still possible in certain of these fibres which may, of course, be less dependent on the sodium pump for control of background $[Na]_i$. Nevertheless, it remains true that, if we eliminate these fibres from consideration, the electrical results of low doses are nearly always opposite to those of high doses.

Sodium pump stimulation

These experiments confirm the results of Cohen *et al.* (1976*b*) and others in showing increased ionic gradients at low doses. Inspection of Table 1 shows that at approximately 2×10^{-7} M current shifts change from outward to inward (e.g. fibres C, D, F, G, K; cf. Fig. 5). If it is assumed that a small degree of net inhibition occurs at higher doses in those fibres with no current change and a positive inotropic effect, then this 'watershed' of around 2×10^{-7} M is seen in all cases. The negative inotropic effect was seen only once at a concentration of 5×10^{-7} M. Our computations also suggest that pump stimulation must surely by now be an accepted effect of low levels of cardiotonic steroids in cardiac muscle and other tissues. Blood (1978) and Noble (1980) have interpreted this in terms of a disinhibition by the drugs of pump sites which are normally inhibited by either steric interaction with neighbouring sites (Blood) or the membrane environment (Noble). The present experiments offer no light on these or alternative possibilities other than to demand an action of low doses additional to inhibition of the sodium pump.

A suggestion made recently is that the increased ionic gradients may be mediated by catecholamines (Hougen & Smith, 1980). The electrical and mechanical effects of catecholamines on the Purkinje fibre are, however, usually in *opposite* directions to the changes seen as a result of low concentrations of cardiotonic steroids (see, for example, Fig. 8). Adrenaline increases the inward current at negative potentials because of a shift in the y_∞ curve in the depolarizing direction (Hauswirth, Noble & Tsien, 1968; Hart, Noble & Shimoni, 1980). The recently reported increase of potassium conductance by adrenaline (Cranefield & Gadsby, 1981) would also increase the inward current at potentials negative to E_K. Thus any outward shift in current due to pump stimulation and reduced cleft [K] would, if catecholamines were involved, need to be sufficiently large to counteract the inward shift at negative voltages due to other effects of the catecholamines before any net outward shift were to be observed. Adrenaline increases twitch tension, probably as a result of a large increase in $\bar{g}_{si}$ (Reuter & Scholz, 1977). The frequent association of a *negative* inotropic effect with outward current shift in our experiments would also argue against the involvement of catecholamines.

Negative inotropic effect

An important result from the experiments is the description of a negative inotropic effect and its relationship with increased ionic gradients. A decrease in tension is what is expected from the 'pump-lag' hypothesis following net pump stimulation (Reuter & Seitz, 1968; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Langer & Serena, 1970; Cohen *et al.* 1976*b*). Poole-Wilson, Galindez & Fry (1979), using 10^{-8}M -ouabain, found a roughly 20% reduction in tension in human atrium after 15 and 30 min, and a

similar reduction in tension in human ventricle and rabbit papillary muscle after 30 min. Negative inotropy has also been found with low doses of ouabain in frog ventricle (F. W. Flitney & J. Singh, personal communication) and in ferret papillary muscle (W. Giles & W. J. Lederer, personal communication). Poole-Wilson *et al.* (1979) have suggested that release of acetylcholine (ACh) may account for the negative inotropy which they found. In most tissues ACh will indeed reduce i_{si}, but the effect on the current–voltage relationship is always an *increase* in inward current at negative potentials (Giles & Noble, 1976; Garnier, Nargeot, Ojeda & Rougier, 1978; Noma & Trautwein, 1978; Hino & Ochi, 1980; Ojeda, Rougier & Tourneur, 1981) which is the opposite to what we have observed with pump stimulation. Furthermore, an increase in ^{42}K efflux was not observed by Poole-Wilson *et al.* (1979) and would be expected from the experiments of Hutter (1957) if ACh release were the mechanism.

Dempsey, McCallum, Kent & Cooper (1971) have also described negative inotropy following 4×10^{-7} M-ouabain in isolated cat papillary muscle, when bulk extracellular [Ca] was less than 0·6 mM. Although we have shown transient negative inotropy in sheep ventricular trabeculae we were unable to confirm their results on lowering $[Ca]_b$ and have no explanation for the discrepancy.

Low-dose positive inotropic effect

We have been unable to find a unifying hypothesis to link increases in tension with changes in membrane currents. Our results confirm the deductions of Blood & Noble (1978) on the need for more than one inotropic response. We have already discussed the inotropy resulting from net pump inhibition; the alternative has been termed the 'low-dose mechanism' because it has been clearly seen only at low doses. It may well, though, exist, but be masked, at high doses. The distinguishing features of this increase in twitch tension are summarized as follows:

(i) It is associated with an outward shift of membrane current. Although this distinguishes it clearly from the 'pump-lag' mechanism pump stimulation is probably not a necessary condition (see below).

(ii) It is of faster onset (2–5 min approximately) than the inotropy of inhibition (10–20 min).

(iii) It is sometimes transient, reversing spontaneously (at least partially) after 5–10 min.

(iv) After wash-off low-dose inotropy always reverses fully, whereas that associated with any more than a minor degree of inward current shift is often only slowly and partially reversible.

(v) The maximum increase in twitch tension seen in association with pump stimulation is an order of magnitude less than is possible in the presence of inhibition.

(vi) No increase in tonic tension has been observed with positive inotropy in the presence of net pump stimulation.

The sole reason that the low-dose mechanism has been so far identified with pump stimulation is that without an outward current shift it is not possible to exclude a rise in $[Na]_i$ and thereby 'pump-lag' as the reason for the increase in tension. For the following reasons pump stimulation need not, however, be causally related:

(i) It is difficult to conceive of a mechanism whereby pump stimulation *per se* could increase twitch tension.

(ii) The dose range over which the low-dose positive inotropic effect was seen is slightly narrower than that for pump stimulation. In particular, pump stimulation (and negative inotropy) was observed on increasing the dose when slightly lower concentrations had give positive inotropy and outward current shift.

(iii) There was no difference between the pattern of tension responses in those fibres showing an early positive inotropic effect without current change and those showing the same early rise in tension with outward current shift.

(iv) The temporal association between the onset of outward current shift and the increase in tension need not, of course, imply a causal link. The onset of both processes may be limited by the rate of cardiotonic steroid binding to the membrane. Fig. 7 is an example of the sort of correlation we have observed. Note that the outward current shift occurs slightly *after* the onset of the rise in tension, and that its reversal is similarly delayed. Reasons for the current change being delayed until after $[Na]_i$ has changed were discussed previously and attributed to slowness of drug binding.

It is probable, then, that we have had to underestimate the frequency of occurrence of the low-dose positive inotropic effect. It would need, for example, the use of a Na-sensitive micro-electrode to distinguish other instances where no current change occurs. One such case may be the tension response in 0·5 mM-Ca_b shown in Fig. 10, where a small rise in tension occurs over the first 8 min to be followed by a much larger rise and pump inhibition. This is exactly similar to the tension rise shown in Fig. 3 of Blood & Noble (1978) and in the first 8 min this rise is probably due to the low-dose mechanism.

As with pump stimulation, some fibres fail to show an early positive inotropic effect (cf. Fig. 2) and this may be related to the initial levels of $[Na]_i$ and $[Ca]_i$. It is clear that lowering $[Ca]_b$ renders the low-dose positive mechanism more likely to occur, although this in itself gives little clue as to its origin. Evidence for a membrane-associated calcium fraction which is released by cardiotonic steroids has been presented by Nayler (1973), Carrier *et al.* (1974), and Gervais, Lane, Anner, Lindenmayer & Schwartz (1977). Our experiments also suggest that it is likely that an additional action of the glycosides in intact myocardial cells is to make more calcium available to the contractile apparatus by a means other than pump inhibition and Na/Ca exchange. This may be linked with a higher-affinity binding site for cardiotonic steroids, evidence for which has been presented by Bentfeld *et al.* (1977) and Erdmann, Philipp & Scholz (1980).

In the clinical situation it is conceivable that any *primary* increase in contractility after glycoside administration may be attributable to the low-dose mechanism, whereas pump inhibition may be more associated with toxic manifestations such as arrhythmias.

We should like to thank the staff of Clays, Buckingham, for their considerable co-operation in providing sheep hearts and especially Taffy James and Len Bagnall. We are grateful to Frank Edwards for help in setting up the computer and to Tony Spindler for his excellent technical support. The dihydro-ouabain was a gift of Hommel AG, Zurich.

G. H. was supported by an MRC Fellowship and a Sidney Perry Junior Research Fellowship at St Peter's College, Oxford. Y.S. was supported by the British Heart Foundation; his present address is Department of Cardiology, Hadassah Medical Centre, Ein Kerem, Jerusalem, Israel. The work was supported by a grant from the British Heart Foundation.

REFERENCES

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

BENTFELD, M., LÜLLMANN, H., PETERS, T. & PROPPE, D. (1977). Interdependence of ion transport and the action of ouabain in heart muscle. *Br. J. Pharmac.* **61**, 19–27.

BLOOD, B. E. (1978). Glycoside induced stimulation of membrane Na–K ATPase – fact or artifact? In *Biophysical Aspects of Cardiac Muscle*, ed. MORAD, M., TABATABAI, T. & SMITH, S. New York & London: Academic Press.

BLOOD, B. E. & NOBLE, D. (1978). Two mechanisms for the inotropic action of ouabain on sheep cardiac Purkinje fiber contractility. In *Biophysical Aspects of Cardiac Muscle*, ed. MORAD, M. TABATABAI, T. & SMITH, S. New York & London: Academic Press.

CARRIER, G. O., LÜLLMANN, H., NEUBAUER, L. & PETERS, T. (1974). The significance of a fast exchanging superficial calcium fraction for the regulation of contractile force in heart muscle. *J. mol. & cell. Cardiol.* **6**, 333–347.

COHEN, I., DAUT, J. & NOBLE, D. (1976*a*). The effects of potassium and temperature on the pace-maker current, i_{K_2}, in Purkinje fibres. *J. Physiol.* **260**, 55–74.

COHEN, I., DAUT, J. & NOBLE, D. (1976*b*). An analysis of the actions of low concentrations of ouabain on membrane currents in Purkinje fibres. *J. Physiol.* **260**, 75–103.

CRANEFIELD, P. F. & GADSBY, D. C. (1981). Isoprenaline increases the potassium permeability of the resting membrane of canine cardiac Purkinje fibres. *J. Physiol.* **318**, 34–35*P*.

DAUT, J. (1980). The effects of dihydro-ouabain on the action potential of cardiac Purkinje fibres. D. Phil. thesis, University of Oxford.

DEITMER, J. W. & ELLIS, D. (1978*a*). Changes in the intracellular sodium activity of sheep heart Purkinje fibres produced by calcium and other divalent cations. *J. Physiol.* **277**, 437–453.

DEITMER, J. W. & ELLIS, D. (1978*b*). The intracellular sodium activity of cardiac Purkinje fibres during inhibition and re-activation of the Na–K pump. *J. Physiol.* **284**, 241–259.

DEMPSEY, P. J., MCCALLUM, Z. T., KENT, K. M. & COOPER, T. (1971). Dissociation of cardiac inotropic and transmembrane action potential effects of ouabain. *J. Pharmac. exp. Ther.* **177**, 78–84.

DIFRANCESCO, D. (1981*a*). A new interpretation of the pace-maker current in calf Purkinje fibres. *J. Physiol.* **314**, 359–376.

DIFRANCESCO, D. (1981*b*). A study of the ionic nature of the pace-maker current in calf Purkinje fibres. *J. Physiol.* **314**, 377–393.

DIFRANCESCO, D. & NOBLE, D. (1980). If 'i_{K_2}' is an inward current, how does it display potassium specificity? *J. Physiol.* **305**, 14*P*–15*P*.

DIFRANCESCO, D. & NOBLE, D. (1981). A model of cardiac electrical activity incorporating restricted extracellular spaces and the sodium–potassium pump. *J. Physiol.* **320**, 25*P*–26*P*.

DIFRANCESCO, D. & NOBLE, D. (1982). Implications of the re-interpretation of i_{K_2} for the modelling of the electrical activity of pacemaker tissues in the heart. In *Cardiac Rate and Rhythm*, ed. BOUMAN, L. N. & JONGSMA, H. J., pp. 93–128. The Hague: Martinus Nijhoff.

EARM, Y., HART, G., NOBLE, D. & SHIMONI, Y. (1980). Simultaneous measurement of tension and voltage-clamp currents in sheep Purkinje fibres at low concentrations of ouabain and strophanthidin. *J. Physiol.* **305**, 63–64*P*.

EDMANDS, R. E., GREENSPAN, K. & FISCH, C. (1967). An electrophysiologic correlate of ouabain inotropy in canine cardiac muscle. *Circulation Res.* **21**, 515–524.

EISNER, D. A. & LEDERER, W. J. (1979). Inotropic and arrhythmogenic effects of potassium-depleted solutions on mammalian cardiac muscle. *J. Physiol.* **294**, 255–277.

EISNER, D. A., LEDERER, W. J. & VAUGHAN-JONES, R. D. (1981). The dependence of sodium pumping and tension on intracellular sodium activity in voltage-clamped sheep Purkinje fibres. *J. Physiol.* **317**, 163–187.

ELLIS, D. (1977). The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibres. *J. Physiol.* **273**, 211–240.

ERDMANN, E., PHILIPP, G. & SCHOLZ, H. (1980). Cardiac glycoside receptor, $(Na^+ + K^+)$-ATPase activity and force of contraction in rat heart. *Biochem. Pharmac.* **29**, 3219–3229.

GADSBY, D. C. (1980). Activation of electrogenic Na^+/K^+ exchange by extracellular K^+ in canine cardiac Purkinje fibres. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4035–4039.

GARNIER, D., NARGEOT, J., OJEDA, C. & ROUGIER, O. (1978). The action of acetylcholine on background conductance in frog atrial trabeculae. *J. Physiol.* **274**, 381–396.

GERVAIS, A., LANE, L. K., ANNER, B. M., LINDENMAYER, G. E. & SCHWARTZ, A. (1977). A possible molecular mechanism of the action of digitalis. *Circulation Res.* **40**, 8–14.

GHYSEL-BURTON, J. & GODFRAIND, T. (1977). Importance of the lactone ring for the action of therapeutic doses of ouabain in guinea-pig atria. *J. Physiol.* **266**, 75–76*P*.

GHYSEL-BURTON, J. & GODFRAIND, T. (1980). Influence of extracellular KCl on the stimulation and the inhibition of the Na–K pump by ouabain. *J. Physiol.* **307**, 65*P*.

GILES, W. & NOBLE, S. J. (1976). Changes in membrane currents in bullfrog atrium produced by acetylcholine. *J. Physiol.* **261**, 103–123.

GODFRAIND, T. & GHYSEL-BURTON, J. (1979). Stimulation and inhibition of the sodium pump by cardiotonic steroids in relation to their binding sites and their inotropic effect on guinea-pig isolated atria. *Br. J. Pharmacol.* **66**, 175–184.

HART, G., NOBLE, D. & SHIMONI, Y. (1980). Adrenaline shifts the voltage dependence of the sodium and potassium components of i_f in sheep Purkinje fibres. *J. Physiol.* **308**, 34–35*P*.

HAUSWIRTH, O., NOBLE, D. & TSIEN, R. W. (1968). Adrenaline: mechanism of action on the pacemaker potential in cardiac Purkinje fibres. *Science, N.Y.* **162**, 916–917.

HINO, N. & OCHI, R. (1980). Effect of acetylcholine on membrane currents in guinea-pig papillary muscle. *J. Physiol.* **307**, 183–197.

HOUGEN, T. J. & SMITH, T. W. (1980). Biphasic effect of cardiac glycosides on the sodium pump: role of catecholamines. *Circulation* **62**, III-987.

HUTTER, O. F. (1957). Mode of action of autonomic transmitters on the heart. *Br. med. Bull.* **13**, 176–180.

ITO, M., HOLLANDER, P. B., MARKS, B. H. & DUTTA, S. (1970). The effects of six cardiac glycosides on the transmembrane potential and contractile characteristics of the right ventricle of guinea pigs. *J. Pharmac. exp. Ther.* **172**, 188–195.

KASS, R. S., LEDERER, W. J., TSIEN, R. W. & WEINGART, R. (1978). Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibres. *J. Physiol.* **281**, 187–208.

KASS, R. S., TSIEN, R. W. & WEINGART, R. (1978). Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibres. *J. Physiol.* **281**, 209–226.

LANGER, G. A. & SERENA, S. D. (1970). Effects of strophanthidin upon contraction and ionic exchange in rabbit ventricular myocardium: relation to control of active state. *J. mol. & cell. Cardiol.* **1**, 65–90.

LEE, C. O., KANG, D. H., SOKOL, J. H. & LEE, K. S. (1980). Relation between intracellular Na ion activity and tension of sheep cardiac Purkinje fibres exposed to dihydro-ouabain. *Biophys. J.* **29**, 315–330.

NAYLER, W. (1973). An effect of ouabain on the superficially-located stores of calcium in cardiac muscle cells. *J. mol. & cell. Cardiol.* **5**, 101–110.

NOBLE, D. (1980). Review: mechanism of action of therapeutic levels of cardiac glycosides. *Cardiovascular Res.* **9**, 495–514.

NOMA, A. & TRAUTWEIN, W. (1978). Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. *Pflügers Arch.* **377**, 193–200.

OJEDA, C., ROUGIER, O. & TOURNEUR, Y. (1981). Effects of Cs on acetylcholine induced current. Is i_{K_1} increased by acetylcholine in frog atrium? *Pflügers Arch.* **391**, 57–59.

PETERS, T., RABEN, R.–H. & WASSERMANN, O. (1974). Evidence for a dissociation between positive inotropic efect and inhibition of the Na^+–K^+–ATPase by ouabain, cassaine and their alkylating derivatives. *Eur. J. Pharmac.* **26**, 166–174.

POOLE-WILSON, P. A., GALINDEZ, E. & FRY, C. H. (1979). Effect of ouabain in therapeutic concentrations on K^+ exchange and contraction of human and rabbit myocardium. *Clin. Sci.* **57**, 415–420.

REUTER, H. & SCHOLZ, H. (1977). A study of the ion selectivity and the kinetic properties of the calcium dependent slow inward current in mammalian cardiac muscle. *J. Physiol.* **264**, 17–47.

REUTER, H. & SEITZ, H. (1968). The dependence of calcium efflux from cardiac muscle on temperature and ionic composition. *J. Physiol.* **195**, 451–470.