J. Physiol. (1976), **263**, *pp.* 73–100
With 11 *text figures*
Printed in Great Britain

TRANSIENT INWARD CURRENT UNDERLYING ARRHYTHMOGENIC EFFECTS OF CARDIOTONIC STEROIDS IN PURKINJE FIBRES

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(*Received 19 December 1975*)

SUMMARY

1. Voltage-clamp experiments were carried out in calf Purkinje fibres to determine the basis of transient depolarizations (TDs) associated with digitalis-induced arrhythmias.

2. Under the influence of strophanthidin, depolarizing clamp pulses were followed by a transient inward current (TI) which was small or absent in untreated preparations. The TI also appeared in the wake of a train of action potentials. It was designated TI because its magnitude and timing were appropriate to account for the TD.

3. Longitudinal voltage non-uniformity during the TI was determined with two voltage-recording micro-electrodes. Although the non-uniformity was not severe, the TI wave form was observed when the voltage difference signal was used to measure membrane current density.

4. Over the diastolic range of potential, the strophanthidin-induced TI appeared superimposed upon the normal pace-maker mechanism, the decay of a potassium current, i_{K_2}. The TI could be dissociated from i_{K_2}, however, by means of its unusual kinetic properties.

5. TIs could also be recorded at holding potentials positive to -55 mV, i.e. outside the range where i_{K_2} deactivation occurs.

6. The TI amplitude showed a slow and strongly sigmoid dependence on the duration of the preceding depolarizing pulse. Stronger depolarizations increased the TI amplitude and speeded its development. Cooling reduced the TI amplitude, while slowing and exaggerating the sigmoid time-dependence.

7. Two clamp pulses in close succession gave additive effects in evoking a subsequent TI. This finding and the sigmoid time-dependence fit with previous observations that TDs are most prominent following a series of closely spaced action potentials.

8. The TI can help generate spontaneous depolarizations in preparations showing the 'low voltage oscillations' which often occur with advanced digitalis toxicity.

INTRODUCTION

When Purkinje fibres are exposed to ouabain or other cardiotonic steroids they develop an enhanced pace-maker potential (Dudel & Trautwein, 1958; Vassalle, Karis & Hoffman, 1962). The increase in diastolic depolarization may lead to spontaneous activity in isolated Purkinje tissue and this effect appears closely correlated with the early stages of digitalis toxicity in intact animals (Rosen, Gelband & Hoffman, 1973). In contrast to Purkinje fibres, isolated ventricular muscle preparations are less prone to the development of automaticity, although this is possible (see Ferrier, 1976). Ventricular muscle is also far less susceptible to the other effects of cardiotonic steroids, such as loss of resting potential, change in action potential duration (Vassalle *et al.* 1962; Kassebaum, 1963) or inhibition of active ion transport (Polimeni & Vassalle, 1971). The greater sensitivity of the specialized conducting tissue suggests that digitalis may produce ventricular extrasystoles and other arrhythmias through its action on the His–Purkinje system.

To understand the basis for the positive chronotropic effect of cardiotonic steroids, it is important to know their action on membrane currents. Recent voltage-clamp experiments (Aronson, Gelles & Hoffman, 1973; Isenberg & Trautwein, 1974) suggest two different mechanisms for the increase in automaticity.

Modification of the pace-maker potassium current. In untreated Purkinje fibres, the pace-maker potential is controlled by the slow decay of a potassium current, i_{K_2}. Aronson *et al.* (1973) found that ouabain reduced the magnitude of i_{K_2}, without altering the voltage dependence of its activation. They suggested that ouabain promotes spontaneous activity by decreasing outward potassium current over the pace-maker range of potentials.

Inhibition of electrogenic sodium transport. Isenberg & Trautwein (1974) found that ouabain or its dihydro-derivative can promptly reduce time-independent outward current. They attributed the effect of electrogenic sodium pumping. Although no attempt was made to explain ouabain-induced automaticity, Isenberg & Trautwein's results are relevant since the fall in outward current should have the same effect as applied depolarizing current in promoting diastolic depolarization (Trautwein & Kassebaum, 1961).

Transient influx of calcium ions. This hypothetical mechanism was not considered in the previous voltage-clamp papers, but was suggested by

Ferrier & Moe (1973) in studying the influence of acetylstrophanthidin on electrical activity. They found that the drug-enhanced diastolic depolarization was directly dependent on the extracellular concentration of calcium, $[Ca]_o$, and that it could be inhibited by manganese ion. They suggested that digitalis-like compounds induce a current flow, probably carried by Ca^{2+} ions, which does not participate in the normal pace-maker. This idea was consistent with earlier observations indicating that the after-potential induced by cardiotonic steroids differed qualitatively from normal pace-maker activity in its enhancement by preceding action potentials (Gandel, Wittenberg, Hogan & Klocke, 1970; Davis, 1973; Rosen, Gelband, Merker & Hoffman, 1973; Ferrier, Saunders & Mendez, 1973).

Ferrier & Moe's hypothesis is particularly interesting since it involves a novel ionic basis for the spontaneous depolarization, rather than a modification of the pre-existing pace-maker process. Our voltage-clamp experiments have, in fact, revealed a transient inward current (TI) that is 'induced' or at least enormously enhanced by cardiotonic steroids. Ion replacement experiments described in a later paper (R. S. Kass, W. J. Lederer, R. W. Tsien & R. Weingart, to be published) will be concerned with the ionic basis of the TI. The present article will describe some of the component's time, and voltage-dependent properties. These kinetic properties allow the TI to dominate the primary arrhythmogenic effect of digitalis-like compounds in Purkinje fibres.

Some of the results have already been briefly reported (Lederer & Tsien, 1975).

METHODS

Experimental procedure. Purkinje fibre bundles were taken from both ventricles of calf hearts. Shortened preparations (1–2 mm) were voltage clamped using the two micro-electrode method of Deck, Kern & Tautwein (1964) with minor modifications (Tsien, 1974*a*). No diastolic extension was applied (cf. Ferrier, 1976). In some experiments (Fig. 4) a third intracellular micro-electrode was used to determine the degree of voltage non-uniformity. The longitudinal voltage difference provided a measure of the membrane current density (Adrian, Chandler & Hodgkin, 1970) and a check on the measurement of total current (Kass & Tsien, 1975).

Solutions and drugs. The modified Tyrode solution had the following ionic composition (mM): 150 Na, 4 K, 5·4 Ca, 0·5 Mg, 10 Tris-maleate (pH 7·2–7·4), 155·8 Cl. The solutions also contained 5 mM glucose and were pre-gassed with 100% O_2. Experiments were carried out between 35 and 37° C except in Fig. 7. During a given voltage-clamp run the temperature was held constant to within $\pm 0{\cdot}2°$ C.

In most of the experiments, strophanthidin (Sigma) was used as the cardiotonic agent. The aglycone is known for its rapid onset of action and ready reversibility in red cells (Sachs, 1974). In Purkinje fibres, the effects of strophanthidin developed over the course of an hour or so, then remained stable over periods long enough to carry out extensive voltage-clamp procedures. Ouabain (Sigma) also evoked TIs but seemed less suitable because it gave progressive increases in the level of toxicity.

RESULTS

Influence of strophanthidin on pace-maker activity

Fig. 1 illustrates the effects of strophanthidin on electrical activity and underlying changes in membrane current. The upper trace in each panel shows a train of ten action potentials, evoked by external stimuli

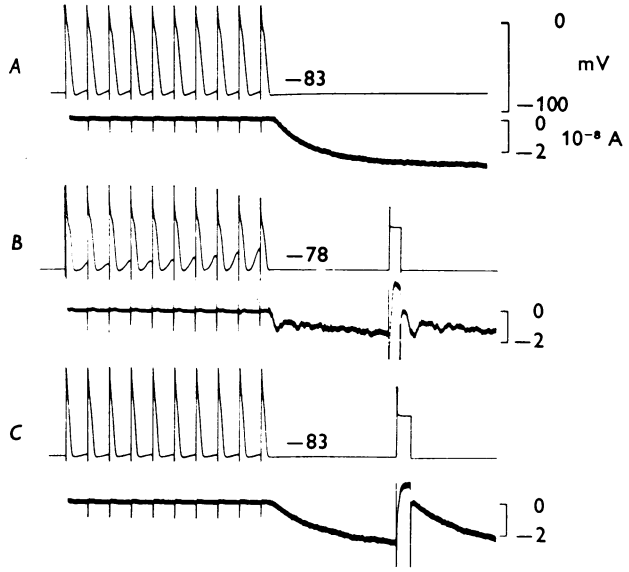

Fig. 1. Effect of 1 μM strophanthidin on electrical activity and membrane current over the pace-maker range of potentials. Each panel shows chart recordings of membrane potential (above) and total membrane current (below). *A*, a train of action potentials was stimulated by external shocks at 0·5 Hz after a rest period of 25 seconds. Voltage-clamp control was imposed following the tenth action potential at the point of maximum repolarization. *B*, depolarizing voltage pulses 47 mV in amplitude lasting about 1 sec were applied in the strophanthidin run of 27 min and later, in *C*, the recovery run, and show respectively the occurrence of the transient inward current (TI) phenomenon and its disappearance after removal of the drug. The increase in current 'noise' seen in the strophanthidin run is also reversible. Preparation 127–2; 35° C; Ca_o = 5·4 mM; total capacitance = 0·063 μF.

at 0·5 Hz. Exposure to strophanthidin (Fig. 1*B*) produced a less negative maximum diastolic potential and also enhanced the diastolic depolarization. Similar enhancement has been observed previously using other cardiotonic steroids (Hogan, Wittenberg & Klocke, 1973; Davis, 1973; Rosen *et al.* 1973; Ferrier *et al.* 1973). These studies demonstrated that cardiotonic steroid-induced pace-maker activity differs from the normal diastolic

depolarization in its striking dependence on preceding activity. In Fig. 1*B*, this beat-dependence appears as a progressive steepening of the diastolic depolarization with successive action potentials. With more rapid stimulation or greater intoxication, the pace-maker potentials can reach threshold and produce spontaneous impulses.

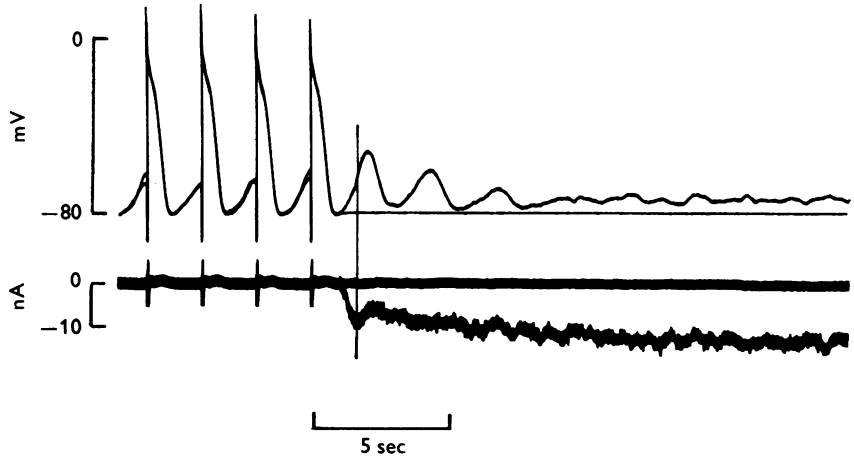

Fig. 2. The transient depolarization (TD) and the TI: a comparison of timing and magnitude. The two superimposed records are from the same experiment shown in Fig. 3.1. The upper panel shows the two records of membrane potential, while the lower panel shows the two corresponding current records. After the tenth action potential of the first run, external stimulation was discontinued and voltage-clamp control was not imposed, allowing the development of transient depolarizations. Following the tenth action potential in the next series, the membrane potential was clamped at the maximum diastolic potential. The vertical bar runs through the peak of the TI and shows its relationship to the TD. The magnitude of the TI is 10 nA and the maximum rate of rise of the TD is 0·06 V/sec.

The right side of Fig. 1 shows the effect of strophanthidin on time-dependent current over the pace-maker range of potentials. In each run, voltage clamp was imposed at the point of maximum repolarization of the last action potential in the train (cf. Vassalle, 1966). Clamping the potential at the maximum diastolic potential resulted in slow changes in membrane current (lower trace in each panel), revealing the processes which generate the diastolic depolarization. In the absence of strophanthidin (Fig. 1*A*, *C*) the current developed slowly and smoothly toward a steady inward level. The time dependence reflects the shutting-off of an outward potassium current, i_{K_2} (Deck & Trautwein, 1964; Vassalle, 1966; Noble & Tsien, 1968).

Strophanthidin-induced transient inward current

Exposure to strophanthidin altered the membrane current in a number of ways (Fig. 1*B*). The most obvious effect was the development of an inward bump on the current record. The TI appeared not long after the clamp had been imposed, and it seemed to be superimposed on a progressive development of net inward current, qualitatively similar to that seen in Fig. 1*A* or *C*. The TI was also recorded in the wake of a depolarizing voltage pulse (*B*). A similar clamp pulse was applied in *C*, after removal of the drug, but did not produce any noticeable inward current transient.

The results in Fig. 1 suggest that the enhancement of the diastolic depolarization might be mediated by the transient inward current. This possibility is examined more closely in Fig. 2, which compares the time course of the TI with the changes in membrane potential which took place when the voltage clamp was not imposed. The thin vertical line marks the peak of the TI. It is evident that the timing of the peak corresponds rather well with the steepest portion of the first transient depolarization following the action potential train. The voltage trace also shows further oscillations in membrane potential but these were not synchronous with any overt change in the membrane current under voltage clamp. No exact correspondence is to be expected. In the case where membrane potential is allowed to vary, each oscillation presumably induces currents which in turn promote the next depolarization. Under voltage clamp, such a sequence fails to take place because repeated depolarizations are prevented.

Is the transient current sufficiently large to account for the enhanced diastolic depolarization? Under conditions of spatial uniformity, the net ionic current (i_1) and the rate of depolarization ($\dot{V}$) should be related by the equation

$$-i_1/c_m = \dot{V}. \tag{1}$$

The capacitance, c_m, may be taken as the total preparation capacitance since the voltage changes are slow enough to be felt by cleft membranes as well as the surface membrane (Fozzard, 1966; Mobley & Page, 1972). To introduce experimentally measured values, it is useful to introduce an approximate version of eqn. (1), namely

$$-i_{peak}/c_m \doteqdot \dot{V}_{max}. \tag{2}$$

Some degree of approximation must enter in because the amplitude of the inward transient (i_{peak}) gives the ionic current at the maximum diastolic potential (-79 mV in this case) while the maximum rate of depolarization occurs at a less negative potential (near -65 mV).

In the present experiment, the measured values were $c_m = 0{\cdot}063\,\mu F$ $-i_{peak} = 10^{-8}\,A$, and $\dot{V}_{max} = 0{\cdot}06\,V/sec$. Since $(10^{-8}\,A)/(0{\cdot}063\,\mu F) = 0{\cdot}16\,V/sec$, the eqn. (2) does hold approximately. At least part of the discrepancy could arise if the resting membrane conductance gave outward current at -65 mV which partially offset the TI. It seems reasonable to conclude, therefore, that TI is appropriate in magnitude, as well as timing, to generate the observed increase in diastolic depolarization.

Ferrier *et al.* (1973) used the term TD (for 'transient depolarization') in describing the depolarizing after potential produced by acetylstrophanthidin and other cardiotonic steroids. We will follow this precedent by using the term TI to refer to the transient inward current induced by strophanthidin.

Other effects of strophanthidin

The results so far support Ferrier & Moe's hypothesis that cardiotonic steroids enhance pace-maker activity by inducing ionic current changes which do not participate in normal activity. This leaves open the question of whether the normally functional ionic components were altered as well. The records in Fig. 1 might be taken as evidence that the slow decay of the pace-maker potassium current had been diminished by strophanthidin. This would be consistent with an earlier report (Aronson *et al.* 1973) that ouabain reduces the magnitude of i_{K_2}. Note, however, that the clamp potential in Fig. 1 varied with the maximum diastolic potential. Since i_{K_2} is steeply voltage-dependent (Noble & Tsien, 1968; Tsien, 1974*a*), the effect of strophanthidin must be distinguished from the effect of membrane potential *per se*.

Fig. 3 compares current traces recorded at the same potential (-79 mV), in the absence of drug (*A* and *B*, *a*) and during the course of exposure to strophanthidin (*A* and *B*, *b–d*). Each of the current records was obtained by imposing the voltage clamp after a train of ten action potentials, as in Fig. 1. Fig. 3*A* shows the last three action potentials in the trains which preceded the corresponding current records (*B*, *a–d*). The voltage records show the progressive change in maximum diastolic potential as well as the development of enhanced pace-maker depolarization.

Altered time-independent current. The various effects of strophanthidin are more readily distinguished by examining the current records. Fig. 3*B* (*b–d*) may be compared with the smooth curves, which are tracings of the control trace (Fig. 3*B*, *a*). Fig. 3*B*(*b*) shows the first effect of strophanthidin, a downward displacement in the current record, with little alteration in the time course of the slow decay. The change in the time-independent background current is consistent with the early effect of dihydro-ouabain, which Isenberg & Trautwein (1974) have attributed

to reduction in outward current carried by the electrogenic sodium pump. Further downward displacement of the current race took place as the drug exposure was continued (Fig. 3*B*, *c*, *d*). This may reflect an increasing degree of pump inhibition, or possibly, changes in the driving force or conductance for passive ionic currents (see Cohen, Daut & Noble, 1976).

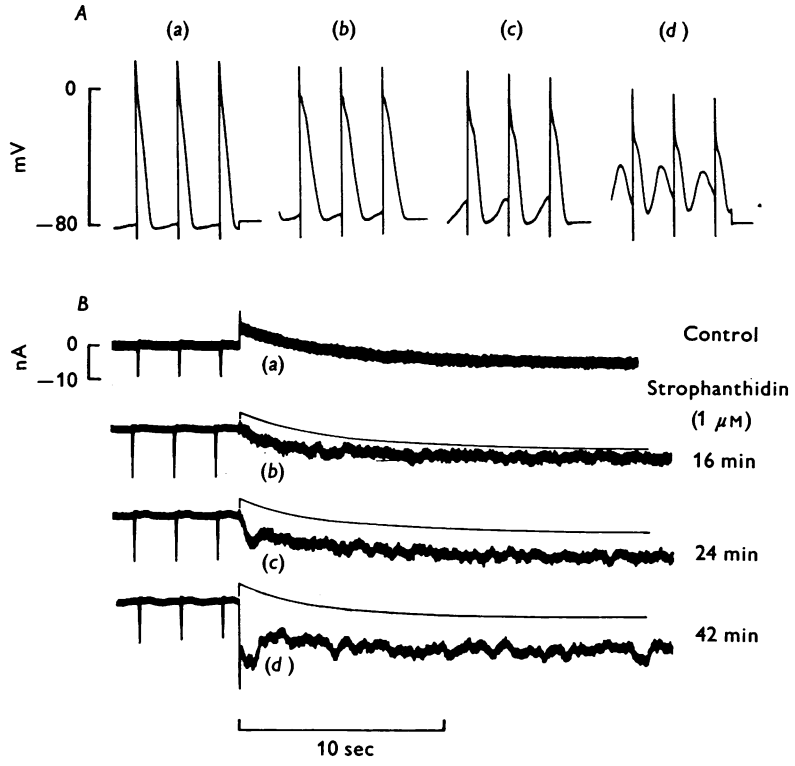

Fig. 3. Progressive effect of strophanthidin on electrical activity and on membrane current. *A* shows the last three action potentials in a series of ten from the same experiment shown in Fig. 3·1. *B*, current records correspond with the action potential records as indicated by the appropriate letters. The action potentials show the usual change in duration and plateau height with progressive intoxication. *A* (*a*) the control record was taken after the strophanthidin had been washed off. There is a marked increase in the diastolic depolarization from *A* (*a*) to (*c*), and in *A* (*d*), TDs are produced following each action potential. Following the last action in a series, the membrane potential was clamped to −79 mV, thus permitting the comparison of the various current records. The control current record from *B* (*a*) has been drawn as a fine line superimposed on the current records of the strophanthidin runs. Three clear effects can be seen developing from *B* (*a*) to (*d*): reduction in outward current; growth of the TI; and increase in current 'noise'.

Fig. 3*B*(*c*) gives the first clear indication of the TI in this set of records. Here, the TI appears as a discrete bump, superimposed on the usual slow time-dependent change. If the TI is set aside, the remaining current record does not show any clear change in the magnitude or time course of the i_{K_2} decay. Despite the absence of such a change, the voltage record (Fig. 3*A*, *c*) displays an obvious increase in diastolic depolarization. The present results differ, therefore, with the view of Aronson *et al.* (1973). These workers attributed the acceleratory effect of ouabain to a diminution of i_{K_2} which would favour slow depolarization by unmasking inward backward current.

A possible explanation of the discrepancy is provided by current trace (Fig. 3*B*, *d*). Here, the toxic effect has developed to the point where it is no longer clear whether or not i_{K_2} has been altered. Fig. 3*B*(*d*) might be interpreted as a reduction in the i_{K_2} decay tail (cf. Aronson *et al.* 1973). Alternatively, it is also possible that the i_{K_2} decay has simply been masked by a rather large TI.

Strophanthidin-induced current fluctuations. The current records in Fig. 3 show a progressive increase in 'noise' during the course of strophanthidin exposure. Full recovery was obtained after removing the drug (compare traces *A* and *C* in Fig. 1). The current 'noise' recorded under voltage clamp corresponds to fluctuations in membrane potential in the absence of voltage clamp (see voltage trace in Fig. 2).

Strophanthidin-induced current fluctuations were clearly observed in most experiments, although the magnitude was somewhat variable. Analysis of the fluctuations in terms of random elementary events (cf. Katz & Miledi, 1972) indicate that the hypothetical unit event is much too large to be accounted for by all-or-nothing blockade of individual sodium pump sites by strophanthidin molecules (Kass, Lederer & Tsien, 1976).

Is the transient inward current an artifact?

It is important at this stage to consider the question of whether the TI is a genuine membrane current. Among the possible sources of error, the most likely possibilities are some sort of mechanical artifact, or an artifact arising from electrical non-uniformity.

Movement artifact? In principle, movement of the micro-electrode tip could give rise to an erroneous voltage signal that would result in an artifactual current wave form under voltage clamp. Visual observations with the dissecting microscope argue against this idea. Large TIs are accompanied by clear after-contractions (cf. Ferrier, 1976) but small TIs produce no visually detectable movement. This observation has been extended by tension recordings in voltage-clamped Purkinje bundles

(R. S. Kass and R. W. Tsien, in preparation). In those cases where the TI is accompanied by an after-contraction, it remains unlikely that the mechanical activity introduces significant error in the observed membrane current. The after-contraction which accompanies the TI is usually much smaller than the twitch during the 'on' of a depolarizing clamp pulse; yet, there is no hint of a TI-like wave form in the current signal at the time of the twitch. Thus, movement artifacts cannot be a general explanation of the TI phenomenon.

Voltage non-uniformity? Another important question is the adequacy of the two micro-electrode voltage-clamp method in measuring the TI. Voltage non-uniformity may invalidate measurements of large, rapid membrane currents (Johnson & Lieberman, 1971), although it may not be serious when studying small, slowly changing membrane currents (Mobley & Page, 1972; Hellam & Studt, 1974). In approaching this problem, we have determined longitudinal non-uniformity during the TI by using the three micro-electrode voltage-clamp technique of Adrian *et al.* (1970). A shortened Purkinje fibre bundle was impaled at two points by voltage recording micro-electrodes, V_1 and V_2 (see inset, Fig. 4). The voltage difference, $V_2 - V_1$, was recorded at high gain, and provides a direct indication of voltage non-uniformity along the length of the preparation. It also serves as a measure of membrane current density at V_1, the point of voltage control. Cable theory for both linear and non-linear membrane characteristics has shown that $(V_2 - V_1)$ is a remarkably good measure of membrane event, i_m, one that is less susceptible to error than the total applied current (I_T). Agreement between these two measures gives a useful indication of their validity (Kass & Tsien, 1975).

Fig. 4 shows simultaneous recordings of $(V_2 - V_1)$ and I_T in a preparation that had been exposed to 2 μM strophanthidin. The upper trace shows the voltage signal V_1. A 4 sec depolarizing pulse to -10 mV was imposed from a holding potential of -50 mV. The middle trace is the $(V_2 - V_1)$ signal and the lower trace is I_T. Both records show a clear TI following the depolarizing pulse, with good agreement in the TI time course. The amplitude of the transient I_T signal (73 nA) also agreed rather well with the amplitude determined from the $(V_2 - V_1)$ record (3 mV $\equiv$ 63 nA). The conversion factor from voltage to current (1 mV $\equiv$ 21 nA) was obtained by measuring the slow current changes during the clamp pulse itself.

The conditions of this experiment were deliberately arranged to produce the worst case for voltage non-uniformity. The TI magnitude was unusually large, and the preparation length (2·14 mm) was longer than that used in other experiments. Nevertheless, the variation in $(V_2 - V_1)$ during the TI was only 3 mV, less than 10 % of the variation in V_1 during the pulse.

The conclusion is that the voltage non-uniformity along the fibre length is large enough to allow measurement of the TI using $(V_2 - V_1)$ but not so great as to invalidate the results using I_T.

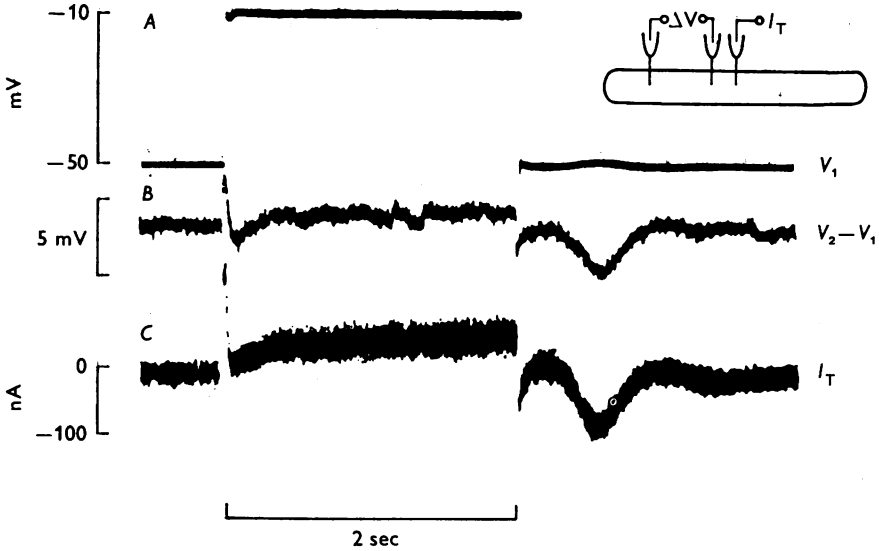

Fig. 4. Measurement of longitudinal voltage decrement during the TI. As the inset indicates, a Purkinje fibre preparation was impaled by three micro-electrodes, labelled V_1, V_2 and I_T (from left to right). *A*, the potential V_1 was controlled by the clamp feed-back circuit, which applied current I_T (*C*). *B*, the voltage difference between V_2 and V_1 is small, indicating that longitudinal voltage non-uniformity is not very serious. However, the $(V_2 - V_1)$ trace does exhibit the wave form of the TI. Over-all preparation length, 2·14 mm. Distance from end of preparation to V_1, 0·36 mm; from V_1 to V_2, 0·50 mm; from V_2 to I_T, 0·21 mm, 2 μM strophanthidin, preparation 150–1, apparent cylindrical area of cell core, 0·0096 cm².

Kinetics of the strophanthidin-induced transient inward current

The evidence presented so far indicates that strophanthidin enhances pace-maker activity principally by inducing a TI. It seemed worth while, therefore, to explore the kinetic requirements for evoking the TI phenomenon. We carried out voltage-clamp experiments to characterize the relationship between the TI and the preceding history of membrane potential, to answer a number of basic questions:

(1) how does the TI vary with the strength and duration of the preceding depolarization? How do the kinetics compare with the kinetics of known current components?

(2) does the TI require action potentials, or, for example, the flow of the excitatory current which accompanies action potentials?

(3) do the kinetic properties of the TI account for previous observations (Davis, 1973; Ferrier *et al.* 1973; Fig. 1) showing the importance of the preceding pattern of activity in eliciting transient depolarizations?

The kinetics of the TI were studied by applying a series of rectangular clamp pulses of varying duration and magnitude. Successive pulses were separated by relatively long intervals (> 10 sec) to ensure that each TI would only be influenced by the immediately preceding clamp pulse but not earlier pulses. This point will be substantiated later (Fig. 11). The use of clamp pulses is somewhat analogous to the procedure of stimulating action potential trains, separated by long rest intervals.

Clamp pulses were applied from a fixed holding potential. In some runs, as in Fig. 1, we employed a relatively negative holding potential (-80 to -60 mV) which keeps the voltage-clamp experiment close to the conditions during the action potential studies. The disadvantage of negative holding potentials is that clamp pulses are invariably followed by the decay of the pace-maker potassium current, i_{K_2}, as well as the TI. This combination of current charges makes it more difficult to perform quantitative analysis. It was convenient, therefore, to carry out some of the kinetic studies with a less negative holding potential, outside the normal diastolic range. Interference from the pace-maker potassium current was avoided, since i_{K_2} remains fully activated at potentials positive to about -50 mV (Noble & Tsien, 1968).

Fig. 5 shows records from an experiment where the kinetics were studied from a holding potential of -41 mV. The left-hand column illustrates current traces for 1 sec clamp pulses to several potential levels within the plateau range. These pulses were too brief to be very effective in eliciting TIs, although some inward transient can be clearly seen for the strongest pulse shown. The right-hand column shows the effect of longer-lasting voltage pulses (5 sec). Here a clear TI is observed for even the weakest clamp depolarization. Increasing the strength of the depolarization from -24 to $+2$ mV enhanced the TI enormously (changes in amplification were needed to keep the current records from going offscale). The increases in the magnitude of the TI are accompanied by a shorter delay in attaining the inward peak, decreasing from 1·4 sec (lower right) to 0·65 sec (upper right).

The records in Fig. 5 show an inward surge of current during the clamp depolarization itself due to the secondary ('slow') inward current (Reuter, 1967; Vitek & Trautwein, 1971). Other current components were largely eliminated by the use of the relatively depolarized holding potential. The fast sodium current is fully inactivated (Weidmann, 1955) and the transient outward chloride current (Dudel, Peper, Rüdel & Trautwein, 1967) is largely inactivated (Fozzard & Hiraoka, 1973, Fig. 10). Thus,

with regard to the second question above, the results indicate that the TI can be evoked independently of at least some of the currents which flow during normal action potentials. This conclusion is supported by ion

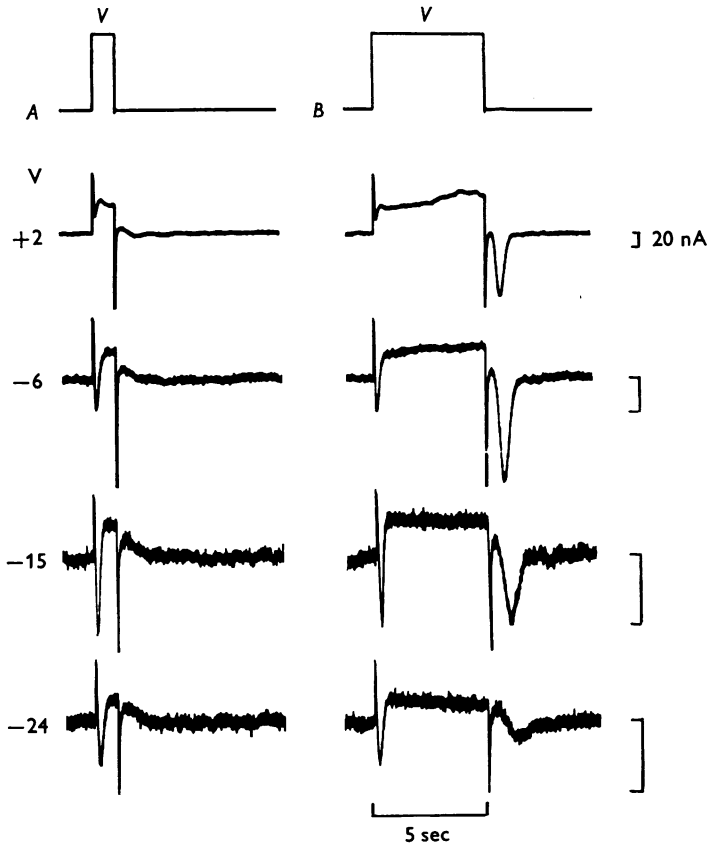

Fig. 5. Effects of depolarizing voltage pulse duration and magnitude on TI size. From a holding potential of -41 mV depolarizing voltage-clamp pulses were imposed for either: A, 1 sec; or B, 5 sec. The range of depolarization ranged from -24 to $+2$ mV as is indicated on the left-hand side of the Figure. The size of the resulting TIs were measured and plotted in Fig. 3·6 along with information from voltage pulses of different durations. Note the variation in scale in the different current records (vertical bar gives 20 nA). 1 μM strophanthidin. Preparation 148–1.

replacement experiments (Kass *et al.* to be published). However, the evidence does not rule out the possibility that sodium or chloride currents, i_{Na} or i_{Cl}, influence the development of the toxic state over a longer period.

Analysis of this experiment was performed by measuring TI amplitude relative to the current base line (see Fig. 5B, top). Fig. 6 shows these

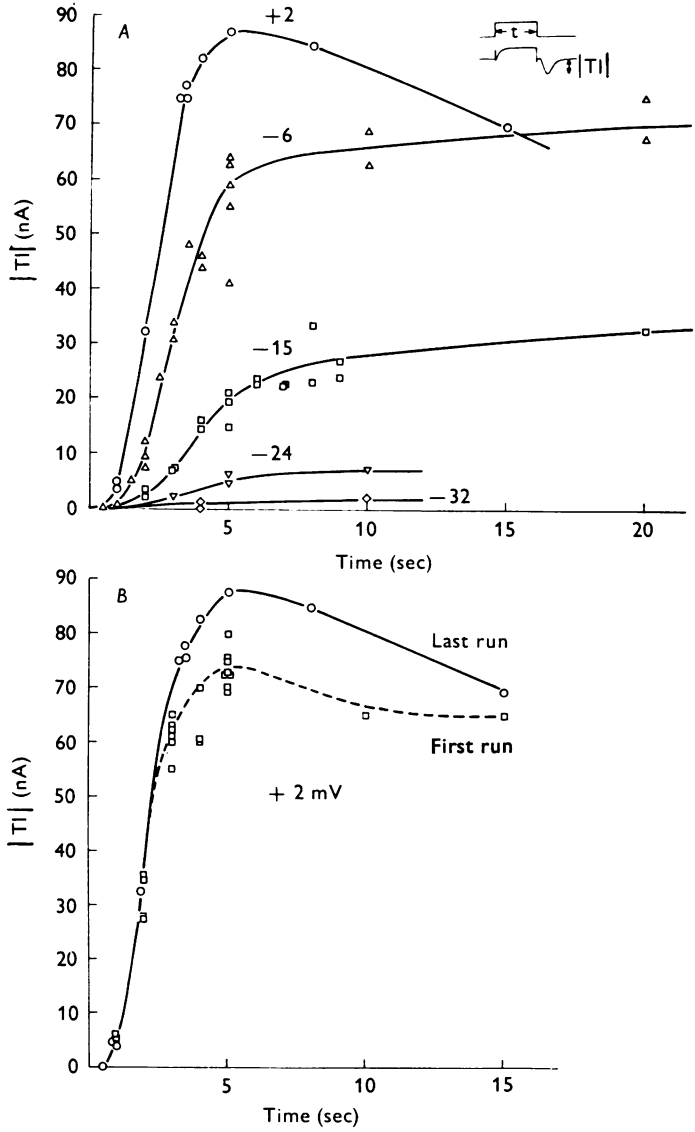

Fig. 6. *A*, graph of TI magnitude as a function of voltage-clamp pulse length (*t*) for five different depolarizing potentials. The inset schematically reveals the method of analysis. From a holding potential of −41 mV, the membrane was depolarized to a potential between −32 and +2 mV for periods up to 20 sec. The resulting TIs were measured and plotted as a function of pulse duration. Sample records are shown in Fig. 5. The five runs were carried out over a period of 22 min.

Fig. 6. *B*, bracketing runs which define the extent of ongoing toxicity during this experiment. Square symbols show data from an earlier run, preceding those illustrated in the top panel. Circles show results obtained in the last run of the series. Although the agreement is not perfect, progressive intoxication is not a serious problem. 1 μM strophanthidin; preparation 148–1.

measurements, and others from the same experiment. The amplitude of the TI is plotted (ordinate) as a function of the duration of the preceding clamp pulse (abscissa). Each increase of the TI is at a particular potential within the plateau range. The increase in TI amplitude follows a sigmoid time course in all cases. Increasing the level of the depolarization speeds the growth of the TI and increases the maximum amplitude that is reached.

The family of curves in Fig. 6 was obtained over a period of 19 min and it is fair to ask whether the results were significantly affected by a progressive increase in the degree of toxicity. This point was checked by bracketing the other runs with two runs at +2 mV (Fig. 6*B*). Some increase in TI amplitude is evident but the time course of development is hardly changed.

The sigmoid onset of the TI was a consistent finding in all of the kinetic experiments, although there were quantitative variations in the amount of delay which may have been due to differences in temperature or the degree of toxicity. The sigmoid time course is noteworthy, because it would seem to distinguish the TI from other slow time-dependent processes in Purkinje fibres, which develop exponentially with time (Noble & Tsien, 1968, 1969). The first question, of course, is whether the sigmoid onset is genuine. Interference from other time-dependent current components must be considered, since this has been misleading in the past (McAllister & Noble, 1966; Noble & Tsien, 1968).

In the present case, interference from other current components was reduced by avoiding potentials where i_{K_2} is dominant. A small 'tail' of outward current was present, however, due to the decay of i_x (Fig. 5*A*, lower traces). Since the measurements were made relative to the current base line, the presence of some outward i_x would be expected to cause a corresponding underestimate of the TI. Fortunately, the magnitude of the error is probably quite small, of the order of a few nA. Some distortion of the kinetic curve may have occurred in Fig. 6 but not enough to account for the marked sigmoidicity observed.

Effect of temperature. Further evidence on the delayed onset is provided by considering the kinetics at lower temperature. Fig. 7*A* shows current records from another preparation, obtained at two temperatures. At 37·5° C, the results were qualitatively similar to those illustrated in Figs. 5 and 6. Some TI appeared after a 1 sec pulse and the TI amplitude increased if the pulse was prolonged. There was a slow increase in net outward current during the pulse due to the onset of i_x (Noble & Tsien, 1969), once again raising the possibility that the decay of i_x may have given outward tail current which in turn caused an underestimate of the TI amplitude.

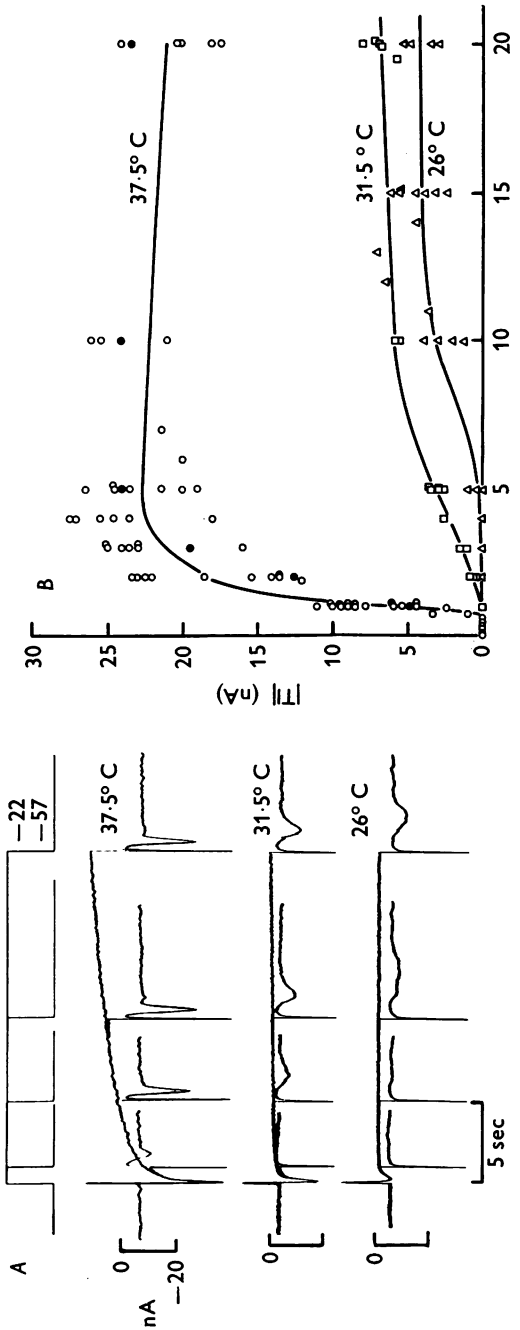

Fig. 7. Effect of temperature on the development of the TI. *A*, shows superimposed current records accompanying depolarizing pulses of varying duration from a holding potential of −57 to −22 mV. *B*, graph plots data from the same experiment, including the results illustrated in the left panels. TI magnitude was measured with respect to the steady current base line and plotted as a function of the duration of the preceeding clamp depolarization. The first run was at 37·5° C (open circles), the second run at 26° C (triangles); the third run at 31·5° C (squares) and the last run at 37·5° C (filled circles). 0·5 μM strophanthidin throughout; preparation J150–1.

This difficulty can be avoided in analysing the results at 26° C. Here, the time-dependent currents during the clamp pulse are largely suppressed. No slow increase in i_x appeared during the 20 sec clamp pulse and no hint of an outward decay tail could be detected after any of the clamp pulses. Under these more favourable circumstances, the delayed appearance of the TI is clear and unambiguous. There is no TI following a 5 sec pulse and a clear TI after the 20 sec pulse.

Analysis from this experiment is illustrated in Fig. 7*B*. Results are included from a run at 31·5° C to show the graded effect of temperature. Cooling markedly delays the appearance of the TI and reduces the maximal amplitude for a very long clamp depolarization.

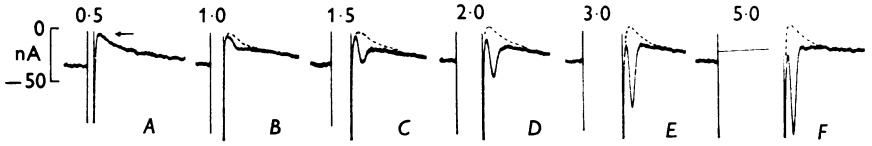

Fig. 8. Separation of TI from a potassium current, i_{K_2}, by varying pulse duration. From a holding potential of −61 mV depolarizing voltage-clamp pulses to +6 mV were imposed for varying periods from 0·3 to 5·0 sec. *A*, (0·5 sec) reflects the deactivation of i_{K_2} without any superimposed TI. The arrow indicates the magnitude of the current tail for a 0·3 sec voltage-clamp pulse. *B–F*, the 0·5 sec TI-free record is superimposed as a dashed line on the current records, following longer depolarizing pulses. As the TI grows larger, it appears to mask the decaying tail of i_{K_2}. 1 μM strophanthidin; preparation 148–1.

Separating the TI from the normal pace-maker mechanism

The slow time course of TI induction provides a convenient method for separating the contributions of the TI and i_{K_2} to current changes over the pace-maker range of potential. Unlike the TI, i_{K_2} turns on rapidly in the plateau range, reaching full activation within 100 msec or so (McAllister & Noble, 1966). Assuming that this characteristic is not seriously altered by strophanthidin, it should be possible to choose a clamp pulse duration long enough to fully activate i_{K_2}, but brief enough not to induce any TI. Obtaining a relatively pure record of i_{K_2} should then allow the quantitative analysis of current records following longer clamp pulses, where the TI and i_{K_2} overlap.

Varying pulse duration. Fig. 8 illustrates this approach using the same preparation as in Figs. 5 and 6. In this part of the experiment, current records were obtained with a holding potential of −61 mV, which was negative enough to cause some i_{K_2} deactivation following a clamp pulse. The membrane was depolarized to +6 mV for varying durations. A

500 msec clamp pulse (Fig. 8*A*) was followed by an exponentially decaying outward tail. An even briefer clamp pulse (300 msec) gave an outward tail of equal amplitude (arrow), thus establishing that i_{K_2} was fully activated at the beginning of the decay tail. There is no trace of any TI in Fig. 8*A*, as expected from the results in Fig. 6.

The TI did appear, however, following longer clamp pulses (Fig. 8*B–F*). This may best be seen by comparing the actual current records with the dashed trace, a replica of the decay in Fig. 8*A*. The difference represents the putative contribution of the TI and it is clear that this contribution increased progressively as the voltage pulse was prolonged. Analysis of the TI amplitude for these and other records gives a measure of the kinetics of onset. The results agree qualitatively with those results already presented (Fig. 6) in showing a markedly sigmoid development with time. The onset was more rapid for the TIs shown in Fig. 8 but this was probably due to a progressive increase in toxicity during the course of the exposure to strophanthidin, rather than any systematic effect of holding potential *per se*.

Varying pulse level. Another way of distinguishing the TI from the decay of i_{K_2} relies upon their voltage dependence. In the steady state, i_{K_2} varies from fully deactivated to fully activated over the potential range between -90 and -50 or -60 mV (Noble & Tsien, 1968).

Depolarization beyond -50 mV produces no greater degree of activation. On the other hand, the turning on of the TI requires depolarizations well into the plateau range, that is, beyond -50 mV. This difference suggests that the TI may be separated from changes in i_{K_2} by varying clamp pulse level.

Fig. 9 illustrates this approach. The upper panel shows an experiment in a strophanthidin-treated preparation, using clamp depolarizations from a holding potential within the pace-maker range. Following depolarizations to -40 or -22 mV, step repolarization to the holding potential (-72 mV) produced a simple exponential decay of i_{K_2}. The amplitude of the decay tail is the same in both cases, indicating that full activation of i_{K_2} had been achieved before the repolarizing step. Increasing the level of the clamp pulse to -6 mV induced the TI, which appears as the large inward transient in the uppermost trace. The algebraic difference between the upper and middle traces thus represents the TI itself, without interference from i_{K_2}.

The results in Figs. 8 and 9 indicate that the TI differs from the normal pace-maker mechanism in requiring a prior depolarization that is both long and strong. The contrasting requirements are interesting because they may help explain the marked dependence of TDs on previous repetitive activity (see Introduction). A train of closely spaced action

potentials may have the same cumulative effect as a long clamp depolarization. Before accepting this explanation, it is important to consider how the effects of separate depolarizations can combine.

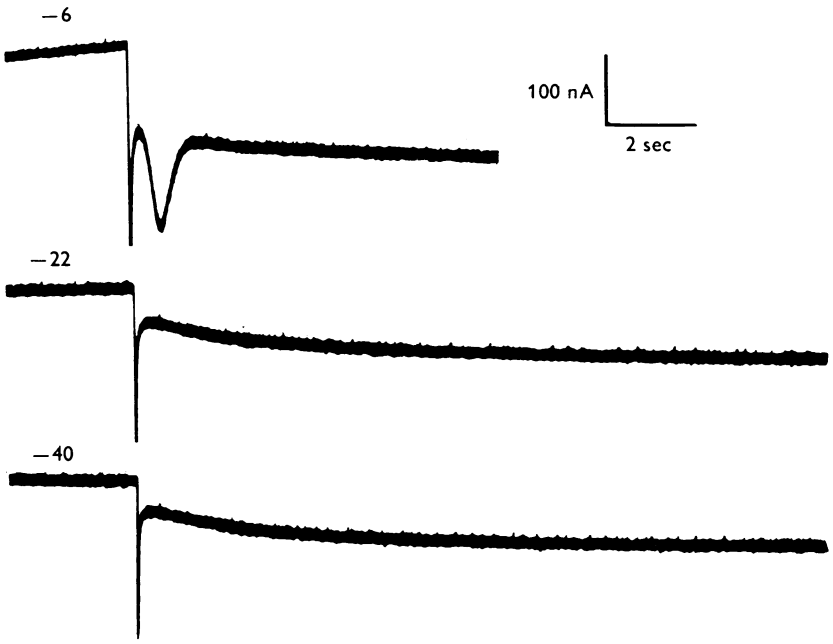

Fig. 9. Separation of TI from i_{K_2} by varying pulse level. Current records accompany step repolarizations to −72 mV, following a preceding 5 sec clamp pulse to −6, −22, or −40 mV. Each of records shows a slow decay of i_{K_2} following the 'off' of the pulse but only the uppermost trace includes a TI as well. 1 μM strophanthidin; preparation 160–5.

Additive effects of successive membrane depolarizations

Previous studies (see Ferrier *et al.* 1973 for references) have shown that spacing between successive action potentials is quite important to their combined effect in giving a TD. Apparently, each action potential leaves behind some after-effect which persists for long enough for summation to occur. In characterizing such behaviour, it is convenient to use voltage-clamp pulses, since these may be separated by a variable interval that is well-defined and easily controlled. Such studies are less clear cut using action potentials, since their shape and duration changes as their proximity is varied.

Fig. 10 illustrates results from two different preparations. The experimental protocol is indicated in the inset. Two clamp pulses, a 4 sec conditioning pulse and a 1 sec test pulse, were separated by a variable

interval, t. The experiment studies the influence of t on the amplitude of the TI following the test pulse.

Fig. 10A shows that the amplitude of the test TI decreased progressively as the interval t was prolonged. The family of points reflects the gradual disappearance of the influence of the conditioning pulse. The data were reasonably fitted by a decaying exponential, with a time constant of 475 msec.

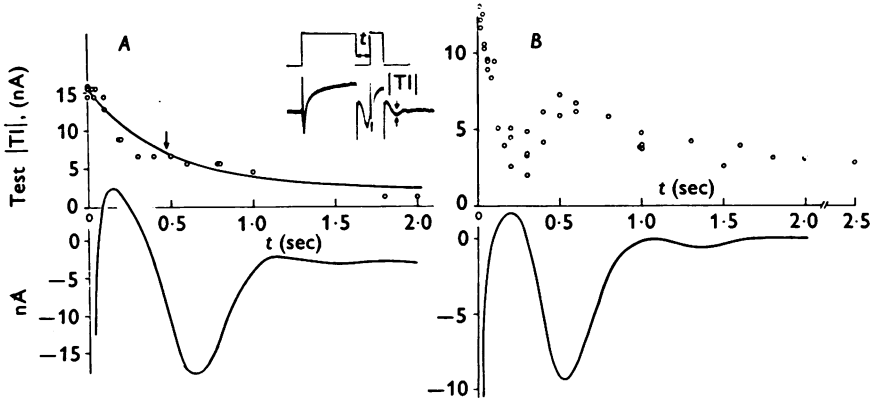

Fig. 10. Cumulative effect of membrane depolarization on induction of the TI. The inset shows the two pulse procedure. A 4 sec conditioning pulse and a 1 sec test pulse were separated by a variable interval, t. The amplitude of the TI which followed the test pulse was measured and plotted against t (graph). The TI following the conditioning pulse is plotted below on the same time scale. A and B were obtained with different preparations and illustrate the range of results observed. A, preparation 157–2; 1 μM strophanthidin; apparent cylindrical area 0·0055 cm^2. B, preparation 156–3; 1 μM strophanthidin; apparent cylindrical area, 0·008 cm^2. Vertical scales adjusted to allow comparison between experiments.

Fig. 10B illustrates a somewhat different result, obtained in another preparation but using the same pulse protocol. In this case, the influence of the conditioning pulse on the test TI does not fall monotonically and a simply decaying exponential would obviously fail to provide a satisfactory fit. The data showed a minimum near t = 0·25 sec and a maximum near t = 0·5 sec. These features seem to mirror the time course of the TI which immediately follows the conditioning pulse ('conditioning TI', below). The resemblance in time course suggests that some of the after-effect can arise from the 'conditioning TI' itself.

This hypothesis is provocative but it provides no clear explanation for the results in Fig. 10A. The monotonic decay in Fig. 10A is particularly

puzzling because the time course and magnitude of the 'conditioning TI' are not very different from that in *B*. These two examples show the opposite extremes of behaviour observed in a total of five preparations. Despite the obvious variability in the time course of the after-effect, all the experiments agreed in demonstrating combined effects of successive membranes depolarizations.

Role of the TI in 'low voltage oscillations' evoked by cardiotonic steroids

Up to now, this paper has focused on the early manifestations of digitalis toxicity, increases in diastolic depolarization which occur without substantial loss of maximum diastolic potential (see Ferrier *et al.* 1973). In more advanced stages of toxicity, Purkinje fibres become partially depolarized and show spontaneous activity (Vassalle *et al.* 1962; Müller, 1963) with characteristically slow upstrokes (see Cranefield, 1975). The spontaneous potentials have been designated 'low voltage oscillations' (Hauswirth, Noble & Tsien, 1969) since they occur positive to -50 mV or so. Over this potential range, the dominant components are the slow inward current (i_{si}) and i_x, not i_{Na} and i_{K_2}, the currents which control the normal pace-maker potential (see Imanishi, 1971; Cranefield, 1975; Kass & Tsien, 1975).

By what means do cardiotonic steroids produce low voltage oscillations? The most obvious factor, loss of membrane potential itself, is probably sufficient to cause some sort of sustained repetitive activity. Thus, Aronson & Cranefield (1974) have shown the ouabain-evoked low voltage oscillations may be mimicked in untreated preparations simply by applying a steady depolarizing current. This experiment reinforces the importance of potential range but leaves open the possibility of some additional mechanism of cardiotonic steroid action. Since the TI can be observed at potentials positive to -50 mV, it seemed possible that it may contribute to generation of low voltage oscillations.

Fig. 11 compares low voltage oscillations in the absence and presence of strophanthidin in two different preparations. These particular records were selected because the spontaneous activity happened to span nearly the same range of potentials in each case. To study the underlying basis of the spontaneous depolarizations, we imposed the voltage clamp at the point of maximum repolarization and observed subsequent variations in membrane current (Vassalle, 1966; cf. Figs. 1–3). In the strophanthidin-free experiment (Fig. 11*A*), clamping the membrane at -48 mV produced a slow monotonic increase in net inward current. Similar behaviour was found by Hauswith *et al.* (1969) and was explained by the decay of i_x. The normal pace-maker potassium current was fully activated at the clamp potential and could not contribute to the slow current.

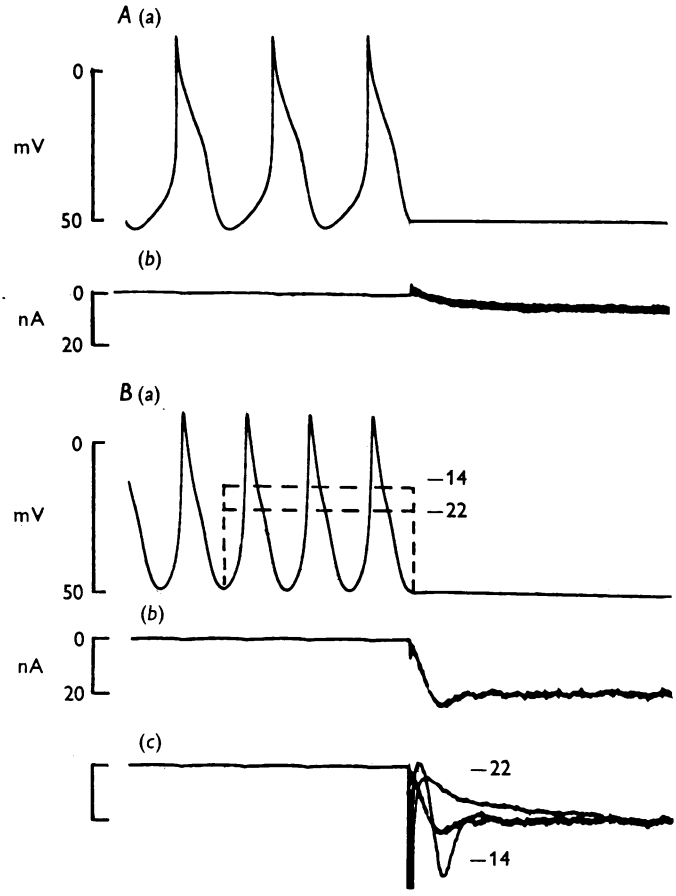

Fig. 11. Spontaneous activity in partially depolarized Purkinje fibres and related membrane current changes. *A*, (*a*) 'low voltage oscillations' in the absence of strophanthidin. (*b*) corresponding membrane current trace. The slow current change occurred when the membrane potential was clamped near the level of maximum repolarization. The preparation was partially depolarized at the beginning of the experiment, but some time after these records were taken it recovered a resting potential negative to −80 mV and gave full-blown action potentials when stimulated. Preparation 163–1; apparent cylindrical area, 0·013 cm². *B*, (*a*) 'low voltage oscillations' in a strophanthidin-intoxicated preparation. (*b*) current trace included a TI when membrane was clamped at level of maximum repolarization. (*c*) current trace in (*b*) is repeated together with current changes following 5 sec clamp pulses to −14 and −22 mV (dashed traces in (*a*)). Preparation 160–5; 1 μM strophanthidin: apparent cylindrical area, 0·014 cm².

Fig. 11*B* illustrates the same procedure in a strophanthidin-treated preparation. In this case, clamping at −49 mV gave a different result, a non-monotonic change in net current. There is an inward peak, which suggests the involvement of the TI. To clarify this involvement, Fig. 11*B* (*c*) repeats the current trace in *B*(*b*) and compares it with other traces (dashed) which show different amounts of TI. These records were produced by step repolarizations to −48 from potentials indicated in *B*(*a*). Repolarization from −14 to −48 mV evoked a clear cut TI which was considerably larger than the TI following the spontaneous potentials themselves. Apparently, the previous 5 sec depolarization to −14 mV was more effective in inducing the TI than the low voltage oscillations, which reach more depolarized levels but only briefly. On the other hand, repolarization from −22 mV produced a smooth monotonic current change with little or no TI, resembling the behaviour in the absence of strophanthidin (Fig. 11*A*). This record is included because it provides a rough estimate of the current changes due to i_x alone. Acting by itself, the decay of i_x would have produced some slow depolarization since background inward current was unmasked. The additional participation of the TI significantly increases the development of net inward current and thus favours more rapid spontaneous depolarization. This in turn leads to an earlier attainment of the threshold for the upstroke, which depends upon activation of the slow inward current (see Cranefield, 1975).

These results suggest that cardiotonic steroids may promote low voltage oscillations by evoking TIs, as well as by producing partial depolarization. Both factors may play a role in the arrhythmogenic influence of these agents in advanced stages of toxicity.

DISCUSSION

This paper has examined the basis of increased pace-maker activity in Purkinje fibres exposed to cardiotonic steroids in relatively high concentrations. Our voltage-clamp experiments showed that the enhanced diastolic depolarization is dominated by a TI. This 'TI' is evoked by exposure to strophanthidin or ouabain but is virtually absent in untreated preparations, playing no part in the normal pace-maker depolarization. Participation of this novel current component distinguishes the effect of digitalis intoxication from the chronotropism of adrenaline or theophylline, which accelerate the pace-maker through changes in i_{K_2} (Hauswirth *et al.* 1969; Tsien 1974*a*, *b*). The contrast in mechanism provides a basic justification for special terms such as 'transient depolarization' (Ferrier *et al.* 1973) or 'low amplitude potential' (Rosen *et al.* 1973) which refer to cardiotonic steroid-enhanced diastolic depolarizations. These

terms may remain useful but it should be remembered that TI-dependent responses can reach threshold and produce full-blown spontaneous impulses that are neither 'low' in amplitude nor so 'transient' in duration.

Contrasts between the transient inward current and the normal pace-maker mechanism

Although the TI dominates the diastolic depolarization in strophanthidin-treated preparations, time-dependent current changes due to decay of i_{K_2} are by no means absent (cf. Aronson *et al.* 1973). This result makes it important to find procedures for dissecting these current components. The next paper (Kass *et al.* to be published) describes variations in ion concentration or pharmacological agents which could be used to distinguish the TI from i_{K_2}. In the present work, we have relied upon the dissimilar time and voltage-dependence of these components in dissecting their contributions to the net membrane current. The kinetic properties of the TI are interesting because they help explain the enhancement of TDs by prior repetitive stimulation (see Introduction), a key finding that originally suggested that digitalis toxicity might involve a novel cellular mechanism. The kinetic experiments are also useful in providing a starting point for future explanations of the basis of the TI itself.

Dependence on repolarization level. Both the TI and i_{K_2} deactivation are evoked by repolarizing voltage steps. However, there are differences in the level of repolarization that is appropriate. Deactivation in i_{K_2} requires repolarization to potential negative to -60 mV or so (see Hauswirth, Noble & Tsien, 1972, Fig. 1). On the other hand, the TI can be evoked by repolarizations over a broad range, including voltages well positive to -60 mV (Fig. 5; Lederer, 1976, Fig. 4.9). Thus, i_{K_2} deactivation cannot participate in 'low voltage oscillations' (Hauswirth *et al.* 1969) whereas the TI can contribute, particularly under the influence of cardiotonic steroids (Fig. 11).

Dependence on prior depolarization level. Deactivation of i_{K_2} depends upon previous activation. In the steady state, full i_{K_2} activation is achieved by depolarizations to levels positive to about -60 mV (Noble & Tsien, 1968). On the other hand, induction of the TI requires potentials positive to -40 or -30 mV (the precise value varies with the degree of intoxication). Thus, by choosing suitable clamp pulses, i_{K_2} may be studied without interference from the TI, even in the presence of cardiotonic steroids (Fig. 9).

Dependence on prior depolarization duration. At any given depolarizing level, there are important differences in the time course of development of i_{K_2} and TI. Activation of i_{K_2} obeys first order kinetics, increasing

exponentially in time, while the induction of the TI follows a markedly sigmoid time course. There are also substantial differences in the absolute speed of activation expressed in terms of the time required for half maximal activation ($t_{\frac{1}{2}}$). At -20 mV and 35-36° C, $t_{\frac{1}{2}}$ for i_{K_2} is roughly 30 msec (McAllister & Noble, 1966, Fig. 2; Hauswirth *et al.* 1972, Fig. 8). At the same potential and temperature, $t_{\frac{1}{2}}$ for the TI might be 3–4 sec (Fig. 6). This disparity was exploited in Fig. 8 to separate the TI from i_{K_2} decay.

Direction of current flow. In the most fundamental sense, the TI differs from the normal pace-maker process because it represents the turning on of an inward current rather than the shutting off of an outward current. The evidence for this claim is presented in a later paper (Kass *et al.* to be published) but is mentioned here for completeness.

Cumulative effects of repetitive drive

The contrasting temporal properties of the TI and i_{K_2} can help explain previously observed contrasts between TDs and normal pace-maker activity. The diastolic depolarization in untreated Purkinje fibres is characteristically depressed by repetitive stimulation, presumably due to activation of electrogenic sodium transport (Vassalle, 1970). This 'overdrive suppression' can be reduced or even reversed under the influence of ouabain, to the point where spontaneous activity is actually facilitated by previous drive. Such 'post-pacing acceleration' was first described in ventricular activity of intact animals (Lown, Cannon & Rossi, 1967; Wittenberg, Streuli & Klocke, 1970) but was soon confirmed in isolated Purkinje fibre preparations (Gandel *et al.* 1970; Davis, 1973). More detailed studies by Ferrier *et al.* (1973) and Ferrier (1976) have carefully characterized the influence of repetitive activity on subsequent transient depolarizations. Trains of driven action potentials were followed by TDs which appeared coupled to the last action potential in the train (e.g. Fig. 2). The amplitude of the TDs could be enhanced by increasing the number or frequency of the driven beats.

These observations are explained, at least qualitatively, by the findings in this paper. A train of action potentials produces a cumulative effect because: a single action potential acts like a relatively brief clamp depolarization, and is by itself a relatively poor activator of the TI (Fig. 5); successive action potentials produce additive effects on TI induction (Fig. 11) and can combine in approaching the effectiveness of a prolonged clamp depolarization.

Possible arrhythmogenic mechanisms

The kinetic properties of the TI seem to account for the features which distinguish the TD from normal pace-maker activity and, consequently, the characteristic 'post-pacing acceleration' in whole ventricle. The parallelism between these phenomena strongly supports the TI as one important mechanism for digitalis arrhythmias. Beat-dependence of the TD cannot be directly explained by other cellular events, such as blockade of electrogenic sodium pump current, reduction in the magnitude of i_{K_2} (see Introduction) or accumulation of potassium in extracellular clefts (Cohen *et al.* 1976). Further work is needed to clarify the various stages in the development of digitalis toxicity, and to sort out the involvement of membrane current changes such as the TI and drug-induced current fluctuations. It will be interesting to see whether all the modifications of membrane current are ultimately dependent upon the interaction of cardiotonic steroids with the sodium pump.

This research was supported by grants HL 13306 and AM 17433 from the U.S. Public Health Service. Some of the experiments formed part of the doctoral thesis of W. J. Lederer. R. W. Tsien is an Established Investigator of the American Heart Association.

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