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ROLE OF CALCIUM IONS IN TRANSIENT INWARD CURRENTS AND AFTERCONTRACTIONS INDUCED BY STROPHANTHIDIN IN CARDIAC PURKINJE FIBRES

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SUMMARY

1. Under the influence of strophanthidin, Purkinje fibres exhibit transient inward current (TI) which contributes to arrhythmogenic activity. Voltage-clamp experiments were carried out to study the role of Ca ions in this phenomenon.

2. The amplitude of TI varied directly with the extracellular Ca concentration, Ca_o. Magnesium ions had an antagonistic effect.

3. TI was closely associated with a phasic increase in force ('aftercontraction'). Like TI, the aftercontraction was evoked by a preceding action potential or by the break of a strong depolarizing pulse.

4. TI and the aftercontraction displayed similar wave forms although peak current preceded peak force by 50–100 msec. Both transients were enhanced by increasing the strength or duration of the preceding depolarization pulse. Both events were slowed as the potential level following the pulse was displaced in the negative direction.

5. TI and the aftercontraction could be evoked in the absence of cardiotonic steroids by strongly elevating Ca_o.

6. Additional experiments were carried out to test the hypothesis that TI reflects an influx of Ca^{2+} ions. Mn inhibited TI but the development and removal of the inhibition lagged far behind the effects on the slow inward current.

7. TI could be suppressed and eventually inverted by varying the membrane potential in the positive direction. The inversion potential averaged -5 mV and was not consistent with a Ca-specific pathway. The aftercontraction was more closely related to the phasic conductance change underlying the current than to the current flow itself.

8. The results are consistent with the idea that an oscillatory release of Ca from an intracellular store is the primary event underlying both the aftercontraction and the conductance change which generates TI. Digitalis intoxication or very high Ca_o may promote such events by elevating intracellular Ca levels.

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INTRODUCTION

Purkinje fibres develop a characteristic depolarizing afterpotential under the influence of toxic concentrations of cardiotonic steroids such as ouabain or strophanthidin. The afterpotential can become large enough to reach threshold and may thus contribute to the development of spontaneous ectopic impulses in the intact heart under a variety of clinical conditions (see Cranefield, 1975, and Ferrier, 1977, for review). Several investigators have found that the depolarizing afterpotential is strongly enhanced by a previous series of closely spaced action potentials (Hogan, Wittenberg & Klocke, 1973; Davis, 1973; Rosen, Gelband, Merker & Hoffman, 1973; Ferrier, Saunders & Mendez, 1973). This behaviour distinguishes the digitalis-induced afterpotential from the normal pace-maker depolarization, and it has led to a number of special terms for the afterpotential, including 'transient depolarization' or 'TD' (Ferrier *et al.* 1973). The current change underlying TD was investigated by Lederer & Tsien (1976), using short calf Purkinje fibres under voltage clamp. The results indicated that TD is generated by a digitalis-induced transient inward current. The transient inward current does not participate in the normal pace-maker depolarization, which is controlled by a distinct K current I_{K_2}. Lederer & Tsien (1976) termed the transient inward current 'TI' because of its role in what Ferrier *et al.* (1973) called TD. We shall use TI as a descriptive name which leaves open the question of whether the transient current is carried by a previously identified ionic pathway.

This paper is concerned with the role of Ca ions in the genesis of TI. The involvement of Ca has been suggested by a number of earlier studies of TD. Ferrier & Moe (1973) showed that TD is enhanced in Ca-rich solutions, and reversibly abolished in Ca-free solution. Transient depolarization is suppressed by agents which are known to inhibit Ca movements, such as Mn ions (Ferrier & Moe, 1973), magnesium ions (Ferrier & Saunders, 1972) and verapamil (Rosen *et al.* 1973). Finally, an aftercontraction accompanies TD in acetylstrophanthidin-treated Purkinje fibres, and shows similar dependence on previous activity (Ferrier, 1976).

In the present experiments, the involvement of Ca ions was explored by direct measurements of TI under voltage clamp. We have examined the effects of Ca_o and inhibitors of Ca currents, as well as the influence of membrane potential itself. Aftercontractions were recorded simultaneously in many of the experiments, and were used to indicate changes in myoplasmic Ca levels. Our results support the idea that a phasic release of Ca from intracellular stores is the primary event which controls the transient inward current across the surface membrane. The following paper (Kass, Tsien & Weingart, 1978) presents evidence that the inward current is largely carried by Na ions.

METHODS

Purkinje fibre bundles were obtained from left and right ventricles of calf hearts. Short preparations (1–2 mm) were voltage-clamped by means of the two-microelectrode method of Deck, Kern & Trautwein (1964). Force was recorded using an Endevco 87102 transducer, kindly lent to us by Dr W. K. Chandler. The preparation was supported by a glass pedestal, with one end attached to the force transducer and the other end fixed to a mechanical anchor. The attachments were made after tying the preparation with fine silk thread to stainless-steel pins whose ends had been bent into hooks. Mechanical vibrations were reduced by a Lansing vibration

isolation system which supported the set-up. The remaining noise on the force trace was largely due to the continuous solution flow and was often reduced by low-pass filtering (corner frequency 10 or 30 Hz). The filtering did not appreciably reduce the amplitude of aftercontractions. Current and force signals received identical filtering in cases where time-to-peak current and time-to-peak force were compared. Current and force were recorded along with voltage and temperature on a four-channel chart recorder (Brush 440). The amplitude of TI or the aftercontraction were measured with respect to a sloping base line drawn tangent to the troughs which flanked the transient peak. This procedure was chosen because it treats the current and force signals in the same manner and specifically emphasized their phasic aspects.

In most of the experiments, the preparations were intoxicated by repetitive stimulation of action potentials in the presence of 1–2 μM-strophanthidin (Sigma). Voltage-clamp runs were carried out at a stage beyond the initial development of the positive inotropic effect, when TD and aftercontractions were clearly enhanced. The composition of the modified Tyrode solution was as follows (mM): 150 Na, 4 K, 5·4 Ca, 0·5 Mg, 155·8 Cl, 10 Tris-maleate (pH 7·2–7·4). The solutions also contained 5 mM-glucose and were pre-gassed with 100% O_2. Ca, Mg and Mn concentrations were varied by including appropriate amounts of the chloride salts, without compensatory changes in other salt concentrations except where specifically indicated. The experiments were carried out at 35–37 °C, with temperature held constant to within $\pm$0·2 °C during a given experiment.

RESULTS

Antagonistic effects of Ca and Mg

Previous studies of transient depolarizations have suggested that the transient inward current might be strongly influenced by Ca and Mg (Ferrier & Saunders, 1972; Ferrier & Moe, 1973). We examined the effects of these ions in voltage-clamp experiments, and found antagonistic effects on the magnitude of TI which are consistent with the earlier work on the transient depolarization.

Fig. 1 shows current records from a voltage-clamp experiment in a preparation exposed to 1 μM-strophanthidin. TI was evoked by using a standard 5 sec clamp pulse to $-$ 32 mV from a holding potential of $-$ 69 mV. Current traces from four consecutive runs are presented in order in panels *A–D*. The simplest comparison involves Fig. 1*C* and *D*. These records show the effect of varying the Ca ion concentration while keeping the total divalent ion concentration fixed.

Fig. 1*C* was obtained in the presence of 5·4 Ca, 0·5 Mg, the standard solution in the present series of voltage-clamp experiments (see Dudel, Peper, Rüdel & Trautwein, 1967). Removal of external Ca (*D*) produced a clear and virtually complete abolition of TI. The reduction in TI amplitude occurred very rapidly: record *D* was taken 2 min after initiating the solution change. The change in TI had already reached a steady state but, at this point, there was still a significant surge of slow inward current. Within another 3 min, this inward surge also disappeared (record not shown). At this stage, Ca_o was restored to 5·4 mM, and within 3 min, TI showed full recovery. The removal of Ca ions was then repeated and it once again abolished TI.

The comparison between Fig. 1*C* and *D* involved an elevation in Mg_o at the same time as a decrease in Ca_o. The effect of varying Mg_o alone is illustrated by comparing Fig. 1*B* and *C*. Reduction of Mg_o from 5·9 to 0·5 mM resulted in a threefold increase in the amplitude of TI. The inhibitory effect of Mg supports Ferrier & Saunders' (1972) preliminary report on transient depolarizations, and also fits with the beneficial effects of this ion in the treatment of digitalis-induced ventricular arrhythmias (Ghani & Smith, 1974).

As a final point, the records in Fig. 1 also provide an illustration of the effect of varying Ca_o while keeping Mg_o constant. Fig. 1*A*, *B* and *D* were obtained with bath Ca concentrations of 10·8, 5·4 and 0 mM-Ca. There is a graded reduction in the amplitude of TI over this broad range of Ca concentration. The variation cannot be attributed to running-down of the preparation since the illustrated results were bracketed by other runs. For example, a run in 5·4 Ca, 5·9 Mg (same solution as in Fig. 1*B*) was carried out before the run illustrated by Fig. 1*A*. This run gave a transient current whose amplitude is indicated by the vertical bar to show the excellent agreement with the transient in Fig. 1*B*. Reversibility after Fig. 1*D* was also checked by even later runs, as mentioned previously.

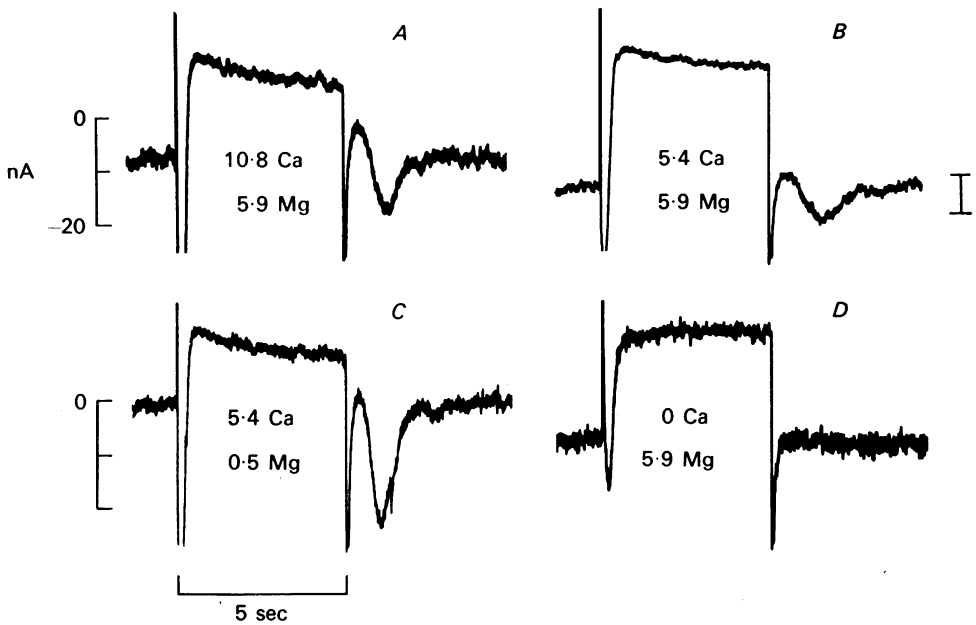


Fig. 1. Antagonistic effects of Ca and Mg on TI magnitude. The four panels show current records under varying ionic conditions during the application of 5 sec voltage clamp pulses from −69 to −32 mV. The bar to the right of *B* indicates the TI magnitude of an earlier run under the same conditions as *B*. Preparation 144-5; 1 μM-strophanthidin; apparent cylindrical area of preparation, 0·0041 cm^2.

The antagonistic effects of Ca and Mg on TI are entirely consistent with the findings of Ferrier & Moe (1973) and Ferrier & Saunders (1972) on transient depolarizations. Our observations go beyond theirs in showing effects on TI, exclusive of possible changes in other diastolic currents such as I_{K_2}. The simplest interpretation of the results is that the current reflects a transient influx of Ca ions across the cell membrane, as suggested by Ferrier & Moe. However, as Ferrier (1976) points out, other interpretations are also possible (see p. 198).

Aftercontractions associated with the transient inward current

It has been known since the work of Bozler (1943) that 'oscillatory afterpotentials' can occur in association with transient increases in mechanical activity. Such after-

contractions, or 'Nachkontraktionen' as they were called by Reiter (1962), have been studied extensively in working heart muscle (see Discussion). Aftercontractions may also be recorded in Purkinje fibres intoxicated with acetylstrophanthidin, and seem closely related to transient depolarizations (Ferrier, 1976). Both events are enhanced by a preceding train of closely spaced action potentials or by stretching the preparation. In earlier voltage-clamp experiments (Lederer & Tsien, 1976), aftercontractions accompanying TI were often observed under the dissecting microscope.

The measurement of contractile force seemed particularly appropriate in exploring the possibility of a genuine Ca current since contractile activity is highly sensitive to the intracellular Ca concentration. Force and membrane current were measured simultaneously in most of the experiments. Fig. 2 illustrates the contractile behaviour

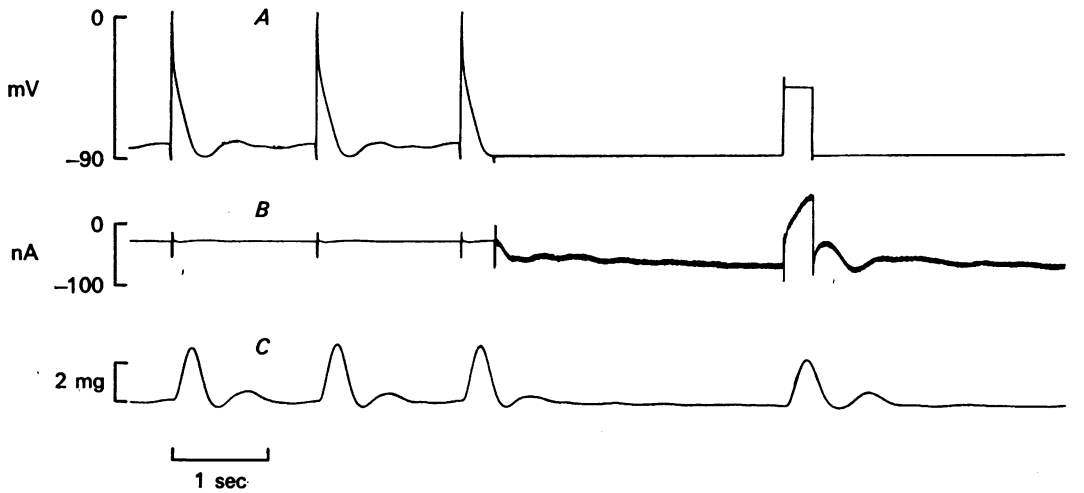


Fig. 2. Toxic effects of strophanthidin on membrane current and contractile activity. Chart records of membrane potential (*A*), membrane current (*B*) and force (*C*). *A*, the last three action potentials from a train of eleven responses. The action potentials were evoked by external shocks and were followed by transient depolarizations *B*, the stimulus artifacts and the presence of a small amount of steady hyperpolarizing current. *C*, twitches accompanying the action potentials and aftercontractions in association with the transient depolarizations. Following the last action potential, the membrane was clamped at the maximum diastolic potential (−88 mV). This evoked TI on the current trace (*B*) as well as an aftercontraction (*C*). The right side of *B* shows a depolarizing clamp pulse from −88 to −44 mV for 300 msec. The depolarizing step gave rise to an inward current surge (*B*) and a twitch (*C*), a repolarizing step evoked TI (*B*) and an aftercontraction (*C*). Preparation R30-1; 1 μM-strophanthidin; apparent cylindrical area, 0·009 cm^2.

of a Purkinje fibre preparation under the influence of strophanthidin. The left side of the Figure shows action potentials (trace *A*) and twitches (trace *C*) which were evoked by externally applied shocks. The first two action potentials were followed by transient depolarizations, which were not large enough in this case to reach the excitatory threshold. As the force trace (*C*) indicates, each action potential was accompanied by a twitch, and each TD was associated with an aftercontraction. The aftercontractions were smaller and slower than the twitches, but their timing and magnitude were rather

consistent from beat to beat. After a third action potential, the voltage clamp was activated just at the point of maximum repolarization (cf. Vassalle, 1966; Lederer & Tsien, 1976, fig. 1). This procedure prevented the transient depolarization, but it resulted in TI (current trace *B*) as well as another aftercontraction. A similar association between TI and the aftercontraction was evident when the membrane was depolarized with a rectangular clamp pulse to −44 mV. The 'on' of the pulse was followed by a twitch, and the 'off' of the pulse produced TI and an after contraction which were similar to those following the action potential. Clamp pulses are a convenient means for evoking the transient events because they can be used under ionic or pharmacological conditions which would abolish action potentials.

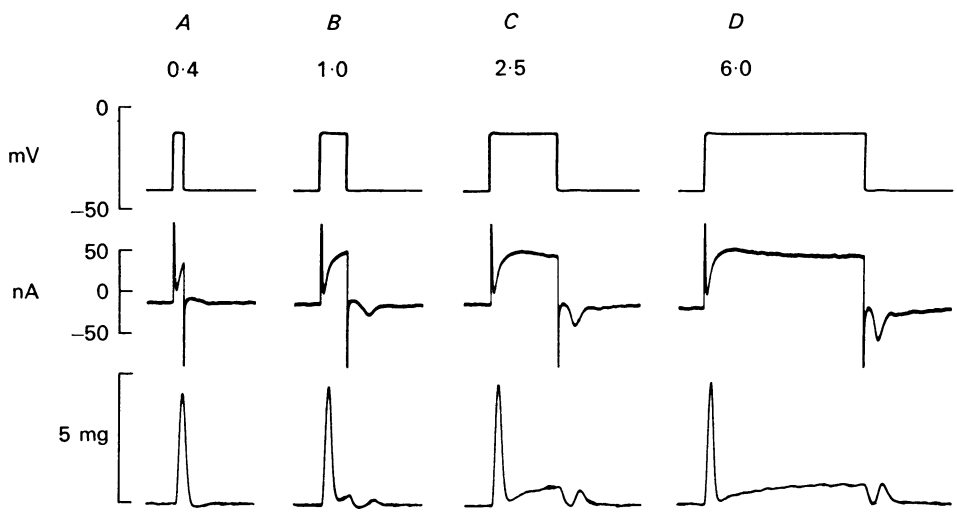


Fig. 3. Effect of varying pulse duration on TI and aftercontraction. The upper records show rectangular voltage pulses from −39 to −13 mV with durations indicated above in seconds. The accompanying TI and aftercontraction are given in the middle and lower traces. TI and aftercontractions grow together as the depolarization is prolonged. Preparation R26-7; 1 μM-strophanthidin; apparent cylindrical area, 0·015 cm^2.

Time- and voltage-dependence of the TI and aftercontraction

We carried out voltage-clamp experiments to compare the kinetic properties of the transient events. Fig. 3 illustrates the influence of varying the duration of depolarizing pulses while keeping the level fixed. A 400 msec pulse to −13 mV triggers a twitch but virtually no TI or aftercontraction. Longer pulses produce a slowly developing component of tonic tension during the depolarization (cf. Fozzard & Gibbons, 1975) and TI and aftercontraction following the termination of the pulse. Analysis of these records and others from the same experiment is shown in Fig. 3*D*. The amplitudes of TI and the aftercontraction increase together as the pulse duration is increased. Both of the transients develop with a markedly sigmoid time course, as reported previously for TI alone (Lederer & Tsien, 1976, fig. 6). This type of correlation between the kinetics of TI and of the aftercontraction was observed over a range of conditions which are not illustrated here. For example, increasing the strength of the depolarization or the degree of intoxication produced

parallel changes in the current and force transients; both conditions favoured a more rapid development and a larger maximal amplitude as pulse duration was progressively increased (see Lederer & Tsien, fig. 6).

Another comparison between TI and the aftercontraction is illustrated in Fig. 4. In this experiment the level and duration of the depolarization remained unchanged, but the level of the subsequent repolarization was varied. Panel A shows superimposed records of repolarizations to increasingly negative potentials. These were accompanied by transient currents (*B*) and aftercontractions (*C*) with progressively later peaks. The repolarization to −43 mV evoked larger TI and larger aftercontraction than the steps to −19 or −69 mV. In all cases, the wave forms of TI and aftercontractions are quite similar.

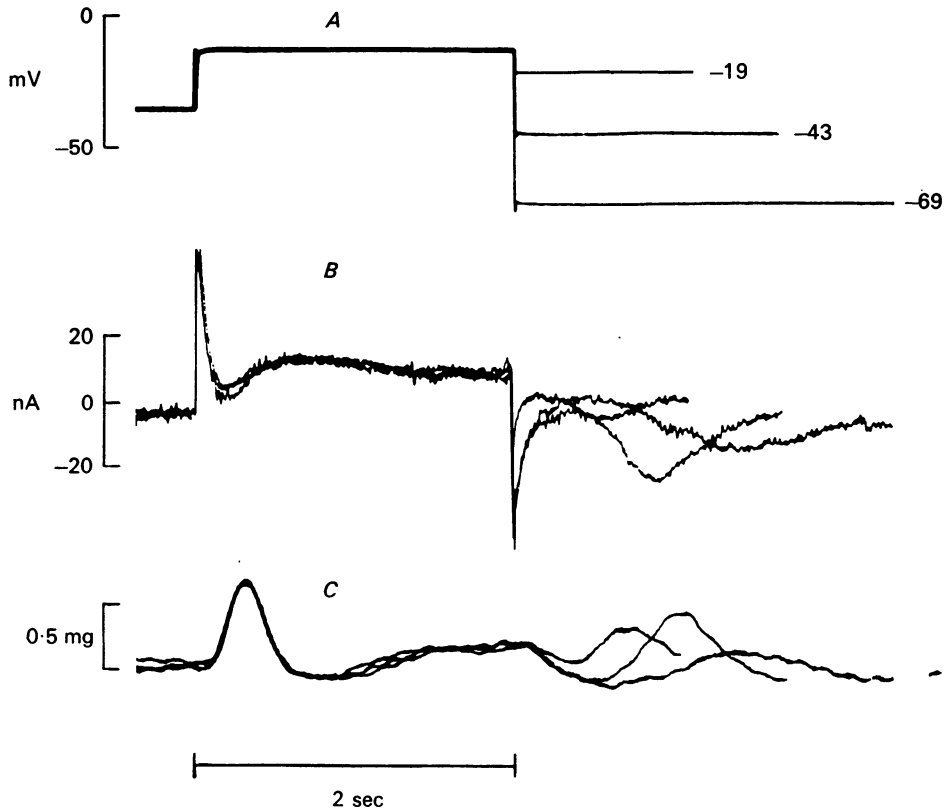


Fig. 4. Dependence of TI and aftercontraction on level of repolarization. *A*, three records of membrane potential which were photographically superimposed. The membrane was depolarized from −34 to −12 mV for 2 sec in each case, then repolarized to the various levels indicated. *B* and *C*, the associated current and force signals. Correspondence with the voltage records is indicated by the length of the traces. Repolarizing steps to increasingly negative potentials produce TI and aftercontractions with progressively delayed peaks. Preparation R11-1; 1 μM-strophanthidin; apparent cylindrical area, 0·015 cm^2.

Analysis of these records and others from the same experiment is presented in Fig. 5. Panel *A* compares the amplitudes of TI and the aftercontraction. Both show a similar dependence on the level of repolarization. Panel *B* plots the time between

the repolarizing step and the peak of TI or the aftercontraction. In both cases, the time-to-peak increased monotonically as the repolarization potential became more negative, with the peak aftercontraction lagging by about 90 msec over most of the voltage range. The delay between peak TI and peak aftercontraction is less clear at the most negative potentials, but this may be due to the error in measuring the time-to-peak of a transient with low amplitude and slow time course. In other experiments, the peak aftercontraction always followed the peak TI with delays ranging from 50 to 140 msec.

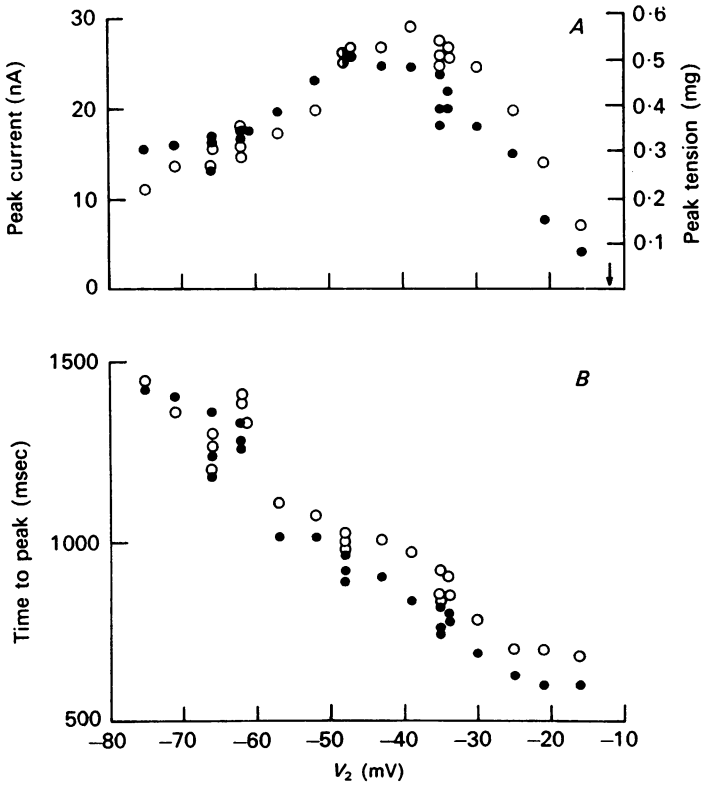


Fig. 5. Effect of repolarization level on the amplitude and time-to-peak of the TI (●) and the aftercontraction (○). Analysis of records in Fig. 4 and others from the same experiment. *A*, amplitude of the transients as a function of repolarization potential. The vertical scales for the TI (left) and aftercontraction (right) were adjusted to match the transient amplitudes at −48 mV. Arrow indicates potential preceding repolarizing step. *B*, time delay between repolarizing step and peak TI or peak aftercontraction. The aftercontraction lags TI by about 90 msec over most of the voltage range.

The temporal relationship between TI and the aftercontraction is approached in another way in Fig. 6. The records were taken from a preparation where the repolarizing step (*A*) evoked not one, but three or four inward current transients (*B*). These transients have the appearance of damped sinusoidal oscillations, and each is associated with a discrete aftercontraction (*C*). Multiple aftercontractions have been reported previously in atrial or ventricular muscle preparations (see Discussion), although not under voltage clamp. We found that the appearance of multiple peaks

was variable from preparation to preparation, but could be enhanced in a given experiment by increasing the degree of cardiotonic steroid intoxication or by using a strong but brief depolarizing pulse.

The correlation between the current and force signals is emphasized by the photographically superimposed records in panel *D*. The current record is simply repeated, while the force record is inverted and advanced by 80 msec. The current and force signals are brought into close register by this procedure. Thus, it is evident that both signals share the same degree of damping, and that their temporal relationship can be adequately described by a single-valued delay.

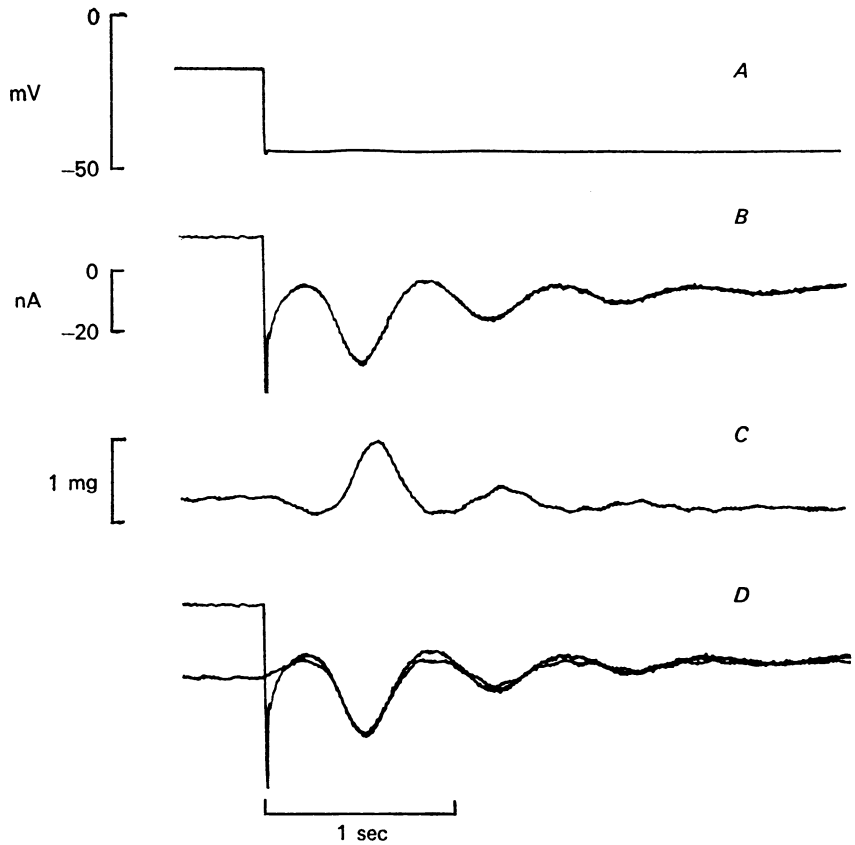


Fig. 6. Temporal relationship between transient inward currents and aftercontractions. A repolarizing step to −44 mV (*A*) terminated a 10 sec depolarizing pulse to −17 mV. The repolarizing step evoked a series of inward current transients (*B*) which were associated with discrete aftercontractions (*C*). In panel *D* TI and aftercontraction records are superimposed, the force record being inverted and advanced by 80 msec. Preparation R9-4; 1 μM-strophanthidin; apparent cylindrical area 0·007 cm^2.

Ca ions and inhibitors of calcium current

The kinetic characteristics of TI and the aftercontraction suggest a close relationship between the two phenomena. We have pursued this point by examining the effects of calcium ions and blockers of Ca current. Fig. 7 shows the results of exposing a Purkinje fibre preparation to Ca-rich solution in the absence of any cardiotonic

steroid. Both TI and the aftercontraction were not seen when a long depolarizing pulse was imposed in the control run in the presence of 5·4 Ca (panel *A*). When the Ca concentration was increased to 18 mM, the same clamp pulse evoked a clearcut transient inward current and a very large aftercontraction (*B*). The current and force transients changed together during the onset of the calcium effect; both amplitudes were maximally increased about 2 min after beginning the change of solutions, and fell off

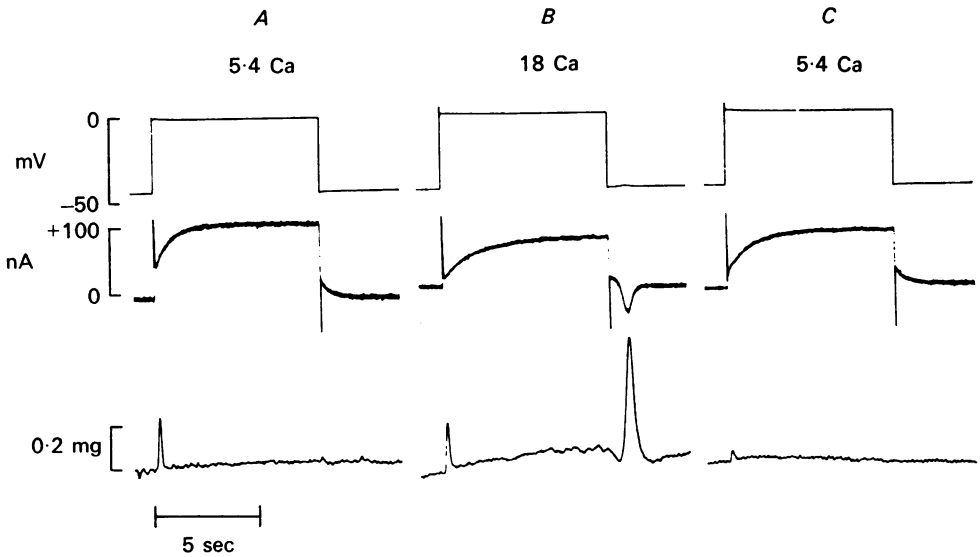


Fig. 7. Effects of Ca-rich solutions in the absence of cardiotonic steroid. Depolarizing clamp pulses from −44 to −2 mV (top) are shown with associated current records (middle) and force records (bottom). *A*, control records in 5·4 mM-Ca Tyrode. *B*, 5 min after increasing Ca to 18 mM. *C*, 18 min after restoring Ca to 5·4 mM. Preparation R12-2; apparent cylindrical area 0·007 cm².

to maintained values which are shown in Fig. 7*B*. TI and the aftercontraction also decreased together during the recovery in 5·4 Ca (*C*). This experiment is consistent with Ferrier & Moe's (1973) demonstration that Ca-rich solutions (12·5 Ca) can evoke TD in the absence of cardiotonic steroid. The influence of high Ca concentration on aftercontractions in Purkinje fibres has not been reported previously, but our results are compatible with earlier studies in atrial or ventricular muscle (see Discussion for references).

Fig. 8 illustrates the effect of D600 on TI and the aftercontraction in the presence of strophanthidin. D600 is a methoxy derivative of verapamil, a drug which has been reported to suppress ouabain-induced TD (Rosen *et al.* 1973). Verapamil and D600 have been used to inhibit Ca currents in heart and other excitable tissues (see Reuter, 1973 for references). In the experiment shown in Fig. 8, D600 (10^{-6} g/ml.) abolished TI and the aftercontraction, as well as the slow inward current and the twitch. During the onset of the D600 effect, TI and the aftercontraction decreased together and were fully inhibited within 4 min. Exposure to Mn ions also produced concomitant decreases in the current and tension transients (results not shown).

Is the aftercontraction triggered by a transient Ca^{2+} influx?

The results presented so far indicate that TI and the aftercontraction are closely correlated but they do not indicate the nature of the relationship or the role of Ca. Two leading possibilities are indicated as follows.

(1) Transient increase in calcium permeability → phasic Ca influx (TI) → mechanical activation (aftercontraction)

(2) Phasic Ca release from intracellular store → Transient inward current carried by some unspecified ion; Mechanical activation (aftercontraction)

The first hypothesis follows the lines of Ferrier & Moe (1973). On this view, TI is carried by Ca^{2+} ions and the aftercontraction is a direct consequence of their interaction with the regulatory proteins of the contractile machinery. The second hypothesis proposes that TI and the aftercontraction are both secondary events. The primary event would be a transient or oscillatory release of Ca^{2+} from an

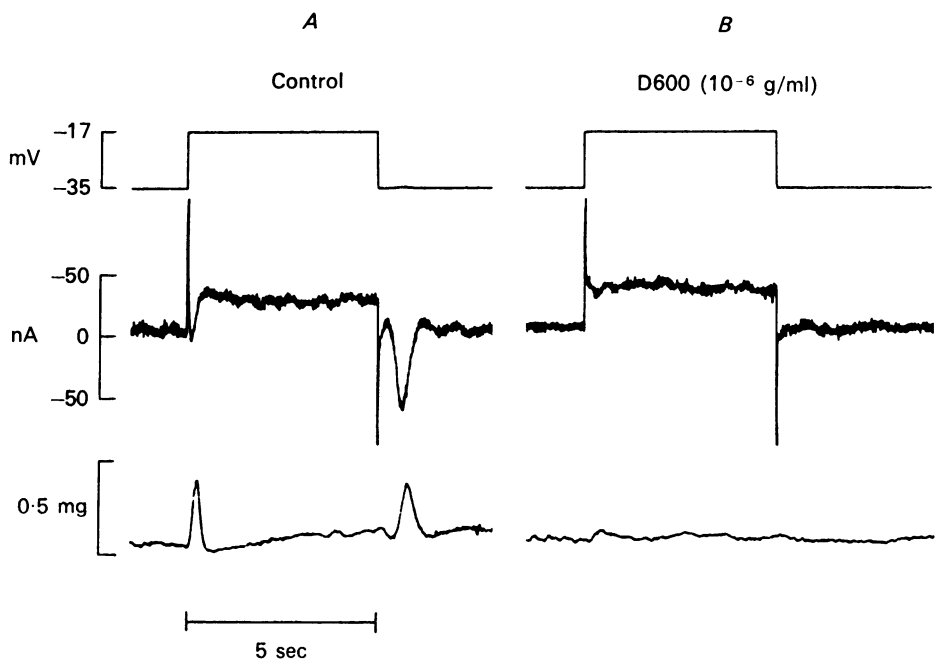


Fig. 8. Inhibition of TI and the aftercontraction by D600. *A*, a depolarizing step from −35 to −17 mV (upper trace) evokes a surge of slow inward current (middle trace) and a twitch (bottom trace). The pulse is terminated by a repolarizing pulse which produces TI and an aftercontraction. *B*, the slow inward current and TI are almost completely abolished by exposure to D600 (10^{-6} g/ml.) for 13 min. The tension trace becomes virtually flat. Preparation R12-2; 1 μM-strophanthidin; apparent cylindrical area, 0·007 cm^2.

intracellular store (see Ferrier, 1976, p. 161). TI would be explained as a current which is regulated by calcium but which need not be carried by Ca^{2+} ions.

Either of these hypothesis are consistent with the results presented thus far. For example, the lag between TI and the aftercontraction (Figs. 5*B*, 6*D*) is consistent with the idea that Ca influx causes the contraction. On the other hand, the lag is also consistent with the second hypothesis if the activation of contraction were slower than activation of membrane current. Similar ambiguity arises in the interpretation of experiments where the bathing calcium concentration is varied. Changes in TI and the aftercontraction could be attributed to direct modification of the putative Ca^{2+} influx (hypothesis 1) or an indirect influence on the amount of releasable Ca^{2+} in intracellular stores (hypothesis 2). The inhibitory action of D600 in the experiment illustrated in Fig. 8 is also open to different interpretations involving the direct blockade of a Ca pathway or an indirect reduction in Ca release from the intracellular store.

Comparison of inhibitory effects on the TI and the slow inward current

The remainder of the paper will be devoted to experiments which help distinguish between the alternative hypotheses. One of the first clues was provided by experiments using relatively low concentrations of Mn ion or D600. The aim was to compare the time course of inhibitory effects on TI and the slow inward current (I_{si}). If the reduction in TI were a secondary result of decreased Ca entry via I_{si}, one might expect the reduction in TI to lag behind the decrease in I_{si}. A disparity in time course might also be expected on recovery.

The results of such an experiment are presented in Fig. 9. Mn was used in most cases because its effects were more readily reversible than those of D600. Panel *A* shows a series of current records which accompanied a standard clamp pulse (Fig. 9*A*, *h*). In the control record (Fig. 9*A*, *a*) and after recovery (*g*), a surge of I_{si} appeared with the 'on' of the pulse and TI appeared following the 'off'. Records *a–d* show the onset of the effect of 2 mM–Mn, and *e–g* illustrate the recovery after Mn was removed from the bathing solution.

Exposure to Mn was followed by a very prompt reduction in the surge of I_{si} (*b*) without noticeable change in TI. Continued exposure (*c*, *d*) produced no further decrease in I_{si}, but did cause a progressive fall in TI amplitude and a slowing of TI time course. The contrast in the development of the inhibitory effects is emphasized by panel *B*, which plots measurements of TI amplitude and the peak slow inward current. The graph shows that reduction of I_{si} was half complete within about 5 min after initiating the solution change, while the reduction in TI displayed half-time of about 20 min. The disparity in time course was even greater during the washout. Restoration of the I_{si} surge was rapid, but half-recovery of TI took more than 1 hr. Thus, at various stages of the manganese run, one or other current was preferentially reduced. An earlier run in the same preparation and two other experiments showed similar results. In one of these experiments force measurements were carried out and showed that the aftercontraction recovered from Mn inhibition with the same time course as TI itself, while the twitch paralleled I_{si} in showing a rapid recovery.

These results do not support the idea that Mn ions simply block a pathway for TI

(cf. Ferrier & Moe, 1973). Since the decrease in the amplitude of TI was closely associated with a slowing of the time-to-peak, some additional change in channel kinetics must also be invoked. A more serious difficulty stems from comparison of the inhibitory effects on TI and I_{si}. One might account for the slow onset of inhibition by postulating that the TI pathway was less sensitive to Mn than I_{si} channels, but this would not explain the more complete inhibition which eventually develops, or the slower recovery when manganese was removed. The results are consistent, on the other hand, with an indirect effect of manganese on intracellular

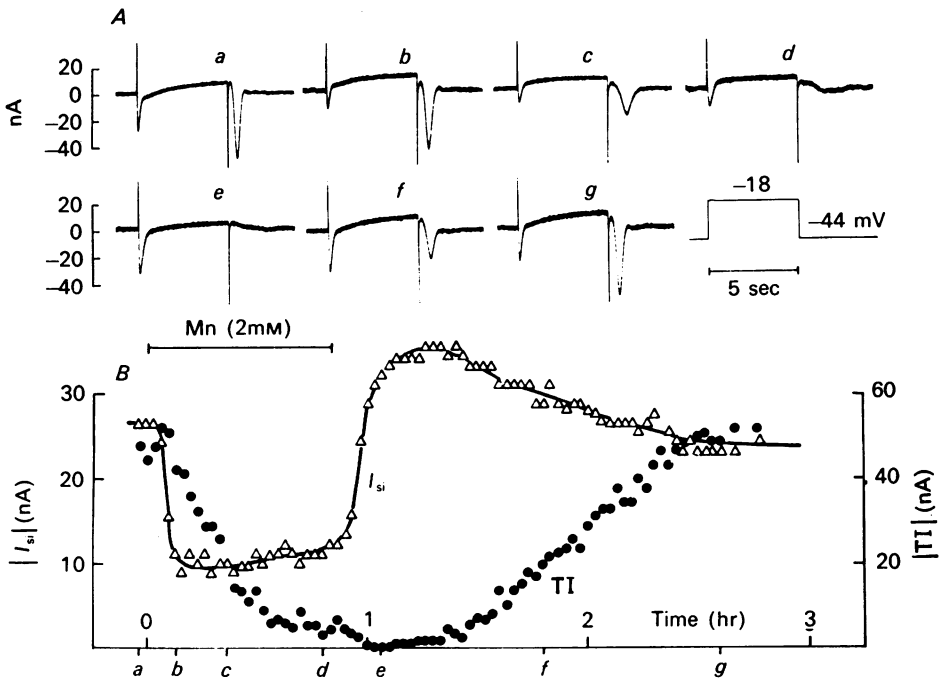


Fig. 9. Inhibition of slow inward current and TI by Mn. *A*, current traces (*a–g*) accompanying a standard clamp pulse. Traces *a–g* were taken at various times indicated on the abscissa below (*B*). Horizontal lines indicate level of I_{si} peak during control run (*a*). *B*, time course of effect of 2 mM-$MnCl_2$ on slow inward current (left ordinate) and TI (right ordinate). Current amplitudes were measured relative to the steady holding current. An earlier run in the same preparation gave very similar results, including the 'overshoot' in the slow inward current magnitude following removal of Mn. Preparation 158-4; 1 μM-strophanthidin; apparent cylindrical area, 0·005 cm^2.

Ca stores. This might be a secondary consequence of the reduction in I_{si}. An intracellular site of Mn action is another possibility which cannot be ruled out since Mn is known to enter cardiac cells (Ochi, 1976; Delahayes, 1975).

Experiments using D600 instead of Mn were less effective in demonstrating disparities in the inhibition of TI and I_{si}. Maximally effective concentrations (10^{-6}–5×10^{-6} g/ml.) promptly abolished I_{si} and TI as in Fig. 8, but the inhibition was poorly reversible. The effect of a rather lower concentration of D600 (5×10^{-8} g/ml.) was studied in a single experiment. Exposure to the drug was followed by a full recovery of I_{si}, but the onset of the inhibitory effect on I_{si} was relatively slow

($t_{\frac{1}{2}} \doteqdot 10$ min) and did not precede the reduction in the t.i. In other respects, the effects of D600 were quantitatively similar to that of Mn. Reduction of the amplitudes of TI and aftercontraction was accompanied by a clearcut increase in the respective time-to-peak. Recovery of TI during the washout of D600 lagged behind the recovery of I_{si}. Like the results with Mn, the effects of D600 seem inconsistent with a straightforward blockade of the pathway which carries TI.

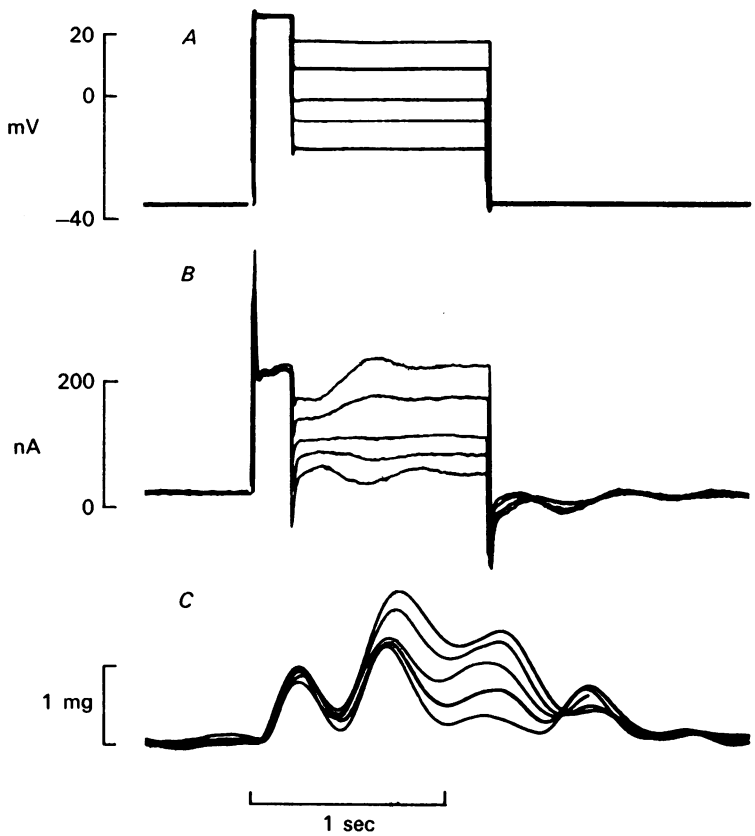


Fig. 10. Voltage-dependent reversal of TI. *A*, superimposed voltage traces from five trials during a single run. Step depolarization to +25 was followed by repolarizations to +17, +9, −1, −8 and −17 mV. *B*, associated records of membrane current (same vertical order as voltage traces). *C*, associated contractile force (same vertical order as voltage traces). For further details, see text. Preparation R22-3; 1 μM-strophanthidin; apparent cylindrical area, 0·009 cm^2.

Voltage-dependent reversal of TI

The results using Ca current inhibitors prompted us to look for other evidence against the calcium influx hypothesis. One direct approach involved showing that aftercontractions could be consistently observed in the absence of transient inward current. A dissociation would be consistent with earlier experiments in ventricular muscle where aftercontractions sometimes appear without detectable afterdepolarization (Reiter, 1962; Ferrier, 1976).

Fig. 10 shows that aftercontractions can be recorded when the inward current transient is suppressed by varying the electrical driving force. The potential was stepped from a holding potential of -37 to $+25$ mV for 200 msec in order to induce TI and aftercontraction. The membrane was then repolarized to various levels. This protocol is similar to that shown in Fig. 4 but here the repolarizations span a different voltage range. Between -20 and $+20$ mV the current signal (*B*) and force signal (*C*) are influenced in rather different ways by varying the voltage level. As the level is displaced toward positive potentials, the aftercontraction grows somewhat larger and is followed by increasing amounts of tonic force. The associated current records show a different kind of voltage-dependence. The transient current is inward at -17 mV, less inward at -8 mV and nearly flat at -1 mV. An outward transient was recorded at $+9$ mV and it becomes larger at $+17$ mV. Fig. 11 shows the analysis of these records (filled symbols) and other data (open symbols) from the same run. The amplitude of the current transient follows a continuous curve which intersects the abscissa at -2 mV. This intercept defines a reversal potential, E_{rev}.

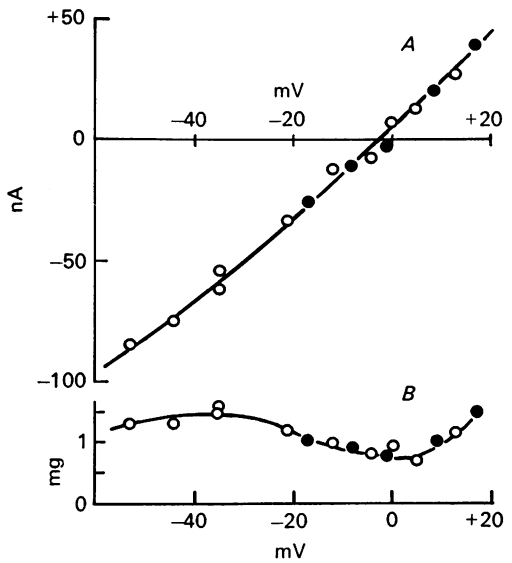


Fig. 11. Influence of repolarization potential (abscissa) on TI (*A*) and the aftercontraction (*B*). Transient amplitudes were measured (see Methods) using results in Fig. 10 (filled symbols) and other records from the same experiment (open symbols). Continuous curves drawn by eye. The reversal potential E_{rev} is given by the voltage intercept in *A*.

Voltage-dependent inversion of TI was demonstrated in a total of thirty-eight runs in fifteen preparations using the procedure illustrated in Fig. 10. The reversal potential was determined by graphical interpolation as in Fig. 11. An average value of $E_{rev} = -4{\cdot}8 \pm 1{\cdot}2$ mV (mean $\pm$ S.E. of mean, $n = 38$) was obtained by pooling the determinations in standard Tyrode solution. Effects of varying the ionic composition of the bathing fluid are presented in the following paper (Kass *et al.* 1978).

The reversal potential results argue against calcium as the predominant carrier of the transient inward current (see Discussion). They are consistent with the idea

that intracellular calcium regulates the membrane permeability of other ions as suggested in hypothesis 2. On this view, the role of Ca in TI might be comparable to the action of a post-junctional membrane. Following the analogy, the transient inward current may be described by the following expression:

$$\mathrm{TI} = \Delta g\,(E - E_{\mathrm{rev}}). \tag{1}$$

Here TI is defined as the transient component of current, whether it is inward or outward. The driving force for the transient current, $(E - E_{\mathrm{rev}})$ is presumed responsible for the polarity of TI. Δg is a phasic or oscillatory increase in membrane conductance, defined, in effect as $\mathrm{TI}/(E - E_{\mathrm{rev}})$. The time course of the conductance change remains similar to the wave form of the aftercontraction even though the driving force changes sign. There is a slight increase in time-to-peak for both current and force transients as the potential is displaced toward positive values (Fig. 10). The strong resemblance between Δg and the aftercontraction is consistent with the idea that both events are expressions of a transient increase in myoplasmic Ca.

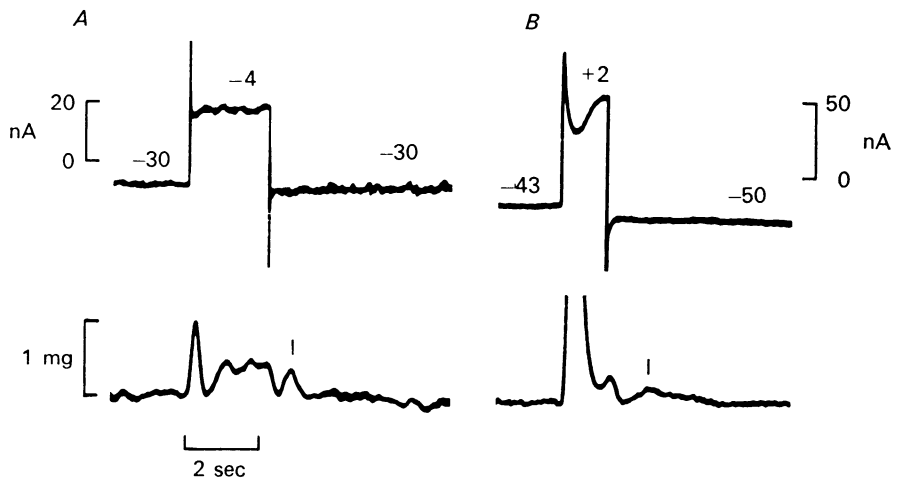


Fig. 12. Aftercontractions without detectable TI at potentials negative to E_{rev}. Current and force records from two different preparations under voltage clamp in the presence of 5·4 mM-Ca but in the absence of strophanthidin. Membrane potentials as indicated (mV). Aftercontractions follow clamp pulses and are indicated by vertical markings but current traces show no corresponding TI. *A*, preparation R21-1. *B*, preparation E10-2. Only part of the twitch is shown in *B* (full amplitude, 4 mg).

Further evidence for intracellular Ca release as the primary event

Aftercontractions can sometimes be observed in ventricular muscle in the absence of detectable transient depolarization (Reiter, 1962; Ferrier, 1976; see Discussion). This result suggests that myoplasmic Ca must rise to a critical threshold level in order to evoke a depolarizing inward current. It provides further support for the idea that the primary transient event is intracellular Ca release and not an electrically detectable entry of Ca across the surface membrane. Arguments along these lines can also be based on observations in Purkinje fibres. We found occasionally small aftercontractions without detectable TI at holding potentials far negative to E_{rev}. Fig. 12 illustrates these results.

In these examples, the dissociation between the aftercontraction and TI depended upon limiting the rise in intracellular Ca, and presumably restricting the transient conductance change. The driving force was significant since TI was seen at the same holding potential when larger Ca transients were evoked following other depolarizing pulses. These results differ from Fig. 10 in the nature of the dissociation between aftercontraction and TI. In Fig. 10, the membrane conductance changes but TI is suppressed because the driving force is near zero.

DISCUSSION

Is TI a Ca current?

The present study was prompted by the hypothesis that TI is a Ca current across the surface membrane (see Ferrier & Moe, 1973; Lederer & Tsien, 1975; Lederer, 1976). A calcium influx was proposed originally by Ferrier & Moe on the basis of effects of Ca_o and Mn on transient depolarizations. Such experiments are thrown into a different light by our finding that TI inverts when the membrane is strongly depolarized (Fig. 10). Since the reversal occurs near -5 mV, far negative to the calcium equilibrium potential, the transient inward current cannot be carried by a Ca specific pathway. In fact, the value of the reversal potential does not correspond to the equilibrium potential of any major ion: it lies negative to E_{Na} and positive to E_K or E_{Cl}. One must consider, therefore, the possibility that TI may be generated by a non-specific conductance, possibly analogous to the acetylcholine-induced conductance at the motor endplate (see Rang, 1975, for review). Determinations of E_{rev} provide a basis for studying the involvement of various ions and will be presented in the next paper (Kass *et al.* 1978). The results indicate that the major carrier of transient inward current is Na.

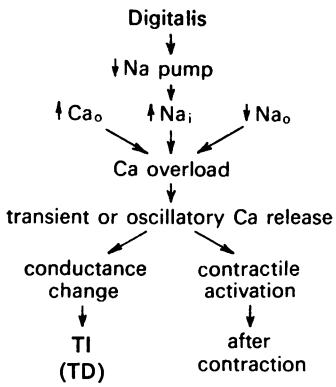
Evidence against artifactual explanations of TI

The inversion of TI at strongly depolarized potentials argues against certain artifactual explanations for the transient phenomena. For example, oscillatory inward currents might have been attributed to uncontrolled voltage oscillations. Gross voltage nonuniformity in either the longitudinal or radial direction might well produce synchronized transients in records of total current and force. However, this type of explanation cannot easily account for the voltage-dependent inversion of transient currents in Figs. 10 or 11. The inversion of TI would require that the hypothetical uncontrolled voltage oscillation be suppressed or even inverted without loss of synchrony with the phasic increases in force. This seems highly unlikely. Thus, the inversion experiment complements other evidence against artifactual explanations. The possibility of gross voltage inhomogeneity along the length of the preparation has been ruled out by direct measurements during transient depolarizations (Ferrier *et al.* 1973) or transient inward currents (Lederer & Tsien, 1976). The idea of radial non-uniformity depends on a current–resistance drop arising from radial current flow through the series resistance offered by narrow intercellular clefts (Johnson & Lieberman, 1971). Rabbit Purkinje strands have clefts 30–50 times wider than those of ungulate preparations (Johnson & Sommer, 1967; Sommer &

Johnson, 1968) and should have much lower radial series resistance. Despite this difference, rabbit Purkinje strands display transient inward currents which are similar to those seen in calf preparations (T. J. Colatsky & S. Siegelbaum, unpublished observations).

Role of Ca_i in arrhythmogenic effects of digitalis

Ca ions may be important to TI because of their relation to the conductance change which underlies the transient current. Our present evidence for this view depends upon the assumption that aftercontractions reflect variations in Ca_i. Aftercontractions show a strong temporal correlation with the conductance change, but not with the magnitude or polarity of current flow during TI. The results in Figs. 10 and 11, and those in the next paper can be accounted for by postulating that intracellular Ca regulates the membrane permeability to certain other ions. The possibility of Ca-regulated ion permeability has already been explored in erythrocytes (e.g. Romero & Whittam, 1971), nervous tissue (e.g. Meech & Standen, 1975; Hagins & Yoshikami, 1974) and cardiac muscle (Isenberg, 1975; Bassingthwaighte, Fry & McGuigan, 1976). Ca injection experiments in gland cells (Lcewenstein, 1975; Petersen & Iwatsuki, 1978) provide a precedent for the idea that intracellular Ca may increase the conductance for a depolarizing current. Such a conductance change is incorporated in the following scheme for explaining certain arrhythmogenic effects of digitalis:



A transient or oscillatory release of calcium from an intracellular store is the key step in this hypothesis. It is taken as the common link between various experimental procedures and the eventual production of TI and aftercontraction. Toxic concentrations of cardiac glycosides or aglycones initiate the sequence of events by inhibiting Na extrusion by the Na–K pump. As the cell gains Na, the Na gradient is reduced, causing a secondary decrease in calcium extrusion by the Ca–Na exchange (Reuter & Seitz, 1968; see Blaustein, 1974, for review). Increases in Na_i may also promote Ca release from mitochondria (Carafoli, Tiozzo, Lugli, Crovetti & Kratzing, 1974; but see also Fabiato & Fabiato, 1973, for contradictory evidence in skinned cardiac cells). Eventually the cells reach a state of 'Ca overload' which is accompanied by transient or oscillatory movements of Ca between intracellular stores and myoplasm. Such Ca movements have already been inferred from force measurements in ventricular cells with disrupted sarcolemmas (Fabiato & Fabiato, 1972; Bloom,

Brady & Langer, 1974) and in intact atrial muscle (Glitsch & Pott, 1975). In the final stages of the scheme the presumed variation in myoplasmic Ca is expressed separately by Ca-activated surface membrane conductance change (TI) and Ca-activated force (aftercontraction).

Effects of other agents

According to the hypothesis, TI may be evoked when intracellular Ca is elevated by procedures other than digitalis intoxication. Ca-rich solutions promote entry of Ca^{2+} ions down their electrochemical gradient. Na-poor solutions reduce the Na gradient and thereby decrease Ca extrusion via the Ca–Na exchange. Both high Ca_o and low Na_o are known to favour 'Ca overload' and, as expected, both conditions promote TI and aftercontractions in the absence of cardiotonic steroids (Fig. 7; figs. 4, 6, Lederer, 1976; fig. 5, Kass *et al.* 1978). Our results in Purkinje fibres are consistent with earlier studies in working myocardial preparations where Ca-rich or Na-poor solutions evoked aftercontractions (Bozler, 1943; Reiter, 1962; Kaufmann, Fleckenstein & Antoni, 1963; Braveny, Šumbera & Kruta, 1966; Jensen & Katzung, 1968; Posner & Berman, 1969; Mascher, 1971; Ryo, 1971; Verdonck, Busselen & Carmeliet, 1972; Glitsch & Pott, 1975). In some investigations, aftercontractions were observed without detectable transient depolarization (Reiter, 1962; Mascher, 1971; Verdonck *et al.* 1972; Ferrier, 1976). This type of dissociation is confirmed by our voltage-clamp experiments (Figs. 10, 12). Thus, the aftercontraction cannot be a direct consequence of the depolarizing potential change, as proposed by Kaufmann *et al.* (1963). The present hypothesis accounts for the close parallelism between mechanical and electrical transients under certain circumstances while allowing for dissociation under other conditions.

Agents which modify the slow inward current are not explicitly included in the scheme, but their actions may also be accounted for by secondary changes in Ca_i. Catecholamines promote aftercontractions in ventricular muscle (Reiter & Schöber, 1965) and the aftercontractions can be accompanied by transient depolarizations (Ryo, 1971; Nathan & Beeler, 1975). These effects could be indirect consequences of the well-known enhancement of Ca influx and I_{si} by adrenergic compounds. Similar reasoning suggests that the suppression of TI and aftercontraction by Mn, verapamil or D600 may be a subsidiary effect of decreased I_{si} and reduced Ca_i. Experiments using a submaximally effective concentration of Mn (Fig. 9) are consistent with this interpretation, since the inhibition of TI clearly lags behind the decrease in I_{si} and restoration of TI is much slower than I_{si} recovery.

Oscillatory behaviour: possible subcellular basis

TI has oscillatory characteristics which have been revealed by a variety of experiments (Lederer & Tsien, 1976). The wave form of TI often resembles a sinusoid and in some experiments, the initial inward transient is followed by a series of progressively diminishing peaks. Additive effects of two depolarizing pulses on the amplitude of subsequent TI may depend on the interval between the pulses in an oscillatory manner. Along with TI, strophanthidin intoxication promotes spontaneous fluctuations in membrane current. These fluctuations can give rise to variations in membrane potential in the absence of voltage clamp. Spectral analysis of

the current fluctuations indicates that they have the same frequency composition as TI itself (Kass, Lederer & Tsien, 1976). The current fluctuations are accompanied by spontaneous variations in force which also have similar periodic characteristics (R. S. Kass, R. W. Tsien & R. Weingart, in preparation). These observations under voltage clamp may be relevant to earlier reports of spontaneous oscillations in mechanical or electrical activity in preparations where membrane potential was allowed to vary (Pappano & Sperelakis, 1969; Winegrad, 1974; Glitsch & Pott, 1975; Akselrod & Lass, 1975; Chapman & Leoty, 1976; Goshima, 1976).

A subcellular basis of such oscillatory activity has been suggested by experiments using cardiac cells whose sarcolemmas have been disrupted (Fabiato & Fabiato, 1972; Bloom *et al.* 1974; Müller, 1976) or removed by mechanical microdissection (Fabiato & Fabiato, 1975). In the presence of light EGTA buffering and appropriate levels of free Ca, these preparations often show cyclic contractile activity. The contractions have been interpreted in terms of periodic Ca movements between the myoplasm and an intracellular store. The store has been identified as the sarcoplasmic reticulum on the basis of effects of non-ionic detergents (Fabiato & Fabiato, 1975) and pharmacological agents such as caffeine, ruthenium red, azide and oligomycin (Bloom *et al.* 1974; Fabiato & Fabiato, 1975). The present results complement the work in skinned cardiac preparations by relating the oscillatory changes in Ca_i to the electrical behaviour of intact cells. In the skinned cell, cyclic variations in Ca can be demonstrated despite the virtual absence of sarcolemma. In the intact cell, on the other hand, surface membrane potential may have an important influence on the oscillatory behaviour. A sudden change in membrane potential seems to synchronize the events which take place spontaneously during the current fluctuations. A net release of Ca is evoked by a repolarizing step, and the magnitude and time course of the release are sensitive to the repolarization level (Fig. 5). This relationship between surface membrane potential and Ca release has interesting implications for excitation–contraction coupling and deserves further study.

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