

## Multiple Effects of Calcium Antagonists on Plateau Currents in Cardiac Purkinje Fibers

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**ABSTRACT** We studied the influence of Mn, La, and D600 on action potentials and plateau currents in cardiac Purkinje fibers. The Ca antagonists each abolished the second inward current, but they failed to act selectively. Voltage clamp experiments revealed two additional effects: decrease of slow outward current ( $i_x$ ) activation, and increase of net outward time-independent plateau current. These effects occurred at inhibitor concentrations used in earlier studies, and were essential to the reconstruction of observed Ca antagonist effects on electrical activity. The inhibitory influence of Mn, La, and D600 on  $i_x$  suggested that  $i_x$  activation might depend upon prior Ca entry. This hypothesis was not supported, however, when  $[Ca]_o$  was varied: elevating  $[Ca]_o$  enhanced Ca entry, but  $i_x$  was nevertheless depressed. Thus, the results suggested instead that Ca antagonists and Ca ions have rather similar effects on  $i_x$ , possibly mediated by changes in membrane surface charge.

### INTRODUCTION

The cardiac action potential plateau is maintained by a delicate balance between inward and outward components of ionic current. The plateau is terminated by a slow change in net ionic current in the outward direction, which tips the balance in favor of repolarization. Although it is generally agreed that voltage- and time-dependent changes in ionic current are crucial (see Noble and Tsien, 1972; cf. Kootsey and Johnson, 1973), disagreement remains regarding the identity of the ionic component which triggers the repolarization process.

In the case of the Purkinje fiber action potential, controversy arises because both the inward and outward components of plateau current show important time dependence. First, there is an inactivating inward current, carried at least in part by calcium ions. This component is conveniently labeled  $i_{s1}$  ("second" or "slow" inward current) and is distinguished from the excitatory sodium current ( $i_{Na}$ ) by its kinetics (Reuter, 1968; Vitek and Trautwein, 1971), its dependence on  $[Ca]_o$  (Vitek and Trautwein, 1971), and its insensitivity to tetrodotoxin (Carmeliet and Vereecke, 1969). In addition to  $i_{s1}$ ,

there is also a slowly activating outward current,  $i_x$ , which is largely carried by potassium ions and which underlies the slow delayed rectification in the plateau range of potentials (Noble and Tsien, 1969).

Earlier studies of plateau current did not anticipate the existence of time dependence in *both* the inward and outward components. It is hardly surprising, therefore, that voltage clamp results were interpreted with emphasis on the importance of either  $i_{si}$  (Reuter, 1967, 1968; Vitek and Trautwein, 1971) or  $i_x$  (Noble and Tsien, 1969), to the exclusion or at least neglect of the other. It is now clear that both components play essential roles in the normal action potential. The activation of  $i_{si}$  generates the secondary depolarization and "notch" configuration that is often observed. The subsequent inactivation of  $i_{si}$ , together with the slow onset of  $i_x$ , combine to give a net change in the total ionic current which terminates the plateau (see McAllister et al., 1975).

The importance of the interplay between  $i_{si}$  and  $i_x$  has been emphasized by studies on the effect of epinephrine and other agents which mimic its action on the Purkinje fiber plateau (Tsien et al., 1972). Epinephrine has two clear-cut effects on the plateau which occur independently of an acceleration in rate: (*a*) the plateau level is elevated toward more positive potentials (Reuter, 1967) and (*b*) the action potential duration is decreased (Giotti et al., 1973). Voltage clamp experiments in short Purkinje fibers demonstrate that effect *a* is accounted for by an increase in the magnitude of  $i_{si}$  (Reuter, 1967), and that *b* is due to an augmentation of  $i_x$  (Tsien et al., 1972). Using a computer model of the action potential (McAllister et al., 1975), further reconstruction work (Tsien, 1973) showed that either of the individual changes in  $i_{si}$  or  $i_x$  would be inappropriate to account for the overall epinephrine effect.

While exploring the effects of epinephrine, it became evident that further study of plateau currents would be expedited by better methods for isolating the individual components. The most obvious method, ionic substitution, is not very helpful: withdrawal of Na or Ca ions, for example, reduces  $i_{si}$  (Vitek and Trautwein, 1971) but also affects potassium permeability (McAllister and Noble, 1966; Noble and Tsien, 1969; Trautwein, 1973). It is natural, therefore, to turn to a pharmacological approach (cf. Hille, 1970). A variety of blocking agents are available, and have been widely used (see Reuter, 1973; Trautwein, 1973). In working heart muscle the second inward current (Ca current) is suppressed by Mn (Hagiwara and Nakajima, 1966; Rougier et al., 1969; Ochi, 1970), La (Katzung et al., 1973), or the organic compounds verapamil and its methoxy- derivative, D600 (Kohlhardt et al., 1972). In Purkinje fibers Mn has previously been used as an inhibitor of  $i_{si}$  (Carmeliet and Vereecke, 1969). In fact, Vitek and Trautwein (1971) have claimed that Mn ions block the slow inward current selectively.

We examined the effects of Mn, La, and D600 on plateau currents, in hope of verifying their specificity as Ca antagonists. Contrary to the claim of Vitek and Trautwein (1971), we found that Mn is far from selective in its actions. While abolishing the second inward current, Mn also reduced the activation of  $i_x$ . The same is true for La and D600. Furthermore, these Ca antagonists also influenced the net time-independent current in the plateau range of potentials. All of these effects took place within the previously employed range of inhibitor concentrations, suggesting that the earlier results may need reinterpretation.

Although this lack of pharmacological specificity was disappointing, the effects of Mn, La, and D600 on  $i_x$  were also interesting because they suggested a possible link between Ca entry and the activation of outward K current. Such a relationship seems to hold in certain nerve cells (see Meech, 1974 for references). We have explored this "Ca entry hypothesis" by studying the influence of  $[Ca]_o$  on  $i_x$ . The results failed to support the Ca entry idea, but suggested instead that many of the effects of Ca antagonists and Ca ions may be due to alterations of external surface charge.

#### METHODS

Purkinje fiber bundles from calf hearts were used in the experiments. Voltage clamp studies were performed in short preparations by means of the two-microelectrode technique of Deck et al. (1964), as described by Tsien (1974 *a*). Action potentials were studied with conventional intracellular microelectrode recording. The preparations were driven by just-suprathreshold external stimuli, at a rate of 0.5 Hz unless otherwise indicated. No difference in electrical activity was found between long bundles and short preparations that were suitable for voltage clamp experiments. Voltage clamp pulses were applied at rates slower than 0.5 Hz (see figure legends) to allow measurement of slow current changes after clamp pulses.

#### *Solutions*

Reported procedures were followed as closely as possible in reexamining the effects of so-called Ca antagonists, Mn, La, and D600. We used previously recommended concentrations of manganous chloride (Baker, Phillipsburg, N.J.), lanthanum chloride (British Drug Houses, Poole, England), and compound D600 ( $\alpha$ -isopropyl- $\alpha$ [(*N*-methyl-*N*-homoveratryl)- $\gamma$ -aminopropyl]-3,4,5-trimethoxyphenylacetonitrile · HCl, Knoll, Ludwigshafen am Rhein, Germany). D600 concentration is given in grams per milliliter to allow direct comparison with the values of Kohlhardt et al. (1972). The formula weight of D600 · HCl is 521 daltons. Solutions containing Mn, La, or D600 were made up from refrigerated stock solutions without compensation for changes in ionic strength or osmolality. The Tyrode solution was very similar to that of Dudel et al. (1967) and contained: 150 mM Na, 4 mM K, 5.4 mM Ca, 0.5 mM Mg, 155.8 mM Cl, 5 mM glucose, and 10 mM Tris-maleate (pH 7.2–7.4). In some experiments, the concentration of CaCl<sub>2</sub> was changed to 1.8 mM or 7.2 mM

(see figure legends). All studies were performed near 36°C and within a given experiment the temperature was kept constant to within  $\pm 0.2^\circ\text{C}$ . Unless otherwise stated, the figures illustrate the effects of the drugs in the steady state (usually achieved after 10–15-min exposure).

#### *Analysis of Time-Dependent Current*

In the voltage clamp experiments the membrane potential was routinely held near  $-40$  mV between clamp pulses. At this level, the fast excitatory current is fully inactivated (Weidmann, 1955 *a*), and the transient outward chloride current is about 90% inactivated (Fozzard and Hiroaka, 1973, Fig. 10). As a result, step depolarizations from the holding potential produced time-dependent current changes which were dominated by inward  $i_{ss}$  and outward  $i_x$ . No detailed analysis of  $i_{ss}$  was carried out since the objective was to abolish this component. Slow outward current ( $i_x$ ) was studied by means of step potential changes within the plateau range (Noble and Tsien, 1969). A depolarizing clamp pulse was followed by a tail of outward current at the holding potential whose amplitude indicated the amount of  $i_x$  conductance turned on at the end of the pulse. Tail amplitudes were measured on highly amplified chart recordings by extrapolating the decay time-course back to the break of the pulse. Interference from inward tails of  $i_{ss}$  was not a serious problem because these decayed much faster than  $i_x$ . Since we were interested in conductance changes over the duration of an action potential, clamp pulses were generally restricted in duration to a fixed value, usually 1 s. Tail amplitudes were plotted as a function of the pulse potential, giving "isochronal" activation curves. These must be distinguished from "steady-state" activation curves (Noble and Tsien, 1969) which require much longer pulses. The approach used here does not fully characterize the kinetics but reduces the extent of potassium accumulation and the number of pulses which must be applied during a given run. Some of the experiments were carried out in partially depolarized preparations but the results were similar to those obtained in preparations with normal resting potentials (see Tsien, 1974 *a*).

## RESULTS

### *Effects of Ca Antagonists on the Action Potential*

Fig. 1 gives an overview of the effects of the Ca antagonists on electrical activity in Purkinje fibers. Two types of stimulated activity are illustrated on the left and right side of each record. The left side of each panel shows action potentials initiated from the normal resting potential. In the presence of D600 (middle panel), the action potential plateau is lower and briefer than in drug-free Tyrode solution (top and bottom panels).

The right side of the various panels shows the responses which resulted after the membrane potential had been deliberately depolarized to about  $-50$  mV by applying outward current through a second microelectrode (bar). From this level in normal Tyrode solution, stimuli evoked the so-called "slow response" which has often been studied in preparations depolarized

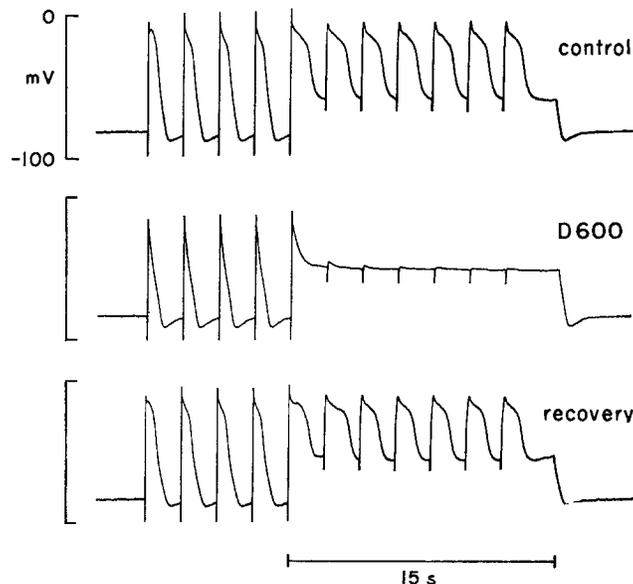


FIGURE 1. Effect of D600 on action potentials and slow responses. Chart recordings of transmembrane potential during the course of a continuous microelectrode impalement. Electrical activity was evoked by trains of external stimuli. During the later part of the train (right) the preparation was partially depolarized by applying outward current (21 nA) through a second intracellular microelectrode. The current pulse lasted 15 s and is indicated by the horizontal bar. The top and middle panels illustrate the activity just before and 5 min after exposure to D600 ( $5 \times 10^{-6}$  g/ml). The bottom panel shows the recovery 1 h after removing the drug. Action potential overshoots were off-scale on the chart recording and shock artifacts appear as downward spikes. Preparation 137-5, 7.2 mM  $[Ca]_o$ .

by potassium-rich solutions (Carmeliet and Vereecke, 1969; Cranefield et al., 1972). Exposure to D600 (middle panel) abolished the slow response and removal of the inhibitor (bottom panel) restored the response to its original waveform. Similar abolition of slow responses was obtained by treating partially depolarized fibers with Mn (10 mM) or La (0.5 mM) as in previous studies (see Aronson and Cranefield, 1973 for references). The Ca antagonists also proved effective in abolishing "low voltage oscillations" (Hauswirth et al., 1969), as expected if the rising phase in this type of activity depended on the second inward current (Aronson and Cranefield, 1974), rather than  $i_{Na}$  as suggested by Hauswirth et al. (1969).

According to the prevailing view, a single action, the selective blockade of the second inward current, can explain both the inhibition of the slow response (Kohlhardt et al., 1972) and the lowering and shortening of the action potential plateau (Vitek and Trautwein, 1971). Other experiments reveal, however, that the actions of the Ca antagonists are not so simple.

*Onset of High Concentration Effects*

Fig. 2 compares the responses to relatively high concentrations of D600 and Mn, respectively, during the course of a single impalement. Oscilloscope records were taken at 3-min intervals during the onset of drug effects. In Fig. 2 B, exposure to Mn (10 mM) lowered and abbreviated the plateau concomitantly. The steady effect (dashed trace) agrees with previous results

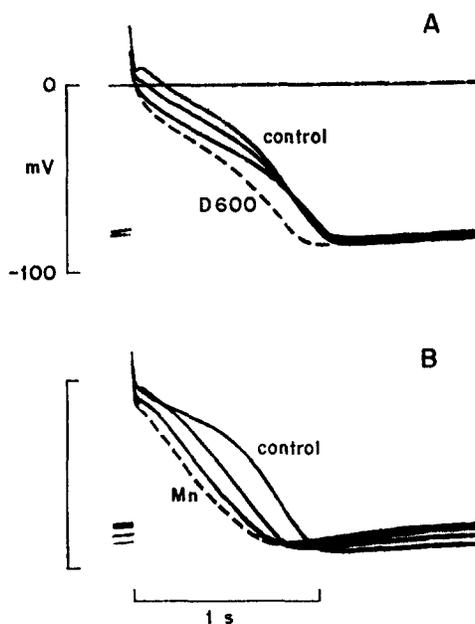


FIGURE 2. Effects of high concentrations of Ca antagonists on the action potential.  $5 \times 10^{-6}$  g/ml D600 and 10 mM Mn were used. Solid traces were oscilloscope records taken before, and 3 and 6 min after onset of drug actions. Dashed traces are steady-state effects (recorded after about 15 min). Records were obtained with a continuous micro-electrode impalement in preparation 128-3, 7.2 mM  $[Ca]_o$ . Note that D600 does not affect the pacemaker depolarization.

of Vitek and Trautwein (1971, Figs. 2 and 6). On the other hand, D600 ( $5 \times 10^{-6}$  g/ml), produced a different pattern of response. Initially, exposure to D600 lowered the plateau and slightly *lengthened* the action potential; during further exposure, the plateau level became even lower, while the action potential duration (APD) decreased. Eventually, no further change took place, and the steady-state response (dashed trace) demonstrated an overall reduction in APD (as in Figs. 1 and 2 A). This contrast in the development of the change in plateau configuration is supplemented by another difference which appeared in the effects on pacemaker activity. As illustrated in Fig. 2,

Mn markedly steepened the diastolic depolarization, while D600 had no effect.

Further explanation of these differences will be given below. The recordings in Fig. 2 already suggest, however, that Mn and D600 do not share identical actions, and therefore, that at least one of these agents fails to act on the second inward current alone.

#### *Influence of Lower Concentrations of D600 or Mn*

Reduced concentrations of Ca antagonists were used in hopes of distinguishing their primary action from other effects. Fig. 3 compares the effects of Mn and D600 at concentrations 10 times lower than the respective levels in Fig. 2. In both instances, the Ca antagonists lowered the plateau level, as before;

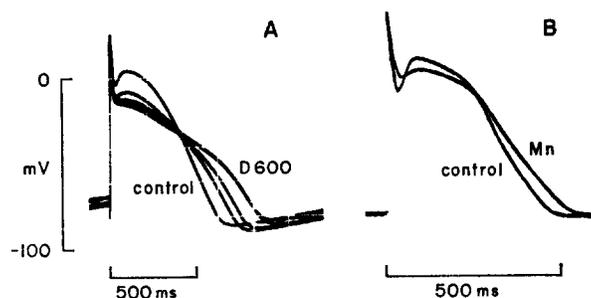


FIGURE 3. Effect of low concentrations of D600 and Mn on action potentials. Oscilloscope records from two preparations. (A) Before, and 6, 8, and 12 min after exposure to D600 ( $5 \times 10^{-7}$  g/ml). Preparation 88-4, driven at 0.5 Hz in 5.4 mM  $[Ca]_o$ . (B) Before and 16 min after beginning exposure to Mn (1 mM). Preparation 141-1, driven at 1 Hz in 7.2 mM  $[Ca]_o$ .

however, the action potential duration was *increased*. This behavior is consistent with the transient lengthening in Fig. 2, and with earlier observations by Rosen et al. (1974) and Cranfield et al. (1974) using verapamil (a compound that is closely related to D600). In our own experiments, prolongation of the action potential accompanied lowering of the plateau at D600 concentrations as low as  $5 \times 10^{-8}$  g/ml (see Table I).

Table I summarizes the results from the action potential experiments. In each experiment, exposure to the Ca antagonist reduced the height of the plateau; however, there was a wide range of effects on APD. The overall pattern supports the view given by Figs. 2 and 3. Thus, D600 in "high" concentration ( $5 \times 10^{-6}$  g/ml) consistently abbreviated APD, while "moderate" levels ( $5 \times 10^{-7}$  g/ml, as recommended by Kohlhardt et al., 1972) gave variable effects, reducing APD in five cases and lengthening it in three others. This concentration dependence is clear enough to indicate that the primary effect of Mn or D600 results in the lowered plateau, and that this

TABLE I  
EFFECTS OF D600, Mn AND La ON ACTION POTENTIAL DURATION

Preparation	Concentration	APD(-60 mV)			ΔAPD
		Control	Test	Recovery	
	<i>mg/liter</i>	<i>ms</i>	<i>ms</i>	<i>ms</i>	%
<b>D600</b>					
111-3	0.05	430	470	430	+9
	0.75	430	480	440	+10
	0.1	430	430	430	0
	0.5	420	480	420	+14
88-4	0.5	510	710	—	+39
91-20	0.5	572	670	—	+17
112-1	0.5	1,460	3,160	1,400	+121
94-6	0.5	740	500	—	-48
122-2	0.5	630	350	590	-43
	0.5	630	340	646	-47
123-1	0.5	590	500	556	-13
130-1	0.5	630	560	—	-11
123-2	0.5	760	580	760	-31
	5.0	680	330	—	-51
128-3	5.0	860	660	820	-21
137-5	5.0	830	570	900	-34
<b>Mn</b>					
141-1	1	395	445	415	+10
	3	420	420	—	0
128-3	1	770	756	750	-1
	10	750	390	750	-48
122-2	10	720	340	730	-53
123-1	10	790	420	790	-47
	10	660	360	660	-45
<b>La</b>					
128-3	0.5	750	670	700	-8
131-1	0.5	610	520	780	-25
134-5	0.5	1,134	760	—	-48

Steady-state effect of exposure to Ca antagonists. Plateau level was lowered in all cases; the accompanying change in action potential duration was measured at -60 mV.  $[Ca]_o$  was either 5.4 or 7.2 mM. Preparations were driven at 0.5 Hz (except 130-1, 0.33 Hz and 141-1, 1 Hz). In cases where recovery was studied, ΔAPD was taken as  $2(\text{test APD})/(\text{control APD} + \text{recovery APD})$ .

action is distinguishable from a less sensitive "side effect" which produces the abbreviated action potential.

#### *Effects of La*

La (0.5 mM) resembled 10 mM Mn in its influence in lowering the plateau, decreasing APD, and steepening the pacemaker depolarization. These effects developed within a few minutes, as found for Mn or D600, but the La effects were less readily reversible.

#### *Voltage Clamp Studies: Altered Plateau Currents*

The action potential results raised questions about the ionic basis of Ca antagonist effects. We therefore carried out voltage clamp experiments in shortened Purkinje fibers. Currents in the plateau range of potentials were studied by applying depolarizing voltage steps from a steady holding potential near  $-40$  mV. Through this procedure, the excitatory sodium current remained inactivated (Weidmann, 1955 *a*) and thus did not interfere with the analysis of the plateau currents  $i_{s,i}$  and  $i_x$ . The outward chloride transient was also largely inactivated (Fozzard and Hiraoka, 1973).

**MULTIPLE EFFECTS OF CA ANTAGONISTS** Fig. 4 illustrates the influence of Mn on currents in the plateau range. The individual records (*a-g*) were taken from seven consecutive runs, obtained alternatively in control (left) and Mn-containing solutions (right). Each record corresponds to the same voltage waveform, a standard 20-mV depolarizing pulse lasting 2 s. In normal Tyrode solution (record *a*) the step depolarization elicited a surge of inward current carried by  $i_{s,i}$  (Reuter, 1968; Vitek and Trautwein, 1971). The second inward current then decayed, and gave way to the slower development of outward current, due to the activation of  $i_x$  (Noble and Tsien, 1969). The effects of Mn on this time-dependent behavior were reversible (compare records *a*, *c*, *e*, and *g*), and very concentration dependent. Exposure to 1 mM Mn (record *b*) simply reduced the surge of second inward current, without appreciably altering the slower development of outward current. On the other hand, higher concentrations exerted a more complex influence (see *d* and *f*). Exposure to 10 mM Mn (*a*) abolished the inward current surge, (*b*) eliminated the slow onset of outward current, and (*c*) shifted the "late" current level in the outward direction. These changes can be explained as follows: *a* is the expected blockade of  $i_{s,i}$ ; *b* is due to the suppression of  $i_x$  (see *Effect of Ca Antagonists on Delayed Rectification*, below); and *c* may be attributed to an alteration of some time-independent current component. This explanation is supported by further evidence that will be presented below.

Record *f* shows that 3 mM Mn produces changes rather similar to 10 mM Mn. Thus, the multiple effects of Mn occur over the range of concentrations which earlier studies have employed in hope of "selectively" modifying

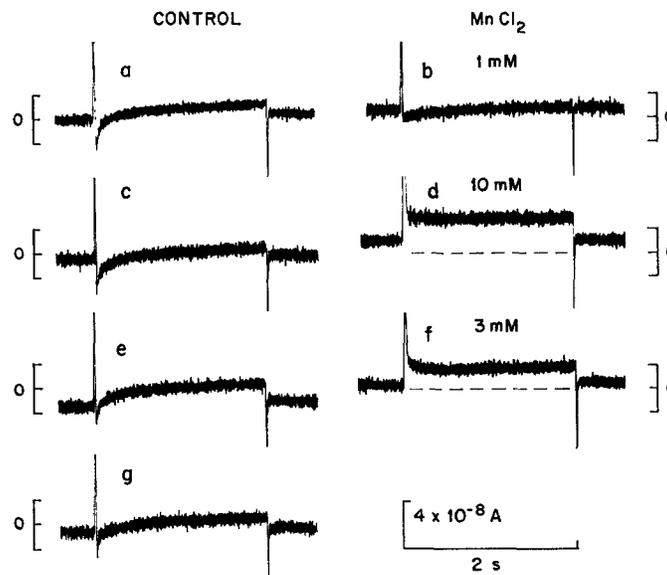


FIGURE 4. Graded effect of Mn on plateau currents. Net membrane current records during a standard depolarizing clamp pulse (from a holding potential of  $-38$  to  $-18$  mV for 2 s). Records *a-g* were taken from consecutive runs in Tyrode solution (left panels) and Mn-containing solution (right panels), and show steady-state effects. These chart recordings were taken at low paper speed and moderately high amplification in order to emphasize changes in late currents. Under these conditions, the amplitude of the inward surge was underestimated by about 25% (cf. Fig. 6 C). Preparation 62-5, 1.8 mM  $[Ca]_o$ , apparent cylindrical area of cell core  $0.011$  cm<sup>2</sup>.

electrical activity. Unfortunately, this lack of pharmacological specificity was also found for the other Ca antagonists, D600 (Fig. 5 B) and La (Fig. 6).

**ALTERED CURRENT-VOLTAGE RELATIONSHIP** The Ca-antagonist effect was characterized in Fig. 4 at a fixed test voltage near  $-20$  mV. Fig. 5 extends the analysis to the entire plateau range of potentials. The results are presented in the form of a current-voltage diagram, with vertical lines to indicate the magnitude of the time-dependent changes in net current. The various filled symbols represent the current at the end of 2-s pulses to different potentials, as plotted along the abscissa.

Panel A was taken from the same experiment as Fig. 4. The current-voltage diagram indicates that the abolition of time-dependent current changes was virtually complete over the entire range of potentials studied both for 3 and 10 mM Mn. However, a graded effect was produced on the late current level (effect *c*, above). Increasing the Mn concentration from 3 to 10 mM made the *i-v* relationship flatter and more outward. Thus it appears that effect *c* may be the least sensitive of the various changes.

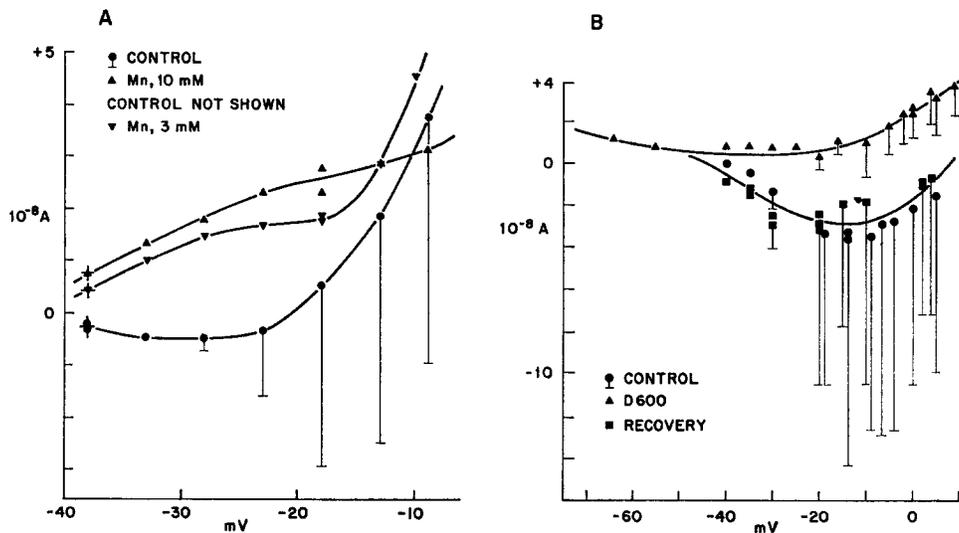


FIGURE 5. Effect of Mn and D600 on the late currents in the plateau range of potentials. The filled symbols plot net currents at the end of clamp steps to the voltages plotted along the abscissa. The vertical bars indicate time-dependent changes in currents during the various clamp pulses. (A) Currents at the end of 2-s clamp steps in control solution, 10 mM Mn, and 3 mM Mn. These were taken from runs *c*, *d*, and *f*, respectively, in the experiment of Fig. 4. (B) Currents at the end of 1-s pulses in control solution, in  $5 \times 10^{-7}$  g/ml D600 and after removal of the drug. Preparation 130-1, 7.2 mM  $[Ca]_o$ , apparent cylindrical area of cell core, 0.008 cm<sup>2</sup>. When referred to this area, the magnitude of the increase in net outward current is about  $4 \mu\text{A}/\text{cm}^2$  at  $-20$  mV.

Fig. 5 A was typical of three experiments in Mn and two in La, and conflicts with an earlier statement that "the N-shape of the outward current voltage relation was not altered by  $Mn^{++}$  ions . . ." (Vitek and Trautwein, 1971, p. 217). We have no explanation for the discrepancy, but we suggest that the present finding would be appropriate to explain the marked decrease in APD which we found (Fig. 2) and which Vitek and Trautwein reported.

Fig. 5 B illustrates the effect of D600 on currents over the plateau range. D600, like Mn, markedly reduced the magnitude of the inward current surge. Some time-dependent current change remained (short vertical lines attached to triangles) but the time dependence was slow and probably due to the slow onset of  $i_x$ . D600 also shifted the late current level in the outward direction. There is some indication in this figure that the shift in late current was confined to the potential range positive to  $-50$  mV (see Discussion).

The effect of D600 on late outward current was clear-cut in Fig. 5 B and in another experiment, but was not apparent in a third experiment at the same concentration ( $5 \times 10^{-7}$  g/ml). On the basis of this limited evidence, it seems possible that the variability in effect *c* may account for the range of effects of D600 on action potential duration (Table I).

*Dissection of Inhibitor Effects*

Fig. 6 shows the action of La, and extends the analysis of the inhibitory effects. As in Fig. 4, a standard depolarizing clamp pulse was used to monitor plateau currents. Panel C shows superimposed oscilloscope records before and during exposure to La (0.5 mM). The inward surge ( $i_{s,i}$ ) and slower onset of outward current ( $i_x$ ) are evident in the control record. La resembled Mn in abolishing both of these time-dependent changes. It also displaced the late current (at the end of the 1-s clamp pulse) in the outward direction.

The increase of the late net current was already pointed out in Figs. 4 and 5, and is a particularly important feature of the Ca antagonist behavior because of its possible influence on the repolarization of the action potential (see Fig. 9). The displacement cannot be accounted for by a change in  $i_x$  since  $i_x$  is an outward component whose activation is actually suppressed (Fig. 7, below). This leaves open the possibility that the change in late current reflects an alteration in some component that is effectively time independent, for example, a potassium "leak" current, or some fraction of  $i_{s,i}$ , which carries steady current (see Discussion). Whatever its explanation, the change in steady current must be large enough to offset the suppression of  $i_x$ . In fact, the estimation of the steady-current displacement requires the elimination of interference from  $i_x$ .

Fig. 6 illustrates a method for dissecting  $i_x$  from the total current signal, which leads to an estimate of the displacement in steady current without interference from  $i_x$  (shaded area in panel C). The first step was to determine the time-course of  $i_x$  activation, during the clamp depolarization. Outward "tails" were used as an index of the prior degree of activation (see Methods). Fig. 6 A plots the tail amplitude (measured at the holding potential,  $-48$  mV) as a function of the duration of the preceding clamp pulse (to  $-22$  mV). The points were fitted by a single exponential,  $\tau_x = 1.25$  s, and they indicate the time-course of  $i_x$  activation at  $-22$  mV.

With this information, it was then possible to determine the contribution of  $i_x$  to the current during step depolarizations to  $-22$  mV. The analysis of the current onset was carried out on a highly amplified chart recording (panel B). The onset was fitted by an exponential curve (solid line) which obeyed the equation

$$i = i_o + (i_x)_\infty [1 - \exp(-t/\tau_x)], \quad (1)$$

where  $i_o = i_\infty - (i_x)_\infty$ .  $i_\infty$  (horizontal line in panel B) was taken as the net outward current at the end of a very long (6 s) clamp pulse. Since the parameters  $i_\infty$  and  $\tau_x$  were already known, the curve fit serves simply to determine the value of  $i_o = i_\infty - (i_x)_\infty$ . This value then corresponds to the steady-state current with  $i_x$  subtracted off, that is, the level of the dashed line in B. The

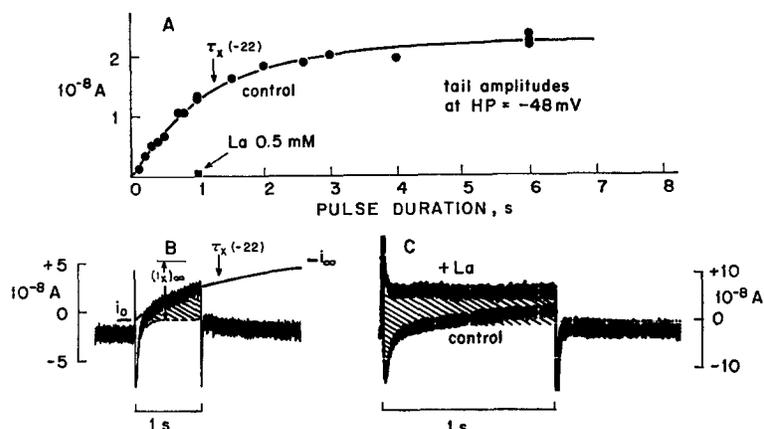


FIGURE 6. Plateau current components and their modification by La. A and B illustrate results in Tyrode solution and the procedure for dissecting off  $i_x$ . C compares the  $i_x$ -free current in Tyrode with the current in the presence of La. (A) Time-course of  $i_x$  activation at  $-22$  mV, as indicated by the growth of outward tails (measured upon return to the holding potential,  $-48$  mV). The envelope of tail amplitudes has been fitted by an exponential (smooth curve) with time constant  $\tau_x = 1.25$  s. (B) Chart recording of membrane current accompanying a 1-s pulse to  $-22$  mV from the holding potential. The peak of the inward surge is partly off-scale. The shaded area indicates the contribution of  $i_x$  to the net current. This was determined by fitting the onset to an exponential (smooth curve), using  $\tau_x$  as determined in A. The steady-state current level ( $i_{\infty}$ ) was taken as the current 6 s after depolarizing to  $-22$  mV. Extrapolating the exponential back to  $t = 0$  determines  $i_0 = i_{\infty} - (i_x)_{\infty}$  as indicated. Suppression of the slow exponential component (that is,  $i_x$ ) leaves the dashed curve, the " $i_x$ -free current." (C) Storage oscilloscope traces showing the effect of La (0.5 mM). The control trace corresponds to the signal in B, but was taken with lower amplification and a faster time base. The lower border of the shaded area corresponds to the dashed line in panel B. The shaded area gives the  $i_x$ -free, La-sensitive current, i.e. (net current in La) - (net current in Tyrode minus  $i_x$ ). Preparation 55-1,  $1.8$  mM  $[Ca]_o$ , apparent cylindrical area of cell core,  $0.011$  cm $^2$ .

shaded area in B then indicates the contribution of  $i_x$  to the total current. The " $i_x$ -free" current signal was left with only a rapid phase of time dependence. In this experiment, the rapid phase was well fitted by a single exponential, time constant 60 m. This value agrees with earlier estimates of Reuter (1968) and Vitek and Trautwein (1971) for  $\tau_f$ , the time constant of inactivation of  $i_{si}$ .

The procedure for subtracting off  $i_x$  could, in principle at least, be repeated for the La trace. In practice, this was not necessary. La in this case suppressed  $i_x$  rather completely, as seen from the flat current record in panel C, and the absence of any outward tail after the standard 1-s clamp pulse (square symbol in panel A). It was immediately possible, therefore, to extract the  $i_x$ -free, La-sensitive current. This is the shaded area in 6 C, which incorporates the shaded area of 6 B.

The magnitude of the steady-current displacement in Fig. 6 C was  $6.3 \mu\text{A}/\text{cm}^2$ . In two other experiments in La, the displacement (without allowance for  $i_x$ ) was 8.7 and  $4.1 \mu\text{A}/\text{cm}^2$ . These values are large by comparison to the net current which flows during the repolarization phase so it is not surprising that La abbreviates the plateau (see *Calculated Action Potentials and Ca Antagonists* below).

#### *Effect of Ca Antagonists on Delayed Rectification*

The nature of the influence of Ca antagonists on  $i_x$  was examined further by studying  $i_x$  activation over a broader range of potentials (Fig. 7). Once again, tails were measured at a fixed holding potential, and used as an index of the conductance that was activated during a preceding clamp pulse. Each panel in Fig. 7 shows the voltage-dependent activation for 1-s pulses (see Methods). Exposure to Mn, La, or D600 gave a marked effect on  $i_x$  tails (filled symbols) that was fairly reversible. Each of these Ca antagonists diminished the tail amplitude (and presumably, the degree of activation) at all potentials, but not in a proportional manner. Similar results were obtained in other experiments using longer clamp pulses (5–10 s). The interpretation of the results and their significance will be left for the Discussion.

**DOES CA ENTRY PROMOTE  $i_x$  ACTIVATION?** Since our original aim was to selectively inhibit  $i_{s,i}$ , it was particularly disappointing to find that the organic Ca antagonist, D600, shared with Mn and La an influence on  $i_x$ . But the similarity of their actions also raised an intriguing question: could the suppression of  $i_x$  be causally linked to inhibition of  $i_{s,i}$ ? These changes would be related, for example, if the entry of calcium ions via  $i_{s,i}$  promoted the subsequent onset of the outward  $i_x$  component. Such a Ca entry hypothesis follows lines suggested by earlier observations in other excitable cells. The role of calcium entry in promoting outward K current has been most dramatically demonstrated by the recent calcium injection experiments of Meech (1972, 1974) in snail neurones and Krnjevic and Lisiewicz (1972) in spinal motoneurones. Others (e.g. McGuigan, 1974) have speculated that calcium entry might play a similar role in ventricular muscle. The hypothesis as applied to  $i_x$  offers the attractive possibility of tying together the increases in both  $i_{s,i}$  and  $i_x$  which are produced by epinephrine in Purkinje fibers (Tsien et al., 1972) and frog atrial trabeculae (Brown and Noble, 1974).

**EFFECT OF CALCIUM IONS ON  $i_x$**  We tested the Ca entry hypothesis by studying the influence of  $[\text{Ca}]_o$  on  $i_x$ . Elevation of external calcium concentration is known to promote Ca entry via the second inward current, judging by the increase in contractile activity observed visually, as well as by electrical measurements (Reuter, 1967; Vitek and Trautwein, 1971). In snail neurones,

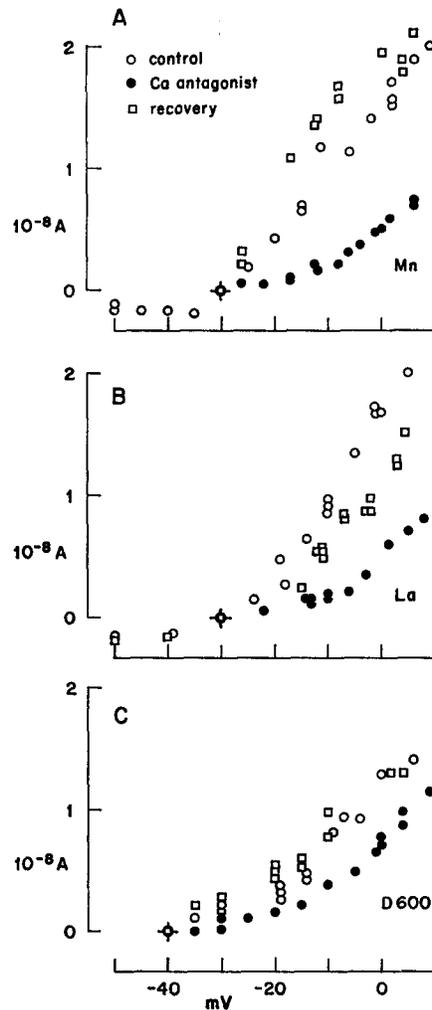


FIGURE 7. Effects of Ca antagonists on  $i_x$  activation. Isochronal activation curves were obtained in a control run (open circles), in drug-containing solution (filled circles), and after removal of the antagonist (open squares). Each symbol corresponds to a single  $i_x$  tail, measured at the holding potential ( $-40$  mV, crossed symbol). The tail amplitude (ordinate) is plotted as a function of the level of the preceding pulse (abscissa). All pulses were 1 s in duration. (A) Effect of Mn (3 mM), preparation 134-2, 7.2 mM  $[Ca]_o$ , clamp pulses applied once every 8 s. (B) Effect of La (0.5 mM), same preparation and conditions as A. (C) Effect of D600 ( $5 \times 10^{-7}$  g/ml), same experiment as in Fig. 5 B, pulses applied once every 7 s.

the hypothesis has been strongly supported by the finding that increasing  $[Ca]_o$  does, in fact, increase outward K current (Meech and Standen, 1974).

Fig. 8 illustrates the effect of fourfold changes in  $[Ca]_o$  on  $i_x$  activation. Data were obtained in 7.2 mM Ca (open circles), then in 1.8 mM Ca (filled

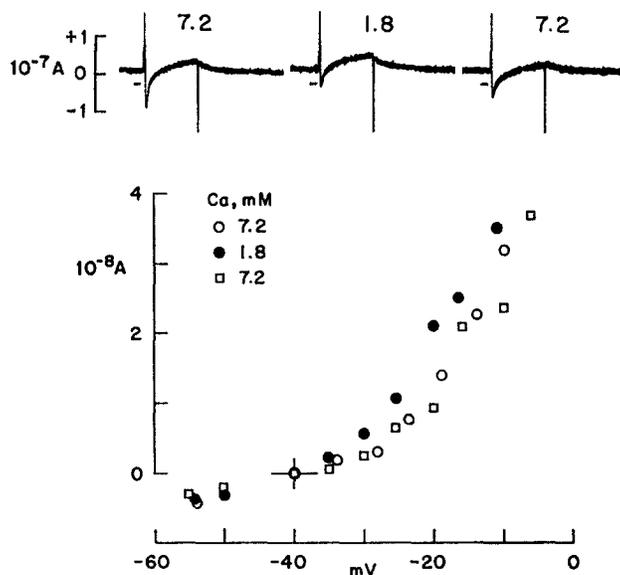


FIGURE 8. Effect of calcium ions on  $i_x$  activation. Isochronal activation curves in 7.2 (open circles), 1.8 (filled circles), and finally 7.2 mM  $[Ca]_o$  (open squares). Tail analysis similar to that in Fig. 7. The symbols plot tail amplitude at the holding potential ( $-40$  mV, crossed symbol) after clamp pulses to various potentials (abscissa). Clamp pulses 2 s in duration were applied once every 10 s. The traces show the membrane current using chart records at low amplification during a standard clamp pulse ( $+25$  mV, 2 s). In 1.8 mM  $[Ca]_o$  the peak inward current (bars) is smaller than in the bracketing runs in 7.2 mM  $[Ca]_o$ . Preparation 141-2, apparent cylindrical area of cell core,  $0.017$  cm $^2$ .

circles), and finally in 7.2 mM Ca (open squares). The inset shows a representative current record from each run, during a standard voltage clamp depolarization pulse. As expected, the inward surge decreased when  $[Ca]_o$  was lowered, and increased when  $[Ca]_o$  was raised. During the same runs,  $i_x$  tails were recorded at higher amplification. Their amplitudes are plotted in isochronal activation curves as in the previous figure. It is evident from Fig. 8 that raising the external calcium concentration reduces the  $i_x$  tail at any given potential within the range considered. This effect was observed in each of a total of five different preparations.

The results show clearly that increases in  $i_{s,i}$  can be accompanied by suppression of  $i_x$  activation. Since the increase in  $i_{s,i}$  is thought to signify increased Ca influx, the Ca entry hypothesis seems highly unlikely for  $i_x$ . It remains to be seen whether the same conclusion applies to potassium current in ventricular muscle (see McGuigan and Bassingthwaite, 1974), or to time-independent K pathways in Purkinje fiber (e.g.,  $i_{K1}$ ). In the present case, some alternative explanation is necessary for the effects of Ca ions and Ca antagonists (see *Change in External Surface Charge?* below).

## DISCUSSION

*Multiple Actions of Ca Antagonists*

Table II summarizes the present results along with earlier evidence showing the influence of Ca ions and Ca antagonists on the excitatory sodium current ( $i_{Na}$ ) and the pacemaker potassium current ( $i_{K_2}$ ). It is evident that at previously prescribed concentrations, neither Mn, La, nor D600 can be considered as a specific blocker of the second inward current.

TABLE II  
INFLUENCE OF Ca IONS AND Ca ANTAGONISTS ON  
MEMBRANE CURRENT COMPONENTS

Agent	Concentration	$i_{si}$	$i_s$	$i_{Na}$	$i_{K_2}$
Ca	7.2 mM (raised from 1.8 mM)	Augments	"Shifts"	Shifts*	Shifts†
Mn	10 mM	Blocks	"Shifts"	Shifts§	Shifts§
La	0.5 mM	Blocks	"Shifts"	Shifts  , ¶	Shifts ¶
D600	$5 \times 10^{-7}$ g/ml (0.96 $\mu$ M)	Blocks	"Shifts"	Shifts**	No effect‡‡

Entries for  $i_{si}$  based on present work and earlier studies cited in the Introduction. For  $i_s$ , "shifts" refers to Figs. 7 and 8 and is subject to reservations about isochronal activation data.

\* Weidmann, 1955 *b*.

† Brown, 1973.

§ Tsien, unpublished.

|| Katzung et al., 1973, in ventricular trabeculae.

¶ Tsien, 1974 *b*.

\*\* Rosen et al., 1974, using verapamil, 10  $\mu$ M.

‡‡ Tsien, 1974 *a*.

No data are available for the transient outward chloride current (Dudel et al., 1967; Fozzard and Hiroaka, 1973).

**EXCITATORY SODIUM CURRENT** All three agents shared some degree of calcium-like "stabilizing action" on  $i_{Na}$  (Weidmann, 1955 *b*; Frankenhaeuser and Hodgkin, 1957). Mn, La, and D600 to varying extents increased the threshold for excitation and decreased the action potential overshoot (see Fig. 2), in agreement with earlier observations in squid nerve (Baker et al., 1973).

**PACEMAKER POTASSIUM CURRENT** In one respect, D600 acts more selectively than the other Ca antagonists. It does not influence the pacemaker potassium current,  $i_{K_2}$  (Tsien, 1974 *b*), and therefore did not steepen the pacemaker depolarization (Fig. 2, bottom Fig. 3). On the other hand, Mn and La each shift the voltage-dependent activation of  $i_{K_2}$  along the potential axis toward less negative values (Tsien, 1974 *b*). The voltage shifts are in the right direction and are large enough to explain the steepening of the pacc-

maker (Fig. 2, top), judging from computer reconstruction work (McAllister et al., 1975, Figs. 15 and 18).

**OUTWARD PLATEAU CURRENT** The changes in  $i_{Na}$  and  $i_{K_2}$  are not serious drawbacks to the use of Ca antagonists in the analysis of plateau currents. However, a major difficulty does arise because of their influence on  $i_x$ , as described in this paper. Although inhibition of  $i_{Na}$  can take place without major changes in  $i_x$  (Fig. 4), the margin of safety is rather narrow, and discouraging for the routine use of the Ca antagonists in voltage clamp analysis.

#### *Change in External Surface Charge?*

The experiments showed that Mn, La, and D600 suppress the onset of  $i_x$ , and furthermore, that elevation of calcium concentration produces a similar effect. Since the Ca entry hypothesis fails to account for these findings (see p. 182), some other explanation must be provided. One interesting possibility is suggested by the fact that  $i_{Na}$ ,  $i_{K_2}$ , and  $i_x$  each seem to be affected through a shift in their voltage-dependent kinetics toward less negative potentials. Such voltage shifts are well-known for permeability changes in excitable membranes, and probably take place through the binding of the ion, e.g.,  $Ca^{++}$  to the membrane surface (Frankenhaeuser and Hodgkin, 1957; see Brown, 1974 for review). Since Mn, La, and D600 are each positively charged, it seems reasonable to propose that they share a similar mechanism of action, involving changes in surface charge.

The kinetics of  $i_x$  were characterized in this paper by isochronal activation curves (see Methods) rather than steady-state curves. Strictly speaking, then, it is a matter of assumption that the results in Figs. 7 and 8 reflect a genuine voltage shift, and not merely an overall slowing of the activation process. If this assumption is valid, the magnitude of the apparent voltage shift in Fig. 8 (5 mV) or the average value in a total of five preparations (6.4 mV) are in good agreement with the 5–6-mV shift found by Weidmann (1955 *b*) for the kinetics of  $i_{Na}$  for the same fourfold change in  $[Ca]_o$ . Similarly, Noble and Tsien (unpublished) and Brown (1973) found an 8-mV shift in the activation of  $i_{K_2}$ . The agreement between these values provides some support for the idea that Ca ions influence  $i_x$  (as well as  $i_{Na}$  and  $i_{K_2}$ ) through a rather uniform alteration in surface charge. It must be admitted, however, that the surface charge idea will be difficult to establish or rule out conclusively, since it is always possible to argue that an agent (e.g., D600) binds near one type of channel ( $i_{Na}$ ) but not near another ( $i_{K_2}$ ).

#### *Influence of Ca Antagonists on Time-Independent Plateau Current*

The voltage clamp experiments revealed another important effect of Mn, La, and D600 which is illustrated in Figs. 4–6. Each agent modified the time-independent current over the plateau range of potentials. The net effect was

a displacement of the late current level in the outward direction. The consequences of this displacement on electrical activity are substantial (see *Calculated Action Potentials and Ca Antagonist Effects*, below), so it is worthwhile to consider its basis.

The first question is whether the increase of net outward current merely reflects an alteration of the time-dependent current components,  $i_{s,i}$  and  $i_s$ , whose sensitivity to Ca antagonists is already apparent. This cannot be the case for  $i_s$ , since  $i_s$  carries outward current over the plateau range, and is clearly diminished by Mn, La, and D600 (Figs. 4–7). The situation is less clear for the second inward current. If  $i_{s,i}$  channels carry inward current in the steady state, blockage of the channels would be expected to contribute to the observed effect.

Suppose that the channels for  $i_{s,i}$  are gated by an activation process,  $d$ , and an inactivation process,  $f$  (see Bassingthwaite and Reuter, 1972). Then, in the steady state,

$$(i_{s,i})_{\infty} = \bar{g}_{s,i} (E - E_{s,i}) d_{\infty} f_{\infty}, \quad (2)$$

where  $\bar{g}_{s,i}$  is the conductance of the fully open channels,  $E_{s,i}$  is the reversal potential, and  $d_{\infty}$  and  $f_{\infty}$  are steady-state values of the corresponding gating variables. The channels will carry significant steady current if the product  $d_{\infty} f_{\infty}$  is appreciable. Experiments in ventricular muscle (Bassingthwaite and Reuter, 1972; New and Trautwein, 1972; Trautwein et al., 1975) suggest that  $d_{\infty} f_{\infty}$  may reach a value of  $(0.4)(0.4) = 0.16$ . This means that the steady-state current could be as large as one-sixth of the maximum possible peak inward current. Now if the inhibitors simply reduced the conductance  $\bar{g}_{s,i}$  (by analogy to the blockage of sodium channels by tetrodotoxin), one would expect that steady current would be diminished *in proportion to* the reduction in transient current.

The trouble with this explanation is that the surge of  $i_{s,i}$  is more sensitive than the displacement in late outward current. In Fig. 4, time-dependent second inward current was more or less abolished by 1 mM Mn, without any appreciable displacement of the late outward current. Similarly, D600 ( $5 \times 10^{-7}$  g/ml) abolished the surge of  $i_{s,i}$  in all cases, but only occasionally altered the time-independent current.

This disparity in effects cannot be reconciled with a simple reduction in  $\bar{g}_{s,i}$ . The results seem to require some sort of dual action. One possibility is that at low concentrations, the inhibitors only interfere with the transient surge of  $i_{s,i}$ , while fully blocking both transient and steady-state current at high concentrations. Another explanation is that the antagonists act on two kinds of second inward current channels: normal  $i_{s,i}$  channels, which carry the transient current and largely inactivate, and a second population of channels,  $i'_{s,i}$ , which lack the property of inactivation, and which are presumed

to be less sensitive to blockage. This latter type of scheme was implicit in the model of McAllister et al. (1975) and will be used in the following section to relate changes in plateau current with alterations in the action potential. It should be emphasized, however, that the nature of the change in steady plateau current remains unclear, and that the net current displacement could also be accounted for by an increase in outward K movement, for example, through an alteration in the time-independent leak pathway,  $i_{K_1}$ .

#### Calculated Action Potentials and Ca Antagonist Effects

Fig. 9 incorporates the observed actions of D600 on membrane currents in an attempted "reconstruction" of its influence on the plateau. D600 was chosen as a focus for this work because it was somewhat more selective than Mn and La in not affecting  $i_{K_2}$ . The comparison of calculated action potentials and experimental records (Figs. 1, 2, and 3) provides a useful check on the voltage clamp results and also serves as a simple summary of the main findings of this paper.

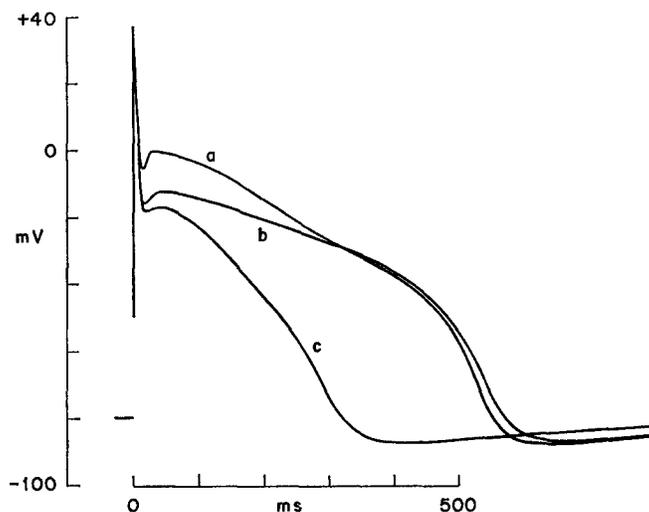


FIGURE 9. Calculated action potentials and reconstruction of D600 effects. (a) Control action potential, using parameter values and initial conditions in McAllister et al. 1975, Figs. 6 and 13. The only change is an increase in  $\bar{g}_{Ca}$  from 0.04 to 0.05 mmho/cm<sup>2</sup>, which prolongs the APD to give better agreement with control recordings in the present experiments. (b) Attempt at simulating the effect of moderate concentrations of D600.  $\bar{g}_{Ca}$  was reduced fourfold relative to *a*, and the rate constants for  $x_1$  and  $x_2$  were displaced by 15 mV toward less negative potentials. (c) Simulation of activity under the influence of high concentrations of Ca antagonist. Their effect on steady plateau current was incorporated by eliminating the contribution of the time-independent plateau current  $i'_{Ca}$ , while leaving other parameters as in *b*.

Membrane action potentials were calculated on an IBM 370 computer (International Business Machines, Armonk, N.Y.) using a Purkinje fiber model (McAllister et al., 1975) that was based on experimental information. Details about parameter values are given in the caption of Fig. 9. Trace *a* is the "control" action potential, and trace *b* is a simulation of the action of D600 at moderate concentrations ( $\leq 5 \times 10^{-7}$  g/ml). Action potential *b* incorporates two effects of the Ca antagonists, and D600 in particular: (*a*) reduction of the magnitude of the second inward current, and (*b*) a voltage shift in the kinetics of  $i_c$ . These changes in plateau current reflect the experimental results in Figs. 5 and 7, respectively. They give rise to a lowering of the plateau and a lengthening of the action potential which resembles, in a qualitative manner at least, the experimental recordings (Fig. 2 A, early effect; Fig. 3).

Trace *c* is a reconstruction of the steady effect of high D600 concentrations ( $\geq 5 \times 10^{-6}$  g/ml). It may also serve as a simulation of the actions of Mn (10 mM) and La (0.5 mM). This calculation included the same two modifications *a* and *b* above, and one additional change: (*c*) displacement of the steady level of plateau current in the outward direction. This was carried out by eliminating the inward plateau current contributed by the time-independent component  $i'_{ss}$ . The magnitude of the displacement (+4.1  $\mu\text{A}/\text{cm}^2$  at  $-30$  mV) was representative of experimental values (Figs. 4–6). The result shows some further reduction in the height of the plateau relative to *b*, and a marked *decrease* in APD, as found in Figs. 1 and 2. Thus, the experimentally observed abbreviation of the action potential can be accounted for by changes in time-independent plateau current (cf. Vitek and Trautwein, 1971).

The modified action potentials in Fig. 9 were calculated by incorporating discrete, and very simple parameter changes. In reality, the effects may not be so clearly separable, and there may also be variability in their importance from one preparation to another (Table I). The reconstruction does illustrate, we believe, one major qualitative conclusion: that each of the effects of the Ca antagonists must be included in an explanation of the observed pattern of changes in the action potential (e.g. Fig. 2 A).

#### *Interpretation of Earlier Studies*

There are some clues that the lack of pharmacological specificity found in Purkinje fibers may also apply to the influence of Ca antagonists in other cardiac preparations. In frog atrial trabeculae, Rougier et al. (1969, p. 93) report that "in some, very rare cases we have noticed for weaker concentrations of manganese (0.5 mM and below) a slight lengthening of the action potential; however, this was accompanied by a decrease in amplitude . . . ." Similarly, in cat papillary muscle, Tritthart et al. (1973) observed a very slight prolongation of APD with D600 ( $10^{-6}$  g/ml) which accompanied the

lowering of plateau height. These results are comparable to Figs. 2 A and 3 in this paper, and suggest the possibility of multiple actions.

A clear-cut and selective blockade of  $i_{si}$  has not yet been demonstrated. Until this goal is achieved, it seems inappropriate to use Ca antagonists like Mn as a means of defining the role of  $i_{si}$  in the normal action potential (cf. Vitek and Trautwein, 1971, p. 217; Prokopczuk et al., 1973). In view of the wide interest in the second inward current and its role in normal action potentials and slow responses (Fig. 1) we hope that future pharmacological efforts will yield better inhibitors and more compelling evidence.

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