On the Mechanism Underlying the Action of D-600 on Slow Inward Current and Tension in Mammalian Myocardium

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SUMMARY D-600, the methoxy derivative of verapamil, is said to affect the force of cardiac contraction and the slow inward current (I_s) specifically by reducing the membrane conductance for Ca^{2+} (g_s). However, it is apparent that many effects of D-600 cannot be adequately explained solely by an effect on g_s. We studied the effects of D-600 on membrane current and tension of cat papillary muscle, using a conventional single sucrose gap voltage clamp technique. The results indicate that D-600 not only reduces the maximal Ca conductance but also, depending on concentration and duration of exposure, alters both the kinetics of the Ca-carrying system and the amplitude of the steady state outward current. No changes in the steady state activation and inactivation variables or in the rate of I_s inactivation were found. However, a substantial increase in the time to peak I_s, as much as 7 times normal, was observed after exposure to D-600 (0.5 \times 10^{-6} to 2.0 \times 10^{-6} M) for at least 20 minutes. Because approximately only 75% of the reduction in I_s induced by D-600 could be attributed to change in the maximum value of g_s (g_s), we conclude that the change in time to peak and about 25% of the reduction in I_s must be due to a change in the activation kinetics of the Ca-carrying system. Calculations suggest that the time to 70% activation of g_s can be prolonged to as much as 10 times normal by prolonged exposure to negatively inotropic concentrations of D-600.

THE ROLE of the Ca-dependent slow inward current (I_s) in both the electrical and mechanical activity of the heart is currently receiving attention. Inward current carried by Ca^{2+} is believed principally responsible for the generation of slowly conducted impulses, for the low amplitude oscillations in the diastolic potential induced by ouabain, and for the regulation of the strength of myocardial contraction. However, precise definition and quantification of both I_s and the outward current in myocardial fibers have been complicated by the overlap in the time courses of these two currents, especially at membrane potentials positive to 0 mV. Therefore, the need for a simple method to either isolate or separate these two components of the membrane current is obvious. A recent study describing the effects of verapamil and some of its analogues suggested that a separation method is available: verapamil and D-600 were said to specifically reduce I_s by reducing the membrane Ca conductance (g_s). However, the effects of D-600 on the resting potential and terminal phases of repolarization of the cardiac action potential as well as on the relationship between stimulus interval and the force of contraction of the myocardium cannot be explained solely by a reduction of g_s.

The results presented here indicate that D-600 not only reduces the maximum Ca conductance (g_s) but also, depending on concentration and duration of exposure, alters the kinetics of the Ca-carrying system and the amplitude of the steady state outward current in cat papillary muscle.

Methods

The effect of racemic D-600 (a-isopropyl-\((N\text{-}methyl-N\text{-}homoveratryl})\text{-}\gamma\text{-}aminopropyl\text{-}3,4,5\text{-}trimethoxyphenylacetonitrile HCl), the methoxy derivative of verapamil (Knoll, Ludwigshafen am Rhein, Germany), on the contraction and membrane currents of cat papillary muscles and ventricular trabeculae was determined using the single sucrose gap voltage clamp method described by New and Trautwein and modified by Trautwein et al. The hearts of young adult cats of either sex were removed under ether narcosis and immediately rinsed in warm oxygenated Tyrode’s solution. Right ventricular trabeculae or papillary muscles having appropriate geometry (diameters of 0.15–0.5 mm and lengths of 2.5–5 mm) were isolated by ligating both ends with a fine silk suture and dissecting them from the heart. A muscle then was placed in the right hand chamber of a muscle bath divided into three chambers with two rubber diaphragms. After securing the right end to a force-displacement transducer (Statham U-2 cell), the muscle was next positioned for experimentation in the three chambers by drawing it into and through holes of appropriate sizes in the rubber diaphragms. The left end of each muscle then was fixed to keep muscle length as constant as possible. Initially all three chambers were perfused with Tyrode’s solution equilibrated with 95% O_2 and 5% CO_2 and...
maintained at 37 ± 1°C. The composition of the Tyrode’s solution (mm) was: NaCl, 140; KCl, 3; MgCl2, 1; CaCl2, 1.8; NaHCO3, 12; Na2HPO4, 0.4; and glucose, 5. To establish the sucrose gap and for voltage clamping, the left chamber was perfused with a Tyrode’s solution in which the NaCl had been replaced with equimolar KCl. The center compartment was perfused with a sucrose solution containing 304 mm sucrose and 0.1 or 0.01 mm CaCl2 dissolved in glass-distilled water. The right chamber served as the test compartment and was perfused with the above-mentioned Tyrode’s solution or with a Tyrode’s solution containing 2 × 10^-7 to 2 × 10^-6 M D-600. The muscles were exposed to D-600 for periods ranging between 20 and 60 minutes.

Membrane potential was detected with 10- to 20-MΩ glass microelectrodes filled with 3 M KCl (tip potentials ≥ 5 mV) and was controlled with a conventional feedback voltage clamp amplifier to supply current to the preparation. A detailed description of the voltage clamp apparatus has been given elsewhere. The membrane was clamped to various potentials at constant rates of 5-12/ min. Voltage, current, and tension were recorded either on film or on FM tape.

Results

**GENERAL EFFECTS OF D-600 ON THE ACTION POTENTIAL, TENSION, AND MEMBRANE CURRENTS**

The resting potential of cat ventricular muscle usually was reduced by 5-10 mV on prolonged exposure (>30 minutes) to D-600 in concentrations of 1 × 10^-6 to 2 × 10^-6 M. Effects on the action potential were somewhat variable. In general the voltage level of the plateau phase was reduced. The rate of repolarization was increased during phase 2 and decreased during phase 3. The overall effect always was a shortening and suppression of the plateau phase, whereas the action potential duration measured at 80% of full repolarization was variable and unpredictable. Sometimes the duration was shorter but in two-thirds of the cases it was at least 20% longer than control duration. D-600 always reduced twitch tension.

Action potentials and tension recorded during a voltage clamp experiment are presented in the left panels of Figure 1. In this case the muscle was exposed to D-600 (5 × 10^-7 M) for 60 minutes; during this time the resting potential declined by about 8 mV and the action potential assumed a triangular shape due to plateau suppression and reduced rate of repolarization. These changes in the resting and action potential can be qualitatively understood by examining the concomitant changes in the membrane currents (Fig. 1, right panels). The magnitude of the downward deflection of the current trace occurring 10-30 msec after the membrane voltage had been changed from -60 to -10 mV was reduced by D-600. The downward deflection of the current trace, representing inward membrane current, resulted from the slow inward current. Following inscription of the maximal inward membrane current, the current slowly decayed and then reversed polarity as the delayed outward current developed. These records indicate that D-600 reduced maximal Ia and the delayed outward current in response to 800-msec voltage clamp steps from -60 to -10 mV. The onset of these drug actions was not immediate, requiring at least 10-15 minutes before any effect could be noted. Exposure times of up to 1 hour were necessary to approach a steady state at concentrations of 0.5 to 2 × 10^-6 M. A reduction by D-600 in the magnitude of Ia, the isochronal delayed outward current, and tension could be observed at all voltage levels between -40 and +20 mV. The progressive changes induced by D-600 are illustrated by the current-voltage and tension-voltage relationships shown in Figure 2A and B. In this instance exposure to D-600 (5 × 10^-7 M) for 1 hour reduced Ia to 30% of control in the voltage range between -10 and +20 mV. The tension-voltage relationship (Fig. 2B) also was reduced by D-600 and a comparison of the Ia-V relationships and the tension-voltage relationships indicates that the percentage decrease in Ia caused by the drug at any given voltage was reflected approximately by the percentage decrease in tension at the same voltage.

Because of the relatively slow development of the membrane effects of D-600 under sucrose gap conditions and our apparent inability to reverse the actions of D-600 by drug washout, control experiments were made to assess the effect of time alone on the membrane currents. The results of three experiments indicated that no significant change in either the membrane current or the resistance between the left and right chambers across the sucrose gap...
CURRENT-VOLTSAGE (A) and tension-voltage (B) relationship before and during exposure to D-600 (5 x 10^{-7} M). From a holding potential of -60 mV the membrane was clamped for 800 msec to potentials $V_c$ (see inset). Slow inward current (here designated as $I_{si}$) was taken as the difference between peak current and the current at 250 msec. $I_{ou}$ refers to the amplitude of outward current at 800 msec with reference to zero current (dashed line).

The current records in Figure 1 show that D-600 has an additional effect on membrane current: the time between the onset of the voltage step and the occurrence of peak $I_{si}$ was lengthened by the drug. A typical example of this action can be seen in Figure 3, which shows superimposed membrane current records before and after exposure for 30 minutes to D-600 (10^{-6} M). Under control conditions the time to peak $I_{si}$ (i.e., the maximal downward deflection of the trace) in response to a voltage step from $-50$ to $-10$ mV was 15 msec and was increased to 35 msec after exposure to D-600. As with the other effects of D-600, the time of onset of this increase in time to peak $I_{si}$ as well as its magnitude were variable from fiber to fiber but always were observable after exposure for 30-40 minutes to 2 x $10^{-6}$ M D-600. The increase in time to peak $I_{si}$ was 50-150% of control at voltage levels between -20 and +20 mV in about 80% of the cases. However, occasionally even greater increases, as much as 7 times greater than control, were observed (see Fig. 7). Observations made on two preparations suggest that the extent of the increase in time to peak $I_{si}$ was nominal after a short rest period of 1-2 minutes or when very long interpulse intervals (3-6 pulses/min) were used. At pulsing rates approaching 60/min the time to peak was always at least 1.5 times that of control.
EFFECT OF D-600 ON THE KINETICS OF THE Ca-CARRYING SYSTEM

Bassingthwaighte and Reuter have proposed that \( I_a \) in cardiac muscle can be described by:

\[
I_a = g_a (E - E_m),
\]

where \( g_a \) is the conductance for slow inward current and \( (E - E_m) \) is the driving force for the slow inward current. In heart a major part of \( I_a \) is apparently carried by \( Ca^{2+} \). The time course of \( g_a \) is given by:

\[
g_a(t) = g_a(0) \cdot \frac{d(t)}{d(0)} \cdot f(t),
\]

where \( g_a(0) \) is the maximum or limiting conductance, \( d \) is a dimensionless activation variable, and \( f \) is a dimensionless inactivation variable. The variables \( d \) and \( f \) proceed at a rate defined by their time constants \( \tau_d \) and \( \tau_f \) to their steady state values, \( d_* \) and \( f_* \), which range in value from 0 to 1 depending on voltage. While it is not certain that the approach is strictly valid, this Hodgkin-Huxley type formulation provides a valuable framework for the study of physiological and pharmacological interventions.

Effect of D-600 on \( f_* \)

The steady state inactivation variable \( f_* \) describes the voltage-dependence of the availability of \( g_a \). Under control conditions it has a sigmoid shape which approaches 1 at -50 mV, 0.5 between -30 and -20 mV, and 0 at 0 mV.\(^3\)\(^-\)\(^9\) What this implies is that \( I_a \) is completely available for activation when the voltage clamp holding potential (or the resting potential under non-clamp conditions) is more negative than -50 mV, and completely unavailable (inactivated) when the holding potential is positive to 0 mV. A shift of \( f_* \) to the left along the voltage axis would reduce \( I_a \) at all voltages.

The voltage clamp sequence used to determine \( f_* \) is shown in the schematic of Figure 4A. The membrane was clamped to 0 mV and repolarized for 2 seconds to potentials \( V_c \) between -10 and -80 mV at a rate of 12/min. The slow inward current system is completely inactivated after 3 seconds at 0 mV. Repolarization for 2 seconds allows a voltage-dependent removal of this inactivation which is reflected by the amplitude of \( I_a \) triggered by the return to 0 mV. These current amplitudes are plotted in Figure 4A, which shows the results of two complete experiments. Following the control determinations (open symbols) D-600 was applied and the preparation was stimulated at 12/min until marked changes were observed in the action potential. Determinations at this time (filled symbols) indicate that the drug reduced \( I_a \) to about 1/4 the control value. The shape of the relationship, however, remained essentially the same. This point is made more clearly in Figure 4B, which shows the normalized averaged data (\( f_* = I_a/I_{max} \)). While there is some scatter in the data, it appears that D-600 has no appreciable effect on \( f_* \).

Effect of D-600 on \( d_* \)

The steady state activation variable \( d_* \) describes the voltage-dependence of the activation of \( g_a \). Under control conditions it is almost a mirror image of \( f_* \), approaching 0 at -50 mV, 0.5 between -30 and -20 mV, and 1 at 0 mV.\(^3\)\(^-\)\(^9\) This implies that a voltage clamp step to -50 mV triggers little \( I_a \) while a step to 0 mV fully activates the current. A shift of \( d_* \) to the right along the voltage axis would reduce \( I_a \) at all voltages.

The method used to determine \( d_* \) involved 30-msec step depolarizations to potentials between -40 and +30 mV (see schematic, Fig. 4A). At the end of the 30-msec step it is assumed that d is near its steady state value \( d_0 \) and some fraction of \( g_a \) is activated. Repolarization back to the holding potential elicits an inward current tail whose amplitude is governed by the relation \( I_a = g_a(E - E_m) \). Assuming that the holding potential, \( E \) and \( E_m \) remain constant, the tail current of \( I_a \) is directly proportional to the \( g_a \) activated by the short depolarizing clamp.

The voltage clamp records of the 30-msec depolarizations to -30, -10 and +10 mV from a holding potential of -50 mV are shown in Figure 5. After obtaining the control determinations, activation runs were repeated after exposure to D-600 (10^-6 M) for 10 and 30 minutes. Of importance is that the time to peak \( I_a \) at 0 mV was approximately twice that of control. B: the steady state inactivation variable was obtained by normalizing the data in A (\( f_* = I_{max}/I_a \)). Mean values of the two experiments before (open symbols) and after (filled symbols) D-600. Curves fit by eye.
back to zero time (i.e., the moment of repolarization) and are plotted in Figure 6A. The normalized data (dwa = 1/Imax) are presented in Figure 6B and indicate that D-600 did not significantly affect the relationship between dwa and membrane voltage.

Effect of D-600 on dwa and on the Rates of Activation and Inactivation

Comparison of control records with those obtained after a 30-minute exposure to D-600 is shown in Figure 5. The data in Figure 6A suggest that in this particular experiment D-600 (10⁻⁶ M for 30 minutes) reduced gsa by about 50% since the amplitude of the tail current is a direct function of gsa which in turn is a function of fi. However, it is also necessary to know whether D-600 affects the time course of inactivation since the gsa existing at the end of the 30-msec test pulse is dependent on how far ft(t) (see Equation 2) has proceeded toward its steady state level.

By selecting a set of membrane current records from an experiment in which D-600 caused no change in the delayed outward current, we were able to estimate the effect of D-600 on ft(t) and gsa. Membrane currents in response to clamp steps from -60 to 0 mV before and after D-600 (10⁻⁶ M) for 30 minutes were superimposed and are shown in the inset of Figure 7. In this instance D-600 markedly reduced Iwa and lengthened the time to peak Iwa without changing the outward current. Therefore, it is reasonable to assume that the change in current resulting from exposure to D-600 was solely the result of change in the determinants of Iwa rather than in the determinants of the delayed outward current. The magnitude and time course of the outward current components were estimated by fitting a straight line to the currents plotted on semilog paper (Fig. 7, dashed line). After subtraction of the outward component from the total current signal, a second component emerged whose decline is well described by a single exponential having a time constant of approximately 90 msec. On the basis of conductance measurements and other evidence (McDonald and Trautwein, in preparation) this exponential is thought to represent the time course of the fi parameter. As shown in Figure 7, D-600 (open triangles) had prolonged fi only nominally, the change being 5% of the control value. D-600 (5 x 10⁻⁷ to 2 x
cases the outward current. These effects did not occur immedi-
ately on exposure to the drug, the time to onset was usually 5 to 15
minutes and periods of up to 1 hour were required to approach a
steady state. During the first 10-30 minutes after the application of D-600, the ampli-
tude of I_{si} was reduced with little change in the time to
peak current. This reduction in current amplitude can be
attributed to a reduction in maximum conductance for
slow inward current, g_{si}. At later times (20-40 minutes)
the drug induced a lengthening in the time to peak I_{si}. It
appears likely that this was due to a large reduction in the
rate of activation. This slower rate of activation combined
with an unchanged (from control) rate of inactivation,
resulted in a decrease in I_{si} which was in addition to the
decrease ascribable to change in g_{si}. After exposure for
30-60 minutes to D-600, I_{si} was usually reduced by 60-
80% and 1/4 to as much as 1/5 of this reduction can be
reasonably attributed to change in the rate of activation.

The extent to which D-600 may reduce the rate of
activation of g_{si} was calculated from the data presented
in Figure 7. The calculations were made by solving Equation
2 for d(t), assuming g_{si} to be fully available and not
inactivating with time [thus f(t) = 1] and applying the
same simplifying assumptions used to define g_{si} and the
time course of the f parameter. The dashed curves in
Figure 8 depict the time course of the activation parameter,
d(t), calculated for the I_{si} component of the currents
shown in the inset of Figure 7 before (filled triangles) and
after (open triangles) D-600. While no pretense is made
with regard to defining the behavior of d(t) mathemati-
cally, the calculation of d(t) indicates that the time re-
quired to fully activate g_{si} was substantially lengthened by
exposure to D-600. Under control conditions after only 5
msec d was already approximately 70% of its maximum
level and 95% after only 10 msec. In contrast, after D-600
more than 60 msec and 120 msec were required to achieve
70% and 95% of the maximal level, respectively. Thus, to
adequately account for the increase in time to the inward
current peak from approximately 10 to 70 msec occurring in
the current records depicted in Figures 7 and 8, we must
conclude that D-600 decreased the rate of activation of g_{si}
to less than 10% of normal.

Discussion

In the present study on cat ventricular muscle, D-600
(0.5 x 10^{-4} to 1 x 10^{-4} M) similarly did not affect
\tau_i in five other experiments employing 2-second clamp steps to potentials between
-20 and +20 mV.

The exponentials describing the time courses of f before
and after D-600 exposure obtained from the time courses of the current before and after D-600 were extrapolated
back to zero time in Figure 7 (solid lines). The intercept of the
f function with zero time yields information on g_{si} if
certain simplifying assumptions are made: (1) the pulse
from -60 to 0 mV activates maximum g_{si}; (2) f is a
reasonable approximation to the inactivation of I_{si} even
during activation; (3) inactivation starts without an appre-
ciable delay when the voltage clamp step is imposed. The
first assumption appears reasonable from the data in Fig-
ure 6. Although there is no information on the second and
third assumptions, they are in keeping with a Hodgkin-
Huxley type formulation. On this basis then, the intercepts of the f curves with the zero time axis indicates that D-600
reduced g_{si} to about 55% of the control value (from 1.27
to 0.72) in this experiment.

The main factor influencing the shape of I_{si} is the prod-
uct d(t) f(t). Since the time course of inactivation did not
seem to be influenced by D-600, it would appear that the
lengthening in time to peak I_{si} by the drug was due to a
greatly reduced rate of activation. The net effect of a
reduction in the rate of activation, combined with an
unchanged rate of inactivation, will be a reduction in the
amplitude of I_{si} that is independent of any change in g_{si}.
This can be appreciated from the data in Figure 7. The
amplitude of I_{si} was reduced by D-600 from 1.05 to 0.28
µA, but the fall in g_{si} from 1.3 to 0.7 units accounts only for
a reduction in I_{si} to approximately 0.57 µA. The
remaining portion of the reduction in I_{si} (0.29 µA) or about
1/3 of the total change in I_{si} must be accounted for by
an additional mechanism involving a change in the rate of
activation of I_{si}. It is unlikely that the apparent reduction
in the rate of activation was due to the reduction of g_{si} per
se since partial voltage-dependent inactivation preceding
pulses to 0 mV does not result in large alterations in the
time to peak I_{si} under control conditions even when the
current amplitude is reduced as much as 75%.

The validity of some of the experiments would be ques-
tionable if the slow inward current system were not fully
restored during the period between test pulses. The time
constant for the restoration process in cat papillary muscle
ordinarily is about 200 msec. While we did not ascertain
whether the restoration process was affected by D-600,
it was clear from the results of two experiments that
restoration was not a factor even at pulsing rates of
30/min. The pulsing rate used in this study was 6-12/min.

![Figure 8](http://circres.ahajournals.org/)
The main effect of D-600 on \(I_{\text{sl}}\), a reduction in \(g_{\text{sl}}\), is in agreement with the conclusion reached by Kohlhardt et al.\(^5\) in an earlier voltage clamp study on cat ventricular muscle. However, their statement that D-600 blocks the "transmembrane Ca conductivity of mammalian myocardial fibers in a highly selective manner" appears to require modification. First, the effect on Ca conductivity can involve a change in the rate of activation as well as a reduction in \(g_{\text{sl}}\). Second, the drug usually but not always reduced the outward current. The latter finding is in agreement with the slight prolongation in action potential duration observed by Tritthart et al.\(^5\) in cat papillary muscle treated with \(2 \times 10^{-6}\) m D-600. The lengthening of the action potential accompanied by a reduced plateau voltage and duration during D-600 treatment does not appear to be restricted to ventricular muscle. A closely related compound, verapamil, has been reported to have similar effects in Purkinje fibers.\(^6\)\(^7\) In a recent voltage clamp study on Purkinje fibers, Kass and Tsien\(^8\) reported that low doses of D-600 (\(\leq 10^{-6}\) m) generally lengthened the action potential duration and reduced the outward current, \(I_{\text{t}}\). However, the effect of the drug on the steady state outward current was variable, in two cases increasing it and in a third reducing it. They felt that this variability might explain why on some occasions similar concentrations of D-600 lengthened the action potential duration and shortened it on others. That the effect of D-600 on the cardiac action potential is not predictable is further illustrated by its action on embryonic chick ventricular cells. In these preparations D-600 (\(10^{-6}\) to \(10^{-5}\) m) suppresses both the plateau phase and the action potential duration.\(^9\) It is conceivable that D-600 may have more than one action on the delayed outward current. Evidence that increasing the intracellular \(Ca^{2+}\) concentration will cause an increase in the late or delayed outward current of sheep Purkinje fibers\(^10\) suggests that a decrease in intracellular \(Ca^{2+}\) concentration might reduce the outward current. The reduction in \(I_{\text{t}}\) and the accompanying decrease in the twitch tension caused by D-600 suggest that the compound may cause a decrease in the intracellular \(Ca^{2+}\) levels and thereby indirectly reduce the delayed outward current. Another action could be one to increase the delayed outward current by an as-yet undefined mechanism. Two such actions could explain the observed qualitative variability in the effect of D-600 on the action potential duration and the delayed outward currents. Possible differences in the effects of each optical isomer on the outward current also for these studies.

These results suggest that studies which employ D-600 to define whether or not the slow inward current is involved with an aspect of cardiac electrical or contractile activity must be interpreted cautiously. D-600, like tetrodotoxin, affects the maximal conductance for the affected current but, unlike tetrodotoxin, also can modify the kinetics of the slow inward current and affect the delayed outward current as well. In addition, effects on other processes involved in regulating intracellular \(Ca^{2+}\) levels cannot be ruled out at this time. Furthermore, because of the rather large effect of D-600 on the \(I_{\text{t}}\) activation kinetics, attention must be paid to experimental design particularly with regard to stimulation rate. At fast stimulation rates, processes dependent on the slow inward current will be much more affected than at slow stimulation rates. Thus, the use of D-600 as a specific blocking agent for \(I_{\text{t}}\) in ventricular muscle seems limited on at least three grounds: (1) it alters the kinetics of the Ca-carrying system; (2) it affects the outward current; and (3) its action is often quite slow in onset and can be both quantitatively and qualitatively variable when used in voltage clamp studies employing the single sucrose gap technique.

References

3. Trautwein W, McDonald TF, Tripathi O: Calcium conductance and tension in mammalian ventricular muscle. Pfluegers Arch 354: 55-74, 1975
On the mechanism underlying the action of D-600 on slow inward current and tension in mammalian myocardium.

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Circ Res. 1977;40:408-414
doi: 10.1161/01.RES.40.4.408

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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