Interaction of D-600 with the transmembrane domain of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase

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Experiments were performed to determine whether the organic Ca\(^{2+}\) channel blocker D-600 (gallopamil), which penetrates into muscle cells, affects sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake by directly inhibiting the light SR Ca\(^{2+}\)-ATPase. We have previously shown that at 10 mM, D-600 inhibits LSR ATP-dependent Ca\(^{2+}\) uptake by 50% but has no effect on ATPase activity (21). These data suggest that the SR Ca\(^{2+}\)-ATPase might be a potential target for D-600. The ATPase activity of the enzyme is associated with its hydrophilic cytoplasmic domain, whereas Ca\(^{2+}\) binding and translocation are associated with the transmembrane domain (18).

In the present experiments, we determined which of the two domains of the ATPase is affected by D-600. Thermal inactivation experiments using the SR Ca\(^{2+}\)-ATPase demonstrated that D-600 decreased the thermal stability of Ca\(^{2+}\) transport but had no effect on the stability of ATPase activity. In addition, D-600 at a concentration of 160 mM did not have any leaking effect of Ca\(^{2+}\) on the Ca\(^{2+}\)-loaded SR. Thermal denaturation profiles of SR membranes revealed that D-600 interacts directly with the transmembrane domain of the Ca\(^{2+}\)-ATPase. No evidence for interaction with the nucleotide domain was obtained. We conclude that the Ca\(^{2+}\) blocker D-600 inhibits the SR Ca\(^{2+}\) pump specifically by interacting with the transmembrane Ca\(^{2+}\)-binding domain of the Ca\(^{2+}\)-ATPase.

IN SKELETAL MUSCLE CELLS the magnitude and time course of the contraction-relaxation cycle depend on the levels of the cytosolic-free Ca\(^{2+}\) concentration, which is controlled by the transverse tubules and sarcoplasmic reticulum (SR) membranes. It has been shown that in skeletal muscle nearly all the voltage-dependent Ca\(^{2+}\) channels are localized in the transverse tubular membrane system (1, 2). The voltage-dependent Ca\(^{2+}\) channels give rise to inward Ca\(^{2+}\) currents (\(I_{Ca}\)) that can be inhibited by a diverse class of organic compounds, conventionally grouped together as Ca\(^{2+}\) antagonists or Ca\(^{2+}\) channel blockers. The interaction of such drugs with voltage-dependent Ca\(^{2+}\) channels has been extensively studied (9–11). It has been proposed that organic Ca\(^{2+}\) channel antagonists exert their inhibitory effect by promoting a type of gating in which the channels are unable to open. Colvin et al. (5) and Wang et al. (24) have shown that relatively high concentrations (0.1 and 3 mM) of verapamil, an organic Ca\(^{2+}\) blocker from the fenylalkylamine series, may interact with the SR. However, they did not determine the mechanism of interaction between verapamil and the SR.

We have previously reported that another type of organic Ca\(^{2+}\) channel blocker, diltiazem, produces up to an 80% twitch potentiation in frog skeletal muscle cells (14). We attributed this potentiation to a decrease in the rate of Ca\(^{2+}\) uptake by the SR. This hypothesis was confirmed using split muscle fibers in which we showed that diltiazem tends to maintain a high level of cytosolic-free Ca\(^{2+}\) by exerting an inhibitory effect on Ca\(^{2+}\) uptake by the SR (12).

The Ca\(^{2+}\) antagonist D-600 is more frequently used than other Ca\(^{2+}\) channel antagonists to study its effect on skeletal muscle \(I_{Ca}\), excitation-contraction coupling, and the mechanisms by which they may produce these effects (1, 2, 6, 8, 12–15, 19, 22). For this reason and for our previous study (21), we chose D-600. It is interesting to note that D-600 has been used more despite the fact that other organic Ca\(^{2+}\) channel blockers are more efficient and widely used in clinical applications. We have recently reported an inhibitory effect of D-600 on force development in skinned skeletal muscle fibers and on Ca\(^{2+}\) uptake by isolated SR (22). These observations, along with the fact that D-600 penetrates into cells (12), may explain the facilitation of excitation-contraction coupling seen with D-600 by other researchers (6).

The present study was undertaken to determine whether the inhibitory effect of D-600 on Ca\(^{2+}\) uptake by the SR is caused by a direct interaction with the SR...
Ca\textsuperscript{2+}-ATPase. The experiments were performed in isolated light SR (LSR) vesicles from rabbit skeletal muscle in which ATPase, Ca\textsuperscript{2+} uptake activities, and Ca\textsuperscript{2+} leakage were measured. We also determined the effect of D-600 on the denaturation profile of the Ca\textsuperscript{2+}-ATPase in LSR. This paper provides evidence of a direct interaction between D-600 and the transmembrane domain of the SR Ca\textsuperscript{2+}-ATPase.

**MATERIALS AND METHODS**

**Preparation of LSR.** Microsomes, isolated from rabbit skeletal muscle, were loaded on a discontinuous sucrose gradient composed of three layers, 25, 27.5, and 35% (wt/vol), containing 20 mM Tris-malate and 1 mM dithiothreitol (DTT), pH 6.8. The microsomes were then centrifuged for 14 h at 23,000 rpm. The lighter bands were discarded, and the pellet was washed and loaded on the top of a second discontinuous sucrose gradient of 28, 32, 35, and 45% (wt/vol). The fraction separated at the 32/35% interface, which contains the highest specific Ca\textsuperscript{2+}-ATPase activity, was collected. This fraction is hereafter referred to as LSR. LSR has a protein content of ~90% Ca\textsuperscript{2+}-ATPase.

**ATPase activity.** ATPase activity in LSR was determined by a colorimetric technique, based on the reaction between P\textsubscript{i} and malachite green (15). Aliquots of 0.003 mg/ml LSR were incubated in a solution containing (in mM): 0.1 CaSO\textsubscript{4}, or 1 EGTA, 5 MgSO\textsubscript{4}, 77 potassium methanosulfonate, 20 Tris-malate, and 1 NaATP, pH 6. The reaction was stopped after 30 min of reaction with a solution containing 0.045% hydrochloride malachite green, 4.2% ammonium molybdate in 4 N HCl, 0.8 ml Triton X-100 (10% for each 100 ml of solution), and 0.25 ml sodium citrate (34%), and the absorbance was read at 660 nm.

**Calcium uptake.** SR Ca\textsuperscript{2+} uptake was determined using the metallochromic indicator Arsenazo III in a Ca\textsuperscript{2+}-free solution containing (in mM) 0.1 CaSO\textsubscript{4}, 5 MgSO\textsubscript{4}, 77 potassium methanosulfonate, 20 Tris-malate, 1 NaATP, and 0.7 Arsenazo III, pH 6.8, as previously described (4). We used sulfates mainly because intracellular chloride in mammalian and in amphibian skeletal muscle is very low (7) and Cl\textsuperscript{−} triggers Ca\textsuperscript{2+} release from SR (20). Ca\textsuperscript{2+} transport was determined from the change in absorbance at 660 nm. Protein (0.05 mg/ml) was added to the reaction solution, and after a 30-s incubation, the reaction was started by adding 1 mM ATP. Ca\textsuperscript{2+} uptake was measured in parallel with the above reaction solution, but using 1 µCi of \textsuperscript{45}CaCl\textsubscript{2}. The Ca\textsuperscript{2+} remaining in the LSR vesicles was determined by filtration, and the radioactivity was measured using a scintillation counter.

**Thermal inactivation.** LSR membranes (2 mg/ml) were incubated in 10% sucrose containing (in mM) 1 DTT, 20 Tris-malate, and 1 CaSO\textsubscript{4}, pH 6.8, and were heated at one of several fixed temperatures in a water bath for 1, 3, 5, 7, or 10 min. Ca\textsuperscript{2+}-ATPase activity and ATP-dependent Ca\textsuperscript{2+} transport at room temperature (25°C) were determined after 30 min of reaction and plotted as a function of incubation time at temperatures between 37 and 53°C. Thermal inactivation and thermal denaturation were used to demonstrate the nature of the interaction between D-600 and the LSR Ca\textsuperscript{2+}-ATPase. The values for Ca\textsuperscript{2+} transport and ATPase activity were normalized with respect to the first value obtained at each temperature after 1 min of incubation. The rate of inactivation (i.e., conversion of active to inactive enzyme) is indicated by the rate constant for inactivation, k, which varies with temperature according to the Arrhenius relation

\[
k = e^{-E_A/(RT)}
\]

where A is the Arrhenius number defined as the frequency factor, E\textsubscript{A} the activation energy, R the gas constant, and T the absolute temperature.

The inactivation temperature (T\textsubscript{i}) is defined as the temperature resulting in half inactivation when the temperature is increased at a rate of 1°C/min. For the thermal inactivation experiments, we heated the sample at a temperature between 35 and 70°C. The value of T\textsubscript{i} reflects the sensitivity of the Ca\textsuperscript{2+}-ATPase to thermal inactivation. T\textsubscript{i} was calculated using Eq. 1, which transforms the rate of inactivation, determined by measurements of activity as a function of time at a constant temperature, to inactivation, determined by measurements of activity as a function of temperature when this is increasing at a constant rate (18). T\textsubscript{i} was determined so that a direct comparison could be made with the characteristics of denaturation, determined by differential scanning calorimetry at a scan rate of 1°C/min (see below) and described by T\textsubscript{m} (the temperature of half denaturation).

**Differential scanning calorimetry.** The thermal denaturation profile of LSR membranes was determined by differential scanning calorimetry (DSC). A high-resolution Microcal MC2 DSC was used to obtain all scans. SR membranes (8–10 mg/ml) were heated at a rate of 1°C/min from 10 to 100°C. The samples were then cooled to 10°C and rescanned. For the DSC experiments, SR was suspended in the absence or presence of 10 to 200 µM of D-600 in the solution used for thermal inactivation. Denaturation was completely irreversible after scanning up to 100°C. Intrinsic baseline curvature was corrected by subtracting the scan from the data scan, and a correction was made for the shift in specific heat on denaturation (C\textsubscript{p}), as previously described (18). DSC scans were deconvoluted assuming irreversible denaturation. This procedure requires that denaturation and inactivation can be approximated by a two-state reaction of the form

\[
f_p(T(t)) = 1 - \exp[-(RT^2/E_A)/(\exp[E_A/(T - T_0)/RT^2])]
\]

where T\textsubscript{0} is the temperature at which k = 1, t is time, and \nu is the scan rate. The derivative of f\textsubscript{p} as a function of temperature is proportional to the excess C\textsubscript{p}. The curves of excess C\textsubscript{p} as a function of temperature were deconvoluted into individual components using a recursive minimization routine (18). The T\textsubscript{m} in the DSC of each component is defined as the temperature at which the area under the individual curve for each component is one-half of the total area.

**RESULTS**

The effect of 10 µM D-600 on the kinetics of ATP-dependent Ca\textsuperscript{2+} uptake by LSR is illustrated in Fig. 1. The complete time course of Ca\textsuperscript{2+} uptake is inhibited by 50% at 30 min. Such an inhibition of Ca\textsuperscript{2+} uptake could reflect an interaction of D-600 with either the Ca\textsuperscript{2+}-ATPase nucleotide-binding domain, with a subsequent inhibition of ATP hydrolysis, or the Ca\textsuperscript{2+}-ATPase trans-
membrane Ca\(^{2+}\)-binding domain, with subsequent inhibition of Ca\(^{2+}\) translocation. The Ca\(^{2+}\)-ATPase activity in LSR was measured to determine whether D-600 affects the ATP hydrolytic activity. As shown in Fig. 2, D-600, at concentrations up to 160 \(\mu\)M, had no inhibitory effect on the ATPase activity. This result suggested that the interaction of D-600 with the ATPase might be at a site other than the nucleotide-binding site without affecting the ATP hydrolytic activity.

**D-600 potentiates the thermal inactivation of Ca\(^{2+}\) uptake, but not ATPase activity.** To investigate in more detail whether D-600 interacts with the ATP-binding domain or the Ca\(^{2+}\)-binding domain, we used thermal analysis techniques (i.e., thermal inactivation and differential scanning calorimetry) that have been previously used to distinguish between these domains in the Ca\(^{2+}\)-ATPase of the SR (18). To determine whether D-600 (10–320 \(\mu\)M) has a direct effect on Ca\(^{2+}\)-uptake activity, we performed thermal inactivation experiments on LSR by measuring how much of the activity at 25°C remained after LSR membranes were incubated for 1, 3, 5, 7, or 10 min at four different temperatures. Figure 3 illustrates the behavior of thermal inactivation of Ca\(^{2+}\) uptake in the absence (A) and presence (B–G) of D-600. The reaction was started by the addition of 1 mM NaATP.
tially with time, demonstrating pseudo first-order kinetics. Figure 3 also shows that the rate of inactivation (slope) of Ca$_{\text{2+}}$ uptake is temperature dependent. At any temperature, inactivation was faster in membranes incubated with D-600 than in those incubated without the drug.

The rate constants of inactivation of Ca$_{\text{2+}}$ uptake shown in Fig. 3 were used to obtain Arrhenius plots (Fig. 4). It is clear from these plots that the rate of thermal inactivation increases dramatically as the concentration of D-600 is increased. Linear regression analysis was used to obtain the activation energy ($E_A$), corresponding to the slope of the curves, and the frequency factor ($A$), corresponding to the intercept.

Figure 5 shows the predicted plots of the derivative of fractional inactivation as a function of temperature ($d\ln a_D/dT$). These plots correspond to the DSC profiles predicted for the protein domain, of which denaturation is responsible for inactivation of Ca$_{\text{2+}}$ uptake. The values of $E_A$, $A$, and $T_i$, where $T_i$ corresponds to the temperature of half-inactivation if temperature were to be increased at 1°C/min, are given in Table 1. $T_i$ was determined from the $f_D$, expressed as a function of temperature ($f_D$ vs. $T$), and calculated from the values of $E_A$ and $A$ obtained from the Arrhenius plots using Eq. 1. As shown in Table 1, the $T_i$ for Ca$_{\text{2+}}$ uptake in the absence of D-600 was 53.2 [0.8°C ($n = 3$)]. Inactivation proceeded with a high-activation energy in excess of 500 KJ/mol, suggesting that a protein conformational change such as partial unfolding is responsible for the inactivation. This table also shows that 10 $\mu$M of D-600 decreased the $T_i$ for inactivation of Ca$_{\text{2+}}$ uptake by 4.9°C, from 53.2 to 48.3 [0.9°C ($n = 3$)]. As the concentration of D-600 was increased up to 160 $\mu$M, $T_i$ progressively decreased, whereas $E_A$ remained at a relatively constant value, suggesting that the mechanism of inactivation remains unchanged at the concentrations tested. When a concentration of 320 $\mu$M of D-600 was used, a sharp decrease in $E_A$ and $T_i$ occurred. These results suggested that at very high concentrations of D-600, the mechanism of inactivation of Ca$_{\text{2+}}$ uptake changes, probably due to an increase in the passive permeability of Ca$_{\text{2+}}$ from the SR.

To rule out the possibility that the effect of D-600 on Ca$_{\text{2+}}$ uptake was due to Ca$_{\text{2+}}$ leakage, we measured passive Ca$_{\text{2+}}$ permeability. Figure 6 shows the amount calculated from the values of $E_A$ and $A$ obtained from the Arrhenius plots using Eq. 1. As shown in Table 1, the $T_i$ for Ca$_{\text{2+}}$ uptake in the absence of D-600 was 53.2 [0.8°C ($n = 3$)]. Inactivation proceeded with a high-activation energy in excess of 500 KJ/mol, suggesting that a protein conformational change such as partial unfolding is responsible for the inactivation. This table also shows that 10 $\mu$M of D-600 decreased the $T_i$ for inactivation of Ca$_{\text{2+}}$ uptake by 4.9°C, from 53.2 to 48.3 [0.9°C ($n = 3$)]. As the concentration of D-600 was increased up to 160 $\mu$M, $T_i$ progressively decreased, whereas $E_A$ remained at a relatively constant value, suggesting that the mechanism of inactivation remains unchanged at the concentrations tested. When a concentration of 320 $\mu$M of D-600 was used, a sharp decrease in $E_A$ and $T_i$ occurred. These results suggested that at very high concentrations of D-600, the mechanism of inactivation of Ca$_{\text{2+}}$ uptake changes, probably due to an increase in the passive permeability of Ca$_{\text{2+}}$ from the SR.

To rule out the possibility that the effect of D-600 on Ca$_{\text{2+}}$ uptake was due to Ca$_{\text{2+}}$ leakage, we measured passive Ca$_{\text{2+}}$ permeability. Figure 6 shows the amount
of Ca$^{2+}$ remaining in LSR vesicles loaded in the absence of D-600 as a function of time. Ca$^{2+}$-loaded vesicles were diluted in a solution containing 2 mM EGTA in the absence and presence of D-600. D-600 had no effect on passive leakage at a concentration of 160 $\mu$M. However, 320 $\mu$M of D-600 produced a sharp decrease in intravesicular Ca$^{2+}$. Although Ca$^{2+}$ leaks faster in the presence of 320 $\mu$M, this does not appear to be the consequence of membrane disruption because addition of the ionophore A-23178 produced an immediate and full Ca$^{2+}$ leakage.

To provide further evidence of a direct interaction between D-600 and the transmembrane domain of the Ca$^{2+}$-ATPase, DSC was used to determine the actual site of interaction.

**Differential scanning calorimetry.** Lepock et al. (18) have shown that the DSC profile obtained from highly purified LSR membranes has two peaks (endotherms) corresponding to the denaturation of the cytosolic nucleotide-binding and the transmembrane Ca$^{2+}$-binding domains of the Ca$^{2+}$-ATPase. Based on these data, we also used LSR membranes to obtain a set of ATPase DSC denaturation profiles at different D-600 concentrations. Figure 7 shows the denaturation profile of LSR in which the $T_m$ values were determined by deconvolution of the two endotherms as previously described (18). Ca$^{2+}$-ATPase denatures in two major endotherms. The main component, A, which corresponds to the denaturation of the cytosolic nucleotide-binding domain, had a $T_m$ of 50°C. This component was not affected by D-600. The second component, B, which corresponds to the denaturation of the transmembrane Ca$^{2+}$-binding domain, had a $T_m$ of 60 [1.01°C (n = 3)]. The $T_m$ of component B was shifted to lower temperatures in the presence of D-600 (10–250 $\mu$M). A concentration of 100 $\mu$M shifted the $T_m$ of component B up to the point at which it overlapped with the $T_m$ of component A. However, this concentration of D-600 did not affect the thermal stability of the nucleotide-binding domain.

The transition temperatures at various concentrations of D-600 calculated from the denaturation profile of the Ca$^{2+}$-ATPase component B are given in Table 2. These results show that the transmembrane domain is the ATPase component affected by D-600. The effect of D-600 on component B is dose dependent at the same concentrations in which Ca$^{2+}$ uptake is inhibited. In addition to the endotherms described for components A and B, an endotherm at ~95°C was detected in some of the scans. This is likely due to the denaturation of an additional protein component. An exotherm (valley) in the region of 70–75°C occurred at high D-600 concentrations. The underlying cause of this exotherm is unknown.

To show that the effect of D-600 on the LSR function, specifically on the Ca$^{2+}$ uptake, is related to a conformational change in the transmembrane domain of the ATPase, we plotted the changes in $T_m$ of component B and the changes in $T_i$ for Ca$^{2+}$ uptake, both as a function of D-600 concentration (Fig. 8). Qualitatively, the curves for $T_m$ and $T_i$ are similar; however, $T_i$ values are higher than $T_m$ values. These thermal analyses results demonstrate that D-600 interacts with the transmembrane domain of the SR Ca$^{2+}$-ATPase, causing a conformational change that may be responsible for sensitizing the thermal inactivation of Ca$^{2+}$ uptake in LSR.

### Table 2. Transition temperature for thermal denaturation of the Ca$^{2+}$-ATPase: component B (Ca$^{2+}$-binding domain)

<table>
<thead>
<tr>
<th>[D-600], $\mu$M</th>
<th>$T_m$, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60.0 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>58.2 ± 0.3</td>
</tr>
<tr>
<td>20</td>
<td>57.4 ± 0.4</td>
</tr>
<tr>
<td>50</td>
<td>52.4 ± 0.4</td>
</tr>
<tr>
<td>100</td>
<td>51.5 ± 0.5</td>
</tr>
<tr>
<td>250</td>
<td>51.6 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SD from 3 experiments.
DISCUSSION

Our results show that D-600 1) inhibits Ca\textsuperscript{2+} uptake in isolated LSR membranes in a dose-dependent manner, 2) does not affect the hydrolytic activity of the Ca\textsuperscript{2+}-ATPase at doses in which Ca\textsuperscript{2+} uptake is inhibited, 3) does not affect LSR passive permeability of Ca\textsuperscript{2+} at concentrations up to 160 \mu M, 4) decreases the thermal stability of the ATP-dependent Ca\textsuperscript{2+}-uptake activity in a dose-dependent manner, and 5) decreases the thermal stability of the transmembrane domain of the Ca\textsuperscript{2+}-ATPase in a dose-dependent manner.

We conclude that the inhibition of Ca\textsuperscript{2+} uptake by D-600 is not the consequence of Ca\textsuperscript{2+} leakage because membrane permeability is not affected by the drug (Fig. 6). These results also lead us to conclude that the organic Ca\textsuperscript{2+} channel blocker D-600 interacts directly with the Ca\textsuperscript{2+}-ATPase at a concentration in which muscle fiber contractility is also affected (21). Assessing from the DSC results and the ATPase hydrolytic activity, D-600 does not affect the nucleotide-binding domain of the ATPase. Instead, the impaired thermal stability of Ca\textsuperscript{2+} uptake and the impaired Ca\textsuperscript{2+}-binding domain indicate that D-600 interacts with the ATPase transmembrane domain. These results confirm and extend our previous finding that D-600 affects SR Ca\textsuperscript{2+} loading in skinned muscle fibers (21). The failure of D-600 to inhibit caffeine-induced Ca\textsuperscript{2+} release from isolated junctional sarcoplasmic reticulum strongly supports the premise that the SR Ca\textsuperscript{2+}-ATPase, rather than the SR Ca\textsuperscript{2+} channel, is affected by this drug (21).

Lepock et al. (18) showed that in the absence of Ca\textsuperscript{2+}, the LSR Ca\textsuperscript{2+}-ATPase denatures as a single component with a T\textsubscript{m} of 48–49°C, and in the presence of Ca\textsuperscript{2+}, a second component appears that denatures at a T\textsubscript{m} of 60°C. These transitions in temperature represent the denaturation of the nucleotide-binding domain of the Ca\textsuperscript{2+}-ATPase (detectable by DSC and with FITC in the absence of Ca\textsuperscript{2+}) and denaturation of the Ca\textsuperscript{2+}-binding domain (detectable by DSC and by tryptophan fluorescence in the presence of Ca\textsuperscript{2+}), respectively (18). Previous studies by Lepock et al. (18) and Cheng and Lepock (3) have shown that a conformational change in a region of the protein closely associated with the Ca\textsuperscript{2+}-binding sites in the transmembrane domain causes uncoupling of Ca\textsuperscript{2+} transport from ATP hydrolysis. This may be due to the unfolding of a conformationally flexible site involved in Ca\textsuperscript{2+} translocation, but not in ATP hydrolysis before the complete unfolding of the Ca\textsuperscript{2+}-ATPase, as has been suggested to occur for a number of other enzymes (23). These data support our conclusion that D-600 specifically affects the transmembrane domain of the Ca\textsuperscript{2+}-ATPase containing the Ca\textsuperscript{2+}-binding sites.

The experiments presented here further support our previous results obtained in skeletal muscle fibers (21, 22) that demonstrate that D-600 has a twitch-potentiating effect. This twitch potentiation (22), observed despite the inhibitory effect of D-600 on contractures at low temperatures (8), shows that early steps in the excitation-contraction coupling chain of events remain functional in the presence of D-600. We had previously suggested that D-600 affects muscle contraction by exerting an inhibitory effect on the SR Ca\textsuperscript{2+}-ATPase (22). A potential mechanism is that D-600 penetrates into the myoplasm in intact fibers and interferes with the excitation-contraction coupling mechanism by inhibiting the SR Ca\textsuperscript{2+}-ATPase. As a result, myoplasmic Ca\textsuperscript{2+} is not completely sequestered into the SR after each twitch. Consequently, cytosolic Ca\textsuperscript{2+} increases slightly after each activation and twitches are thus potentiated (19, 22).

Colvin et al. (5) reported that high concentrations (1 mM) of Ca\textsuperscript{2+} channel blockers (dihydropyridines) stimulated the Ca\textsuperscript{2+}-ATPase activity in isolated SR from cardiac and skeletal muscle, but no such effect was observed at lower concentrations. Wang et al. (24) also observed that verapamil, felodipine, and diltiazem at a concentration of 40 \mu M produced a 40–60% activation of the SR Ca\textsuperscript{2+}-ATPase. As we show in the present studies, 320 \mu M of the organic Ca\textsuperscript{2+} channel blocker D-600 causes LSR Ca\textsuperscript{2+} leakage (Fig. 6). Thus the data of Colvin et al. (5) and Wang et al. (24) could not be interpreted as the result of an increased SR Ca\textsuperscript{2+} leakage caused by extremely high Ca\textsuperscript{2+} channel blocker concentrations that produce a stimulated activity of the Ca\textsuperscript{2+}-ATPase. Another consequence of the increased Ca\textsuperscript{2+} permeability at 320 \mu M of D-600 is a decrement in the E\textsubscript{A} of the Ca\textsuperscript{2+}-uptake activity (Fig. 5). These increments in the ATPase activity produced by these Ca\textsuperscript{2+} channel blockers are not consistent with the fact that at the same doses, these Ca\textsuperscript{2+} channel blockers inhibit Ca\textsuperscript{2+} uptake in SR (5, 19–22, 24). We recently found that 10–160 M diltiazem does not have any effect on the Ca\textsuperscript{2+}-ATPase activity in SR of fast-twitch rabbit skeletal muscle (12). Another consequence of the increased passive Ca\textsuperscript{2+} permeability at 320 \mu M of D-600 is a decrement in the activation energy, E\textsubscript{A}, of the Ca\textsuperscript{2+}-uptake activity (Fig. 5).

Based on the observations described in this paper, we propose that the effect of <80 \mu M D-600 on skeletal muscle fiber contractility can be explained by a direct interaction with the LSR Ca\textsuperscript{2+}-ATPase, specifically with the transmembrane Ca\textsuperscript{2+}-binding domain. This is in agreement with the fact that D-600, similar to other Ca\textsuperscript{2+} channel blockers, is hydrophobic and capable of being incorporated into the membrane matrix (15). Our results, however, cannot distinguish whether D-600 binds to the transmembrane helices of the ATPase or disrupts the lipid bilayer neighboring the transmembrane domain, subsequently interfering with Ca\textsuperscript{2+} translocation. Our results also show that D-600, at concentrations up to 160 \mu M, does not have any effect on SR Ca\textsuperscript{2+} leakage. This strongly suggests that D-600 does not affect the lipid bilayer of the SR membrane, but rather affects the area closely associated with the transmembrane domain.

We cannot exclude the possibility that, in addition to its effect on Ca\textsuperscript{2+}-uptake inhibition (22), D-600 may also inhibit the T-tubular membrane voltage sensor with a mechanism similar to that described for Ca\textsuperscript{2+}.
channel blockage in single dialyzed heart cells (17). Thus the effect of D-600 in skeletal muscle appears to be complex. In addition to its Ca\(^{2+}\) channel-blocking effect, which has been shown to play no role in excitation-contraction coupling (2, 13), D-600 also inhibits the SR Ca\(^{2+}\) transport system. The inhibition of the Ca\(^{2+}\)-ATPase by D-600 could by itself explain the effects of this drug on the excitation-contraction coupling mechanism.

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