Block of Contracture in Skinned Frog Skeletal Muscle Fibers by Calcium Antagonists

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ABSTRACT   The ability of a number of calcium antagonistic drugs including nitrendipine, D600, and D890 to block contractures in single skinned (sarcolemma removed) muscle fibers of the frog *Rana pipiens* has been characterized. Contractures were initiated by ionic substitution, which is thought to depolarize resealed transverse tubules in this preparation. Depolarization of the transverse tubules is the physiological trigger for the release of calcium ion from the sarcoplasmic reticulum and thus of contractile protein activation. Since the transverse tubular membrane potential cannot be measured in this preparation, tension development is used as a measure of activation. Once stimulated, fibers become inactivated and do not respond to a second stimulus unless allowed to recover or reprime (Fill and Best, 1988). Fibers exposed to calcium antagonists while fully inactivated do not recover from inactivation (became blocked or paralyzed). The extent of drug-induced block was quantified by comparing the height of individual contractures. Reprimed fibers were significantly less sensitive to block by both nitrendipine (10°C) and D600 (10 and 22°C) than were inactivated fibers. Addition of D600 to fibers recovering from inactivation stopped further recovery, confirming preferential interaction of the drug with the inactivated state. A concerted model that assumed coupled transitions of independent drug-binding sites from the reprimed to the inactivated state adequately described the data obtained from reprimed fibers. Photoreversal of drug action left fibers inactivated even though the drug was initially added to fibers in the reprimed state. This result is consistent with the prediction from the model. The estimated K_I for D600 (at 10° and 22°C) and for D890 (at 10°C) was ~10 μM. The estimated K_I for nitrendipine paralysis of inactivated fibers at 10°C was 16 nM. The sensitivity of reprimed fibers to paralysis by D600 and D890 was similar. However, inactivated fibers were significantly less sensitive to the membrane-impermeant derivative (D890) than to the permeant species (D600), which suggests a change in the drug-binding site or its environment during the inactivation process. The enantomeric dihydropyridines (+) and (−) 202-791, reported to be calcium channel agonists and antagonists, respectively, both caused paralysis, which suggests that blockade of a transverse tubular mem-

brane calcium flux is not the mechanism responsible for antagonist-induced paralysis. The data support a model of excitation-contraction coupling involving transverse tubular proteins that bind calcium antagonists.

INTRODUCTION

In skeletal muscle, plasmalemmal calcium channels are located mainly in the membrane of the transverse tubules (T membrane, Nicola-Siri et al., 1980; Almers and Palade, 1981). Presumably these channels account for the influx of calcium that occurs during trains of twitches and K contractures (Bianchi and Shanes, 1959; Curtis, 1966). However, the role T membrane calcium channels and their associated calcium flux play in the coupling of T membrane depolarization and calcium release from the sarcoplasmic reticulum (SR) is unclear. The channel kinetics are too slow for a significant calcium conductance to be activated during a short duration stimulus such as an action potential (Sanchez and Stefani, 1978; Almers and Palade, 1981) and elimination of the calcium flux does not immediately affect twitch activation in whole cells (Armstrong et al., 1972; Nicola-Siri et al., 1980; Gonzalez-Serratos et al., 1982). Thus, an influx of calcium does not seem to be a prerequisite for contractile activation. It is therefore somewhat surprising that certain calcium channel antagonists (agents that block calcium channels) cause paralysis of skeletal muscle fibers.

Eisenberg et al. (1983) first described the effects of one such calcium channel antagonist, D600 (methoxyverapamil). They demonstrated that single twitches stimulated by action potentials were not affected by the drug. However, when fibers were stimulated by prolonged depolarizations caused by elevated extracellular potassium, and exposed to 30 μM D600 at 7°C they produced a single contracture after which the fibers were paralyzed. Once paralysis was produced neither direct electrical stimulation nor additional application of potassium resulted in contractile activation. Eisenberg et al. (1983) also demonstrated that paralyzed fibers contract in response to caffeine, have normal resting membrane potentials, and conduct action potentials normally. Since the SR membrane is not affected by the drug (McCleskey, 1985), D600 most likely interacts with the mechanism that links T membrane depolarization to SR calcium release.

The work of Eisenberg et al. (1983) prompted Hui and co-workers (1984, 1987) to explore the effect of D600 on intramembrane charge movement, nonlinear capacity currents thought to be a manifestation of some step in the excitation of contraction (Schneider and Chandler, 1973). They showed that charge movement is not present in D600 paralyzed fibers and suggested that D600 may act by immobilizing charge. Since Chandler et al. (1976) have shown that immobilization of charge movement is associated with contractile inactivation, D600 may act by stabilizing fibers in the inactivated state. This notion is supported by the work of Caputo and Balaños (1987) and Berwe et al. (1987) who studied the interaction of the drug with force development in voltage-clamped amphibian fibers.

Similar effects in skeletal muscle have been seen with the dihydropyridines (DHP), a diverse class of calcium channel antagonists and agonists. Binding studies on isolated T membrane indicate that this membrane is rich in dihydropyridine receptors (Curtis and Catterall, 1984). Lamb (1986) has shown that the DHPs inhibit charge

movement in mammalian fibers. Brum and Rios (1986) demonstrated that DHPs enhance the inactivation process in voltage-clamped amphibian fibers. They have also provided strong evidence (Rios and Brum, 1987) that the DHP receptor may be part of the molecule that generates charge movements.

Recently, Donaldson et al. (1984) reported that D600 and its impermeant analogue D890 block contractures in skinned (sarcolemma removed) muscle fibers from rabbit. The characteristics of block in skinned mammalian fibers were similar to those reported for intact amphibian fibers. An initial contracture in the presence of drug was necessary before paralysis was induced.

In this study we have explored in detail the ability of several DHPs, D600, and D890 to block contracture in skinned amphibian fibers stimulated by ionic substitution. Our goal was to localize the site of action of these drugs as well as to define the mechanism by which they interfere with contractile activation.

Preliminary results of these experiments have appeared in an abstract (Fill and Best, 1987).

METHODS

Fiber Preparation

The techniques used in this study have been described in detail previously (Fill and Best, 1988).

Single fibers from the semitendinosus muscle (dorsal head) of the frog *Rana pipiens berlandieri* were manually skinned (sarcolemma removed) under light mineral oil. An aluminum foil clip was attached to each end of the skinned fiber. The fiber was then mounted in a photoelectric tension transducer. Fiber diameter was recorded at slack length after which a 20% stretch was applied. The fiber was then suspended in any of several solutions that filled depressions (1.5 ml) in a temperature-controlled, anodized aluminum plate. The fiber could be moved between solutions in less than 2 s. Due to the photo sensitivity of some of the drugs used, all experiments were performed under sodium vapor lighting.

Solutions

Solution recipes were generated by a computer program based on one originally written by Donaldson and Kerrick (1975) that solves the multiple equilibrium reactions necessary to describe solutions containing several ligands. Binding constants used in the program were adjusted for temperature (10 or 22°C) as described by Godt and Lindley (1982). The composition of the solutions are shown in Table I. To minimize the osmotic effects described by Mobley (1979), the product of monovalent cation and anion concentrations remained constant (368 mM^2). Fibers were never exposed to elevated levels of EGTA and calcium ion. Instead, all solutions contained a pCa of 7.3 (10 μM EGTA) to mimic the physiological calcium concentration in the myoplasm. It is assumed that the calcium load in the SR was maintained near physiological levels.

All drugs, except D890, were dissolved in polyethylene glycol (PEG) before being added to the solutions. The PEG concentration in any solution never exceeded 1%. Control experiments indicated that 1% PEG (vol/vol) had no effect on the contractile properties of the skinned fibers nor on release stimulated by ionic substitution. The lipophilic drugs used in this study were found to bind readily to plexiglass and plastic bottles. Therefore, drug-containing solutions were stored in glass. The d600 and D890 were a gift from Knoll Ag (Ludwigschafen, Germany). Nitrendipine and nisoldipine were obtained from Miles Laboratories,

Inc. (New Haven, CT). The two enantiomers, (±)202-791, were supplied by Sandoz Ltd. (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Data Collection

Once skinned, fibers were immediately mounted in the tension transducer. An initial contracture was elicited by an ionic substitution during which choline chloride was substituted for potassium propionate as the major monovalent ion in the bathing solutions (see Table I). It has been proposed that the T tubules reseal and become polarized after the skinning process and that ionic substitution causes a depolarization of the T tubules, thus triggering calcium release (Constantin and Podolsky, 1967; Donaldson, 1985; Stephenson, 1978; Volpe and Stephenson, 1986, Fill and Best, 1988). Evidence for the existence of resealed, polarized T tubules in this preparation includes the ability of blockers of the Na-K-ATPase and iono-

TABLE I

Composition of Solutions

	Choline chloride	Potassium-propionate	Choline-propionate
Standard solutions			
Recovery	4	92	—
Stimulating	92	4	—
Solutions of variable ionic composition			
	5	73.6	17.4
	5.5	66.9	23.6
	6	61.3	28.7
	7	52.6	36.4
	8	46.0	42.0
	10	36.8	49.2
	20	18.4	57.6
	37	9.9	49.1

Concentrations are in millimoles per liter. All solutions contained 2.11 Na_2ATP, 5.2 Na_2Cp, 0.0031 $CaCl_2$, 0.05 $Tris_2EGTA$ (pCa = 7.3), 14.6 TrisOH, 3.22 $MgSO_4$, and 27 MOPS buffer (pH = 7.0). The calculated concentration of MgATP was 2.0 and of free Mg^{2+} it was 1.0. The ionic strength of all solutions was 150 mM.

phores that should collapse ion gradients to make fibers unresponsive to ionic substitution (Donaldson, 1985; Volpe and Stephenson, 1986). Selective extraction of T membranes by saponin also renders fibers unresponsive (Donaldson, 1985). We have shown previously (Fill and Best, 1988) that once a contracture is initiated fibers became inactivated and will not respond to a second stimulation unless allowed to recover (reprime) in normal resting solution. Repriming is a time-dependent process that is sensitive to the amount of choline chloride in the recovery solution. Contractures of nearly identical height can be elicited by ionic substitution after identical recovery periods in the same solution. If the duration of the recovery period is shortened, the peak height of the next contracture is diminished. In this study, fibers were placed in the normal resting (recovery) solution as soon as the contracture initiated by the ionic substitution had decayed. The length of the period during which fibers remained in the recovery solution depended on the temperature at which experiments were being performed. Fibers remained in the recovery solution for 2 min at 22°C and for 4 min at 10°C. The duration of the recovery intervals used in these experiments represent the optimal

recovery period at each temperature. At either temperature, four to five consecutive contractures of nearly identical height could be elicited from a fiber as long as the recovery interval between contractures remained constant.

Data were collected only from the first few contractures of each fiber. The data collection protocol consisted of a control contracture followed by a test contracture. Experiments were routinely ended by exposing the fiber to caffeine. This allowed normalization of the control release stimulated by ionic substitution and also served to show the paralyzed fibers contained releasable calcium (see Fig. 1). The peak height of the control contracture averaged 71.3 ± 10.7% (n = 34) of a maximal caffeine (10 mM) contracture from the same fiber. This compares with 72.0 ± 9.1% found in an earlier study (Fill and Best, 1988). All drugs were initially applied to the fibers in the recovery solution during the interval between the control and test contracture. The peak height of the test contracture was determined as a percentage of the peak height of the control contracture. Percent block was calculated as 100% minus the percent control. Only one data point was obtained from each fiber.

Photoreversal of Drug Action

Intense illumination was used to rapidly reverse the action of the light-sensitive DHP nisoldipine. The output of six fiber optic lamps and two projection bulbs were directed at the fiber. Direct measurement of light intensity at the fiber is technically complex and was not necessary to interpret the results of the experiment. The temperature of the solution near the fiber was monitored continuously and did not vary appreciably from 10°C during the experiment.

Curve Fitting

Curves were generated using a nonlinear least-squares fitting program based on the Marquardt (1963) algorithm.

A concerted binding scheme involving two noninteracting drug-binding sites (Monod et al., 1965; Karlin, 1967; Colquhoun, 1973) was used to describe the majority of the data obtained from reprimed fibers. The equation used to fit the data is given in the Discussion as Eq. 3. Since a simplified equation analogous to Eq. 3 was not available to describe drug action on inactivated fibers, the Hill equation was used to fit the concentration dependency of paralysis in inactivated fibers (as well as the nitrendipine data at 22°C):

$$\text{Percent block} = \frac{[D]^y}{[D]^y + Q} \times 100 \tag{1}$$

where Q and y are empirically determined contants, and [D] stands for drug concentration.

The significance of differences between parameters determined by fitting the same function to different data were tested using Student's t test ($P < 0.01$).

RESULTS

D600 Application to Reprimed and Inactivated Fibers

In this study, drugs were applied to fibers that were in one of two states, which we shall call reprimed and inactivated. We have previously shown that after a contracture stimulated by ionic substitution, skinned fibers will not respond to a second stimulus unless allowed to recover in normal resting solution for several minutes (Fill and Best, 1988). We shall refer to fibers that are fully recovered and thus capa-

ble of responding to ionic substitution as being reprimed. We shall use inactivated to refer to the state immediately following a contracture when a fiber is unable to respond to a second stimulus. The record in Fig. 1 A illustrates the repriming of a fiber after an initial contracture. Immediately after the first contracture the fiber is inactivated and will not respond to a second stimulus for several seconds. The ability of the fiber to respond to a second stimulus returns with a half time of about 30 s at 20°C.

An experiment demonstrating effects of D600 application to both reprimed and inactivated fibers is shown in Fig. 1 B. The top record demonstrates the effect of
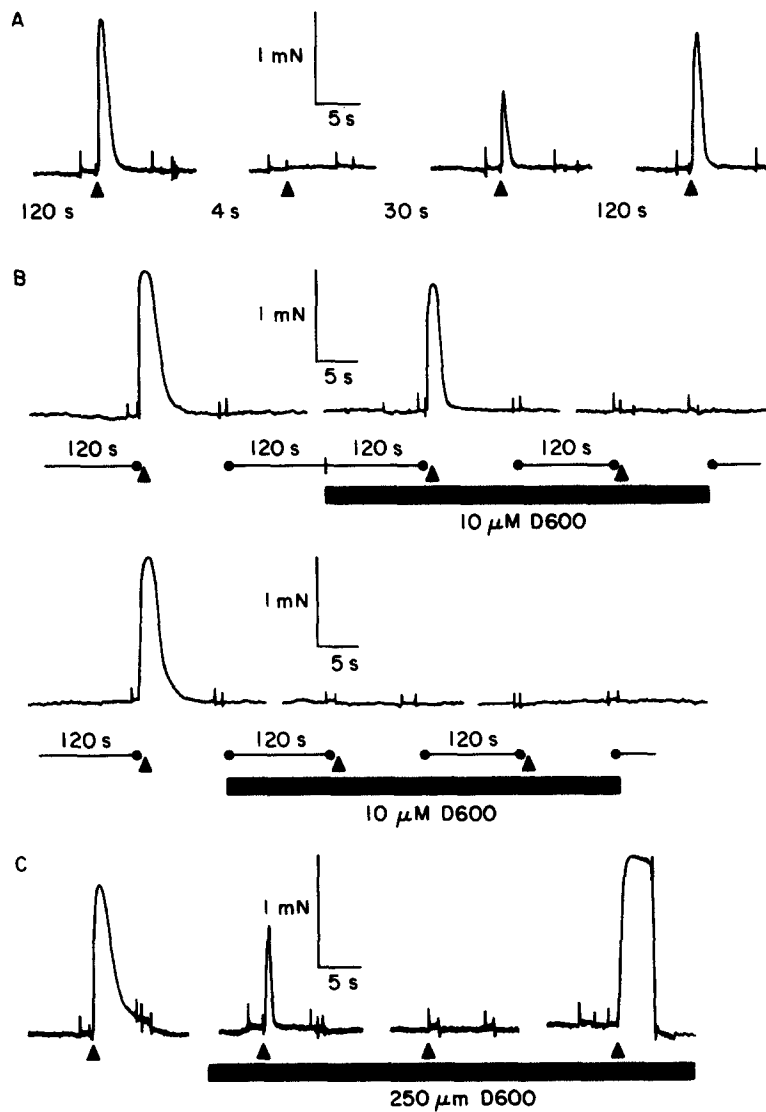

FIGURE 1

D600 (10 μM at 22°C) on a reprimed fiber. After the initial control contracture, the fiber was allowed to recover for 120 s. Once reprimed, D600 was applied to the fiber. The next stimulation resulted in a contracture of nearly the same height as that of the control. However, after this contracture, the fiber did not respond to further stimulation as if the drug blocked contractile repriming. A use-dependent block of contractile recovery by D600 is consistent with the results of Eisenberg et al. (1983) in intact cells and those of Donaldson et al. (1984) in skinned mammalian fibers. The bottom record in Fig. 1 *B* demonstrates the effect of D600 on an inactivated fiber. Immediately after the control contracture, before contractile repriming begins and while the fiber was still inactivated, the drug was applied. Unlike the reprimed fiber, the next stimulation elicited no response. Thus, fully reprimed fibers required a single conditioning contracture to be blocked by 10 μM D600 at 20°C, while inactivated fibers did not. After washing the drug out from both reprimed and inactivated fibers, contractures could be regained after extended repriming periods. The tracings in Fig. 1 *C*. show that a blocked fiber that will not respond to stimulation by ionic substitution does give a vigorous contracture when exposed to caffeine.

D600 Interrupts the Repriming Process

To further explore the relationship between drug action and repriming, D600 was applied to fibers during the repriming process (Fig. 2). In these experiments, the repriming interval "X" was varied while the drug exposure time (60 s) remained constant. The first contracture in each trace in Fig. 2 is a control elicited after a 120-s repriming period. The second contracture followed a variable repriming interval and a 60-s exposure to D600. Since the drug was applied to the fiber in the recovery solution, additional repriming might have occurred during the 60-s drug exposure period. The open bar at the end of each record represents the expected peak height of a contracture after a repriming period of Xs. The hatched bar repre-

FIGURE 1. (*Opposite*) The effect of D600 (10 μM) on ionically stimulated skinned fibers (20°C). The solid bar indicates when drug was present. Arrows indicate stimulation by ionic substitution. The first contracture in each tension record is a control contracture after a 120-s recovery period. (*A*) Records demonstrating inactivation and repriming. Immediately after the initial contracture (4 s), a second stimulus would not cause a response, indicating that the fiber was in the inactivated state. The fiber's ability to respond then recovered over time, with complete recovery after 120 s at this temperature. We refer to this recovery process as repriming. Control contracture is 24.3 N/cm^2. (*B*) The effects of D600 on reprimed and inactivated fibers. The time elapsed during the breaks in the tension records are represented by the line segments directly below them. The top record demonstrates the effect of D600 on a reprimed fiber. Application of D600 to a reprimed fiber does not block the first postdrug response, but does eliminate further contracture. Control contracture is 27.1 N/cm^2. The bottom record demonstrates the effect of D600 on a mechanically inactivated fiber. Application of D600 to an inactivated fiber blocks the next, and all subsequent, contractures. After washing the drug out from both reprimed and inactivated fibers, contractures (not shown) were regained. Similar results were obtained at 10°C. The control contracture is 25.8 N/cm^2. (*C*) Fibers made nonresponsive to stimulation by ionic substitution through exposure to D600 still responded to caffeine (final arrow).

sents the expected peak height of a contracture after a repriming period of X plus 60 s (data for both open and hatched bars redrawn from Fill and Best, 1988). In the top record, the fiber was allowed to reprime for a total of 75 s. During the first 15 s of this period D600 was not present. The height of the second contracture was com-
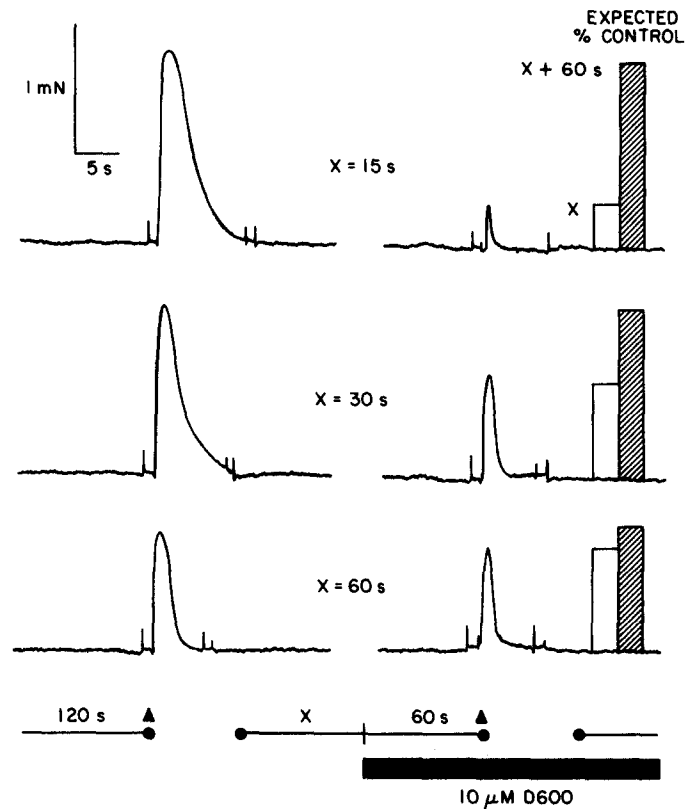

FIGURE 2. D600 interrupts the repriming process. Tension records demonstrating the effect of applying D600 during the repriming process. The experimental protocol is shown at the bottom. The repriming interval X was varied, while drug exposure time was constant at 60 s. The open bars represent the expected contracture height after a repriming interval of X seconds. The hatched bars represent the expected contracture height after a repriming interval of X + 60 s. Each series of contractures is taken from a separate fiber. In the top record the fiber was allowed to reprime for a total of 75 s. During the first 15 s, D600 was not present. The height of the second contracture is close to that of the open bar. The same is true for each of the other records. Thus, the degree of repriming is what would be expected if the fibers only reprimed when drug was not present. Control contractures (top to bottom) were 20.6, 22.4, and 23.7 N/cm^2.

pared to that of the bars at the end of the record. It is clear that the height of the second contracture is close to that of the open bar. The degree of repriming, indicated by the height of the second contracture, is what would be expected for a fiber reprimed for 15 s rather than one reprimed for 75 s. Similar results with different drug-free repriming intervals are shown in the other two records.

Data collected from 36 experiments of this type are summarized in Fig. 3. The solid and dashed lines were drawn from the same data used to generate the bars in Fig. 2. The extent of repriming expected during the interval X (open bars in Fig. 2) is represented by the solid line. The extent of repriming expected during the interval X plus 60 s (hatched bars in Fig. 2) is represented by the dashed line. The data points represent the measured degree of repriming that occurred during the interval between contractures. The degree of repriming is very close to what would be expected if the fiber reprimed only when the drug was not present.

The data presented in Fig. 1 suggest that D600 produces block by interacting preferentially with the inactivated state of the fiber to prevent further repriming. If this is true, then in partially reprimed fibers one would expect D600 paralysis to be incomplete. Furthermore, the extent of block should depend on the degree of
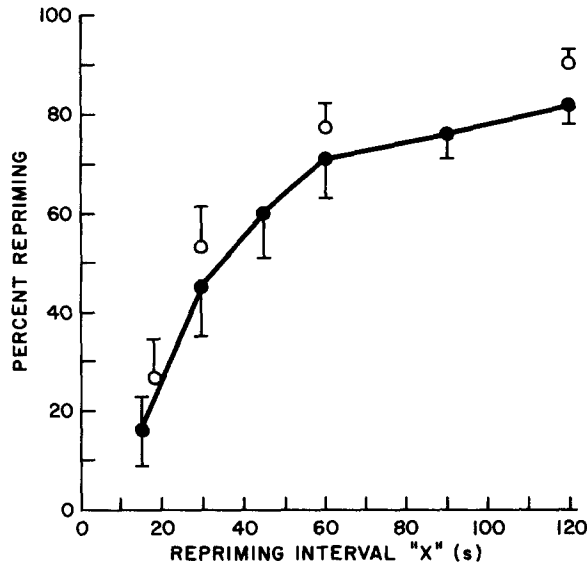

FIGURE 3. Summary of data collected as described in Fig. 2 (closed symbols). The degree of repriming, measured as the height of the test contracture, is plotted as closed symbols against the repriming intervals indicated. The repriming intervals plotted are those that occurred without drug present (X in Fig. 2). Points are averages of at least six determinations (n ± SD). Open symbols are controls redrawn from data in Fill and Best, (1988) and indicate the normal recovery of fibers under identical conditions. Note that the degree of repriming was never greater than that which occurred in the controls. These results suggest that repriming did not occur in the presence of D600.

repriming that occurred before D600 was applied. Figs. 2 and 3 demonstrate that fibers reprime normally until 10 μM D600 is applied. Thus, these data suggest that saturating D600 interrupts or prevents the process of repriming by interacting with the inactivated state of the fiber.

The D600 Concentration-Effect Relationship

The relationship between D600 concentration and paralysis was determined. Both reprimed and inactivated fibers were exposed to several concentrations of D600 at 10 and 22°C. The concentration-effect relationships are shown in Fig. 4. At both temperatures inactivated fibers (circles) were always more susceptible to D600 than

were reprimed fibers (triangles). Inactivated fibers at 10°C (open symbols) were more susceptible to D600 paralysis than they were at 22°C (closed symbols). In reprimed skinned fibers, however, the concentration dependence of D600 paralysis was not temperature dependent. The D600 concentration-effect data indicate that the process of inactivation greatly increases the susceptibility of fibers to D600 paralysis.
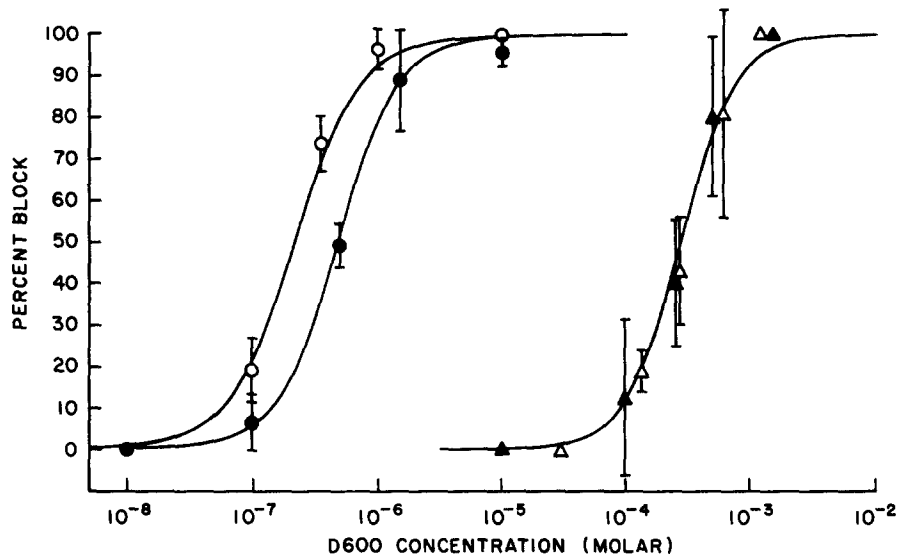

FIGURE 4.   D600 concentration-effect relationships. Data from reprimed fibers at 10°C (△) or 22°C (▲) and from inactivated fibers at 10°C (O) and 22°C (●). Inactivated fibers were more sensitive to drug than were reprimed fibers. Paralysis of inactivated fibers was temperature sensitive, while paralysis of reprimed fibers was not. Change in temperature did not effect the shape of the concentration-effect relationship for either reprimed or inactivated fibers. The data points represent the average of five or more determinations and the error bars are the standard deviation. Data from reprimed fibers were fit using the concerted binding scheme described in the text. At 10°C the fitted parameters for the reprimed curves were $n = 2.0 \pm 0.1$ and $K_I = 9.9 \pm 0.8$ μM. For 22°C they were $n = 2.0 \pm 0.1$ and $K_I = 9.1 \pm 0.7$. The parameters are not significantly different. The data from inactivated fibers were fit using the Hill equation with $y = 2.1 \pm 0.0$ and $Q = 0.04 \pm 0.02$ at 10°C, and $y = 2.2 \pm 0.1$ and $Q = 0.3 \pm 0.08$ at 22°C. The Q values are significantly different.

The Process of Inactivation Changes the Accessibility of the D600/D890 Binding Site

Since D600 is highly lipid soluble, it is unclear whether its binding sites are on the extracellular or myoplasmic side of the T membrane. This issue has been resolved using D890, a membrane-impermeable derivative of D600. These two compounds compete for the same binding site (Janis and Triggle, 1984).

The process of inactivation causes measurable changes in the affinity of fibers for D600. To investigate whether this change in affinity affects the accessibility of D890

to the D600/D890 binding site, the concentration-effect relationships of D890 at 10°C were determined (Fig. 5). These data were compared with the D600 data collected at 10°C (redrawn from Fig. 4). Reprimed fibers (triangles) had very similar affinities for both drugs. In inactivated fibers (circles), however, the affinity for D890 (closed symbols) is approximately two orders of magnitude less than that for D600 (open symbols).
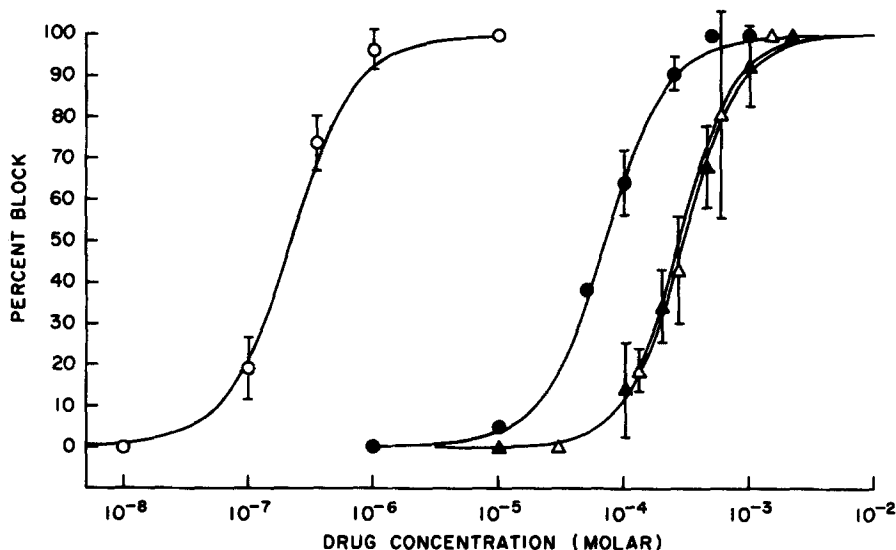

FIGURE 5.   The process of inactivation changes the accessibility of the D600/D890 binding site. D600 and D890 were each applied to reprimed and inactivated fibers at 10°C. Data points are averages (±SD) of more than five determinations. The D600 concentration-effect data (open symbols) is redrawn from Fig. 4. Reprimed fibers (triangles) have similar affinities for both drugs. Inactivated fibers (circles), however, have much greater affinity for D600 (open symbols) than they do for D890 (closed symbols). Since D890 is a membrane-impermeable derivative of D600 (a lipid soluble compound), these data suggest that the D600/D890 binding site in reprimed fibers is accessible from the myoplasm, but that the process of inactivation changes the accessibility of the site. D890 data from reprimed fibers fit with the concerted model ($n = 2.0 \pm 0.0$, $K_1 = 9.0 \pm 0.4$). Filled parameters are not statistically different from those used to describe the D600 data at 10°C. D890 data from inactivated fibers fit with the Hill equation ($y = 1.5 \pm 0.4$, $Q = 500 \pm 6$). Only the Q value is significantly different from the parameters used to describe the D600 data.

The Nitrendipine Concentration-Effect Relationship

Recent evidence suggests that DHPs are capable of paralyzing intact skeletal muscle cells by interrupting charge movements. (Lamb, 1986; Rios and Brum, 1987). We compared the sensitivity of fibers to the paralyzing effects of nitrendipine and D600. The concentration-effect relationship for nitrendipine was determined for both inactivated and reprimed fibers (Fig. 6). At 22°C, the nature of nitrendipine paralysis was quite different from that described for D600. Specifically, nitrendipine appeared to paralyze both reprimed and inactivated fibers equally well (filled sym-

bols). Furthermore, these data were adequately described by a simple 1:1 binding scheme ($K_d = 1$ μM). Nitrendipine paralysis at 10°C, however, was similar to that described for D600. At 10°C inactivated fibers were tenfold more susceptible to nitrendipine paralysis than were reprimed fibers (open symbols) and the curves shown were generated by the same model that was used to fit the D600 data. These results indicate that dropping the temperature from 22 to 10°C changes the nature of nitrendipine paralysis. At 22°C the block seems to be independent of the repriming process, since both inactivated and reprimed fibers are affected equally. At
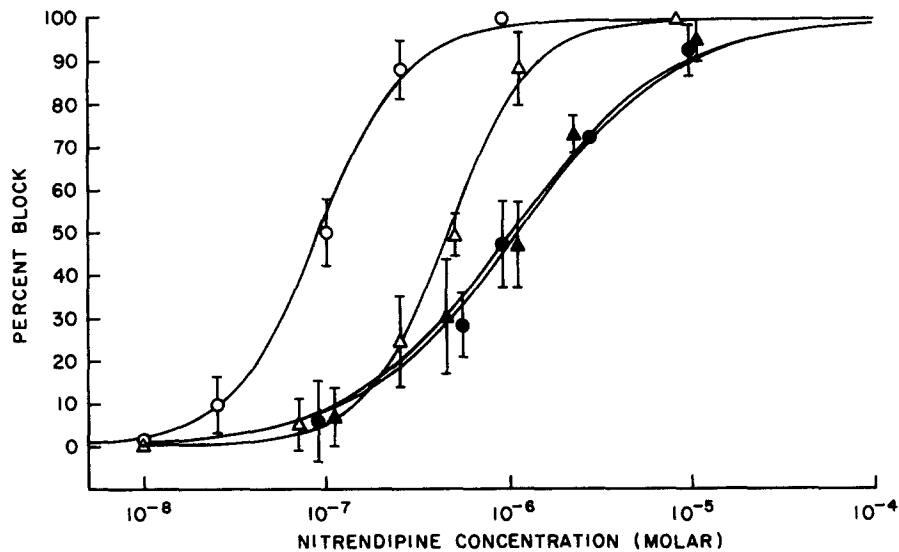

FIGURE 6. Nitrendipine concentration-effect relationships. Data collected on both reprimed (triangles) and inactivated (circles) fibers at 22 (filled symbols) and 10°C (open symbols). The data points represent the average of five or more determinations. Error bars represent the standard deviation. At 10°C inactivated fibers are more susceptible to nitrendipine paralysis than reprimed fibers. At 22°C inactivated and reprimed fibers seem equally susceptible to nitrendipine paralysis. Data collected at 22°C were best described by a 1:1 binding scheme (Hill equation, $y = 1.01 \pm 0.06$, $Q = 1.02 \pm 0.14$ for reprimed fibers, $y = 0.99 \pm 0.06$, $Q = 1.00 \pm 0.14$ for inactivated fibers). The fitted parameters are not statistically different. At 10°C, data from inactivated fibers were fit using the concerted model ($n = 2.08 \pm 0.03$, $K_1 = 0.016 \pm 0.002$) and those from reprimed fibers were fit using the Hill equation ($y = 2.04 \pm 0.01$, $Q = 0.008 \pm 0.01$).

10°C, nitrendipine acts in a D600-like fashion in that inactivated fibers were more susceptible to paralysis than were reprimed fibers.

Reprimed Fibers Are Drawn into the Inactivated State

We have demonstrated that reprimed as well as inactivated skinned fibers are paralyzed by DHPs and D600, although with significantly different sensitivity as the inactivated state is more sensitive to drug. To explain this observation, we assume a model in which the inactivated and reprimed states are in equilibrium (see the Dis-

cussion). When drug is applied to a partially reprimed fiber it should preferentially interact with those binding sites that are in the inactivated state, thus altering the equilibrium between the inactivated and reprimed states.

To directly test this idea we have determined the state in which fibers are when paralyzed, by using the extremely light-sensitive DHP nisoldipine. Sanguinetti and Kass (1984) have shown that the pharmacological action of nisoldipine can be rap-
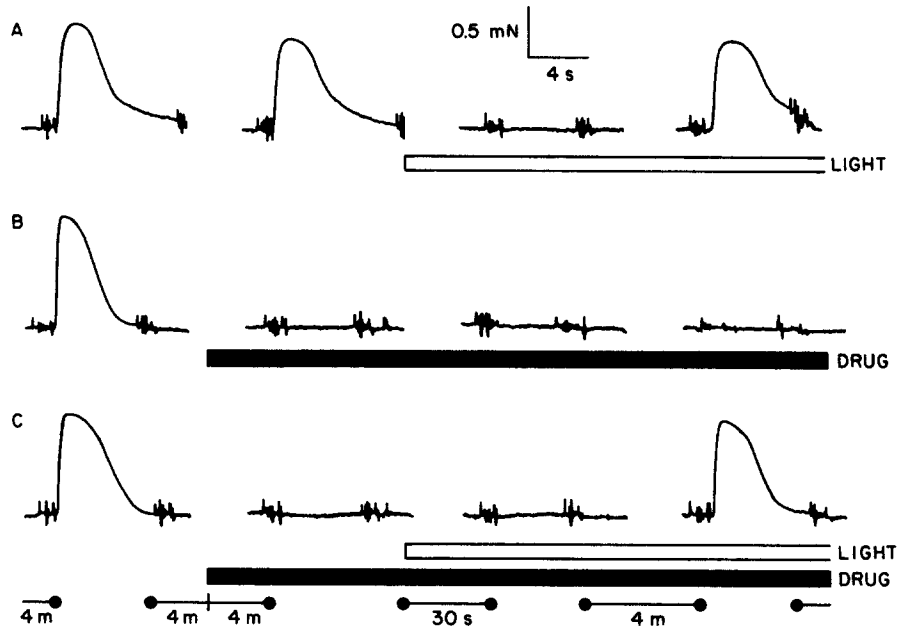

FIGURE 7.   Rapid reversal of drug action reveals that paralyzed fibers are in the inactivated state. Line segments (bottom) show time elapsed between stimuli for all records. The open bar indicates intense illumination and the filled bar indicates presence of 2.5 μM nisoldipine. Calibration bars for all records are shown at top. Eight experiments (all at 10°C) were performed with identical results. (*A*) Tension record demonstrating that fibers are unaffected by the illumination. As expected at 10°C, no repriming occurs after 30 s and substantial repriming occurs after 4 min. (*B*) Tension record demonstrating nisoldipine paralysis. Nisoldipine was applied after the fiber was allowed to reprime. At this concentration and temperature the fiber was completely paralyzed. (*C*) A reprimed fiber was paralyzed by nisoldipine (as in *B*). The action of the drug was reversed by intense illumination. If reprimed fibers, when paralyzed, remain in the reprimed state, the fiber should have been at least partially reprimed during the 30-s illumination. Instead, the fiber had to be reprimed before a response could be elicited.

idly reversed by intense illumination. Reprimed fibers were exposed to a saturating concentration (2.5 μM) of nisoldipine (Fig. 7). Once the fiber was paralyzed, the action of the drug was reversed by intense illumination. The contractile properties as well as the repriming process were unaffected by the illumination (Fig. 7 *A*). Suppose that immediately preceding the illumination period, the paralyzed fiber were in the reprimed state. Then, after illumination and destruction of the drug, the fiber

should have been in the drug-free reprimed state, and a contracture would have been elicited upon stimulation. Conversely, if the paralyzed fiber were in the inactivated state, then no contracture would be elicited after illumination until a suitable repriming period had elapsed.

Reprimed fibers that had been paralyzed by nisoldipine were unresponsive to stimulation after 30 s of illumination (Fig. 7 B). After the repriming period (4 min), stimulation resulted in a large contracture (Fig. 7 C). Thus, after exposure to 2.5 μM nisoldipine, fibers must be allowed to reprime before they become sensitive to ionic
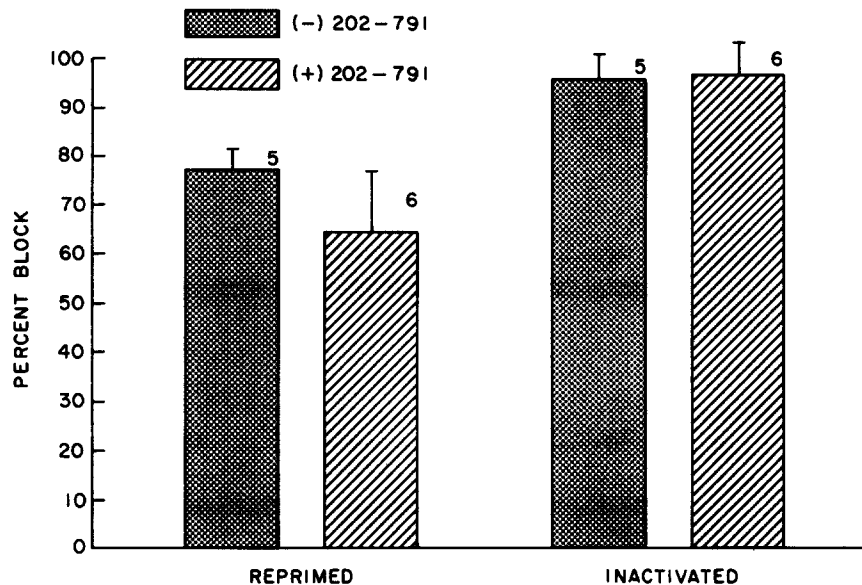

FIGURE 8. The calcium channel agonist (+) 202–791 blocks excitation-contraction coupling. Both enantiomers of the dihydropyridine 202–791 were applied to reprimed and inactivated fibers at 10°C. The (+) enantiomer is a calcium channel agonist and the (−) enantiomer is an antagonist. All experiments were performed with 1 μM drug. Data are averages (±SD). The number of determinations is indicated for each bar. The agonist (hatched bars) and the antagonist (filled bars) had similar effects of both reprimed and inactivated fibers. Inactivated fibers were always more susceptible to paralysis than were reprimed fibers. Since the agonist and antagonist both cause paralysis, these results suggest that block of calcium conductance in the T membrane is not necessary for paralysis.

substitution. Although the fiber was initially reprimed when drug was applied, drug binding seems to have drawn the fiber into the inactivated state.

It is possible that the initial period of illumination (30 s) did not totally reverse the action of the drug. The following points argue against this notion. The degree of repriming that occurred in 4 min was the same before and after paralysis. If the 30-s illumination was not sufficient to reverse most of the drug's action, the fibers would not have reprimed normally. Consistent with this argument is the observation that in control experiments (data not shown) lower intensity light resulted in incomplete

repriming during the 4-min period after illumination was terminated. Since no contractile response could be elicited, we conclude that the fiber was in the inactivated state when it was paralyzed. Similar results were obtained from seven other fibers.

Paralysis Not Due to Blockade of a Calcium Conductance

To determine if blockade of a calcium flux from resealed T tubules into the myoplasmic space contributed to the abolition of tension, a calcium channel agonist was used. These experiments used the optically pure stereoisomers of the DHP 202-791. The enantiomer (+)202-791 is reported to be a voltage independent, calcium channel activator while (−)202-791 is a calcium channel blocker (Williams et al., 1985). Each enantiomer (1 μM) was applied to inactivated and reprimed fibers at 10°C. Both (−)202-791 and (+)202-791 paralyzed fibers (Fig. 8). Like the D600 and nitrendipine data, inactivated fibers were more susceptible to paralysis by either enantiomer than were the reprimed fibers.

DISCUSSION

Calcium Antagonists Stabilize the Inactivated State in Skinned Fibers

In this study, calcium antagonistic drugs were applied to reprimed and inactivated amphibian skinned fibers. We define inactivated as the state fibers are in immediately after contracture induced by ionic substitution in which a second stimulus does not trigger a response. Reprimed refers to the state fibers are in after the time-dependent process of recovery from inactivation has occurred. For 10 μM D600, block of reprimed fibers was similar to that described by Eisenberg et al. (1983) in intact cells and by Donaldson et al. (1984) in skinned mammalian fibers. A conditioning contracture was necessary in the presence of drug before block was seen. Block of inactivated fibers, however, was quite different. A conditioning contracture was not necessary to paralyze an inactivated fiber. One explanation for this result is that the conditioning contracture needed to paralyze reprimed skinned fibers in the presence of D600 puts the fibers into the inactivated state and that the affinity for the drug in this state is much higher than in the reprimed state. The concentration-effect relationships for D600, D890, and nitrendipine support this notion since they indicate that the sensitivity of inactivated fibers to these drugs is greater than that of reprimed fibers. Furthermore, caffeine contractures were not affected by the presence of drug. Caffeine is thought to stimulate the SR directly, thus, drug-induced paralysis most likely affects a step in the process that couples T tubule stimulation and activation of SR calcium channels. The results suggest that calcium antagonists cause paralysis of skinned fibers by binding to T tubule membranes and that they interact preferentially with the inactivated state.

DHP binding studies on isolated T membrane receptor proteins reveal a 1:1 binding stoichiometry (Fosset et al., 1983; Borsotto et al., 1984). In this study, however, a simple 1:1 binding scheme did not adequately describe the majority of the data obtained from reprimed fibers as it could not account for the steepness of the concentration-effect relationship. Since drug sensitivity depended on the condition of the fiber, reprimed or inactivated, when drug was applied, multistate binding schemes were of particular interest.

We assumed the following simplified model which is based on a similar scheme used to describe intact fibers by Berwe et al. (1987).

$$K_R \downarrow \begin{array}{c} R \rightarrow A \rightarrow I \\ \\ RD \end{array} \downarrow K_I \qquad ID$$

(Scheme 1)
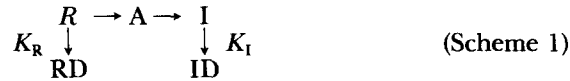
R, A, and I refer to the reprimed, active, and inactivated states, respectively, and K_R and K_I are the dissociation constants for the equilibrium binding of drug (D) to reprimed and inactivated fibers. The activated state corresponds to the time of calcium release. We assume that the transition between R and A is rapid and that drug binding is not affected. Thus, we use the size of a contraction as a probe of the fractional occupancy of the R state.

We have tested this model directly by determining in which state reprimed fibers are when paralyzed (see Results). When drug is applied to a fiber it will preferentially interact with whichever state has the lowest dissociation constant and this will alter the equilibrium between the R and I states. In the special case appropriate to those experiments where drug is applied to reprimed fibers and ($K_I \ll K_R$), the bound state, ID, should dominate (Karlin, 1967; Colquhoun, 1973). The photoinactivation of nisoldipine block (Fig. 7) shows clearly that adding drug to reprimed fibers changes the equilibrium such that the fibers are in the inactivated state when drug action is removed. This result is consistent with the model shown above.

To fit the concentration effect data, an independent subunit scheme was initially most attractive since binding studies (Fosset et al., 1983; Borsotto et al., 1984; Brandt et al., 1985) suggest that the DHP binding sites behave independently. This scheme postulates that in order for an increment of inactivation to occur a certain number (n) of promoters must be in the I state, and that the state of each promoter is independent of all others. The data were adequately fit by this scheme only if "n" was equal to ~40. As this seems intuitively unlikely, a model assuming independent protomers was not used. A concerted binding scheme involving two binding sites was found to describe the data for reprimed fibers adequately (except for nitrendipine at 22°C) and was used to generate the binding curves shown in the Results.

The concerted scheme postulates that two or more protomers, each containing a single drug-binding site, are linked together to form an oligomer. These oligomers can be in either of two states, termed here the R and I states. The transition from one state to the other is an all or none event; that is, all protomers of the oligomer undergo the same transition. Drug-binding sites are assumed to be independent. We can then define the percent of protomers in the inactivated state with or without bound drug, as percent block:

$$\text{Percent block} = 1 \bigg/ \left\{ 1 + L\left[\left(1 + \frac{[D]}{K_R} \right) \bigg/ \left(1 + \frac{[D]}{K_I} \right) \right]^n \right\} \times 100 \qquad (2)$$

where L represents the equilibrium constant between the R and I states [R]/[I]. The variable n represents the number of protomers linked together to form one oligomer. Percent block relates the actual response of the fiber to the maximal response of fully reprimed fibers in the drug-free state.

When the R state is favored ($L \gg 0$) and the affinity for drug binding to the I state

is much greater than that for the R state ($K_I/K_R \ll 0$) the following, simplified equation with three free parameters holds:

$$\text{Percent block} = [D]^n/([D]^n + LK_I^n) \times 100 \tag{3}$$

Under these conditions only the R and ID states will have significant occupancy. This equation was used to describe the effect of drug on reprimed fibers (except for nitrendipine at 22°C). We assumed a value of $L = 100$ based on observations of fiber repriming previously described in detail (Fill and Best, 1988; see also Fig. 2), which suggest that for the repriming interval and solutions used in this study, fiber recovery should have been maximal with little inactivation remaining. Values of L below ~80 gave noticeably poor fits.

The Locus of the Drug-binding Site

Nitrendipine and D600 are highly lipid soluble. Therefore, even though the drugs were added to the myofilament space in this study, their binding sites could have been on either surface of the T membrane. We have investigated this issue by using D890, a membrane-impermeable derivative of D600. Both D890 and D600 paralyzed skinned fibers with equal affinity when applied to the myoplasmic side of the T membrane of reprimed fibers. This confirms the observations of Donaldson et al. (1984) in skinned mammalian fibers, and suggests that the drug-binding site is accessible from the myoplasm. A similar conclusion was drawn by Hui and Milton (1987) from observations on intact frog muscle fibers. However, when D890 was applied to inactivated fibers, its ability to induce paralysis was reduced compared with D600. The change in the binding affinity for these drugs suggests that the process of inactivation induces some change in the binding site or its environment that affects D890 binding more than D600 binding. This change might involve a translocation of the binding site from a myoplasmic-accessible state to a state in which the site is more accessible to a lipid-soluble drug. For instance, the binding site might move from the myoplasmic surface of the T membrane to a location within the lipid of the T membrane. Alternatively, a nonspecific effect on the lipid environment might account for the results.

Role of the Calcium Antagonists

One possible mode of action of the calcium channel antagonists is to alter a calcium conductance in the T membrane, which is necessary for fiber activation. However, since a calcium channel agonist could also produce paralysis, blockade of the T membrane calcium conductance does not seem to be a tenable hypothesis to account for the effects of the calcium antagonists described in this study. A similar conclusion was reached by Hui and Milton (1987). This notion is also consistent with the bulk of the data in intact cells (Spiecker et al., 1979; McCleskey, 1985). The majority of the DHP receptors in the T membrane may not be functional calcium channels (Schwartz et al., 1985). Rios and Brum (1987) have suggested that these extra receptors may be involved in the voltage-sensing step of the coupling process linking T membrane depolarization to SR calcium release. The DHP receptor is structurally similar to sodium channels and contains a domain that has been tentatively identified as a voltage-sensing region (Takahashi et al., 1987; Tanabe et al.,

1987). If this voltage-sensing mechanism is a component of the coupling process, calcium channel antagonists might disrupt its function and thus paralyze the fiber (Rios and Brum, 1987). Nitrendipine paralysis at 22°C may involve a nonspecific action of the drug at the high concentrations (Yatani and Brown, 1985) required to see an effect. One possible action of nitrendipine at these concentrations might be to block T membrane potassium channels which are thought to play a critical role in the stimulus, ionic substitution, used in this study.

Intact Fiber Considerations

The isolated DHP receptor protein from T tubules can have either high or low binding affinity (Ptasienski et al., 1985). Bean (1984), in a study of DHP block of calcium currents, has shown that the DHP receptor has high affinity if the calcium channels are held in the inactivated state. He explained his results using an extension of the modulated receptor hypothesis which postulates that DHP binds tightly to the inactivated calcium channel and weakly to the resting state of the channel. A similar scheme involving high affinity binding of drug to inactivated muscle fibers was used to explain the effects of nifedipine on charge movements and calcium release by Rios and Brum (1987) and of D600 on contractures by Berwe et al. (1987). Preferential interaction of drug with inactivated skinned fibers was clearly demonstrated in this study and drug effects in skinned fibers are similar to those reported in intact cells. The shift in the apparent dissociation constant for D600 (Fig. 4), was approximately three orders of magnitude. An effect of this size was predicted by Berwe et al. (1987) from the drug-induced shift in the steady state restoration curve of intact fibers. The K_D of about 7 nM (7–11°C) calculated by Rios and Brum (1987) for nifedipine binding compares favorably with the value of 16 nM for K_1 used to fit the nitrendipine data at 10°C reported in this study. Finally, the temperature dependency of drug action we have demonstrated is consistent with a temperature dependency of paralysis in intact fibers first described by Eisenberg et al. (1983). The close similarities between the block of contractures in skinned fibers reported in this paper and paralysis of intact fibers from a variety of laboratories suggests a similar mode of action of the drugs in the two preparations.

We have shown in skinned fibers that nitrendipine causes block in a fashion similar to D600 in that inactivated fibers are more susceptible to paralysis than are reprimed fibers. We have also demonstrated that the effect of the nitrendipine and D600 on contracture is not related to drug concentration by a simple 1:1 relationship. Assuming the drug receptor is actually part of the molecule that gives rise to charge movements (Rios and Brum, 1987) we propose that the charge movement molecule exists in a multimeric form and that transitions between the reprimed and inactivated states in intact cells involve concerted transformations of multiple drug-binding sites. This is consistent with electron micrographs that show arrays of multimetric particles in the T membrane (Franzini-Armstrong and Nunzi, 1983). A logical extension of this argument is that the activation of calcium release (R → A), is also a concerted transition. Interestingly, the concerted model yields an inconsistency between the best fit value for the dissociation constant of drug binding and the apparent K_D estimated from the 50% point of the concentration-response curve

(for nitrendipine at 10°C, K_I = 16 nm, $K_{50\%}$ ≈ 100 nm; see Fig. 6). This may explain previously published differences between drug action and binding.

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