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PARALYSIS OF FROG SKELETAL MUSCLE FIBRES BY THE CALCIUM ANTAGONIST D-600

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SUMMARY

1. The Ca^{2+} channel blocker D-600 (methoxyverapamil) paralyses single muscle fibres of the frog: fibres exposed to the drug at 7 °C give a single K^+ contracture after which they are paralysed, unable to contract in response to electrical stimulation or further applications of K^+.

2. Paralysed fibres contract in response to caffeine and have normal resting potentials and action potentials.

3. Fibres treated with D-600 at 22 °C are not paralysed. Paralysed fibres warmed to 22 °C recover contractile properties: they twitch and give K^+ contractures. Other workers have shown that D-600 blocks a Ca^{2+} channel at room temperature; thus, the paralytic action of D-600 is probably mediated by a different membrane protein, perhaps a different Ca^{2+} channel from that blocked at room temperature.

4. These results suggest that the binding of D-600 can disrupt the mechanism coupling electrical potential changes across the T membrane to Ca^{2+} release from the sarcoplasmic reticulum.

INTRODUCTION

Excitation of the surface membrane of skeletal muscle is linked to the contraction of sarcomeres by a chain of mechanisms. Much attention has been paid to the role of Ca^{2+} in these mechanisms (Lüttgau & Spiecker, 1979; Almers, Fink & Palade, 1981; Cota & Stefani, 1981; Huerta & Stefani, 1981; Gonzalez-Serratos, Valle-Aguilera, Lathrop & de Carmen Garcia, 1982; see the reviews and citations in Endo, 1977; Rasmussen, 1981 and Bianchi & Frank, 1981). One would expect drugs called Ca^{2+} antagonists (Triggle, 1981) to have some effect on excitation–contraction coupling, if Ca^{2+} movements across the surface or tubular membrane were intimately involved, or if the drugs could reach and block the release site for Ca^{2+} in the sarcoplasmic reticulum (s.r.). In fact, one might expect such drugs to block contraction in either case.

The Ca^{2+} antagonist D-600 (methoxyverapamil, a tertiary amine) has been shown to block Ca^{2+} currents in skeletal muscle (Sanchez & Stefani, 1978; Almers & Palade, 1981; Almers *et al.* 1981; Gonzalez-Serratos *et al.* 1982) with minor effects on K^+ contractures (Huerta & Stefani, 1981; Lüttgau & Spiecker, 1979) and other indices

of excitation–contraction coupling (Dörrscheidt-Käfer, 1977; Griffiths & Taylor, 1983). Ca^{2+} antagonists have more dramatic actions on cardiac muscle (see Hescheler, Pelzer, Trube & Trautwein, 1982, Pelzer, Trautwein & McDonald, 1982 and references cited there; compare slow-tonic skeletal muscle: Kaumann & Uchitel, 1976; Huerta & Stefani, 1981). Excitation–contraction coupling in cardiac and skeletal-twitch fibres apparently differs in this respect, as in several others. These differences are puzzling given the similar structures, functions, and biological roles of excitation–contraction coupling in the two kinds of muscle.

We report here that D-600, applied in a specific manner, paralyses skeletal muscle fibres with quite minor changes in the other properties we have examined. Our main observation is that a single frog twitch-fibre exposed to D-600 at 7 °C contracts only once in response to a solution rich in K^+. After the first K^+ contracture, the fibre does not contract again in response to K^+, nor does it twitch in response to electrical stimulation. The paralysed fibre gives a normal response to caffeine, suggesting that the s.r. of paralysed fibres contains and is able to release normal amounts of Ca^{2+} (see the review of Endo, 1977). After exposure to D-600 and the 'conditioning' K^+ contracture, the action potential, the resting membrane potential, and the ability of the membrane potential to respond to K^+ are quite normal. The paralytic effect of D-600 depends on temperature: treatment with D-600 at 22 °C does not produce paralysis; furthermore, paralysis, produced in the cold, disappears if the preparation is warmed to room temperature. Since D-600 blocks Ca^{2+} channels at room temperature (Almers *et al.* 1981), it seems likely that the paralytic action of D-600 is mediated by a different membrane protein.

Only a few agents are known to block excitation–contraction coupling in skeletal muscle and most of these act incompletely or have other effects (see the reviews by Sandow, 1965; Caputo, 1978). D-600 is a potent and convenient uncoupler. The resulting immobilized preparation should be useful to investigators of the non-contractile properties of skeletal muscle. Investigation of the uncoupling should provide insight into the molecular mechanisms of excitation–contraction coupling.

METHODS

All experiments measuring tension were performed on single fibres dissected from the tibialis anterior muscle of the frog *Rana temporaria*. The fibres were held to a strain gauge by clips made of aluminium foil attached to the tendons close to the ends of the single fibre. Each fibre was mounted in a bath which allowed solution changes in less than 1 s, as judged by the change of colour when dye was present in one of the solutions. Electrical measurements were made from bundles of fibres from the tibialis anterior muscle or from surface fibres of the sartorius muscle with micropipettes of resistance 18–30 MΩ when filled with 3 M-KCl. Electrical measurements from paralysed fibres did not pose special difficulties. In fact, penetrations seemed easier (i.e. the resting potential was established more abruptly) and the resting potential seemed to be more stable than in normal fibres.

Each fibre was soaked in normal Ringer solution (Table 1) for at least 1 h; twitches were elicited and several contractures (at least two, and usually three) were recorded in response to the sudden application of K^+-rich solutions: solutions 25, 40, 60 and 190 K of Table 1. The solutions were designed to have $[K^+][Cl^-]$ product close to normal, so the fibre volume, and internal concentrations of K^+ and Cl^-, remained close to normal (Hodgkin & Horowicz, 1959, 1960*a*). The 190K solution was designed to ensure complete activation of the fibre (Hodgkin & Horowicz, 1960*b*; Lüttgau & Spiecker, 1979). Only if the K^+ contractures and twitches were similar to those reported previously

with these solutions (originally, Hodgkin & Horowicz, 1960*b*; recently reviewed in Caputo, 1978, and Lüttgau & Moisescu, 1978) did the experiment proceed. Dr J. R. Lopez kindly told us that the lifespan of preparations repeatedly exposed to K^+-rich solutions seems to be increased if the K^+ solution is not present during the final phase of relaxation. Thus, the normal Ringer solution was re-applied to a fibre soon after the plateau phase of the K^+ contracture. The resulting inflexion can be seen in the relaxation phase of most of our tension records.

TABLE 1. Composition of solutions

Solution	Na^+	K^+	Ca^{2+}	Cl^-	$CH_3SO_3^-$
NR	120·2	2·5	1·8	121	0
25 K	95·2	25	1·8	12	106
40 K	80·2	40	1·8	7·5	111
60 K	60·2	60	1·8	5·0	113
100 K	20·2	100	1·8	3·6	115
190 K	0	195	1·8	3·6	190

Concentrations are given in mM to 3 significant figures. $CH_3SO_3^-$ is methane sulphonate, not to be confused with methyl sulphate. All solutions contained 0·85 mM-$H_2PO_4^-$ and 2·15 mM-HPO_4^{2-} before they were adjusted to pH 7·2 by the addition of small amounts of HPO_4^{2-}.

The Ca^{2+} antagonist D-600 is a racemic mixture of the tertiary amine methoxyverapamil (Hescheler *et al.* 1982) kindly provided by Knoll AG. Most experiments were done with a concentration of 30 μM since that was the concentration used by Almers *et al.* 1981 (caption to their Fig. 3) to block Ca^{2+} currents in a similar preparation. At a concentration of 0·5 μM the drug did not block contractions; at a concentration of 2 μM contracture tension was reduced to 10% of normal.

RESULTS

The paralysing action of D-600 is shown in Fig. 1. Experiments reported in this paper were performed between 5 and 7 °C, unless otherwise stated. After dissection and soaking in normal Ringer solution, a single fibre was stimulated, eliciting the twitch marked with a T in the figure. K^+-rich solutions (solutions 25, 60 and 190 K of Table 1) were applied, producing normal K^+ contractures in healthy fibres. The fibre was then exposed for 20 min to a normal Ringer solution containing D-600. All solutions containing D-600 had a concentration of 30 μM unless otherwise stated. A subsequent conditioning contracture, produced by solution 190 K containing D-600 appears normal in amplitude and duration, although our procedure of terminating contractures in Ringer solution obscures small changes in duration. In a few experiments with 100 μM-D-600, the conditioning contracture was clearly prolonged. D-600 decreases the Ca^{2+} current flowing inward across the T membrane, reducing the depletion of Ca^{2+} expected in the tubular lumen (Almers *et al.* 1981).

After the conditioning contracture in D-600, intra- or extracellular electrical stimulation produced no twitch or tetanic tension; no movement could be seen under the stereomicroscope. Subsequent application of 190 K solution produced no tension or movement but application of caffeine (3 mM; 7 °C) produced a normal caffeine contracture.

Fig. 1 illustrates two of the twenty-three experiments performed, all of which showed similar results. We never observed any active tension in response to stimulation or application of 190 K solution in fibres in which D-600 had been applied before and during the conditioning contracture. Fibres remained paralysed after long

soaks (up to 4 h) in normal Ringer solution, despite repeated electrical stimulation or repeated applications of 190 K solution.

Some experiments were done to investigate the role of temperature, since other workers, experimenting usually at warmer temperatures, have not reported paralysis induced by D-600. Fig. 2 shows an experiment in which the fibre was paralysed in

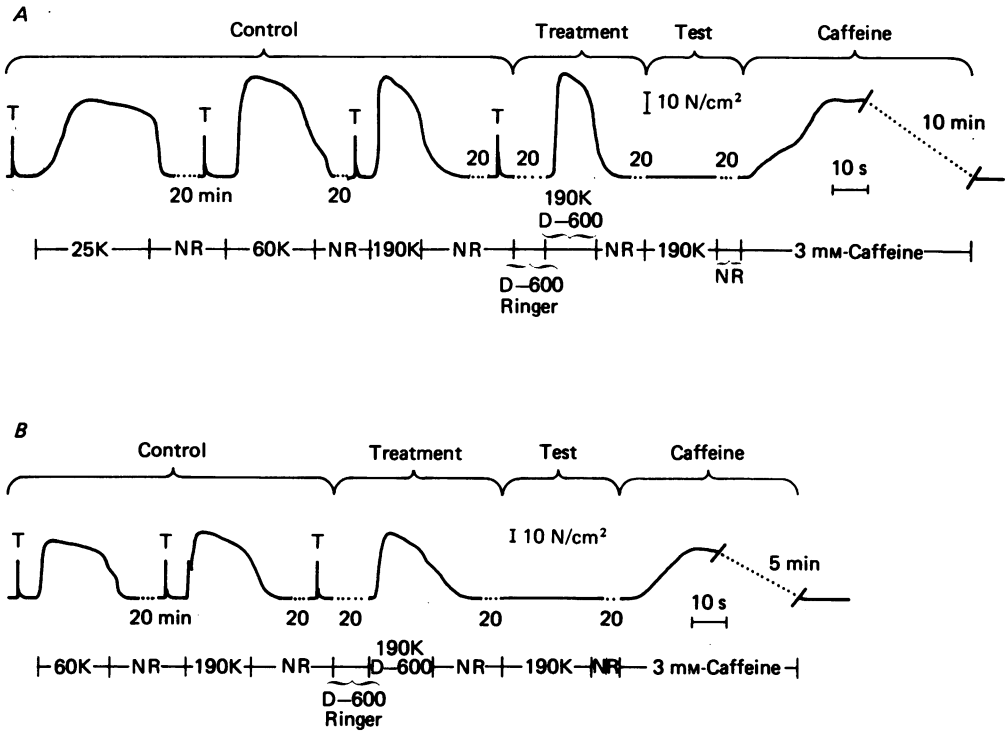


Fig. 1. The paralysing action of D-600 treatment in the cold. After a series of contractures to test the health of the fibre, 30 μM-D-600 was applied for 20 min in normal Ringer solution. A subsequent contracture, elicited by the 190 K solution containing D-600, was quite normal (see text). Subsequently, the fibre was paralysed, unable to give twitches or K^+ contractures. Application of 3 mM-caffeine produced a contracture.

the cold as previously described: the health of the fibre was tested by eliciting twitches and K^+ contractures with the 25, 60 and 190 K solutions; D-600 was applied for 20 min in Ringer solution; and a 190 K solution containing D-600 was applied in a conditioning contracture. Subsequent stimulation or application of 190 K solution did not produce movement or tension, as long as the fibre was kept cold. When the fibre was warmed to 22 °C, however, contractures of normal peak tension, but shortened duration (Lüttgau & Spiecker, 1979; Huerta & Stefani, 1981) were found. Experiments were also carried out entirely at room temperature. Fibres were soaked at 22 °C in normal Ringer solution containing D-600, exposed to a conditioning-contracture solution of 190 K plus D-600, and then tested. No paralysis was seen. Thus, the paralytic action of D-600 is temperature dependent.

D-600 was applied to the fibre in a number of other ways. Fig. 3 shows that a prolonged application of the drug (4 h) did not block a subsequent K^+ contracture.

After that contracture the fibre was paralysed. Fig. 4*B* shows that brief application of D-600, followed by a conditioning contracture, produces paralysis.

Fig. 4*A* illustrates an experiment in which the conditioning contracture was performed in the absence of D-600. The health of the fibre was tested as usual, D-600 was applied, but in this experiment the fibre was rinsed in normal Ringer solution

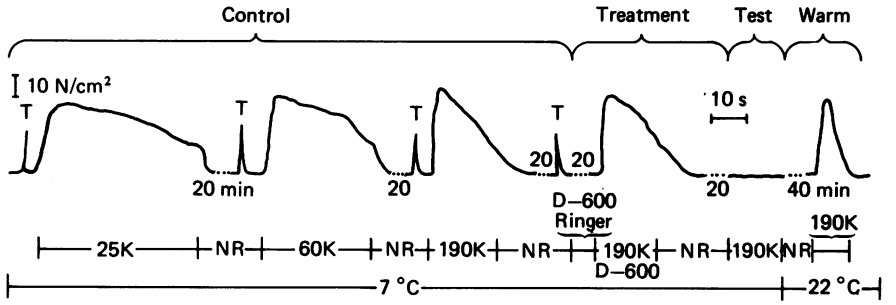


Fig. 2. The effect of temperature. After the usual series of contractures to test the health of the fibre, D-600 was applied before and during a conditioning contracture. The fibre was then warmed to 22 °C. A subsequent application of 190 K solution produced a contracture of shortened duration.

(*without* D-600) before the conditioning contracture. When 190 K solution *without* D-600 was subsequently applied, a conditioning contracture was observed, after which the fibre was paralysed.

Several durations of drug soaks and Ringer solution rinses were tried. The drug concentration was kept at 30 μM during the soak, because one might anticipate interesting but distracting complexities if a less than saturating drug concentration were used. If the drug application and rinse each lasted 20 s, the fibre showed no sign of recovery from paralysis after repeated electrical stimulation or repeated application of 190 K solution. If the drug application was 10 s and the rinse lasted 1 min, however, repeated application of 190 K solution produced recovery. Our apparatus does not permit accurate control of the duration of soak and rinse but the shortest durations, some 2–5 s, still produced paralysis although they were barely long enough to allow diffusion into the T system.

We conclude then that the paralytic action of the conditioning contracture requires only brief prior exposure of the fibre to D-600; the drug need not be present during the conditioning contracture.

A few experiments were done to determine the dependence of paralysis on the concentration of D-600. If 0·5 μM-D-600 was applied, a subsequent K^+ contracture did not paralyse the fibre. Application at a concentration of 2 μM, followed by a K^+ contracture, reduced the peak tension in a subsequent contracture to 10 % of normal.

Some fibres were stimulated while in D-600 solution before confrontation with a K^+-rich solution. Complex changes were seen in the pattern of fatique observed in twitches at 1/s and in tetani, changes which might well be produced by a use-dependent change in the conductances underlying the action potential (Griffiths & Taylor, 1983). We did not pursue these changes using the action-potential recording or voltage-clamp analysis necessary to determine the underlying mechanisms.

If D-600 drastically modified the electrical properties of muscle fibres, our results

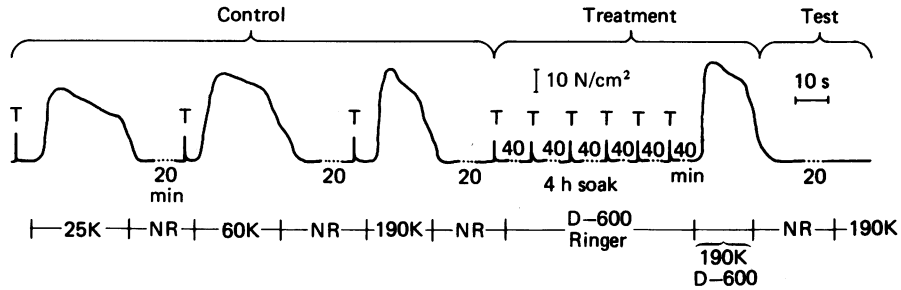
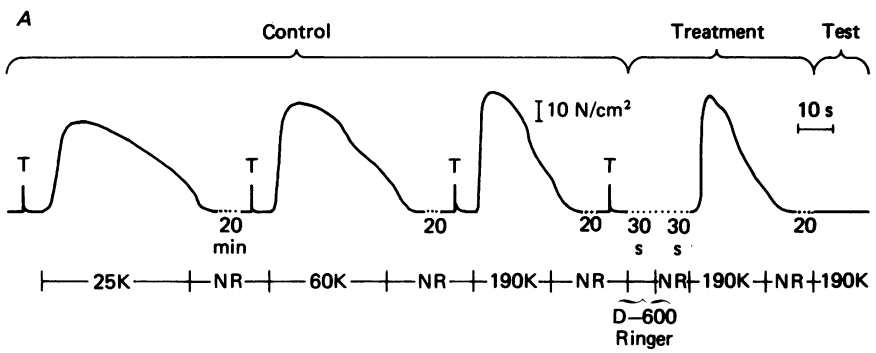


Fig. 3. Prolonged soak. After the usual series of contractures to test the health of the fibre, D-600 was applied for 4 h. The fibre gave a conditioning contracture in response to 190 K solution containing D-600; subsequently, the fibre was paralysed.



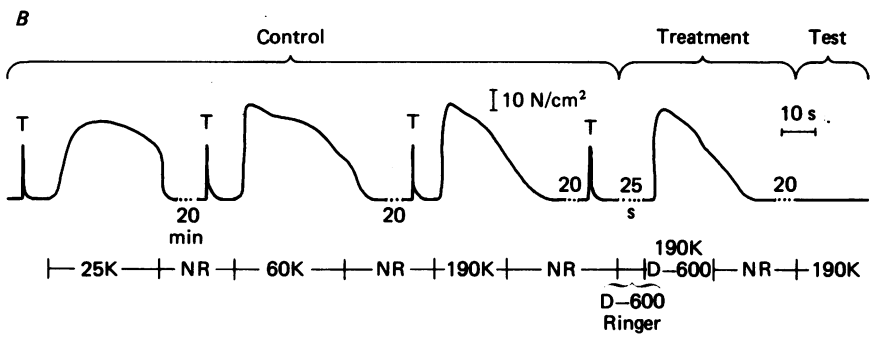


Fig. 4. *A*, conditioning contractures in the absence of D-600. After the usual set of contractures to test the health of the fibre, D-600 was applied for 30 s. The fibre was then rinsed in normal Ringer solution (NR) *without* D-600 for 30 s. A conditioning contracture, elicited by 190 K *without* D-600, produced a paralysed fibre unable to contract in response to subsequent application of 190 K. *B*, brief application of D-600. After the usual set of contractures, the fibre was exposed to D-600 for 25 s. A subsequent conditioning contracture, elicited by 190 K solution containing D-600, produced a paralysed fibre unable to contract in response to subsequent application of 190 K solution.

could be explained without a direct effect on excitation–contraction coupling. Experiments were done on bundles of fibres from the tibialis anterior to rule out the obvious possibilities. The resting potential of paralysed fibres was quite normal: ten fibres from four preparations had a resting potential of $-86{\cdot}2 \pm 1{\cdot}5$ mV (mean $\pm$ S.E. of the mean, $n = 10$) whereas nine normal fibres from the same four preparations had

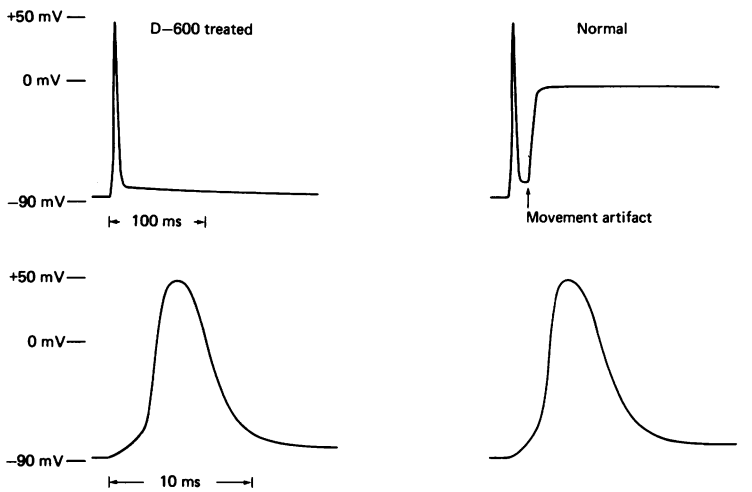


Fig 5. The action potential in normal and paralysed surface fibres of the sartorius muscle, shown at two different time scales. The twitch of the normal fibre knocked the micro-electrode out of the fibre, producing the movement artifact shown in the upper right-hand panel. The maximum rate of rise of the action potential in paralysed fibres was 130 V/s, in normal fibres 160 V/s, at 7 °C. The records were traced from digitized records.

a resting potential of $-90{\cdot}3 \pm 0{\cdot}8$ mV. Even this small depolarization is probably a result of experimental damage associated with micro-electrode penetration. Paralysed fibres, not previously penetrated in normal Ringer solution, had a resting potential of $-89{\cdot}5 \pm 0{\cdot}9$ mV. Paralysed fibres also depolarized as expected in K^+-rich solutions: thirteen fibres from three paralysed preparations bathed in 190 K solution had a resting potential of $-2{\cdot}9 \pm 1{\cdot}5$ mV.

The D-600 treatment previously described had to be changed when experiments were done on the sartorius muscle, presumably because of the thickness of the whole muscle and the consequent diffusion delays. A modified procedure produced complete paralysis of fibres in the surface layers of the sartorius, when intracellular electrical stimulation or 190 K solution was applied, as judged by observation in the microscope of muscles stretched 30 % beyond slack length. In this modified procedure, D-600 was applied in normal Ringer solution for 30 min; a conditioning contracture was then produced by a 20 s application of the 190 K solution containing D-600; afterwards, the muscle was allowed to recover for 30 min in normal Ringer solution containing D-600. The contracture and recovery solutions were applied sequentially twice more, producing a total of three conditioning contractures. After this triple conditioning, the surface layers were paralysed and the action potentials shown in Fig. 5 were

recorded from a surface fibre. As expected, this procedure did not paralyse fibres deep within the sartorius, because of the brief application of the 190 K solution.

DISCUSSION

This paper documents the paralytic action of D-600, when applied and tested in the cold, following a conditioning K^+ contracture. The paralysis is total and entirely reproducible. The resulting immobilized preparation of skeletal muscle may prove useful. The electrical properties of the paralysed fibres are quite normal. The image of paralysed fibres in the electron microscope is indistinguishable from that of normal fibres, including the qualitative appearance of the membranes of the T system, terminal cisternae, and pillars (unpublished observations, R. Milton & B. Eisenberg).

Some thoughts about the mechanism of the paralytic action are unavoidable, although detailed analysis requires investigation of the critical effect of temperature, using sophisticated techniques to measure the electrical structure (Eisenberg & Mathias, 1980), non-linear charge movement (Chandler, Rakowski & Schneider, 1976; Horowicz & Schneider, 1981; Hui, 1982), and transient change in Ca^{2+} concentration (Baylor, Chandler & Marshall, 1981; Taylor, Lopez, Griffiths, Trube & Cecchi, 1982) in the sarcoplasm of paralysed fibres. Thoughts about mechanism are constrained by our results with caffeine, which indicate that the s.r. of paralysed fibres contains and is able to release normal amounts of Ca^{2+}, and by our early results (Hui, Milton & Eisenberg, 1983) showing that non-linear charge movement is absent in paralysed fibres.

$$\text{Binding site} + \underset{\text{Bathing solution}}{\text{D-600}} \rightleftharpoons \text{D-600/binding site} \underset{\text{Warm temperature}}{\overset{K^+ \text{ Contracture}}{\rightleftharpoons}} \text{blocking site for coupling}$$

The above scheme serves as a summary of our results and the beginning of an explanation. We choose not to seek an explanation in terms of the phenomena called activation, inactivation, priming, or repriming; rather, we seek explanations in terms of specific, if hypothetical molecular mechanisms located in defined membranes (cf. Miyamoto & Racker, 1982).

We postulate a binding site for D-600 in the T membrane, a tight enough binding site so that a 20 s rinse in Ringer solution will not remove the bound drug. Binding of drug to this site does *not* prevent contraction. Rather, it allows a subsequent conditioning contracture to paralyse the fibre. Thus, we include a second site, a blocking site at which D-600 itself (or a complex of receptor and D-600) prevents excitation–contraction coupling. The first site may well be simply a lipid moiety in the T membrane, since D-600 is known to be lipid soluble and to have pharmacological actions dependent on that property (Miledi & Parker, 1980; Hescheler *et al.* 1982). But the lipid solubility of the drug does not in itself (i.e. in the absence of activation) permit access to the blocking site: a 4 h soak does not block a subsequent K^+ contracture.

It may be necessary to complicate the model further to account quantitatively for the effects of prolonged soaks and rinses in drug-containing and drug-free solutions.

It will surely be necessary to expand this model to something more comprehensive if the other phenomena of excitation–contraction coupling are to be included. The decisive effect of temperature begs for a decisive explanation, perhaps in terms of the steep temperature dependence of 'active' transport systems such as the Ca^{2+} pump or the Na^+/Ca^{2+} exchanger (see the paper of Caputo, 1972, on the steep temperature dependence of contractures in normal fibres). But the model shown on p. 502 may be able to explain the temperature effect as a simple consequence of temperature-dependent rate constants.

The location and molecular nature of the sites shown in the scheme on p. 502, if they exist at all, are significant properties of the central mechanism of excitation–contraction coupling, the mechanism coupling depolarization of the T membrane to Ca^{2+} release from the terminal cisternae (t.c.) of the s.r. For example, if D-600 acts by blocking a Ca^{2+} release site *in the t.c. membrane*, it must gain access to that site in a few seconds, either during the conditioning contracture or during the previous period of drug application. One supposes that a conductive connexion between T lumen and s.r., perhaps through the pillars connecting these structures (Somlyo, 1979; Eisenberg, Mathias & Gilai, 1979; Eisenberg & Eisenberg, 1982), would be needed to explain rapid access of D-600 to the t.c. membrane, although it is possible that the lipid solubility of the drug would allow sufficiently rapid movement through the T membrane. The electrical model of T–s.r. coupling (Mathias, Rae & Eisenberg, 1979; Mathias, Levis & Eisenberg, 1980) includes a conductive connexion between T system and s.r., as well as a Ca^{2+} release site in the t.c. membrane, which might be subject to D-600 blockade.

On the other hand, the blocking site might be a Ca^{2+} channel in the T membrane, intimately involved in excitation–contraction coupling, as postulated in the trigger Ca^{2+} model of excitation–contraction coupling (reviewed in Endo, 1977; Rasmussen, 1981; and Bianchi & Frank, 1982; see Lüttgau & Spiecker, 1979; Gonzalez-Serratos *et al.* 1982). The trigger Ca^{2+} hypothesis requires that a component of Ca^{2+} influx be the link between T membrane deplorization and Ca^{2+} release from the s.r. For example, Ca^{2+} flux through a 'contraction' channel might produce a substantial local concentration of Ca^{2+} in the space between T membrane and t.c., thus inducing pillar formation or Ca^{2+} release from the s.r. Such a contraction channel, if it exists, is *not* likely to be the Ca^{2+} channel responsible for most of the Ca^{2+} current studied by Sanchez & Stefani, 1978; Almers & Palade, 1981; Almers *et al.* 1981; and Gonzalez-Serratos *et al.* 1982. That Ca^{2+} channel is blocked by D-600 at room temperature, but a putative contraction channel, responsible for the movement of trigger Ca^{2+}, is *only* blocked by D-600 in the cold, according to our results.

Our experiments *cannot* be taken as evidence for the trigger Ca^{2+} model, however, unless a component of Ca^{2+} current (or a component of unidirectional Ca^{2+} influx) can be experimentally correlated with paralysis by D-600. The trigger component must be present in contracting fibres, but absent in paralysed fibres. Even if such a component of current or flux is found, one must explain (Miyamoto & Racker, 1981) the puzzling finding that low concentrations of extracellular Ca^{2+} block contraction in cardiac muscle, and frog slow-tonic muscle fibres, while vanishing concentrations of Ca^{2+} have negligible effects on skeletal muscle.

The blocking site in the scheme on p. 502, might be located in the T membrane and

still not be a Ca^{2+} channel of the usual sort. The site in the T membrane could be chemically similar to (or evolutionarily derived from) a Ca^{2+} channel without actually being part of a channel for current flow. In the remote control model of excitation–contraction coupling (Schneider & Chandler, 1973; Chandler, Rakowski & Schneider, 1976), the blocking site could be part of the rigid rod, linking charge movement in the T membrane to Ca^{2+} release from the terminal cisternae. In the electrical model of T–s.r. coupling (Mathias *et al.* 1979, 1980), the blocking site might control the conductance of channels linking T lumen to t.c. If the blocking site is in the T membrane, both models suggest that at least a component of non-linear charge movement (Huang, 1982; Hui, 1982) is modified in paralysed fibres. Our early results (Hui *et al.* 1983) indicate that non-linear charge movement is absent in paralysed fibres.

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