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CALCIUM CHANNELS AND INTRACELLULAR CALCIUM RELEASE ARE PHARMACOLOGICALLY DIFFERENT IN FROG SKELETAL MUSCLE

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SUMMARY

1. The pharmacology of Ca^{2+} channels and intracellular Ca^{2+} release from the sarcoplasmic reticulum (s.r.) were compared by injecting Ca^{2+} channel blockers into the cytoplasm and observing contraction under voltage clamp of frog skeletal muscle fibres, a preparation that contracts only in response to Ca^{2+} release from the s.r.

2. A method for quantifying intracellular injections by co-injecting a fluorescent dye is described.

3. Nifedipine injected into cells blocks Ca^{2+} current through the cell membrane showing that nifedipine is active when applied to the cytoplasmic side of the membrane in which Ca^{2+} channels are located.

4. Neither the presence of Ca^{2+} channel blockers in the extracellular medium nor 24 h incubation in nifedipine and D-600 affect contraction.

5. Nifedipine and D-600 injected to intracellular concentrations much greater than necessary to block Ca^{2+} channels do not affect contraction.

6. The presence of 30 μM-D-600 during K^+ contractures caused paralysis but 20 μM-nifedipine did not. Thus, contracture-dependent D-600 paralysis is not due to blockade of the transverse tubule Ca^{2+} channel.

7. It is concluded that: (a) a functioning Ca^{2+} channel on the cell membrane is not necessary to trigger Ca^{2+} release from the s.r.; (b) s.r. Ca^{2+} release and Ca^{2+} channels are pharmacologically different.

INTRODUCTION

Contraction in skeletal muscle requires the release of Ca^{2+} into the cytoplasm from an intracellular organelle, the sarcoplasmic reticulum (s.r.), in response to depolarization of the transverse tubule membrane, a system of cell membrane invaginations. The two major unanswered questions in this process are: (1) what is the nature of communication between the transverse tubules and the s.r.? and (2) what is the nature of the mechanism for Ca^{2+} release from the s.r.?

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The two known pathways for Ca^{2+} entry into the cytoplasm are from sarcoplasmic reticulum Ca^{2+} release and sarcolemmal Ca^{2+} channels located in the transverse tubules (Stanfield, 1977; Nicola-Siri, Sanchez & Stefani, 1980; Almers, Fink & Palade, 1981; Fosset, Jaimovich, Delpont & Lazdunski, 1983; Glossman, Ferry & Boschek, 1983; Curtis & Catterall, 1984). The main purpose of this study is to test whether Ca^{2+} release from the s.r. is pharmacologically similar to transverse tubule Ca^{2+} channels. A motivation for this work was the knowledge that tetracaine blocks both s.r. Ca^{2+} release (Lüttgau & Oetliker, 1968; Almers & Best, 1976; Almers, 1976) and Ca^{2+} channels (Almers *et al.* 1981).

Ca^{2+} channels in the transverse tubules are also blocked by extracellular application of nifedipine, D-600 and Ni^{2+} (Almers *et al.* 1981, Almers & McCleskey, 1984). The present study compares the effects of these blockers on transverse tubule Ca^{2+} channels and on Ca^{2+} release from the s.r. Even after intracellular injection to concentrations 100 times higher than required to block Ca^{2+} channels, the blockers failed to have any effects on excitation–contraction coupling that could be related to their known action on Ca^{2+} channels.

The results of this paper have been described in an abstract (McCleskey & Almers, 1981).

METHODS

Micro-electrode experiments

A two-micro-electrode voltage clamp was used on sartorius muscle from *Rana temporaria* frogs only for contraction experiments. The electronics are standard and described elsewhere (Almers, 1972). The electrode used for passing current was filled with 2 M-K citrate and had a resistance of 3–5 MΩ. The voltage electrode was filled with 3 M-KCl (resistance 5–20 MΩ) or, when used for intracellular injection, 0·2 M-KCl plus the injection material. Electrodes filled with 0·2 M-KCl were bevelled to reduce resistance to about 30 MΩ. Current and voltage electrodes were inserted within 50 μm of each other and 200–300 μm from the end of the cell at the pelvic tendon. The cell was not used unless the rise time of a 1 ms pulse to +100 mV was 250 μs or less. Bath temperature was held at 4–6 °C. The holding potential was −100 mV.

Contraction was observed under 400× magnification allowing the detection of movement of a few sarcomeres in the vicinity of the voltage electrode. Pulses were applied every 5 s and adjusted by the observer starting from subthreshold levels. The duration was varied for pulses to 0, 50 and 100 mV whereas the potential was varied for pulses of 10, 100 and 1000 ms durations. Threshold durations were recorded to an accuracy of 0·1 ms and threshold potentials to 1 mV. Increasing the duration or potential one or two units above threshold would generally cause a strong, unambiguous contraction except in the case of 1000 ms pulses, in which case contraction was so gradual as to be difficult to observe. Observation of contraction in the Vaseline-gap voltage clamp was more difficult both because of poorer optics and the tendency of the cell to contract as a unit rather than as a few isolated sarcomeres.

Vaseline-gap experiments

Segments of single fibres were dissected from the semitendinosus muscle of *Rana temporaria* and placed in a chamber as described by Hille & Campbell (1976). Fibres used for contraction studies were held in the end pools to prevent them from shortening. The length of cell being voltage clamped (gap width) was 300–500 μm for measuring the Ca^{2+} current (I_{Ca}) and 200 μm or less for observation of contraction.

Electronics are as described by Hille & Campbell (1976) with some modifications as described by Almers & Palade (1981). Leak and capacity currents were subtracted from the total current by an analog circuit of conventional design (Hille & Campbell, 1976). Current records were filtered at 100 Hz by a low-pass 4-pole Bessel filter and photographed from the oscilloscope screen.

Bath temperature for contraction experiments was 4–6 °C; Ca^{2+} current experiments were done at room temperature. The holding potential was −100 mV unless otherwise noted.

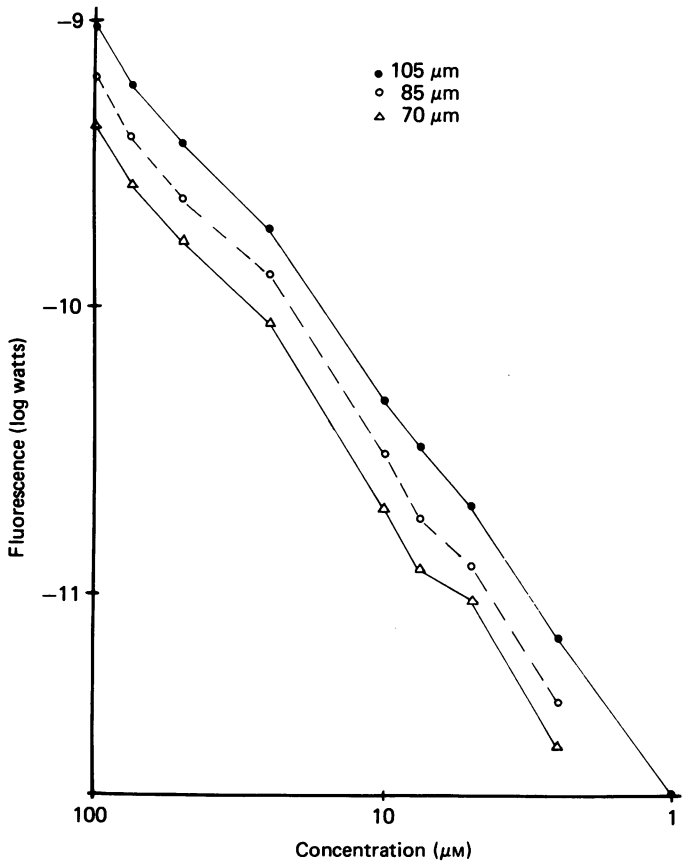

Fig. 1. Fluorescence references prepared for three different fibre diameters with a 260 μm diameter light spot. Abscissa: concentration of fluorescein in the reference pipettes. Ordinate: logarithm of fluorescent light intensity in watts 20 s after the onset of illumination. Points are averages of three readings each from a different pipette. Background fluorescence was 6×10^{-12} W. Peak-to-peak noise when filtered at 1 Hz was equivalent to $0{\cdot}6 \times 10^{-12}$ W.

Injection

Solution in the micro-electrode was pressure-injected into cells. The pressure system included a N_2 tank with a regulator, a miniature toggle valve (Clippard Instruments, Cincinnati, OH) which switched between regulator and atmospheric pressures, and an electrode holder with a pressure-injection side-arm (WPI Instruments Inc., New Haven, CT).

The standard components of the injected solution were: 200 mM-KCl, 1 mM-fluorescein, the test compound and, in some cases, ethanol. The solution for the control injection contained all but the test compound. Electrodes were generally bevelled and always filled with filtered solution immediately before use. Injection was done immediately upon entering the cell to reduce tip clogging and the injection was observed under fluorescence optics when possible. In the micro-electrode voltage-clamp experiments, the voltage electrode was used for injection. The beginning of data gathering would start no later than 5 min after the end of the injection.

Fluorescence assay of micro-injection

Intracellular concentration of the injected Ca^{2+} channel blocker was estimated by measuring the intracellular fluorescence of a co-injected dye, fluorescein. The drug concentration is calculated by

assuming the drug:dye concentration ratio in the cell to be the same as in the injecting electrode. It is necessary to use pressure injection since the technique assumes that bulk fluid from the electrode is placed in the cell.

Epifluorescence was used throughout to image a 260 μm diameter (micro-electrode experiments) or 70 μm diameter (Vaseline-gap experiments) spot of blue light onto the fibre through a dry objective (Leitz LL20 ×, numerical aperture 0·4, working distance 6 mm). The excitation beam was filtered with a Leitz KP490 band-pass filter, a Leitz TK510 dichroic mirror, and heat filters. The emission beam was filtered with the dichroic mirror and a 530–700 nm band-pass filter system. The photodiode was a HAV-4000A (E.G. & G. Inc., Salem, MA). The light source in micro-electrode experiments was a quartz halogen lamp; Vaseline-gap experiments used a 100 W mercury arc lamp (Osram HB0–100) driven with a current of 5 A.

Fig. 1 shows examples of reference curves. References are obtained by taking fluorescence readings from micropipettes with long shanks at a region of appropriate diameter (Endo, 1966). The pipette is filled with a solution containing a known fluorescein concentration, 200 mM-KCl, and 10 mM-phosphate buffer (pH 7).

The reading from a cell was usually taken at the end of an experiment, about 15–20 min after injection. Since diffusion from the injection site occurs throughout this time, the reading underestimates the fluorescein concentration present during the experiment. The possibility that lipid-soluble drugs would simply diffuse out of the cell during this time was considered and controlled for by comparing results with and without drug in the extracellular medium. It is also possible that the drugs do not diffuse after the injection because of local binding sites. In this case, the fluorescein technique underestimates the local drug concentration.

TABLE 1. End-pool solutions (all concentrations mM)

Solution	K^+	Ca^{2+}	Mg^{2+}	TEA^{+}*	Cl^-	Aspartate$^-$	EGTA	Na_2ATP	MOPS	pCa
A	—	—	—	160	—	—	80	—	5	< 9
B	110	0·069	6·5	—	13	110	0·2	0·5	10	7·2
C	97	1·4	7·4	—	18	97	5	0·5	15	7·2

* Tetraethylammonium.

Solutions

The extracellular solution for all strength–duration experiments was Ringer solution (115 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$, 3 mM-Na phosphate, pH 7·2) with 10^{-6} M-tetrodotoxin (TTX). Divalent ions were added as Cl^- salts to Ringer solution with 3 mM-morpholinopropanesulphonic acid (MOPS) substituted for the phosphate buffer. D-600 and nifedipine were added to Ringer solution containing 1 % ethanol (174 mM).

The extracellular solution for Ca^{2+} currents was 10 mM $Ca(CH_3SO_3^-)_2$, 110 mM-tetramethylammonium (TMA^+)-$CH_3SO_3^-$, 10 mM-TMA-MOPS (pH 7). The methanesulphonate ($CH_3SO_3^-$) salts were prepared by titrating $CaCO_3$ or tetramethylammonium hydroxide with methanesulphonic acid. The solution used during single fibre dissection was 75 mM-K_2SO_4, 5 mM-$CaSO_4$, 2·5 mM-MOPS at pH 7.

The end-pool solution for measuring Ca^{2+} current in the Vaseline gap was solution A, Table 1. End-pool solutions for contraction experiments in the Vaseline gap are B and C. Solutions B and C have the same calculated free Ca^{2+} and Mg^{2+} concentrations (pCa 7·2 and pMg 2·2, calculated as by Portzehl, Caldwell & Ruegg, 1964) but differ in total Ca^{2+}, total Mg^{2+}, and EGTA concentrations. All end-pool solutions were pH 7.

Nifedipine was obtained from Pfizer and Delbay Pharmaceuticals. The stock solution was 15 mM-nifedipine in ethanol. Nifedipine solutions were light-protected and experiments were done in red light. The blue light used for fluorescence observation and measurement was not used on a nifedipine-injected cell until the data had been gathered. Injection solutions contained about 50 % ethanol, and nifedipine–Ringer solution contained 150 μM total nifedipine in a 1 % ethanol solution.

D-600 was obtained from Knoll Pharmaceuticals and Dr Robert Eisenberg. The stock solution was 50 mM-D-600 in ethanol. D-600 injection solutions contained about 50 % ethanol, and D-600 Ringer solution contained 1 % ethanol.

RESULTS

Strength–duration curves as an assay for excitation–contraction coupling

In Fig. 2, depolarizing steps of varying amplitudes and durations were applied to the cell membrane, while the fibre was observed through a compound microscope to see whether or not it responded with a contraction. Each point indicates the duration of, and potential reached during, a potential step which was just sufficient to elicit a barely visible contraction. A quantitative interpretation of the resulting 'strength–duration curve' has been given (Adrian, Chandler & Hodgkin, 1969), but for the present purpose it is assumed only that each pulse producing a threshold contraction caused the cytoplasmic free Ca^{2+} concentration to rise to a value which, though unknown, is the same for each point. This has been confirmed by intracellular Ca^{2+} measurements under voltage clamp, using the Ca^{2+}-sensitive dye, Arsenazo-III (Miledi, Parker & Zhu, 1983). At negative potentials, the curve becomes nearly horizontal, defining a rheobase or steady state where the rates of Ca^{2+} release from, and of uptake by, the s.r., are equal. At more positive potentials, release during the pulse is larger than uptake. At extreme positive potentials, the curves become nearly vertical, or voltage-independent, suggesting that the voltage dependence of Ca^{2+}-release has reached saturation and release is activated maximally. It is assumed that under this condition, the rate of release is much greater than uptake, consistent with more direct measurements with Ca^{2+}-sensitive dyes (Miledi, Parker & Schalow, 1977; Kovacs, Rios & Schneider, 1979; Baylor, Chandler & Marshall, 1983). Therefore the 'minimum stimulus duration', namely the threshold duration of a pulse to 100 mV, may be taken as a measure of the rate at which the maximally activated s.r. can release Ca^{2+}. If at 100 mV, Ca^{2+} uptake is ignored and mixing is complete,

$$[Ca^{2+}]_{threshold} = (d[Ca^{2+}]/dt)\Delta t,$$

where $d[Ca^{2+}]/dt$ is the release rate, Δt is the minimum stimulus duration, and $[Ca^{2+}]_{threshold}$ is the minimum cytoplasmic Ca^{2+} concentration necessary for contraction. Thus, under these assumptions, the minimum stimulus duration is inversely proportional to the Ca^{2+} release rate at a potential that presumably leads to maximal activation of the release process. The threshold duration of a pulse to +100 mV should be taken as an approximation to the true minimum stimulus duration since the curve is generally not perfectly vertical.

It was found that the minimum stimulus duration for a cell in the Vaseline-gap voltage clamp shifted from 4·8 to 22 ms when the holding potential was changed from −100 to −63 mV. Diminishing the holding potential is expected to decrease Ca^{2+} release from the s.r. by inactivating the voltage sensor responsible for detecting transverse tubule depolarization (Hodgkin & Horowicz, 1960). The dependence of the strength–duration curve on holding potential has been demonstrated by Horowicz & Schneider (1981). A similar change in the strength–duration curve was seen with tetracaine (Almers & Best, 1976), a substance thought to block intracellular Ca^{2+} release.

Prolongation of the minimum stimulus duration was also seen when the Ca^{2+} buffering ability of the cytoplasm was increased by either direct injection of EGTA or by allowing added EGTA to diffuse up the cut ends of fibres in the Vaseline-gap

clamp. These various control experiments indicate that the minimum stimulus duration is prolonged by manipulations which prevent an increase of intracellular free Ca^{2+}. Thus, the strength–duration curve is suitable for detecting alterations in Ca^{2+} release from the s.r. when Ca^{2+} channel blockers are injected into the cytoplasm.

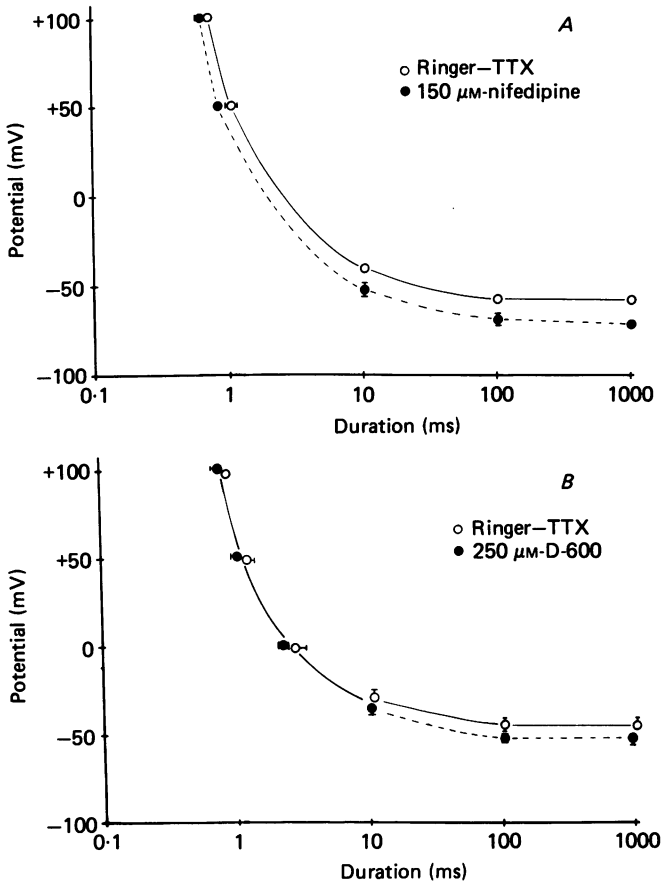

Fig. 2. Block of the transverse tubule Ca^{2+} channel does not inhibit intracellular Ca^{2+} release. Micro-electrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Open symbols: averages from fibres in TTX–Ringer solution + 1 % ethanol. Filled symbols: averages from fibres in 1 % ethanol–Ringer solution + 150 μM-nifedipine (*A*) or 250 μM-D-600 (*B*) within 90 min of application of drug. Each panel is an experiment with one sartorius muscle and points are averages from three to six fibres. Error bars indicate standard deviation when larger than the symbol. *A:* 3/6/80. *B:* 4/28/80.

Effects on contraction by Ca^{2+} channel blockers in the extracellular medium

Ca^{2+} current in skeletal muscle is 50 % blocked by 0·9 μM-nifedipine (Almers & McCleskey, 1984) and by 14 μM-D-600 (Almers, McCleskey & Palade, 1984). Fig. 2 shows strength–duration curves recorded in the absence and presence of large doses of nifedipine and D-600 in the extracellular medium using a two-micro-electrode voltage clamp on intact fibres. Each panel represents data from one sartorius muscle

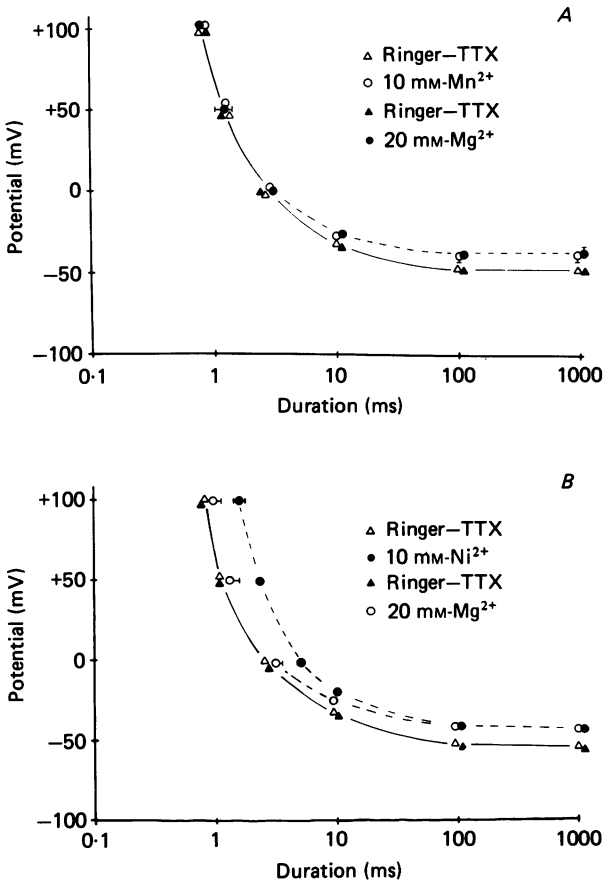

Fig. 3. Effects of inorganic Ca^{2+} channel blockers on the strength–duration curve. Micro-electrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Triangles: control measurements in Ringer solution + 10^{-6} M-TTX. Open circles: 20 mM-Mg^{2+} added to the TTX–Ringer solution. 10 mM-Mn^{2+} (*A*) or -Ni^{2+} (*B*) added to the TTX–Ringer solution. Each panel represents an experiment from one sartorius muscle and points are averages from three to seven fibres. Error bars indicate standard deviation when larger than the symbol. *A:* the order of application of solutions is: (1) Ringer solution, (2) Mn^{2+}, (3) Ringer solution, (4) Mg^{2+} (4/11/80). *B:* the order is: (1) Ringer solution, (2) Ni^{2+}, (3) Ringer solution, (4) Mg^{2+} (5/16/80).

and each point is the average from at least three cells under the same conditions. It is seen that frog muscle tolerates large doses of nifedipine and D-600 without significant effect on the minimum stimulus duration. The mechanisms which trigger Ca^{2+} release during contraction are unaffected by the presence of these Ca^{2+} channel blockers in the extracellular medium. The rheobase is decreased in the presence of either drug, as if the drugs enhanced contraction in response to long duration pulses (see also Dörrscheidt-Käfer, 1977). Since these drugs are lipid-soluble and permeate cell membranes (Bondi, 1978; Pang & Sperelakis, 1983), they may reach the s.r. and could cause the effect by inhibiting Ca^{2+} uptake into the s.r.

Ni^{2+} and Mn^{2+} block the Ca^{2+} channel (Almers *et al.* 1984) and provide a means of testing the effects of extracellular Ca^{2+} channel blockers without the possible complication of intracellular effects. Fig. 3*B* shows that 10 mM-Ni^{2+} slightly increases the minimum stimulus duration; this effect was seen in two other experiments and was significant in all three. Since this shift did not occur with nifedipine, D-600, or Mn^{2+}, it is not due to Ca^{2+} channel blockade. Since the shift is not seen with 20 mM-Mg^{2+} on this muscle or with 10 mM-Mn^{2+} on the muscle in Fig. 3*A*, it is not a non-specific effect seen with all divalent cations. Apparently, Ni^{2+} has an inhibitory effect upon Ca^{2+} release that is unrelated to its role as a Ca^{2+} channel blocker.

An increase in rheobase is seen with Ni^{2+}, Mn^{2+}, and Mg^{2+}. The effect appears to be a general property of divalent ions; it is consistent with the possibility that surface charge effects of the divalent ions require depolarization to more positive potentials to induce contraction (Lüttgau, 1963; Costantin, 1968).

Is D-600 paralysis due to Ca^{2+} channel blockade?

Frog skeletal muscle fibres at cold temperatures become paralysed after a K^+ contracture in the presence of 30 μM-D-600 (Eisenberg, McCarthy & Milton, 1983). Could active Ca^{2+} channels in the transverse tubules be necessary for excitation–contraction coupling under conditions of intense stimulation?

Fig. 4*A* shows D-600 paralysis as it is seen with measurements of contraction threshold. A strength–duration curve, labelled 1, was measured on a sartorius muscle in Ringer solution + 10^{-6} M-TTX before exposure to D-600. The muscle was then exposed to 30 μM-D-600 for 2·5 h during which time it underwent three K^+ contractures of 10 min duration (see inset). The muscle was then returned to Ringer solution + TTX. Three cells were tested within an hour after the last contracture (time 2) and none was seen to contract with pulses to +100 mV for more than 1 s. However, these cells underwent contracture when their membranes were purposely damaged with a micro-electrode, indicating that the contractile proteins still functioned. Recovery from paralysis was noticeable within 7 h after the K^+ contractures (curve 3) and nearly complete after 23 h (curve 4). In an experiment on the contralateral sartorius, D-600 was present during the recovery period. Recovery after 27 h was virtually identical with or without D-600 (with D-600 minimum stimulus duration = 3·0 ± 0·9 ms, $n = 4$), indicating that recovery cannot be due to diffusion of D-600 from a restricted space.

Fig. 4*B* shows that contracture-dependent paralysis does not occur in the presence of nifedipine. Three K^+ contractures in the presence of 20 μM-nifedipine caused no significant change in the minimum stimulus duration. A similar experiment with 20 mM-Ni^{2+} present during the K^+ contractures also failed to cause paralysis. At these concentrations, Ni^{2+} and nifedipine are expected to cause nearly complete block of the Ca^{2+} channel of the transverse tubules. It is concluded that D-600 paralysis is not caused by block of the transverse tubule Ca^{2+} channel since other blocking agents do not cause the same effect.

Two other experiments were concerned with the effect of a low-Ca^{2+} solution upon recovery from paralysis. D-600 paralysis was induced as above and the muscles were placed in a recovery solution in which the calculated free Ca^{2+} concentration was 35 μM-(Ringer solution with 1·8 mM-total Ca^{2+} and 11·1 mM-nitrilotriacetic acid buffered to pH 7 with 3 mM-MOPS). In both experiments three fibres were tested

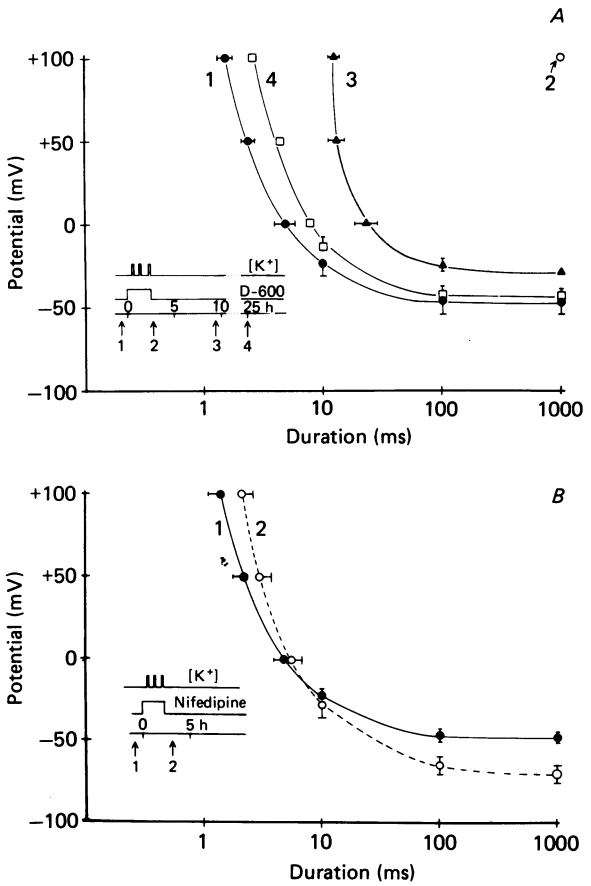

Fig. 4. Effects of K^+-contractures in the presence of 30 μM-D-600 (*A*) or 20 μM-nifedipine (*B*). Micro-electrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Insets indicate the time courses of the experiments as described in the text and numbers on curves correspond to times indicated on the insets. The K^+-contracture solution contained the drug in: 190 mM-K^+-methanesulphonate, 1·8 mM-$CaCl_2$, 3 mM-Na phosphate (pH 7·2). Temperatures were 7–9 °C for measurements and 1 °C during the recovery period. Each panel represents an experiment from one sartorius muscle and points are averages from three to seven fibres. Error bars indicate standard deviation when this is larger than the symbol. *A:* 12/6/82, muscle 1. *B:* 11/17/82, muscle 2.

after about 24 h in the low-Ca^{2+} recovery solution and none contracted in response to 1 s pulses to +100 mV. Apparently, low external Ca^{2+} concentration prevents or slows recovery from D-600 paralysis. By itself, prolonged incubation in low external Ca^{2+} does not cause paralysis. Paralysis was not seen in a muscle which underwent three K^+ contractures in the absence of D-600 before being placed in the low-Ca^{2+} recovery solution. The minimum stimulus duration was $1{\cdot}5 \pm 0{\cdot}1$ ms ($n = 3$) before the contractures, $5{\cdot}4 \pm 0{\cdot}8$ ms ($n = 3$) in 35 μM-Ca^{2+} within 75 min after the contractures, and $4{\cdot}1 \pm 2{\cdot}9$ ms ($n = 3$) after 25 h in 35 μM-Ca^{2+}. Though recovery may require extracellular Ca^{2+}, it does not require functional Ca^{2+} channels in the transverse

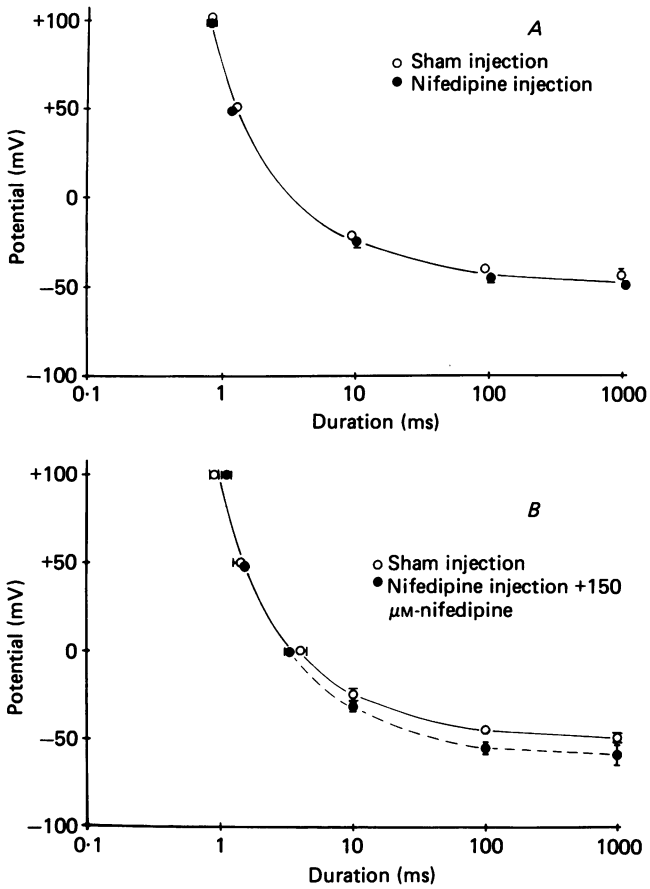

Fig. 5. Nifedipine injected into cells does not inhibit intracellular Ca^{2+} release. Microelectrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Open symbols: averages from fibres injected with carrier solution containing: 200 mM-KCl, 0·83 mM-fluorescein, 66 % ethanol. Filled symbols: averages from fibres injected with 5 mM-nifedipine in carrier solution. Each panel is an experiment from one sartorius muscle and points are averages from three to five fibres. Error bars indicate standard deviation when larger than the symbol. *A:* estimates of intracellular nifedipine concentrations are: 100, 240, and 360 μM in the three fibres tested (1/28/80). *B:* similar experiment to *A* but with 150 μM-total nifedipine in the extracellular solution during and after nifedipine injections. Estimates of intracellular nifedipine concentrations are: 60, 60, 75, 100 and 350 μM in the five fibres tested (3/14/80).

tubules, since recovery from paralysis can occur while most of these channels are blocked by nifedipine. In one muscle, the minimum stimulus duration was $1{\cdot}6 \pm 0{\cdot}3$ ms ($n = 4$) before D-600 induced complete paralysis (three fibres); substantial recovery had taken place after 25 h in Ringer solution + 20 μM-nifedipine (minimum stimulus duration $5{\cdot}4 \pm 3{\cdot}1$ ms, $n = 4$).

In summary, the experiments of the last two sections have failed to find a role for the Ca^{2+} channel of the transverse tubules in excitation–contraction coupling. An active transverse tubule Ca^{2+} channel is not necessary to trigger s.r. Ca^{2+} release, and block of the Ca^{2+} channel is not relevant to D-600 paralysis.

Effects on contraction by Ca^{2+} channel blockers in the cytoplasm

Fig. 5*A* compares strength–duration curves after injection of an ethanol carrier solution with or without 5 mM-nifedipine. If dissolved completely, the injected nifedipine would have led to intracellular concentrations ranging from 100 to 360 μM as measured by the fluorescence assay. These concentrations are more than 100 times higher than required to block half the Ca^{2+} channels in the cell membrane, yet no inhibition of contraction is evident. It is possible that, unlike the fluorescein used to quantify the injection, the nifedipine escaped across the surface membrane before it could inhibit contraction. Fig. 5*B* tests for this possibility. The injection protocol is the same as in Fig. 5*A* except that the muscle is bathed in Ringer solution saturated with nifedipine during and after the nifedipine injection. The decrease in rheobase is expected due to the presence of nifedipine in the extracellular solution (Fig. 2*A*). Evidently, the presence of high concentrations of nifedipine in the cytoplasm does not inhibit intracellular Ca^{2+} release.

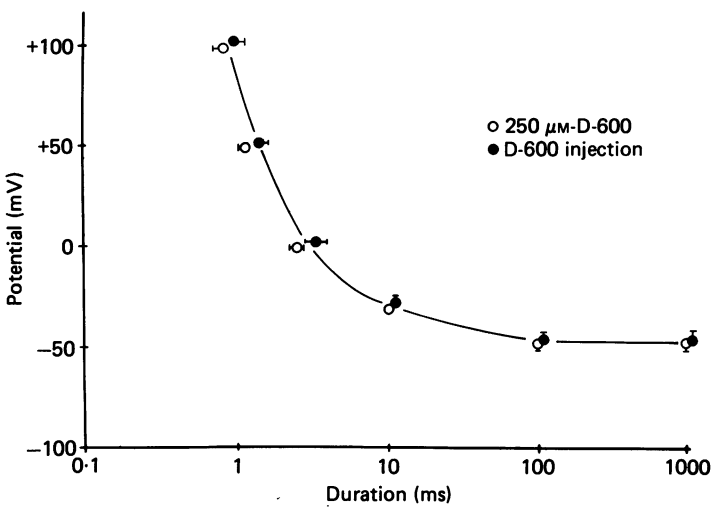

Fig. 6. D-600 injected into cells does not inhibit intracellular Ca^{2+} release. Micro-electrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Extracellular solution throughout: Ringer solution + 250 μM-D-600 + 10^{-6} M-TTX. Open symbols: averages from four fibres without injection. Filled symbol: averages from four fibres with injection of 25 mM-D-600, 1 mM-fluorescein, 200 mM-KCl in 50% ethanol. Error bars indicate standard deviation when larger than the symbol. Estimates of intracellular D-600 concentration: 425, 625, 375 and 250 μM (4/28/80).

Fig. 6 shows a similar experiment with D-600. 250 μM-D-600 is in the extracellular medium for both the control measurements and measurements following D-600 injection. Once again, no effect on the minimum stimulus duration is seen even though D-600 was injected into four cells to concentrations no less than 250 μM.

Another experiment, not shown here, concerned the effect of Ni^{2+} injection on the strength–duration curve. Injection of very high concentrations of Ni^{2+} led to

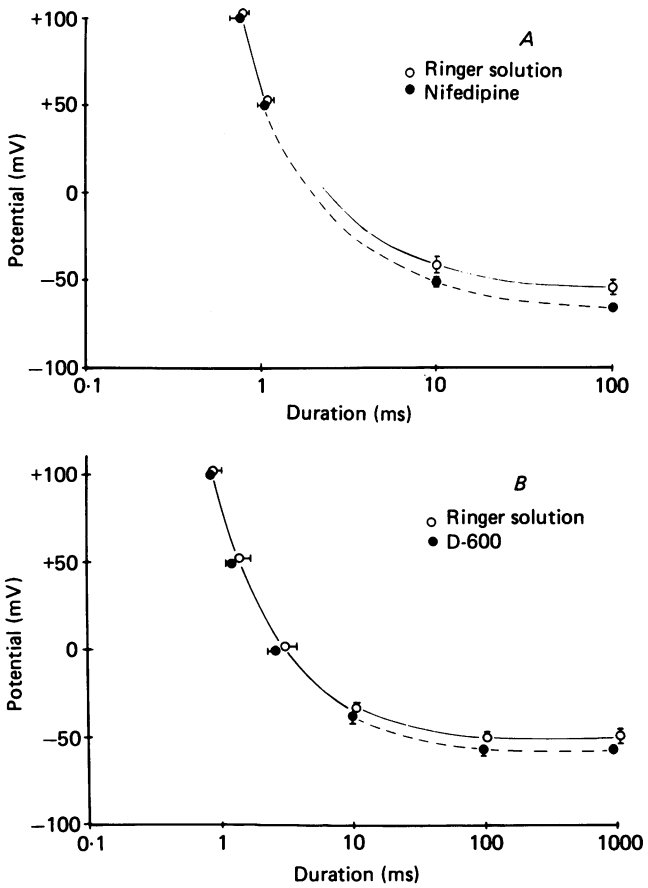

Fig. 7. 24 h incubation in 165 μM-nifedipine (*A*) or 250 μM-D-600 (*B*) does not inhibit intracellular Ca^{2+} release. Micro-electrode voltage clamp. Abscissa: duration of pulses causing threshold contraction. Ordinate: potential reached during threshold pulses. Incubations at 8 °C for 24–30 h. Experiments done in the incubation solution with 10^{-6} M-TTX added. Open symbols: averages from a sartorius incubated in Ringer solution + 0·5 % ethanol. Filled symbols: averages from the contralateral sartorius incubated in ethanol–Ringer solution + the drug. Each panel represents an experiment using contralateral sartorii from one frog; each point is an average from three to seven fibres. Error bars indicate standard deviation when larger than the symbol. *A:* 10/4/79. *B:* 4/22/80.

contractures. None the less, five cells on one muscle were successfully injected to achieve intracellular Ni^{2+} concentrations ranging from 2·5 to 9 mM, concentrations which are all above the 1 mM-external half-blockage concentrations for Ca^{2+} current (Almers *et al.* 1984). The average minimum stimulus duration for the Ni^{2+}-injected cells was $1{\cdot}2 \pm 0{\cdot}2$ ms while that for three cells injected with a similar solution not containing Ni^{2+} was $1{\cdot}1 \pm 0{\cdot}1$ ms.

Fig. 7 presents two experiments in which muscles underwent 24 h incubation in D-600 or nifedipine. Judged by results with radiolabelled verapamil and nitrendipine, this should be sufficient time for the drugs to equilibrate with the cytoplasm (Bondi, 1978; L. M. Schwarz, W. Almers & L. Bolles, personal communication). In Fig. 7*A*

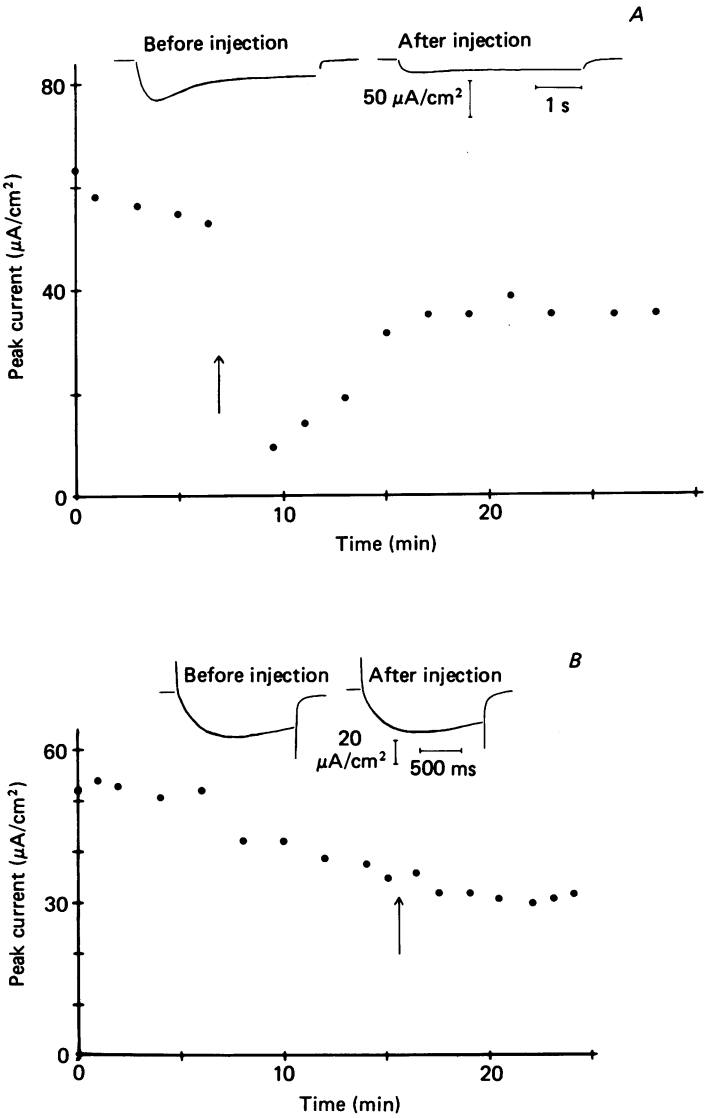

Fig. 8. *A:* nifedipine injection blocks Ca^{2+} current. Vaseline-gap voltage clamp. Ends cut in solution *A*. Vertical axis: peak Ca^{2+} current in response to a pulse to +20 mV from a holding potential of −70 mV. At the arrow, an electrode injects 7·5 mM-nifedipine, 2 mM-fluorescein, 200 mM-KCl, 50 % ethanol. The intracellular fluorescein concentration 7 min after the injection was 30 μM; the calculated nifedipine concentration at this time is then 110 μM. This would be an over-estimate if, as expected, nifedipine passes through the membrane. Inset shows Ca^{2+} currents recorded at $t = 5$ min and $t = 9·5$ min (5/21/81, fibre 2). *B:* injection of carrier solution without nifedipine. Protocol as above, except the injection electrode does not include nifedipine and pulses are to +10 mV and of shorter duration. Intracellular fluorescein concentration 4 min after the injection was 20 μM. Inset shows Ca^{2+} currents at $t = 15$ min and $t = 17·5$ min (5/2/81, fibre 3).

a sartorius muscle was incubated for more than 24 h in a Ringer solution containing 165 μM-total nifedipine while the contralateral sartorius was incubated without nifedipine. The minimum stimulus duration is the same for both muscles, and the small decrease in rheobase is expected even without long incubation (Fig. 2). A similar result is observed in Fig. 7*B*, in which contralateral sartorii were incubated with and without 250 μM-D-600. Evidently, equilibration of nifedipine and D-600 throughout a muscle does not affect Ca^{2+} release from the s.r.

Block of transverse tubule Ca^{2+} channels by micro-injection of nifedipine

Micro-injection applies Ca^{2+} channel blockers to the cytoplasmic face of the s.r. This might fail to block s.r. Ca^{2+} channels if it was required that blockers be applied to the lumen of the s.r. Fig. 8 shows that nifedipine blocks transverse tubule Ca^{2+} channels when applied to either side of the cell membrane. The insets show Ca^{2+} currents recorded from fibres in the Vaseline-gap clamp before and after micro-injection. Nifedipine injection (Fig. 8*A*) caused a decrease in current while there was no decrease in current accompanying injection of carrier solution without nifedipine (Fig. 8*B*). The graphs plot peak Ca^{2+} current against time. The arrow marks the time at which a micro-electrode enters the cell and pressure-injects solution. Block of Ca^{2+} current gradually is relieved, presumably due to nifedipine diffusion from the injection area.

Since a decline of current might be due to cell damage, injection experiments were repeated many times. Injections without drug were accomplished on four cells. The ratios of the current amplitude of the two records immediately after and before the injection ranged from 0·91 to 1·02. Nifedipine injections were done on nine cells and the ratios of current amplitude after and before injection ranged from 0·12 to 0·40. In two experiments there were several minutes between electrode penetration and successful injection; the Ca^{2+} current did not decrease until the injection occurred.

Along with other work regarding extracellular nifedipine (Almers *et al.* 1981; Almers & McCleskey, 1984), these experiments show that Ca^{2+} channel block occurs with application of nifedipine to either side of the membrane across which the Ca^{2+} current passes. However, they do not prove that the drug acts at an intracellular site. Since nifedipine is soluble in non-polar solvents and probably membrane permeant (Pang & Sperelakis, 1983), it may have permeated into the transverse tubule system and bound to an external site.

D-600 injections were accomplished with four cells. The ratios of current amplitude after and before injection ranged from 0·30 to 0·64. While there was a decrease in current following each injection, no cell survived more than 5 min after an injection. The D-600 results support analogous experiments on cardiac muscle (Hescheler, Pelzer, Trube & Trautwein, 1982) and snail neurones (Walden, Witte, Speckmann & Elger, 1984) which do indicate an intracellular site for D-600. Intracellular D-600 appears to block Ca^{2+} current.

DISCUSSION

The search for the s.r. Ca^{2+} release site

The molecule responsible for Ca^{2+} release from the s.r. has not been identified or described. Isolation and reconstitution of s.r. proteins has led to the suggestion of a Ca^{2+} ionophore which is part of the s.r. Ca^{2+} pump (Racker & Eytan, 1975) but there is no evidence that this protein is responsible for voltage-dependent Ca^{2+} release. Fusion of s.r. vesicles to lipid bilayers demonstrated the presence of a channel permeant to monovalent cations, but no Ca^{2+} permeable channel was found (Miller, 1978).

[^{3}H]nitrendipine is reported to bind to s.r.-enriched membrane fractions from rabbit skeletal muscle with a dissociation constant of about 1 nM (Fairhurst, Thayer, Colker & Beatty, 1983). A heavy s.r. fraction was distinguished from the light s.r. fraction by the presence of calsequestrin and it was found that the heavy s.r. was relatively enriched in nitrendipine binding sites but that both fractions showed specific binding. This is in conflict with two other reports. Fosset *et al.* (1983) found no binding to rabbit s.r. but found the highest concentration of nitrendipine sites reported for any tissue on rabbit and frog transverse tubule membranes. Sarmiento, Janis, Colvin, Triggle & Katz (1983) also failed to find nitrendipine binding sites on skeletal and cardiac s.r. Neither Fosset *et al.* (1983) nor Sarmiento *et al.* (1983) reported attempts to distinguish light and heavy s.r. Fairhurst *et al.* (1983) did not assay for transverse tubule markers in the s.r. preparations.

The Ca^{2+} release site of the s.r. is pharmacologically distinct from conventional Ca^{2+} channels

The purpose of the present study was to compare the pharmacology of transverse tubule Ca^{2+} channels and s.r. Ca^{2+} release in intact cells, where the s.r. receives the proper physiological stimulus for Ca^{2+} release. Micro-injection of high concentrations of nifedipine, D-600, or Ni^{2+} into the cytoplasm did not block s.r. Ca^{2+} release. One possibility was that application of Ca^{2+} channel blockers to only the cytoplasmic face of the s.r. might be insufficient to block s.r. channels. This would be expected if blockers must be applied to the high-Ca^{2+} side of the membrane. Experiments such as those in Fig. 8 showed that micro-injection of nifedipine or D-600 blocks transverse tubule Ca^{2+} channels (see also Hescheler *et al.* 1982 and Walden *et al.* 1984). Thus, nifedipine and D-600 block conventional Ca^{2+} channels when applied to either side of the membrane in which the channels are located but these drugs do not block Ca^{2+} release from the s.r. when applied to the cytoplasmic face of the s.r.

Another control (Fig. 7) showed that 24 h incubation in D-600 or nifedipine does not block s.r. Ca^{2+} release. The lipophilic Ca^{2+} channel blockers verapamil and nitrendipine equilibrate throughout sartorius muscles within 4 h (Bondi, 1978; L. M. Schwartz, W. Almers & L. Bolles, personal communication), so D-600 and nifedipine might well enter the lumen of the s.r. in a 24 h incubation.

The possibility that nitrendipine binds to s.r. membrane would not necessarily conflict with the failure of nifedipine to block Ca^{2+} release. Nitrendipine binds to areas of rat brain rich in synaptic junctions (Murphy, Gould & Snyder, 1982) while 30 μM-nifedipine does not block voltage-dependent Ca^{2+} uptake into synaptosomes

(Nachshen & Blaustein, 1979). There is a 100-fold discrepancy between binding of dihydropyridines to membrane fractions and block of Ca^{2+} current by nitrendipine in cardiac muscle (Lee & Tsien, 1983; Bellemann, Ferry, Lübbeck & Glossmann, 1981; Bolger, Gengo, Luchowski, Siegel, Triggle & Janis, 1982) and nifedipine in skeletal muscle (Almers & McCleskey, 1984; Ferry, Goll & Glossmann, 1983; Fosset *et al.* 1983). Evidently the relation between nitrendipine binding and Ca^{2+} channel block in these tissues is still unclear.

Ca^{2+} channel blockers in the extracellular medium

The possibility that current through transverse tubule Ca^{2+} channels might trigger Ca^{2+}-induced Ca^{2+} release from the s.r. (Ford & Podolsky, 1970; Endo, Tanaka & Ogawa, 1970) is unlikely since contraction persists in Ca^{2+}-free Ringer solution containing 5 mM-EGTA (Armstrong, Bezanilla & Horowicz, 1972). The theory was revived by Barrett & Barrett (1978) when they detected reversible loss of contractility in 80 mM-EGTA; they suggested that 5 mM-EGTA may have been insufficient to remove Ca^{2+} from the transverse tubules.

The present results with Ca^{2+} channel blockers in the extracellular solution allow the conclusion that the transverse tubule Ca^{2+} channel is not necessary to trigger s.r. Ca^{2+} release. The same conclusion was reached by Dörrscheidt-Käfer (1977), based upon D-600 experiments before the pharmacology of the skeletal muscle Ca^{2+} channel was known. Analogous experiments using diltiazem concentrations which were too low to cause immediate block of Ca^{2+} current have been done (Gonzales-Serratos, Valle-Aguilera, Lathrop & del Carmen Garcia, 1982).

Of four Ca^{2+} channel blockers tested, only Ni^{2+} appears to inhibit s.r. Ca^{2+} release. Ni^{2+} has been observed to slow gating in several ionic channels: the opening rate of Na^+ channels (Conti, Hille, Neumcke, Nonner & Stämpfli, 1976) and the mean open time of acetylcholine channels (Magleby & Weinstock, 1980) are increased in the presence of Ni^{2+}. Possibly, Ni^{2+} slows gating of the voltage sensor for excitation–contraction coupling. Recent work on heart muscle has shown that Ni^{2+} in the presence of TTX will suppress tension (Klitzner & Morad, 1983); since the effect was not reversed by increasing Ca^{2+} concentration, the authors suggested that the effect is independent of Ca^{2+} channel blockade.

Eisenberg *et al.* (1983) have demonstrated paralysis of frog skeletal muscle in the presence of D-600 after the muscle undergoes K^+ contractures in the cold. Related results with D-600 indicate a decrease in peak tension of successive K^+ contractures at warm temperatures (Kaumann & Uchitel, 1976) and a decrease in tetanic tension (Gallant, 1983). The present experiments test whether D-600 paralysis indicates a role for the transverse tubule Ca^{2+} channel in excitation–contraction coupling. The results showed that D-600 paralysis is not due to blockade of the transverse tubule Ca^{2+} channel since nifedipine and Ni^{2+} did not cause the same effect. A promising alternative is suggested by Hui, Milton & Eisenberg (1983) who found that charge movement (Schneider & Chandler, 1973) is eliminated during D-600 paralysis. Their finding suggests that D-600 paralysis is due to a defect in the voltage-sensing mechanism that regulates Ca^{2+} release.

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