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EFFECTS OF GALLOPAMIL ON CALCIUM RELEASE AND INTRAMEMBRANE CHARGE MOVEMENTS IN FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Intramembrane charge movements and changes in intracellular Ca^{2+} concentration were studied in voltage clamp experiments on cut twitch muscle fibres of the frog. The restoration from inactivation caused by steady depolarization and its modification by the phenylalkylamine Ca^{2+} channel antagonist gallopamil (D600, 10–30 μM) were investigated.

2. D600 prevented the restoration from inactivation of Ca^{2+} release which normally occurred at −80 mV. In D600 Ca^{2+} release recovered from inactivation at −120 mV.

3. D600 did not alter the characteristics of intramembrane charge movements in the depolarized fibre (charge 2) but the increase in the amount of mobile charge in the test voltage range above −60 mV, which normally occurs after changing the holding potential to −80 mV, was suppressed. The charge movement characteristics of D600-paralysed fibres, which were held at −80 mV, equalled those of normal depolarized and inactivated fibres.

4. Control records for the charge movement analysis were always obtained by voltage steps above 0 mV. Using the 'conventional' control in the potential range between −80 and −160 mV led to an underestimation and a kinetic deformation of charge movements in D600-treated fibres, which was due to various amounts of non-linear charge in the control.

5. Like the restoration of Ca^{2+} release at −80 mV in normal fibres the recovery from paralysis at −120 mV in D600-treated fibres was accompanied by a significant increase in mobile charge in the potential range positive of −60 mV. Both Ca^{2+} release and charge movement at test potentials above −60 mV recovered with almost identical time course.

6. Restoration of Ca^{2+} release at a holding potential of −80 mV in normal fibres or at −120 mV in D600-treated fibres could not be clearly correlated to charge movement changes in the voltage range negative of −60 mV (charge 2).

7. Our results are consistent with a voltage-dependent inhibitory effect of D600 on the charge displacement that controls Ca^{2+} release from the sarcoplasmic reticulum but provide little evidence for a conversion of charge 2 into the charge that is involved in the control of Ca^{2+} release.

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INTRODUCTION

In skeletal muscle, Ca^{2+} antagonists of the phenylalkylamine and dihydropyridine type affect not only the transmembrane Ca^{2+} current but also excitation–contraction (E–C) coupling (Berwe, Gottschalk & Lüttgau, 1987; Rios & Brum, 1987). Since E–C coupling remains functional when the Ca^{2+} influx is eliminated by other means (Lüttgau & Spiecker, 1979; Brum, Rios & Stefani, 1988*a*; Brum, Fitts, Pizarro & Rios, 1987, 1988*b*) the effect could not be explained by a simple block of inward permeation of extracellular Ca^{2+}. Present knowledge offers two possible explanations. Either Ca^{2+} antagonists affect two distinct but structurally similar types of molecules, one functioning as Ca^{2+} channel and the other as voltage sensor for E–C coupling, or the Ca^{2+} channel molecule itself plays a second role as a voltage sensor for E–C coupling (Bean, 1986; Rios & Brum, 1987; Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kojima, Matsuo, Hirose & Numa, 1987).

Particularly well documented is the effect of phenylalkylamine drugs on E–C coupling. Eisenberg, McCarthy & Milton (1983) showed that at low temperatures and micromolar concentrations of D600 (gallopamil) E–C coupling remained in a permanently refractory state ('paralysis') after a single potassium-induced contracture. Subsequently Hui, Milton & Eisenberg (1984) showed that intramembrane charge movements were inhibited in paralysed fibres. These intramembrane charge movements are believed to reflect voltage-dependent conformational changes of the voltage sensor for E–C coupling (Schneider & Chandler, 1973). D600-induced paralysis was counteracted by raising the temperature (Eisenberg *et al.* 1983), raising the extracellular calcium concentration (Siebler & Schmidt, 1987) or by hyperpolarization reflecting a shift to more negative potentials of the voltage dependence of restoration (Berwe *et al.* 1987). Voltage dependence could also be demonstrated in skinned fibre experiments, where D600 inhibited contraction at much lower concentrations when the sealed T-tubules were depolarized (Fill & Best, 1989). Likewise Caputo & Bolanos (1989) showed a stronger D600 effect on charge movements in partially depolarized fibres, compared to fibres held at -100 mV. Finally Pizarro, Brum, Fill, Fitts, Rodriguez, Uribe & Rios (1988) demonstrated a use-dependent suppression of charge movement, intracellular Ca^{2+} release, and Ca^{2+} current by repeated depolarizations (see also Fill, Fitts, Pizarro & Rios, 1987; Fill, Fitts, Pizarro, Rodriguez & Rios, 1988).

In the present paper we combined charge movement measurements with simultaneous measurements of intracellular Ca^{2+} to study the effect of D600 in view of the proposed causal relationship between the two signals and the reported effects of the drug on both charge movement and E–C coupling. We carried out measurements in the refractory state caused by prolonged depolarization, stimulated by previous indications that a charge movement component (charge 2) measurable in this condition originates from the inactivated voltage sensor (Brum & Rios, 1987) and we studied restoration from refractoriness after polarization to potentials equal to or larger than the normal resting potential. Our results gave little evidence for the proposed function of charge 2 but showed close parallels between the behaviour of a large fraction of the intramembrane charge movements and Ca^{2+} release. They are consistent with the idea that D600 prevents recovery of those charge movements that remotely control calcium release from the sarcoplasmic reticulum.

A preliminary report of part of the results has been published (Melzer & Pohl, 1987).

METHODS

Preparation

We used twitch muscle fibres of m. semitendinosus or m. ileofibularis of European grass frogs (*Rana temporaria*), which were killed by decapitation. Dissection was started after the fibres had

TABLE 1. Solutions

	Relaxing	Internal	External
K^+	138·6	—	—
Cs^+	—	130·2	10
Na^+	5	14·5	5
Ca^{2+}	—	0·008	7·3
Mg^{2+}	2	5·5	—
TEA	—	—	150
Cl^-	4	11	—
Glutamate	120	108	—
Sulphate	—	—	87·3
Tris maleate	5	17·7	5
EGTA	0·01	0·1	—
ATP	—	5	—
Glucose	—	5·6	—
APIII	—	0·8	—
TTX	—	—	0·00031

All numbers are total concentrations in mM. Abbreviations: EGTA, ethyleneglycol-bis (β-aminoethylether) *N,N,N',N'*-tetraacetic acid; APIII, Antipyrylazo III; TEA tetraethylammonium; ATP, adenosine 5'-triphosphate; TTX, tetrodotoxin.

contracted and spontaneously relaxed in a solution with a high potassium concentration (relaxing solution, Table 1). The procedures for mounting and voltage-clamping cut fibres in the double-Vaseline-gap system were essentially the same as those described by Kovacs, Rios & Schneider (1983). The segment of the fibre in the external pool (middle pool C, Kovacs *et al.* 1983), which was voltage-clamped, had a length of between 450 and 900 μm. The fibres were stretched to sarcomere lengths ranging from 3·4 to 4·7 μm to prevent movement. The chamber was screwed onto a cooling stage consisting of a circular Peltier device (800-3953, Cambion) with a tap water circuit as a heat sink. The temperature was recorded using a thermistor positioned at 12 mm from the fibre. Temperature readings were corrected for the temperature gradient between the fibre and the thermistor caused by the water-immersion microscope objective.

Solutions

The solutions were almost identical with those used previously in cut fibre experiments (Kovacs *et al.* 1983). The constituents are listed in Table 1. Sucrose (10 mM) was added to the external solution in part of the experiments to prevent a slight swelling of the fibre. The Ca^{2+} activity in the external solution was found to be 4·1 mM when measuring it with a Ca^{2+}-sensitive electrode (93-20, Orion). Racemic gallopamil (D600-HCl) was a gift from Knoll-AG (Ludwigshafen, FRG). It was added to the external solution from a 10 mM stock solution.

Optical measurements

The metallochromic indicator dye Antipyrylazo III was used for measuring myoplasmic Ca^{2+} transients. For fibre observation and optical recording we used a system based on a Zeiss ACM microscope equipped with a long working distance water-immersion objective (40/0·75 W, model 461702, Zeiss) which was electrically insulated from the microscope body by a plastic adapter. The light source was a 12 V, 100 W halogen bulb (HLX 64625, Osram), which was connected to a

stabilized power supply (JQE 15-25/M, Kepco). Transmitted light intensities of a roughly 100×50 μm area on the fibre were simultaneously measured at 700 and 850 nm after beam splitting using silicon photodiodes (UV-215 BG, EG&G). The experimental layout and the procedures of photometric recording, deriving the Antipyrylazo absorbance change and calculating changes in the free calcium concentration, were similar to those described in previous publications (Kovacs *et al.* 1983; Melzer, Rios & Schneider, 1986*a*).

For determining the rate of Ca^{2+} release using transients of free Ca^{2+} we used the general procedures described by Melzer, Rios & Schneider (1987). In part of the experiments we made use of the fact that a good approximation of the peak rate of Ca^{2+} release is $E\mathrm{d}Ca/\mathrm{d}t$ with $E = 1 + (D_T)^2/K_D + E_I$ (Melzer *et al.* 1986*a*). D_T is the indicator dye concentration at the time of the test pulse, K_D is the second-order dissociation constant of the indicator for Ca^{2+} (17500 μM^2) and E_I an empirical approximate estimate of the ratio of Ca^{2+} bound to fast equilibrating binding sites to free Ca^{2+} in the myoplasm. For E_I we used a value of 20 which is close to the average value in *R. temporaria* determined in a number of other experiments (D. Feldmeyer, W. Melzer & B. Pohl, unpublished observations).

Data acquisition

Experiments were performed on-line with a minicomputer (SMS 1000, Scientific Micro Systems Inc., equipped with LSI-11/73 processor). For pulse generation we used a 12-bit D–A converter (DT2766, Data Translation); for data sampling a 12-bit A–D converter with DMA capability (DT2782, Data Translation). Timing was controlled by a real time clock (GTSC 306, Grant Technology Systems Corp.). The voltage clamp command pulse was rounded by passing a filter stage with a 0·1 and, in some cases, a 0·6 ms time constant to reduce the size of the capacitive transient. The two light intensity signals passed 4-pole low-pass Butterworth filters (VBF/8, Kemo) set to 1 kHz corner frequency before entering the A–D converter, the current signal passed a transient subtractor stage to eliminate a large fraction of the linear capacitive transient and was filtered at 1 kHz using a passive RC circuit. Each digital record consisted of 240 sampling points (taken at intervals of 1 or 2 ms) and of fifteen additional points which contained information about pulse configuration, DC light, temperature etc., allowing an automatic analysis of the records. For each sample, the A–D converter performed a channel scan on twelve of its sixteen input channels in the DMA mode at its maximum sampling rate (125 kHz). The two light signals and the current and voltage signal were each fed to three A–D channels simultaneously (i, $i+4$, and $i+8$, with $i = 1, \ldots 4$) and the three recordings were averaged to reduce noise caused by pick-up on the transmission lines. On three further channels, temperature and the two resting light intensities at 700 and 850 nm were recorded using the average of 200 successive samples. The signals were stored on floppy discs and processed further by using home-made analysis programmes written in FORTRAN.

Charge movement analysis

In principle, we followed the procedure described by Melzer, Schneider, Simon & Szücs (1986*b*). A mean linear capacitive transient was derived from control currents obtained with 100 ms clamp pulses (1 ms sampling interval) in an appropriate voltage range (see below). Straight sloping lines were fitted to the last fifty points of the current during the pulse and during a 100-point interval after pulse off. The lines were extrapolated to the preceding pulse edge and subtracted from the records. The absolute values of the remaining 'on' and 'off' capacitive current transients were averaged after shifting the 'off' response in time so that the first point of the 'on' and the first point of the 'off' coincided. The mean linear capacitive control was subtracted after appropriate scaling from the 'on' and the 'off' response of the test current. The ionic leak current was removed by subtracting a line fitted to the end of the 'on' and 'off' response as described above for the control currents, leaving the non-linear capacitive currents. Here we assumed that fifty data points after the pulse edge were sufficient for the charge movement current to relax completely.

The slope of the baseline fitted was always very small. In a variety of cases, where the duration of the control pulses matched those of the test pulse we subtracted the scaled control current from the test current and derived the total non-linear current to verify that any steady non-linear component was small.

To determine the charge movements during the 'on' and 'off' response the non-linear capacitive current records were integrated and normalized by the apparent linear capacitance, which was derived from the control records.

For all experiments described in this paper, we chose pulses from 0 to +60 mV as controls, following the procedure described by Brum & Rios (1987). The controls were usually averages of twenty individual records and two controls bracketed a series of test pulses. Linear temporal interpolation was performed between the two bracketing control transients to account for any gradual change in membrane capacity. Alternatively, just one control was used for capacity correction of a series of test currents when they were recorded during a short period of time after or before the control. Bracketing controls were generally not employed when studying restoration of Ca^{2+} release and charge movements after setting the membrane to an appropriately negative holding potential, because in reprimed fibres there is no practically attainable voltage range where the membrane capacitance is constant. In those experiments we determined the linear capacitative transient shortly before polarizing and used this control for correcting records obtained after polarization as has been done in previous work (Brum & Rios, 1987).

We frequently used a pulse protocol to study charge movements in a voltage range remote from the actual holding potential (Brum & Rios, 1987). For this purpose we applied a pre-pulse short enough not to change the state of adaptation (inactivation, restoration) of the voltage sensor for E–C coupling to provide a new baseline for the test pulse. We applied the pre-pulse 100 ms before data acquisition started. Since the test pulse started ten data acquisition intervals after the beginning of sampling, the total length of the pre-pulse became 110 ms at a sampling interval of 1 ms and 120 ms when a sampling interval of 2 ms was used.

The validity of the measuring system was tested by applying the charge movement analysis procedure to a passive electrical circuit which simulated linear capacitance and resistances of a cut fibre in the double gap.

RESULTS

Restoration in normal fibres

In most of our experiments we studied restoration from inactivation at −80 mV where, according to Berwe *et al.* (1987), a large effect of micromolar concentrations of D600 on the degree of restoration can be expected.

Figure 1*A* shows an experiment in which charge movement and Ca^{2+} release were explored in a normal fibre by depolarizing and hyperpolarizing test pulses of 200 ms duration applied from a baseline potential of −50 mV (attained by a 120 ms pre-pulse). With the positive pulses we attempted to single out the charge movement fraction that moves in the voltage range where Ca^{2+} release occurs after recovery from inactivation since −50 mV is close to the threshold of Ca^{2+} release (see also Melzer *et al.* 1986*b*); the negative pulses were applied to detect any possible change occurring in the subthreshold voltage region which may accompany restoration of Ca^{2+} release. This was done in view of the hypothesis that charge 2 which can be moved in the negative voltage region gets converted during restoration to another charge species (charge 1) which is moved in a more positive voltage region (Brum & Rios, 1987).

When stepping to potentials more negative than about −100 mV in depolarized fibres the 'on' area of the non-linear transient current often became substantially larger than the 'off' area. This phenomenon was first described by Brum & Rios (1987) and was thoroughly investigated by Brum *et al.* (1988*b*). The areas of 'on' and 'off' became equal when a conditioning pulse to −190 mV was applied before the test pulse (Brum *et al.* 1988*b*). The effect of the pre-pulse was exclusively on the 'on' response, while the 'off' response was unchanged, indicating that 'on'–'off' inequality was presumably due to a deactivating ionic current which contaminated selectively the 'on' component. Based on these results we followed the procedure of Brum *et al.* (1988*b*) and used the 'off' areas to measure the charge movement associated with hyperpolarizing pulses.

At a holding potential of 0 mV, when Ca^{2+} release was inactivated (records not

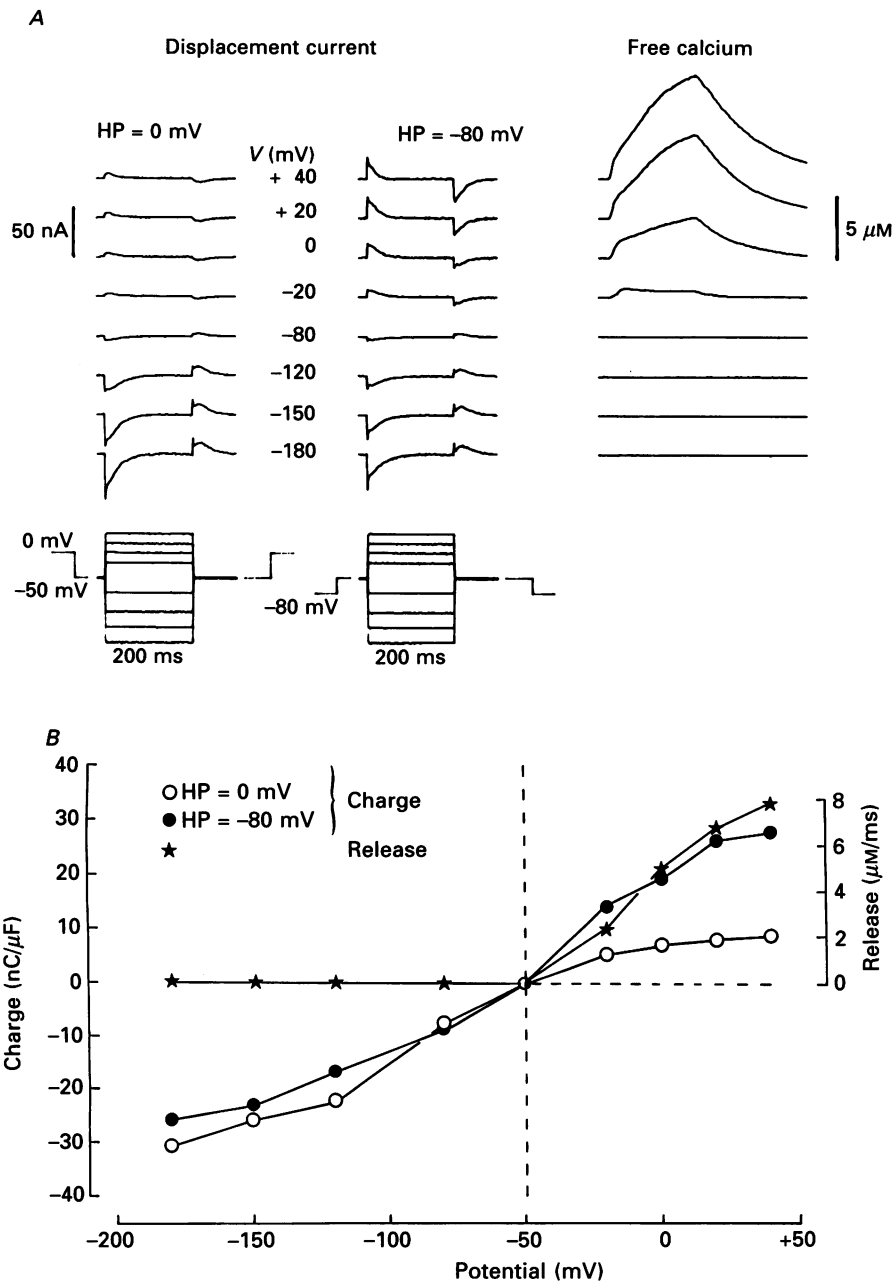


Fig. 1. Effect of polarization to −80 mV on charge movement distribution and Ca^{2+} release in a normal fibre. *A*, charge movement records (left and middle column) for two holding potentials (0 and −80 mV) and intracellular Ca^{2+} transients corresponding to the polarized condition (at 0 mV holding potential there was no change in free Ca^{2+} for any of the test pulses). Before each test pulse was applied to the potential values indicated in the figure, a pre-pulse of 120 ms duration established a baseline potential of −50 mV (see also text). Thus, at both holding potentials the test pulses to corresponding potentials had the same orientation. The records at −180 mV test potential (holding potential, 0 and

shown), most of the non-linear charge displacement took place below −50 mV (left column of Fig. 1). After polarizing the membrane for somewhat more than 25 min to −80 mV, the same test pulses were repeated. Calcium was released (right column) by the depolarizing pulses and the charge displaced by these pulses was substantially greater (middle column). The pre-pulse to −50 mV itself did not release Ca^{2+} (not shown).

The charge–voltage relations for the two holding potentials (Fig. 1*B*) were referred to the baseline potential of −50 mV to emphasize changes in the two potential intervals on either side of this point. In the depolarized state, the charge–voltage relation was steepest at around −100 mV and a total charge of about 40 nC/μF was moved over the potential range from −180 to +40 mV. This closely resembles the distribution in depolarized twitch fibres of *R. pipiens* described by Brum & Rios (1987).

Restoration of Ca^{2+} release (★, see legend of Fig. 1) by polarizing to −80 mV was accompanied by a change of the charge distribution showing a small decline of charge below −50 mV and a larger increase above the release threshold.

The charge distribution as a whole was less steep and the amount of charge that could be displaced was larger in the polarized fibre. A further quantification of the changes occurring in control fibres on polarization will be given in the context of Fig. 4.

D600 effect on charge 2

Brum & Rios (1987) suggested a two-step process of restoration from inactivation, including a fast voltage-dependent transition, followed by a slow voltage-independent transition. The fast voltage-dependent transition was attributed to the non-linear charge movements (charge 2; Adrian, Chandler & Rakowski, 1976; Brum & Rios, 1987), which are measurable in a depolarized fibre (see Fig. 1). According to this hypothesis, charge 2 provides information about the voltage-sensing element of E–C coupling while the release machinery is inactivated. If charge 2 originates from the voltage sensor, then the suppression of restoration by D600 may involve an alteration of the charge 2 characteristics. We tested this possibility in fifteen fibres where D600 was added while the membrane was held depolarized. After waiting for at least 30 min we applied test pulses of either 100 or 200 ms duration starting from the holding potential of 0 mV to increasingly negative potentials.

Charge as a function of voltage was fitted by eqn (1), which describes the potential-dependent distribution of charged particles between two positions in the membrane (Boltzmann distribution):

−80 mV) and at 0, +20, and +40 mV (holding potential, −80 mV) were single measurements, all other records were averages of two to five sweeps. *B*, charge and peak rate of Ca^{2+} release (determined using method 1 of Melzer *et al.* 1987) plotted *versus* test pulse potential. For potentials below −50 mV (negative test pulses) the 'off' charge only was used (see text), for potentials above −50 mV (positive test pulses) the average of 'on' and 'off' charge was plotted. Fibre 154, diameter 130 μm, segment length 595 μm, effective capacitance 16·8 nF, temperature 8 °C, Antipyrylazo concentration 601–637 μM, sarcomere length 4·6 μm.

$$Q = Q_{max} \frac{1}{1+\exp((\bar{V}-V)/k)}. \quad (1)$$

Here Q_{max} is the maximum amount of charge, $\bar{V}$ the voltage at which half of Q_{max} is moved, and k determines the voltage sensitivity. The mean values of these free parameters are listed in Table 2. The value of 26·1 mV for the parameter k corresponds to an apparent valency of 0·93, assuming transfer of the charge across the whole membrane thickness. The parameter values were very close to those obtained with the same pulse protocol in drug-free depolarized fibres of *Rana temporaria* ($Q_{max} = 42{\cdot}2$ nC/μF, $\bar{V} = -103{\cdot}9$ mV, $k = 22{\cdot}9$ mV, D. Feldmeyer, W. Melzer & B. Pohl, unpublished observations).

The time course of the non-linear current records did not follow a simple exponential decline but exhibited a sigmoidal decline (Fig. 2*A*) and in some cases a clear rising phase, which could be an indication of a more complex mechanism than a simple two-state transition. In addition, a rapid spike-like component became often discernible during the first one or two milliseconds in the 'on' as well as in the 'off' transient (see Fig. 2*A*). Similar fast components were reported by Caputo & Bolanos (1989) who attributed this to part of the sodium gating current. Since the fast phase contributed only a small fraction to the total time integral of the current, including it in the charge movement estimate meant introducing only a small potential error.

TABLE 2. Charge movements at 0 and −80 mV holding potential (V_H) in the presence of D600

	Q_{max} (nC/μF)	k (mV)	$\bar{V}$ (mV)	V_H (mV)
Mean ($n = 15$)	42·5	26·1	−104·8	0
(S.D.)	(7·6)	(2·3)	(6·1)	—
Mean ($n = 5$)	44·1	25·6	−101·0	−80
(S.D.)	(6·8)	(5·9)	(7·7)	—

The kinetics of the charge movements appeared to be unaltered by D600. We fitted single exponentials to the 'off' displacement currents starting 5–10 ms after the end of the pulse for steps from 0 mV to potentials between −120 and −180 mV. The average time constant was 9·4 ± 3·2 (S.D.) ms for the fifteen fibres used for Table 2, while it was 8·7 ± 3·5 (S.D.) ms in fourteen control fibres (average temperature 9·5 and 8·8 °C, respectively). Therefore D600 does not seem to affect charge 2 in depolarized fibres.

Charge movements in paralysed fibres

Berwe *et al.* (1987) showed that at 5 μM-D600, the holding potential had to be shifted by about 30 mV to more negative values to get the same degree of restoration. At −70 mV there was no restoration at all within a 10 min restoration period. In agreement with these results, we hardly found any restoration of Ca^{2+} release at −80 mV when using 10–30 μM-D600. Of twenty experiments in which the effect of D600 on restoration of Ca^{2+} release was tested, two showed a significant recovery at −80 mV, five showed only small indications of Ca^{2+} release and thirteen

exhibited no Ca^{2+} release at all. Under normal conditions restoration of Ca^{2+} release was almost complete within the first 2 min after repolarization to this potential (see also Figs 5 and 6). Thus we chose −80 mV as a convenient potential to study D600 paralysis in comparison to the changes occurring in control fibres during recovery from inactivation.

The experiment, described in Fig. 2, investigated the question whether the dipolar characteristics of the membrane changed with the polarization state of the fibre when Ca^{2+} release remained suppressed by D600.

Figure 2*A* shows a sequence of records obtained with hyperpolarizing test pulses at 0 mV holding potential about 30 min before polarization. The records of Fig. 2*B* were obtained about 20 min after polarization to −80 mV. Pre-pulses to a baseline potential of 0 mV were applied for better comparability with the currents measured prior to changing the holding potential. From the figure, it becomes evident that both amplitude and time course of the records differ little at the two different holding potentials.

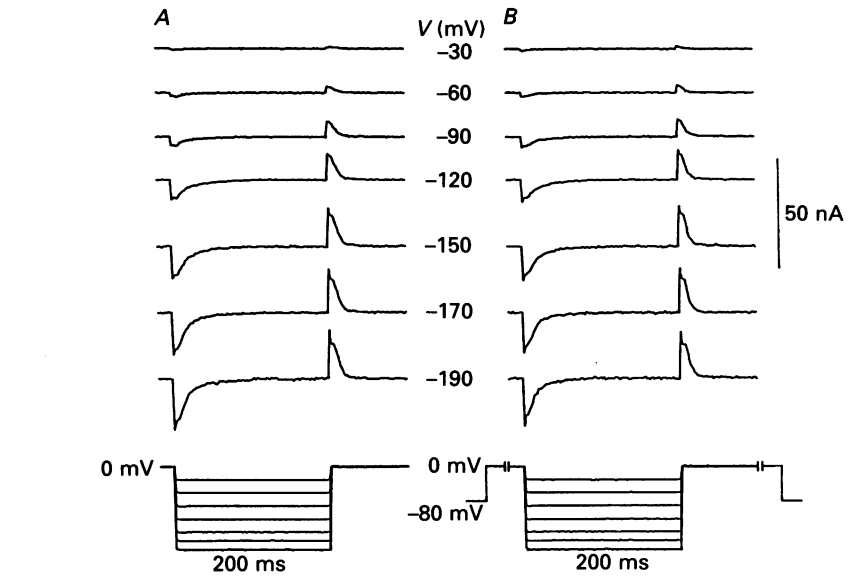
After polarizing to −80 mV and noting that there was no restoration of Ca^{2+} release we continued to apply groups of control pulses from 0 to +60 mV (on top of pre-pulses from −80 to 0 mV) at intervals of about 10 min. The control currents remained very stable over a time period of 1 h with a maximum change of 1·2% of the total capacitive charge, which corresponds to 0·7 nC/μF linear capacitance measured before polarization.

Figure 2*C* shows that the charge–voltage curves at the two holding potentials are very similar in D600. The continuous line is a fit of eqn (1) to both sets of data points (holding potential 0 and −80 mV). The best-fit parameters are given in the figure legend. Table 2 gives the mean values of the best-fit parameters for five fibres for which the charge–voltage relation was determined at −80 mV while Ca^{2+} release was completely paralysed by D600. There was no significant deviation (*t* test, 1% error level) from the corresponding parameters obtained at 0 mV holding potential (Table 2). Therefore it can be concluded that, in the paralysed state, the muscle fibre membrane has dipolar characteristics (i.e. charge 2 type of intramembrane charge movements) identical with those of a normal depolarized and inactivated fibre.

Importance of the control voltage range

Hui *et al.* (1984) carried out the first investigation of charge movements in D600-paralysed muscle fibres. They applied test pulses in the voltage range from −80 to 0 mV and reported a complete immobilization of charge. This differs from our findings. On average (using the data of Table 2) a non-linear charge of 13·5 nC/μF was moved between −80 and 0 mV in our experiments on paralysed fibres, which is 30·6% of the total non-linear charge. Hui *et al.* (1984) used the voltage range negative of −80 mV for controls where the charge–voltage relation of Fig. 2*C* shows a significant non-linear charge displacement. Non-linear charge in the control will lead to an underestimation of the total non-linear charge.

Figure 3 demonstrates this for an experiment in which the pulse procedure of Hui & Milton (1987) was applied. In this investigation, test pulses lasting 100 ms were applied from −80 mV to various more positive potentials; control pulses were applied from −160 mV and always had the same amplitude as the corresponding test



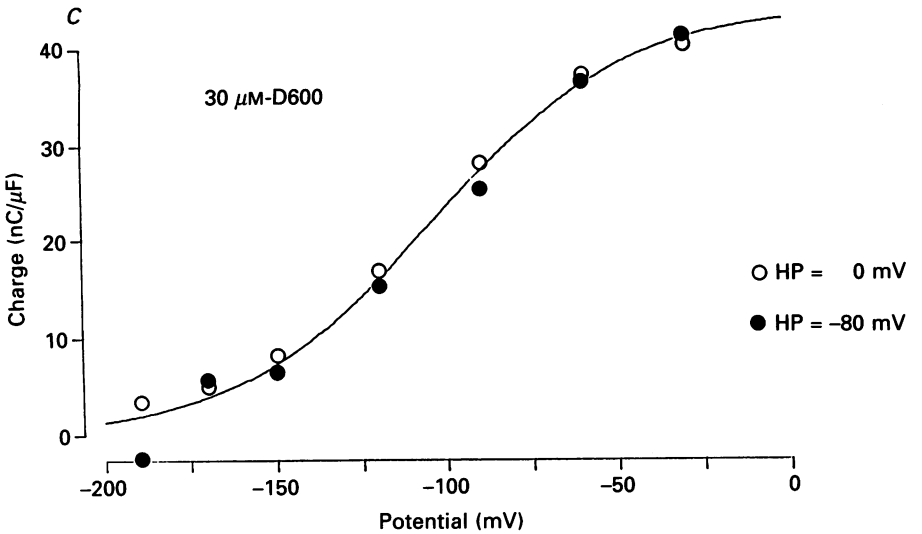


Fig. 2. Charge movements at 0 and −80 mV holding potential in the presence of 30 μM-D600. *A*, hyperpolarizing test pulses of 200 ms duration were applied to the potentials indicated in the figure. The pulses were applied 33–28 min before polarizing the fibre and more than 30 min after application of D600. *B*, same test pulses as in *A*, 18–23 min after polarization to −80 mV; 120 ms before each test pulse a pre-pulse was applied to 0 mV. Only the response to the test pulses was sampled. No Ca^{2+} transient occurred during the pre-pulse. *C*, voltage dependence of 'off' charge for 0 mV (○) and −80 mV (●) holding potential using the data of *A* and *B*. The continuous line represents the best fit to both sets of data points using eqn (1). Best-fit parameter values: Q_{max} = 43 nC/μF, k = 27·2 mV, $\bar{V}$ = −106·3 mV. Fibre 136, diameter 79 μm, segment length 645 μm, effective capacitance 6·1 nF, temperature 10 °C, sarcomere length 4·1 μm.

pulse. The records in Fig. 3*A* and *B* were the results of an analysis using the positive control (0 to +60 mV) while otherwise duplicating the pulse protocol of Hui & Milton (1987). The only difference was a pre-pulse duration of 110 ms instead of 50 ms. The records of Fig. 3*C* were obtained by subtracting the records of Fig. 3*B* from the corresponding records of equal test pulse amplitude in Fig. 3*A*. Therefore, this shows the charge movement estimates that would have been obtained if the pulses in the voltage range negative of −80 mV (Fig. 3*B*) had been used as controls. Even though we did not find complete cancellation, an underestimation of mobile charge is obvious. It is interesting to note that the charge movement record for the largest pulse obtained in this way shows transients in the opposite direction than expected from the test pulse orientation. Such 'reversed charge movements' were also described by Hui & Milton (1987, see their Fig. 6) and were explained by an outward ionic tail current of unidentified origin. Instead, we suggest that it can be explained by the fact that the non-linear charge moved by the control pulse is greater than that moved by the test pulse.

Recovery from inactivation and paralysis

In the series of experiments shown in Fig. 4 we observed the changes which occurred when fibres were transformed (by steady polarization) from either the inactivated or the paralysed state to a state where Ca^{2+} release was again possible.

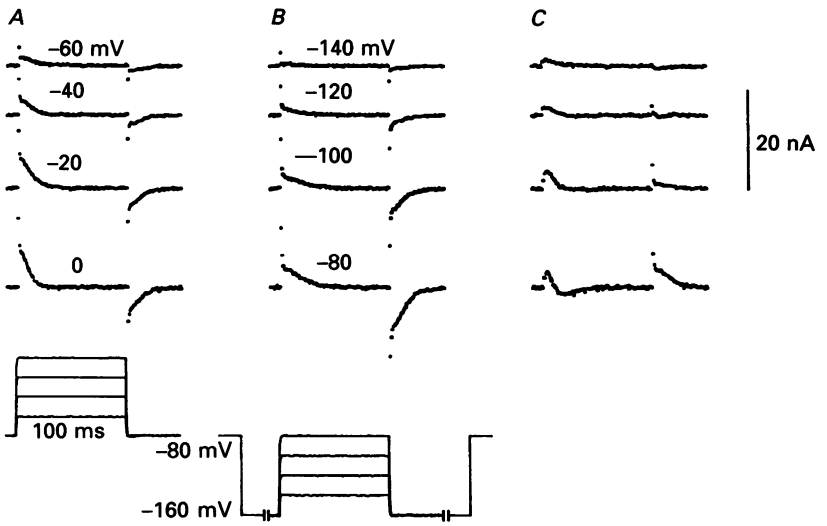


Fig. 3. Inadequacy of the 'conventional' control for charge movement analysis in D600-paralysed fibres. *A* and *B*, the records were obtained more than 96 min after application of 30 μM-D600 and 36–42 min after polarization to −80 mV. Before each test pulse in *B*, a 110 ms pre-pulse provided a baseline potential of −160 mV. The currents were analysed using bracketing 100 ms control pulses from 0 to +60 mV as described in the Methods section. *C*, these records were obtained by subtracting from each record of sequence *A* the corresponding record with equal pulse amplitude of sequence *B* following a procedure almost identical with the one described by Hui & Milton (1987). Note in this case that 'reversed charge movements' can be observed as a consequence of non-linear charge in the voltage range from −160 to −80 mV (see text). Same fibre as in Fig. 2.

To detect changes in the charge distribution as a response to steady polarization we used a pulse protocol (Fig. 4*A*) similar to the one applied by Brum & Rios (1987), spanning two different voltage regions by a positive and a negative test pulse from a common baseline voltage, which was attained by a pre-pulse (for experimental details see the figure legend).

The vertical arrows in Fig. 4*B* indicate the voltage excursions and the arrow-heads point to the average test pulse voltage. The peak voltage for the positive pulse varied in these experiments from +5 to +20 mV and for the negative pulse from −140 to −160 mV. The open columns indicate the amount of non-linear charge moved by the test pulses according to the scale on the left. The shaded columns indicate the estimates found for the peak rate of Ca^{2+} release for the positive test pulse using the approximation $E\mathrm{d}Ca/\mathrm{d}t$ (see Methods section).

Normal fibres (Fig. 4*B*, left side) which were polarized to −80 mV usually showed a substantial increase in the charge moved above −60 mV which ran parallel to a restoration of Ca^{2+} release. The charge moved by the negative test pulse declined, on average, very little (2·8 nC/μF or 11·5% of the initial value). When applying a *t* test for paired data this change of the charge in the negative voltage range was not significant at the 1% error level.

In earlier experiments, we had occasionally seen that some normal fibres released no Ca^{2+} when polarized to −100 mV (D. Feldmeyer, W. Melzer & B. Pohl, unpublished data). Among the fibres used for the present investigation there were two for which polarization to −80 mV caused no restoration of Ca^{2+} release. These fibres which were included in the average of Fig. 4*B* showed no notable change in the charge movements either. Among the seven fibres in this sequence of experiments in which we studied restoration at −80 mV in the presence of 20 and 30 μM-D600 (Fig. 4*B*, right side), there were only two which showed a barely visible trace of Ca^{2+} release within the observation time after polarization (7–80 min).

Even though practically no restoration of Ca^{2+} release occurred in D600 at −80 mV a small increase of charge (2·7 nC/μF) was notable, which is much less than the 10·1 nC/μF increase under control conditions. Hyperpolarization to −120 mV led to a recovery of Ca^{2+} release and a further increase of the charge moved by the positive test pulse by 6·9 nC/μF.

As in normal fibres the charge moved by the negative test pulse declined only slightly. The average decrease at −120 mV was 2·7 nC/μF which was not significant at the 1% level according to a *t* test.

Time dependence of restoration

Figure 4*B* illustrates the state of recovery as a consequence of polarization after a sufficiently long waiting period and represents conditions approaching a steady state. In a further series of experiments, we tried to resolve the time course of restoration from inactivation and from paralysis. For this purpose, the pulse protocols described in conjunction with Fig. 4 were applied at various times after polarization to the respective holding potential.

Figure 5 shows restoration of Ca^{2+} transients from inactivation in a normal fibre (*A*) and in one that had been exposed to 30 μM-D600 for more than 15 min at the time when the first change in holding potential was carried out (*B*). The test pulses had 100 ms duration and went to +5 mV in panel *A* and to +20 mV in panel *B*, each

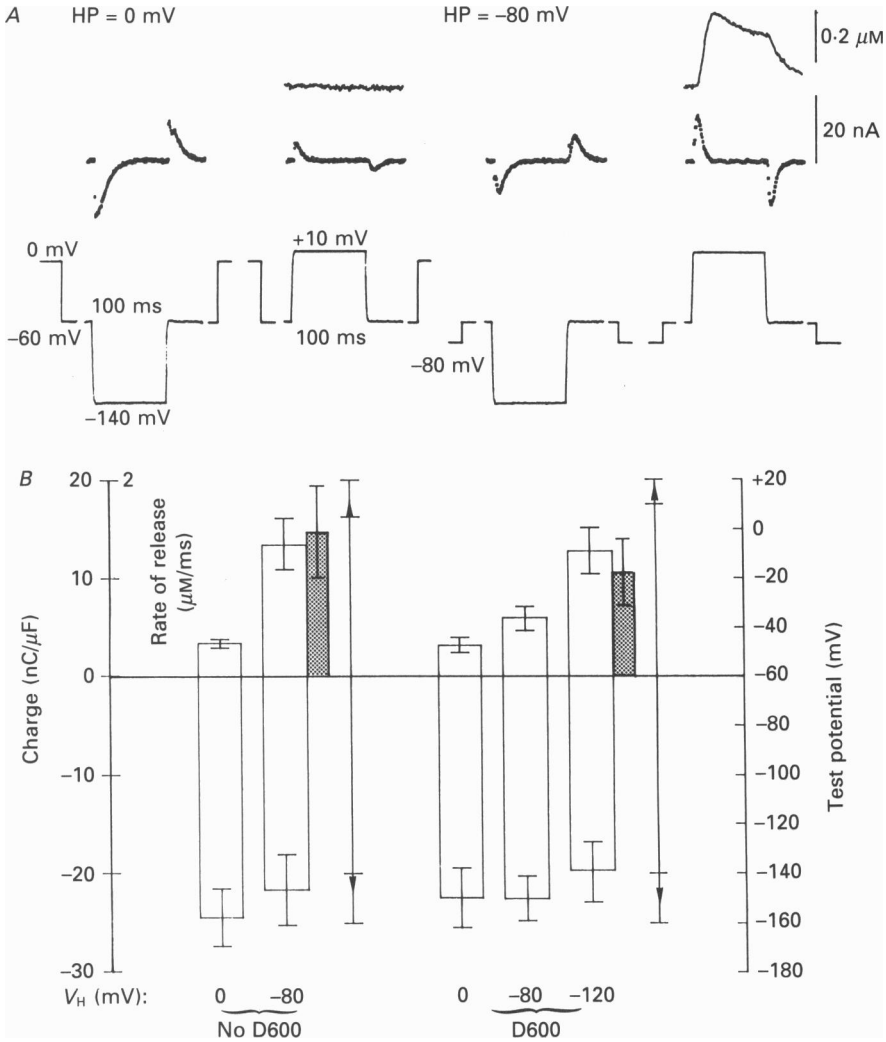


Fig. 4. Restoration of charge movement and Ca^{2+} release in normal and D600-paralysed fibres. *A*, illustration of the pulse protocol used to study charge movements in the voltage range above and below −60 mV at two different holding potentials (0 and −80 mV). The top traces show changes of free calcium concentration for the positive pulses. Each test pulse was applied from a baseline level of −60 mV obtained by a 110 ms pre-pulse. Fibre 103, diameter 103 μm, segment length 595 μm, effective capacitance 12·4 nF, temperature 8 °C, Antipyrylazo concentrations 571 and 671 mM, sarcomere length 3·8 μm. *B*, suppression of restoration of charge and Ca^{2+} release by D600. The open columns represent the average charge (scale on the left) moved by positive and negative test pulses starting from a reference potential of −60 mV at the holding potentials indicated in the figure (0, −80, −120 mV). The shaded columns indicate the mean of the peak rate of Ca^{2+} release (scale on the left). The procedure shown in *A* was used. The behaviour of nine control fibres was compared to that of seven fibres, one of which was treated with 20 and the rest with 30 μM-D600. The arrows point to the average potential (scale on the right) used in these experiments. The error bars indicate maximum and minimum pulse amplitude and the standard error of the mean (S.E.M.) of charge and rate of release respectively.

from a pre-pulse level of −60 mV. Clearly, while a rather fast recovery of the Ca^{2+} transients took place in the control fibre at −80 mV, there was no sign of recovery within the observation period of 11 min in the D600-treated fibre. On the other hand, polarization to −120 mV led to recovery but with a much slower time course than

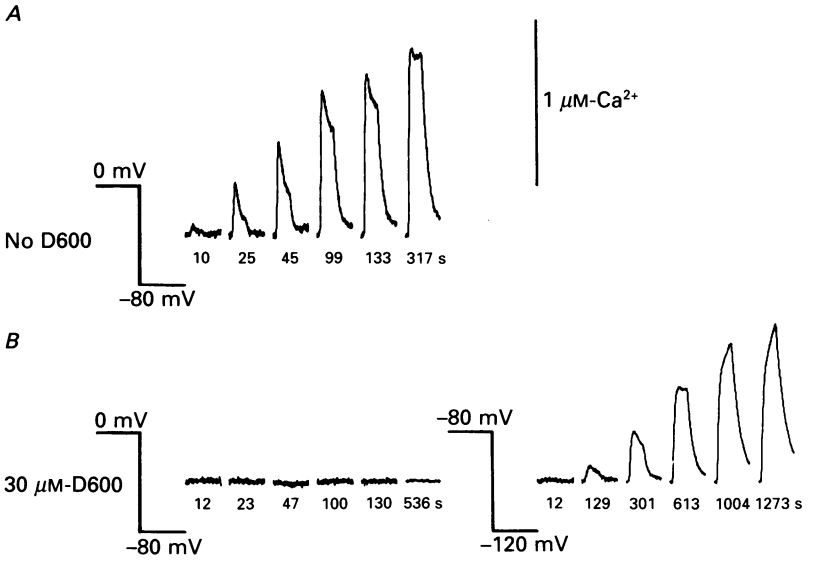


Fig. 5. Restoration of Ca^{2+} transients upon polarization and its retardation by D600. Positive test pulses (to +5 mV in *A* and to +20 mV in *B*) on top of a pre-pulse level of −60 mV (pulse protocol see Fig. 5*A*) were applied at different times after a change of the holding potential. The test pulse duration was 100 ms. *A*, normal fibre; *B*, D600-treated fibre. Same vertical scale. 30 μM-D600 had been applied in the depolarized state more than 14 min prior to polarization to −80 mV and more than 25 min before the subsequent polarization to −120 mV. Fibre 105 (*A*) and 145 (*B*), diameter 104 μm (*A*) and 100 μm (*B*), segment length 645 μm (*A* and *B*), effective capacitance 9 nF (*A*) and 7·7 nF (*B*), temperature 8·9 °C (*A*) and 8·5 °C (*B*), Antipyrylazo concentration 502–535 μM (*A*) and 534–765 μM (*B*), sarcomere length 4·4 μm (*A*) and 4·5 μm (*B*).

in the control fibre at the more positive holding potential. The half-times of restoration to an approximately steady level ranged from 10 to 80 s in normal fibres (at −80 mV) and from 80 to more than 500 s in D600-treated fibres (at −120 mV). There was no great difference in the speed of restoration from paralysis when a fibre was polarized directly to −120 mV or when it was held at −80 mV for several minutes before polarizing further to −120 mV.

The experiments which were referred to in Fig. 5 are again shown in Fig. 6. The latter shows the charge moved by the positive test pulse (●) and by a negative test pulse (■) applied at times indicated on the abscissa (for details of the pulse protocol, see the figure legend). The open circles represent the maximum derivative of the free Ca^{2+} transient as a measure of peak Ca^{2+} release (see Methods section). These values were arbitrarily scaled so that they coincided with charge movement at the last point. The bottom trace in the figure represents the changes in the holding potential which were carried out during the experiments. As long as Ca^{2+} release remained paralysed at −80 mV there was no change in the charge movements. Polarization to

−120 mV led to a progressive increase of the charge moved by the positive pulse and Ca^{2+} release recovered with a very similar time course. There was, however, no change detectable in the charge moved by the negative test pulse in this experiment. The control fibre, too (Fig. 6*A*), showed almost co-temporal restoration of Ca^{2+} release and of charge movement caused by the positive test pulse and a slight decline of charge occurred in the negative voltage region, which is consistent with the steady-state results given in Fig. 4*B*.

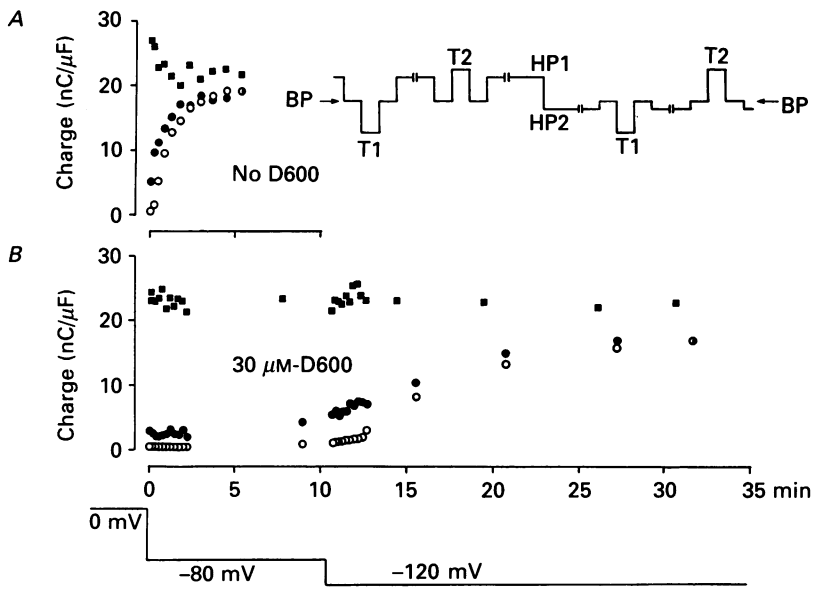


Fig. 6. Time course of charge movement restoration and recovery of Ca^{2+} release. *A*, charge moved at the 'off' for a negative test pulse to −140 mV (■, T1) and average of 'on' and 'off' charge moved by a positive pulse to +5 mV (●, T2), each starting from a baseline potential (BP) of −60 mV (see inset for pulse protocol). The holding potential (HP) was changed at 0 min from 0 mV to −80 mV. ○, peak dCa/dt for the positive pulses (T2) as a measure of the maximum rate of Ca^{2+} release. The values were arbitrarily scaled such that the last value coincided with the corresponding charge value. *B*, same procedure as in *A* applied to a fibre treated with 30 μM-D600. T1 was to −150 mV and T2 to +20 mV. Polarization to −80 mV was followed by further polarization to −120 mV after 11 min. The baseline potential (BP) of −60 mV was established by a pre-pulse prior to each test pulse. Test pulse duration was 100 ms (T1 and T2) in *A* and 200 ms (T1) and 100 ms (T2) in *B*. Same experiments as in Fig. 5.

DISCUSSION

D600 stabilizes the inactive condition

We investigated paralysis of Ca^{2+} release caused by 10–30 μM-D600 at temperatures close to 10 °C. Under these conditions we found either very little or no restoration at a holding potential of −80 mV while with a few exceptions (see below), a rather rapid restoration of Ca^{2+} transients occurred in the absence of the drug (see Fig. 5). Hyperpolarization to −120 mV led to recovery in agreement with the

reported shift of the voltage dependence of restoration to more negative potentials (Berwe *et al.* 1987). Restoration of Ca^{2+} release either in normal fibres or in paralysed fibres after hyperpolarization was always accompanied by a significant increase of mobile intramembrane charge in the voltage range positive of −60 mV. When a fibre failed to recover from inactivation this charge increase was also absent. The very similar time course of restoration of both Ca^{2+} release and charge movement indicates a close causality and supports the view that much of this charge belongs to the voltage sensor of the control mechanism for Ca^{2+} release.

The time course of restoration of both Ca^{2+} release and charge movement at −120 mV were retarded almost equally by D600 (Fig. 6*B*). When holding paralysed fibres at −80 mV we found that the membrane basically exhibits the charge movement characteristics of a normal depolarized fibre. This means that D600 stabilizes the conditions of the inactive state and provides further evidence for the view that the voltage-sensing element of E–C coupling is a target for phenylalkylamines (Berwe *et al.* 1987) and organic Ca^{2+} antagonists in general (Rios & Brum, 1987; Tanabe *et al.* 1987) and that the control mechanism for intracellular Ca^{2+} release in skeletal muscle possesses a voltage sensor which is structurally similar to the voltage-sensing domain of L-type Ca^{2+} channels (Tanabe *et al.* 1987). It is also consistent with the hypothesis that the molecule responsible for the slow Ca^{2+} current in skeletal muscle has a dual function as a voltage-dependent Ca^{2+} channel and as a voltage-sensing device for E–C coupling (see Agnew, 1987); this hypothesis has recently been strengthened by the restoration of E–C coupling and the slow Ca^{2+} current after an injection of complementary DNA encoding the dihydropyridine receptor into myotubes of dysgenic mice (Tanabe, Beam, Powell & Numa, 1988).

The conclusion that D600 stabilizes the conditions of the inactive state is in agreement with the investigation of Hui *et al.* (1984) and Hui & Milton (1987). However, there is a quantitative difference with respect to the amount of charge that can be moved in the inactive (or paralysed) condition, almost certainly resulting from the different ways of correcting for linear capacitive current transients (see Fig. 3). Charge movements determined in paralysed fibres using the positive control have the characteristics of charge 2, described by Brum & Rios (1987), with a mid-point at a rather negative potential (in our case, −101 mV). Control pulses obtained anywhere between −50 and −150 mV will therefore include significant amounts of non-linear charge, and thus lead to an underestimation of the actual charge movement and probably distort the measurement of their kinetics (see Fig. 3).

Brum & Rios (1987) suggested that charge 2 originates from a modified form of the voltage sensor, reflecting its transition between two inactive states. The model of Brum & Rios (1987) predicts – and their experimental results confirm – a simultaneous decrease of charge 2 movement (mid-point −115 mV, Brum & Rios, 1987) and increase of charge 1 movement (mid-point about −24 mV, Melzer *et al.* 1986*b*) during restoration of Ca^{2+} release at −100 mV. Charge 2 associated with the inactive voltage sensor mode was supposed to be converted on restoration to charge 1, the charge movement which is associated with activation and displaced at more positive potentials. In support of this idea, Caputo & Bolanos (1989) reported a shift of the mid-point voltage for mobile charge to more positive potentials by about 60 mV with approximate conservation of the maximum amount of charge. On the other hand

Lamb (1987) found that in mammalian skeletal muscle, the charge movements measurable below −70 mV did not change after polarization arguing against a charge 1–charge 2 interconversion. Our results are in between these two extremes.

At −80 mV holding potential (and in another series of experiments at −100 mV, D. Feldmeyer, W. Melzer & B. Pohl, unpublished observations), we occasionally found a decrease of the charge moved in the negative voltage range (Melzer & Pohl, 1987), but it was on average much smaller than the increase at more positive potentials (Figs 1 and 4*B*), which indicates that more total charge can be moved in the polarized state of the membrane than in the depolarized state and that a significant amount of non-linear charge (charge 2) remains in the negative voltage range after polarization. This observation is not compatible with a simple shift of the charge–voltage relation as a consequence of restoration and suggests that any interconversion which might occur between charge 1 and charge 2 is not 1:1.

We have no explanation for the variability in these different investigations. With the pulse programme used for the experiments of Fig. 4*B* the decrease of charge 2 might be underestimated due to possible overlap with the restoration of 'subthreshold charge' originating from transitions between resting states preceding the active state (Melzer *et al.* 1986*b*). Another possible source is the uncertainty concerning the potential distribution in the membrane area under the Vaseline seal, where a more depolarized state should prevail. This problem has been circumvented recently by using a novel four-gap chamber (Rios, Pizarro & Brum, 1989). The decrease of charge 2 on polarization to −90 mV recorded with this device was between 30 and 60% (Rios, Pizarro & Brum, 1989) which is definitely more than we found (on average, 12% after polarization to −80 mV) but which nevertheless indicates that a variable but substantial fraction of charge 2 persists.

Possible mechanisms of paralysis by D600

It has been pointed out by Berwe *et al.* (1987) that the shift to more negative potentials of the voltage dependence of force restoration caused by D600 can be explained by a binding of the drug to the voltage sensor molecule when it is in the inactive conformational state. This explanation follows a voltage-modulated receptor model which has previously been suggested for dihydropyridine binding to heart calcium channels (Bean, 1984). Recent experiments by Erdmann & Lüttgau (1989) using the pure stereoisomers of the more potent phenylalkylamine devapamil (D888) are consistent with this explanation. According to this model the potential shift $\Delta\bar{V}$ caused by a drug concentration S is

$$\Delta\bar{V} = k \ln (1 + S/K_s), \tag{2}$$

where K_s is the dissociation constant of the drug-binding reaction and k the parameter which determines the steepness when describing the voltage-dependent equilibrium between resting and inactive state by a Boltzmann distribution of the form of eqn (1) (Erdmann & Lüttgau, 1989).

Figure 5*B* indicates a $\Delta\bar{V}$ of about −40 mV in agreement with the results by Berwe *et al.* (1987). Using the k value of −6·66 mV determined by Brum *et al.* (1988) for the steady-state inactivation of charge movement and the concentration of 30 μM-D600 eqn (2) leads to a dissociation constant of 74 nM. In binding studies on cell-free

systems Goll, Ferry, Striessnig, Schober & Glossmann (1984) found values of 12 nM(−)-D600 and 55 nM(+)-D600 for the dihydrophyridine receptor of guinea-pig skeletal muscle and Reynolds, Snowman & Snyder (1986) reported a value of 22·5 nM racemic D600 for rabbit muscle.

The cyclic four-state model of Brum & Rios (1987) proposed that charge 2 moves during a first rapid restoration step following repolarization of the membrane to a sufficiently negative potential, which brings the system transiently into an intermediate inactive state. If D600 was selectively bound to only one of several inactive states, namely that favoured by a depolarized potential, this could explain the voltage-dependent retardation of restoration. Yet in this case one should expect a concentration-dependent shift of the charge–voltage relation for charge 2 to more negative potentials. In agreement with Caputo & Bolanos (1989) this was not observed in our experiments. No shift of the charge distribution would occur if D600 bound with the same affinity to both postulated inactive states (see Brum *et al.* 1988*b*). In addition, one has to assume that the drug binding does not immobilize the voltage sensor, since kinetics and maximum amount of charge were the same. The retarding drug effect on restoration would then simply originate from reducing the concentration of the substrate for the subsequent slow (and possibly voltage-independent) restoration reaction postulated by Brum & Rios (1987).

Apart from reducing the substrate for the slow restoration reaction, D600 could alternatively disturb this reaction directly. The molecular mechanism of inactivation and restoration is not known; it may involve only allosteric changes or a covalent modification, such as protein phosphorylation. It is interesting to note that even in apparently normal fibres a condition similar to D600 paralysis can sometimes be observed (see description of results in the context of Fig. 4). This might indicate that the phenylalkylamines enhance or mimic an intrinsic modification process. Given the obvious similarity of the voltage sensor of E–C coupling to L-type calcium channels, which are known to be heavily regulated in other preparations (for review see Hosey & Lazdunski, 1988), further work should be directed specifically to this question.

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