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CHARGE MOVEMENTS NEAR THE MECHANICAL THRESHOLD IN SKELETAL MUSCLE OF *RANA TEMPORARIA*

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SUMMARY

1. Charge movement was investigated over a range of potentials close to the mechanical threshold in voltage-clamped frog skeletal muscle.

2. The delayed (q_γ) component of the charging currents appeared with a time course lasting well over 100 ms at around −50 to −40 mV, but the currents became larger and faster with further depolarization.

3. The slow charging current was investigated using a 10 mV probe step intercepting the time course of these currents. This procedure showed that the charging currents could last as long as 100–300 ms.

4. The total charge was conserved when the charging current was small and prolonged.

5. The results can be related directly to earlier findings concerning contractile activation of muscle by applied voltage steps to potentials near threshold (Adrian, Chandler & Hodgkin, 1969).

INTRODUCTION

It has been suggested on a number of occasions (Adrian & Peres, 1979; Huang, 1981*a*, 1982; Hui, 1983) that the non-linear charge movement in skeletal muscle is made up of several components. One of these (q_γ) has slow and complex kinetics, especially at membrane potentials close to the mechanical threshold. A number of parallels between the behaviour of q_γ and the generation of tension have led to the suggestion that movement of q_γ is a necessary step in activation of contraction (Adrian & Peres, 1979; Huang, 1982).

The experiments described here extend the investigation of charging currents at voltages close to the mechanical threshold. They were prompted by earlier findings concerning the strength duration behaviour of contractile activation reported by Adrian, Chandler & Hodgkin (1969). In that paper it was shown that the magnitude and duration of a large potential step required to cause a contraction in a muscle fibre (poisoned with tetrodotoxin to eliminate any action potential) could be described by a model involving a first-order rate process for the redistribution of a charged activator as a result of a changed membrane potential. However, a surprising result of those experiments was that a long depolarization to within a very few millivolts of rheobase threshold appeared to have very little effect on the duration of a large second depolarization needed to produce a contraction. In terms of the hypothesis

that a finite quantity of charged activator must be moved to cause a contraction, this result suggests that near rheobase threshold the charge moved is very steeply dependent on the membrane potential but that its rate of movement is very slow. Adrian *et al.* (1969) made the suggestion that these very slow movements of activator in the potential range close to rheobase might in fact be regenerative, the result of an uptake rate inversely related to activator concentrations over a range of subthreshold activator levels.

Slow charge movements and therefore small charging currents present difficulties in measurement because of residual ionic currents. In order to estimate the transient current (and its integral, the charge) associated with a particular change in potential, it is necessary to know the value of the ionic current which flows. In general this will be easier where the potential is returned to an original level (the 'off' of a step) than where it is moved to some new level (the 'on' of a step). Particular attention has therefore been given in these experiments to 'on' 'off' equality of charge movement and to deviations from equality.

We conclude that near the mechanical threshold large transfers of charge take place at rates which make their detection difficult and that since these may last as long as 300 ms at 3–4 °C, studies using pulse durations shorter than this may well have wrongly characterized the foot of the steady-state curve of non-linear charge *versus* membrane potential (Q–V curve) (Adrian & Almers, 1976; Schneider & Chandler, 1973).

METHODS

A three-micro-electrode voltage clamp of the pelvic end of frog (*Rana temporaria*) sartorius muscle fibres employed experimental apparatus fully described elsewhere (Adrian & Rakowski, 1978). What follows is therefore only a summary of the experimental layout. Glass micro-electrodes, resistance 4–10 MΩ were inserted 375 (voltage control electrode, V_1), 750 (second voltage electrode, V_2) and 875 μm (current injection electrode, I_0) from the end of the fibre respectively. Potential recording (V_1 and V_2) electrodes were filled with 3 M-KCl, and the current injection electrode with 2 M-K citrate. The membrane current i_m to the various imposed potential steps,

$$i_m(t) = \frac{d}{6l^2 R_i}[V_2(t) - V_1(t)],$$

was examined. The linear membrane and cable constants were measured by 10 mV depolarizing steps applied from the holding potential of −90 mV. The values of the length constant λ, the internal longitudinal resistance r_i and the membrane resistance of unit length of fibre r_m were calculated from the steady values of the potentials, V_1 and V_2, and the injected current, I_0, at the end of the 10 mV step. The diameter, d, of the fibre studied and its specific membrane constants R_m and C_m were computed employing a value of the internal sarcoplasmic resistivity, R_i, of 391 Ω.cm in hypertonic solution, at 2 °C, and a Q_{10} of 1·37 (Hodgkin & Nakajima, 1972). The capacitative charge moved in response to the potential steps applied was computed as the Simpson's rule integral of the transient part of the current at the beginning and at the end of each potential step $\int[i_m(t) - g_m \Delta V_1(t)]\,dt$ (Adrian & Almers, 1974). The above computations were performed on arrays representing $V_1(t)$, $V_2(t)-V_1(t)$, and $I_0(t)$ obtained by twelve-bit analog-to-digital conversion, after filtering through three-pole Butterworth filters set at a corner frequency of 1 kHz, and sampled using a PDP 11/10E computer (Digital Equipment Corporation, Maynard, MA, U.S.A.) with a Model 502 interface (Cambridge Electronic Design, Cambridge). Four to six sweeps were averaged in each record. Repeated control sweeps in the course of the experiments checked the condition and stability of the fibre: sampling intervals varied according to the pulse procedure used, but never fell outside the Nyquist sampling limitations set by the filter.

Fibres were studied at 3–4 °C in the following solution, at neutral pH: Rb_2SO_4, 5 mM; tetraethylammonium $(TEA)_2$ sulphate, 80 mM; (TEA)Cl, 15 mM; $CaSO_4$, 8 mM; tetrodotoxin (TTX), 2×10^{-7} M; Tris buffer, 3 mM; sucrose, 350 mM. Where the pulse procedure entailed

depolarization of the fibre to beyond −30 to −20 mV, 20 mM-$CoSO_4$ was added in order to suppress transient Ca^{2+} currents, and the sucrose concentration was adjusted to maintain constant hyperosmolarity. Additionally, where some experiments required bringing the zero-current potential for the delayed rectifier close to −90 mV (the holding potential) the concentration of Rb^+ was reduced to 2·5 mM.

RESULTS

The experiments were designed to test the existence of slowly relaxing transient currents extending over hundreds of milliseconds at certain potentials: this is a period substantially longer than has been examined hitherto. The major difficulty faced therefore was the demonstration of small transient currents against background currents reflecting leak admittances, and the exclusion of the possibility that the slow changes so found reflect development of ionic (as opposed to capacitative) currents in the course of a prolonged voltage-clamp step. Each of these problems are considered with the description of the procedures that follow.

Single large pulses

In the simplest experiments, the muscle fibres were subjected to potential steps from the holding potential of −90 mV up to a series of more positive test potentials. In control records, 10 mV potential steps were applied at the holding voltage. Each record was made up by averaging five sweeps. The non-linear charging currents were determined by comparing the test transients with the control records scaled by the ratio of the test/control potential excursions (cf. Adrian & Almers, 1976). The voltage steps were of two durations: 80 ms (as used by Schneider & Chandler, 1973) or 160 ms. The charge movements to these two durations of voltage step are shown superimposed in Fig. 1. A range of potentials between −53 mV and −40 mV are displayed. The vertical arrows mark the time at which the voltage steps ended. The horizontal arrows give the value of the transient at the ends of the 'on' steps, and, at test potentials of −43 and −40 mV show that the relaxation of the charge movement is less complete at the end of the short (80 ms) pulse than at the end of the long pulse. It is at these potentials that the slow q_γ components of the charging currents were present. The difference in the extents of the decays was reflected in the smaller sizes of the 'off' tails (arrowheads) when the pulse length was short: this is what would be expected had less charge movement been achieved in the shorter step, than for the long step.

To determine the amount of charge moved in the 'on' part of the response would require a knowledge of the size of the leak current. As the charge movements at the voltages examined had decay components that had durations of the same magnitude as the pulse length, these could not be determined. However, as has been reported on earlier occasions (Adrian & Almers, 1976; Adrian & Peres, 1979), 'off' transients were always rapid, even if slow components occurred in the 'on' responses, and therefore the charging currents responding to the 'off' parts of the voltage step could be integrated to give a reliable estimate of the charge moved. Fig. 2 plots this charge for three fibres for a range of test potentials between −70 mV and −40 mV. The data for each fibre is shown separately for clarity, with the horizontal arrow in each case indicating zero net charge. When the pulse lengths were at 80 ms, the foot of the dependence of charge (circles and dashed lines) was relatively smooth, and the values

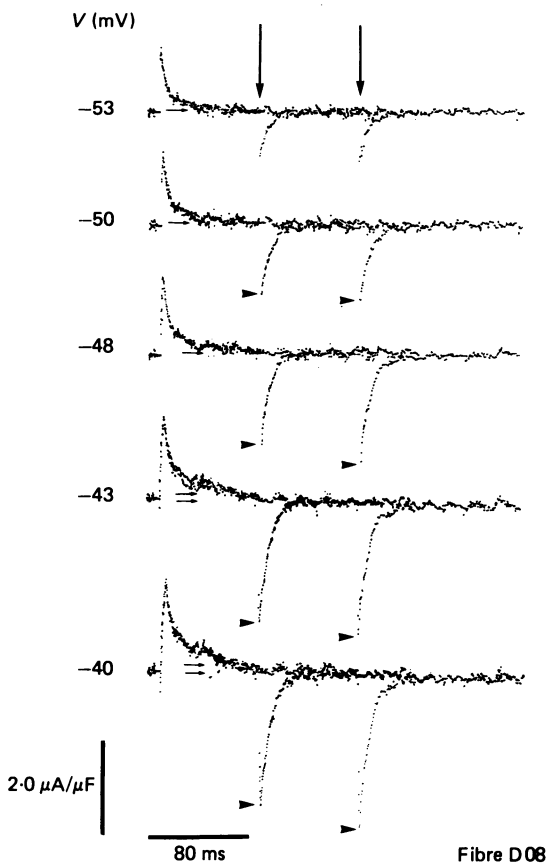

Fig. 1. Charge movements in response to potential steps of duration either 80 or 160 ms to a range of test potentials, V. The vertical arrows mark the end of the imposed step. The horizontal arrows indicate the extent to which the charging current has decayed at the end of the pulse. This differed between long and short pulses at -43 and -40 mV, and was reflected in the different sizes of the 'off' responses (arrowheads) resulting from the two kinds of pulse. Fibre cable constants: $R_i = 336\ \Omega.\text{cm}$; $\lambda = 4{\cdot}2$ mm; $r_i = 2931\ \text{k}\Omega/\text{cm}$; diam. $= 121\ \mu\text{m}$; $r_m = 516{\cdot}5\ \text{k}\Omega.\text{cm}$; $R_m = 19{\cdot}60\ \text{k}\Omega.\text{cm}^2$; $C_m = 13{\cdot}6\ \mu\text{F}/\text{cm}^2$; temp. $= 3{\cdot}9$ °C.

were in agreement with the findings of Schneider & Chandler (1973) where a similar procedure was used, although, in the present experiments, the integration of the 'off' responses was over a longer interval of 130 ms. However, consistently larger values for the charge moved were obtained if a longer pulse length was used to the same test potentials. Thus the integrals of the 'off' charge after a 160 ms long pulse (continuous lines in Fig. 2) gave a dependence of charge that rose more steeply with voltage. Points resulting from a long integration over 260 ms (squares) and a short integration of 130 ms (triangles) are shown: these values were similar. Such results would be expected if one assumed, at the foot of the non-linear Q *versus* V curve, that the charge movements have kinetics sufficiently slow for an 'on' pulse of 80 ms to be not long enough for the transients to reach a steady state. The findings obtained at the longer pulse length resemble the steeper increases with voltage in the

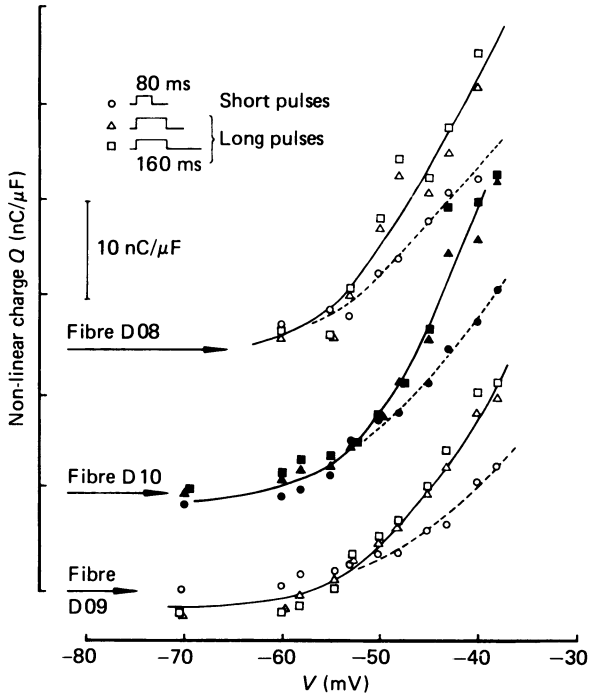

Fig. 2. The foot of the dependence of non-linear charge upon membrane potential in three muscle fibres deduced from integrating the 'off' currents in response to either the 80 ms (circles) or 160 ms (squares and triangles) long pulses. In the latter case integrations over an interval of 130 ms (triangles) or 260 ms (squares) gave similar results. The horizontal arrows denote the zero non-linear charge for each fibre. Fibre cable constants were as follows. Fibre D08: see Fig. 1 legend; fibre D09: $R_i = 340\ \Omega.\text{cm}$; $\lambda = 3{\cdot}31$ mm; $r_i = 3308\ \text{k}\Omega/\text{cm}$; diam. = 114 μm; $r_m = 362\ \text{k}\Omega.\text{cm}$; $R_m = 13{\cdot}05\ \text{k}\Omega.\text{cm}^2$; $C_m = 16{\cdot}8\ \mu\text{F}/\text{cm}^2$; temp. = 3·5 °C; fibre D10: $R_i = 339\ \Omega.\text{cm}$; $\lambda = 5{\cdot}89$ mm; $r_i = 2465\ \text{k}\Omega/\text{cm}$; diam. = 132 μm; $r_m = 857\ \text{k}\Omega.\text{cm}$; $R_m = 36{\cdot}62\ \text{k}\Omega.\text{cm}^2$; $C_m = 28{\cdot}1\ \mu\text{F}/\text{cm}^2$; temp. = 3·6 °C.

charge–voltage curve reported by Adrian & Almers (1976) who used pulse lengths of 120 ms. These experiments therefore resolve the apparent discrepancy between these two papers.

The above effects occurred only in the −50 to −40 mV potential range; stronger depolarizations resulted in charging currents with much more rapid kinetics. Fig. 3 shows one such fibre that was subjected to voltage steps of varying duration from −90 mV to −20 mV. The charge movements obtained are shown at high gain in Fig. 3*B*; their 'off' tails appeared to be similar in size at all the pulse lengths tested, which was between 60 and 130 ms. The total non-linear charge obtained from integrating the 'off' responses was constant for all the potential steps tested (Fig. 3*C*). Admittedly, at these potentials, appreciable delayed outward K^+ current occurred in the transient and gradually inactivated with successive sweeps (Fig. 3*B*). However, any contribution that 'tail' currents might make to the 'off' transients was minimized by reducing the bath Rb^+ to 2·5 mM, thereby bringing the membrane reversal potential to close to the −90 mV holding potential. Additionally any small Ca^{2+} current that might occur was reduced by adding 20 mM-Co^{2+}, with the

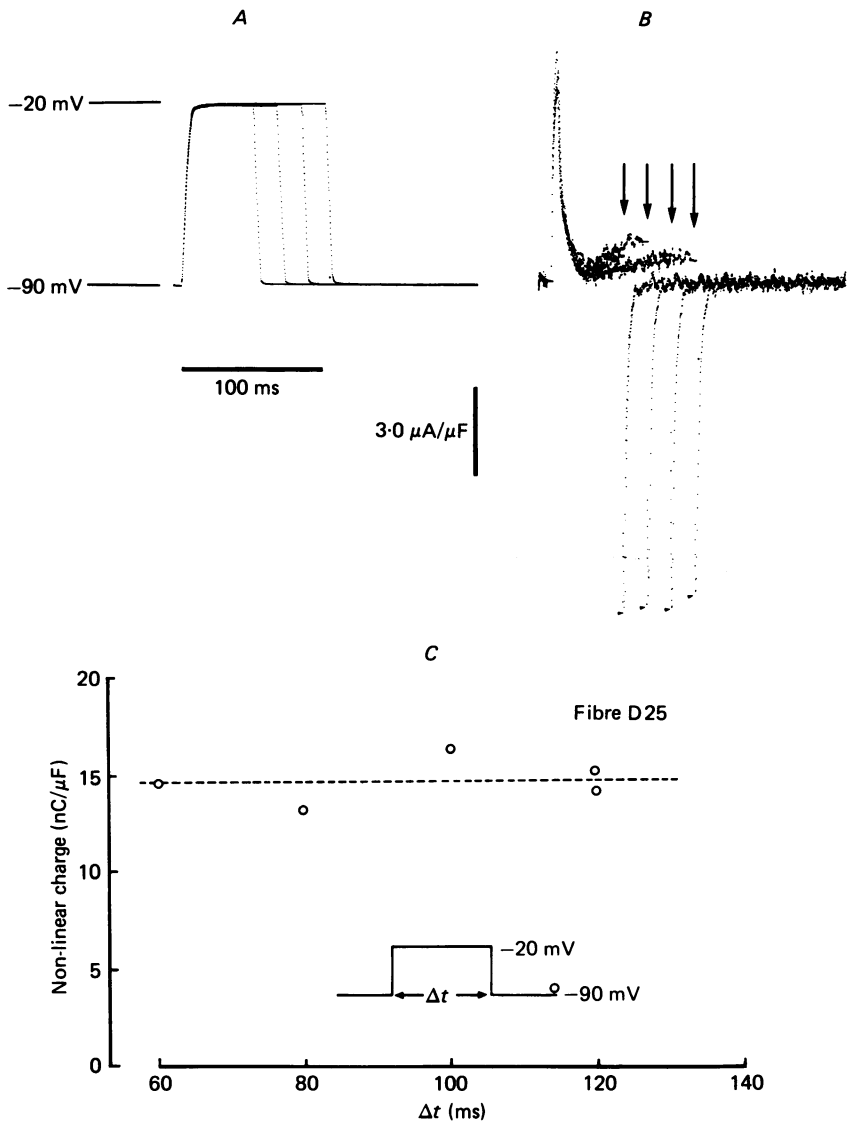

Fig. 3. When large voltage pulses from −90 to −20 mV of varying duration Δt were applied (*A*), the non-linear transients showed marked delayed outward currents (*B*), but the total charge as obtained by integrating the 'off' transients (*C*), remained constant for all the pulse lengths. Fibre cable constants: $R_i = 342\ \Omega.\text{cm}$; $\lambda = 2{\cdot}19$ mm; $r_i = 14587\ \text{k}\Omega/\text{cm}$; diam. = 55 μm; $r_m = 697{\cdot}3\ \text{k}\Omega.\text{cm}$; $R_m = 11{\cdot}97\ \text{k}\Omega.\text{cm}^2$; $C_m = 8{\cdot}3\ \mu\text{F}/\text{cm}^2$; temp. = 3·3 °C.

appropriate adjustments in sucrose concentrations to preserve osmolarity (see Horowicz & Schneider, 1981).

Double-pulse procedures

In the previous experiments, it was not possible to obtain a reliable estimate of the transient part of the 'on' current as its slow decline made it difficult to find the

leak conductances, and so only the 'off' non-linear charge was considered. In consequence 'on' 'off' equality of the charge could not be tested. Such a demonstration would be useful to indicate that the slow changes observed were capacitative in nature and that variations in the size of the 'off' tails did not actually reflect activation of an ionic current.

The experiments described in this section do test for this conservation of charge. In addition, they assess both the range of voltages and the approximate time scales over which the slow decays took place. They involved using a slightly more complicated pulse procedure which incorporated a method for estimating reliably the leak component of the recorded current.

The procedure was as follows: a long pulse, which will be referred to as the 'test' pulse, was applied to the fibre. This had a duration of 920 ms, and entailed a voltage excursion from the −90 mV holding potential, to a test voltage, which was raised in 2 mV increments in the potential range between about −55 and −40 mV. A small 10 mV depolarizing step of 105 ms constant duration was superimposed upon the test pulse. The current (I_0), clamp voltage (V_1) and $V_1 - V_2$ channels were sampled at 1 ms intervals over a 1280 ms sampling window for later analysis and display. Each record was an average of the results from five sweeps. Each experimental run began and ended with application of 10 mV steps at the holding potential to find the linear cable constants from the current responses and so assess the condition and stability of the fibre. Each experimental run explored test voltages in the range around −55 to −40 mV, and employed a probe step imposed either 100 or 400 ms after the onset of the test step. Successive runs employed alternating 100 and 400 ms delays in the probe step; this checked that differences between the two cases were genuine and so excluded the possibility of the effects of drifts in fibre condition.

In subsequent analysis, the steady current expected from leak admittances was estimated from the records at the end of the test pulse, by which time the 'off' recoveries from the end of the probe step had completely decayed. As the voltage at the probe pulse was within 10 mV of that of the test pulse, it is likely that the leak admittance would be similar at both potentials. Consequently, the transient (capacitative) part of the entire trace should be obtained simply by subtracting the voltage array, scaled to the value of the leak current, from the current ($V_1 - V_2$) arrays. Fig. 4*A* superimposes the current traces with the voltage (V_1) arrays so scaled. Even in these records of total charging current, the delayed (q_γ) components of the non-linear charge were clearly visible (arrows) in the test, or the probe responses, as well as their running integrals. The subtraction should give the purely transient part of the record, and subsequent integration (Fig. 4*B*) should then give the amount of polarization as a function of time.

The values of the integrals at the following times are of particular interest: (i) after the onset of the test step, and just before imposition of the probe step, (ii) at the end of the probe step, (iii) after full recovery from the 'off' of the probe step and (iv) after full recovery from the test pulse. After the last stage in the procedure one would expect the net charge moved to be close to zero: this was indeed the case in the running integrals (Fig. 4*B*). This net conservation of charge implies that the phenomena reported here are likely to be capacitative in origin rather than the results of ionic currents (Chandler, Rakowski & Schneider, 1976*a*; Huang, 1983*a*).

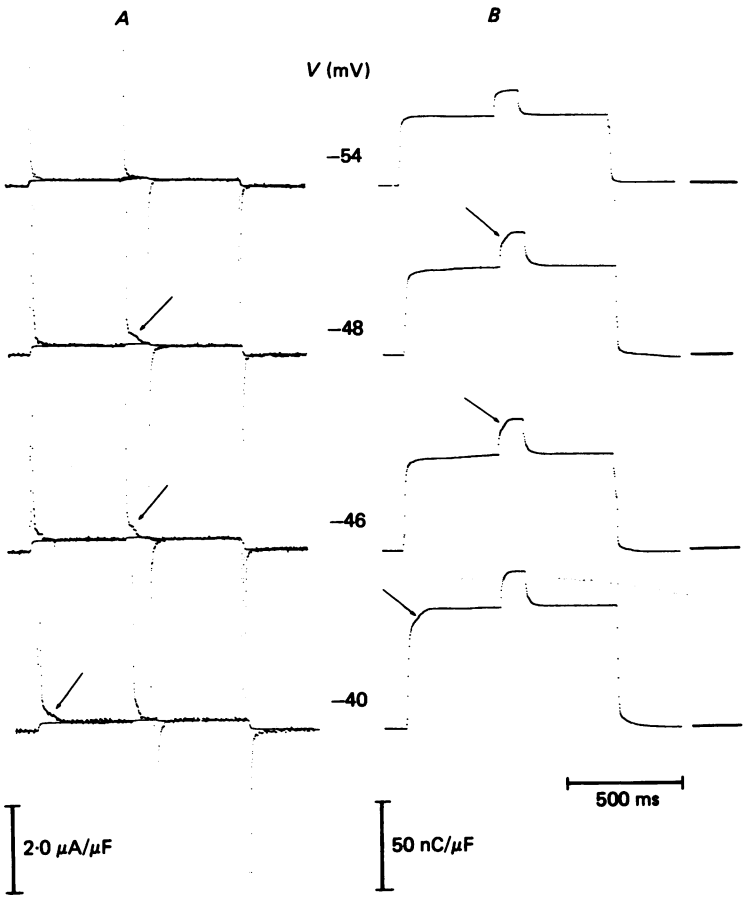

Fig. 4. *A*, the total membrane current obtained in response to a pulse procedure made up of a probe 10 mV depolarizing step applied 400 ms after the imposition of a test pulse to the indicated voltages. The voltage traces scaled to the final steady current obtained in response to the test step are superimposed; these were subtracted from the arrays of current before obtaining *B*, running integrals of total charge. These traces confirm net charge conservation at the end of the pulse cycle. Portions of the traces attributable to the q_γ charge movement component are arrowed. The horizontal lines at the ends of the traces indicate the zero level. Fibre cable constants: $R_i = 339\ \Omega.\text{cm}$; $\lambda = 3{\cdot}6$ mm; $r_i = 6457\ \text{k}\Omega/\text{cm}$; diam. $= 82\ \mu\text{m}$; $r_m = 836{\cdot}3\ \text{k}\Omega.\text{cm}$; $R_m = 21{\cdot}48\ \text{k}\Omega.\text{cm}^2$; $C_m = 4{\cdot}9\ \mu\text{F}/\text{cm}^2$; temp. $= 3{\cdot}6$ °C.

Fulfilment of the condition that the integral of the transient current is zero at the end of the sequence of pulses suggests that the transient current is very largely capacitative in nature. The fibres were studied in a voltage range where delayed rectifier currents were not detectable even during the test pulse, but there remains a possibility that a small part of the final 'off' transient might be the result of a tail current in a time-dependent channel which had opened during the pulse sequence. We detected 'tail' currents which could be attributed to the delayed rectifier and to the Ca^{2+} channel when large depolarizations were attempted, but we believe that such tail currents were negligibly small in the experiments which explored the behaviour of q_γ at its threshold. Nevertheless in this kind of analysis of 'on' and 'off' transients it becomes increasingly important to be able to detect, and ideally to suppress altogether, any 'tail' current through time-dependent conductance channels.

However, in the course of the procedure, the relative amounts of charge moving at the different stages might well be affected by the timing of the probe step. In Fig. 4*B*, the timing of the voltage steps was such that the membrane could relax fully before the next pulse was imposed. In consequence, in addition to the conservation of charge by the end of the entire procedure, the 'on' charge equalled the 'off' charge both before and after the probe step. In contrast, imposing the probe step 100 ms after the test step intercepted the current decay obtained in response to the test step. As a result (Fig. 5), the charge that had not yet moved became part of the response to the 'on' of the *probe* step; this made the charge moved by the probe step appear larger, and so the total charge before the probe step was smaller than its final value after this pulse, as indicated in the Figure.

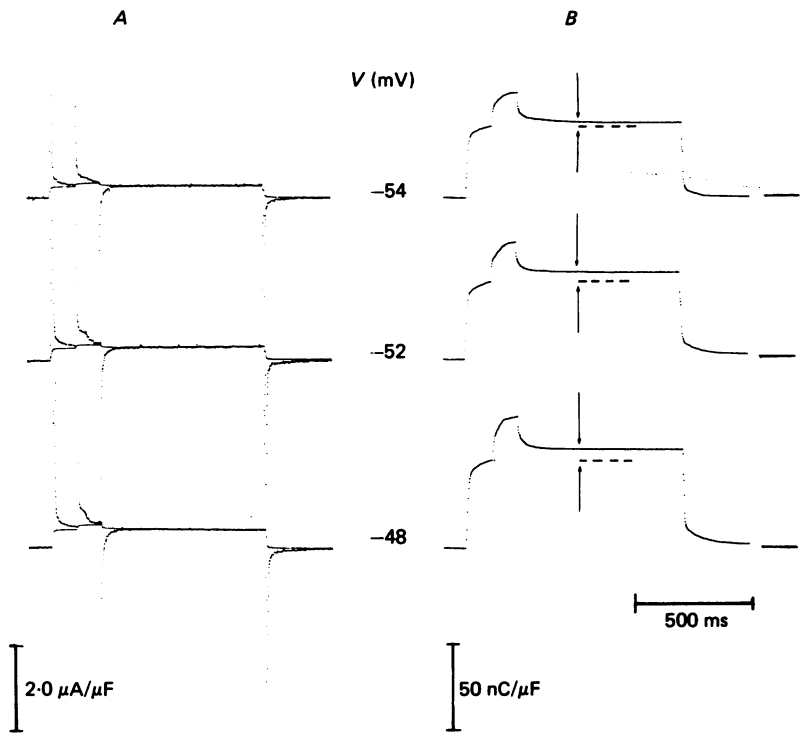

Fig. 5. *A*, same fibre and procedure as in Fig. 4 except that the probe pulse was imposed 100 ms after the onset of the test pulse, thereby interrupting the test response before its full decay. *B*, in consequence, in the integrals, part of the latter decay became incorporated into the probe response, and so more charge appeared to move in the 'on' than in the 'off' of the probe response. Nevertheless there was still charge conservation at the end of the pulse cycle: the horizontal lines at the ends of the traces denote the zero level. The dotted lines denote the charge immediately before the beginning of the probe pulse.

Therefore, although a slow transient might be difficult to see directly, one could assess its presence by observing its effect upon a response to a probe step imposed at a suitable time.

Figs. 6–9 illustrate the results of investigating a range of 'test' voltages between −55 and −38 mV in 2 mV increments. Figs. 6 and 8 plot the total charge moved

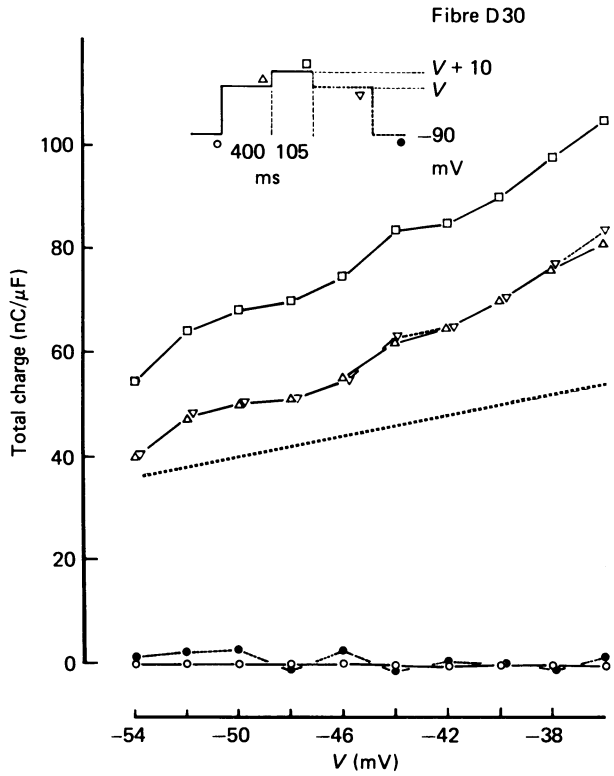

Fig. 6. Total charge at different stages of the pulse procedure shown in the inset, (i) at the beginning of the procedure (○), (ii) after the test pulse but just before imposition of the probe pulse (△), (iii) at the end of the probe pulse (□), (iv) at the end of the test pulse (▽), and (v) at the end of the pulse cycle (●). At *all* stages of the procedure, the steady-state charge is a single-valued function of the membrane voltage. The dotted line gives the expected charge stored in the linear capacitance at the test voltages.

through each successive part of the experimental procedure. The transients in Figs. 7 and 9 are of total charging current, after the leak component had been subtracted. The latter was determined from relaxed transients over the last 100 ms of the test pulse. Total charge, the sum of both non-linear and linear transients, is shown. They are shown at high gain to emphasize the smaller but slower q_γ transients (arrowed). In Fig. 7 these delayed transients are clearly visible first in the probe responses in the −50 mV traces. However, with further depolarization, slow events become discernible in the test responses; these become larger and more rapid, until they resembled the transients that first appeared in response to the probe pulses.

Recovery from the test step was complete if the probe step was applied 400 ms or more after the test step. Under such conditions (Fig. 6) the membrane would be in a steady state both before and after the probe pulse, and so the total charge moved within the membrane would be the same. This expectation was confirmed (Fig. 6, triangles). There was no evidence of any hysteresis resulting from the direction from which the final test potential was reached, even with this close investigation of the membrane in 2 mV steps. This also is consistent with the charge involved being capacitative in origin.

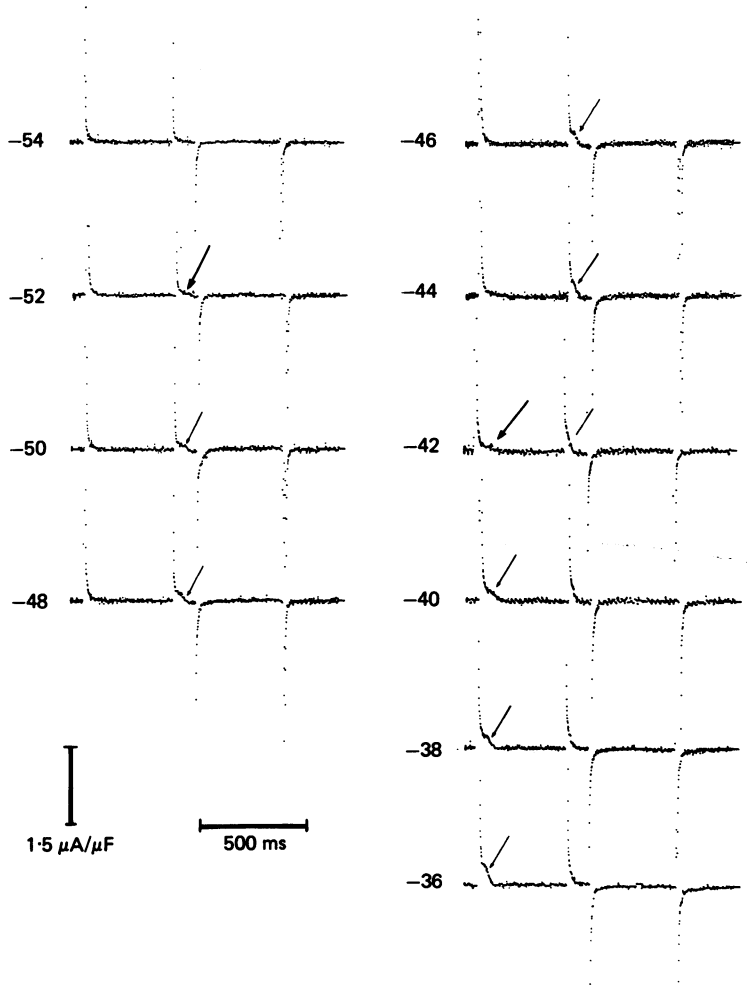

Fig. 7. Traces of the transient parts of the currents obtained from the pulse procedure illustrated in Fig. 6, shown at high gain, which emphasizes the slower non-linear components, but in which the large initial fast (linear) transients are not fully represented. Distinguishable q_γ relaxations (arrowed) appeared first in the probe response when the test voltage was −52 mV, and then in the test response at −42 mV. In this particular spacing of the pulses, the charging currents had fully decayed before imposition of the following pulse.

In contrast, when the probe pulse was imposed 100 ms after the test step, it would appear that the test response had not been able to complete its decay before interception by the probe pulse (Figs. 8 and 9). Again (Fig. 9) portions of the relaxation attributable to q_γ in the transients are arrowed. However, over a limited voltage range, the 'on' of the probe response appeared increased by what might have been part of the original relaxation to the test pulse. This was borne out in the plots of the total charge moved at each stage of the step protocol. Thus the charge just before the probe pulse was smaller than after the recovery from the 'off' of the probe step between test potentials of −52 and −42 mV (Fig. 8). This is precisely as would be expected had the decay of the 'on' response to the *test* step been incomplete at

the onset of the *probe* step, and therefore had become incorporated into the probe response. This effect occurred only through a limited range of voltages close to the threshold of the q_γ charge. Stronger depolarization resulted in the kinetics of the q_γ component becoming far more rapid and thereby completing its decay within 100 ms (Fig. 9).

It therefore emerges from these experiments that the q_γ transient is extremely slow over a narrow range of potentials near the mechanical threshold, taking 100–400 ms to relax, but that its kinetics rapidly become faster even with 5–10 mV further depolarization.

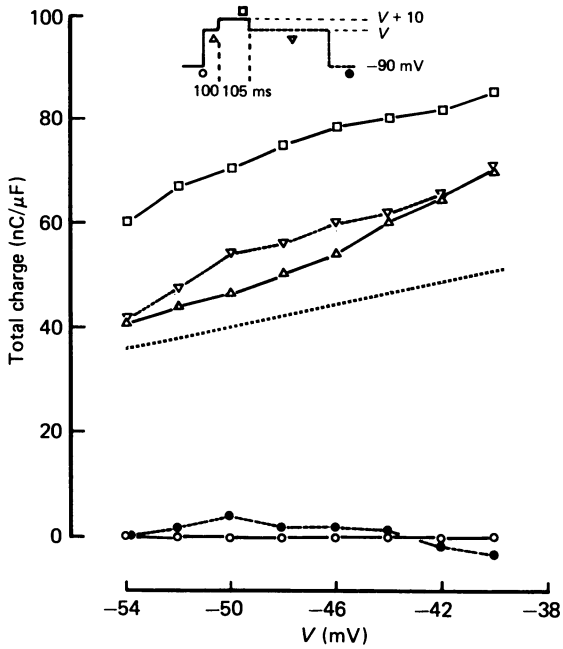

Fig. 8. Fibre, pulse procedure and symbols as in Fig. 6, except that the probe pulse was imposed a briefer time (100 ms) after the onset of the test pulse. At voltages between −52 and −42 mV, the 100 ms interval between imposition of test and probe steps is insufficient for full decay of the charging current. This results in a charge moved (△) less than had the system been fully allowed to relax at the test voltage concerned after the probe pulse (▽). The dotted line gives the charge attributable to the linear capacitance at the test voltage.

Small-pulse procedures

The final series of experiments tested particular predictions that would arise from the conclusion above. They also provided a simple indication of the time course of these slow processes. The transient to a 10 mV probe step, duration 105 ms, was measured. Again, this was superimposed upon a test voltage the responses to which were not sampled, but a range of delays between 50 and 300 ms were explored. This protocol allowed for closer sampling (200 μs) over a shorter time scale (216 ms) and the non-linear charge could be obtained by comparing the test response with a control response to a 10 mV step at −90 mV. The total charge moving in response to the

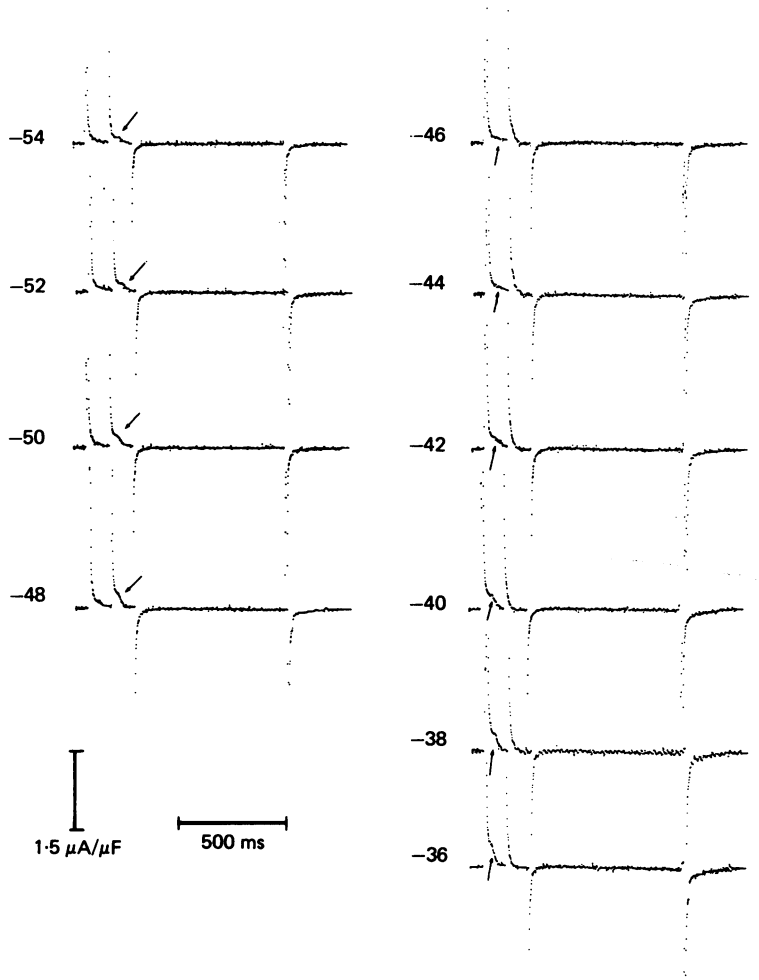

Fig. 9. Transient parts of the total current obtained in response to the voltage steps shown in Fig. 8. When only 100 ms separates the onset of the test and the probe steps, the imposition of the probe step intercepts an incomplete decay of the q_γ part of the charging current (arrowed) to the test step at certain voltages. In consequence the 'on' response of the probe step is increased in size.

small steps was calculated precisely as described by Adrian & Peres (1979) and so, in the 'on' responses the estimate of the leak current had to be based upon the final value of the charging current for the small imposed voltage step. The resulting integrations of 'on' and 'off' charge (Fig. 10) are expressed normalized to the linear charge obtained by a 10 mV step at -90 mV (C_T/C_C) in Fig. 11.

At a test voltage of -47 mV, the integration of the extra charge rose slowly with time as would be expected had a slow (q_γ) relaxation been present. It did not reach a steady value before imposition of the 'off' step (Fig. 10, left). At the end of the 'off' step the net charge moved appeared to be less than zero. This applied at all the delays of the probe pulse that were tested, and Fig. 11 (circles) shows that 'on' integrals were consistently less than the 'off' integrals.

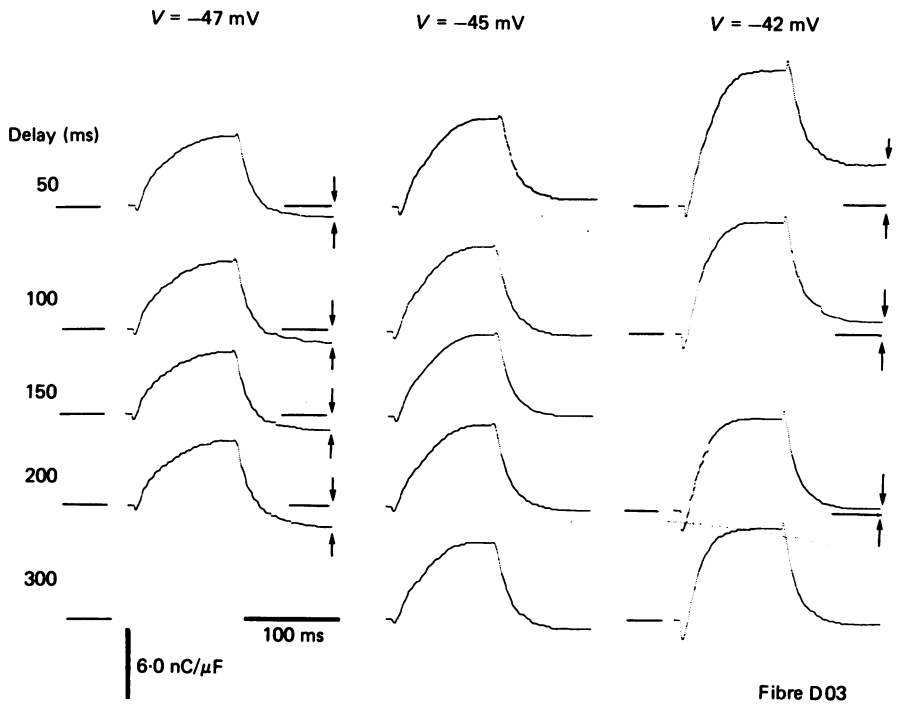

Fig. 10. Running integrals (see text) of the non-linear transients obtained in response to 10 mV probe steps imposed at different delays after the fibre had been clamped to three closely spaced test voltages, V, from a −90 mV holding potential. At −47 mV the integral had not reached a steady value by the end of the 'on' step, and there appeared a net negative charge at the end of the 'off'. At −45 mV the integrals reach a steady value and there is over-all charge conservation. At test voltages of −42 mV, at short delays, part of the relaxation to the test pulse becomes included within the 'on' response, whose integral consequently exceeds that of the 'off'. (Adrian & Almers (1974) show that the integral of the transient part of the current (see Methods) is independent of the shape of the voltage step and depends only on its size. The immediate deflexions at the 'on' and the 'off' of the records imply small differences in the rate of change of potential of the control and test steps. Steady levels should be unaffected by these differences.) Cable constants: $R_i = 335\ \Omega.\text{cm}$; $\lambda = 4{\cdot}12$ mm; $r_i = 4157\ \text{k}\Omega/\text{cm}$; diam. = 101 μm; $r_m = 706\ \text{k}\Omega.\text{cm}$; $R_m = 22{\cdot}45\ \text{k}\Omega.\text{cm}^2$; $C_m = 12{\cdot}5\ \mu\text{F}/\text{cm}^2$; temp. = 4·0 °C.

In the light of earlier suggestions, it seemed from these findings as though the 'on' capacitative charge had been underestimated, which would be expected from an over-estimation of the leak current. This situation could be the result of the relaxation extending over an interval of a similar or longer duration than that of the imposed pulse, as would occur at the first appearance of the q_γ component in the probe step.

When the test voltage was −45 mV, the integrals in Fig. 10 (middle) implied an over-all conservation of charge: this equality of 'on' and 'off' charge is plotted in Fig. 11*B*, and was the case whatever the delay in the probe pulse. These findings are consistent with a more rapid relaxation of q_γ charge in which a steady state was achieved before the end of the probe step. In such a situation a correct estimate of

leak current would be possible. The integrals obtained would then accurately reflect capacitative charge, and so 'on' 'off' equality would now result.

Finally, when the muscle was subjected to a test potential of −42 mV, the amount of charge moved by the 'on' part of the probe step was larger than the 'off' charge (Fig. 10, right), and as a result there appeared a net movement of charge at the end of the pulse. The integrations reached steady values at both the 'on' and 'off' steps. Fig. 11*C* shows that the 'off' integrals were relatively constant through the delays tested; this would be consistent with a steady state having been achieved in all the cases at the onset of the 'off' part of the voltage step.

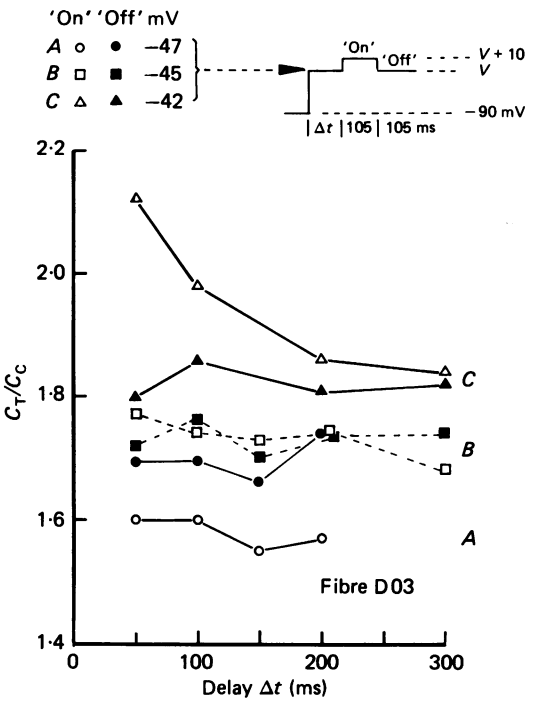

Fig. 11. The 'on' and 'off' integrals from the procedure given in Fig. 10, expressed as a ratio to the linear charge measured from a 10 mV step at −90 mV, at different delays Δt after the onset of the test pulse, at three closely spaced voltages near threshold.

However, the 'on' integrals usually exceeded the 'off' integrals, by an amount that was greater the shorter the time between the onset of probe and test pulses (Fig. 11*C*). In the light of the previous findings, this observation would also be expected if the depolarization to −42 mV resulted in appearance of a slow q_γ transient in the test step that was intercepted by the onset of the probe pulse and so became incorporated into the 'on' portion of the probe response. The difference between the sizes of the 'on' and the 'off' integrals can be used, therefore, to assess the extent of the relaxation in the test step, and the results in Fig. 11 are consistent with a q_γ relaxation extending over 200–300 ms.

These three very different findings occurred over a voltage interval of only 5 mV; again this reflects the very marked voltage dependence of the processes involved.

DISCUSSION

The possible relationship between charge movements and the regulation of contractile activation has been discussed on a number of earlier occasions (Almers, 1978). More recently evidence has become available for the existence of several components of this potential-dependent charge. One component, called 'q_γ' has been of particular interest; this is distinguishable from the rest of the transient current as a delayed component at certain potentials close to the mechanical threshold (Adrian & Peres, 1979). Furthermore, the steep potential dependence, and the susceptibility of q_γ to tetracaine (Huang, 1981*a*, 1982) and to Dantrolene sodium (Hui, 1983) parallel similar characteristics in the production of tension in skeletal muscle. These earlier observations make it likely that, of the three components of charge known so far (q_α, q_β and q_γ; Adrian & Peres, 1979), it is the q_γ component that is the most likely to have a causal relationship with the activation of Ca^{2+} release.

This paper investigates the delayed relaxations of the voltage-dependent charge at potentials close to the contractile threshold. In the low-chloride, high-sulphate, tetraethylammonium-containing solutions employed in these experiments, this occurs when the membrane potential is depolarized to around −50 mV (Huang, 1981*b*). It demonstrates for the first time that these relaxations may extend for as long as 300 ms; this is substantially longer than was previously thought the case. However, these prolonged charge movements occur only over a very narrow range of potentials close to the threshold for contraction where the delayed component first appears, and to be demonstrated clearly the membrane has to be explored with small increments of potential (~2 mV steps). With depolarizations beyond the mechanical threshold, even by as little as 5–10 mV, these relaxations become considerably faster, giving those features of q_γ that have already been reported (Adrian & Peres, 1979; Huang, 1981*a*).

These results bear specifically upon some earlier findings concerning mechanical activation. At 4 °C and at membrane potentials positive to −10 mV, Adrian *et al.* (1969) showed that the strength–duration relation for the initiation of contraction could be explained if a certain concentration of an 'activator' was required, and that the rate of release of the 'activator' beyond −30 mV was directly proportional to the difference between the membrane potential and −30 mV. The strength–duration curve between the rheobase threshold and −10 mV could then be accounted for by supposing that the transfer of 'activator' from 'store' to 'site of action' was governed by a first-order process with rate constants having exponential dependence on membrane potential. Miledi, Parker & Zhu (1983) have proposed a similar model to account for Arsenazo III signals from voltage-clamped muscle fibres. The magnitudes of the rate constants derived from strength–duration measurements are similar to the rates for non-linear charge movement and it has seemed plausible (Chandler, Rakowski & Schneider, 1976*b*) to equate the two processes.

Models of the kind proposed by Adrian *et al.* (1969) predict the steady-state concentration of 'activator' at the 'site of action' in response to long lasting depolarization, and in particular to depolarizations short of the rheobase mechanical threshold. When these predictions were tested by applying a brief but large depolarization at the end of a long, just threshold, depolarization, it was found that

in terms of the model it was impossible, by means of the long subthreshold pulse, to produce an 'activator' concentration more than 50 % of its threshold value. Adrian *et al.* (1969) postulated a mechanism involving a range of 'activator' concentrations which could inhibit the rate of uptake of 'activator' (i.e. removal of 'activator' from its 'site of action').

An alternative explanation is suggested by the present experiments. In what follows we shall make the assumption that charge movement, in particular the component of charge movement called q_γ, can be equated to the movement of 'activator'. If, as we have here shown, the movement of q_γ is initially very slow, taking as long as 300–400 ms to become complete, in the range of voltages where the charge moves slowly, the depolarizations used by Adrian *et al.* (1969) to determine rheobase threshold would have been too short to achieve a steady-state 'activator' concentration. Since the movement of q_γ accelerates steeply with depolarization, at some depolarizations the long pulses used by Adrian *et al.* (1969) (pulse duration 75 ms) would have been long enough to achieve a threshold concentration of 'activator'. But the voltage so determined would not have been the true rheobase. An effect of this kind could have made the apparent steady-state dependence of 'activator' on potential much steeper than the actual dependence. Since the actual dependence is already steep, the apparent excluded range of 'activator' values (50–100 % of its threshold values) could have been the result of finite experimental step size.

In terms of the assumed identity of charge movement and 'activator' we found no evidence for the mechanism which Adrian *et al.* (1969) postulated to account for the excluded steady-state concentrations. If the back-reaction rate for charge movement were N-shaped, as they suggested for the 'activator' movement, the steady-state curve of q_γ *versus* V would be double-valued over some range of voltage. We found no evidence of steady-state hysteresis in experiments of the kind shown in Figs. 6 and 7.

The parallels between the behaviour of q_γ and the postulated 'activator' are certainly striking, but it should be remembered that the behaviour of the 'activator' is derived from strength–duration measurements which can be fitted by a good many different models. Moreover, as has been pointed out, there are uncertainties and difficulties about measuring charge movements, particularly in the region of mechanical threshold. The separation of the charge movement into its components is neither straightforward nor self-evident. But even if q_γ proves to be unconnected with contraction activation its behaviour is striking. It appears to represent the response of an intramembrane structure or molecule to changes in electric field and the response has high-order kinetics that alter in time scale by at least an order of magnitude over a 10 mV interval. It has also a very steep dependence on the field in the steady state (Adrian & Peres, 1979; Huang, 1982; Duane & Huang, 1982). Such behaviour is difficult to explain in simple terms, but if it is indeed the case that this component of charge is the result of configurational change in one or more membrane-bound regulatory proteins, which are known to possess cooperative mechanisms affecting their activities (Monod, Wyman & Changeux, 1965; Huang, 1983*b*), results of this kind may not be surprising.

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