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DIFFERENTIAL EFFECTS OF RYANODINE AND TETRACAINE ON CHARGE MOVEMENT AND CALCIUM TRANSIENTS IN FROG SKELETAL MUSCLE

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SUMMARY

1. Charge movement and myoplasmic calcium transients were simultaneously recorded from frog skeletal muscle fibres by using the double-seal Vaseline-gap technique. Calcium transients were monitored with the fluorescent indicator Rhod-2.

2. Ryanodine modified the kinetics and the total amount of charge moved during depolarizing pulses (Q_{on}), while it did not significantly modify the charge after repolarization (Q_{off}). The extracellular application of 100 μM-ryanodine elicited a temporary initial increase of the delayed component of charge movement (Q_γ) and the calcium transient. Both phenomena were later blocked with the same temporal course and to the same extent.

3. The blockade of Q_γ and the calcium transient was also observed with ryanodine concentrations of 1–10 μM. For membrane potentials positive to -10 mV, the Q_{on} measured was larger in the presence of ryanodine; Q_{off} was not modified.

4. Tetracaine (400–500 μM) blocked a similar delayed component of Q_{on}, identified as Q_γ, as well as the calcium transient monitored simultaneously. This effect was observed in the first minutes after the addition of tetracaine to the extracellular solution.

5. Tetracaine blocked a faster initial component of Q_{on} for voltages positive to -10 mV, corresponding to the voltage range of activation of the calcium current. At these same membrane potentials, Q_{off} was also reduced to a similar extent to Q_{on}.

6. Ryanodine and tetracaine showed different effects on calcium current. Whereas the slow calcium current was not modified upon the addition of ryanodine, it was completely blocked in the presence of tetracaine. The blockade of the slow calcium current made evident the fast calcium current. The effects of tetracaine on the charge movement, the calcium transient and the slow calcium current were reversible.

7. These results suggest that ryanodine and tetracaine may act at different sites. Ryanodine exerts its effect on the sarcoplasmic reticulum ryanodine receptor, blocking calcium release and Q_γ, while tetracaine at these concentrations may affect the release channel and the dihydropyridine receptor, causing a blockade of the charge movement, calcium transient and calcium current.

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INTRODUCTION

Charge movement in frog skeletal muscle, initially described by Schneider & Chandler (1973), has two major components in polarized cells: a fast one termed Q_β, and a slower delayed one termed Q_γ (Adrian & Peres, 1979; Huang, 1981, 1982). Tetracaine has been used to isolate Q_β and Q_γ since it has a selective blocking action on the slower delayed component (Huang, 1982; Hui, 1983). Q_γ has been related to the calcium release from the sarcoplasmic reticulum (SR) and muscle contraction; the steep voltage dependence of Q_γ resembles that of tension generation (Hui, 1983; Hui & Chandler, 1988) and is also similar to calcium-related optical signals (Baylor, Chandler & Marshall, 1979; Miledi, Nakajima, Parker & Takahashi, 1981; Vergara & Caputo, 1983; Maylie, Irving, Sizto & Chandler, 1987; Csernoch, Huang, Szücs & Kovács, 1988).

The SR calcium release channel has a high affinity for the plant alkaloid ryanodine (dissociation constant (K_d) 27 nM) (Pessah, Waterhouse & Casida, 1985) which, at submicromolar concentrations, decreases the channel amplitude and increases the open probability almost to unity (Rousseau, Smith & Meissner, 1987). In skeletal muscle, ryanodine causes a progressive and irreversible contracture (Edwards, Weiant, Slocombe & Roeder, 1948; Fryer, Lamb & Neering, 1989), due to the fact that the SR calcium release channel is stabilized in an open state, with an associated increase of the intracellular free calcium concentration (Su, 1986). Despite the close relationship between charge movement and the gating of the release channel, Fryer *et al.* (1989) found that rat single fibres contracted in the presence of 10 μM-ryanodine and that charge movement 'was virtually unaffected just prior to the fibre contracting irreversibly'.

Recently, it has been reported that the SR calcium release channel from skeletal muscle is also inhibited by tetracaine (Xu, Jones & Meissner, 1990). Concentrations larger than 1 mM fully inhibited the Ca^{2+}-gated release channel, while tetracaine at 500 μM induced prolonged closed intervals with bursts of rapid channel openings and closings. This is in agreement with the blocking effect of tetracaine on the calcium transients in skeletal muscle (Vergara & Caputo, 1983; Csernoch, Huang, Szücs & Kovács, 1988). Furthermore, tetracaine reduced the amount of charge movement in frog skeletal muscle by blocking specifically Q_γ (Huang, 1982; Hui, 1983). In skeletal muscle of mammals, tetracaine blocked a component of charge movement only under hypertonic solutions (Lamb, 1986) but not in isotonic solutions (Hollingworth, Marshall & Robson, 1990).

These findings further support the relationship between Q_γ and calcium release from the SR, and raise the question whether ryanodine and tetracaine exert their effect at the same level or have different targets. If they affect the same molecule(s) directly linked with charge movement, then either one would have the same effect on this phenomenon. Accordingly, if the charge movement is closely related to the gating of the slow calcium channel (Ríos & Brum, 1987; Tanabe, Beam, Powell & Numa, 1988) the calcium current would also be affected by these drugs. It would be interesting to study whether affecting the SR release channel would have an effect on the dihydropyridine-sensitive charge movement, since morphological studies suggest a close relationship of the structures in the feet of the tubular–sarcoplasmic reticulum junction (Franzini-Armstrong & Nunzi, 1983).

This paper shows evidence that ryanodine blocks a delayed component of the charge movement that corresponds to Q_γ and blocks most of the calcium release from the SR without major modifications in the calcium currents. Tetracaine blocks a similar delayed component of the charge movement in addition to another constituent of the charge, probably related to the calcium current.

METHODS

Experiments were carried out in single skeletal muscle fibres isolated from the semitendinosus muscle of the frog (*Rana pipiens sphenocephala*). Frogs were killed by decapitation and the spinal chord and brain tissue were rapidly destroyed with a needle. Fibres were voltage clamped following the same procedures and circuitry for the double Vaseline-gap technique described in Francini & Stefani (1989). Briefly, the fibres were dissected free in a chamber containing dissecting solution (see below for all solutions) and then transferred to the experimental chamber with mounting solution. Thereafter the external surface of the fibre was divided into three electrically insulated compartments by using Vaseline threads and a coverslip. Solutions were then replaced by the extracellular solution in the central pool and by the intracellular one in the end pools. Fibres were mounted at their resting length since the intracellular solution contained 15 mM-EGTA to prevent mechanical artifacts. The pools were electrically connected to wells containing 3 M-KCl via agar bridges equilibrated with the corresponding solution of each pool. Ag–AgCl pellets provided the electrical connection between the wells and the amplifier.

Intramembrane charge movement was recorded from a holding potential of −90 mV with pulses from 50 to 125 ms, in 10 mV steps up to 30 mV. Linear components of the membrane current were subtracted by appropriate scaling of depolarizing control pulses from a membrane potential of −120 mV. The control pulses were 1/4 of the amplitude of the test pulses and were delivered prior to each one of these. In some cases, there was no need to correct the subtracted records with a sloping baseline to quantitate the charge. When necessary, it was done in the second half during the pulses. Calcium currents were recorded with longer pulses (250–300 ms).

Calcium transient recording. Calcium transients were simultaneously recorded with the fluorescent dye Rhod-2 (Molecular Probes, Inc., Eugene, OR, USA) added to the intracellular solution in concentrations of 50–100 μM. Cuvette calibrations of this dye with the same intracellular solution were adequately fitted to a 1:1 calcium–dye reaction. The fitted K_d was about 1·5 μM for calcium, which is very similar to the one previously reported (Minta, Kao & Tsien, 1989). The dye sensitivity was adequate to record the transients even with EGTA inside the cells. Fibres were epi-illuminated with a light source of a 100 W tungsten–halogen lamp. The beam of light initially passed through an excitation filter centred at 546±30 nm (Omega Optical Inc., Brattleboro, VT, USA), and then reflected by a long-pass dichroic beamsplitter centred at 560 nm (at 45 deg). The calcium–dye reaction emitted light at a longer wavelength which was recorded after passing through a third emission filter centred at 590±35 nm. The signal was collected by a photodiode and measured as voltage change. The background fluorescence (F_o) of the experimental chamber and the Vaseline was measured immediately after the addition of the dye and later subtracted from the signal traces. Fluorescence due to the entry of the dye (F_t) was monitored along the experiment and was used to normalize the response due to the emission from the calcium–dye reaction (ΔF). Calcium transients are expressed as $\Delta F\, F_b^{-1}$, where $F_b = F_t - F_o$.

Solutions. The following solutions were used (all in mM). Dissecting solution: K_2SO_4, 101; $CaSO_4$, 2; $MgSO_4$, 2; K-HEPES, 5. Mounting solution: potassium glutamate, 120; $MgCl_2$, 2; Na_2-EGTA, 0·1; K-HEPES, 5. Extracellular solution for charge movement recording: tetraethylammonium-methanesulphonate, 105; $CaCl_2$, 2; $CoCl_2$, 8; tetraethylammonium (TEA)-HEPES, 5; tetrodotoxin, 10^{-3}; 3,4-diaminopyridine, 1. For calcium current recordings we used 10 mM-$CaCl_2$ without $CoCl_2$. Intracellular solution: caesium glutamate, 70; Cs_2-EGTA, 15; $MgCl_2$, 6·9; Cs-HEPES, 10; glucose, 5; Na_2-ATP, 5; phosphocreatine (disodium salt), 5. In all solutions pH was adjusted to 7·0. Osmolarity was corrected to 260 mosM with sucrose when necessary. The temperature (17 °C, or 4 °C for calcium transient recording) was monitored with a thermistor probe placed in the middle pool of the chamber.

Chemicals. Ryanodine (Calbiochem Co., La Jolla, CA, USA) was added from stock solutions to the extracellular solution. The concentrations of ryanodine used ranged from 1 to 100 μM. Only one

concentration of ryanodine was used per fibre since its effect was not reversible. Tetracaine (Sigma Chemical Co., St. Louis, MO, USA) was diluted in distilled water and added from a 20 mM stock solution; it was used at 400–500 μM.

Passive electrical properties. The electrical properties of the fibres and the series resistance were calculated according to the model proposed by Irving, Maylie, Sizto & Chandler (1987). The ratio $r_e:(r_e+r_i)$, where r_e and r_i are the external resistance per unit length under the seal and the internal

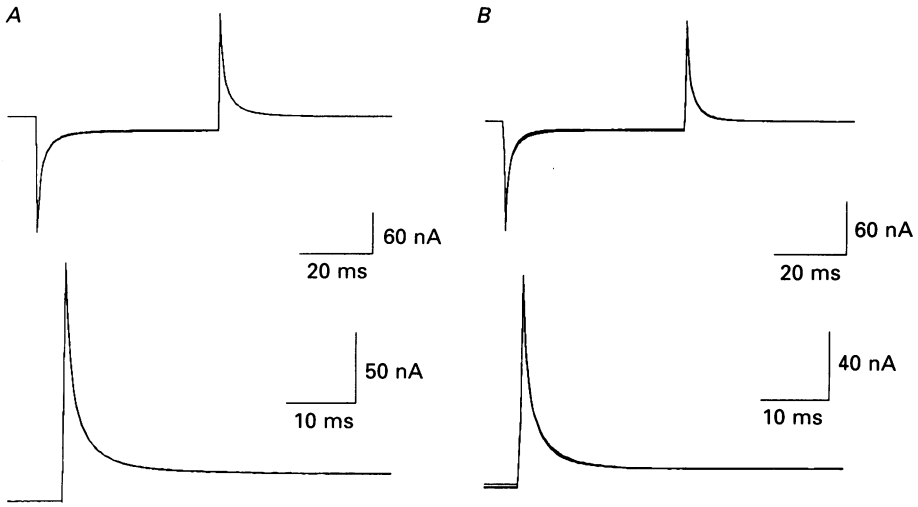

Fig. 1. Membrane linear capacitance. Superimposed trace in two fibres before and at different times after the addition of 100 μM-ryanodine (*A*, 25 min), or 400 μM-tetracaine (*B*, 35 min). Records were obtained with a hyperpolarizing pulse of 20 mV from the holding potential of −90 mV. The membranes linear capacitance was calculated by integrating the current traces shown below. Values of the input capacity remained unmodified during the experiment; 22·1 nF in *A* and 18·4 nF in *B*.

resistance per unit length respectively, ranged from 0·980 to 0·995 (mean 0·989±0·003, S.E.M.; $n = 13$), which indicates the quality of the Vaseline seals. Measurement of the charge movement is critically dependent on the capacitance of the fibres. Some fibres showed a progressive decrease in the amount of membrane linear capacitance and slowness of its kinetics. Under these circumstances, the charge movement could be normalized to the new fibre's capacitance, but changes in the time course of the capacity transient broaden the charge movement leading to a miscalculation and interpretation of the effect. Thus, we included in this study only the fibres whose linear capacitance did not show significant changes during the experiment. Figure 1 shows superimposed traces of capacitive currents recorded before and at different times after the addition of ryanodine (100 μM; *A*) or tetracaine (400 μM; *B*) in two different fibres. Traces were obtained with a pulse to −110 mV from the holding potential (−90 mV). Membrane capacitance was measured as the area under the transient at the end of the pulses (enlarged in the traces below). The fibres used had a maximum reduction in membrane capacitance of 5% without modification in the kinetics.

RESULTS

Effect of ryanodine on charge movement and calcium transients

Figure 2 shows the time course of the effect of 100 μM-ryanodine on charge movement and calcium transients for a pulse to −20 mV. The two upper traces represent the charge movement (left column) and the calcium transient (right column) in control conditions. Charge movement during the pulse (Q_{on}) at this

membrane potential shows a fast component possibly due to the Na^+ channel gating current (Vergara & Cahalan, 1978; Rojas & Suárez-Isla, 1980), followed by Q_β and ultimately a delayed hump, Q_γ. The calcium transient rises to a maximum briefly after the depolarization and then slowly declines. After the pulse, the transient

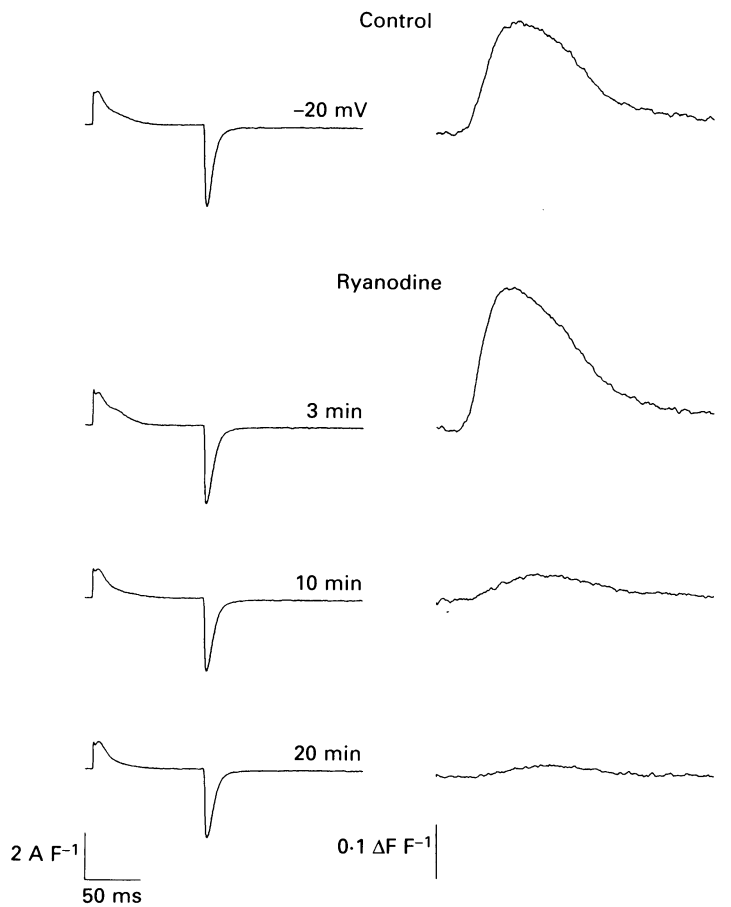

Fig. 2. Effect of ryanodine on charge movement and calcium transients as a function of time. Upper traces show charge movement (left column) and calcium transient (right column) recorded simultaneously under control conditions for a pulse to −20 mV. Charge movement and calcium transients were recorded at different times (indicated by the numbers between the traces) after the addition of 100 μM-ryanodine. Q_{on} and Q_{off} values (nC μF^{-1}) were: control, 33·9 and 34·9; 3 min, 35·8 and 35·9; 10 min, 28·3 and 35·0. Ryanodine caused an initial increment of the delayed component of charge movement and the calcium transient (3 min) and then a parallel inhibition of both processes.

decays quasi-exponentially towards the baseline. Three minutes after adding 100 μM-ryanodine to the extracellular solution, the delayed component of the charge movement became more prominent and the calcium transient was larger and with a faster rising phase which indicates a potentiated SR Ca^{2+} release. The initial components of the charge movement did not show major modifications. As time progressed, ryanodine caused a gradual blockage of the delayed charge component

and the calcium transient. The maximum effect was observed 30–40 min after exposure to this drug and then remained stable. At lower ryanodine concentrations (1, 5 and 10 μM) the blockade of the charge movement developed more slowly than at 100 μM.

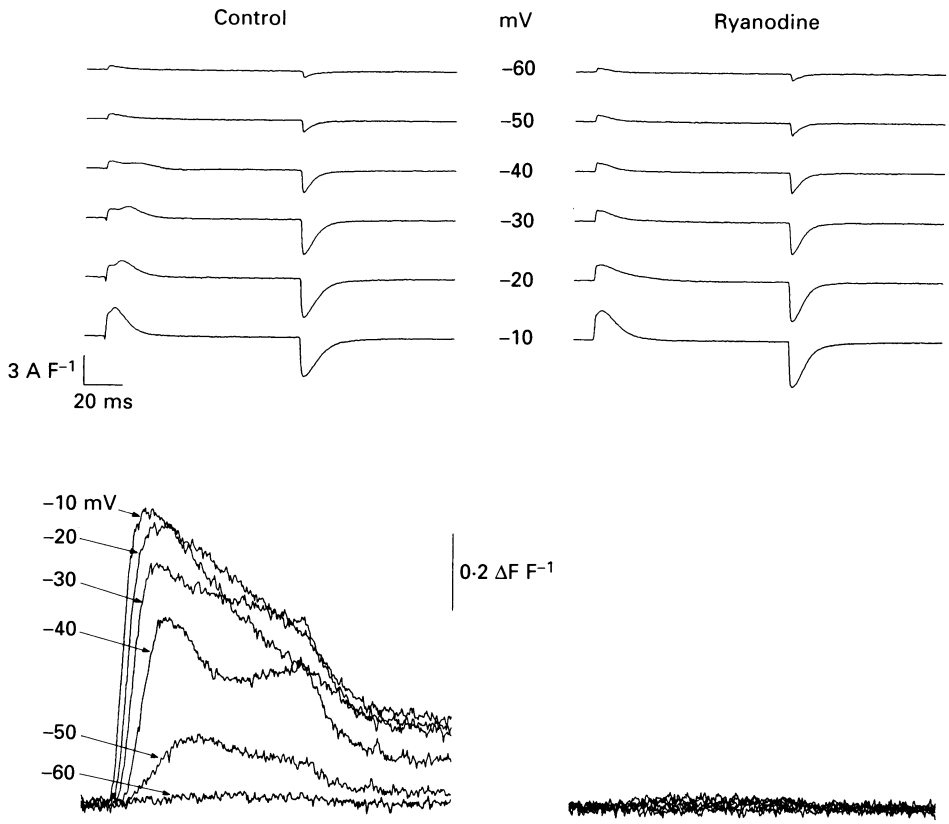

Fig. 3. Blockage of charge movement and calcium transients by ryanodine at different potentials. Control records of charge movement (left column, upper traces) and calcium transients (lower traces) at several membrane potentials, indicated by the numbers next to the traces. Charge movement has a clear delayed component under control conditions, which was blocked by 100 μM-ryanodine (right records, upper traces). The calcium transient was blocked almost totally (lower traces).

In order to determine whether the effect of ryanodine was due to a real decrement of Q_γ or to a shift in the voltage dependence of this component, we examined the charge movement in the voltage range from -80 to 30 mV. Figure 3 presents the evidence that favours the first possibility. Records in the left column were obtained under control conditions and those in the right 30 min after the exposure to 100 μM-ryanodine. Traces below show the calcium transients simultaneously recorded. The numbers near the traces indicate the membrane potential. Control records clearly have Q_β at all those voltages, while Q_γ becomes noticeable at -40 mV for this fibre. For larger depolarizations, Q_γ appears earlier and merges with Q_β. After the addition of ryanodine, the first component of the charge movement persists with the same

characteristics and Q_γ has vanished. The charge movement after the end of the pulse (Q_{off}) remained practically unaffected. The decay of the Q_{on} was faster when Q_γ was present. Compare, for instance, the records at −20 mV; the decay of the charge movement in ryanodine is slower.

The calcium transients were also eliminated by ryanodine, as shown in the lower records (right panel). The parallel changes in charge movement and calcium transients with ryanodine indicate that Q_γ and the calcium release from the SR are tightly related. Since both processes are affected at the same time and to a similar extent, we cannot elucidate whether the calcium release is a consequence or a cause of Q_γ, as discussed before by other investigators (Horowicz & Schneider, 1981; Hui, 1983; Melzer, Schneider, Simon & Szücs, 1986; Csernoch *et al.* 1988). These effects were observed in seven fibres with 1–10 μM- and in five fibres with 100 μM-ryanodine.

Charge movement blocked by ryanodine

To evaluate the components of the charge movement affected by ryanodine, the records in Fig. 4*A* illustrate the ryanodine-sensitive component which was obtained by subtracting the charge movement in ryanodine from that in the control. The fibre had been exposed to 5 μM-ryanodine for 20 min. The component blocked by ryanodine for potentials between −40 and −20 mV has the same characteristics as Q_γ, namely, it has a delayed onset and the peak is reached earlier with stronger depolarizations. This component is similar to the one blocked by repetitive stimulation of muscle fibres in the presence of high intracellular EGTA (García, Pizarro, Ríos & Stefani, 1991) or with caffeine (Kovács, Magyar, Csernoch & Szücs, 1990). For more positive potentials (−10 and 20 mV), the blockade of the upward component (Q_γ) is followed by a negative component that corresponds to an increase of charge in the presence of this drug. Ryanodine did not greatly affect the charge upon repolarization.

As mentioned before, the decay of the charge was slowed following the application of ryanodine. This can also be visualized from the different records in this figure as a downward deflection after the delayed component was blocked. The graphs in Fig. 4*B* show the total amount of Q_{on} (left graph) and Q_{off} (right graph) charge moved under control conditions (○) and after the addition of ryanodine (●) for the same fibre. Note that the values obtained for the Q_{on} charge with ryanodine are smaller for voltages from −60 to −30 mV. However, for larger potentials (0–30 mV) these values are greater than in the control. A two-state Boltzmann distribution of the form $Q = Q_{max}/(1+\exp(V_{\frac{1}{2}} - V/k))$ was fitted to the average of the charge Q_{on} for three different fibres. Q_{max} is the charge moved at saturation, $V_{\frac{1}{2}}$ is the mid-point potential, and k is the slope of the curve. The fitting gave the following values: control, $Q_{max} = 31{\cdot}9$ nC μF^{-1}, $V_{\frac{1}{2}} = -31{\cdot}8$ mV, $k = 14{\cdot}4$ mV; after ryanodine, $Q_{max} = 46{\cdot}8$ nC μF^{-1}, $V_{\frac{1}{2}} = -24{\cdot}8$ mV, $k = 14{\cdot}7$ mV. From these parameters and from the curve in the figure, it is clear that ryanodine caused a change in $V_{\frac{1}{2}}$ due to the blockade of Q_γ. The value of the charge blocked at −30 mV was $4{\cdot}8 \pm 1{\cdot}7$ nC μF^{-1} ($n = 3$). As a difference with the Q_{on} charge, the amount and voltage dependence of the Q_{off} charge was not modified.

Effect of tetracaine on charge movement and calcium transients

We further explored the action of tetracaine on charge movement to compare its effect with that of ryanodine. Tetracaine was used at 400 and 500 μM. With concentrations of 2 and 4 mM, as used in intact muscle fibres, we observed a reduction

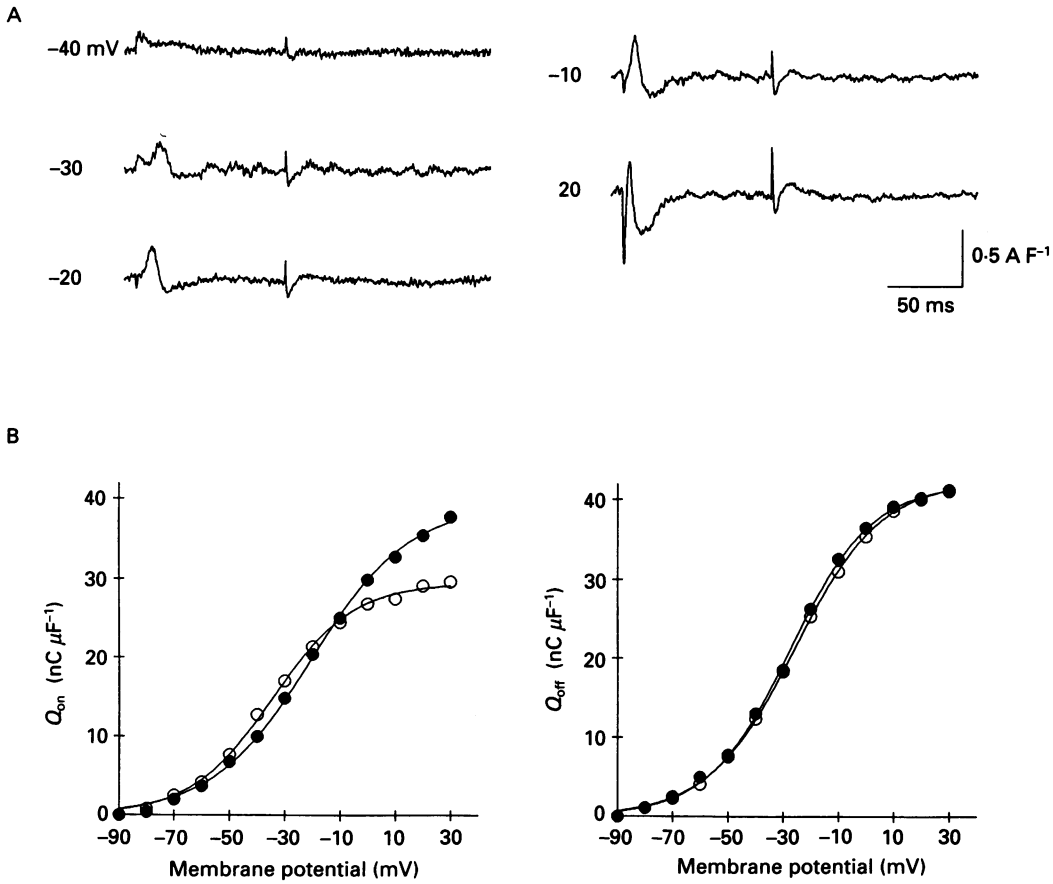

Fig. 4. Component of the charge movement blocked by ryanodine. *A*, the component of the charge sensitive to ryanodine was isolated by subtracting ryanodine from control records. In this case the fibre has been exposed to 5 μM-ryanodine for 20 min. *B*, charge–voltage relationships for the charge moved during the pulse (left) and after the pulse (right). ○, control. ●, charge recorded in the presence of ryanodine.

of 30–40 % in the membrane linear capacitance, which is in agreement with previous findings reported by Csernoch *et al.* (1988) in cut frog muscle fibres and by Lamb (1986) in rabbit fibres. Figure 5 shows typical records obtained from the same fibre under control conditions (left column) and immediately after the addition of 400 μM-tetracaine (middle column). Note the blockage of the delayed component Q_γ of the charge (−30 and −20 mV), and the reduction in the charge moved after the end of the pulses. The column on the right shows the components blocked by tetracaine (difference). The difference records showed: (1) at potentials from −40 to −20 mV, there is a blockade of the delayed charge component which is similar to the one found

with ryanodine at equivalent voltages (see Fig. 4); (2) at −10 mV tetracaine also blocked a faster component of the charge movement; (3) at more positive potentials the blocked charge components became indistinguishable by their time course due to merging of Q_β with a much faster Q_γ and (4) charge movement was always blocked upon repolarization. These effects were seen in three other fibres.

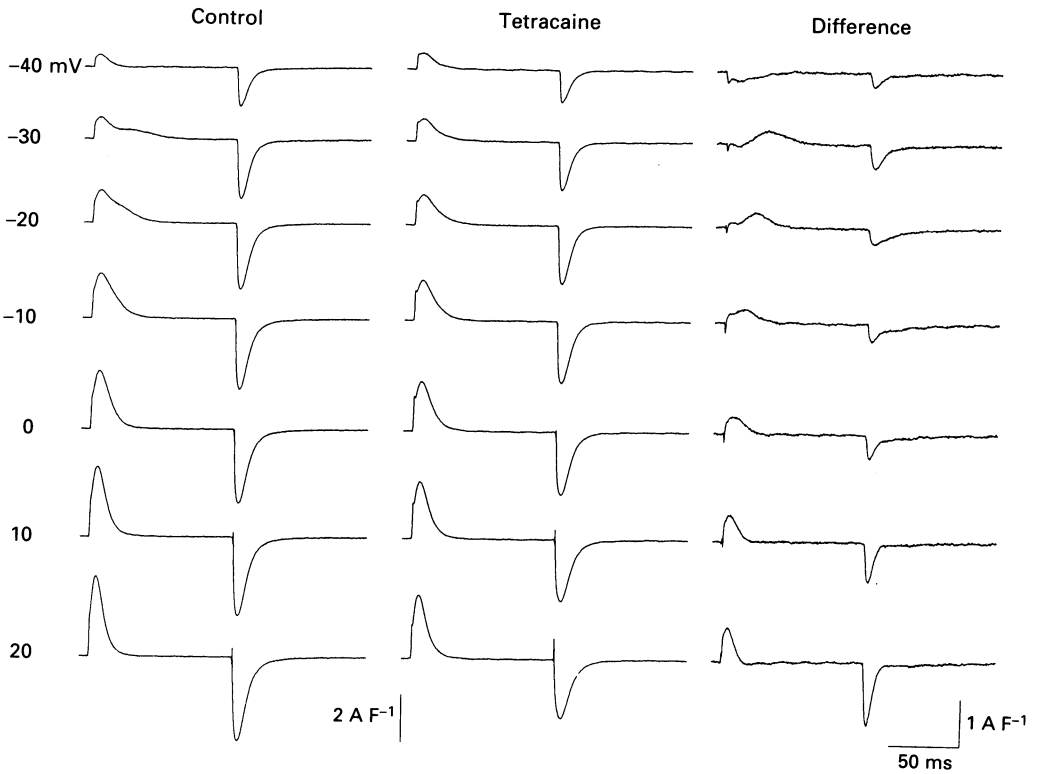

Fig. 5. Effect of tetracaine on charge movement. Control records of charge movement (left column) showing the Q_β and the Q_γ components. Immediately after the addition of 400 μM-tetracaine (middle column) the Q_γ component and an extra charge for larger potentials were blocked, as evidenced by the difference between the two sets of records, shown on the right column.

The reduction by tetracaine in the amount of charge moved for potentials larger than −30 mV is in agreement with previous reports (Vergara & Caputo, 1983; Lamb, 1986). Fitting the experimental data from four fibres to a Boltzmann distribution gave the following values for Q_{on} charges: control, $Q_{max} = 35{\cdot}4$ nC μF^{-1}, $V_{\frac{1}{2}} = -26{\cdot}9$ mV, $k = 12{\cdot}3$ mV; after tetracaine, $Q_{max} = 30{\cdot}7$ nC μF^{-1}, $V_{\frac{1}{2}} = -28{\cdot}9$ mV, $k = 15{\cdot}7$ mV.

Figure 6 illustrates the effect of tetracaine on charge movement (upper traces) and the corresponding calcium transient (lower traces) for pulses to −30 and −20 mV. Together with the elimination of the delayed component of the charge movement, we observed an abolition of the calcium transient immediately after the application of tetracaine. The charge blocked (right column) has the same characteristics as in the last figure. The effect of tetracaine on the calcium transient is different from the one

observed with ryanodine: the calcium transient increased in the presence of ryanodine in the first minutes after its addition and then slowly decreased until it became very small and remained in this new state (30–40 min).

In summary, ryanodine and tetracaine have differences in their action on charge movement. Tetracaine reduced at all potentials and to a similar degree the Q_{on} and

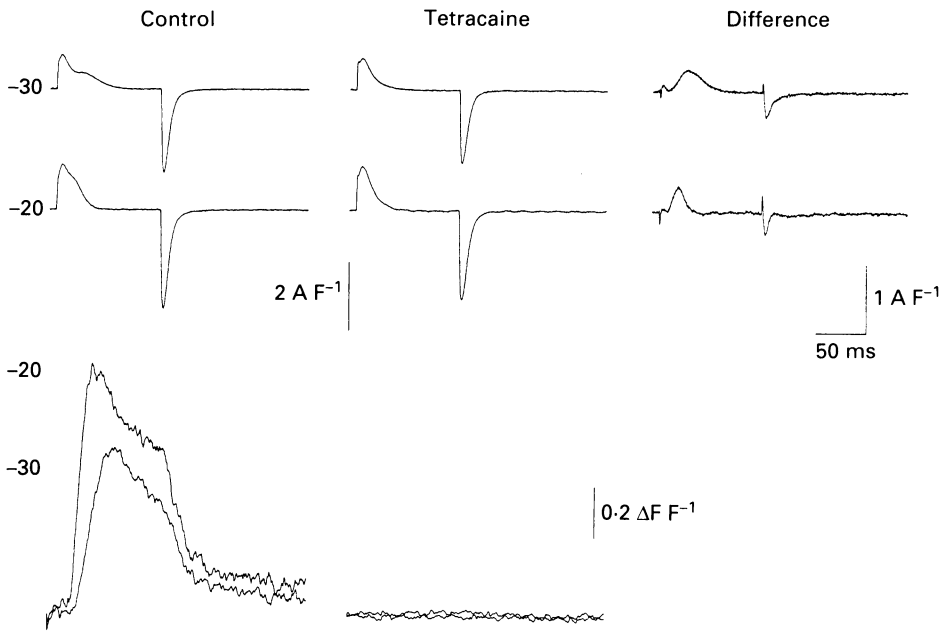

Fig. 6. Blockage of the calcium transient and Q_γ by tetracaine. Control records (left column) of charge movement at −30 and −20 mV showing the Q_β and Q_γ (upper traces) and calcium transients at the same voltages (lower traces) indicating that the calcium release process is active. After the addition of 400 μM-tetracaine (middle records) both the Q_γ component in the charge movement and the transient are completely blocked. The tetracaine-sensitive charge at these voltages is shown in the right column.

Q_{off} charge movement. On the other hand the action of ryanodine depended on the stimulating voltage. For potentials more negative than −30 mV it reduced, as tetracaine did, the delayed component of the Q_{on} charge movement which corresponds to Q_γ. At more positive potentials than −20 mV ryanodine increased the charge during the pulse. Ryanodine did not significantly affect the Q_{off} charge for all stimulating potentials.

Effect of ryanodine and tetracaine on the calcium currents

As part of the charge movement has been associated with the calcium channel gating current (Lamb & Walsh, 1987; Ríos & Brum, 1987; Tanabe *et al.* 1988), we studied the action of ryanodine and tetracaine on calcium currents. Figure 7 shows that 100 μM-ryanodine (upper traces) did not have any effect on the calcium current even 20 min after its addition.

On the other hand, tetracaine had a clear effect on the calcium currents (Fig. 7, lower traces). Soon (3 min), after the addition of 400 μM-tetracaine, the slow calcium

current was completely blocked and the fast low-threshold current became noticeable (Cota & Stefani, 1986). The fast calcium current was not affected after long exposure to tetracaine and it was later blocked by 1 mM-Cd^{2+}. Together with the blockade of the slow calcium current, the charge movement was reduced. The effect

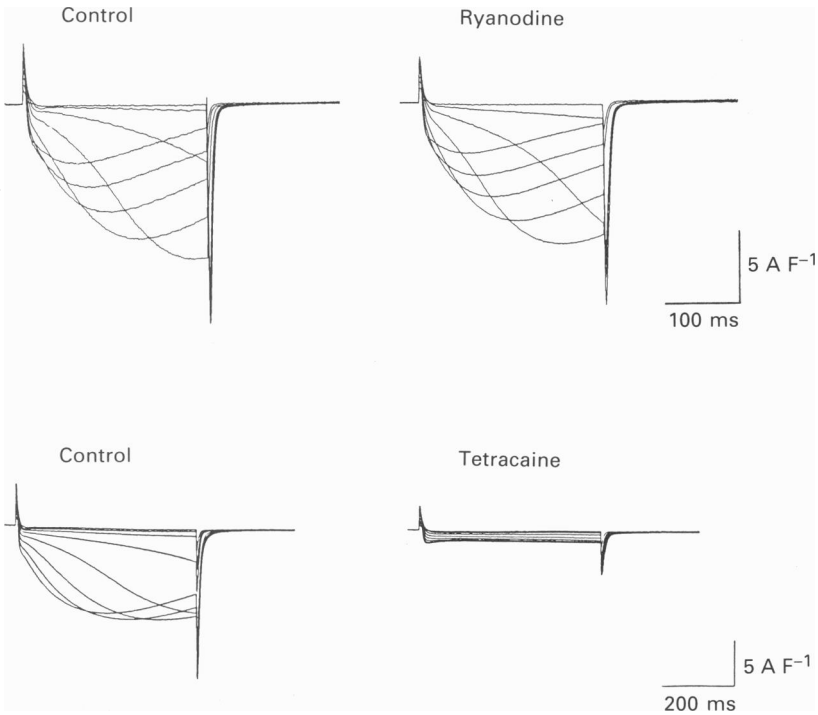

Fig. 7. Effect of ryanodine and tetracaine on the calcium currents. Calcium currents recorded in control conditions (left column) and after the addition of 100 μM-ryanodine (right, upper traces) or 400 μM-tetracaine (right, lower traces) in two different fibres. Ryanodine did not cause major modifications of the calcium current but blocked part of the charge movement. Tetracaine blocked some of the charge movement and fully inhibited the slow calcium current, leaving only the fast current which was later blocked by Cd^{2+}.

of tetracaine was reversible. These observations suggest different sites of action for tetracaine and ryanodine.

DISCUSSION

These experiments compared the effects of ryanodine and tetracaine on charge movement, calcium transients, and calcium currents in segments of skeletal muscle fibres voltage-clamped with the Vaseline-gap technique.

Ryanodine-sensitive charge movement

Ryanodine has a dual effect on skeletal muscle voltage-induced Ca^{2+} transients and the delayed component of charge movement: initially they are potentiated and after a period of incubation they are blocked (Figs 2 and 3). The blockade effect of

ryanodine was present at all the concentrations tested (1–100 μM), the effect being faster with higher concentrations (Fig. 3). The initial potentiation may be explained by the reported stimulating effect of lower ryanodine concentrations on the calcium release channel: an increase of the open probability with the establishment of a permanent long open state of lower conductance (Rousseau *et al.* 1987). Lower intracellular concentrations of ryanodine may be reached in single fibre experiments soon after the addition of this drug. Higher concentrations of ryanodine will finally block the Ca^{2+} release channel with the resulting reduction of the Ca^{2+} transient and blockade of the delayed component of the charge movement. This component had all the characteristics expected for Q_γ (Fig. 4); delayed onset after depolarizations, faster peak with stronger pulses, tetracaine sensitivity and similar voltage dependence (corrected for our extracellular concentration of divalent cations). The blockade of calcium release from the SR, monitored with the calcium-sensitive fluorescent dye, indicates that this component is closely associated with the gating of the SR Ca^{2+} release channel, and is in agreement with the inhibition by ryanodine of the calcium release from heavy SR vesicles (Meissner, 1986).

There are two current hypotheses about the nature of the charge movement components. The first one postulates that Q_β and Q_γ originate from a different subset of voltage sensors (cf. Huang & Peachey, 1989), while the other one proposes that both Q_β and Q_γ arise from the same voltage sensors and that Q_γ is a consequence of calcium release, calcium causing a local neutralization of negative charges on the myoplasmic side of the transverse tubular membrane. The decay phase and the negative deflection of Q_γ would correspond to the reduction in the local Ca^{2+} concentration which will reduce the local transmembrane potential (Csernoch, Uribe, Rodríguez, Pizarro & Ríos, 1989; Pizarro, Csernoch, Uribe, Rodríguez & Ríos, 1991). In agreement with this hypothesis, García *et al.* (1991) showed that repetitive stimulation with high intracellular EGTA blocked the SR Ca^{2+} release process and the delayed component of the charge movement that had the characteristics of Q_γ. In the event that only one set is present, all the voltage-sensitive particles would be in the 'activated' state for membrane potentials larger than 0–10 mV, where Q_{max} is reached. Thus, calcium release from the SR would not cause extra charge movement due to local depolarization and Q_γ would become smaller at these potentials.

Our results show that the component of charge movement blocked by ryanodine has a similar voltage dependence to that expected for Q_γ, and it can be speculated then, that the blockage of the calcium release by ryanodine may be similar to the effect of high intracellular EGTA. As in the presence of high intracellular EGTA, the action of ryanodine was predominant on the charge movement during the pulse, while the charge at the end of the pulse was unaffected. The increase in the amount of charge moved at potentials more positive than -10 mV caused by ryanodine may be tentatively explained by the presence of a negative phase following the decay of the charge movement. This downward deflection of the charge movement has been attributed to the removal of Ca^{2+} from a Ca^{2+} binding site located on the cytoplasmic side of the voltage sensor; thus after the blockade of Ca^{2+} release by high intracellular EGTA and ryanodine, Q_γ is blocked and the decay of the charge becomes slower, and for large depolarizations the absence of the negative phase in the charge movement results in an increase in measured charge (Csernoch, Pizarro, Uribe, Rodríguez & Ríos, 1991; García *et al.* 1991; Pizarro *et al.* 1991).

Another possibility to explain the effect of ryanodine would be the existence of two subsets of voltage sensors and that the one responsible for Q_γ is coupled to the ryanodine receptor in such a way that when the receptor is 'locked' in the closed state, the sensor would become immobile. However, with these experiments we cannot discriminate between these two possibilities.

Tetracaine-sensitive charge movement

In this paper we confirmed previous reports on the blocking effect of the delayed component of the charge movement (Q_γ) by tetracaine in the cut fibre preparation (Fig. 5; Vergara & Caputo, 1983; Csernoch *et al.* 1988). It was also seen that high concentrations of tetracaine (2–4 mM), like those used in intact fibres, are toxic for this preparation. The same results have been obtained in mammalian muscle, although tetracaine blocks Q_γ only in hypertonic solutions (Lamb, 1986; Hollingworth *et al.* 1990).

The question remains whether tetracaine, in addition to the blockade of Q_γ, blocks some other component of charge movement (e.g. Q_β). At potentials (< -30 mV) where the delayed component is evident, its action on the Q_{on} is indistinguishable from the one of ryanodine and high intracellular EGTA (García *et al.* 1991) and it could be explained by a blockade of the calcium release channel. However, the fact that tetracaine also reduced Q_{off}, suggests that it must directly act on the macromolecules responsible for the charge movement. In keeping with this line of thinking, tetracaine at similar concentrations also blocked the dihydropyridine (DHP)-sensitive tubular Ca^{2+} current (see next section).

As opposed to the effect of ryanodine on the calcium transient, tetracaine inhibited the calcium release immediately after its addition to the extracellular solution. This may be due to the slow association rate of ryanodine with its receptor, as shown in binding studies (Pessah, Francini, Scales, Waterhouse & Casida, 1986). Furthermore, tetracaine suppressed the calcium transient completely in a reversible way.

Differential effect of ryanodine and tetracaine on the calcium current

Ryanodine did not have any effect on the calcium currents even after 20 min (Fig. 7). This lack of effect is contradictory to the results obtained in single-channel recording from rabbit tubular calcium channels incorporated into planar lipid bilayers (Valdivia & Coronado, 1989). In those experiments, ryanodine inhibited the opening of DHP-sensitive calcium channels activated by the agonist Bay K 8644. The discrepancy may be due to the use of a different species (amphibian *vs.* mammalian) and electrophysiological technique (Vaseline seal *vs.* lipid bilayer).

Opposite to ryanodine, tetracaine totally blocked the DHP-sensitive slow calcium current in a reversible way. The differential effect of tetracaine and ryanodine on calcium currents supports the view that ryanodine is mainly acting on the calcium release channel while tetracaine, in addition to the calcium release channel, also acts on membrane macromolecules associated with charge movement. Furthermore, the fact that ryanodine can block the Q_γ component without affecting the calcium current suggests that Q_γ is not associated with the calcium current gating charge. Recent evidence strongly indicates that skeletal muscle DHP-receptors are the macromolecular basis for the DHP-sensitive calcium current and charge movement and that a fraction of the charge movement might be the gating charge of the tubular

calcium channel (Lamb & Walsh, 1987; Ríos & Brum, 1987; Tanabe *et al.* 1988). The reported blockade by tetracaine of the calcium current and charge movement suggests that a tetracaine-sensitive component other than Q_γ might be associated with the gating of the calcium channel.

In summary, the reported findings can be explained in the following way: (1) ryanodine affects Q_γ which is a consequence of SR calcium release; (2) tetracaine has a dual effect; it blocks the SR calcium release channel as ryanodine and it also has a direct effect on charge movement associated with the DHP-receptor, which would be part of the voltage sensor for excitation–contraction coupling and the gating charge of the calcium current.

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