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DIFFERENTIAL EFFECTS OF TETRACAINE ON TWO KINETIC COMPONENTS OF CALCIUM RELEASE IN FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Intramembrane charge movements and changes in intracellular calcium concentration were recorded simultaneously in voltage clamped cut skeletal muscle fibres of the frog in the presence and absence of tetracaine.

2. Extracellular application of 20 μM tetracaine reduced the increase in myoplasmic [Ca^{2+}]. The effect on the underlying calcium release flux from the sarcoplasmic reticulum was to suppress the peak of the release while sparing the steady level attained at the end of 100 ms clamp depolarizations.

3. While the peak of the release flux at corresponding voltages was reduced by 62% after the addition of tetracaine, the rate of inactivation was the same when the pulses elicited release fluxes of similar amplitude.

4. Higher concentrations of tetracaine, 0·2 mM, abolished the calcium signal in stretched fibres whereas in slack fibres this concentration left a non-inactivating calcium release flux.

5. Lowering the extracellular pH antagonized the effect of the drug both on charge movements and on calcium signals. The permanently charged analogue tetracaine methobromide lacked effects on excitation–contraction coupling.

6. These results imply that the two kinetic components of calcium release flux have very different tetracaine sensitivities. They are also consistent with an intracellular site of action of the drug at low concentration. Taken together they strongly suggest that the inactivating and non-inactivating components of calcium release correspond to different pathways: one that inactivates, is sensitive to tetracaine and is controlled by calcium, and another that does not inactivate, is much less sensitive to tetracaine and is directly controlled by voltage.

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INTRODUCTION

The local anaesthetic tetracaine is a well-known inhibitor of excitation–contraction (EC) coupling events in skeletal muscle. Applied in the millimolar range this drug suppresses K^+-induced contractures in intact fibres (Caputo, 1972). In the same preparation tetracaine treatment was found to abolish the delayed component of charge movement, termed Q_γ (Huang, 1980, 1981; Hui, 1982). This finding originated the notion that this abolition was at the core of the excitation–contraction coupling effect of the drug. Q_γ suppression was also observed in cut fibres (Vergara & Caputo, 1983). When myoplasmic Ca^{2+} transients were recorded simultaneously they were also suppressed by the drug (Vergara & Caputo, 1983; Csernoch, Huang, Szücs & Kovács, 1988; Csernoch, Pizarro, Uribe, Rodríguez & Ríos, 1991). On the basis of these dual effects the view has emerged that tetracaine is a specific inhibitor of a voltage sensor underlying Q_γ; this voltage sensor is necessary for release and does not work when bound to tetracaine, hence the inhibition of calcium release.

Studies on subcellular preparations of skeletal muscle, however, suggest a different mechanism. In skinned fibres tetracaine inhibits calcium-induced calcium release, and does it much more effectively than procaine (Endo, 1985). In isolated triads tetracaine inhibits release independently of the triggering stimulus (Antoniu, Kim, Morii & Ikemoto, 1985). Tetracaine has also been found to reduce the probability of the open state of the calcium release channel reconstituted in bilayers (Xu, Jones & Meissner, 1990; Bull & Marengo, 1990). This suggests that the EC coupling inhibition by tetracaine is primarily a block of the release channel, rather than an effect on the voltage sensor.

How can a primary block of calcium release explain the effect on Q_γ? Recent observations of suppression of Q_γ by primary antagonists of calcium release suggest that Q_γ is a consequence of calcium release (Csernoch *et al.* 1991; García, Pizarro, Ríos & Stefani, 1991; Szücs, Csernoch, Magyar & Kovács, 1991; Pizarro, Csernoch, Uribe, Rodríguez & Ríos, 1991). The proposed mechanism assumes the existence of Ca^{2+}-binding sites, close to the voltage sensors, on the myoplasmic face of the transverse-tubular membrane. Ca^{2+}, released from the sarcoplasmic reticulum, binds to these sites and causes an increase in local potential, which determines the delayed component of charge movement. In this view, the effect of tetracaine on Q_γ is a predictable consequence of its release-blocking action.

Here we report a study of the effect of tetracaine on the calcium transient and the underlying release flux in voltage clamped frog cut fibres. The present studies were carried out with lower concentrations of tetracaine than used in the classical studies. The use of these lower concentrations allowed us to demonstrate that the two components of calcium release flux, the inactivating peak and the maintained steady level (Melzer, Ríos & Schneider, 1984), have different sensitivities to tetracaine. We also provide evidence of an intracellular site of action of the drug. An abstract on some aspects of these results has been published (Pizarro, Csernoch, Uribe & Ríos, 1989).

METHODS

The experiments presented in this paper were carried out on cut segments of fast twitch skeletal muscle fibres singly dissected from the semitendinosus muscle of the frog *Rana pipiens*. Frogs were

kept in an aquarium at 15 °C and fed regularly with live crickets. Before muscle dissection they were killed by decapitation under deep anaesthesia (achieved by immersion in water plus 10% ethanol). Singly dissected fibres were voltage clamped in a double Vaseline-gap apparatus. Intramembrane charge movement currents and increases in myoplasmic [Ca^{2+}] (calcium transients), elicited by depolarizing pulses from a negative holding potential (V_h, −90 mV) were measured simultaneously. The fibres were mounted stretched to 4 μm per sarcomere, to avoid the contraction that would otherwise have been caused by the calcium transients. The calcium transients were monitored optically with the absorbance dye Antipyrylazo III (Ap III) diffused into the fibres from the cut ends. Details of the technique for recording charge movements and calcium transients, as well as for pulse generation and data acquisition, have been given elsewhere (Kovács, Ríos & Schneider, 1983; Brum & Ríos, 1987; Brum, Ríos & Stefani, 1988). Calcium release flux was calculated from the recorded calcium transients by the method of Melzer, Ríos & Schneider (1987) modified by Brum, Ríos & Schneider (1988). Release flux records were corrected for depletion of calcium in the sarcoplasmic reticulum (SR) (Schneider, Simon & Szücs, 1987).

The external solution, bathing the working fibre segment in the middle pool, contained (mM): 126 TEA CH_3SO_3; 2 $Ca(CH_3SO_3)_2$ or $Cd(CH_3SO_3)_2$; and 5 TEA Tris-maleate. Both solutions contained in addition 1 μM TTX and 1 mM 3,4-diaminopyridine. The Cd^{2+}-containing solution also had 0·1 mM La $(CH_3SO_3)_3$ and 0·5 mM anthracene-9-carboxylic acid. These solutions are respectively termed '2 Ca' and 'Cd-La-A9C'. The internal solution, bathing the cut fibre ends contained (mM): 102·5 caesium glutamate; 5·5 $MgCl_2$; 5 ATP (as sodium salt); 4·5 sodium Tris-maleate; 13·2 caesium Tris-maleate; 0·1 EGTA; 0·8 Ap III and 1 g l^{-1} of glucose. $CaCl_2$ (8 μM) was added for a nominal 50 nM [Ca^{2+}]. The pH was adjusted (to 7·0 when not stated otherwise) by the addition of TEAOH or HCH_3SO_3. The temperature was 13 or 14 °C.

RESULTS

Low tetracaine suppresses the peak of calcium release

Early in our work (Csernoch *et al.* 1988, 1991) we realized that concentrations of tetracaine much lower than those used in previous studies were sufficient to cause large effects. Figure 1 shows the effect of 20 μM tetracaine applied extracellularly on myoplasmic calcium transients (records at top) and calculated release flux (bottom), elicited by a depolarizing clamp pulse to −40 mV (left), or to +20 mV. (The calculation of release fluxes involves estimating, by procedures described before, parameters that characterize the processes that remove calcium from the myoplasm. The parameter values that best described the removal processes in reference (legend of Fig. 1) also worked well in the presence of tetracaine, indicating that the drug had no significant effects on calcium removal.) Release flux records were corrected for SR depletion (Schneider *et al.* 1987). The main effect of tetracaine is a selective reduction of the inactivating (or peak) component of release. The final level of release flux at −40 mV was the same before and after the application of tetracaine. For the bigger depolarization the value of the release flux at the end of the pulse was greater in tetracaine, but the steady level of flux had not been reached by the end of the shorter pulse.

Averaged results from three fibres are plotted in Fig. 2. The steady level was measured at the end of a 100 ms depolarization by taking the mean of the values from the last 5 ms. Since the pulses to higher voltages were of 50 ms or shorter, the graph of steady levels (at bottom) extends over a more limited range of voltages than the graph of peaks (at top). Peak calcium release flux was reduced by nearly the same factor at every voltage tested while the steady level was not affected.

The results shown in Fig. 2 were confirmed in two other fibres studied in a narrower voltage range. The effects of 20 μM tetracaine on release waveforms of similar peak value (near 4 or 5 μM ms^{-1}) in five different cells are listed in Table 1. The peak was

greatly reduced (62% on average) while the effect on the final level was not statistically significant. We recently reported that the same concentration of tetracaine, 20 μM, suppressed a relatively small amount of charge movement with a delayed time course that we identified as the Q_γ component (Csernoch *et al.* 1991; Szücs *et al.* 1991).

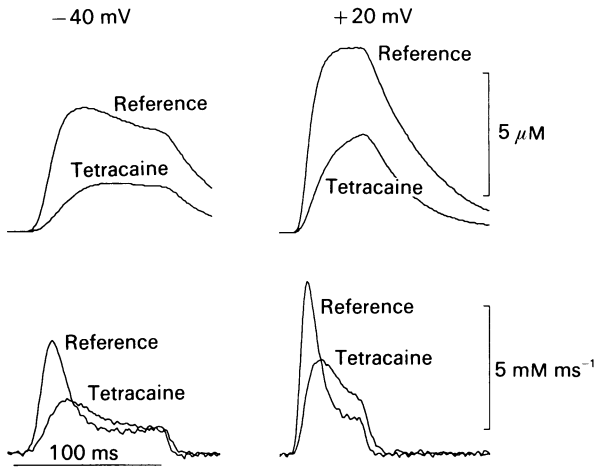

Fig. 1. Effect of 20 μM tetracaine on calcium transients and calcium release flux. Top records are myoplasmic calcium transients obtained at two different voltages before the application and in the presence of tetracaine, bottom records are the corresponding calcium release fluxes. Responses elicited by a clamp depolarization to −40 mV (left), or a depolarization to +20 mV. Fibre 497; external solution, Cd-La-A9C; linear capacitance, 9·9 nF; diameter, 74 μm; sarcomere length, 4·1 μm; temperature, 14 °C. The [dye] was between 696 and 784 μM when the reference records were obtained and between 1160 and 1210 μM when the records in tetracaine were obtained. The release flux was calculated as described in the Methods with the following parameter values, valid for all fibres in the present paper: $k_{off,CaTN}$, 1200 s^{-1}; $k_{on,CaTN}$, 125 $\mu M^{-1} s^{-1}$; $k_{on,CaParv}$, 100 $\mu M^{-1} s^{-1}$; $k_{on,MgParv}$, 0·03 $\mu M^{-1} s^{-1}$; $[Ca^{2+}]_i$, 50 nM; [TN], 240 μM; $[Mg^{2+}]$, 900 μM. Other parameters were selected with least-squares computer procedures to best fit the 'offs' of sets of calcium transients, and had the following values: $k_{off,CaParv}$, 0·84 s^{-1}; $k_{off,MgParv}$, 1·65 s^{-1}; M, 1200 $\mu M\ s^{-1}$; $K_{D,pump}$, 1 μM; [Parv], 1200 μM. Abbreviations used here and throughout are as follows: Parv, parvalbumin; TN, troponin; M, maximum pump transport rate; $K_{D,pump}$, dissociation constant of Ca^{2+}-binding sites on the pump; k_{on} and k_{off}, rate constants of the reactions indicated with subscripts.

High tetracaine spares a component of Ca^{2+} release

A higher concentration of tetracaine (0·2 mM) was tried in five fibres stretched to more than 4 μm per sarcomere; this treatment completely abolished the calcium transient.

The same concentration of tetracaine was also applied to slack fibres that were able to contract before the application of the drug. Figure 3 shows a typical experiment in a slack fibre. In these fibres the calcium transients were drastically reduced but not completely abolished. The traces on the left were taken before the drug was applied; contractile movement, causing a downward artifact in the record at −65 mV (thicker trace), prevented the application of pulses to a higher voltage. Traces on the right were obtained in the presence of 0·2 mM tetracaine. Substantial calcium transients were recorded for pulses positive to −60 mV. With high voltage pulses the

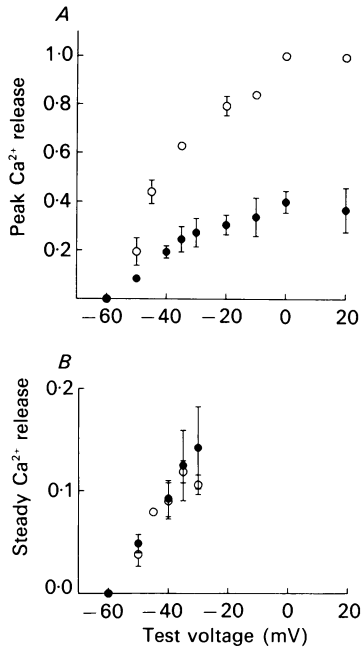

Fig. 2. Voltage dependence of calcium release flux in reference and tetracaine-treated conditions. *A*, peak of calcium release flux measured before the application of 20 μM tetracaine (○) and in the presence of the drug (●) plotted against test pulse voltage. *B*, steady release flux measured as the average during the last 5 ms of the pulse. Points are averages of measurements in three fibres, error bars represent the S.E.M. All values in each fibre are normalized to the peak release flux at 0 mV in reference. Fibres 496, 497, 498, all in external solution Cd-La-A9C at 14 °C. Data for 496 given in Fig. 5, for 497 in Fig. 1. Fibre 498: linear capacitance, 8·8 nF; diameter, 72 μm; sarcomere length, 4·0 μm; $k_{off,CaParv}$, 1·00 s^{-1}; $k_{off,MgParv}$, 7·67 s^{-1}; M, 390 $\mu M\ s^{-1}$; $K_{D,pump}$, 1 μM; [Parv], 847 μM.

TABLE 1. Effect of tetracaine on release flux at different test potentials

	Reference			20 μM tetracaine	
Fibre (mV)	V_{test}	Peak	Steady level ($\mu M\ ms^{-1}$)	Peak	Steady level ($\mu M\ ms^{-2}$)
486	−40	3·7	0·69	2·19	0·64
496	−50	4·9	1·03	1·77	0·66
497	−40	4·51	0·92	2·15	1·04
498	−45	4·07	0·69	1·02	0·55
541	−55	5·4	0·71	1·39	0·72
Average		4·56	0·81	1·7	0·72
S.E.M.		0·33	0·08	0·25	0·09

Pulses (100 ms long) to the test potential (V_{test}) indicated in the second column were applied to the fibres immediately before and immediately after drug application. The steady level was measured as the average of the calcium release flux waveform during the last 5 ms of the pulse. Their means before and in the presence of the drug were not statistically different (t test, $P > 0·4$). Fibre 486; external solution, Cd-La-A9C; linear capacitance, 12·4 nF; diameter, 84 μm; sarcomere length, 4·3 μm; temperature, 13 °C; $k_{off,CaParv}$, 1·00 s^{-1}; $k_{off,MgParv}$, 4·34 s^{-1}; M, 887 $\mu M\ s^{-1}$; $K_{D,pump}$, 1 μM; [Parv], 500 μM. Data for the other fibres given in legends of Figs 1, 2 and 6.

calcium level attained was high enough to elicit contraction, as revealed by downward movement artifacts at −10 and −20 mV. The movement was visible through the microscope.

Most interestingly, the calcium transients left in 0·2 mM tetracaine had a characteristic ramp-like time course. As shown in Fig. 3, right panel, at all the

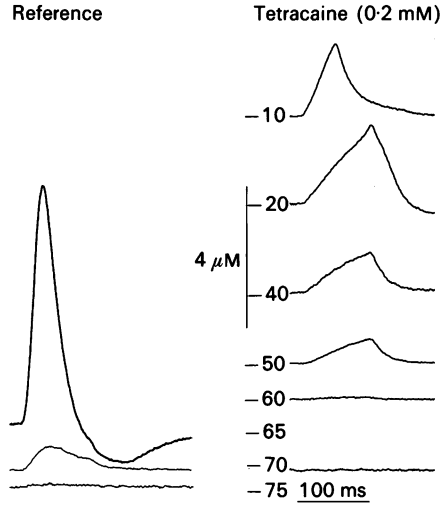

Fig. 3. Calcium transients in the presence of 0·2 mM tetracaine in a slack fibre. Left panel (reference), calcium transients measured before drug application. Since the fibre moved with a depolarization to −65 mV (thicker trace), larger pulses were not applied. Records in right panel were obtained after the application of 0·2 mM tetracaine. Pulse voltages are listed near the records (no pulses to −75 and −65 mV were applied in tetracaine). Fibre 452; external solution, 2 Ca; linear capacitance, 17·9 nF; diameter, 110 μm; sarcomere length, 2·6 μm; temperature, 13 °C. The [dye] was between 182 and 412 μM.

voltages tested the calcium transient continued to rise at an almost steady rate throughout the pulse. This was in sharp contrast with the transients before drug treatment (left), which went through a maximum and then declined during the pulse. Note that this was the case even for the calcium transient elicited by the pulse to −70 mV, which had no movement artifacts. As shown in Fig. 4*A*, in this particular record $[Ca^{2+}]$ was lower than that reached at −20 mV in the presence of tetracaine, in which the calcium transient did not decline until the pulse was terminated.

This dramatic change in calcium transient kinetics corresponds to large changes in the kinetics of calcium release. Figure 4*B* shows the calcium release flux waveforms calculated from the calcium transient at −70 mV before drug treatment and at −40 and −20 mV in the presence of tetracaine. The reference release waveform had the typical early peak followed by a relaxation to a lower level.

It is likely that the phase of inactivation following the peak of calcium release is mediated by the increase in myoplasmic $[Ca^{2+}]$ (Schneider & Simon, 1988; Simon, Klein & Schneider, 1991). However, the release waveforms in the presence of tetracaine did not show a clear inactivation even though the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) reached was somewhat bigger for the pulse to −40 mV in tetracaine than at

−70 mV in reference. Therefore, the lack of inactivation in tetracaine could not be the consequence of a lower $[Ca^{2+}]_i$ as reported by the dye.

Since the dye reports an average $[Ca^{2+}]_i$ and the local $[Ca^{2+}]$ near the release sites is probably much greater (Pizarro *et al.* 1991) it is still possible that the local $[Ca^{2+}]_i$

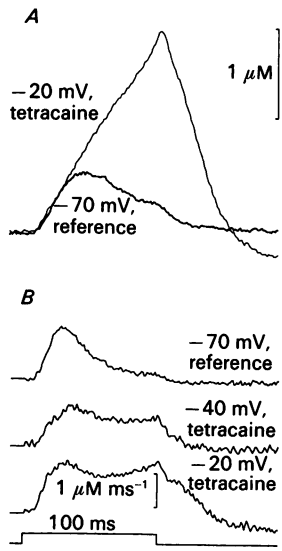

Fig. 4. Calcium release flux in the presence of 0·2 mM tetracaine. *A*, superimposed calcium transients elicited by a pulse to −70 mV before the drug was applied and to −20 mV in the presence of tetracaine, showing that the initial rate of rise is roughly the same for both transients. *B*, calcium release flux calculated from the records in *A*. The release flux in response to a pulse to −40 mV in 0·2 mM tetracaine is also shown. Same fibre and calcium transients as in Fig. 3. $k_{off,CaParv}$, 1·00 s^{-1}; $k_{off,MgParv}$, 2·50 s^{-1}; M, 1910 μM s^{-1}; $K_{D,pump}$, 1 μM; [Parv], 873 μM.

was greater in reference, thus justifying the greater inactivation of calcium release. Csernoch *et al.* (1991) have shown under simple assumptions that the maximum local $[Ca^{2+}]_i$ near the release sites, the peak of calcium release flux and the maximum of the time derivative of the $[Ca^{2+}]_i$ reported by the dye are all roughly proportional. The calcium transients at −70 mV in reference and at −20 mV in tetracaine are plotted together in Fig. 4*A* to show that their maximum rate of rise is approximately the same. Hence, the local $[Ca^{2+}]_i$ could not have been very different in these cases. Thus the absent or reduced inactivation was not due to lower calcium levels attained in tetracaine-treated fibres. Records with similar characteristics were recorded in five other fibres.

Low tetracaine does not affect inactivation

The results presented so far amount to a pharmacological difference between the inactivating and the steady parts of the release waveform and suggest (hypothesis 1), that the different components occur through physically distinct release pathways – different channels or separately gated processes in the same channel.

An alternative interpretation (hypothesis 2) is that the drug has two different actions: to block the release channel with low affinity and to hinder the inactivation process with higher affinity, thus those channels which are not blocked inactivate less. In this hypothesis the equality of maintained levels of release in reference and in the presence of 20 μM tetracaine is fortuitous.

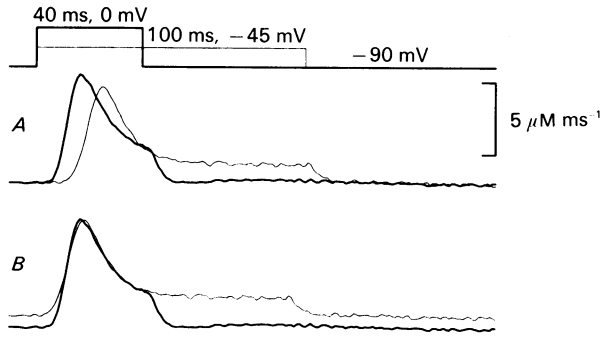

Fig. 5. Inactivation of calcium release flux in 20 μM tetracaine. Top, voltage command pulses. *A*, calcium release flux in reference (thin trace) and in 20 μM tetracaine. The pulses, represented schematically at the top, were selected to elicit release fluxes with a similar amplitude of the inactivating component. *B*, the release flux in reference was shifted by 7 ms to earlier in time and by 0·8 μM ms^{-1} to compare the inactivation phases of both records. External solution, Cd-La-A9C; linear capacitance, 12·5 nF; diameter, 82 μm; sarcomere length, 4 μm; temperature, 4 °C. Same fibre as in Figs 1 and 2 of Csernoch *et al.* (1991); the reference record is the same as the record at −45 mV in Fig. 1 of that paper; the corresponding calcium transient is in Fig. 2 of the same paper and the model parameters used to calculate release from the calcium transient are listed in Table 2 of Csernoch *et al.* (1991).

Experiments were carried out to decide between the two hypotheses. The essence of hypothesis 2 is that tetracaine blocks at high concentration and slows inactivation at low concentration. A simple prediction is that records with similar amplitude of the inactivating component should inactivate more slowly in tetracaine. Hypothesis 1 predicts instead that channels not blocked should be similar to reference channels. These predictions were tested comparing release records obtained with pulses that were high enough in tetracaine to match their inactivating portion with reference records at a lower voltage. One such pair is shown in Fig. 5*A*, the pulses used are schematically represented at the top. The pulse to 0 mV in 20 μM tetracaine (thick traces) elicited a release with a slightly greater peak than the pulse to −45 mV in reference. The difference in peak height, however, seemed to match almost exactly the difference in steady component, consequently the amplitude of the inactivating component was very similar in the two situations. This is demonstrated in panel *B* where the reference record has been shifted both vertically and horizontally to allow better comparison of its inactivating portion with that of the record in tetracaine. The almost perfect superposition of inactivation (found also in the other two experiments where adequate pulses were applied) demonstrates that the release channels not blocked by the drug inactivate with normal kinetics, and is consistent with hypothesis 1 and inconsistent with hypothesis 2.

The effect of tetracaine is intracellular

The fact that tetracaine blocks only one of the two kinetic components of release, even though both components are voltage dependent, contradicts a mechanism in which tetracaine affects release as a consequence of blockade of charge movement at

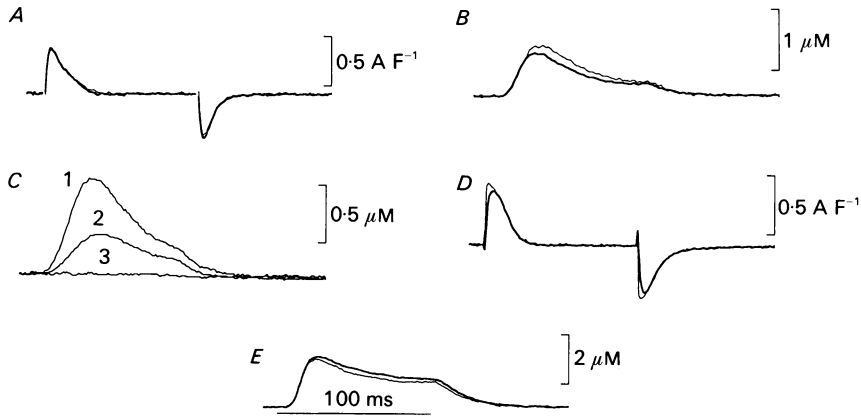

Fig. 6. *A* and *B*, effect of 0·2 mM tetracaine at extracellular pH of 5·3. Intramembrane charge movement currents (*A*) and calcium transients (*B*), in reference (thin traces) and in the presence of 0·2 mM tetracaine at extracellular pH of 5·3 (thick traces). Fibre 541; external solution, 2 Ca; linear capacitance, 11 nF; diameter, 80 μm; sarcomere length, 3·8 μm; temperature, 13 °C; [dye], 471 μM. *C*, effect on calcium transients of the same concentration of tetracaine at pH 5·8. Records labelled 1 and 2 correspond to reference and drug-treated conditions respectively. Record 3 was obtained in 0·2 μM tetracaine at extracellular pH 7·0. Fibre 532; external solution, 2 Ca; linear capacitance; 9·14 nF; diameter, 75 μm; sarcomere length, 3·6 μm; temperature, 13 °C; [dye] between 409 μM (record 1) and 469 μM (3). *D* and *E*, effect of 0·2 mM tetracaine methobromide applied extracellularly. Intramembrane charge movements (*D*) and calcium transients (*E*), in reference (thin traces) and in the presence of the drug (thick traces). Fibre 539; external solution, 2 Ca (pH 7); linear capacitance, 7·98 mF; diameter, 70 μm; sarcomere length, 3·9 μm; temperature, 13 °C; [dye], 344 μM (in reference) and 389 μM.

the voltage sensors, and is more simply explained as a primary intracellular effect on release channels. It was therefore of interest to attempt to localize the binding site responsible for the low concentration effects of tetracaine.

Tetracaine is a weak base with a pK ($-\log$ of the deprotonation equilibrium constant) of 8·9, thus, at the pH of our external solution 80% of the drug is protonated. If the effect was intracellular or required partition in the membrane lipid, it would depend on the concentration of the neutral form of the drug. Acidification of the external saline should then antagonize the effect of tetracaine. We tested this prediction in several experiments represented in Fig. 6. Records in panels *A* and *B* were taken at an extracellular pH of 5·3, both before (thin traces) and during drug treatment (thick traces); 0·2 mM tetracaine at this pH had no effect on charge movement currents (*A*) and a minor effect on the calcium transients (*B*).

Calcium transients recorded in a different fibre at higher pH levels are shown in panel *C*. Record 1 was measured in reference solution at pH 5·8, then 0·2 mM

tetracaine was applied at the same pH and record 2 was obtained. Record 3 is the calcium transient in the same fibre after the pH was changed to 7·0 in the presence of the same concentration of tetracaine. The last intervention completely abolished the calcium transient (as normally observed for this concentration and pH in stretched fibres). At pH 5·8 the calcium transient was reduced less than at 7·0 but more than in the experiments at 5·3 (panel *B*). Experiments with 0·2 mM tetracaine were carried out at pH 5·8 and 7·0 in three fibres, with similar results.

In an independent set of experiments we studied the effect on charge movement and calcium transients of tetracaine methobromide (4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ester methobromide), a permanently charged analogue of tetracaine (kindly provided to us by Dr Jonathan Cohen, Washington University, St Louis). This drug, added to the extracellular solution, had no significant effect on any of these phenomena in any of the three fibres in which it was tested. Representative records of charge movement and Ca^{2+} transients are shown in Fig. 6*D* and *E*.

It is interesting to note that 0·2 mM tetracaine under these conditions (low pH) had no significant effect on charge movement currents (Fig. 6*A*), suggesting that if there is a direct effect of the drug on the voltage sensor the site is reached through the membrane lipid phase.

The experiments in this group therefore indicate that the primary site of tetracaine action at these lower concentrations is either intracellular or is accessed from the lipid phase.

DISCUSSION

Two distinct pathways of calcium release

This work provides indirect evidence of the existence of two separate components in calcium release. One of them is tetracaine sensitive, is responsible for the peak of the release waveform and inactivates. The other is spared by this drug at lower concentrations, and is still clearly detectable at higher concentrations of drug in slack fibres.

The observation of a ramp-like transient of free $[Ca^{2+}]$ in tetracaine, which can be interpreted as the persistence of a maintained component of release flux, is consistent with earlier observations by Almers (1976) and Almers & Best (1976) on the effects of the drug on contraction of single fibres. The tetracaine-sensitive component of calcium release flux is, in all likelihood, calcium-induced calcium release. The pharmacology of this process observed in skinned fibres, isolated triads and reconstituted channels, argues strongly in this direction. Most importantly, Jacquemond, Csernoch, Klein & Schneider (1991) showed that intracellular injection of the fast calcium buffer bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) in frog cut fibres abolishes the peak of calcium release and spares the non-inactivating component. The effects of BAPTA are remarkably similar to those of tetracaine reported in this paper and both sets of experiments paint a consistent picture of two release processes, separately controlled by voltage and Ca^{2+}. In apparent contradiction, Baylor & Hollingworth (1988) found that the injection of fura-2 increased Ca^{2+} release in intact fibres. This result may be reconciled, however, as the total buffer concentration attained was substantially less than that used by

Jacquemond *et al.* (1991), and presumably insufficient to attenuate the local increase in $[Ca^{2+}]_i$ to a level that does not induce Ca^{2+} release.

Ríos & Pizarro (1988) proposed that these two release processes correspond to physically different channels, based on the peculiar stoichiometry suggested by Block, Imagawa, Campbell & Franzini-Armstrong (1988) of calcium release channels facing '*jT* tetrads' (putative voltage sensors) alternating with channels without tetrads. In their view, the channels facing tetrads would be under direct control by transverse-tubular membrane voltage, whereas the others would be controlled by Ca^{2+}. The first set of channels would open first and remain open for the duration of the pulse, originating the maintained component of release. The second set would be activated by Ca^{2+} released through the first set, and would also undergo Ca^{2+}-dependent inactivation, originating the peak component of release. This view is entirely consistent with the effects of low tetracaine, which would be a specific blocker of the calcium-induced calcium release mechanism (or channel).

Alternatively, it is possible to imagine a dual release mechanism with a single-channel protein. The channel would have two open states. A state of low conductance (open 1) would be accessible by depolarization of the T-membrane. A second open state (open 2), of high conductance, would be reached in a Ca^{2+}-dependent transition. Such a model would have a number of complexities: since release channels can be opened by Ca^{2+} without the intervention of voltage (reviewed by Ríos & Pizarro, 1991) open 2 should be directly accessible from closed states. To make this consistent with the present results, tetracaine at 20 μM should specifically block the transition into open 2 while leaving the equilibrium of the 'closed $\Leftrightarrow$ open 1' transition unchanged. Complex mechanisms of this sort are possible, especially in view of the structural complexity of the release channel protein, as suggested for instance in the three-dimensional reconstructions of Wagenknecht, Grassucci, Frank, Saito, Inui & Fleischer (1989).

Localization of the site of action of tetracaine

The pH experiments as well as those with the charged analogue indicate the existence of a hydrophobic barrier in the diffusion pathway of the drug to its site of action. Similar pH dependence of the effect of tetracaine on potassium contractures was observed in intact fibres by Sánchez & Caputo (1987). These results suggest an intracellular site of action at low concentrations which, as discussed in the previous section, is most likely localized on the release channel itself. Further evidence in this direction is the similarity of the effects of tetracaine on both charge movements and calcium release, to those of blockers of the release channel like Ruthenium Red (Csernoch *et al.* 1991) and ryanodine (García, Avila-Sakar & Stefani, 1992), as well as other interventions intended to alter release flux directly (Csernoch *et al.* 1991; Szücs *et al.* 1991).

Tetracaine effects on charge movement

The mechanism of the inhibition of charge movement by tetracaine has been controversial. Thus, the charge suppressed by 1–4 mM of the drug has been used as a definition of the component Q_γ (Hui, 1983; Huang, 1989). On the other hand, we

have provided evidence that its effect at 20 μM is primarily on the calcium release channels (Csernoch *et al.* 1991; Szücs *et al.* 1991; Pizarro *et al.* 1991) and that the effect on Q_γ is secondary, indicative of a feedback influence of the released Ca^{2+} on the voltage sensor. The present results, suggesting a primary effect of tetracaine on a distinct component of calcium-triggered calcium release, constitute additional evidence that myoplasmic Ca^{2+} feeds back on the voltage sensor to cause Q_γ and/or determine its kinetic properties.

Higher concentrations of the drug clearly suppressed the inactivating component of the release too. In the framework of the dual pathway model outlined above, this would be interpreted as a block of the voltage-controlled release pathway. This could either be due to a lower affinity binding site on these channels – the change in affinity might be caused by the interaction of the release channel with the DHP receptor –, to the existence of another, lower affinity target site for tetracaine on the DHP receptor, or both. The question cannot be decided on the basis of these experiments. Since García *et al.* (1992) showed that 0·4 mM tetracaine completely abolished the slow calcium current, there is no doubt that the drug can interact with the dihydropyridine receptor.

The present results together with the recent work of Jacquemond *et al.* (1991) are evidence of a calcium-induced mechanism contributing to physiological calcium release. This, together with the effects of released calcium on the voltage sensor, either causing or modifying the charge movement component Q_γ (Csernoch *et al.* 1991; Jong, Pape & Chandler, 1992), brings to two the number of positive feedback processes apparently at work in the control of calcium release. An emerging question of major physiological significance is how these two potentially self-sustaining mechanisms remain under tight control by membrane voltage.

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REFERENCES

ALMERS, W. (1976). Differential effects of tetracaine on delayed potassium channels and displacement currents in frog skeletal muscle. *Journal of Physiology* **262**, 613–637.

ALMERS, W. & BEST, P. M. (1976). Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *Journal of Physiology* **262**, 583–611.

ANTONIU, B., KIM, D. H., MORII, M. & IKEMOTO, M. (1985). Inhibitors of Ca release from the isolated sarcoplasmic reticulum. I. Ca channel blockers. *Biochimica et Biophysica Acta* **816**, 9–17.

BAYLOR, S. M. & HOLLINGWORTH, S. (1988). Fura-2 calcium transients in frog skeletal muscle fibres. *Journal of Physiology* **403**, 151–192.

BLOCK, B. A., IMAGAWA, T., CAMPBELL, K. P. & FRANZINI-ARMSTRONG, C. (1988). Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *Journal of Cell Biology* **107**, 2587–2600.

BRUM, G. & RÍOS, E. (1987). Intramembrane charge movement in frog skeletal muscle fibres: properties of charge 2. *Journal of Physiology* **387**, 489–517.

BRUM, G., RÍOS, E. & SCHNEIDER, M. F. (1988). A quantitative model of calcium removal from the myoplasmic solution. (Appendix to Brum, G., Ríos, E. & Stefani, E. (1988). Effects of extracellular calcium on the calcium movements of excitation–contraction coupling in skeletal muscle fibres.) *Journal of Physiology* **398**, 441–473.

BRUM, G., RÍOS, E. & STEFANI, E. (1988). Effects on extracellular calcium on the calcium movements of excitation–contraction coupling in skeletal muscle fibres. *Journal of Physiology* **398**, 441–473.

BULL, R. & MARENGO, J. J. (1990). Effects of tetracaine on calcium channels from sarcoplasmic reticulum. *Biophysical Journal* **57**, 343a.

CAPUTO, C. (1972). The time course of potassium contractures of single muscle fibres. *Journal of Physiology* **223**, 483–505.

CSERNOCH, L., HUANG, C. L.-H., SZÜCS, G. & KOVÁCS, L. (1988). Differential effects of tetracaine on charge movements and Ca signals in frog skeletal muscle. *Journal of General Physiology* **96**, 601–612.

CSERNOCH, L., PIZARRO, G., URIBE, I., RODRÍGUEZ, M. & RÍOS, E. (1991). Interfering with calcium release suppresses I γ, the delayed component of intramembranous charge movement. *Journal of General Physiology* **97**, 845–884.

ENDO, M. (1985). Calcium release from the sarcoplasmic reticulum. *Current Topics in Membrane Transport* **25**, 181–230.

GARCÍA, J., AVILA-SAKAR, A. J. & STEFANI, E. (1991). Differential effects of ryanodine and tetracaine on charge movement and calcium transients in frog skeletal muscle. *Journal of Physiology* **440**, 403–417.

GARCÍA, J., PIZARRO, G., RÍOS, E. & STEFANI, E. (1991). Effect of the Ca buffer EGTA on the delayed component of charge of skeletal muscle. *Journal of General Physiology* **97**, 885–896.

HUANG, C. L.-H. (1980). Charge movement components in skeletal muscle. *Journal of Physiology* **304**, 31–32*P*.

HUANG, C. L.-H. (1981). Effects of local anaesthetics on the relationship between charge movements and contractile thresholds in frog skeletal muscle. *Journal of Physiology* **320**, 381–391.

HUANG, C. L.-H. (1989). Intramembrane charge movements in skeletal muscle. *Physiological Reviews* **68**, 1197–1247.

HUI, C. S. (1982). Pharmacological dissection of charge movement in frog skeletal muscle fibers. *Biophysical Journal* **39**, 119–122.

HUI, C. S. (1983). Pharmacological studies of charge movements in frog skeletal muscle. *Journal of Physiology* **337**, 509–529.

JACQUEMOND, V., CSERNOCH, L., KLEIN, M. G. & SCHNEIDER, M. F. (1991). Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophysical Journal* **60**, 867–874.

JONG, D.-S., PAPE, P. C. & CHANDLER, W. K. (1992). Effects of sarcoplasmic reticulum (SR) calcium depletion on intramembranous charge movement in frog cut muscle fibers. *Biophysical Journal* **61**, A130.

KOVÁCS, L., RÍOS, E. & SCHNEIDER, M. F. (1983). Measurements and modification of free calcium transients in frog skeletal muscle fibres by a metallochromic indicator dye. *Journal of Physiology* **343**, 161–196.

MELZER, W., RÍOS, E. & SCHNEIDER, M. F. (1984). Time course of calcium release and removal in skeletal muscle fibers. *Biophysical Journal* **45**, 637–641.

MELZER, W., RÍOS, E. & SCHNEIDER, M. F. (1987). A general procedure for determining calcium release in skeletal muscle fibers. *Biophysical Journal* **51**, 849–864.

PIZARRO, G., CSERNOCH, L., URIBE, I. & RÍOS, E. (1989). Tetracaine and pathways of Ca^{2+} release in skeletal muscle. *Biophysical Journal* **55**, 88a.

PIZARRO, G., CSERNOCH, L., URIBE, I., RODRÍGUEZ, M. & RÍOS, E. (1991). The relationship between Q_γ and Ca release from the sarcoplasmic reticulum in skeletal muscle. *Journal of General Physiology* **97**, 913–947.

RÍOS, E. & PIZARRO, G. (1988). Voltage sensors and calcium channels of excitation–contraction coupling. *News in Physiological Sciences* **3**, 223–227.

RÍOS, E. & PIZARRO, G. (1991). Voltage sensor of excitation–contraction coupling in skeletal muscle. *Physiological Reviews* **71**, 849–908.

SÁNCHEZ, M. E. & CAPUTO, C. (1987). Effects of local anaesthetics and pH on depolarization–contraction coupling of *Rana pipiens* muscle fibres. *Acta Cientifica Venezolana* **38**, 170–179.

SCHNEIDER, M. F. & SIMON, B. J. (1988). Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. *Journal of Physiology* **405**, 727–745.

SCHNEIDER, M. F., SIMON, B. J. & SZÜCS, G. (1987). Depletion of calcium from the sarcoplasmic reticulum during calcium release in frog skeletal muscle. *Journal of Physiology* **392**, 167–192.

SIMON, B. J., KLEIN, M. G. & SCHNEIDER, M. F. (1991). Calcium dependence of inactivation of calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. *Journal of General Physiology* **97**, 437–471.

SZÜCS, G., CSERNOCH, L., MAGYAR, J. & KOVÁCS, L. (1991). Contraction threshold and the 'hump' component of charge movement in frog skeletal muscle. *Journal of General Physiology* **97**, 897–911.

VERGARA, J. & CAPUTO, C. (1983). Effects of tetracaine on charge movements and calcium signals in frog skeletal muscle fibers. *Proceedings of the National Academy of Sciences of the USA* **80**, 1477–1481.

WAGENKNECHT, T., GRASSUCCI, T., FRANK, J., SAITO, A., INUI, M. & FLEISCHER, S. (1989). Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature* **338**, 167–170.

XU, L., JONES, R. V. & MEISSNER, G. (1990). Tetracaine inhibition of the Ca release channel from skeletal sarcoplasmic reticulum. *Biophysical Journal* **57**, 170a.