J. Physiol. (1976), **262**, *pp.* 583–611
With 11 *text-figures*
Printed in Great Britain

EFFECTS OF TETRACAINE ON DISPLACEMENT CURRENTS AND CONTRACTION OF FROG SKELETAL MUSCLE

BY W. ALMERS AND P. M. BEST*

From the Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195, U.S.A. and the Physiological Laboratory, Cambridge CB2 3EG

(*Received 31 March 1976*)

SUMMARY

1. The kinetics of mechanical activation of intact fibres were examined with a voltage-clamp technique. Tetracaine (2 mM) increases fifteen- to seventyfold the time required to produce a just visible contraction by cell membrane depolarization.

2. Displacement currents thought to be related to contractile activation remain in 2 mM tetracaine. Their characteristics are virtually identical to those found in the absence of the drug. Displacement currents also remain in fibres immobilized by treatment with 10 mM formaldehyde.

3. Despite its effect on contraction of intact fibres, tetracaine does not diminish contraction tension when Ca is applied directly to the contractile proteins of 'skinned' muscle fibres. The sensitivity of the myofilaments to Ca^{2+} also remains undiminished.

4. When acting on intact fibres the drug must therefore inhibit Ca^{2+}-release from the sarcoplasmic reticulum. It is estimated that 2 mM tetracaine diminishes more than tenfold the capacity for Ca^{2+}-release in response to cell membrane depolarization.

5. If muscle displacement currents represent events linking depolarization to Ca^{2+}-release, then tetracaine must be able to block the release without affecting the potential-sensing portion of the release regulating mechanism.

6. Further experiments on skinned fibres show that tetracaine blocks or greatly diminishes caffeine contractions, but that Cl-induced contractions of normal amplitude are still possible.

* Present address: Department of Pharmacology, University of Berne, Switzerland.

INTRODUCTION

The local anaesthetic tetracaine inhibits contraction of intact skeletal muscle fibres at millimolar concentrations, be it caffeine-induced contractions (Feinstein, 1963; Bianchi & Bolton, 1967; Lüttgau & Oetliker, 1968) or the more physiological contractile response to cell membrane depolarization (Lüttgau & Oetliker, 1968). Both types of contraction ultimately depend on release of Ca^{2+} from the sarcoplasmic reticulum into the myoplasm. In an important paper, Lüttgau & Oetliker (1968) have examined the interaction between tetracaine, caffeine and sarcolemmal membrane potential and have suggested that tetracaine and caffeine act at a common site, possibly at the 'triadic junction' (Franzini-Armstrong, 1970), where transverse tubular system and sarcoplasmic reticulum meet in close apposition.

At the triadic junction, the two membrane systems probably interact in such a way that a membrane potential change in the tubules produces Ca^{2+}-release from the sarcoplasmic reticulum. A plausible hypothesis as to the mechanism of this interaction now exists. Chandler, Rakowski & Schneider (1976*b*) have suggested that the triadic gap is bridged by macromolecules which sense the membrane potential across the tubule membrane with one end and with the other open or close Ca-permeable pores in the neighbouring reticulum. From such a hypothesis, one would expect the tubule membrane to contain molecular dipoles or charged, membrane-bound molecular particles which can re-orient in response to membrane potential changes and in doing so cause an electric current, a 'displacement current'. Displacement currents of the expected kind have been recorded (Schneider & Chandler, 1973; Chandler, Rakowski & Schneider, 1976*a*; Adrian & Almers, 1976 *a*, *b*) and their characteristics indicate the presence in the cell membrane of polar molecules carrying sufficient charge or dipole moment to respond strongly to potential changes like action potentials.

Here, we report efforts to characterize the action of tetracaine at concentrations sufficient to block the contractile response to cell membrane depolarizations of physiological magnitude. Our experiments show that the drug neither interferes with the contractile proteins nor affects the displacement currents in any substantial way (see also Almers, 1976). Therefore tetracaine probably acts by inhibiting a final step in contractile activation, namely Ca^{2+} release, rather than blocking the release-activating signal as it travels from the transverse tubules to the sarcoplasmic reticulum. Preliminary acounts of this work have been included elsewhere (Almers, Adrian & Levinson, 1975; Almers & Best 1976).

METHODS

Electrical measurements

Unless indicated otherwise experiments were carried out at 2–4° C on sartorius muscles of the frog *Rana temporaria*, mostly with the three micro-electrode voltage clamp technique of Adrian, Chandler & Hodgkin (1970). Two electrodes were inserted at distances l and $2l$ from the pelvic end of a muscle fibre in order to record the membrane potentials V_1 and V_2, respectively, with respect to a micro-electrode which sensed the potential of the bath. The bath electrode was placed within 1 mm from the electrode recording V_1. All of these electrodes were filled with 3 M-KCl, had resistances from 7 to 20 MΩ and tip potentials less than 6 mV when measured in Ringer fluid. A third intracelluar micro-electrode was filled with 2 M-citrate, had a resistance of 3–10 MΩ and was inserted at distance $2l+l'$ from the end of the fibre; l' varied between 50 and 100 μm. By injecting the appropriate current through this electrode V_1 was controlled ('voltage-clamped') by electronic feed-back. The current-delivering electrode was electrically shielded to within 1–2 mm of its tip by applying a grounded coat of conductive paint, and on top of that another coat of insulating varnish (Valdiosera, Clausen & Eisenberg, 1974*a*).

Controlling V_1 by electronic feed-back, one can measure the membrane current density I_m at distance l from the fibre end by

$$I_m = \frac{a}{3l^2 R_i} \Delta V, \qquad (1)$$

where a is the fibre radius, R_i the myoplasmic resistivity and $\Delta V = (V_2 - V_1)$ is the voltage decrement along the fibre (Adrian *et al.* 1970). When necessary, an average value of $a = 40$ μm was assumed, and $R_i = 279$–299 Ω cm, depending on the exact temperature (Hodgkin & Nakajima, 1972).

Measurement of displacement currents. The experimental protocol (Adrian & Almers 1976*b*) was to impose in succession five small 'control steps,' usually from −108 to −92 mV, and then one 'test step' of equal duration going from −100 mV to some test potential. During control and test steps, measurements of membrane potential and current were made once every 0·4 msec each and the values stored in a PDP-8 digital computer (Digital Equipment Corp., Maynard, Mass.) Each of these measurements was the average of eight samples taken by the analogue-to-digital converter at 50 μsec intervals. Currents during the five control steps were summed and used to correct the membrane current during the 'test step' for capacitive and leakage admittances at −100 mV. The correction was done by multiplying the summed currents during the control pulse by $T/5C$, and subtracting the result point for point from the current during the test pulse; T is the amplitude of the V_1-excursion during the test pulse, C that during the control pulse. The sequence of five control and one test pulse was repeated four-times for signal averaging. After finishing such a *block* of four sequences, the signal- averaged and leakage- and capacity-corrected current during the test pulse was digitally 'smoothed' (see Almers, 1976) in order to reduce high-frequency noise and then displayed on an oscilloscope and photographed. The charge carried by the transient portion of this current was determined by subtracting horizontal base lines and evaluating the time integral of the remainder with a planimeter. Sometimes ionic currents appeared to increase during a test pulse; in that case a sloping base-line fit to the current trace during the final portion of the pulse was subtracted before determining the time integral of the remainder. Interval between control and test pulses was 1 sec, interval between successive blocks was 35–60 sec. The potential between blocks was held at −100 mV; test potentials were varied randomly.

The transient currents remaining after the subtraction will be called 'displacement currents'; they are capacitive currents (Chandler *et al.* 1976*a*) which flow in excess of the conventional capacitive current and represent the re-orientation of dipolar or charged molecules inside the membrane. Rationale and limitations of this experimental approach have been discussed elsewhere (Adrian & Almers, 1976*a*, *b*; Almers *et al.* 1975). Briefly, the cell membrane capacity is viewed as consisting of two components, one independent of potential as it arises in the lipid bilayer portion of the membrane, the other subject to dielectric saturation at extreme potentials because it results from polar membrane constituents. At the strongly negative potentials prevailing during the control pulse, polar membrane constituents of physiological interest are for the most part immobilized by the electric field; at the less negative potentials during the test pulse, however, they can re-orient and produce current. To the extent that re-orientation during the control pulse is negligible, the transients remaining after the subtraction procedure are a complete version of the currents due to polar membrane constituents.

Membrane capacity. For each *block* the effective cell membrance capacity C_{eff} was measured from the signal-averaged current- and voltage displacements during control pulses by

$$C_{\text{eff}} = \frac{a}{3l^2 R_i V_1(\infty)} \int_0^\infty \left[V_2(t) - \frac{V_2(\infty)}{V_1(\infty)} V_1(t) \right] \mathrm{d}t, \qquad (2)$$

where the fibre is assumed to be a circular cylinder of radius a and C_{eff} is referred to unit cylinder surface. All variables refer to a control pulse starting or terminating at $t = 0$; $V_1(\infty)$ and $V_2(\infty)$ are final values of the voltages $V_1(t)$ and $V_2(t)$ at distances l and $2l$ from the end (Adrian & Almers, 1976*a*). $V_1(t)$ is the potential controlled by feed-back; for both test and control pulses, it was an exponentially delayed step with time constant 0·5 msec. Membrane capacities obtained during and after the control pulse were averaged. In general, membrane currents were referred to C_{eff}; if this is done, the factor $a/3l^2R_i$ cancels out and knowledge of a and R_i is unnecessary.

Solutions. All electrical measurements were done in Cl^--free media and in order to wash out intracellular chloride ions, muscles were immersed for 30–60 min (20° C) in solution C (Table 1*A*) containing K^+ instead of Rb^+ in order to facilitate chloride escape. Muscles were then ready for use in one of the experimental solutions (A or B in Table 1*A*). All solutions contained 4×10^{-7} M tetrodotoxin (Sankyo) and 1·5 mM-Na phosphate buffer adjusted to pH 7·0–7·2. The two main components of muscle membrane's resting conductance – Cl conductance and the inwardly rectifying K channel – are absent in these media, and the membrane conductance is so low (see legends of Table 2 for values) that one would expect d.c. potential changes to spread uniformly into the transverse tubular system (Adrian & Almers, 1974). Also, muscles were somewhat depolarized in the experimental media, and in order to remove various kinds of refractoriness caused by this depolarized state, each fibre was voltage-clamped at −100 mV for 3–7 min before measurements were started.

Mechanical measurements on 'skinned' muscle fibres

The experimental procedure followed that of Hellam & Podolsky (1969). Fibre bundles from the dorsal head of the semitendinosus muscle were blotted and transferred into silicone oil. Relaxing solution (RS in Table 1*B*), a virtually Ca^{2+}-free, adenosine triphosphate (ATP)-containing medium, was injected into the silicone fluid so it surrounded the fibre bundle. Pieces of single fibres were isolated and the sarcolemma removed mechanically. The 'skinned' fibre segment, 0·5–1·5 mm in length, was mounted at just above slack length (resting tension about 1 mg) between two tiny stainless-steel clamps. One of the clamps was attached to a photo-electric

transducer (Hellam & Podolsky, 1969) producing an output of about 35 V/g tension at less than 1 mm/g compliance. Fibre diameter was measured at this stage. Once mounted, the fibre was lifted out of the silicone oil into a small well containing relaxing solution. From the relaxing solution, the fibre could be transferred to various other solutions by pulling the small well of relaxing solution out from underneath and substituting one filled with the test solution. In this way solutions could be changed within 2–5 sec. Phototransducer output was continuously monitored with a pen recorder (Hewlett-Packard 7702A).

TABLE 1. Composition of solutions

A. Intact fibres

Solution type	Na^+	TEA^+	K^+	Rb^+	SO_4^{2-}	$CaSO_4$
A	180	0	0	10	95	5
B	0	180	0	10	95	5
C	180	0	10	0	95	5

B. Skinned fibres, pCa^{2+}–tension relationship

Solution type	Imidazole	Na_2ATP	Mg	Ca	K_2EGTA	K
pCa = 3·6	82·7	2·3	3·6	7·5	7·0	19·9
3·8	83·2	2·2		7·3		20·1
4·0	83·6	2·2		7·2		20·3
4·2	83·8	2·1		7·0		20·3
4·4	84·0			6·9		20·4
4·6	84·1			6·8		
4·8	84·1			6·7		
5·0	84·2			6·5		
5·3			3·7	6·0		
5·5				5·5		
RS			4·1	0·08*		

C. Skinned fibres, Ca^{2+} release experiments

pCa = 4·5	83·2	3·1	2·2	7·1	7·0	19·8
6·5	94·8	2·8	2·2	0·02*	0·05	34·3
RS	83·4	2·8	2·2	0·08*	7·0	20·4

All concentrations in mM. All solutions adjusted to pH 7. Where no entry, concentration as above. In *B* and *C*, solutions contained 15·7 mM-K_2-creatine phosphate besides the listed ingredients and the anion accompanying K^+, Ca^{2+} and Mg^{2+} was chloride. Solution pCa = 6·5 in *C* was an exception; it contained propionate instead of chloride and only 15·1 mM-K_2-creatine phosphate. * Estimated contamination from distilled water and reagents.

Bathing solutions for skinned fibres. Details of their composition are given in Table 1*B*, *C*. Following the procedure of Donaldson & Kerrick (1975), published equilibrium dissociation constants were used in designing solutions of ionic stength 150 mM, [MgATP] = 2 mM, ($[K]^+ + [Na]^+$) = 70 mM and the Ca^{2+} activities given in the Table. Solutions in Table 1*B* were used to determine the pCa–tension relationship for the contractile proteins; the anion accompanying Mg^{2+}, Ca^{2+}, and K^+ was chloride and the composition was chosen such that $[Mg]^{2+}$ = 1 mM. The solutions in Table 1*C*

contained only 0·1 mM ionized Mg and propionate instead of Cl^-. Solutions labelled 'pCa = 6·5' or 'RS' in Tables 1*B*, *C* contained no added Ca^{2+}; their pCa (8–9 for RS) was calculated from [EGTA] and estimates of Ca^{2+} contamination from distilled water and reagents. Besides the listed ingredients, all solutions contained 15 u./ml. of creatine phosphokinase and 15·7 mM-K_2-creatine phosphate. The solution pCa = 6·5 in Table 1*C* was an exception and contained only 15·1 mM-K_2-creatine phosphate.

RESULTS

Kinetics of mechanical activation

Following the approach of Adrian, Chandler & Hodgkin (1969), a point along the end of the fibre was voltage-clamped with two intracellular micro-electrodes, one for applying current, and another to measure the membrane potential. Rectangular depolarizing pulses were applied from a holding potential of -100 mV and the fibres observed under a dissection microscope of 100× magnification. This relatively crude method of observation will not resolve radial non-uniformities in contractile activation, but results obtained with it are virtually identical to what one observes with optics of higher resolution (Costantin, 1974). For a given pulse duration, the pulse amplitude was adjusted until a just visible contraction resulted under the two intracellular electrodes. Together, pulse duration and amplitude give a point on one of the strength–duration curves shown in Fig. 1. When the potential during the pulse was negative, the pulse amplitude for a just visible contraction was well defined, usually to within a millivolt. At positive potentials, contraction is less sensitive to the membrane potential, and the pulse duration was adjusted at fixed amplitude.

Fig. 1 summarizes a number of such experiments in isotonic Na_2SO_4-saline without (circles) and with 2 mM tetracaine (triangles). Estimates of the rheobasic threshold potentials for contraction in the three conditions are, ± S.E. of mean: control, $-35·6 \pm 1·6$ mV ($n = 8$, pulse duration 0·4 sec 5° C) 2 mM tetracaine, $-22·8 \pm 1·5$ mV ($n = 5$, pulse duration 1·95 sec, 5° C) and $-20·0 \pm 2·9$ mV ($n = 5$, pulse duration, 0·5 sec, 19·5° C). The difference between the values in the cold is significant at the $P < 0·05$ level (*t* test). Slightly more negative rheobase potentials might have been obtained with longer pulses; however, contraction in that case was difficult to control experimentally, as it depends exceedingly steeply on the membrane potential (Hodgkin & Horowicz, 1960).

Cell membrane depolarization can still produce mechanical activity in 2 mM tetracaine, but Fig. 1 shows that at 5° C, depolarizations on the average need to last 15–70 times longer. Though the experiment does not exclude that full tetanic tension might result in tetracaine if the cell membrane were depolarized far enough and for long enough, Fig. 1

supports the observation of Lüttgau & Oetliker (1968) that the drug is an effective inhibitor of the contractile response to cell membrane depolarization. No matter how strong, a depolarization will not cause contraction at 5° C if it lasts less than about 70 msec. If a normal action potential were possible in 2 mM tetracaine, it would fail to cause mechanical activity by a large margin.

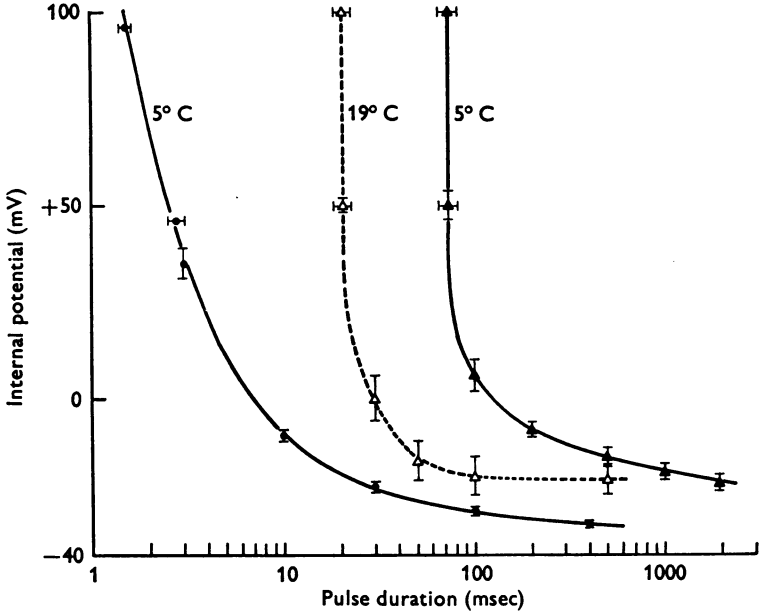

Fig. 1. Strength–duration curves in the absence (○) and presence of 2 mM tetracaine (▲ at 5° C, △ at 19·5° C). Filled symbols are averages from six fibres each, error bars give S.E. of means. The curves summarize results from an equal number of fibres in each of three muscles first without and then with tetracaine. Open symbols are averages on four fibres from a muscle from the same animal as one used for measurements in the cold. Rectangular pulses were passed to the voltage clamp without exponential delay in these experiments. Even so, short pulses to +40 mV and beyond deviated noticeably from being square; their equivalent duration was taken as the time between the two instances where the voltage trace crossed a level midway between −35 mV and the maximal potential during the pulse. Muscles were mounted at their *in situ* length which was measured before dissection when the legs of the frog made an angle of 90°. Voltage electrode 375 μm, current electrode 425 μm from the pelvic ends of fibres. Solution A.

When interpreting strength curves, one considers that a just visible contraction will result whenever the myoplasmic Ca^{2+} concentration has risen to a certain value which, though unknown, is assumed constant for a given experimental condition. In that sense, measurement of strength–duration curves is a 'null method' where points on a given curve represent

conditions where a similar myoplasmic [Ca^{2+}] has been reached. In normal fibres, threshold pulses to positive potentials become so short that signal propagation down the transverse tubules or, if applicable, the relaxation times of the displacement currents are likely to be dominant factors. In tetracaine, both tubular potential spread and re-distribution of charge (Fig. 2) will have gone to completion at times much less than required for activating contraction. The vertically rising branches of the curves in tetracaine therefore demonstrate that the voltage dependence of mechanical activation saturates at positive potentials (see also Adrian, Chandler & Rakowski, 1976). At saturating potentials, contractile activation requires $72{\cdot}3 \pm 8{\cdot}5$ msec at 5° C ($n = 6$) and $20{\cdot}3 \pm 2{\cdot}1$ msec at 19·5° C ($n = 4$); figures are given ± S.E. of mean and refer to measurements at +100 mV. In 2 mM tetracaine, the apparent 'temperature coefficient' (Q_{10}) for activation at 100 mV is therefore 2·4.

Intracellular tetracaine concentration. Since the drug may act from the inside of the cell, its concentration there is of interest. We believe that in our measurements, internal and external drug concentrations were virtually equal. Tertiary amine local anaesthetics in their uncharged form are so highly permeant (permeability coefficients of 1 cm/sec or more by analogy with uncharged salicylic acid; Gutknecht & Tosteson, 1973) that drug access to the cell interior will be rate-limited by unstirred layers rather than the cell membrane (e.g. McLaughlin, 1975). One expects therefore that in surface fibres of a sartorius muscle, the free base will reach equal internal and external concentrations within minutes of external application. To the extent that external and internal pH (normally neutral, Bianchi & Bolton, 1967) are the same, also the protonated form of the drug will reach equal concentration inside and outside. However, the drug may change the internal pH, and the remainder of this discussion will give reasons for our belief that such changes, if present, will have been too slow and too small to affect our experimental results.

The first drug effect after external application will be an internal alkalinization, since free base entering the cell will capture protons. In our experiments, nearly 2 mM internal protons will have been bound by the drug since tetracaine ($pK\hat{a} = 8{\cdot}24$, Bianchi & Bolton, 1967) is about 95% protonated at neutral pH. With a myoplasmic buffering capacity of about 30 m-equiv/pH unit (Furusawa & Kerridge, 1927; cat skeletal muscle) the alkalinization will have amounted to somewhat less than 0·1 pH unit. If the protonated form of the drug, tetracaine$^+$, is permeant, then acidification will follow alkalinization. Charged drug molecules will be drawn into the cell by the membrane potential, deprotonate inside and leave the cell again as free base. The drug will therefore act as a proton carrier tending to dissipate electrochemical proton gradients at a rate depending, among other factors, on the membrane permeability to tetracaine$^+$. The tetracaine$^+$-permeability can be estimated from results obtained by McLaughlin (1975) on artificial membranes made from negatively charged lipids. In Fig. 9 of his paper, the membrane conductance at pH 7 due to 10 mM tetracaine$^+$ could have been no more than $1{\cdot}6 \times 10^{-6}$ mho/cm^2. This would translate into a permeability coefficient of about 4×10^{-8} cm/sec, roughly 10–70 times less than muscle membrane's Cl permeability (4×10^{-6} cm/sec if referred to sarcolemmal membrane only; Hodgkin & Horowicz, 1959). The correct value for muscle membrane may well be substantially less because, for instance, muscle membrane is probably not composed purely of negatively charged lipid. We find an average membrane potential of

−43 mV for tetracaine-poisoned fibres in solutions A or B of Table 1*A*, and assuming an initially neutral pH inside and out, one can use constant-field theory (Goldman, 1943) to calculate that muscle fibres in our experiments may have gained 1 mM of protons every 36 min. It would then have taken about 70 min to reverse the initial proton loss due to entry of free base. Thereafter, intracellular pH may have declined

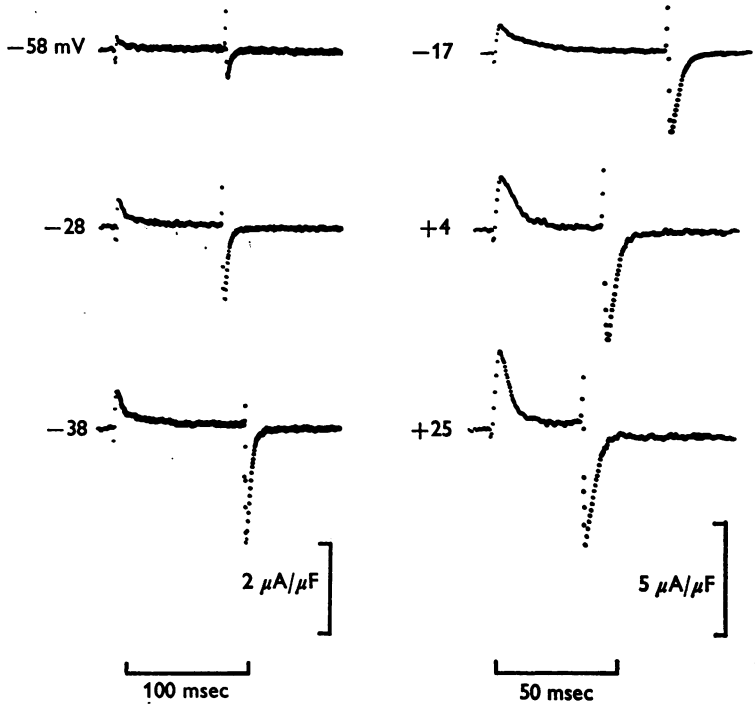

Fig. 2. Membrane currents in solution B + 2 mM tetracaine, corrected for capacity and leakage at −100 mV as described in Methods. Beginnings of outward (upward) and inward transients coincide with beginnings and ends of test pulses to the potentials indicated next to each trace (mV). The aberrant points at the beginning of inward transients result from imperfect constancy of pulse duration or imperfect voltage control; the points were included in forming the time integral of the transient by considering that each point covers a time interval of 0·4 msec. Control pulses from −110 to −84 mV; holding potential −100 mV. Electrode spacing l = 375 μm. 1 μA/μF corresponds to 2·25 mV. Fibre 40-13.

to a value of about 6·2, and [tetracaine$^+$] inside risen to 6 times the external concentration. Both drug and protons would then have been at electrochemical equilibrium. However, with a myoplasmic buffer capacity of 30 m-equiv/pH unit, this process would have taken place with a time constant of about 18 hr. Furthermore, these considerations neglect the metabolic processes which normally maintain sizeable electrochemical proton gradients across cell membranes (e.g. Thomas, 1976) and would have opposed internal acidification in our experiments.

Experimental evidence, so far as it goes, confirms these expectations. Bianchi & Bolton (1967) found that 30 min–1 hr after application of lidocaine or procaine to sartorius muscles, the drug content per unit fibre volume was 1·12 and 1·26 times the external concentration. Internal pH was still elevated at that time, indicating that acidification is indeed either slow or absent and that the excess drug uptake probably represents adsorption to the various membrane sytems in a skeletal muscle fibre. Our own experiments were carried out between 15 min and 2 hr after drug application, and neither strength–duration curves nor the parameters describing delayed K^+-channels (Almers, 1976) showed systematic variation over that period. In conclusion, any deviations of internal pH from its normal value were probably negligible and without effect on our experimental results.

Displacement currents in tetracaine

Given the large inhibitory effects of tetracaine on contractile activation it seemed of interest whether the drug affected the displacement currents thought to play a role in the regulation of Ca^{2+}-release into the myoplasm. Fig. 2 shows membrane currents during test pulses to the indicated potentials, corrected for capacitive and leakage admittances at -100 mV. 2 mM tetracaine was present, and as expected from the strength–duration curve, none of the depolarizing pulses caused contraction. Even so, displacement current transients remain; all records show an outward current transient which occurred during the pulse ('on-transient') and an inward transient after the pulse ('off transient'). For a given pulse, on- and off-current transients carry approximately equal charge; small differences (absolute value $1{\cdot}44 \pm 2{\cdot}21$ nC/μF standard deviation, nineteen measurements, maximal difference was 6·53 nC/μF at $+55$ mV) are attributed to noise, to uncertainties in forming the time integral of the transients and to residual time-dependent ionic currents at positive potentials.

The experiment is analysed in Fig. 3 which shows the charge carried by the current transients (top) and the relaxation rates of the transients (bottom) as a function of membrane potential. Following Schneider & Chandler (1973), the charge distribution function in Fig. 3 is described by

$$Q = \frac{Q_{max}}{1+\exp\left[-(V-\bar{V})/k\right]}, \tag{3}$$

where Q is the steady-state charge displacement during a test step to the potential V, Q_{max} the charge displacement at saturating (positive) potentials and k and $\bar{V}$ are adjustable parameters. Eqn. (3) would result, for instance if the charged particles (dipoles) giving rise to the displacement currents could distribute themselves between only two positions (orientations) in the membrane. Then $\bar{V}$ would be the potential of equal distribution (transition potential) and 24 mV/k would equal the effective valency of the particle (Schneider & Chandler, 1973) or twice the charge on each end of a dipole rod as long as the membrane is thick (Almers *et al.* 1975). The curve

in Fig. 3 (top) is a least-squares fit of eqn. (3) to the data points with $Q_{max} = 22{\cdot}7$ nC/μF, $\bar{V} = -31{\cdot}8$ mV and $k = 10{\cdot}5$ mV; this value for k would correspond to an 'effective particle valency' of 2·3.

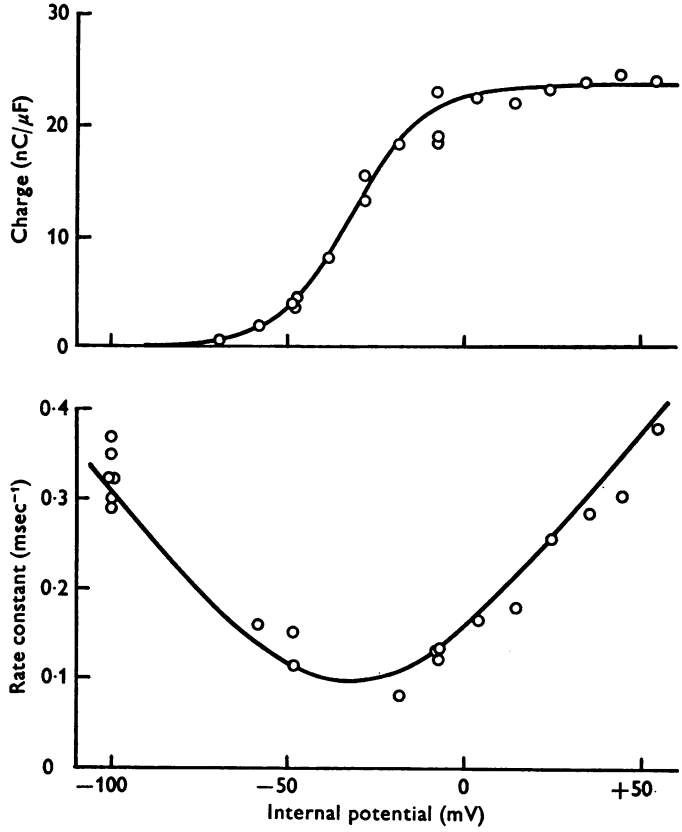

Fig. 3. Top: steady-state charge displacement (ordinate) versus the potential V_1 (abscissa) during test pulses from −100 mV. Charge displacements were obtained by averaging the time integrals of the current transients during and after a test pulse. Bottom: reciprocal rate constants determined by fitting exponential decays to the current transients from 3 to 5 msec onwards after the voltage change. Points at −100 mV from transients at the ends of pulses to potentials up to −28 mV, all others from transients during pulses. For significance of curves see text. Same experiments as in Fig. 2.

Least-squares fitting. We used a modification of a program written by Dr Bertil Hille following the *Patternsearch* procedure described, e.g. in Colquhoun (1971). Data points to be fitted were averages of charges carried by transients during (Q_{on}) and after (Q_{off}) the pulse, and ideally each data point should be weighted by the reciprocal of its variance. The minimum variance was assumed to be 0·25 (nC/μF)^2,

but since on- and off-charges often disagreed by more than compatible with this value, we estimated the variance s^2 of each data point by

$$s^2 \simeq \frac{Q_{on}^2 - 2Q_{on}Q_{off} + Q_{off}^2}{2}. \quad (4)$$

This relation would be an estimate of the variance in a sample of two taken from a normally distributed population. Though eqn. (4) is probably not very accurate, we felt that this approach was better than weighting each point equally, particularly since the discrepancies between 'on' and 'off' charges increased at large potential excursions.

Relaxation rates were obtained by fitting exponential decays to the current transients, using semilogarithmic plots and calculating reciprocal time constants from them. Although only data points in excess of 3–5 msec after a potential change were considered, single exponentials sometimes fitted poorly, particularly in the range -45 to -20 mV. Better fits could have been obtained with sums of two exponentials, but this was not explored further since the only purpose of the kinetic analysis in Fig. 3 was to provide a basis of comparison with other data. Failure of single exponentials to fit current transients is also observed in the absence of tetracaine (Adrian & Almers, 1976*b*; Almers *et al.* 1975). The continuous curve in the bottom part of Fig. 3 was drawn according to (rate constant) $= \alpha + \beta$ where α and β are assumed to be forward- and backward rate constants for particle- or dipole-movement and given by

$$\alpha = \frac{\bar{\alpha}(V - \bar{V})/k}{1 - \exp[(\bar{V} - V)/k]}, \quad (5)$$

$$\beta = \frac{\bar{\alpha}(\bar{V} - V)/k}{1 - \exp[(V - \bar{V})/k]} \quad (6)$$

(see Chandler *et al.* 1976*a*). With k and $\bar{V}$ given from the charge distribution curve, $\bar{\alpha}$ is the only adjustable parameter and was 47·3/sec in Fig. 3; each rate measurement was weighted equally.

Table 2*A* sumarizes data from experiments of this sort. Comparison with the entries in part *C* of Table 2 shows that with the possible exception of $\bar{V}$, the characteristics of displacement currents in isotonic tetracaine-containing solution appear nearly identical to those found in the absence of the drug in hypertonic media. The more negative values for $\bar{V}$ in the latter case could be a consequence of the increased myoplasmic ionic strength expected in that condition; tetracaine itself can contribute no more than about 5 mV to the shift (Almers, 1976). In summary, 2 mM tetracaine has only small effects, if any, on size, kinetics or other properties of the displacement currents. Other data leading to the same conclusion are described in the following paper.

Charge movement at contraction threshold. A few measurements of

TABLE 2. Displacement currents in immobilized fibres

Fibre no.	Q_{max} (nC/μF)	$\bar{V}$ (mV)	k (mV)	$\bar{\alpha}$ (sec^{-1})	C_m (μF/cm^2)
		A. Immobilization by 2 mM tetracaine			
38-3-2	33·1	−18·7	13·16	35·9	6·24
40-3-1	22·7	−31·8	10·48	47·3	7·63
40-3-2	29·5	−29·2	12·56	49·4	5·32
40-3-3	29·1	−27·4	15·47	50·1	7·46
Mean ±	28·6	−26·8	12·92	45·7	6·66
S.E. of mean	2·5	3·3	1·19	3·8	0·63
		B. Immobilization by formaldehyde treatment			
10-1-3	23·1	−31·7	15·8	48·7	9·76
11-1-3	16·2	−44·5	15·3	—	6·66
11-1-4	9·4	−41·4	12·7	—	6·58
40-4-1	21·8	−19·2	19·8	—	8·02
40-4-2	13·3	−20·7	20·2	—	7·34
Mean ±	16·8	−31·5	16·8	—	7·67
S.E. of mean	2·9	5·8	1·6	—	0·65
		C. Immobilization by hypertonic media			
Mean[1] ±	32·0	−48·6	12·6	—	8·56[2]
S.E. of mean	3·0	2·6	1·5	—	1·06
Mean[3] ±	24·5	−44·1	7·8	53[4]	—
S.E. of mean	1·7	3·4	0·7	—	—

First column gives fibre reference; fibres from the same muscle have the same first three digits. Second to fourth columns give parameters for the charge distribution curve determined by least-squares fit of eqn. (3) to the data. $\bar{\alpha}$ was calculated from individual rate measurements $1/\tau$ by

$$\bar{\alpha} = \frac{k}{\tau(\bar{V}-V)} \tanh\left(\frac{\bar{V}-V}{2k}\right), \tag{8}$$

where τ was obtained by fitting decaying exponentials to current transients starting 3–6 msec after a voltage clamp, and $\bar{V}$ and k are as given in the third and fourth columns. Entries for $\bar{\alpha}$ in column (6) are the means from all determinations in a given fibre. The last column gives membrane capacity at −100 mV obtained from charging transients during the control pulse by eqn. (2). In referring capacities to membrane areas, fibres were assumed to be circular cylinders of radius $a = 40$ μm and the capacity was referred to unit cylinder surface. Leakage resistance per unit cylinder surface could be estimated from the final value of ΔV during control pulses; they were on average 15·4 kΩ cm^2 in B. These values are lower limits and may have been twice as high since no allowance was made for impalement leaks around the microelectrodes. (1) Results of Adrian & Almers (1976*b*) obtained in solution B (Table 1) made 2·5 times hypertonic by adding 350 mM sucrose. (2) Obtained from charging transients during the control pulse as follows. Mean value of the myoplasmic resistance r_i per cm fibre length was 13·4 ± 5·0 MΩ/cm in five fibres in hypertonic solution B at 4° C; this gave a capacity per unit fibre length of 0·215 μF/cm. To obtain C_m from this value, the radius of the unshrunken fibre was assumed to be $a = 40$ μm as above. (3) Obtained by Chandler *et al.* (1976*a*) in Ringer fluid containing TEA^+ instead of sodium and made 3–4 times hypertonic by adding sucrose. (4) Kinetic analysis of one fibre. Electrode spacing for *A*,*B*: $l = 300$–375 μm; temperature 2–4° C.

ment currents were made in sodium sulphate saline (solution A) in the absence of tetracaine. The average charge displacement slightly below the rheobasic contraction threshold was $8{\cdot}66 \pm 0{\cdot}31$ nC/μF ($n = 5$, test pulses to -37 mV). In two instances, contraction threshold and charge movement

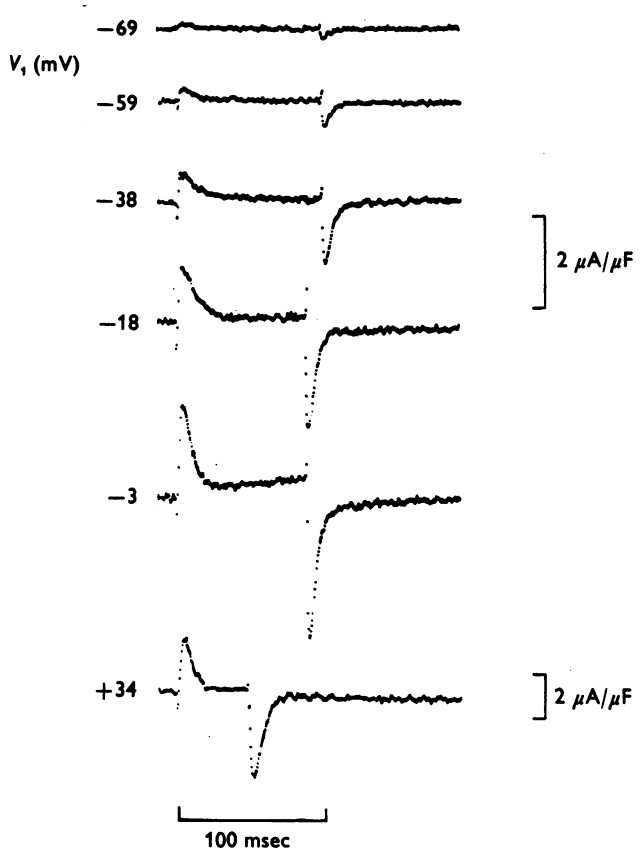

Fig. 4. Membrane currents in solution B after treatment with solution C containing 10 mM formaldehyde. Currents were corrected for leakage and capacity measured with control pulses from -100 to $-92{\cdot}5$ mV. Electrode spacing $l = 300$ μm, temperature 3° C, 1 μA/μF corresponds to 1·92 to 2·25 mV. Fibre 10-1-3.

were measured in the same fibre. Both fibres gave just visible contractions after 120 msec pulses to -37 mV and charge displacements at -37 mV of 7·0 nC/μF (fibre 40-1-2) and 7·7 nC/μF (fibre 40-1-3). This represents about 22–30 % of the maximum (Table 2*A* and *C*), in fair agreement with results of Adrian *et al.* (1976). When 2 mM tetracaine was added, charge displacement at the new rheobasic contraction threshold of -23 mV was of the order of 14 nC/μF; this value was interpolated from measurements

at -17 mV ($17{\cdot}0 \pm 1{\cdot}7$ nC/μF; $n = 5$) and at -27 mV ($10{\cdot}7 \pm 1{\cdot}1$ nC/μF, $n = 5$). Charge displacement at contractile rheobase in 2 mM tetracaine is therefore about half the maximum.

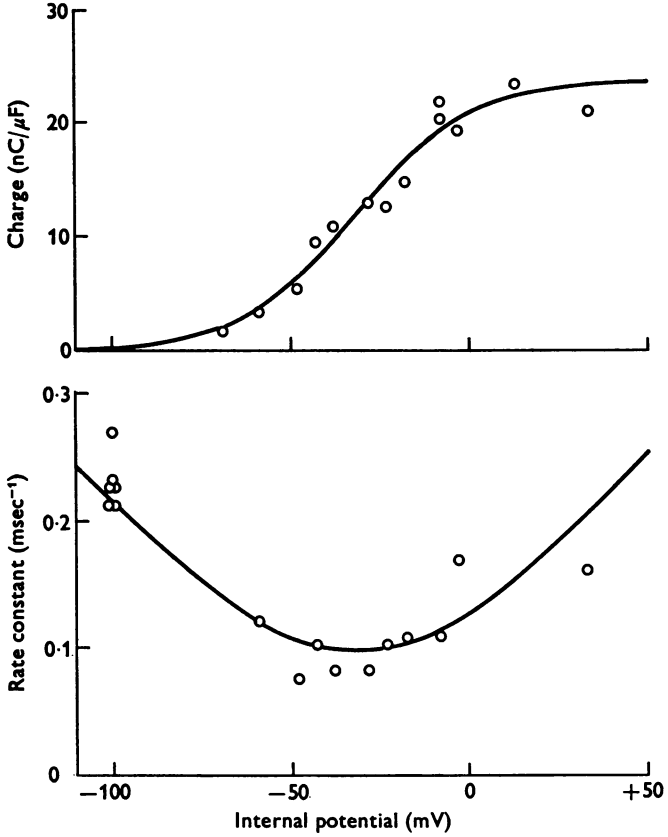

Fig. 5. Top: charge displacement (ordinate) against potential (V_1) during the test pulse (abscissa). Bottom: reciprocal time constants with which current transients decayed. Formaldehyde-treated fibre. Details of data analysis as in Fig. 3. Same experiment as in Fig. 4.

Displacement currents after formaldehyde treatment

The contractile response to depolarization can also be blocked by small amounts of the fixative formaldehyde (Hutter, 1969). After dissection and removal of intracellular chloride in solution C, 10 mM formaldehyde was added from a 43% formalin stock solution. Thirty minutes later, the medium was replaced with formaldehyde-free TEA^+-sulphate (solution B), the muscle cooled to 4° C and measurement started. Fig. 4 shows records of displacement currents from such an experiment. None of the ten fibres treated and investigated in this way showed signs of movement.

Nevertheless, displacement currents remain, and resemble those found in fibres immobilized with tetracaine or hypertonic solutions.

In Fig. 5, charge displacement (top) and reciprocal time constants of transients (bottom) are plotted against membrane potential. The top curve is a least-squares fit of eqn. (3) with $Q_{max} = 23{\cdot}1$ nC/μF, $\bar{V} = -31{\cdot}7$ mV and $k = 15{\cdot}8$ mV. The current transients in Fig. 4 were well fitted by single exponentials over the entire voltage range. The bottom curve is given by (rate constant) = $\alpha+\beta$ with α and β as in eqn. (5) and (6), k and $\bar{V}$ as given above and $\bar{\alpha} = 48{\cdot}7$/sec. Results from four other experiments of the kind are given in Table 2*B*. Compared to the results in tetracaine or hypertonic solutions, Q_{max} appears diminished and k may be slightly increased. In the one experiment where a kinetic analysis was made (Fig. 5) the kinetic parameter $\bar{\alpha}$ appeared normal. In summary formaldehyde is another agent which can block contraction without abolishing displacement currents.

Charge distribution curves after formaldehyde treatment were sometimes less steep and gave larger values of k than fibres immobilized by hypertonic solutions or in tetracaine. One can therefore question whether charge displacement during the control pulse was in fact negligible. Allowing for this, one can write

$$Q = Q^*(V) - (V - V_H)\frac{dQ^*(V_H)}{dV} - Q^*(V_H), \quad (7)$$

where V_H is the holding potential at which control pulses are taken and $Q^*(V)$ is the charge distribution curve (Adrian & Almers, 1976*b*). If $Q^*(V)$ is given by eqn. (3) with approximately the above parameters, measurements at $V_H = -100$ mV would lead one to underestimate k and Q_{max}, the latter by about 12%. In the experiment of Fig. 2 and 3 such effects would have been negligible.

Effect of tetracaine on the contractile proteins

Since tetracaine inhibits contraction in intact fibres without substantial effect on displacement currents, it seemed of interest whether the drug had any effect on 'skinned' fibres where the sarcolemma had been removed mechanically. In the presence of MgATP and other substances normally contained in the myoplasm, contraction is regulated by the Ca^{2+}-concentration around the myofibrils (Ebashi & Endo, 1968), and 'skinned' fibres have the advantage that this Ca^{2+}-concentration can be varied directly. The left part of Fig. 6 shows tension developed by an unpoisoned fibre upon transfer from a virtually Ca^{2+}-free 'relaxing solution' (RS in Table 1) to one containing 0·25 mM-Ca^{2+} (pCa = 3·6). At that Ca^{2+}-concentration tension development is maximal in the bathing solutions used here (see Donaldson & Kerrick, 1975, and Fig. 8). Average values of maximal tension ± S.E. of mean in skinned fibres were $1{\cdot}8 \pm 0{\cdot}5$ kg/cm^2 ($n = 5$, *Rana pipiens*) and $1{\cdot}5 \pm 0{\cdot}6$ kg/cm^2 ($n = 5$, *Rana temporaria*) which is

satisfactorily close to the tetanic tension of an intact fibre (2–4 kg/cm^2; see for example Hodgkin & Horowicz, 1960). The second record in Fig. 6 was obtained 5 min after addition of 2 mM tetracaine. Final tension at pCa = 3·6 was virtually equal to that in the absence of the drug. In seven other experiments of this sort (*Rana pipiens*) the maximal tension developed in tetracaine was 1·5 ± 0·04 times that without the drug. Caputo (1976) has recently obtained a similar result on chemically skinned fibres.

TABLE 3. Effect of Ca^{2+}-concentration on tension

pCa	Control	Tetracaine
	A. Rana pipiens	
5·5	11·0 ± 3·3 (6)*	27·9 ± 7·7 (9)
5·3	11·0 ± 3·9 (8)	21·3 ± 6·3 (6)
5·0	26·0 ± 5·0 (13)	61·0 ± 5·0 (10)
4·8	53·7 ± 2·7 (5)	78·0 ± 2·8 (6)
4·6	70·8 ± 1·7 (6)	84·9 ± 3·2 (5)
4·4	69·8 ± 7·4 (11)	87·0 ± 11·6 (7)
4·3	78·9 ± 2·9 (5)	97·0 ± 1·3 (4)
4·2	96·0 ± 3·3 (7)	93·0 ± 6·3 (4)
4·0	100† (5)	100† (5)
3·8	100† (5)	100† (5)
	B. Rana temporaria	
5·5	—	35·0 ± 2·9 (5)
5·0	—	72·7 ± 3·6 (4)
4·8	52·0 ± 6·3 (8)	72·5 ± 1·0 (2)
4·3	78·7 ± 7·8 (4)	

* Mean ± S.E. of mean (sample size).

† Tension never measurably different from values at pCa = 3·6. Standard error of mean assumed 1·4%.

All values give the final tension as a percentage of the tension recorded at pCa = 3·6.

Experiments such as those in Fig. 6 demonstrate that tetracaine does not diminish the maximal force when Ca^{2+} is applied directly to the contractile proteins. Fig. 7 shows tension development at submaximal Ca^{2+}-concentrations. All three traces show tension first at pCa = 4·8 and then at the saturating level of pCa = 3·6; the middle trace starts 5 min after addition, the right trace 5 min after withdrawal of 2 mM tetracaine. In the presence of the drug, tension at pCa = 4·8 is a larger fraction of the maximal value. Fig. 8 and Table 3 show data at intermediate concentrations; the experiments were similar to that shown in Fig. 7 except that measurements with and without tetracaine were in general done on different fibres. Throughout, final tension is given as a fraction of the

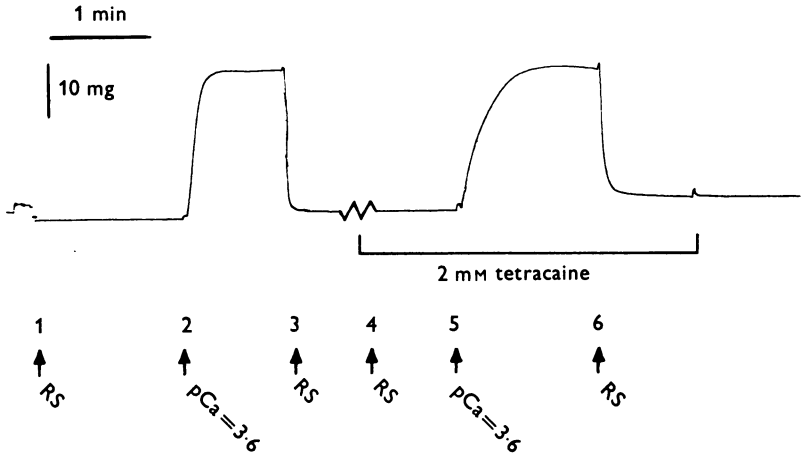

Fig. 6. Tension produced by a skinned fibre maximally activated by Ca^{2+} in the absence and presence of 2 mM tetracaine. At arrows, the bathing solution was changed to that indicated beneath (see Table 1*B*). Fibre radius 20 μm, *Rana pipiens*, temperature 20° C.

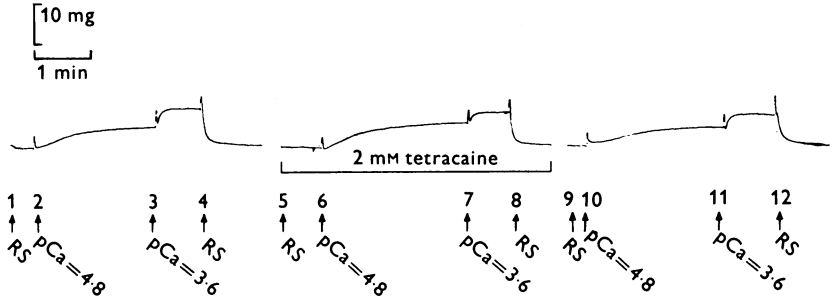

Fig. 7. Effect of Ca^{2+} concentration on contracture tension of a skinned fibre. Segments of a continuous tracing. Records in the presence of tetracaine were obtained 5 min after addition of the drug, the following trace 5 min after tetracaine withdrawal. Note that in tetracaine, the tension at pCa = 4·8 is a greater fraction of maximal tension than in the absence of the drug. All solutions as in Table 1*B*. Fibre radius 19·5 μm, *Rana pipiens*, temperature 20° C.

value measured at pCa = 3·6. The curves in Fig. 8 are least-squares fits to the equation (Hill, 1913)

$$\frac{T}{T_{max}} = \frac{[Ca^{2+}]^n}{K+[Ca^{2+}]^n}, \tag{8}$$

where T is the tension at a given Ca^{2+}-concentration, T_{max} the maximal tension and n and K are adjustable parameters. This equation would result, for instance, if n Ca^{2+}-ions combined simultaneously with a

receptor R to form a complex RCa_n^{2+} with equilibrium constant K, and if the tension T were linearly proportional to the number of such complexes. There is no evidence for either of these assumptions; we use eqn. (8) only as a convenient way to describe our data quantitatively, and because it allows us to compare them with those of other authors. In the control

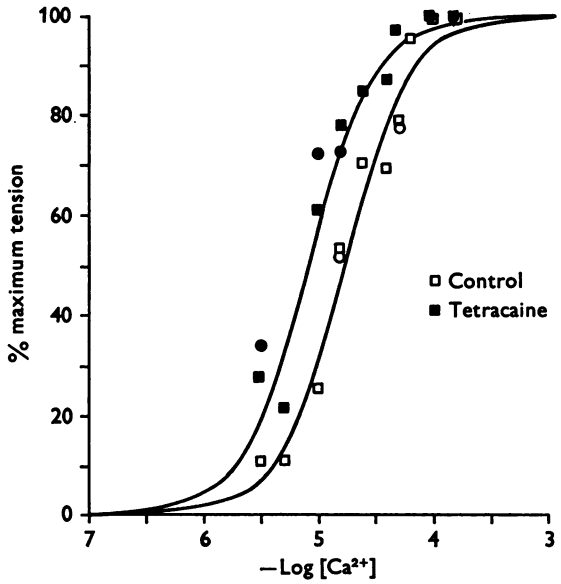

Fig. 8. Contracture tension as a fraction of maximum (measured at pCa = 3·6) plotted against Ca^{2+} concentration of bathing medium. Each point is the mean of measurements on 5–13 fibres; these means are given also in Table 3 together with other details. Curves are least-squares fits of eqn. (8) to the data points from *Rana pipiens*; each point was weighted by $1/s^2$ where s is the S.E. of the mean for each data point. Parameters are $K = 114{\cdot}4\ \mu M^n$, $n = 1{\cdot}74$ in the control and $K = 43{\cdot}5\ \mu M^n$, $n = 1{\cdot}8$ in the presence of 2 mM tetracaine. The midpoints of the curves are at pCa = 5·09 in tetracaine and pCa = 4·82 in the control, indicating a drug-induced shift to about twofold lower Ca^{2+} concentrations. Circles, *Rana temporaria*; squares *Rana pipiens*. Temperature 20° C.

situation, our values for K and n ($114{\cdot}5\ \mu m^n$ and 1·738 respectively) agree reasonably well with those found by Donaldson & Kerrick (1975) in similar experiments. In 2 mM tetracaine, n is unaltered but K slightly diminished as suggested already by Fig. 7. The midpoints of the control and tetracaine curves are at pCa 4·8 and 5·1, respectively, The drug therefore seems to produce a shift to about twofold lower Ca^{2+}-concentrations. The differences at pCa = 4·6, 4·8 and 5·0 are statistically significant at the $P < 0{\cdot}01$ level (t test).

It seemed possible that the shift in Fig. 8 resulted from interaction of the drug with the Ca^{2+}-buffer system. For instance, a reaction of the type tetracaine$^+$ + H.EGTA^{2-}⇌(tetracaine.H.EGTA$^-$) with a dissociation constant in the millimolar range could explain the observed shift as a displacement of Ca^{2+} from the CaEGTA complex. An attempt was made to test the possibility using the murexide absorption spectrum as an indicator of the ionized Ca-concentration (Ohnishi & Ebashi, 1963). Adding 4 mM tetracaine to a solution of pCa = 5·0 (Table 1*B*) produced no detectable change in absorption by murexide, and from the resolution of the apparatus it is estimated that any increase in $[Ca^{2+}]$ caused by 4 mM tetracaine must have been by less than about 8 μM (P. M. Best, unpublished). Any increase in $[Ca^{2+}]$ produced by 2 mM tetracaine was probably less than half that amount; therefore the shift in Fig. 8 probably represents a genuine effect on the contractile proteins.

Time course of contractures. Especially at low $[Ca^{2+}]$, the time course of tension development is much slower than expected for radial diffusion of the CaEGTA-complex into the fibre. Hellam & Podolsky (1969) have reported that at millimolar concentrations the dissociation rate of the complex is less than the rate at which one might expect the dissociating Ca^{2+} to be accumulated by the sarcoplasmic reticulum. The approach towards steady tension in Figs. 6 and 7 would then reflect a slow Ca^{2+} -loading of the reticulum to a level where Ca^{2+}-leak from the reticulum equals Ca^{2+}-uptake and no further net accumulation is possible. Accepting this view, we make the plausible but untested assumption that when tension is steady, Ca^{2+}-uptake by the reticulum has stopped or fallen to levels too low to disturb the (radially uniform) Ca^{2+}-EGTA buffer system.

In Fig. 6 tetracaine appeared to slow tension development. Such a finding could be explained if tetracaine, like procaine (Thorens & Endo, 1975) blocked 'Ca-induced Ca release' from the sarcoplasmic reticulum, a release mechanism observed in skeletal (Ford & Podolsky, 1972*b*) as well as cardiac muscle (Kerrick & Best, 1974). The rapid rise of tension in the normal fibre could occur whenever exposure to the pCa = 3·8 solution triggers further Ca^{2+}-release from the reticulum, thereby rapidly establishing a saturating Ca^{2+}-concentration. When Ca-induced release is blocked as it might be in tetracaine, steady state would not be attained until net uptake by the reticulum has fallen to low levels.

Tetracaine and Ca^{2+}-release from the sarcoplasmic reticulum

Since tetracaine does not appear to incapacitate the contractile proteins, its effect on intact fibres can only result from blocking Ca^{2+}-release out of the sarcoplasmic reticulum. Therefore, Ca^{2+}-release in skinned fibres was investigated using solutions weakly buffered with EGTA. In weakly buffered solutions, Ca^{2+}-release from the reticulum can be sufficient to override the buffer system and cause contraction. In Fig. 9 (top trace), the sarcoplasmic reticulum of a skinned fibre was loaded with Ca^{2+} in a strongly buffered solution of pCa = 4·5 (Table 1*C*) causing the first contraction. Upon return to a weakly buffered solution of subthreshold

Ca^{2+}-concentration (pCa 6·5) the fibre relaxed. Addition of 5 mM caffeine to this Ca^{2+}-loaded fibre caused contraction which at pCa = 6·5 indicates Ca^{2+}-release from internal stores. The contraction eventually subsided,

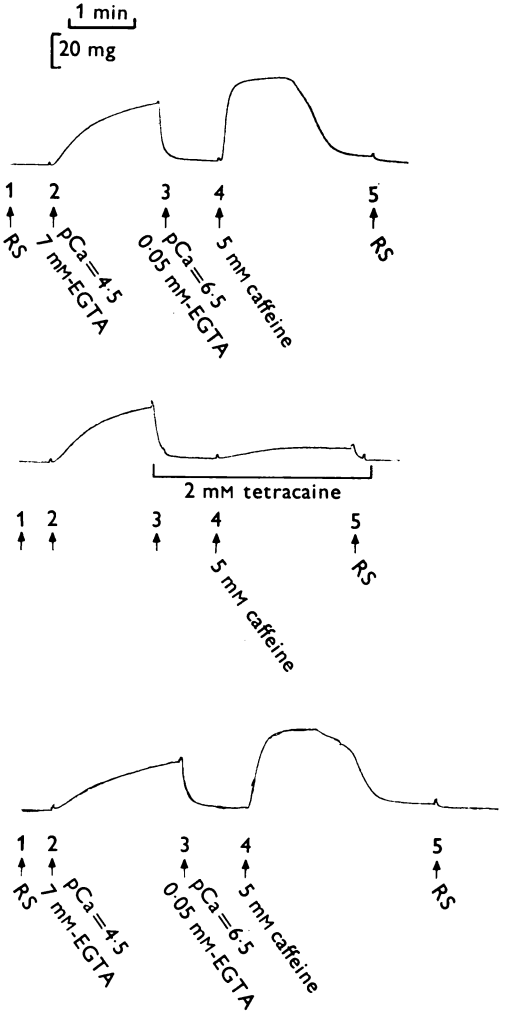

Fig. 9. Reversible inhibition by tetracaine of caffeine contractures in a skinned fibre. All solutions as in Table 1*C*. Tetracaine was present as indicated in the middle trace. Solution changes at arrows 1–3 in the middle trace as in the top trace. Fibre radius 30 μm, *Rana pipiens*, 20° C.

presumably because all the Ca^{2+} accumulated by the reticulum had been released and absorbed by the buffer system. 2 mM tetracaine reversibly diminished the caffeine contracture (middle and bottom trace). The experiment was tried 15 times, and tetracaine always reduced tension

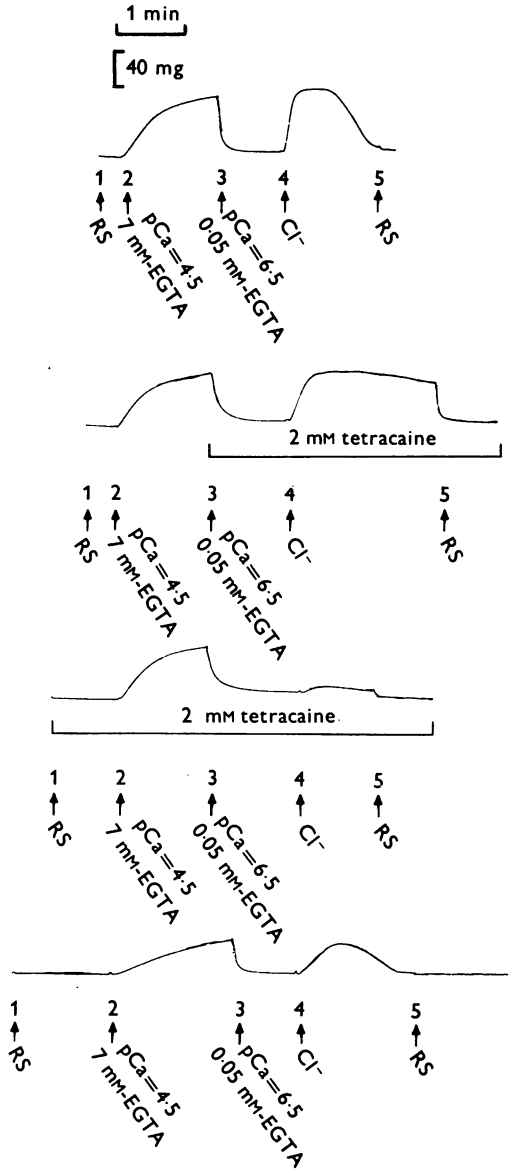

Fig. 10. Effect of tetracaine on chloride-induced contractures. All solutions as in Table 1*C*. At arrows marked '4', the propionate contained in the bathing solution was exchanged for Cl. Tetracaine applied in the two middle traces as indicated. Notice that tetracaine does not diminish Cl^--induced tension (second trace) unless the drug is applied during the period allowed for Ca^{2+}-loading of the reticulum (third trace). Fibre radius 29·5 μm, *Rana pipiens*, 20° C.

during a caffeine contraction to less than 20% of the control value. Inhibition by tetracaine was less pronounced in these experiments than in intact fibres (Feinstein, 1963; Lüttgau & Oetliker, 1968). Perhaps the Ca^{2+} causing the residual 'caffeine contraction' in tetracaine came not from the sarcoplasmic reticulum but was contained in the bathing medium. Possibly the caffeine was slightly contaminated with Ca^{2+}, or interacted with other ingredients of the solution.

One can also induce Ca^{2+}-release in skinned fibres by raising the ambient Cl^--concentration (Endo & Nakajima, 1973). The experiment in Fig. 10 was similar to that in Fig. 9. The sarcoplasmic reticulum was allowed to accumulate Ca^{2+} at pCa = 4·5 until immersion in a weakly buffered, Cl^--free solution of pCa = 6·5 produced relaxation. Propionate in the solution was then replaced by chloride and a large contracture ensued. As Endo & Nakajima suggested, this 'chloride-induced Ca^{2+}-release' could be explained if the membrane of the reticulum were Cl^--permeable; sudden elevation of extra-reticular $[Cl^-]$ could then make the internal potential of the reticulum more negative and possibly produce an increase in Ca^{2+}-permeability.

As is seen in the second trace, tetracaine does not greatly diminish the response. In eleven experiments of the kind, peak tension of the Cl response in 2 mM tetracaine was always within 15% of the control value. The drug will almost certainly have reached its site of action within the 60 sec allowed for equilibration since Lüttgau & Oetliker found 0·1 mM of the drug sufficient to relax an intact fibre of 110 μm diameter within 20 sec from a caffeine contraction; sufficiently rapid action of tetracaine is also indicated by Fig. 9. Therefore it appears that chloride-induced Ca^{2+}-release could still take place in the presence of tetracaine. It is possible, however, that the release rate was less in the second trace of Fig. 10; for example, tension rose more slowly, possibly because at a lower release rate it took longer to saturate the contractile proteins. Also, relaxation was slower, again perhaps because the amount of Ca^{2+} accumulated by the reticulum was expanded at a lesser rate. Nevertheless, it is clear that tetracaine affects Cl^--induced release less than that induced by 5 mM caffeine.

The third trace shows that tetracaine can have a large effect on the Cl^--induced contraction if the drug is present during the loading process. This brings to mind a report by Johnson & Inesi (1969) that tetracaine at sufficient concentration can block Ca^{2+}-accumulation by the reticulum of rabbit skeletal muscle. It is difficult to say from Fig. 10 to what extent Ca^{2+} uptake was inhibited. The bottom trace shows that inhibition of Ca^{2+}-loading by tetracaine is at least partially reversible.

DISCUSSION

Our results confirm that tetracaine inhibits the contractile response to cell membrane depolarization in intact muscle. In skinned fibres, however, this inhibition was observed only when contraction depended on calcium release from internal stores as, for example, during exposure to caffeine. When Ca^{2+} was applied directly to the contractile proteins, 2 mM tetracaine diminished neither the force developed by a skinned fibre nor its sensitivity to Ca. It therefore appears that tetracaine blocks contraction in intact fibres by inhibiting Ca release from the sarcoplasmic reticulum. This inhibition of Ca release is not accompanied by loss or conspicuous alterations of the displacement currents which are thought to represent a cell membrane potential-dependent step in the regulation of Ca release. Still, our experiments rule out neither an involvement of the displacement currents in this process nor even a direct linkage (Chandler *et al.* 1976 *b*) between the molecules giving rise to displacement currents and the Ca^{2+} release sites on the sarcoplasmic reticulum. For example, the drug tetrodotoxin can block ion transport through Na channels in nerve membranes without effect on what are thought to be the 'gating currents' accompanying the opening and closing of these channels (Armstrong & Bezanilla, 1974). Yet it is generally thought that the ion-transporting part of the Na channel is part of the same molecules as, or at least structurally linked to, the moieties which open and close the channel. Be that as it may, if the displacement currents observed in muscle *are* involved in regulating Ca release our experiments show that it is possible to inhibit Ca release without affecting the release-regulating signal between transverse tubules and sarcoplasmic reticulum.

Residual mechanical activity in the presence of tetracaine

Contraction and Ca release can still occur to some extent in 2 mM tetracaine (Figs. 1, 10) and remain dependent on the cell membrane potential (Fig. 1). One therefore asks to what degree Ca release had been blocked in our experiments. Although a definitive answer will probably not be given until we have more direct methods for measuring Ca release, there are indirect arguments suggesting that inhibition was substantial.

In order to produce contraction in a tetracaine-poisoned intact fibre, depolarizing pulses at 5° C must last so long that neither potential spread down the transverse tubules (Valdiosera *et al.* 1974*b*) nor the relaxation of displacement current transients can determine the minimum stimulus duration for contractile activation. Much more likely, this duration is determined by the rate at which Ca can be released from the tetracaine-

poisoned sarcoplasmic reticulum, and the fact that strength–duration curves become essentially vertical at positive potentials indicates that the potential dependence of Ca release saturates, as does the curve relating charge displacement to cell membrane potential (Fig. 3). Potentials positive to about 40 mV may therefore be assumed to fully activate whatever fraction of the potential-dependent release mechanism remains intact in 2 mM tetracaine. Since the voltage dependence of contraction saturates at similar potentials also in partially refractory, but unpoisoned fibres (Adrian *et al.* 1976) one can compare the strength–duration curves of Fig. 1 in presence and absence of the drug. Such a comparison would suggest that 2 mM tetracaine diminishes Ca release twenty-fivefold since at 50 mV it takes about 25 times longer in tetracaine to supply a threshold amount of Ca to the myoplasm. This estimate relies on assuming that: (*a*) Ca release by a single release site is a constant except as affected by tetracaine; (*b*) Ca uptake by the sarcoplasmic reticulum can be ignored here; (*c*) the minimum amount of Ca needed for contractile activation is not affected by the drug; and (*d*) that Ca release starts and stops without delay at beginning and end of a depolarizing pulse. There is at present no evidence for or against assumption (*a*); (*b*) is correct at least for normal fibres since by Ford & Podolsky's (1972*a*) measurements, one would expect the uptake rate at 21° C to be of the order of 0·28 mM/sec at subthreshold Ca^{2+} concentrations and therefore only 0·063 mM/sec at 5° C (Weber, Herz & Reiss, 1966). This is small compared to a release rate of at least 2·5 mM/sec obtained if one followed Hill's (1949) suggestion that muscle at 0° C is maximally activated within 40 msec after a stimulus and that at least 0·1 mM-Ca^{2+} (Ebashi & Endo, 1968) has been supplied to the myofilaments by that time. In the presence of tetracaine, Ca^{2+} uptake is inhibited but may not be negligible; tetracaine inhibition of Ca^{2+} release may therefore be somewhat less than twenty-fivefold. Assumption (*c*) would be true if over the concentration range experienced by a fibre during threshold activation, the majority of myoplasmic Ca binding sites were involved in activating crossbridges. Assumption (*d*) is wrong, at least if displacement currents regulate and precede Ca^{2+} release, but the assumption need not introduce much error if one chooses membrane potentials where on- and off-displacement transients have similar time courses.

In order to allow for the kinetics of charge displacement which may influence Ca^{2+} release, we consider that the release rate Φ is linearly proportional to the time integral of the displacement current. Since at extreme potentials the currents are fairly well fitted by single exponentials,

$$\Phi = \Phi_{max} \frac{Q}{Q_{max}} [1 - \exp(-t/\tau_{on})], \quad 0 \leqslant t \leqslant d, \tag{9}$$

$$\Phi = \Phi_{max}\frac{Q}{Q_{max}}[1-\exp(-d/\tau_{on})]\exp[-(t-d)/\tau_{off}], \quad d \leqslant t. \tag{10}$$

Φ_{max} is the release rate when all mobile dipoles or charges are in the depolarized configuration, τ_{on} the time constant for displacement currents during depolarization, τ_{off} that during repolarization and d the duration of the depolarizing pulse. Eqn. (9) refers to Ca^{2+} release during the pulse, eqn. (10) to that after the pulse while some release sites may still be active. The total amount, R, of Ca^{2+} released as a result of the

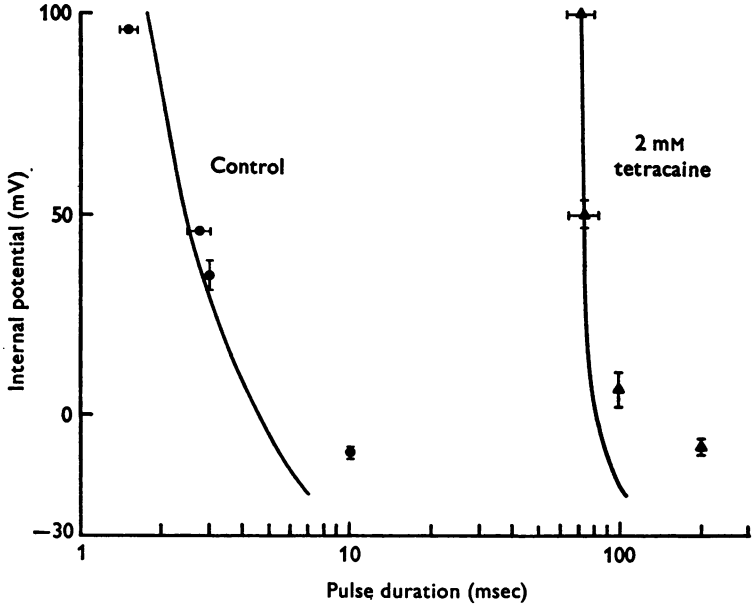

Fig. 11. Partial reconstruction of strength–duration curves of Fig. 1 using displacement current parameters. Curves were drawn by eqn. (12) with R/Φ_{max} = 2·8 msec in the absence and 74·5 msec in the presence of 2 mM tetracaine, i.e. assuming a 26·6-fold reduction in Ca^{2+}-release capacity. Parameters for displacement currents were taken from the experiment 40-3-1 (Table 2) which gave the most complete data at positive potentials.

pulse is obtained by integrating eqns. (9) and (10) with respect to time and adding the results. Eqn. (9) is integrated from $t = 0$ to $t = d$ and eqn. (10) from $t = d$ to infinity:

$$R = \int_0^\infty \Phi\, dt = \Phi_{max}\frac{Q}{Q_{max}}\{d+(\tau_{off}-\tau_{on})[1-\exp(-d/\tau_{on})]\}. \tag{11}$$

Let the subscripts T and N refer to measurements with 2 mM tetracaine and under normal conditions; any effects of the drug on displacement currents are neglected. If the amount released at threshold is not changed by tetracaine, $R_N = R_T$ and

$$\frac{\Phi_{max,N}}{\Phi_{max,T}} = \frac{d_T+(\tau_{off}-\tau_{on})[1-\exp(-d_T/\tau_{on})]}{d_N+(\tau_{off}-\tau_{on})[1-\exp(-d_N/\tau_{on})]}, \tag{12}$$

For τ_{on} = 2·86 msec at +45 mV and τ_{off} = 3·22 msec at −100 mV (curve in Fig. 3), for d_N = 2·8 msec and d_T = 74 msec, both at +46 mV (see Fig. 1) the ratio in eqn. (13) indicates that tetracaine reduces capacity for Ca^{2+}-release twenty-fivefold as estimated above. Tubular delays are neglected here.

At more positive potentials, the minimum stimulus durations with and without tetracaine diverge. An effect of this kind is expected as τ_{on} diminishes. The curves in Fig. 11 were drawn by eqn. (11) taking the voltage dependence of Q/Q_{max} and τ_{on} from Fig. 3 and solving iteratively for d. Given R/Φ_{max} and that it is reduced about twenty-fivefold in tetracaine, the analysis contains no further free parameters. The calculated strength–duration curves reproduce the main features of the data at positive potentials, suggesting that the kinetics of displacement currents are approximately consistent with a direct proportionality between Ca^{2+} release by the reticulum and the fraction of charges or mobile dipoles in the depolarized configuration. The treatment fails at negative potentials, perhaps partly because Ca^{2+}-uptake by the sarcoplasmic reticulum has been neglected.

Another possible consequence of tetracaine's inhibition of Ca^{2+}-release is a change in rheobasic threshold for contraction, and it is of interest whether a twenty-fivefold reduction in release rate is consistent with the relatively small threshold shift from -36 to -23 mV observed here. At potentials just below contraction rheobase, Ca^{2+}-uptake by the sarcoplasmic reticulum must equal the release, since otherwise the myoplasmic Ca concentration would eventually rise above threshold. Therefore, even a large inhibition of Ca^{2+}-release may fail to produce large shifts in rheobase potential if the uptake is inhibited also. Tetracaine-induced inhibition of Ca^{2+}-uptake is strongly suggested by Fig. 10 and Johnson & Inesi (1969; fragmented sarcoplasmic reticulum from rabbit skeletal muscle) found that 2 mM tetracaine reduces steady-state Ca^{2+}-accumulation sixfold. Moreover, the drug appears to double the sensitivity of contractile proteins to Ca^{2+}, at least at suprathreshold concentrations, and Ca^{2+}-uptake at rheobase may therefore have occurred at twice lower myoplasmic Ca^{2+}-levels. Since at sufficiently low concentration, Ca^{2+}-uptake varies in linear proportion to the ambient $[Ca^{2+}]$ (Weber, 1971; Ford & Podolsky, 1972*a*) it is easy to imagine that at rheobase, the uptake (and therefore also release) was $2 \times 6 = 12$ times less with 2 mM tetracaine than without. This effect is still smaller than the twenty-fivefold inhibition of maximal Ca^{2+}-release rate estimated by comparing minimum stimulus durations. However, the drug shifted the rheobase potential by 13 mV, suggesting that the rheobasic Ca^{2+}-release in tetracaine, though diminished in absolute terms, may have been a larger fraction of the maximum than in normal fibres.

In summary, both minimum stimulus durations and rheobasic contraction thresholds are consistent with the view that 2 mM tetracaine diminished more than tenfold the capacity for Ca^{2+}-release from the sarcoplasmic reticulum in response to cell membrane depolarization.

Some of the experiments reported here were done while one of us (W.A.) was on leave of absence at the Physiological Laboratory in Cambridge, England and it is a pleasure to thank Dr R. H. Adrian for his kind hospitality. We are grateful to Drs A. M. Gordon and W. G. L. Kerrick for loan of equipment and to Drs Gordon,

Sue K. Donaldson and B. Hille for helpful discussions and for reading the manuscripts. P.M.B. was a Research Fellow of the Washington State Heart Association. Supported by USPHS grant no. AM17803.

REFERENCES

ADRIAN, R. H. & ALMERS, W. (1974). Membrane capacity measurements on frog skeletal muscle in media of low ion content. *J. Physiol.* **237**, 573–605.

ADRIAN, R. H. & ALMERS, W. (1976*a*). The voltage dependence of membrane capacity. *J. Physiol.* **254**, 317–338.

ADRIAN, R. H. & ALMERS W. (1976*b*). Charge movement in the membrane of striated muscle. *J. Physiol.* **254**, 339–361.

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1969). The kinetics of mechanical activation in frog muscle. *J. Physiol.* **204**, 207–230.

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1970). Voltage clamp experiments in striated muscle fibres. *J. Physiol.* **208**, 607–644.

ADRIAN, R. H., CHANDLER, W. K. & RAKOWSKI, R. F. (1976). Charge movement and mechanical repriming in skeletal muscle. *J. Physiol.* **254**, 361–388.

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

ALMERS, W., ADRIAN, R. H. & LEVINSON, S. R. (1975). Some dielectric properties of muscle membrane and their possible importance for excitation-contraction coupling. *Ann. N.Y. Acad. Sci.* **264**, 278–292.

ALMERS, W. & BEST, P. M. (1976). Effects of tetracaine on contraction and 'gating currents' in frog skeletal muscle. *Biophys. J.* **16**, 152*a*.

ARMSTRONG, C. M. & BEZANILLA, F. (1974). Charge movement associated with the opening and closing of the activation gates of the Na channels, *J. gen. Physiol.* **63**, 533–552.

BIANCHI, C. P. & BOLTON, T. C. (1967). Action of local anesthetics on coupling systems in muscle. *J. Pharmac. exp. Ther.* **157**, 388–405.

CAPUTO, C. (1976). The effect of caffeine and tetracaine on the time course of potassium contractures of single muscle fibres. *J. Physiol.* **255**, 191–208.

CHANDLER, W. K., RAKOWSKI, R. F. & SCHNEIDER, M. F. (1976*a*). A non-linear voltage dependent charge movement in frog skeletal muscle. *J. Physiol.* **254**, 245–283.

CHANDLER, W. K., RAKOWSKI, R. F. & SCHNEIDER, M. F. (1976*b*). Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol.* **254**, 285–316.

COLQUHOUN, D. (1971). *Lectures on Biostatistics*. Oxford: Clarendon Press.

COSTANTIN, L. L. (1974). Contractile activation in frog skeletal muscle. *J. gen. Physiol.* **63**, 657–674.

DONALDSON, S. K. B. & KERRICK, W. G. L. (1975). Characterization of the effects of Mg^{2+} on Ca^{2+}- and Sr^{2+}-activated tension generation of skinned skeletal muscle fibres. *J. gen. Physiol.* **66**, 427–444.

EBASHI, S. & ENDO, M. (1968). Calcium ion and muscle contraction. *Prog. Biophys. molec. Biol.* **18**, 123–183.

ENDO, M. & NAKAJIMA, Y. (1973). Release of calcium induced by 'depolarization' of the sarcoplasmic reticulum membrane. *Nature, New Biol.* **246**, 216–218.

FRANZINI-ARMSTRONG, C. (1970). Studies of the triad. I. Structure of the junction in frog twitch fibres. *J. Cell Biol.* **47**, 488–499.

FEINSTEIN, M. B. (1963). Inhibition of caffeine rigor and radiocalcium movements by local anesthetics in frog sartorius muscle. *J. gen. Physiol.* **47**, 151–172.

FORD, L. E. & PODOLSKY, R. J. (1972*a*). Calcium uptake and force development by skinned muscle fibres in EGTA buffered solutions. *J. Physiol.* **223**, 1–19.

FORD, L. E. & PODOLSKY, R. J. (1972*b*). Intracellular calcium movements in skinned muscle fibres. *J. Physiol.* **223**, 21–33.

FURUSAWA, K. & KERRIDGE, P. M. T. (1927). The hydrogen ion concentration of the muscle of the cat. *J. Physiol.* **63**, 33–44.

GOLDMAN, D. E. (1943). Potential, impedance and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

GUTKNECHT, J. & TOSTESON, D. C. (1973). Diffusion of weak acids across lipid bilayer membranes: effects of chemical reactions in unstirred layers. *Science, N.Y.* **182**, 1258–1261.

HELLAM, D. C. & PODOLSKY, R. J. (1969). Force measurements in skinned muscle fibres. *J. Physiol.* **200**, 807–819.

HILL, A. V. (1913). The combinations of haemoglobin with oxygen and with carbon monoxide. I. *Biochem. J.* **7**, 471–480.

HILL, A. V. (1949). The abrupt transition from rest to activity in muscle. *Proc. R. Soc. Lond.* B. **136**, 399–420.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & HOROWICZ. (1960). The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. *J. Physiol.* **153**, 370–385.

HODGKIN, A. L. & NAKAJIMA, S. (1972). The effects of diameter on the electrical constants of frog skeletal muscle fibres. *J. Physiol.* **221**, 105–120.

HUTTER, O. F. (1969). Potassium conductance of skeletal muscle treated with formaldehyde. *Nature, Lond.* **224**, 1215–1216.

JOHNSON, P. N. & INESI, G. (1969). The effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharmac. exp. Ther.* **169**, 308–314.

KERRICK, W. G. L. & BEST, P. M. (1974). Calcium ion release in mechanically disrupted heart cells. *Science, N.Y.* **183**, 435–437.

LÜTTGAU, H. C. & OETLIKER, H. (1968). The action of caffeine on the activation of the contractile mechanism in striated muscle fibres. *J. Physiol.* **194**, 51–74.

MCLAUGHLIN, S. (1975). Local anaesthetics and the electrical properties of phospholipid bilayer membranes. In *Progress in Anesthesiology*, vol. 1. *Molecular Mechanisms of Anesthetics*, ed. FINK, B. R. New York: Raven Press.

OHNISHI, T. & EBASHI, S. (1964). Spectrophotometrical measurement of instantaneous calcium binding of the relaxing factor in muscle. *J. Biochem.* **54**, 506–511.

SCHNEIDER, M. F. & CHANDLER, W. K. (1973). Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature, Lond.* **242**, 244–246.

THORENS, S. & ENDO, M. (1975). Calcium-induced calcium release and 'depolarization-'induced calcium release: their physiological significance. *Proc. Japan Acad.* **51**, 473–478.

THOMAS, R. C. (1976). The effect of carbon dioxide on the intracellular pH and buffering power of snail neurones. *J. Physiol.* **255**, 715–736.

VALDIOSERA, R., CLAUSEN, C. & EISENBERG, R. S. (1974*a*). Measurement of the impedance of frog skeletal muscle fibres. *Biophys. J.* **14**, 295–315.

VALDIOSERA, R., CLAUSEN, C. & EISENBERG, R. S. (1974*b*). Impedance of frog skeletal muscle fibres in various solutions. *J. gen. Physiol.* **63**, 460–491.

WEBER, A. (1971). Regulatory mechanisms of the calcium transport system of fragmented rabbit sarcoplasmic reticulum. I. The effect of accumulated calcium on transport and adenosine triphosphate hydrolysis. *J. gen. Physiol.* **57**, 50–63.

WEBER, A., HERZ, R. & REISS, I. (1966). Study of the kinetics of calcium transport by isolated fragment sarcoplasmic reticulum. *Biochem. Z.* **345**, 329–369.