Fura-2 diffusion and its use as an indicator of transient free calcium changes in single striated muscle cells

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Two questions bearing on the use of fura-2 to measure transient changes in intracellular Ca^{2+} concentration have been addressed. To investigate fura-2 intracellular binding, the amounts of fura-2 and [^{14}C]glycine in *Balanus nubilus* myofibrillar bundles after loading were determined and their intracellular apparent diffusion constants measured. No significant fura-2 immobilisation occurs under the conditions used. The apparent diffusion constant for fura-2 in aqueous solution was determined. The relationship between half-time for relaxation of force and fura-2 fluorescence transients, and intracellular fura-2 concentration, in voltage-clamped single muscle fibres was examined. Significant buffering of the Ca^{2+} transient occurred at fura-2 concentrations above $\sim 6\ \mu M$.

Fura-2 *Diffusion* *(Striated muscle)* *Ca^{2+}* *Fluorescence* *Muscle contraction*

1. INTRODUCTION

A variety of intracellular processes in a wide range of cells are regulated by the Ca^{2+} concentration they are exposed to. The measurement of intracellular Ca^{2+} concentrations (Ca_i^{2+}) is therefore important. The fluorescent Ca^{2+} indicator fura-2 [1] is becoming widely used for the measurement of Ca_i^{2+} in various preparations [2–5]. A potential problem for the use of this indicator, particularly in the measurement of fast changes in Ca_i^{2+} such as those occurring in striated and cardiac muscle, is its high affinity for Ca^{2+}, in the 200 nM range [1]. The measurement of the concentration at which the indicator will introduce significant Ca^{2+} buffering intracellularly, with a consequent slowing of the time course of transient force and Ca_i^{2+} changes, is therefore necessary if such fast changes are to be measured.

A problem in the quantification of measurements made with many metallochromic dyes, however, has been one of intracellular indicator binding (see e.g. [6]). Grynkiewicz et al. [1] report a possible small component of fura-2 free acid (fura-2) binding to red cell membranes. If fura-2 is to be used for quantitative measurements, the possibility of intracellular binding must then be examined more carefully.

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2. MATERIALS AND METHODS

2.1. *Measurement of fura-2 diffusion in aqueous solution*

The rate of diffusion of fura-2 (K Tes buffered, pH 7.00; Molecular Probes, OR, USA) in solution was measured in a capillary (length = 80 mm, diameter = 0.6 mm). The capillary was clamped horizontally in a Prior micromanipulator, allowing axial movement of the capillary under a 10× microscope objective (0.25 NA; Gillert & Sibert, Microinstruments, Oxford, England). A second capillary (diameter approx. 0.2 mm) sealed to a 1 μl Hamilton syringe (V.A. Howe, London) was used to inject 0.3 μl of 1.5 mM fura-2 by displacement into a solution (LRS) of ionic composition similar to the barnacle intracellular environment

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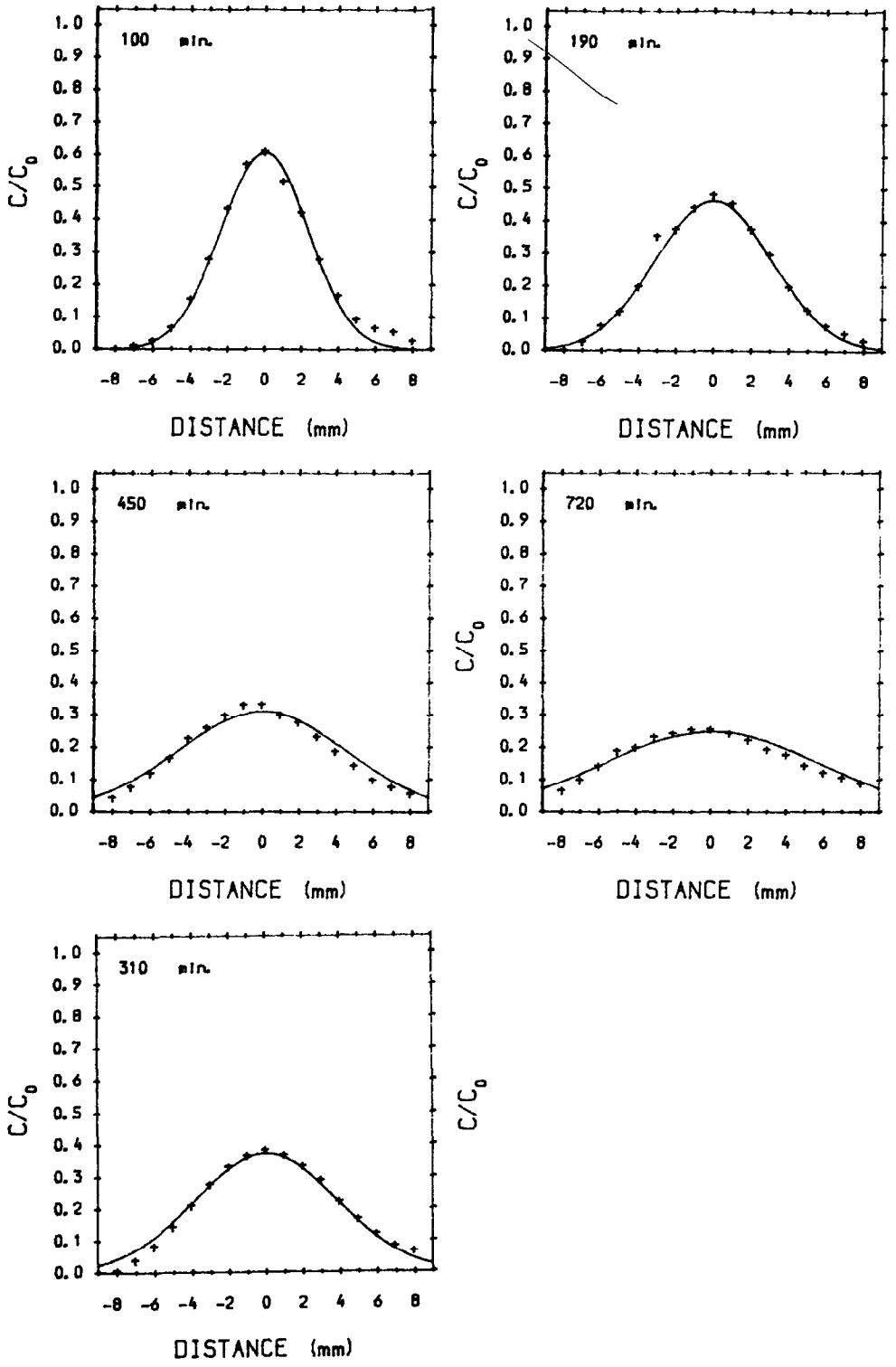

Fig.1. Longitudinal diffusion of fura-2 in aqueous solution with time. Fura-2 was initially injected between -1.8 and $+1.8$ mm. The solid lines represent eqn 1 fitted to the data points with $D' = 4.7 \times 10^{-6}$ cm$^2 \cdot$s^{-1}.

((mM) 0.1 EGTA, 60 Tes, 5.6 $MgCl_2$, 5 ATP (Mg^{2+} free), 0.08 M ionic strength) in the first capillary. Fura-2 was excited at 380 nm by light from a 150 W xenon lamp (Wotan XBOgs, Applied Photophysics, London), passing through a monochromator (2 mm slitwidth) and conducted at 90° to the objective onto the capillary by a 5 mm diameter liquid light guide (Oriel Scientific, Kingston-upon-Thames, Surrey). Fluorescence was recorded through the objective and a 500 nm cut-on filter (Corning 3.73) onto a photomultiplier (PM) tube (9781B; Thorn EMI, England).

2.2. *Myofibrillar bundle efflux experiments*

Myofibrillar bundles for the efflux experiments were dissected from single fibres under paraffin oil (medium grade; BDH, Poole, England). A bundle was glued by means of cyanoacrylate adhesive (Superglue; Loctite, Welwyn Garden City, England) to two glass rods and submerged in a 145 μl cuvette in the solution changer. Diffusion from myofibrillar bundles was measured by changing solutions at given time intervals for a bundle previously loaded for 1 h in 145 μM LRS containing 100 μM [^{14}C]glycine and 150 μM fura-2. The solution fura-2 concentration was measured in a fluorimeter (Shimadzu RF540; V.A. Howe, London), while [^{14}C]glycine concentration was measured in a Perkin Elmer scintillation counter (Ready Solve EP Scintillation Fluid; Beckman, High Wycombe, England). No significant quenching by fura-2 at the concentrations used was observed.

All experiments were done at room temperature (20°C). The apparent dissociation constant for fura-2 under simulated intracellular conditions (LRS) was determined to be 456 nM.

2.3. *Single muscle fibres*

Single muscle fibres of 2.5 – 3 cm length from the barnacle (*Balanus nubilus*) were cannulated and axially microinjected with fura-2. Intracellular fura-2 concentrations ranged from 6 to 180 μM. The fibres used had resting membrane potentials in the range −45 to −60 mV (uncorrected for junction potentials). A voltage-clamp electrode, consisting of a 100 μm diameter platinised platinum wire glued to a 100 μm (outside diameter) glass KCl-filled capillary, containing a 25 μm bright platinum wire was then inserted axially along the muscle fibre. In one case a fibre was stimulated by constant current pulses, otherwise fibre stimulation occurred under voltage clamp. Force was recorded with a force transducer (AE801; Aksjeselskapet Mikro-elektronikk, Horten, Norway), with a 7 mm steel pin glued to the strain-sensitive leaf, protruding 5 mm from the tip. The indicator was excited at 340, 360 or 380 nm by light from the xenon light source passing through the monochromator and conducted onto the preparation by a 5 mm liquid light guide optically coupled to a quartz rod of the same diameter. Light was collected at 90° to the exciting light through a 5 mm × 1 m silica light guide (Barr & Stroud, Glasgow) and a 500 nm (10 nm bandwidth) bandpass filter onto a PM tube (9502B; Thorn EMI, Ruislip, England). Output from the photomultiplier was passed through a current-to-voltage converter (20 ms time constant).

3. RESULTS

3.1. *Fura-2 diffusion in aqueous solution*

The value of the apparent diffusion constant (D'), in the absence of any significant binding or tortuosity factors, was determined by measuring the diffusion of fura-2 with time from the site of injection in aqueous solution. The results of such an experiment are shown in fig.1. The injecting capillary was introduced from the end shown as the positive distance, apparent by the slight perturbation of the profile at the shorter times. The data points were fitted by eye to eqn 1, describing diffusion with time and distance from an extended initial distribution [7]:

$$\frac{C}{C_0} = \frac{1}{2}\sum_{i=1}^{n} C_i\left\{\mathrm{erf}\frac{x-h_2}{2\sqrt{Dt}} - \mathrm{erf}\frac{x-h_1}{2\sqrt{Dt}}\right\} \qquad (1)$$

where n is the number of regions of different initial concentration C_i over which the diffusing substance is initially distributed, x is the distance from the centre of the site of injection, C_0 is the concentration at distance $x = 0$, $t = 0$, and h_1 and h_2 are the upper and lower bounds of the region C_i, respectively. Fitting routines were not required as an obvious difference in the goodness of fit was apparent with a change of D' as small as 0.5×10^{-6} cm$^2 \cdot$s^{-1}. The value of D' giving the best fit to the data was 4.7×10^{-6} cm$^2 \cdot$s^{-1}. The average

value of D' found for two experiments was $3.9 \times 10^{-6} \pm 0.6 \times 10^{-6}$ cm$^2 \cdot$s^{-1}.

3.2. *Fura-2 and [^{14}C]glycine diffusion from myofibrillar bundles*

The results of a representative experiment to measure fura-2 and [^{14}C]glycine efflux are shown in fig.2. The first 3 s of efflux were discounted to eliminate the contribution of effluent carried over from the loading solution. The intercept with the ordinate (M_0) was determined by extrapolating the curve fitted to the data points by eqn 2 to time zero. Eqn 2 describes the amount of diffusing substance (M) entering or leaving a cylinder of radius a (in this case the bundle radius) in time t.

$$\frac{M}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4}{a^2\alpha_n^2} \exp(-D\alpha_n^2 t) \qquad (2)$$

where M_∞ is the amount after infinite time and the α_n are the positive roots of the Bessel function of order zero $J_0(a\alpha_n) = 0$. No significant difference was found between the fit using eqn 2 and that obtained by use of the solution of the diffusion equation obtained by use of the Laplace transform [7,8].

The best fit values M_0 and D' were determined by use of a least squares, two variable fitting routine. The points and curves of fig.2 represent the renormalised efflux curves after determination of M_0. The D' values for [^{14}C]glycine and fura-2 were found to be $7.5 \times 10^{-7} \pm 0.6 \times 10^{-7}$ cm$^2 \cdot$s^{-1} ($n = 3$) and $3.9 \times 10^{-7} \pm 0.4 \times 10^{-7}$ cm$^2 \cdot$s^{-1} ($n = 6$), respectively. The fibre space occupied by fura-2 was 28 ± 2% ($n = 2$) of that occupied by [^{14}C]glycine.

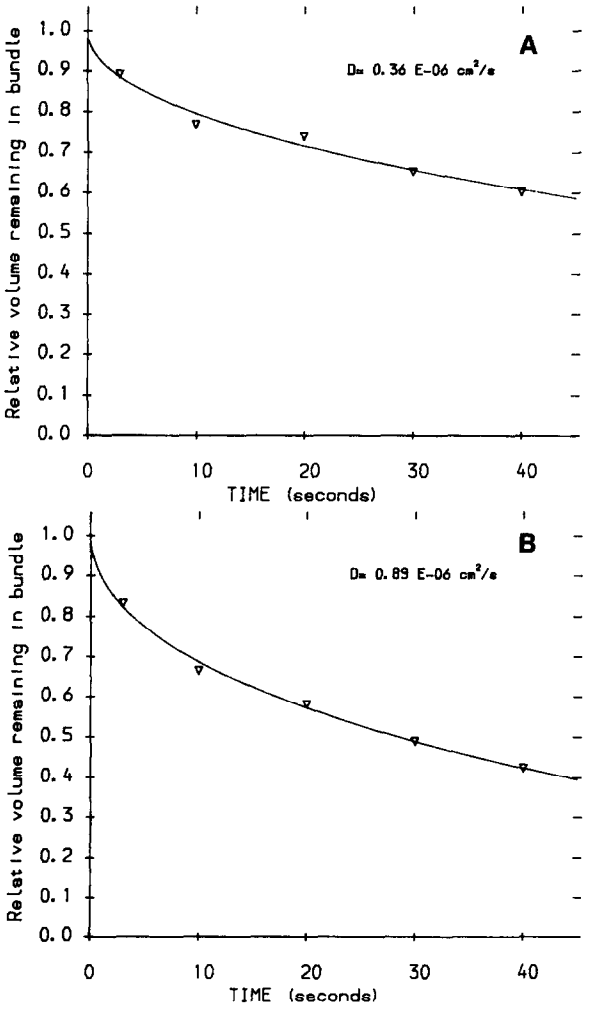

Fig.2. Efflux of fura-2 (A) and [^{14}C]glycine (B) from a myofibrillar bundle with time. The solid lines are eqn 2 fitted to the data points.

3.3. *Fura-2 fluorescence and force recorded in single muscle fibres*

Fig.3 shows the emission spectrum of a barnacle single muscle fibre before (trace a) and after (trace b) injection of fura-2. Subtraction of the two traces (trace c) shows the increase in fibre fluorescence due to the presence of fura-2. A very large increase in fluorescence (peak at 505 nm) over fibre autofluorescence is apparent. The emission spectrum of fura-2 in LRS in a cuvette is very similar to that of trace c in fig.3 and reaches a peak value at 500 nm. Transient changes in force and fluorescence in response to depolarising pulses under voltage clamp were recorded from 21 single muscle fibres injected to a range of intracellular fura-2 concentrations (6 – 165 μM). Special care was taken to inject the fibres along their entire length, in order to obtain a uniform distribution of the indicator axially. The transient changes in force and fluorescence in response to a 40 mV depolarising voltage-clamp pulse from one such fibre are illustrated in fig.4. Upon excitation at 340 nm an increase in fluorescence is seen, while a decrease may be observed upon excitation at 380 nm. No significant change in fluorescence was measured upon excitation around 358 nm, in-

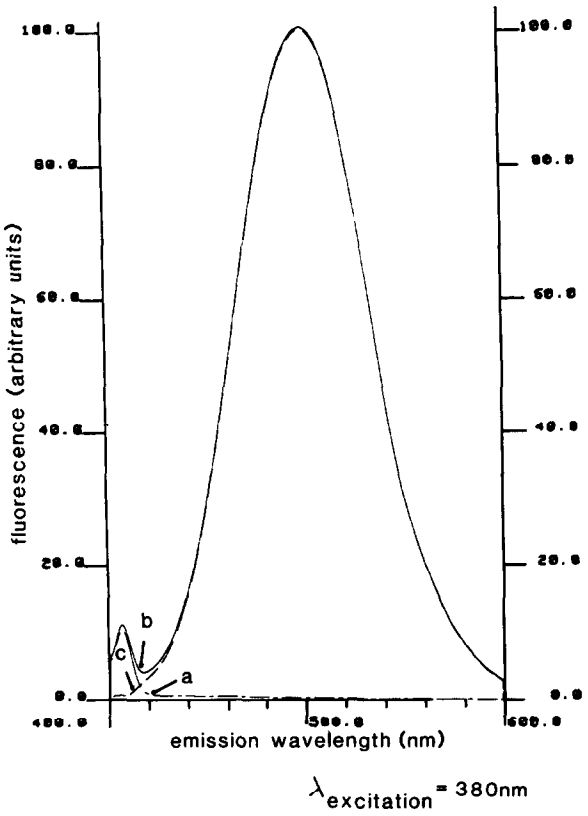

Fig.3. Emission spectrum of a barnacle single muscle fibre. Before (a) and after (b) injection of fura-2 (100 μM). (c) Difference between (a) and (b).

dicating that there was no significant contribution from Ca^{2+}-independent fluorescence changes, such as movement artifacts, to these transients.

The relations between the times for half-maximal decay ($t_{1/2}$) of force and fluorescence and fibre intracellular fura-2 concentration are illustrated in fig.5, for all the fibres studied. Each point represents the mean $t_{1/2}$ for force or fluorescence for one fibre, plotted against intracellular fura-2 concentration. It is apparent from fig.5 that the $t_{1/2}$ for both force and fluorescence decay increases with increasing intracellular fura-2 concentration. The mean force $t_{1/2}$ point on the ordinate of fig.5 (at 200 ms) is that of an uninjected fibre at this temperature. Experimentally, there was no relationship between either the intensity of stimulation or the force developed and the $t_{1/2}$ of fluorescence decay for any given fibre.

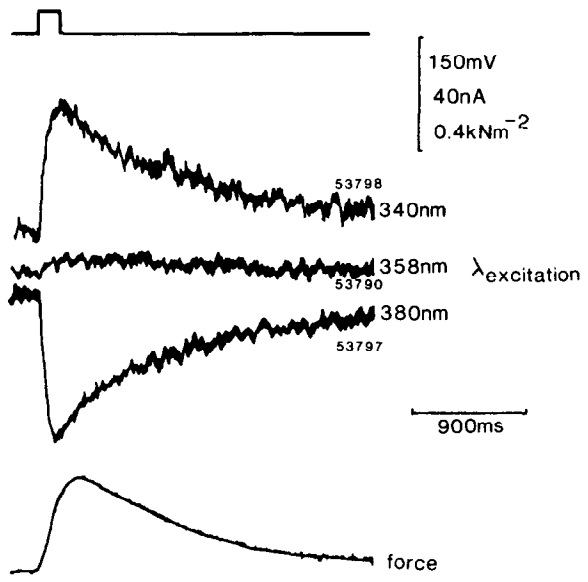

Fig.4. Transient changes in force and fluorescence from a barnacle single muscle fibre injected with 165 μM fura-2, in response to a 40 mV depolarising voltage-clamp pulse. Resting potential and holding potential were −37 mV.

Two effects of increasing the intracellular fura-2 concentration are shown in table 1. Firstly, there is a 4-fold increase in the mean $t_{1/2}$ for fluorescence decay with an increase in fura-2 concentration from 6 to 120 μM, while the $t_{1/2}$ for force increases 3.5-fold. The $t_{1/2}$ for fluorescence at 6 μM fura-2 is very close to that calculated from the deconvoluted aequorin signal (200 ms at 11–12°C [9]).

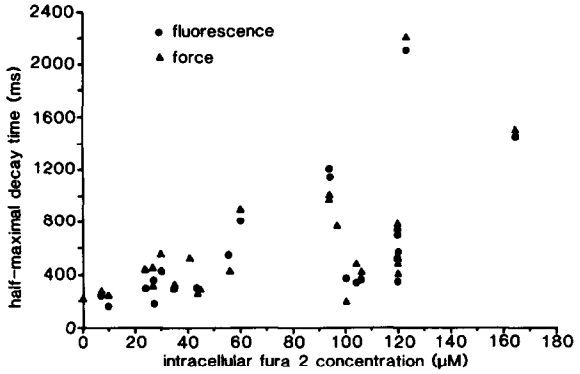

Fig.5. Plot of the times for half-maximal decay of force (▲) and fura-2 fluorescence (●) against intracellular fura-2 concentration for 21 single muscle fibres.

Table 1

Analysis of times for half-maximal decay of force and fluorescence at different $[fura\text{-}2]_i$

$[Fura\text{-}2]_i$	Experiment		n	Mean half-maximal decay time ± SE (ms)
120 μM	2.4.85F1 2.4.85F2 26.4.85F1	force	55	861.8 ± 69.9
	2.5.85F2 22.5.85F1	fluorescence	55	810.8 ± 81.3
30 μM	3.4.85F1 11.4.85F1	force	48	435.0 ± 15.3
	1.5.85F1 2.5.85F1	fluorescence	48	303.9 ± 13.4
6 μM	11.4.85F2	force	24	249.8 ± 8.2
	22.5.85F2	fluorescence	14	193.2 ± 10.8

Temperature 20°C

Statistical analysis

Significant difference between concentrations compared to experiments (analysis of variance): $p < 0.01$

Significance of difference

		120 μM		30 μM		6 μM	
		Force	Fluorescence	Force	Fluorescence	Force	Fluorescence
120 μM	force	–					
	fluorescence	n.s.	–				
30 μM	force	<0.01	<0.01	–			
	fluorescence	<0.01	<0.01	<0.01	–		
6 μM	force	<0.01	<0.01	<0.01	<0.01	–	
	fluorescence	<0.01	<0.01	<0.01	<0.01	<0.01	–

As the apparent dissociation constant of Ca^{2+} from aequorin is in the high micromolar range [10], no significant buffering is to be expected from this indicator in arthropod fibre (but see [11] for frog). Secondly, at the higher intracellular fura-2 concentrations examined (30 and 120 μM), the $t_{1/2}$ for force became significantly different from that for fluorescence.

4. DISCUSSION

Despite the widespread use of fura-2 and quin2, the fluorescent Ca^{2+} indicator with a similar Ca^{2+} affinity [12], little is known directly about their effect on the intracellular environment by their Ca^{2+} buffering capacity. An important reason for this must be the difficulties associated with accurate measurement of intracellular concentration of the indicator and the control of the amount of indicator entering the small cells in which most of the measurements are performed. One buffering effect on Ca^{2+} influx in squid axons has been described [13]. Quin2 injected at high intracellular concentrations (~300 μM), like other non-fluorescent chelators such as EGTA, EDTA, NTA (nitrilotriacetic acid) and BAPTA (bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), selectively inhibit Ca_o/Na_i exchange. In our case, introduction of fura-2 by microinjection along the entire length of a large striated muscle fibre allows accurate control of the final intracellular concentration obtained, permitting a direct determination

of the effects of intracellular fura-2 on transient changes in force and fluorescence during rapid transient changes in free Ca^{2+} associated with activation processes.

To assess the extent of buffering of the Ca^{2+} transient by fura-2, measurements of transient changes in free Ca^{2+} using aequorin in the same preparation, under similar conditions [9], may serve as a point of reference. In these experiments aequorin was microinjected by replacement along the entire length of the muscle fibre to a final concentration of ~1 μM. The time course of the force transient upon injection of aequorin was the same as that before injection, indicating the introduction of a negligible amount of additional Ca^{2+} buffering power by the aequorin. This method of injection makes it very likely that any perturbation of the Ca^{2+} transient would be reflected in the force transient. The $t_{1/2}$ for decay of fura-2 fluorescence at concentrations lower than 6 μM is not significantly different from that of the Ca_i^{2+} transients measured with aequorin. Also, at these low fura-2 concentrations, the $t_{1/2}$ of the force transient approaches that measured in an uninjected fibre. It is thus only at concentrations as low as 25 μM that fura-2 measures the time course of the free Ca^{2+} change accurately.

The time course of the Ca^{2+} transient and thus the exact relationship between fluorescence $t_{1/2}$ and intracellular fura-2 concentration depends on the properties of the Ca^{2+} release and uptake reactions. Part of the explanation for the larger scatter in the data points at higher fura-2 concentrations in fig.5 would then be the inter-fibre variability of these properties, which becomes apparent when at higher fura-2 concentrations, a greater Ca^{2+} load is placed upon them.

The diffusion of fura-2 from myofibrillar bundles was compared with that of [^{14}C]glycine. The curve fitting procedure allowed determination of the space occupied by the two compounds in the loaded bundle, as well as the apparent diffusion coefficient for diffusion of the compounds from the bundle. The similarity between the D' value for glycine, an uncharged small molecule (at pH 7.00) which is not expected to show significant intracellular binding [14], and that for fura-2 indicates that fura-2 also is not bound or accumulated in restricted intracellular spaces to any significant extent. The space occupied by [^{14}C]glycine is 72 ± 2% larger than that occupied by fura-2. The difference in fibre space occupied by [^{14}C]glycine and fura-2 is consistent with the report by Stephenson et al. [14], who concluded that anions were excluded to a greater or lesser extent from the myofilament space, depending on the amount of charge carried and the ionic strength of the solutions. At the ionic strength used in this study (0.08 M) the fura-2 penta anion (at pH 7.00) would be expected to be excluded by more than the 41% Stephenson et al. [14] found for $EGTA^{2-}$. A significant exclusion (20% or more) for fura-2 at ionic strengths up to 0.356 M at pH 7.00 is to be expected.

The diffusion constant for fura-2 diffusion in aqueous solution was determined to be 5.0×10^{-6} cm$^2 \cdot$s^{-1} at 20°C and 0.08 M ionic strength. This compares to a value of around 9×10^{-6} cm$^2 \cdot$s^{-1} for glycine in water at 20°C [15]. A lower D' would be expected for fura-2 as this molecule will have a larger Stokes radius than glycine (M_r 830 for fura-2 and M_r 75 for glycine), which is related to D by the Stokes-Einstein equation:

$$D = kT/6\pi nr \quad (3)$$

where k is the Boltzmann constant, T is the absolute temperature, n is the viscosity of the medium and r is the particle radius. Also, the 5 negative charges on fura-2 at pH 7.00 would further decrease D' for fura-2 as Longsworth [16] found D and charge on the molecule to be inversely related. The D' for bundle diffusion is reduced to about 10% of that in free solution for both fura-2 and [^{14}C]glycine. This parallel behaviour is further evidence that fura-2 penta anion shows no appreciable intracellular immobilisation. The large reduction in D' for the bundle for both substances compared with their values in free solution is probably due to the higher viscosity of the diffusing medium inside the fibre. Eqn 3 predicts a decrease in D' under such conditions.

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