Fluorescence changes from single striated muscle fibres injected with labelled troponin C (TnCDANZ)

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A fluorescently labelled derivative of the calcium binding subunit of troponin, TnC, has been injected into isolated striated muscle fibres from the barnacle *Balanus nubilus*. The Ca^{2+} affinity of isolated TnC is close to that of intact troponin when located in the thin filament. Excitation of the TnCDANZ within the muscle cell (325nm) revealed a marked fluorescence at 510 nm and was similar to that observed in vitro, which was absent at 400 or 600 nm after subtraction of the fibre autofluorescence. High Ca^{2+} salines increased the fluorescence at 510 nm by roughly 2 times. Single voltage clamp pulses produced a rapid rise in fluorescence at 510 nm after allowing for any non-specific changes at 400 nm, and this signal preceded force development by approx. 55 ms at 22° C. It reached a maximum at the same time as force and subsequently decayed more slowly. The fluorescence signal increased in magnitude with increase in stimulus intensity. These results suggest that Ca^{2+} attaches rapidly to the contractile filament, but is lost relatively slowly and imply a slow decay of the activation process.

Muscle *Troponin C* *Fluorescence* *Ca^{2+}* *Contraction*

1. INTRODUCTION

Although it has been established that the free calcium concentration within muscle cells changes rapidly upon activation [1–4] little is known of the time course of calcium bound to the contractile system as a result of this transient free calcium event.

Recently, a dansylaziridine-labelled subunit of whole troponin, troponin C (TnCDANZ) has been described [5] which shows a 2.1-fold increase in fluorescence at 510 nm when calcium ions occupy the calcium-specific sites of this moiety [5] on excitation at 325 nm. The affinity of TnCDANZ (K_{Ca} 3.3×10^5 M^{-1}) increases at the calcium specific sites by ~10 times when incorporated into intact troponin, but decreases by a factor of ~10 times when whole troponin is incorporated onto the actin filaments [6]. Hence the affinity for calcium and the time course of the consequent fluorescence change of free TnCDANZ should be similar to that of whole troponin on the contractile filaments.

Here we report upon the first use of this labelled troponin C in cells and document the time course of the fluorescence change of free TnCDANZ compared to the time course of isometric force development from a single muscle fibre under voltage-clamp control.

2. MATERIALS AND METHODS

Single muscle fibres from the barnacle (*B. nubilus*) were isolated and cannulated [7] and axially microinjected with a stock solution of TnCDANZ (13 mM nominally) in 100 mM KCl (pH 7.0). The volume injected was 0.4 μl, so that after dilution the final intracellular concentration was in the range 50–150 μM. The cells used in these experiments had membrane potentials in the

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range −45 to −60 mV. They were stimulated with an axially inserted electrode assembly where the current electrode was 100 μm diam. platinised Pt wire and the voltage sensing part was a 100 μm capillary filled with 0.5 M KCl and containing a 25 μm diam. bright Pt wire [7,8]. The voltage clamp circuit was standard and force was recorded with an Akers force transducer (sensitivity 12 mN·V^{-1}; resonance frequency 4 kHz). Fibre fluorescence was excited at 325 nm with a Liconix He-Cd laser (nominally 1 mW at 325 nm but operating at about 250 μW under feedback control) beamed via a front-surfaced mirror and quartz light guide onto the preparation. A section of frosted UV-compatible plastic cuvette was used as light diffuser to reduce the intensity of the laser spot and also to act as a beam spreader. The light was collected by a 5 mm × 1 m standard light guide (Barr & Stroud, Glasgow), passed through a filter assembly with interference filters (12.5 mm diam.) at 440, 520 and 600 nm, halfband width (10 nm) (Dietrich Optics, USA), and via a 5-mm silica rod light guide to the 10-mm sensitive face plate of an EMI 9502B photomultiplier tube arranged in an anode ground configuration with the HV at −800 to −1000 V (Keithley, Reading). Output from the photomultiplier was passed through a current-to-voltage converter with a 10-ms time constant and the data stored on a Nicolet 1170 system with magnetic tape store.

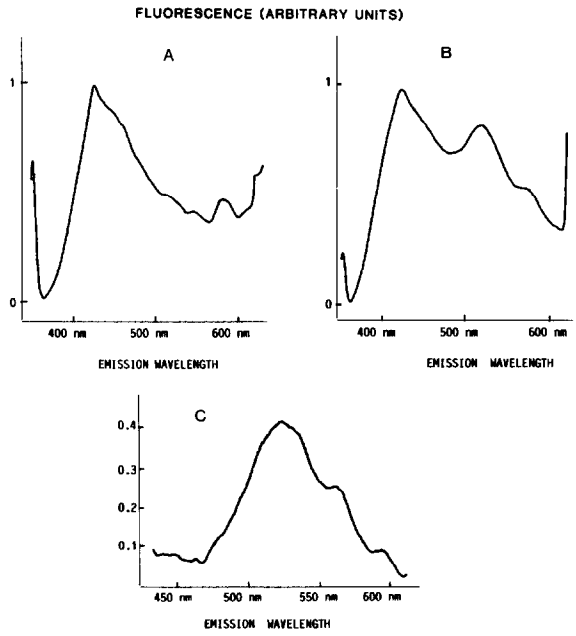

Fig.1. Fluorescence spectrum obtained from a single muscle fibre on excitation at 325 nm (Spectrophysics Spectrofluorimeter, 5 mm slit width, quartz cuvette). A. Spectrum obtained from native fibre (autofluorescence). B. Spectrum obtained after injection of TnCDANZ into the same fibre. 1 h was allowed for diffusion of TnCDANZ from the region of injection. C. Subtraction of A from B. Temp. 17°C, $[TnCDANZ]_i$ = 15 μM, 22.6.84.

3. RESULTS

In fig.1a, the fibre autofluorescence in the absence of injected TnCDANZ is indicated upon excitation at 325 nm in a conventional spectrofluorimeter assay. The fibre fluorescence shows a maximum at ~420 nm followed by a gradual decline to reach a value at 600 nm which is ~50% of that observed at 420 nm (fig.1a). In the presence of injected TnCDANZ the fibre shows a marked additional fluorescence maximum at 520 nm (fig.1b,c) which is close to that observed for Ca–TnCDANZ complex in free solution [5] at the physiological Mg^{2+} ion concentration expected within intact muscle cells [9,10].

This result indicated that the emission peak of the Ca-dependent TnCDANZ signal could be assessed satisfactorily at ~500 nm in intact cells, while a control wavelength for non-specific fluorescence changes could be either 600 or 450 nm, where the TnCDANZ signal was small. Usually either 440 or 400 nm wavelength was chosen, as these values are close to the maximum of the fibre autofluorescence and should therefore be a sensitive index of the non-specific effects if present. All records have been corrected for background fluorescence from the chamber.

Typical fluorescence signals obtained from a fibre on stimulation are shown in fig.2. Records 2a and c were obtained before injection of the cell with TnCDANZ. The scaled subtraction of fluorescence at 410 and 490 nm yields no net signal. The fibre was then injected with labelled troponin C, and after allowing time (1–2 h) for radial diffusion of the label from the site of injection (see [7]), records b and d were obtained. Scaled subtraction of fluorescence at 410 and 490 nm now yields a net signal. This difference

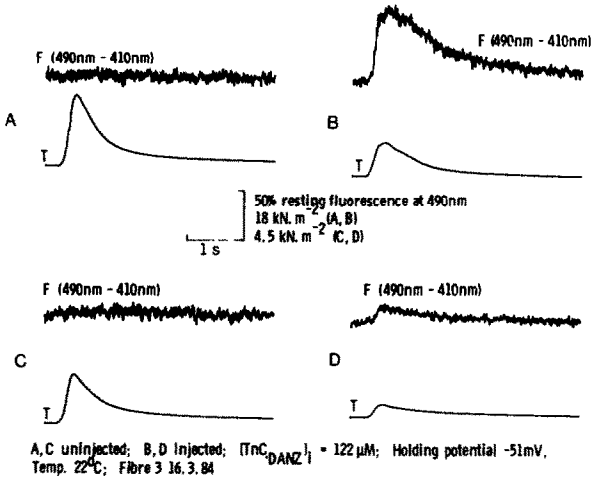

Fig.2. Tension (T) and fluorescence (F) changes recorded from a barnacle muscle fibre under voltage clamp control. A and C, prior to TnCDANZ injection, B and D, after injection. Fluorescence records were obtained by scaled subtraction of records obtained at 410 and 490 nm on excitation at 325 nm. Depolarising pulses, 200 ms duration. Subtractions performed on responses with similar peak force values. Temp. 22°C. [TnCDANZ]$_i$ = 122 μM 16.3.84. F3.

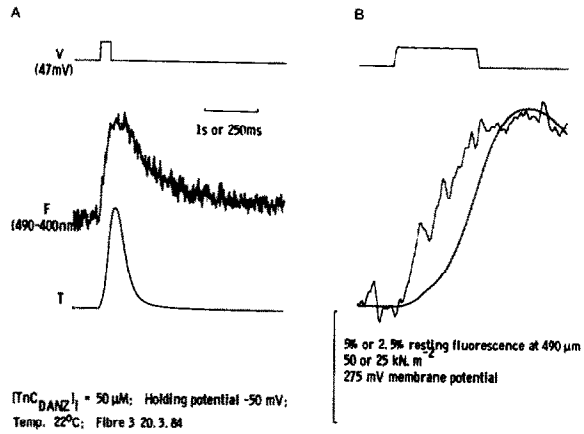

Fig.3. Time course of tension and fluorescence responses to stimulation. Left panel, membrane potential (V), fluorescence signal after scaled subtraction (F) and tension (T). Right panel, same response, but displayed on a 4× faster time trace. [TnCDANZ]$_i$ = 50 μM. Holding potential −50 mV. Temp. 22°C, 20.3.84. F3.

signal and the associated force response upon application of a depolarizing pulse both increase with increasing stimulus intensity. The time course of the fluorescence difference signal compared to force development can be assessed most easily in fig.2b, but is more clearly illustrated in the data presented in fig.3, where the experimental records have been displayed at two different sweep speeds. The faster sweep record, fig.3b, indicates that the fluorescence record rises faster than force. The $t/2$ of fluorescence rise precedes force by 55 ms (n = 15) and the fluorescence increase is virtually complete when force is still only 50% of its peak value, while the peak of force and fluorescence reach a maximum value at similar times.

Since the fibre difference fluorescence (490–410 nm) does not show the same signal during stimulation in a TnCDANZ-injected fibre as in the uninjected fibre, this result suggests that the signal from the injected cell may well arise, considering fig.1c, from a change in TnCDANZ fluorescence as a result of calcium ions being bound at the 'calcium-specific' sites of this protein subunit. A possible objection to this interpretation could be that the fluorescence signal obtained at these two wavelengths may not arise from the same regions of the cell, thus rendering the scaled subtraction procedure inappropriate. For example, the incident excitation beam might not penetrate beyond the surface of the cell or alternatively the fluorescence signal from one or both measured wavelengths may be strongly absorbed before it reaches the fibre surface. Both of these possibilities would lead to a signal derived principally from the surface of the preparation. In addition, either wavelength could contain differing degrees of contribution from peripherally located fluorescent groups again leading to a signal originating primarily from the fibre surface. To test these possibilities, the autofluorescence signal from an uninjected fibre was measured as a function of fibre radius and the results were plotted as a log–log graph (fig.4). If the fibre is circular in cross-section, then a maximum value of 2 should be obtained for the slope of this relation if the fluorescence arises from the volume of the preparation, and a value of 1 if only from the surface. The results obtained show a similar dependence on fibre radius of fluorescence at 520 and 440 nm, suggesting that they arise from similar regions of the cell, and the slope for the relationship is close to the value of 2 predicted for a cell volume dependency at both wavelengths.

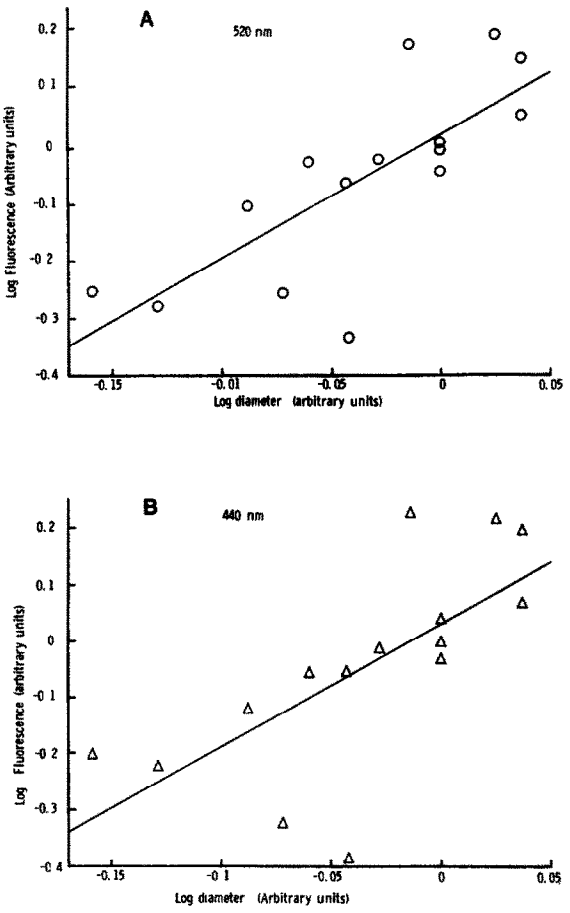

Fig.4. Log–log plot of fibre diameter against fluorescence intensity for fibre autofluorescence at 520 nm (upper panel) and 440 nm (lower panel) on excitation at 325 nm. Combined data from 3 fibres. Fluorescence and diameter expressed as a fraction of a reference level in each case. Line fitted by linear regression, $M_{520} = 2.20$, $M_{440} = 2.175$. Temp. 22°C.

Finally, in an attempt to examine the likely kinetics of the TnCDANZ signal in response to the free calcium change, the occupancy of the low affinity calcium binding sites following a step change in free calcium concentration was computed, on the assumption of simple competition between calcium and magnesium ions at this binding site. In this preparation physiological free Mg^{2+} is ~ 5 mM, and the forward rate constant for magnesium binding can be assumed to lie in the range $10^4 - 10^5$ $M^{-1} \cdot s^{-1}$ [12]. Rate constants governing calcium binding were taken from [11,12]. The calcium specific sites were, surprisingly 50% occupied with magnesium at 5 mM and although the magnesium displacement reaction is likely to be rapid it may be slow enough to produce some reduction in the Ca^{2+} 'on' rate to the magnesium occupied sites compared to the apo-calcium sites for a step-change in free Ca^{2+} at high levels of Ca^{2+} activation (fig.5). These simulations suggest that the Ca^{2+} on rates might be reduced at extreme values to the same range as rate constant values predicted from the rise time of the TnCDANZ fluorescence signal in the intact muscle fibre (fig.3b). However, there is strong evidence to suggest that the interaction of calcium and magnesium at the low affinity sites might be more complex than simple competition (see [14]), in which case the value of the 'off' rate constant for Ca^{2+} would be increased.

4. DISCUSSION

Here, we report upon the use, within an isolated muscle cell, of the fluorescently labelled calcium regulatory subunit troponin C (TnCDANZ). The affinity of TnCDANZ at the calcium specific sites is increased 10 times upon incorporation into intact troponin, yet decreases again by a factor of 10 when whole troponin is incorporated onto the actin filament in rabbit [6]. Hence the affinity of isolated rabbit-derived TnCDANZ should be similar to that of rabbit troponin on the actin filament, and the time course of the fluorescence change from rabbit TnCDANZ in solution should give some indication of the likely time course of calcium bound to rabbit troponin on the thin filament. The affinity of TnCDANZ measured in vitro in the presence of physiological concentrations of Mg^{2+} [9,10] may well be different from that in vivo and possibly different from barnacle troponin barnacle troponin [13], and dual regulation and phosphorylation may have a role to play in activation in this tissue. Nevertheless, the signal must give an indication of the time course of low affinity site occupancy.

The data presented here indicate that electrical stimulation of TnCDANZ-injected barnacle muscle fibres causes a net change in fluorescence at 520 nm after subtraction of non-specific effects monitored at 440 nm. This is consistent with the view that a fluorescence change would be expected

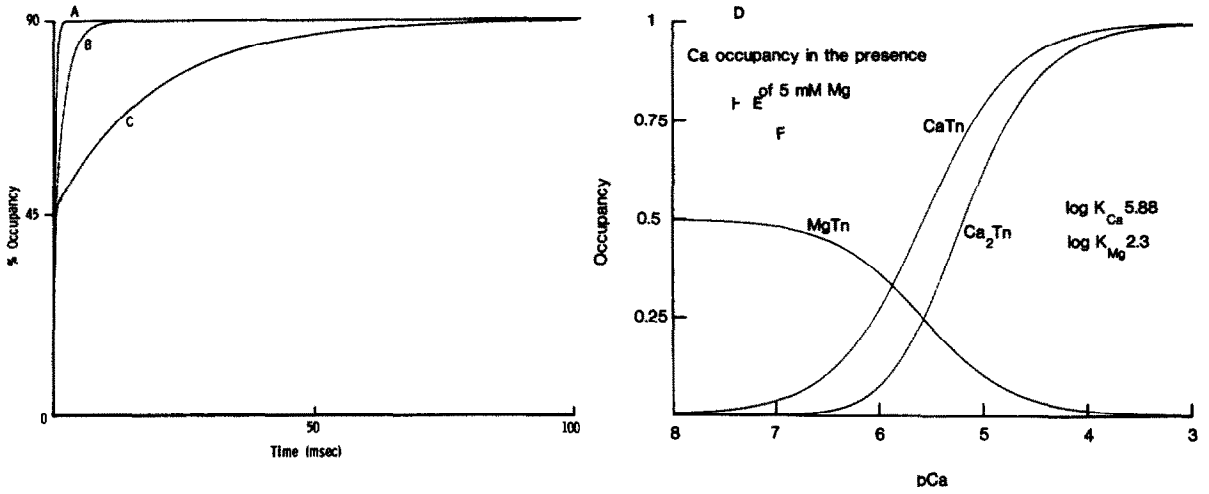

Fig.5. Computer predictions of site occupancy as a function of calcium and magnesium ion concentrations. Left, time course of Ca^{2+} binding to TnCDANZ in the presence and absence of 5 mM Mg^{2+}. Free Ca^{2+} change as step function of suitable amplitude giving 90% of maximal activation in the presence (curves B and C) or absence (curve A) of Mg^{2+}. $k_{on}^{Ca} = 10^8\ M^{-1}\cdot s^{-1}$, $k_{off}^{Ca} = 1.3 \times 10^2\ s^{-1}$, $k_{on}^{Mg} = 10^5\ M^{-1}\cdot s^{-1}$ (curve B) or $10^4\ M^{-1}\cdot s^{-1}$ (curve C), $k_{off}^{Mg} = 500\ s^{-1}$ (curve B) or $50\ s^{-1}$ (curve C). Right, equilibrium on occupancy as a function of free calcium concentration [Mg^{2+}] 5 mM, pK_{Ca} 5.88, pK_{Mg} 2.3. Gear method used for numerical integration [23].

at this wavelength as a result of calcium binding to the injected TnCDANZ. Furthermore, experiments performed on uninjected fibres which were subsequently injected with TnCDANZ showed that this signal at 520 nm was absent before TnCDANZ injection. The reported dependence of fluorescence intensity upon the square of the fibre radius further supports this view, since it would appear that injected TnCDANZ would be excited to fluoresce throughout the fibre cross-section, and that the non-specific fluorescence at 440 nm should arise from the same region of the cell as the TnCDANZ signal. Localised membrane damage of injected fibres exposing the indicator to a raised (10 mM) Ca^{2+} concentration caused a roughly 2-fold increase in fluorescence at 520 nm after correction for non-specific changes at 440 nm, as would be expected from the in vitro behaviour of TnCDANZ. These observations add further support to the notion that, in intact fibres, the fluorescence at 520 nm after correction for non-specific changes at 440 nm is as would be expected from the in vitro behaviour of TnCDANZ upon the binding of Ca^{2+}.

Experimentally, the fluorescence change from free TnCDANZ upon stimulation is not radically different from that of force development in these barnacle muscle fibres. This is most clearly illustrated in fig.3a where the peak of the fluorescence difference signal and that of isometrically recorded force occur at about the same moment. There are, however, differences in the time course of the two parameters. The rising phase of the fluorescence difference signal usually precedes force development and is complete at a time when force is only 50% developed (fig.3b). This would suggest that the calcium bound to isolated TnCDANZ and putatively to the troponin on the intact thin filament, occurs substantially before the onset of force. This finding would not be in discord with observations which indicate that, in frog skeletal muscle, cross-bridge movement, measured in terms of the change in the equatorial X-ray diffraction intensity ratios ($I_{I,O}$, $I_{I,I}$), substantially precedes force development [15,16]. Likewise, fibre stiffness, a measure of cross-bridge attachment, also precedes force development in single frog fibres at 5°C [17]. Nevertheless, the time course of the development of the fluorescence change is slower than would have been predicted simply from the published 'on' rate constants for the calcium specific sites (k_{on} $10^8\ M^{-1}\cdot s^{-1}$). One possible reason for this

discrepancy is that the on rate for calcium to the calcium-specific sites could be limited by a relatively slow magnesium displacement reaction. At equilibrium, calculations suggest that some 50% of the sites were occupied by magnesium ions when the free magnesium ion concentration was set at 5 mM, the likely value in these cells. Computer simulation indicated that at the expected rate of magnesium displacement for such low-affinity sites ($50 - 500\ s^{-1}$) there may be slowing of the calcium on rate to the Mg^{2+}-occupied sites (fig.5, left), although the rate to the apo-sites was, as expected, unaffected. The extent of the slowing might be sufficient to explain some of the slowing of the rate of rise of fluorescence observed in the intact cells, although the nature of the magnesium–calcium ion interaction at the low affinity sites must be fully elucidated first, and may be more complex than simple competition (see [14]). Another notion was that the magnesium displacement rate constant could be much altered by the ionic environment within the muscle cell but this possibility has not so far been examined. In addition, it will be of great interest to employ another indicator within the cell whose response to free calcium changes is well documented, such as aequorin [3,4,7] so that the signal from both the aequorin and TnCDANZ signal can be recorded in addition to the force response.

Perhaps the most interesting feature of the present results is the observation that the net change in fluorescence at 520 nm decays more slowly than force in all records so far examined. This fact is well demonstrated in fig.3a. The half time for the decay of fluorescence is on average 2.0 × that of force at 22°C.

Hence the Ca^{2+} attached to the Ca^{2+}-specific sites of TnCDANZ, and also presumably to intact troponin on the thin filament, dissociates more slowly than the decline of force. This is not in discord with mechanical findings on single frog muscle fibres, where it is recognised that the exponential fall of force observed after the 'knee' of force, represents essentially sarcomeric disorganisation and does not necessarily represent true decline of activation at the level of individual sarcomeres. This is clearly shown in experiments using the sarcomere clamp [18] where the isometric fall of force, preceding the knee of tension, can be considerably prolonged by maintaining sarcomeric homogeneity. These experiments, if directly applicable to this muscle fibre preparation, would support the notion that this slow decay process may well involve a component or reaction which is dependent upon the relatively slow release of calcium from the contractile system.

Support for this notion also comes from the description of a prolonged 'tail' to the aequorin light response and hence free calcium, seen both in single barnacle [8,19] and frog muscle fibres [20,21]. In barnacle fibres a potentiation of a second force response is observed for an identical depolarizing pulse provided that the elevated light 'tail' is present [19,22].

Again, this matter will be more clearly resolved when experiments can be performed where both aequorin and TnCDANZ are injected into the same muscle fibres so that the response from both these indicators can be directly compared to the time course of the decay isometric force response, and when the response is studied in other muscle fibre preparations, for example, frog.

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