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The effects of reported Ca^{2+} sensitisers on the rates of Ca^{2+} release from cardiac troponin C and the troponin-tropomyosin complex

[1]Susan J. Smith & Paul J. England

Department of Cellular Pharmacology, Smith, Kline and French Research Limited, The Frythe, Welwyn, Herts. AL6 9AR

1 The calcium sensitivity of force production of cardiac muscle fibres is altered by certain drugs. The sites of action of three such compounds (pimobendan, sulmazole, isomazole) within the myofibril have been investigated. Calmodulin antagonists, perhexilene and bepridil, which have been shown to alter the calcium dependence of myofibrillar ATPase activity and oxmetidine, an H_2-receptor antagonist which binds to calmodulin, were also studied.

2 The rates of dissociation of calcium from both the regulatory and high affinity sites on bovine isolated cardiac troponin C (cTnC) were measured in a stopped-flow fluorimeter. The rates of dissociation were found to be $136.5 \pm 16\,s^{-1}$ and $1.3 \pm 0.20\,s^{-1}$ (mean $\pm$ s.e.mean, $n = 11$ determinations; conditions: 100 mM KCl, 10 mM MOPS, 3 mM $MgCl_2$, 0.1 mM dithriothreitol, pH 7.0, 15°C). Sulmazole, isomazole and perhexiline (final concentration of 50 μM) had no effect on the rate of Ca^{2+} dissociation from the regulatory Ca^{2+} site, indicating that these compounds do not act on cTnC directly.

3 The rate of dissociation of Ca^{2+} from the regulatory site was slightly reduced (~20%) by pimobendan (50 and 100 μM) and was somewhat increased by oxmetidine (28% at 100 μM).

4 Bepridil (25 μM) reduced the rate of dissociation by 50%, indicating a direct effect of bepridil on TnC.

5 Sulmazole, isomazole, perhexiline, pimobendan (50 μM) and bepridil (25 μM) were without effect on the rate of dissociation of Ca^{2+} from the high affinity Ca^{2+}/Mg^{2+} sites. Oxmetidine caused 24% decrease in the rate of Ca^{2+} dissociation from these sites.

6 The rate of dissociation of Ca^{2+} from the regulatory site on the complex of troponin-tropomyosin (TnTm) was measured. Sulmazole and pimobendan (50 μM) were without effect on the rate of dissociation of Ca^{2+} from the regulatory site in the protein complex, and isomazole (50 μM) caused only a slight reduction (23%). Perhexiline (50 μM) or bepridil (10 μM) reduced the rate of Ca^{2+} dissociation by about 50%. The rate of dissociation of Ca^{2+} from the high affinity Ca^{2+}/Mg^{2+} sites was not altered by sulmazole, isomazole, or pimobendan (50 μM), but was decreased ~35% by perhexiline (50 μM) or bepridil (10 μM).

Introduction

Drugs which increase the force of contraction of the failing heart may have therapeutic benefit in congestive heart failure. One possible mechanism of action of such drugs may be to increase the Ca^{2+} sensitivity of the contractile proteins (Adelstein & Eisenberg, 1980; Ruegg, 1987; Wetzel & Hauel, 1988).

Regulation of cardiac contractility involves Ca^{2+}-dependent changes in the actin-bound troponin-tropomyosin (TnTm) complex (reviewed in Zot & Potter, 1987). The troponin complex (Tn) is composed of three subunits. Troponin C (TnC) is the Ca^{2+}-binding subunit. Cardiac muscle TnC has two high affinity, metal binding sites in the C-terminal region which can bind Ca^{2+} or Mg^{2+}, and one low affinity, Ca^{2+} binding site near the N-terminus. Skeletal muscle TnC has two high affinity, C-terminal sites, and two low affinity Ca^{2+} specific N-terminal sites (van Eerd & Takahashi, 1975). Troponin I (TnI) the inhibitory subunit which inhibits actin-myosin interaction, can be phosphorylated in heart by adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase. Troponin T (TnT) enhances the interaction of the other subunits with tropomyosin.

In cardiac muscle, the free Ca^{2+} concentration reaches about 1 μM during systole (Allen & Kurihara, 1980). This is only sufficient to produce half-maximal activation of force (Fabiato, 1983). A drug which increases the Ca^{2+} occupancy of the low affinity Ca^{2+} specific site of the Tn complex may, therefore, increase the interaction of actin and myosin and, hence, produce a greater force at a given level of cytoplasmic Ca^{2+} (Johnson *et al.*, 1979).

Cardiotonic agents have been identified which appear to act directly on the contractile proteins, increasing their sensitivity to Ca^{2+} (Wetzel & Hauel, 1988). This is shown by an increase in the force produced by skinned cardiac tissue preparations at a submaximal Ca^{2+} concentration, reflecting an alteration in the affinity of regulatory Ca^{2+} specific sites which are involved in activation of force production (Pan & Solaro, 1987).

In vitro, Ca^{2+} sensitising agents have been identified by their effects on the adenosine 5′-triphosphatase (ATPase) activity of washed myofibrils, which retain the basic thick and thin filament arrangement of the contractile proteins in intact muscle. Measurement of myofibrillar ATPase has been favoured as an *in vitro* screen for Ca^{2+} sensitising agents, because of its apparent simplicity and the ease of protein preparation and assay. However, it is becoming apparent that the data from these assays may not predict the likely effects of the drugs in muscle. This may be because the behaviour of the myofibrils during the ATPase assay is complex and ill defined (discussed in Smith & England, 1989). In general, compounds may modify V_{max} or basal ATPase activity in addition to any effects on Ca^{2+} sensitivity (Silver *et al.*, 1985). These results suggest that the compounds identified as Ca^{2+} sensitisers *in vivo* may act at sites in addition to those on the troponin complex.

In order to facilitate the development of more specific drugs, it is important to determine where they act within the contractile apparatus. Here, we have used systems of increasing complexity to define the sites of action of compounds

[1] Author for correspondence.

within the TnTm complex. We have compared the effects of compounds known to be Ca^{2+} sensitisers on the rates of Ca^{2+} release from Ca^{2+} binding sites on isolated TnC or on TnTm.

The rates of Ca^{2+} release from sites on isolated cardiac TnC have been measured by stop-flow with a fluorescent chelator. This identifies agents which increase the Ca^{2+} affinity of TnC directly.

The rates of Ca^{2+} release from the low affinity Ca^{2+}-specific and high affinity Ca^{2+}/Mg^{2+} sites of the cardiac TnTm complex have also been measured. TnI labelled covalently with a fluorescent probe incorporated into the TnTm complex was found to indicate the Ca^{2+} occupancy of these sites. This provides an assay for Ca^{2+}-sensitizing compounds which act on some component of the TnTm complex.

With these approaches, we have investigated the sites of action of the calcium sensitisers (reviewed by Wetzel & Hauel, 1988) pimobendan, sulmazole and isomazole, and calmodulin antagonists, perhexiline and bepridil (Silver *et al.*, 1985).

A combination of the methods described here can be used for the first step in the development of an accurate structure-activity relationship that will allow rational drug design of better Ca^{2+} sensitising compounds, acting on known components of the TnTm complex.

Methods

Protein preparation

TnC was prepared from beef heart essentially as described in Szynkiewicz *et al.* (1985) by ion exchange chromatography of urea-extracted, EDTA-washed cardiac myofibrils. A protease inhibitor cocktail was included throughout (50 μM phenylmethyl sulphonyl fluoride, 1 $\mu g\,ml^{-1}$ each of leupeptin, pepstatin and antipain). Purification by ion exchange chromatography was found to be more effective than calcium-dependent hydrophobic interaction chromatography. The appropriate eluate from DEAE Sephadex A25 was bound to a Pharmacia FPLC Mono-Q column and eluted with a gradient of 0 to 1 M NaCl in 20 mM Tris HCl, 0.5 mM dithiothreitol (DTT), pH 7.5 at 4°C. TnC was eluted from 0.35 to 0.5 M NaCl. The purity of the protein was determined by its mobility on 15% polyacrylamide 0.1% SDS gels (Laemmli, 1970). To reduce calcium contamination of the protein, it was dialysed against 50 mM Tris, 1 mM EGTA, 0.5 mM DTT at pH 8.5 and finally into Milli-Q water containing 1 mM DTT, and freeze dried.

TnC concentration was determined from its absorbance at 276 nm after subtraction of the absorbance at 320 nm due to light scattering by use of M_r 18,500 and extinction coefficient of $0.3\,cm^2\,mg^{-1}$ (Szynkiewicz *et al.*, 1985).

Crude TnTm was prepared from beef heart by ammonium sulphate precipitation of an extract from an acetone powder of myofibrils (Stull & Buss, 1977). The purified material was diluted 2 fold in glycerol and stored at −20°C.

Preparation of fluorescently labelled troponin I in the troponin-tropomyosin complex

Crude TnTm was dialysed free of mercaptoethanol with a large excess of buffer. Protein (40–60 μM) in KCl (30 mM), MOPS (10 mM) at pH 7.0 was labelled at a cysteine residue by addition of 4-(N-iodoacetoxyethyl-N-methyl)-7-nitrobenz-2-oxa-1,3-diazole (IANBD) in ethanol (40–60 μM final concentration) at 4°C in the dark. The reaction with IANBD was terminated after 6 h by addition of 10 fold excess of DTT followed by extensive dialysis in the dark against KCl (100 mM), MOPS (10 mM), DTT (0.1 mM) at pH 7.0. The resulting suspension was clarified by centrifugation (Trybus & Taylor, 1980).

The extent of labelling was determined from the spectrum of the reacted protein after dialysis, with extinction coefficients of $E_{278}\,mg\,ml^{-1} = 0.38$ for TnTm and $E_{480}\,mM = 25$ for IANBD. The low degree of labelling (0.27 and 0.41 $mol\,mol^{-1}$ TnTm) found for two preparations was expected as conditions were chosen to label only one of the two cysteine groups on TnI (Leszyk *et al.*, 1988). The localisation of the label on TnI was confirmed by running samples of the modified protein on SDS polyacrylamide gels (15%) (Trybus & Taylor, 1980) and comparing the position of fluorescent and protein bands.

Kinetic methods

Transient measurements were carried out in a Hi-Tech (Salisbury, UK) stopped flow instrument with a dead time of 3.0 ms, at 15°C. The rate constants of Ca^{2+} dissociation from TnC were measured directly by use of the fluorescent chelator 2-[[2-bis(carboxymethyl)]-amino-5-methylphenoxy]-methyl]-6-methoxy-8-bis(carboxymethyl)]-aminoquinoline, Quin-2 (Rosenfeld & Taylor, 1985a). Ca^{2+} depleted TnC (20 μM) was mixed with an excess of Quin-2 (480 μM). Excitation was from a Xenon lamp (Wotan 150 W/S or equivalent) and the incident beam passed through a monochromator at 339 nm (5 nm band pass). The increase in fluorescence as calcium bound to Quin-2 was monitored at 90° to the incident beam with a 455 nm cut-off filter. Measurements of the rate of Ca^{2+} release over a three fold range of Quin-2 concentrations (80–240 μM final) gave similar results. Control experiments in which Ca^{2+} was mixed with Quin-2 indicated that Ca^{2+} binding to the chelator occurred in the dead time of the instrument.

The rate constants of Ca^{2+} dissociation from the TnTm complex were measured with Quin-2 as above. The fluorescence of IANBD-labelled TnI within the TnTm complex was monitored with excitation at 490 nm and a 500 nm cut off filter. The fluorescence decreased with time as IANBD-TnTm (2.6 μM) was mixed with excess EGTA (20 mM) to chelate the released Ca^{2+}.

The rate of Ca^{2+} release from the Quin-2 Ca^{2+} complex on mixing with 20 mM EGTA was determined, in the presence of excess Quin-2, to be $32.5\,s^{-1}$ at 15°C. This is similar to the value determined by Rosenfeld & Taylor (1985a) for EDTA. This rate was not affected by the compounds used in the study.

In experiments where compounds were present, they were preincubated with TnC or TnTm for 15 min at room temperature before being loaded into the stop flow.

Binding of Ca^{2+} to Quin-2 is accompanied by an increase in fluorescence on excitation at 339 nm and a smaller decrease in fluorescence on excitation at 380 nm (Grynkiewicz *et al.*, 1985). Since some of the compounds to be tested show considerable absorbance at 339 nm, the excitation wavelength for Quin-2 fluorescence, the light available to excite fluorescence in the assay was frequently reduced in the presence of the test compound (inner filter effect). To avoid this problem, fluorescence can be excited at a longer wavelength (380 nm). This was done for drugs such as pimobendan and sulmazole and the decrease in fluorescence of Quin-2 on release of Ca^{2+} from TnC was monitored. Control experiments showed that the rates of Ca^{2+} release from TnC determined at either wavelength in the absence of drug were the same. The rates of chelation of Ca^{2+} by EGTA from the Quin-2 Ca^{2+} complex were identical when monitored either at 339 nm or at 380 nm.

However, the signal observed as Ca^{2+} was released from TnC and bound to Quin-2 was smaller for excitation at 380 nm (about 2.5 fold reduction), compared to that with excitation at 339 nm. Therefore for experiments at 380 nm, the slit width was increased to 1 nm and the voltage on the photomultiplier tube was adjusted to obtain approximately the same size signal at either excitation wavelength.

Drugs

Drugs used were pimobendan (UD-CG 115) and sulmazole (AR-L 115), kindly provided by Karl Thomae, isomazole generously donated by Eli Lilly and oxmetidine (SK&F 92994).

Bepridil and perhexiline were obtained from Sigma Chemical Corp.

Data collection and analysis were by standard methods. Two hundred 12 bit data points were collected per record. Four or more data traces were averaged, after the traces which showed obvious artefacts were discarded and the averaged traces fitted. The fluorescence change has an exponential time-dependence of the type $I(t) = Ie^{-kt}$ where k is the rate of Ca^{2+} release and I is the fluorescence at time t. For Ca^{2+} release from sites with two off rates, the time dependence was fitted to using two exponential terms. A Simplex algorithm was used in an iterative process to minimise the deviation of the data from the theoretical curve by adjustment of the chosen parameters. For a satisfactory fit, a residual plot showing the deviation of the data from the fitted curve indicated that the residuals were randomly distributed.

Results

Measurement of dissociation of Ca^{2+} from troponin C

The rate of dissociation of Ca^{2+} from TnC was measured by monitoring the increase in fluorescence on mixing with Quin-2. The transient collected over 100 ms was biphasic and fitted to a double exponential equation. In the example shown in Figure 1a, the solid line through the data corresponds to two rates of $162\,s^{-1}$ and $1.6\,s^{-1}$. The rate of the slower process was determined more accurately to be $0.6\,s^{-1}$ by collection of data for longer times (5 s, Figure 1b).

The effects of pimobendan (50 or 100 μM), sulmazole (50 μM), isomazole (50 μM), perhexilene (50 or 100 μM), bepridil (25 μM) and oxmetidine (100 μM) on the rate of Ca^{2+} dissociation from TnC were tested in similar experiments. The results are shown in Table 1. In some cases, the spectroscopic properties of the compounds themselves limited the maximum concentration which could be tested. Data for sulmazole and pimobendan were collected with excitation at 380 nm.

As shown in Table 1, sulmazole, isomazole and perhexiline at final concentrations of 50 μM had no effect on the rate of Ca^{2+} dissociation from the low affinity site of TnC. The rate of dissociation was slightly reduced by pimobendan ($\sim$20% at 50 or 100 μM) and was somewhat increased by oxmetidine ($\sim$30% at 100 μM).

Only bepridil had a major effect on the rate of dissociation of Ca^{2+} from the low affinity Ca^{2+} binding site. Bepridil (25 μM) reduced the rate of dissociation by $\sim$50%. This increased affinity of the regulatory site for Ca^{2+} occurred without any significant change in the affinity of the Ca^{2+}/Mg^{2+} high affinity sites.

Sulmazole, isomazole, perhexiline or pimobendan (50 μM) also had no effect on the rate of Ca^{2+} dissociation from these Ca^{2+}/Mg^{2+} sites. Oxmetidine caused a slight decrease in dissociation rate (24%).

Jaquet & Heilmeyer (1987) have suggested that dimerization of cardiac TnC occurs over the range 5–10 μM under essentially similar conditions to those used here. Dimerized protein has a lower Ca^{2+} affinity than the monomeric protein as determined by measurements of Ca^{2+} binding, with the apparent Ca^{2+} affinity of TnC decreasing three fold over this range of protein concentration. If this were due to effects on the low affinity site, the Ca^{2+} off-rate from this site would be expected to be increased at high protein concentration. We measured the rate of Ca^{2+} dissociation from TnC over a wide concentration range (10–60 μM TnC final). There was no increase in the rate of dissociation from the low affinity site (<10%) and at most a 25% increase in the rate of dissociation from the high affinity sites. The extent of dimerization of the protein would be expected to range from 24% to >85% dimers, over this range of TnC concentration.

Jaquet & Heilmeyer (1987) suggested that drugs which alter the extent of dimerization of TnC would cause an apparent

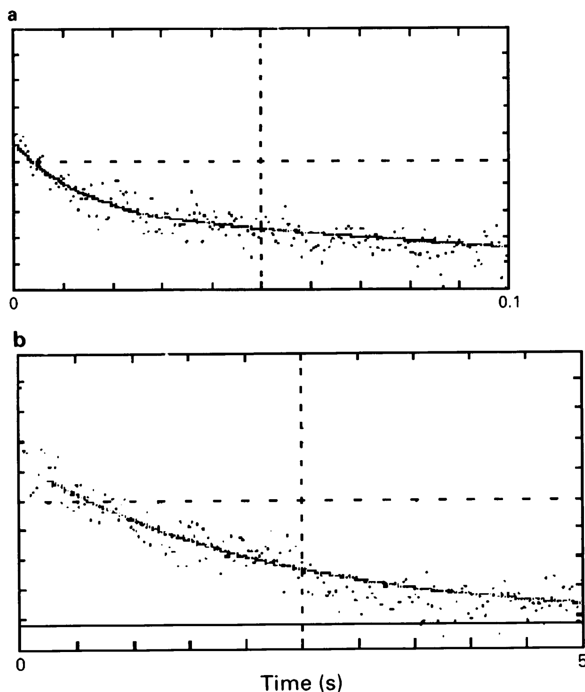

Figure 1 Stop flow traces of calcium dissociation from cardiac troponin C (cTnC). The data are the average of 5 traces. (a) The fluorescence signal collected for 0.1 s can be fitted by two exponential terms with rates $162\,s^{-1}$ and $1.6\,s^{-1}$. The fast phase contributes approximately one third of the amplitude of the fluorescence change allowing for the dead time of the stop flow. (b) Fluorescence signal collected for 5 s. The solid line corresponds to the rate of $0.6\,s^{-1}$. Full scale 0.25 V. Vertical axes are photomultiplier voltage changes representing fluorescence changes in arbitrary units. Lamp voltage is adjusted so that the overall voltage change is less than 2 V. Conditions: excitation 339 nm, emission > 475 nm. Composition of solution in mM: KCl 100, MOPS 10, $MgCl_2$ 3, dithiothreitol 0.1, pH 7.0 at 15°C. Final concentrations of cTnC 10 μM, added Ca^{2+} 50 μM, Quin-2 240 μM.

Table 1 The effects of Ca^{2+} sensitising compounds on the rates of Ca^{2+} release from low and high affinity sites on bovine cardiac troponin C (TnC)

	Rate of dissociation of Ca^{2+} from TnC as % vehicle control	
	Low affinity site	*High affinity sites*
Control	100%	100%
Sulmazole (50 μM)	97%	93%
Isomazole (50 μM)	109%	111%
Pimobendan (50 μM)	84%	105%
(100 μM)	79%	95%
Oxmetidine (100 μM)	128%	76%
Perhexiline (50 μM)	101%	93%
(100 μM)	101%	96%
Bepridil (25 μM)	47%	104%

Conditions are given in the legend to Figure 1, except that measurements for sulmazole and pimobendan were made by use of excitation at 380 nm and by monitoring the rate of decrease in Quin-2 fluorescence. Values for the rate of dissociation of Ca^{2+} from the high affinity sites are taken from fits of data collected for 5 s. The values are given relative to vehicle controls in the same experiments, (perhexiline, 0.05% ethanol, pimobendan, 0.025% ethanol). The concentrations of ethanol present had no effects on rates of dissociation of Ca^{2+}. For each experiment, 4 to 10 data traces were averaged. The results shown are means of such data from two experiments with different batches of TnC.

Control value was $136.5 \pm 16\,s^{-1}$ and $1.3 \pm 0.2\,s^{-1}$ for rates of dissociation from the low affinity and high affinity Ca^{2+} binding sites (mean $\pm$ s.e.mean, $n = 11$).

alteration in the calcium affinity of the protein. Since the effects of any dimerization of the protein on its Ca^{2+} affinity appear small under our conditions, we have not investigated this further.

Measurement of dissociation of Ca^{2+} from troponin tropomyosin complex

Measurements of Ca^{2+} dissociation from sites in TnTm complex were made to identify Ca^{2+} sensitisers which do not act on TnC directly and to determine the effects of protein-protein interactions on the drug protein complex.

The rate of dissociation of Ca^{2+} from the TnTm complex was measured from the increase in fluorescence on mixing with excess Quin-2 (Figure 2a). The solid line through the data is a fit to a single exponential equation with a rate of $23\,s^{-1}$, corresponding to dissociation from the low affinity site. Fluorescence transients collected for 5 s (data not shown) showed a slower process at $1.2\,s^{-1}$ corresponding to dissociation of Ca^{2+} from the high affinity sites. Thus, the Ca^{2+} binding properties of the high affinity sites of cardiac TnC are not affected by incorporation into the TnTm complex, whereas the rate of release from the low affinity site is reduced some 10 fold. Similar effects have been shown for skeletal TnC (Rosenfeld & Taylor, 1986b).

Measurement of Ca^{2+} release from TnTm by chelation with Quin-2, as described above, gave data that were difficult to analyse, particularly when drugs were present. It was unclear whether this was due to the optical or biochemical properties of the system. To overcome this problem, the rates of dissociation of Ca^{2+} were measured by monitoring the changes in fluorescence of IANBD on TnI incorporated into the TnTm complex (Trybus & Taylor, 1980). The large change in fluorescence on Ca^{2+} release from the complex and the low fluorescence background make this a sensitive technique. None of the compounds tested absorbs at the excitation wavelength of IANBD (490 nm).

To determine whether the fluorescent modification of TnI with IANBD altered the Ca^{2+} binding properties of the TnTm complex into which it was incorporated, the rate of dissociation of Ca^{2+} from the fluorescently labelled complex was also measured as described above. The increase in fluorescence of the calcium chelator and indicator on release of Ca^{2+} from the complex is shown in Figure 2b. The solid line through the data corresponds to a rate of $22\,s^{-1}$ for Ca^{2+} release from the low affinity site. Fluorescence transients collected for longer times (data not shown) showed a slower process at $0.9\,s^{-1}$ corresponding to dissociation from the high affinity sites. These values are the same as those found for the unmodified protein complex under the same conditions. Hence the Ca^{2+} binding properties of the TnTm complex are not modified by fluorescently-labelling the TnI.

In parallel experiments, the fluorescence of IANBD was monitored (Figure 3) as Ca^{2+} was released from the labelled TnTm by mixing with excess chelator EGTA. The solid line through the data corresponds to a fit to a double exponential equation with rates of $22\,s^{-1}$ and $1.8\,s^{-1}$. These rates are the same as those of Ca^{2+} release monitored in the experiment described above, and indicate that the probe on TnI measures Ca^{2+} release from the low and high affinity sites of TnC within the TnTm complex. In contrast, the fluorescence changes of IANBD-labelled skeletal TnI incorporated into skeletal TnTm occur only on Ca^{2+} release from the low affinity Ca^{2+} sites of the TnC component of the complex (Rosenfeld & Taylor, 1985b).

Figure 4 shows data from a stopped flow experiment in which changes in fluorescence of IANBD labelled TnI within the TnTm complex were measured as Ca^{2+} was released from the complex on mixing with excess EGTA (10 mM final). Figure 4a shows a control trace. The solid line through the data is the fit to two exponential terms giving two rates $12.7\,s^{-1}$ and $1.4\,s^{-1}$ for the release from the low and high affinity Ca^{2+} sites respectively. Figure 4b shows a trace from the same experiment in the presence of 50 μM perhexiline, which can be fitted to give two rates of $6.5\,s^{-1}$ and $0.9\,s^{-1}$ for the release from the low and high affinity Ca^{2+} sites respectively.

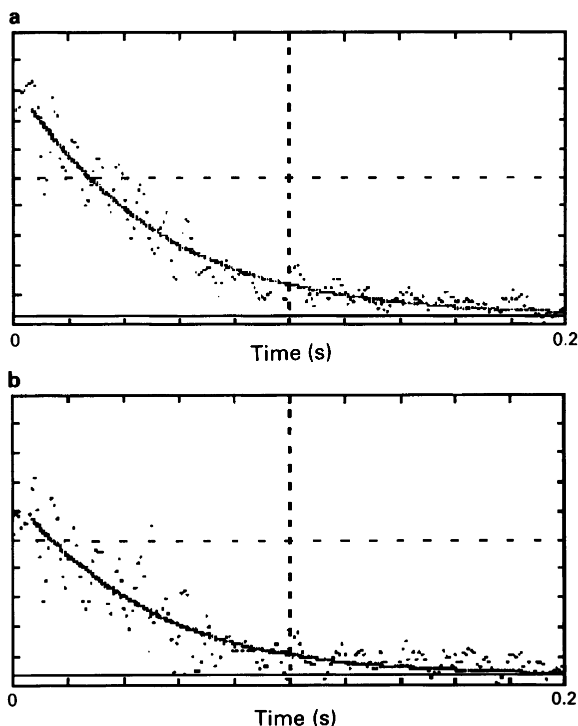

Figure 2 Rate of Ca^{2+} release from unlabelled and 4-(N-iodoacetoxyethyl-N-methyl)-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labelled troponin-tropomyosin (TnTm) (a and b). Data from stop-flow Quin-2 Ca^{2+} chelation experiment in which TnTm is mixed with Quin-2. Average of 4 runs. Final concentrations: Quin-2 240 μM, TnTm (unlabelled (a), or labelled with IANBD (b)) 5 μM, Ca^{2+} 25 μM added, $MgCl_2$ 3 mM, KCl 0.1 M, MOPS 10 mM, dithiothreitol 0.1 mM, pH 7.0 at 15°C. Excitation of Quin-2 fluorescence at 339 nm, emission > 490 nm. The solid lines through the data correspond to a fit to a single exponential equation with rates of $23\,s^{-1}$ for (a) (unlablled TnTm), and $22\,s^{-1}$ for (b) (IANBD-labelled TnTm). Full scale 0.25 V.

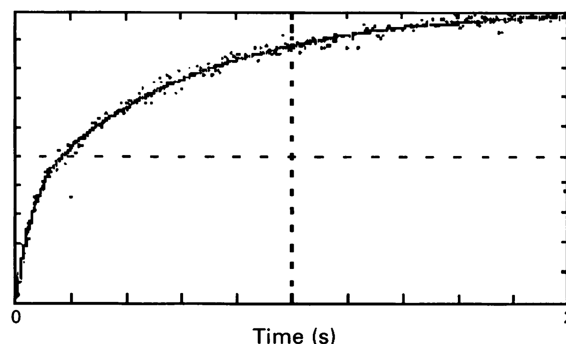

Figure 3 Rate of fluorescent transitions of IANBD-labelled (TnTm). Data from a stop-flow experiment in which changes were measured in fluorescence of 4-(N-iodoacetoxyethyl-N-methyl)-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labelled troponin I (TnI) within the troponin-tropomyosin (TnTm) complex as Ca^{2+} is released and binds to EGTA. Average of 4 runs. Final concentrations: EGTA 10 mM, IANBD-labelled TnTm 1.3 μM, Ca^{2+} 50 μM added, $MgCl_2$ 3 mM, KCl 0.1 M, MOPS 10 mM, dithiothreitol 0.1 mM, pH 7.0 at 15°C. Excitation of IANBD fluorescence at 490 nm, emission > 530 nm. The solid lines through the data correspond to a fit to a double exponential equation with rates of $22\,s^{-1}$ and $1.8\,s^{-1}$ with relative fractional amplitudes of 0.34 and 0.66. Full scale 0.5 V.

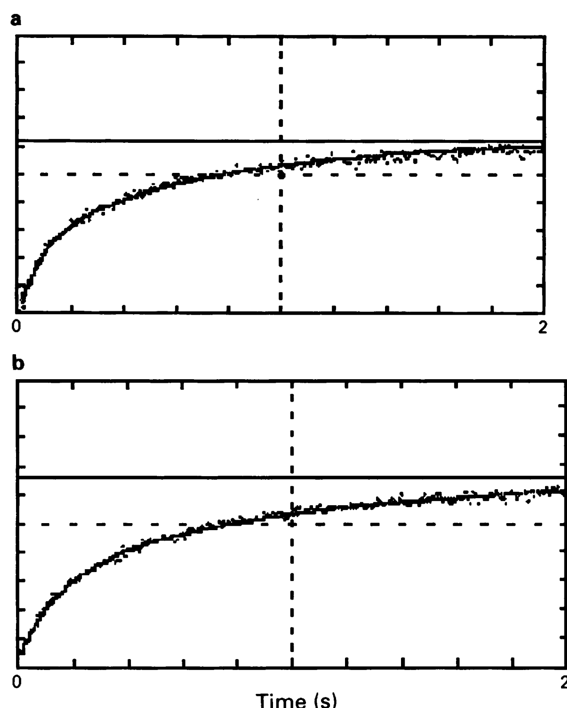

Figure 4 Stop flow traces of calcium dissociation from 4-(N-iodoacetoxyethyl-N-methyl)-7-nitrobenz-2-oxa-1,3-diazole (IANBD) labelled troponin-tropomyosin (TnTm) in the presence and absence of perhexiline. The data are the average of 4 traces. (a) The fluorescence signal in the absence of perhexiline can be fitted by two exponential terms with rates approximately $12.75\,s^{-1}$ and $1.4\,s^{-1}$. (b) The fluorescence signal in the presence of $50\,\mu M$ perhexiline (final) can be fitted by two exponential terms with rates of $6.5\,s^{-1}$ and $0.9\,s^{-1}$. Conditions: as in legend to Figure 3. Full scale 1 V.

The rates of Ca^{2+} release from the TnTm complex labelled with IANBD on TnI have been measured in the presence of a number of compounds shown to be 'calcium sensitisers'. The results are shown in Table 2. Sulmazole and pimobendan ($50\,\mu M$) were without effect on the rate of dissociation of Ca^{2+} from the regulatory site in the protein complex and isomazole ($50\,\mu M$) caused only a slight reduction ($\sim 20\%$). None of these compounds modified the rate of Ca^{2+} release from the high affinity sites. Perhexiline ($50\,\mu M$) or bepridil ($10\,\mu M$) reduced the rate of Ca^{2+} dissociation from the low affinity of sites of TnTm by about 50% and that of the high affinity sites by about 35%.

Table 2 The effects of calcium sensitising compounds on the rates of Ca^{2+} release from low and high affinity sites on fluorescently labelled bovine cardiac troponin-tropomyosin (TnTm) complex

	Rate of dissociation of Ca^{2+} from TnTm as % vehicle control	
	Low affinity site	*High affinity sites*
Control	100%	100%
Sulmazole ($50\,\mu M$)	108%	107%
Isomazole ($50\,\mu M$)	77%	96%
Pimobendan ($50\,\mu M$)	112%	92%
Perhexiline ($50\,\mu M$)	51.5%	66%
Bepridil ($10\,\mu M$)	53.5%	67.5%

The values are given relative to vehicle control. For isomazole and sulmazole, the values are given relative to Ca^{2+} release rates of $18.15 \pm 1.1\,s^{-1}$ and $1.8 \pm 0.1\,s^{-1}$ for the low and high affinity sites (mean $\pm$ s.e.mean, $n = 6$). For perhexiline, pimobendan and bepridil, the vehicle was 0.1% ethanol. The control rates of Ca^{2+} release were $16.2 \pm 0.5\,s^{-1}$ and $1.9 \pm 0.05\,s^{-1}$ respectively for the low and high affinity sites (mean $\pm$ s.e.mean, $n = 4$). The conditions for the experiments are given in the legend to Figure 3.

Discussion

The effects of known Ca^{2+} sensitisers on the rates of Ca^{2+} release from cardiac troponin C and troponin-tropomyosin complex

The aim of the work described here was to develop new assays which would pinpoint the sites of action of Ca^{2+} sensitisers within the TnTm complex.

The interaction of Ca^{2+} with Ca^{2+} binding proteins is generally considered to be fast and to occur at rates approaching those for diffusion limited bimolecular reaction. If this is so, it is unlikely that the rate of Ca^{2+} binding to TnC will be altered by the presence of a Ca^{2+} sensitiser. Hence, the change in Ca^{2+} affinity expected for a Ca^{2+} sensitiser will result from a reduction in the rate of release of Ca^{2+}.

In practice the binding step is too fast to measure and a subsequent conformational change in the protein could occur, but itself be too fast to be detected (Rosenfeld & Taylor, 1985a). Of course in this case it is possible that the presence of bound drug could change the rate of this subsequent conformational change. If this is so, any change in Ca^{2+} release rate that we measure will not completely reflect the overall change in Ca^{2+} affinity of the protein.

It is difficult to extrapolate directly from changes in force production of skinned muscle fibres caused by Ca^{2+} sensitisers to estimate the increased Ca^{2+} affinity expected. However, it seems probable that the reduction in rate of Ca^{2+} release from TnC or TnTm will be small. This method of direct measurement of the rate of Ca^{2+} release from TnC cannot resolve changes of less than 20% accurately. This is of sufficient accuracy to detect changes of Ca^{2+} off-rate likely to be of pharmacological significance. For example, the change in Ca^{2+} affinity of the regulatory site of cardiac TnC caused by calmidazolium (El Saleh & Solaro, 1987) could correspond to a decrease in Ca^{2+} off-rate from $240\,s^{-1}$ to $100\,s^{-1}$, which would be detected in our experiments.

The method used to measure the rate of Ca^{2+} release from TnTm has a higher signal to noise ratio and hence better resolution.

We began by testing the effects of a range of known Ca^{2+} sensitisers on the rates of Ca^{2+} release from cardiac TnC and TnTm. For some compounds, such as sulmazole and pimobendan, their effects on Ca^{2+} release could not explain their effects *in vivo* and *in vitro*.

(i) Sulmazole (AR-L 115) is a phosphodiesterase isoenzyme type III inhibitor with additional inotropic effects (Ahn *et al.*, 1986) attributed to a direct effect on myofibrils (Solaro & Ruegg, 1982). In the presence of sulmazole, the Ca^{2+}-dependence of myofibrillar ATPase activity is shifted such that there is an increase in activity at submaximal Ca^{2+} concentrations and increased Ca^{2+} binding to the myofibrils. More recent evidence suggests that the Ca^{2+} sensitising properties of sulmazole are stereospecific, whereas the other inotropic effects due to inhibition of phosphodiesterase and Na^+-K^+ ATPase activity and other additional mechanisms of action are not (van Meel *et al.*, 1988). The present experiments, with the racemate, show that sulmazole ($50\,\mu M$) does not increase the Ca^{2+} affinity of isolated TnC or TnTm complex. However, it may act on some other component of the contractile apparatus *in vivo* to produce a Ca^{2+} sensitising effect.

(ii) Pimobendan (UD-CG 115 BS) has been shown to have Ca^{2+} sensitising effects on skinned cardiac preparations from guinea-pig and dog, and human papillary muscle (Ruegg *et al.*, 1984; Fritsche *et al.*, 1986; Fujino *et al.*, 1988a). These effects are mainly due to the L-isomer (Fujino *et al.*, 1988b). The racemic mixture is without effect on myofibrillar ATPase

activity at concentrations up to 100 μM. However, measurements of Ca^{2+} binding to skinned dog fibres show that it increases the affinity of the regulatory Ca^{2+} binding sites by two to three fold at 50 μM and decreases the affinity of the Ca^{2+}/Mg^{2+} sites by about 20%.

Here we have looked at the effects of pimobendan on the isolated contractile proteins. We found that the rate of Ca^{2+} release from the regulatory site of bovine cardiac TnC was slightly decreased in the presence of racemic pimobendan. The rate of Ca^{2+} release from the high affinity sites was not changed.

We have failed to confirm the observation that pimobendan decreases the overall Ca^{2+} affinity of bovine TnC (Jaquet & Heilmeyer, 1987). We also found that pimobendan had no effect on the rate of Ca^{2+} release from either low or high affinity sites of TnTm.

Thus, there is a difference in the effects of pimobendan on the Ca^{2+} affinity of dog cardiac fibres and on the bovine isolated proteins. This may be due to a difference in the action of pimobendan in different species. Such a difference in inotropic effects of the demethylated metabolite of pimobendan UD-CG-212 Cl, has been observed (Shibasaki & Endoh, 1987). Alternatively, pimobendan may have specific effects only on the regulatory complex as it functions *in vivo*.

In contrast to our observations with sulmazole and pimobendan, the Ca^{2+} sensitising effects of some compounds could be explained by their effects on TnC and TnTm.

(iii) Isomazole (LY-175326) is one of a series of compounds designed around sulmazole, which has been shown to be a potent inotrope in some species including man (Lues *et al.*, 1988), with a Ca^{2+} sensitising action on skinned fibres. Our observations show that the Ca^{2+} sensitising effect of isomazole may be due to its action on TnTm to increase the Ca^{2+} affinity of the regulatory site of TnC. We show that isomazole does not act directly on isolated TnC.

Calmodulin is another key Ca^{2+} binding protein (Cheung, 1980), which is highly homologous with TnC. The effects of a number of compounds known to bind to calmodulin were tested to determine whether they modified Ca^{2+} binding to TnC or TnTm.

(iv) Calmodulin antagonists, such as perhexiline and calmidazolium have been shown to stimulate myofibrillar ATPase activity at submaximal Ca^{2+} concentrations (Silver *et al.*, 1985). The concentrations giving 50% stimulation at 2 μM Ca^{2+} were found to be 90 μM perhexiline or 9 μM calmidazolium. Perhexiline causes an increase not only in myofibrillar ATPase rate at submaximal Ca^{2+} but also an increase in basal ATPase in the absence of Ca^{2+} (Silver *et al.*, 1985; Smith & England, 1989). Here we have found that perhexiline (50 μM) reduced the rate of dissociation of Ca^{2+} from the TnTm complex, affecting both types of site. Surprisingly, in view of the high degree of homology between calmodulin and TnC, perhexiline had no effect on the Ca^{2+} affinity of TnC alone. In contrast, calmidazolium, another calmodulin antagonist, does have the expected direct effect to increase the Ca^{2+} affinity of the low affinity Ca^{2+} site on cardiac troponin C (cTnC) (El-Saleh & Solaro, 1987). Possible sites of action of perhexiline within the TnTm complex are being investigated further.

Bepridil, another calmodulin antagonist has been shown to increase both the Ca^{2+} sensitivity of dog heart myofibrillar ATPase and Ca^{2+} binding to cTnC (Solaro *et al.*, 1986). The decrease in rate of release of Ca^{2+} from the regulatory low affinity site observed here correlates well with an overall 4 fold increase in the total Ca^{2+} affinity of cTnC in the presence of 100 μM bepridil observed by these authors. Bepridil also decreased the rate of release of Ca^{2+} from the low affinity site in TnTm, as expected from its action on TnC. Bepridil had little effect on the rate of release of the Ca^{2+} from the high affinity sites of TnC alone. However, when TnC was incorporated in the TnTm complex, this rate was decreased.

Oxmetidine, an H_2-receptor antagonist, binds to calmodulin and to skeletal muscle TnC (Reid, 1986 and personal communication). However, its effects on the rates of Ca^{2+} release from the low affinity site and high affinity sites of cardiac TnC are the opposite of those that are expected of a compound acting as a Ca^{2+} sensitiser. Indeed, there are no data showing that it is cardiotonic.

The design of more specific Ca^{2+} sensitisers

The aim of the work described here was to develop new assays which would accurately pinpoint the sites of action of Ca^{2+} sensitisers. However, from the data presented it is apparent that a number of compounds which are 'calcium sensitisers' *in vivo* do not appear to modify the Ca^{2+} affinity of the TnTm complex *in vitro*. This may be because the compounds act by some other mechanism *in vivo*. Another possibility must be considered. The available assays, including those developed here, may fail to predict 'Ca^{2+} sensitisers' because the model systems lack the mechanical coupling of the muscle.

The Ca^{2+} affinity of TnC is itself determined by the nature of crossbridge interactions within the thin filament (actin and the regulatory proteins). Actively-cycling crossbridges are expected to increase the Ca^{2+} affinity of the Ca^{2+} specific site(s) of TnC (Hill, 1984), although the effect may not be large. An indirect estimate indicated a 10 fold increase in the apparent Ca^{2+} affinity of the TnC complex, as skeletal muscle fibres went from a relaxed to a fully contracted state (Guth & Potter, 1987). However, these experiments relied on changes in the fluorescence of modified TnC incorporated into skinned fibres to measure changes in Ca^{2+} affinity of that protein. Direct measurement of Ca^{2+} binding to active fibres failed to confirm this change in affinity (Pan & Solaro, 1987), suggesting that the effect of mechanical activity on the Ca^{2+} affinity of Tn is probably not large.

Identifying the target protein is the key step that will allow rational drug design of more specific Ca^{2+} sensitisers. The methods developed in this study provide information on where the compounds bind within the myofibril and can be extended to the complexes of TnC-TnI and TnC-TnI-TnT. This will allow more precise pinpointing of the location of the drug binding site. The next step is the characterization of the binding site by developing a structure-activity relationship. For TnC which has a suitably low molecular weight, we have been able to investigate the bepridil binding site in some detail by use of ^1H-n.m.r. and the 3D structures of the homologous proteins, skeletal TnC and calmodulin (Smith *et al.*, 1988).

The approaches described here provide *in vitro* assays to develop new compounds which interact with the different components of TnTm to increase the affinity for Ca^{2+}. It remains to be seen whether such compounds are Ca^{2+} sensitisers *in vivo*.

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