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Photolysis of the novel inotropes EMD 57033 and EMD 57439: evidence that Ca^{2+} sensitization and phosphodiesterase inhibition depend upon the same enantiomeric site

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1 We studied the effects of flash photolysis on the novel enantiomeric cardiac inotropes EMD 57033 (a calcium sensitizer) and EMD 57439 (a phosphodiesterase III inhibitor) in rat isolated ventricular trabeculae.

2 In skinned trabeculae, EMD 57439 had no effect on force production, consistent with lack of an active cyclic AMP system in this preparation. In contrast, EMD 57033 potentiated force at partial and maximal activation. A single flash of near u.v. light given at partial activation (30–70%) reduced force potentiation by 52.4±5.2%. No effect was produced by flashes in the presence of EMD 57439 or in the absence of either drug.

3 The time course of relaxation induced by EMD 57033 photolysis was indistinguishable from that obtained on deactivating the muscle by rapidly lowering Ca^{2+} using photolysis of the caged chelator of calcium, diazo-2.

4 In intact, twitching trabeculae, EMD 57033 increased diastolic and peak force and slowed relaxation. These effects were simultaneously reduced by a light flash. In these muscles EMD 57439 reduced force, without affecting the twitch time course. These effects were also reduced by a light flash.

5 The u.v. absorbance spectra of EMD 57033 and EMD 57439 were identical. After photolysis optical density decreased substantially and the peak shifted from 320 nm to 280 nm.

6 The proton n.m.r. spectra of these compounds were identical. The main change post-photolysis was a decrease in the proton signal associated with the enantiomeric carbon atom.

7 This novel manipulation of the molecular structure of EMD 57033 and EMD 57439 within an experiment thus provides direct evidence linking calcium sensitization to a particular molecular structure. The three main effects of the sensitizer on the twitch were simultaneously abolished and may be mechanistically linked. Flash photolysis may be a useful tool for further investigations of the actions of these compounds. In particular, flash photolysis of the sensitizer represents a novel method of rapidly deactivating cardiac muscle.

Keywords: Calcium sensitivity; inotropic agents; cardiac muscle; cardiotonic agents; EMD 57033; EMD 57439; photolysis

Introduction

Chronic congestive heart failure affects up to 2% of the population and carries a prognosis which is worse than that for many types of cancer. The majority of cases are caused by loss of functioning myocardium due to ischaemic damage. Thus pharmacological enhancement of the contractility of remaining myocardium seems an appropriate aim (Lee & Allen, 1995). Unfortunately, conventional positive inotropic agents such as digitalis, catecholamines and phosphodiesterase inhibitors have not successfully reduced mortality (Curfman, 1991). An important reason for this is that their use is associated with enhanced susceptibility to life-threatening cardiac arrhythmias. All these agents increase the strength of the heart by increasing myoplasmic free Ca^{2+} concentration (Ca_i), an inotropic mechanism with a narrow therapeutic ratio and well-documented arrhythmogenic potential (Lee & Allen, 1993).

Increasing the Ca^{2+} sensitivity of the cardiac myofilaments is an alternative inotropic mechanism which would be expected to avoid this problem. However, the use of 'Ca^{2+} sensitizer' drugs might suffer from a different potential side-effect, slowing of relaxation. This has been observed in *in vitro* studies of intact muscle (White *et al.*, 1993) and in principle might impair diastolic filling of the heart, reducing the benefits of increased myocardial contractility. This slowing of relaxation in the presence of a Ca^{2+} sensitizer could be due to a slower release of Ca^{2+} from troponin C over the period when Ca_i is falling during relaxation. To examine this possibility, we proposed to examine the mechanisms responsible for slowed relaxation in the presence of the Ca^{2+} sensitizer, EMD 57033 (5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2*H*-1,3,4-thiadiazin-2-one; Figure 1). This compound (a thiadiazinone) has previously been shown to produce its inotropic effect in intact cardiac muscle by enhancing myofilament Ca^{2+} sensitivity (White *et al.*, 1993; Solaro *et al.*, 1993) and is the active Ca^{2+} sensitizing component of the racemic mixture, EMD 53998 (Lues *et al.*, 1993). Our intended methodology was to lower the Ca^{2+} concentration rapidly in a skinned muscle preparation using diazo-2, a photolabile 'caged' chelator of Ca^{2+} (Adams *et al.*, 1989), in order to measure the intrinsic relaxation speed of the myofibrils in the absence and presence of the drug. However, during control experiments we discovered that EMD 57033 itself is rapidly inactivated by the near u.v. light flash needed to photolyse diazo-2. EMD 57033 is interesting in that its enantiomer (EMD 57439) is also an inotropic agent, but possesses very little Ca^{2+} sensitizing ability (Lues *et al.*, 1993). Instead EMD 57439 alters contractility by inhibiting phosphodiesterase (PDE III). This provides a unique control for studies in intact cardiac muscle.

In the experiments reported here, we have used several techniques to investigate the inotropic effects of these com-

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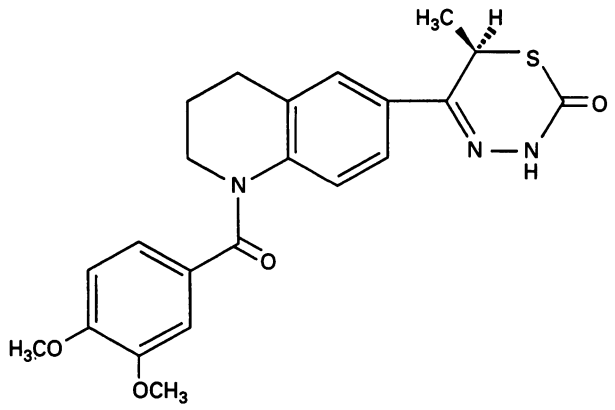

Figure 1 The structure of EMD 57033 (shown) and EMD 57439 indicating the position of the chiral carbon atom.

pounds and their rapid inactivation by a near u.v. light flash. The results indicate that the quite different inotropic effects exhibited by these compounds depend on the same enantiomeric site. Preliminary accounts of this work have been presented previously (Kentish *et al.*, 1994; Lee *et al.*, 1994).

Methods

Male Wistar rats (~250 g) were killed by cervical dislocation. The heart was rapidly removed and washed in Tyrode solution at room temperature (22°C). This solution contained (mM): Na^+ 135, K^+ 5, Ca^{2+} 1, Mg^{2+} 1, Cl^- 100, HCO_3^- 20, HPO_4^{2-} 1, SO_4^{2-} 1, acetate 20, glucose 10, and insulin 5 $u\,l^{-1}$ (40 nM). The solution was equilibrated with 95% O_2/5% CO_2 to give a pH of 7.36. The experiments were performed on isolated ventricular trabeculae (diameter 100–200 μm, length 1–2 mm) which were dissected from the right ventricle. During isolation, butanedione monoxime (25 mM) was present in the solution in order to minimize damage during dissection (Mulieri *et al.*, 1989). The trabeculae were mounted with silk snares in a muscle bath, were superfused with Tyrode at ~1 ml min^{-1} and were field stimulated continually at 0.1 Hz via platinum electrodes. The muscle bath consisted of a channel (1 mm wide, 2 mm deep and 3 cm long) with sides made from glass cover slips. Force was measured by an isometric transducer (Akers AE801) and was recorded on a chart recorded and on a computer, using a Digidata 1200 A/D board and PClamp software (Axon Instruments). PClamp also supplied the trigger pulses for the stimulator and for the flashlamp. Once twitch force had stabilized, a series of 16 twitches was recorded, with a flash given to the muscle midway through each series. The flash (1 ms, 100 mJ) was supplied by a flashlamp (HiTech Scientific) fitted with a UG11 filter (giving a 50% passband of 310–380 nm when fitted with a glass coverslip to attenuate u.v. wavelengths). At the beginning of an experiment the flash was aligned using light-sensitive paper, so that the entire trabecula was covered by the flash. The timing of the flash pulse was adjusted so that the flash occurred early during twitch relaxation (e.g. Figure 3). After several control series had been recorded, the solution was replaced with Tyrode containing 5–20 μM EMD 57033 or EMD 57439. After a new stable twitch force had been reached, the flash protocol described above was repeated several times. All experiments were carried out at 22°C.

In another set of experiments we investigated the effects of EMD photolysis on the myofilaments in skinned fibre preparations. For these experiments, the cell membranes of the trabeculae were permeabilized by bathing the muscles in 1% Triton X-100 + 0.1 mM phenylmethylsulphonylfluoride (PMSF) in relaxing solution for 30 min. Relaxing solution contained (mM): K propionate 83, N,N-*bis*(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) 100, $MgATP^{2-}$ 5, Mg^{2+} 1, Na_2 phosphocreatine 10, Pi 1, EGTA 1 ($[Ca^{2+}] < 10$ nM), glutathione 5, Na azide 5, leupeptin 5 μM; ionic strength 0.20 M; pH 7.10 at 22°C (modified from Palmer & Kentish, 1994). All chemicals were from Sigma, with the exception of Triton X-100 and propionic acid (Fluka). Addition of the protease inhibitors PMSF and leupeptin were found to minimise the deterioration of force production that is normally found with skinned fibres. After skinning, the muscles were cycled through a sequence of: relaxing solution (R), pre-activating solution (PA), activating solution (A), then relaxing solution (see Figure 2). Pre-activating solution was as relaxing solution but with no EGTA ($[Ca^{2+}]$ ~0.1 μM); activating solution was as relaxing solution but contained 0–1.1 mM Ca ($[Ca^{2+}] = 10$ nM – 200 μM). The flashlamp was triggered when force had stabilized in activating solution (Figure 2). After several solution cycles, the muscles were transferred to relaxing solution containing EMD 57033 or EMD 57439 for 5 min before the above protocol was repeated, with the test drug present in all solutions. We also measured the intrinsic rate of relaxation of the myofilaments, using flash photolysis of diazo-2 (Adams *et al.*, 1989). In this case, activating solution had no EGTA but contained 0.25 mM diazo-2 (Molecular Probes, Eugene, Oregon, U.S.A.) and 0–0.25 mM Ca. Again, the flash was triggered once Ca^{2+}-activated force had stabilised (Figure 2e). The relaxation timecourse was fitted to a double exponential function using Clampfit (Axon Instruments).

EMD 57033 and EMD 57439 were supplied by E. Merck. They were prepared as 10 mM stock solutions in DMSO. DMSO alone at the concentrations present in the superfusing solution (0.05%–0.2%) had no inotropic effect on either the skinned or intact trabeculae, and exposing muscles in the presence of DMSO to a near u.v. light flash also did not alter force production.

Analysis of the absorption spectrum of the drugs was carried out using an LKB Ultraspec II spectrophotometer. Photolysis of the drugs (20 μM solution in water) was achieved by continuous exposure to the unfocussed beam from a 75 W xenon arc lamp fitted with a UG11 filter + coverslip (50% passband = 310–380 nm), and was complete after 40 min. (We were unable to measure the spectrum of the EMD compounds after photolysis in the muscle bath itself, because too little compound was present in the 5 μl volume of solution exposed to the flash. Increasing the concentration of compound to try to overcome this problem would have substantially reduced the extent of photolysis because of the high absorbance of solutions of these compounds).

^1H n.m.r. spectra were obtained using a Bruker AMX 400 spectrometer. Spectra were recorded using standard one-dimensional experiments. The acquisition time for each scan was 2.7 s over a spectral width of 6024 Hz. A recycle time of 3 s was left between scans and the flip angle applied was approximately 30°. The drugs were dissolved in dry, deuterated DMSO. For the control spectra, solutions containing approximately 1 mM EMD 57033 and EMD 57439 were used. Due to the path length of the cuvette used for exposure to the arc lamp and the absorbance of the solutions, lower concentrations (approximately 50 μM) had to be used in order to achieve sufficient photolysis. However, it was also necessary to achieve a compromise between drug concentrations low enough to allow adequate photolysis, yet high enough to result in a satisfactory n.m.r. spectrum. At the concentration chosen, substantial photolysis was achieved by exposure to the u.v. arc lamp for 30–40 min and a clear n.m.r. result was obtained.

Results

Figure 2a shows the effect of a near u.v. light flash on the Ca^{2+}-activated force produced by skinned cardiac muscle in the presence of the Ca^{2+} sensitizer, EMD 57033 (5 μM). At the $[Ca^{2+}]$ used (0.42 μM), no force was generated in the absence

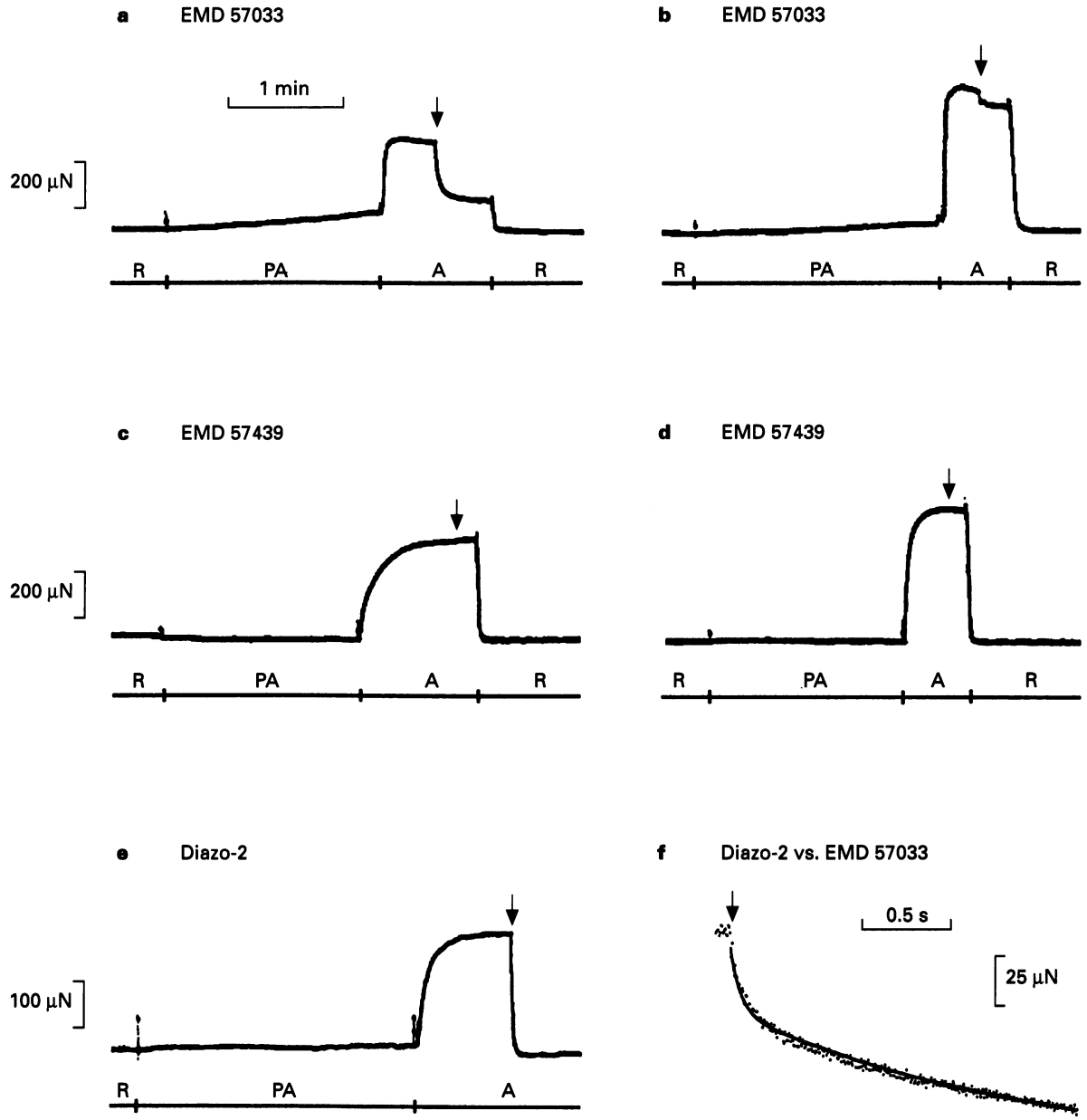

Figure 2 The effects of near-u.v. flashes of light (arrows) during Ca^{2+} activation of skinned trabeculae from the rat right ventricle. (a, b) Flash in 5 μM EMD 57033 at (a) partial activation (0.42 μM Ca^{2+}) and (b) full activation (36 μM Ca^{2+}). Note the flash-induced reduction of force. R = relaxing solution, PA = pre-activating solution, A = activating solution (see text for details). (c, d) Flash in 5 μM EMD 57439 at (c) partial activation (2.0 μM Ca^{2+}) and (d) full activation (36 μM Ca^{2+}). (e) Flash of diazo-2 at partial Ca^{2+} activation (1.8 μM Ca^{2+} initially; 0.25 mM diazo-2). The fall of [Ca^{2+}] produced by diazo-2 photolysis relaxes the muscle completely. (f) Comparison of the early phase of the relaxations after flash of 5 μM EMD 57033 and diazo-2. Points show data for EMD photolysis (for clarity, only 1 in 10 of the recorded points are shown); the line is the bi-exponential fit to the data for diazo-2. One muscle was used for panels (a-d) another for panels (e) and (f).

of EMD 57033 (not shown) but in the presence of the drug the force amounted to 67% of the maximum force, as a result of the increased Ca^{2+} sensitivity of the myofibrils in the presence of EMD 57033. (Note that this effect also caused some activation of force in the pre-activating solution, PA). When a near-u.v. light flash was directed onto the muscle (arrow), force fell rapidly by about 70%, even though the [Ca^{2+}] of the activating solution was unchanged. This indicates that the drug had been partially inactivated by the flash, leading to a reduction in its sensitizing effect on the myofilaments. In 6 muscles at an initial level of 30–70% activation, the drug-induced potentiation of force was reduced by 52.4 ± 5.2% (mean ± s.e.) by one flash. We found that a flash also caused a small relaxation at maximum activation (9.6 ± 1.1%, $n = 5$; Figure 2b). This is consistent with flash-induced inactivation of the relatively small potentiating effect of the drug on maximum Ca^{2+}-activated force (Lues *et al.*, 1993). In these experiments 5 μM EMD 57033 caused potentiation of maximum force by 25.5 ± 5.5% ($n = 6$). The fact that photolysis caused a much greater fall of force at partial activation is expected and is due to the shape of the activation curve. Partial activation is where the Ca^{2+} sensitizing action of the drug has its greatest proportional effect on force.

With the PDE inhibitor, EMD 57439 (5 μM), a different result was obtained. As expected, this enantiomer had no effect on Ca^{2+}-activated force in the skinned muscle, consistent with the lack of an active cyclic AMP system in this preparation. Furthermore, a flash in the presence of EMD 57439 did not affect force, whether the muscle was partially activated (Figure 2c) or fully activated (Figure 2d; $n = 3$). Note that this result

does not indicate that EMD 57439 is not affected by the flash. Since it produces no inotropic effect in this skinned fibre preparation, there was no reversal of inotropic effect to be seen. However, these results do show that the flash-induced decrease in force seen with EMD 57033 was not merely due to degradation of the muscles by exposure to near-u.v. light. In additional control experiments, a similar lack of effect was seen when a flash was given in the absence of either drug or with the DMSO vehicle alone (not shown).

The relaxation produced by photolysis of EMD 57033 was bi-exponential, showing fast and slow components with rate constants of $13.3 \pm 0.9\ s^{-1}$ and $0.85 \pm 0.05\ s^{-1}$ respectively ($n=6$; Figure 2f). Since the fast component is thought to be a measure of the intrinsic rate of myofilament relaxation, it seemed possible that the faster rate reflected the time course of the reduction of Ca^{2+} activation of the myofilaments. It was therefore of interest to compare this rate with that induced by a rapid (~1 ms) drop in solution $[Ca^{2+}]$. This was achieved by flash photolysis of diazo-2, a chelator of Ca^{2+} that has an increased affinity for Ca^{2+} after photolysis. Diazo-2 photolysis also produced rapid relaxation of the muscle (Figure 2e). As Figure 2f illustrates, the time course of early relaxation after diazo-2 photolysis was very similar to that seen following photolysis of EMD 57033. In fact, the fast rate constant for relaxation after diazo-2 photolysis ($14.0 \pm 1.7\ s^{-1}$, $n=6$) was not significantly different ($P=0.72$) from that after photolysis of EMD 57033 (above) measured in the same muscles and at the same initial level of Ca^{2+} activation. Thus de-activation of the myofilaments produced by reducing $[Ca^{2+}]$ or photolysing EMD 57033 had time courses that were indistinguishable. This finding also indicates that the relaxation rate upon photolysis of EMD 57033 was limited by the rate of myofilament de-activation rather than by the rate of degradation of the drug.

We next investigated whether all of the reported actions of EMD 57033 in intact cardiac muscle could also be reduced by a near-u.v. light flash. During a series of electrically-stimulated twitches in an intact trabecula, a flash was given just after peak force so that any effect on relaxation could be studied. Figure 3 shows results obtained in Tyrode solution alone (Figure 3a) or in Tyrode solution plus 5 μM EMD 57033 (Figure 3b). For clarity, only three twitches are shown, with the flash given during the middle twitch (2). In the control experiment (Figure 3a), the flash produced no effect on force. Following the addition of EMD 57033 (Figure 3b) developed force increased and relaxation was prolonged. In addition, diastolic force was elevated in 4 out of 6 muscles exposed to 5 μM EMD 57033. These features have all been noted previously (White *et al.*, 1993; Solaro *et al.*, 1993) and are consistent with an inotropic effect due to increased myofilament Ca^{2+} sensitivity.

As can be seen, a single light flash given during twitch 2 caused several simultaneous effects. Force declined, relaxation rate increased and the elevated diastolic force was abolished. The effects began immediately and took approximately 300 ms for completion (compare twitches 2 and 3). After the addition of 5 μM EMD 57033, peak twitch force was potentiated to $201.5 \pm 33.8\%$ ($n=4$) of control and a single flash reduced this to $138.6 \pm 29.5\%$. Thus the potentiation of force due to the drug was reduced by 62% by a flash. In the series of twitches following the flash, force rose again at a mean rate of 2% per twitch, due to the wash-in of fresh, non-photolysed drug into the muscle. Thus the 62% reduction of force potentiation due to a flash was underestimated by 2%. If flashes were given in consecutive twitches, the effects of EMD 57033 disappeared after 4–5 flashes (not shown). Results similar to those in Figure 3 were obtained at 10 μM and 20 μM EMD 57033 ($n=9$), except that with these higher concentrations elevation of diastolic force was always more pronounced. These findings show that photolysis of EMD 57033 occurs in intact as well as in skinned cardiac muscle and that each flash probably inactivates more than 50% of the drug in the muscle. The fact that all three effects of the drug on the twitch were reduced simultaneously suggests that these effects share a common mechanism.

Figure 4 shows results obtained with the PDE inhibitor EMD 57439 (10 μM) in an intact trabecula. In control twitches (Figure 4a), a flash during twitch 2 had no effect. Somewhat surprisingly, in the rat trabeculae used in these experiments application of EMD 57439 caused a negative inotropic effect

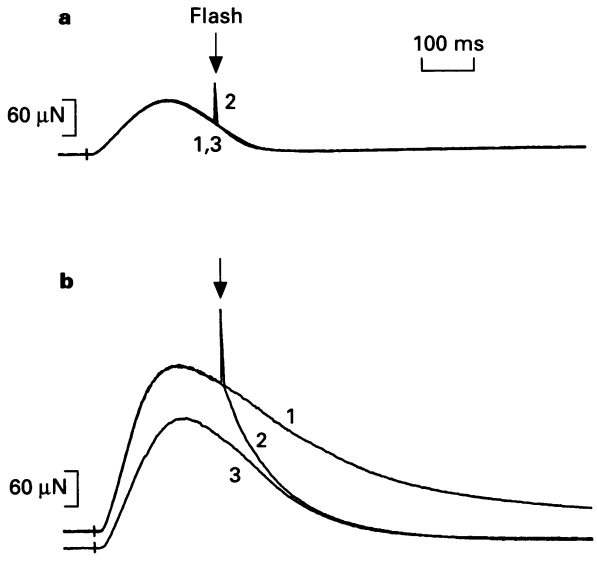

Figure 3 The effects of a near-u.v. flash (arrow) on the positive inotropic actions of EMD 57033 in an intact cardiac trabecula. Small vertical bars indicate the timing of the stimulus. Numbers refer to three consecutive twitches, with a flash given during twitch 2 (the spike is an artifact due to the light-sensitivity of the force transducer). (a) Control (no drug). (b) In the presence of 5 μM EMD 57033. The flash accelerates relaxation and decreases diastolic and systolic forces. Similar results were seen in 10 other preparations with 5–20 μM EMD 57033.

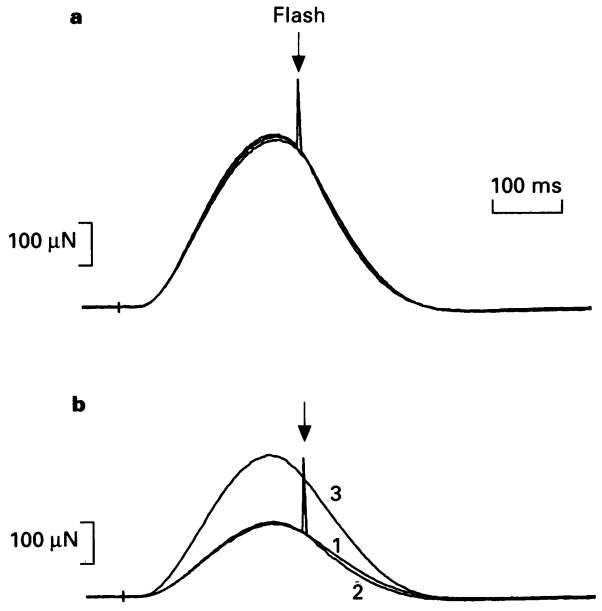

Figure 4 The effects of a near-u.v. flash on the negative inotropic action of EMD 57439 in an intact cardiac trabecula. Numbers refer to three consecutive twitches, with a flash given during twitch 2. Other details as in Figure 3. (a) Control. (b) In the presence of 10 μM EMD 57439. Twitch force is increased in twitch 3, 10 s after the flash. Similar results were seen in 3 other preparations.

(compare twitch 1 in Figure 4b with Figure 4a). For example, with 10 μM EMD 57439 peak force fell to 43.8±8.5% of control (mean±s.e., $n=4$). Neither twitch duration nor diastolic force was significantly altered. These effects are not typical of those caused by PDE inhibition in most preparations, where a positive inotropic effect accompanied by abbreviation of the twitch is usually seen (White *et al.*, 1993; Solaro *et al.*, 1993). Possible reasons for these findings are considered in the discussion. However, the fact that force decreased with this enantiomer in our intact muscle preparation provides an excellent control for non-specific effects of u.v. light on the intact muscles in the presence of the drugs. In Figure 4b, the light flash during twitch 2 accelerated relaxation slightly (though this was not consistently observed). However, by the next twitch 10 s later (trace 3), force had substantially recovered towards control. In 4 muscles, force recovered following a flash to 66.0±10.0% of control, i.e. the force inhibition produced by EMD 57439 was decreased by 44% by the flash (this recovery was underestimated by approximately 4% due to wash-in of fresh drug).

In view of the photo-labile effects of the EMD enantiomers seen in experiments using skinned and intact muscle, we investigated whether physico-chemical changes could be detected in the molecules after exposure to near-u.v. light of the same wavelength range as that used in the muscle experiments. In one approach, we measured the absorption spectra of the compounds before and after exposure to near-u.v. light. The results for the Ca^{2+} sensitiser EMD 57033 are shown in Figure 5. The unphotolysed drug had an absorption maximum at 320 nm, and showed substantial absorption over most of the 310–380 nm range of the flash lamp. As can be seen, following 45 min continuous exposure to the unfocussed beam from the xenon arc lamp (310–380 nm) there was a clear change in the spectrum, with a decrease of optical density and a shift of the peak to 280 nm. This confirms the interpretation that the molecule is altered by exposure to near-u.v. light. Similar results with respect to the absorption spectrum and changes after exposure to the xenon arc lamp were seen with EMD 57439 (not shown).

In order to investigate the molecular changes produced by photolysis we measured the proton n.m.r. spectra of EMD 57033 and EMD 57439 before and after photolysis. The top trace in Figure 6 shows the n.m.r. proton spectrum of EMD 57033. The spectrum of EMD 57439 was identical. Following photolysis obvious differences were present in the spectra indicating both alterations in the parent molecule and (presumably) addition of breakdown products to the solution. The most relevant part of the spectrum is shown expanded in the lower three traces of Figure 6. The quartet at approximately 4.7 p.p.m. is of particular interest since this is the signal associated with the hydrogen attached to the chiral carbon atom.

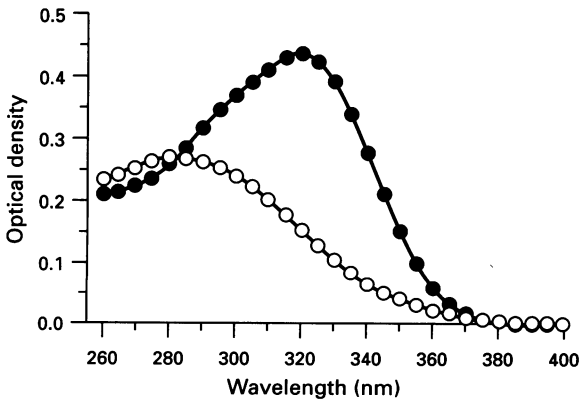

Figure 5 The absorption spectrum of EMD 57033 before (●) and after (○) 45 min exposure to 310–380 nm light from a continuous arc lamp. EMD 57033 (20 μM) with 0.2% DMSO in water, 1 cm light path.

The signal at approximately 7 p.p.m. is associated with the majority of hydrogen atoms attached to other aromatic rings within the molecule and acts as a useful reference. Given the fact that the two molecules cause strikingly different biological effects yet differ chemically only in the orientation of the groups attached to the chiral carbon atom, it seemed likely that photolysis would affect this part of the molecule. As can be seen in Figure 6 partial photolysis of both drugs caused a marked reduction in the relative intensity of the proton signal associated with the enantiomeric carbon atom. This is fully consistent with alteration of the molecule at the enantiomeric site by near-u.v. light.

Discussion

The use of flash photolysis to activate or de-activate synthetic light-sensitive ('caged') molecules has proved to be a fertile method for investigating subcellular physiology (Adams & Tsien, 1993). Because of the potential importance of twitch prolongation which can be caused by novel Ca^{2+} sensitizing inotropes (Lee & Allen 1993), we initially intended to use diazo-2, a caged chelator of Ca^{2+}, to investigate mechanisms underlying the slowed relaxation seen in the presence of one of these drugs (EMD 57033). However, as demonstrated here, we found that EMD 57033 and its enantiomer EMD 57439 are themselves photolabile when exposed to light of 310–400 nm wavelength: their effects on skinned and intact muscles are reduced, the absorption spectrum is altered substantially and the proton n.m.r. spectrum shows loss of the signal associated with the enantiomeric carbon atom. In this molecule the heterocycle containing the N-N bond (the thiadiazinone ring) appears most likely to undergo photolysis by u.v. light of the wavelength used and our results are fully consistent with this. As expected, both enantiomers appeared to undergo the same photolytic reaction. Further work would be necessary to establish the precise chemical alteration produced in the molecule by u.v. light. However, a likely possibility appears to be a sigmatropic rearrangement involving loss of carbonyl sulphide (CSO) and/or nitrogen (N_2) from the thiadiazinone ring. This would result in destruction of the chiral centre, loss of the signal at 4.7 p.p.m and would also be expected to abolish the biological actions, as observed.

One interesting feature of these enantiomers is their markedly different effect in both intact and skinned cardiac muscle. EMD 57439 is a PDE inhibitor with very little myofilament Ca^{2+}-sensitizing activity, while EMD 57033 acts predominantly as a Ca^{2+} sensitizer, but also possesses some PDE inhibitory activity (Lues *et al.*, 1993). However, in species such as rat or ferret, where PDE activity is relatively low (Alousi *et al.*, 1983), the inotropic effect of EMD 57033 in intact muscle is due to enhanced Ca^{2+} sensitivity (White *et al.*, 1993; Solaro *et al.*, 1993). With regard to its mechanism of action, the evidence suggests that EMD 57033 increases myofibrillar Ca^{2+} sensitivity through a direct action on the cross-bridges, probably by increasing their rate of attachment (Strauss *et al.*, 1992; Leijendekker & Herzig, 1992; Solaro *et al.*, 1993; Simnett *et al.*, 1994; Dobrunz *et al.*, 1995). Ca^{2+} binding to the regulatory site of TnC is not affected by EMD 57033 (Solaro *et al.*, 1993; Pan & Johnson, 1996). In fact, the actions of EMD on both maximum force and Ca^{2+} sensitivity in skinned fibres can be explained quantitatively by an increase in the ratio of the attachment rate constant (f) to the detachment rate constant (g) for cross-bridges, with no change in the Ca^{2+} affinity of TnC (Dobrunz *et al.*, 1995).

Since EMD 57033 directly affects the cross-bridges, but not Ca^{2+} binding, it follows the rate of relaxation in skinned fibres after photolysis of EMD 57033 is determined by the net rate of cross-bridge detachment, rather than by the rate of dissociation of Ca^{2+} from TnC. Interestingly, we found that the rate of this relaxation was indistinguishable from that produced by rapidly lowering $[Ca^{2+}]$ using diazo-2, which suggests that the relaxation rate when $[Ca^{2+}]$ is decreased rapidly is also limited

by the rate of cross-bridge dissociation. (This interpretation assumes that the rate of relaxation after EMD photolysis is limited by the rate of myofilament de-activation rather than by the rate of destruction of the drug, but most photolytic reactions occur within a few milliseconds or less). It may also be noted that photolysis of this compound offers a novel alternative method of rapidly reducing myofibrillar activation.

In intact trabeculae, EMD 57033 caused three effects: increased peak twitch force, increased resting tension and slowed relaxation. The effects on twitch and resting force are readily explained by the action of the drug to increase myofilament Ca^{2+} sensitivity. The slowed relaxation could be due to a slower rate of Ca^{2+} release from troponin C, although this would be expected to alter Ca^{2+} binding. Alternatively it could

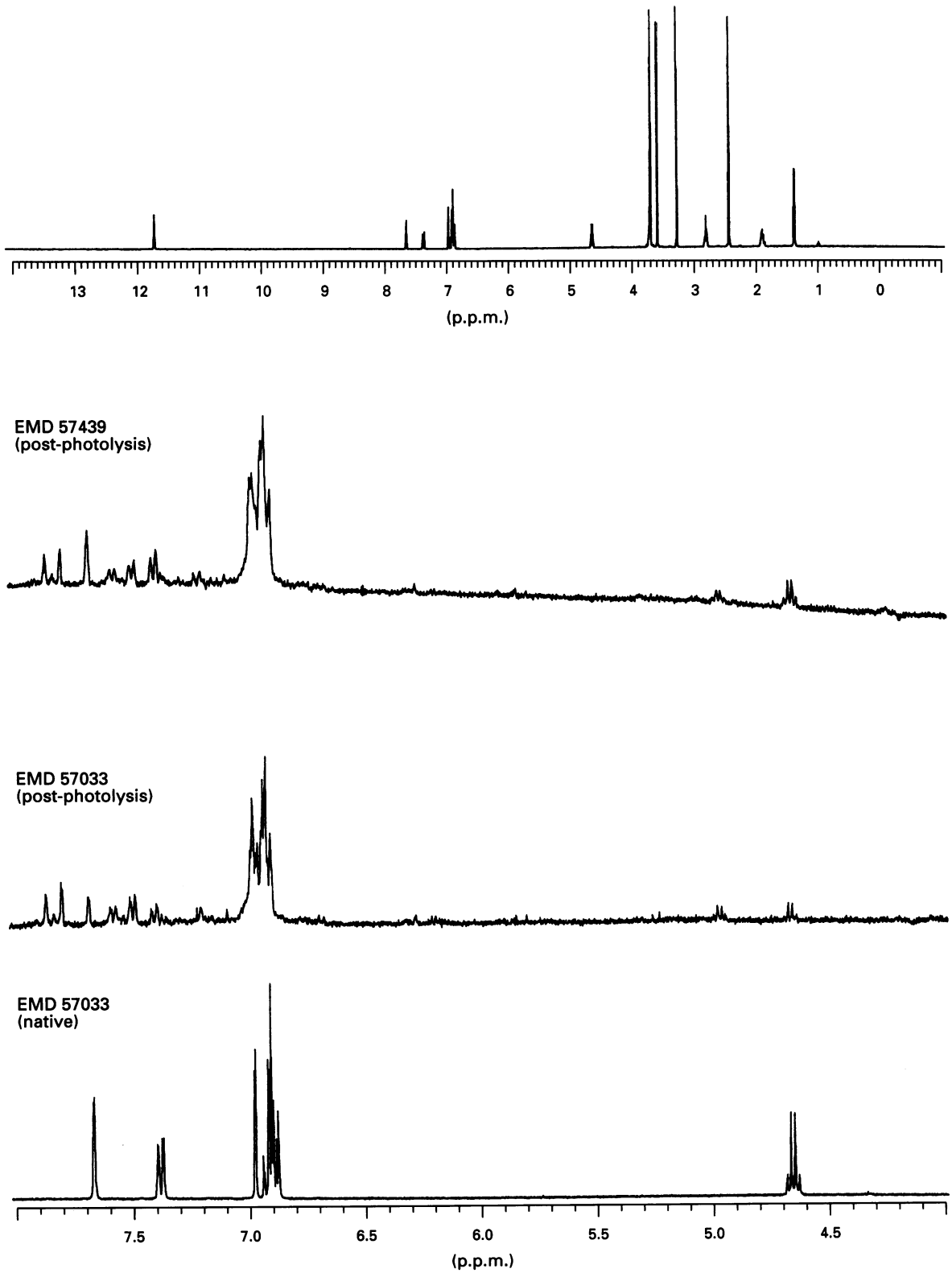

Figure 6 Top trace: proton n.m.r. spectrum of native EMD 57033. Lower three traces: expanded proton n.m.r. spectra of EMD 57439 and EMD 57033 after photolysis and of native EMD 57033 for comparison. The signal due to the enantiomeric carbon atom is seen at a chemical shift of 4.7 p.p.m. After photolysis, note the relative loss of this peak compared with the aromatic signal at 7 p.p.m.

be another consequence of the raised Ca^{2+} sensitivity, since this would cause force to follow more closely the later part of the fall of Ca_i during diastole. Of particular interest was the finding that all three effects were simultaneously abolished by photolysis of the drug. This implies that EMD 57033 interacts with the muscle in a way which directly produces all these effects, and is consistent with (but does not prove) the idea that all three effects may be due to raised Ca^{2+} sensitivity.

A number of studies have been carried out using flash photolysis of caged compounds to investigate the mechanisms of action of EMD 57033. Simnett *et al.* (1993) reported that EMD 57033 slightly increased the relaxation rate produced in skinned muscle by flash photolysis of diazo-2. In a subsequent paper (Simnett *et al.*, 1994) the same group found that EMD 57033 accelerated myofilament activation after photolysis of caged Ca^{2+} (nitr-5). Arner *et al.* (1995) reported that EMD 53998 (the racemic mixture composed of EMD 57033 and EMD 57439) speeded myofilament activation following photolysis of caged ATP. Since photolysis of EMD 57033 will, by itself, cause relaxation of the muscle (Figure 2a), our findings emphasize the importance of using rigorous controls in conjuction with flash photolysis if results are to be correctly interpreted. In the case where photolysis of the caged compound activates the muscle (Simnett *et al.*, 1994; Arner *et al.*, 1995), the overall effects of photolysis of EMD 57033 are difficult to predict, but it is clear that the acceleration of activation reported by Simnett *et al.* (1994) and Arner *et al.* (1995) would have been considerably underestimated, since it would result from the post-photolysis, rather than the initial, concentration of drug. A more serious problem is apparent in studies where the effects of EMD 57033 on the rate of relaxation are studied. Simnett *et al.* (1993) reported that EMD 57033 slightly increased the rate of myofilament relaxation in guinea-pig skinned trabeculae produced by diazo-2 photolysis. However, it is possible that this acceleration was due to the simultaneous relaxing effects of EMD 57033 photolysis and diazo-2 photolysis. Although it has been suggested that significant photolysis of EMD 57033 is avoided by the flash (100 mJ) from a pulsed laser operating at 347 nm (Simnett *et al.*, 1994), the results of the present study raise concerns that this may not be the case. First, EMD 57033 shows considerable absorption at 347 nm (Figure 5). Second, the control for photolysis of the drug itself was carried out at maximum Ca^{2+} activation (Simnett *et al.*, 1994). It was found that force declined by 8.4%, which is comparable to our results under these conditions (Figure 2b) and is thus consistent with the substantial photolysis of the drug, especially since 10 μM EMD 57033 produces only 18% force enhancement at maximum activation in guinea-pig trabeculae (Venturer-Clapier *et al.*, 1992). Our results demonstrate that when using diazo-2 (or other photolabile probes of cellular function), the effects of EMD 57033 photolysis should be checked at partial activation, since this is where the drug produces its greatest proportional effect and is also in the activation range covered after diazo-2 photolysis.

The results we present here, obtained by the novel approach of direct manipulation of the structure of EMD 57033 within an experiment, provide direct evidence linking Ca^{2+} sensitizing activity to a specific molecular structure. The fact that the enantiomer of this chemical structure (EMD 57439) confers a different inotropic mechanism (PDE inhibition) provides the opportunity for an interesting comparison. Unexpectedly, it was a consistent observation in our preparations that this compound had a negative inotropic effect and did not abbreviate the twitch. It is not clear why this should be the case. It was apparently not due to insensitivity of the muscle to increased [cyclic AMP], since the application of the membrane-permeant analogue dibutyryl cyclic AMP (3 mM) produced the expected large potentiation of force and shortening of the twitch (not shown). The negative inotropy was clearly not due to a direct inhibitory effect of EMD 57439 on the myofibrils, since the drug did not affect the force produced by skinned muscles. One possibility is that the sarcoplasmic reticulum in these small muscle preparations was Ca^{2+}-loaded close to full capacity, so that when the drug was applied it caused 'Ca^{2+} overload', a well-known side effect of PDE inhibitors which can cause a negative inotropic effect (Orchard *et al.*, 1983). However, we did not observe the diastolic oscillations that are characteristic of Ca^{2+} overload, nor was there a rise in force during wash-in of the drug. Another possibility which could account for the observed fall of force in this preparation is that EMD 57439 produced phosphorylation of troponin I mediated by cyclic AMP-dependent protein kinase, but did not affect the Ca^{2+} fluxes across the sarcolemmal or sarcoplasmic reticulum membranes. Troponin I phosphorylation causes a reduction in Ca^{2+} sensitivity (Ray & England, 1976), which would then be manifest as a negative inotropic effect. Whatever the precise mechanism, the finding that the effects of photolysis did not become apparent until the next twitch (10 s later) is consistent with an indirect effect such as that mediated via cyclic AMP and may reflect the time needed for elevated [cyclic AMP] to fall after inhibition of PDE activity was relieved by photolysis of the drug. Also, the fact that EMD 57439 consistently caused a negative inotropic effect allows a unique control for non-specific degradative effects of photolysis on intact muscle preparations in the presence of the drugs. With EMD 57439 a marked positive inotropic effect was seen after photolysis, i.e. there was removal of the inhibition of force. Since this is the opposite of the effect seen with EMD 57033, non-specific effects are effectively excluded.

In conclusion, our observations support a myofilament-based mechanism of action for the inotropic effect of EMD 57033 in intact heart muscle and show that its effects on twitch force, relaxation rate and resting tension are closely related. Inactivation by near-u.v. light is associated with alteration of the molecule at the enantiomeric carbon atom, a structure which appears crucial to both Ca^{2+} sensitization and PDE III inhibition. Whether there are common underlying mechanistic features linking Ca^{2+} sensitizing action and PDE inhibition remains to be determined. A possible link lies in the fact that both muscle activation and PDE activity depend on the binding of adenine nucleotides (to cross-bridges and PDE respectively). Flash photolysis of EMD 57033 represents a novel method of rapidly deactivating cardiac muscle. This technology may also prove useful in further investigations of the mechanisms of action of these compounds.

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