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Photosensitization of oesophageal smooth muscle by 3-NO_2-1,4-dihydropyridines: evidence for two cyclic GMP-dependent effector pathways

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1 Photoactivated mechanical responses that resulted from exposure to 3-NO_2-1,4-dihydropyridines (3-NO_2-DHPs) or NO-donors were examined in rat isolated oesophageal smooth muscle with a view to determining the role of calcium and cyclic GMP.

2 Isometric contractile force was recorded in preparations bathed in normal Tyrode or 110 mM K^+-depolarizing solution. Exposure to (+)-PN 202 791, (±)-Bay K 8644 and (−)-PN 2020 791 or the photodegradable NO-donors, sodium nitroprusside (SNP), streptozotocin (STZ) and sodium nitrite photosensitized precontracted tunica muscularis mucosae preparations in a concentration-dependent fashion. Photosensitizing potency followed the order: (+)-PN 202 791 > (±)-Bay K 8644 > (−)-PN 202 791 > SNP > STZ > $NaNO_2$.

3 A low amplitude, slow photorelaxation (slope: 1 mg s^{-1}) was obtained with the L-channel antagonists (−)-PN 202 791 and (+)-Bay K 4407. Photosensitization by the agonist enantiomers (+)-PN 202 791 and (−)-Bay K 5407, as well as racemic Bay K 8644, was mimicked by NO donors and showed at least three different components, consisting of (i) a fast relaxation (slope: 140 mg s^{-1}), (ii) a fast 'off-contraction', and (iii) a delayed slow relaxation. The fast components, but not the delayed slow relaxation, were abolished by blockade of L-type voltage-operated calcium channels, chelation of extracellular calcium and skinning of the plasmalemma, suggesting their mediation by a process linked to calcium entry through L-channels.

4 Both cyclopiazonic acid (3-30 μM) and ryanodine (30 μM) inhibited the fast reponse. This inhibition was accelerated in the presence of extracellular calcium and resembled that seen in tissues exposed to the calcium ionophore A 23187 (1 μM). In calcium depleted tissues, cyclopiazonic acid (3 μM) prevented restoration of the *cis*-dioxolane-induced contraction following re-exposure to a calcium containing high K^+ buffer, but failed to inhibit the photoresponse.

5 Both the fast and slow relaxations were potentiated by zaprinast (10 μM) and inhibited by LY 83583 (10 μM). However, in calcium-depleted, calyculin A-precontracted preparations only the slow relaxation was evident.

6 The present results support the conclusion that: (i) functional L-channels are required for the expression of the fast components of the 3-NO_2-DHP- or NO-donor-induced photoresponse, (ii) NO photorelease followed by activation of soluble guanylyl cyclase is responsible for the photosensitizing activity of 3-NO_2-DHPs and (iii) regulation of the contractile proteins via cyclic GMP-dependent phosphorylation may underlie the slow relaxation.

Keywords: Guanylyl cyclase; 3-NO_2-1,4-dihydropyridines; L-channel; nitric oxide; oesophagus; sarcoplasmic reticulum; tunica muscularis mucosae

Introduction

The 1,4-dihydropyridines (DHPs) enjoy widespread use as ligands at voltage-operated calcium channels of the L-type. A less-known property of some members of this class of compounds is to sensitize smooth muscle to the relaxant effect of u.v. (Mikkelsen *et al.*, 1985a,b) or polychromatic (Triggle & Bieger, 1990) light irradiation. Exposure to DHPs either enhances the endogenous photorelaxation (PhR) in vascular smooth muscle (Golenhofen *et al.*, 1990; Triggle & Bieger, 1990) or photosensitizes *de novo* those preparations lacking an intrinsic response (Golenhofen *et al.*, 1990).

Preliminary structure-activity relationship (SAR) studies of DHPs suggest that only nitro-substituted DHPs are effective photosensitizers of smooth muscle preparations (Golenhofen *et al.*, 1990; Triggle *et al.*, 1991). The action spectrum of PhR determined in the presence of Bay K 8644 and absorbance of this compound in aqueous solution both show a peak around 410 nm (Golenhofen *et al.*, 1990). Light absorption by the DHP molecule may therefore be the initial step leading to PhR. Based on these observations, the DHP-induced PhR has been attributed to photolytic release of nitric oxide (NO) from the 3-NO_2-DHP molecule (Golenhofen *et al.*, 1990) or to the formation of an intermediate nitrosyl radical (Triggle & Bieger, 1990; Triggle *et al.*, 1991). Photolytic NO release from the 3-NO_2-DHP molecule has recently been measured by a chemiluminescence technique (Bauer & Fung, 1994).

NO-mediated relaxation in smooth muscle has been linked to activation of soluble guanylyl cyclase (sGC) (Ignarro, 1991). Photosensitization of aortic smooth muscle by 3-NO_2-DHP is accompanied by an enhanced production of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Triggle *et al.*, 1991; Baik *et al.*, 1994) and does not require the presence of extracellular Ca^{2+} (Triggle & Bieger, 1990). Since cyclic GMP plays an important part in regulating intracellular free calcium levels in smooth muscle (Lincoln, 1989), the present investigation was undertaken to clarify the role of intracellular Ca^{2+} ions in the generation of 3-NO_2-DHP-induced PhR. In particular, it appeared to be of interest (i) to compare photosensitizers pos-

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sessing either agonist or antagonist activity at L-channels with photosensitizers acting via NO-release and (ii) to examine the Ca^{2+}-dependence of their respective actions.

Since 'microsomal' Ca^{2+}-binding has been implicated as a possible mechanism for PhR (McGonigle & Tallarida, 1980) and cyclic GMP-dependent kinases could stimulate the Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR) (Raeymaekers *et al.*, 1988), we further investigated the effect of agents known to disrupt calcium storage or release from the SR. The smooth muscle preparation chosen was the oesophageal tunica muscularis mucosae (TMM) of the rat, because it is virtually devoid of intrinsic responsiveness to light, but known to have a DHP L-channel antagonist-sensitive mechanism of relaxation (Akbarali *et al.*, 1988b) and a nitrergic innervation (Will *et al.*, 1990).

A preliminary account of this work has appeared previously (Bieger *et al.*, 1993).

Methods

Oesophageal smooth muscle preparations were obtained from Sprague-Dawley rats (250–350 g) following anaesthesia with an intraperitoneal injection of urethane (1.5 g kg^{-1}) and exsanguination from the common carotids. The entire oesophagus was excised and the tunica muscularis mucosae (TMM) isolated by microdissection from the striated tunica muscularis externa. Tissues were dissected under white light (0.6 mW cm^{-2}) in oxygenated Tyrode solution containing (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 12.0, NaH_2PO_4 0.42, D-glucose 5.6. Muscle preparations (1–1.5 cm in length) were mounted under a preload of 0.3 g in glass jacketed organ baths and maintained in oxygenated Tyrode buffer (95% O_2 + 5% CO_2) at 37°C and pH 7.4.

To record and analyze fast changes in tension, force transducers were connected with a Digi-Med tension force analyzer (model 200), kindly made available by Micro-Med (U.S.A.). Data were stored as digital information using a PC 486DX2/50. Traces were analyzed using the software Acknowledge for Windows (Biopac System Inc., U.S.A.) or imported into SigmaPlot (Jandel Scientific, U.S.A.) for graphic reproduction.

A depolarizing, nominally Ca^{2+}-free Tyrode solution was prepared by substituting an equimolar concentration of KCl for NaCl to obtain a final potassium concentration of 110 mM, with $CaCl_2$ being omitted from the solution.

Muscle tissues were induced to generate active tonus by means of the following protocols: (i) incubation with the muscarinic cholinoceptor agonist *cis*-dioxolane (CD) at the EC_{100} 0.3 μM in normal Tyrode solution; (ii) incubation in 110 mM K^+ depolarizing solution containing 30 μM calcium; (iii) incubation in 110 mM K^+, nominally calcium free solution containing 0.3 μM CD. The relaxant effect of isoprenaline (Iso, 0.3 μM) was tested at the end of most experiments to ensure that the capability of the tissues to relax in response to a nonphotic stimulus was preserved.

Skinning of the TMM was carried out with β-escin (50 μM) for 20–30 min (Kobayashi *et al.*, 1989) in the presence of a modified solution containing (in mM): KCl 126, NaCl 7, $MgCl_2$ 5, ATP 2.5, 3-[N-morpholino]propanesulphonic acid (MOPS) 10 and EGTA 1.0 at 22°C and pH 7.0 (pCa < 8). Contraction was induced by increasing the Ca^{2+} : EGTA ratio ($[EGTA]_{total}$ = 0.1 mM). The apparent binding constant of the Ca^{2+}-EGTA complex was considered to be 2.52×10^6 M^{-1} at 22°C and pH 7.0. The concentrations of free calcium shown in the results were calculated by the computer program ALEX, the update version of SPECS (Fabiato & Fabiato, 1979; Fabiato, 1988).

Calcium-depletion of the sarcoplasmic reticulum (SR) was achieved by repetitive stimulation of the TMM preparation with CD (0.3 μM) in 110 K^+ nominally calcium-free buffer containing 30 μM of the calcium chelator BAPTA.

Photostimulation was carried out by means of a timer-controlled polychromatic halogen light source (EJL, 200W, General Electric). Photostimuli (0.9 W cm^{-2}) were delivered for 10 s at intervals of 2–5 min through a fibre-optic light guide positioned 10 mm from the preparation. Intensity was determined by means of a radiometer (IL 1700, Ealing) and measured at the tip of the light guide.

Drugs and chemicals

Streptozotocin was obtained from Calbiochem (U.S.A.); (+)-cis-dioxolane (CD), N^G-nitro-L-arginine methyl ester and ryanodine from RBI (U.S.A.); the calcium ionophore A23187, adenosine 5'-triphosphate (disodium salt from equine muscle), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), calyculin A, cyclopiazonic acid, β-escin, ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), indomethacin, isoprenaline hydrochloride (Iso), methscopolamine (MSCP), 3-[N-morpholino]propanesulphonic acid (MOPS), and ouabain from Sigma (U.S.A.). Felodipine (Hässle, Sweden); LY 83583 (6-(phenylamino)-5,8-quinolinedione; Lilly Res. Laboratories, U.S.A.); racemic Bay K 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-[2-(trifluoromethyl)-phenyl]-5-pyridine carboxylic acid methyl ester) and its (−)-and(+)-enantiomers Bay K 5407 and Bay K 4407, respectively (Miles Inc., U.S.A.); zaprinast (M&B 22948; Rhône-Poulenc Inc., U.K.); (+)-and(−)-PN 202 791 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-3-nitro-5-pyridine carboxylate; Sandoz Inc., Switzerland) were generously donated by their manufacturers.

Stock solutions of DHPs, zaprinast, LY 83583 and ryanodine were prepared in anhydrous ethanol; corresponding volumes of the solvent were routinely tested to rule out a vehicle effect.

Presentation of results and statistics

The polygraph traces shown in the figures are representative of data replicated in 4–6 experiments. Traces were digitized with a Logitech scanner for graphic reproduction. The small squares above each trace represent the 10 s photostimulation. Unless noted otherwise, all drug effects shown were replicated at least four times. Statistical results are expressed as mean ± s.e.mean, n represents the number of experiments carried out for each mean. Statistical analysis of the data was performed by Student's t test for paired observations at $P < 0.05$.

Results

Photoresponse induced by 3-NO_2-DHP L-channel agonist/antagonist enantiomeric pairs and NO donors

TMM preparations did not show a measurable intrinsic response to irradiation, irrespective of their contractile state. After exposure to 3-NO_2-DHP or NO-donors, precontracted preparations became responsive to photostimulation. The photo-induced response showed identical features either in CD-precontracted tissues bathed in normal Tyrode or in 110 mM K^+, nominally calcium-free medium (with the exceptions noted below). Irregular fluctuations in tonus occurred when CD (≤0.1 μM)-precontracted preparations were exposed to DHP agonists in Tyrode solution. In contrast, CD- or calcium-induced tonus in depolarizing solution was maintained at a steady level for several hours. Therefore, the majority of experiments were carried out in a depolarizing solution.

When exposed in a K^+-depolarizing solution to the 3-NO_2-DHP agonists (+)-PN 202 791 (Figure 1a) and Bay K 5407 (not illustrated), CD-precontracted tissues showed a characteristic photoresponse consisting of a transient fast relaxation, followed by an off-contraction. In some preparations, a late relaxation of low amplitude occurred before the pre-existing tonus level recovered (Figure 1a and Figure 2a). A virtually identical response was obtained with racemic Bay

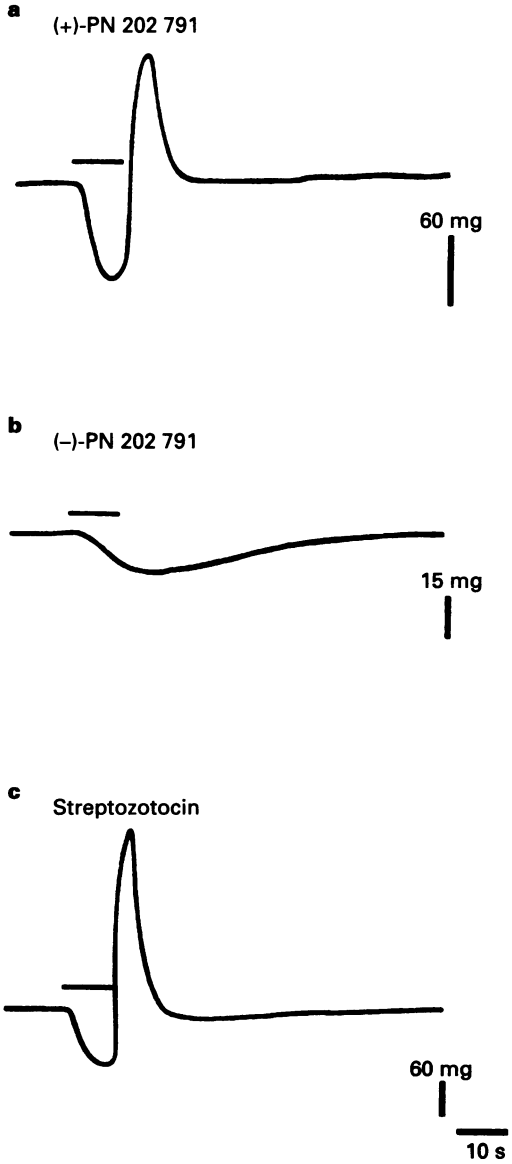

Figure 1 Photoresponse induced by: (a) dihydropyridine (DHP) L-channel agonist (+)-PN 202 791 (1 μM); (b) its L-channel antagonistic enantiomer (−)-PN 202 791 (1 μM); and (c) the NO-donor streptozotocin (1 mM). Tracings showing response of individual tissues are representative of 6–8 independent experiments replicated in each group on separate tunica muscularis mucusa preparations. All experiments were carried out at similar *cis*-dioxolane (0.3 μM)-induced tonus levels ($\approx$180 mg) in 110 mM K^+-depolarizing buffer. Duration of photostimulus is indicated by horizontal bar above each trace.

K 8644 and the NO donors streptozotocin (Figure 1c), sodium nitroprusside (SNP) and sodium nitrite (not illustrated). The amplitude of the fast relaxation as well as the amplitude and slope of the off-response increased with stimulus duration whereas the slope of the fast relaxation remained constant around 140 mg s^{-1} (Figure 2). The fast relaxation was a transient response that reached its nadir within 2.6$\pm$0.2 s and then faded rapidly (escape). The escape was more evident at irradiation periods $\geq$10 s and did not show any irradiation-time dependence (Figure 2). A temporal dissociation between the preceding transient relaxation and the following off-contraction was obtained by photostimulating with longer irradiation intervals (Figure 2a). The off-contraction was seen only under two conditions: at submaximal levels of CD (<0.1 μM)-induced tonus in normal Tyrode buffer or at $[Ca^{2+}]$ < 1 mM in K^+-depolarizing medium, indicative of mechanical saturation when tissues were fully contracted.

The DHP antagonists (−)-PN 202 791 (Figure 1b) and Bay

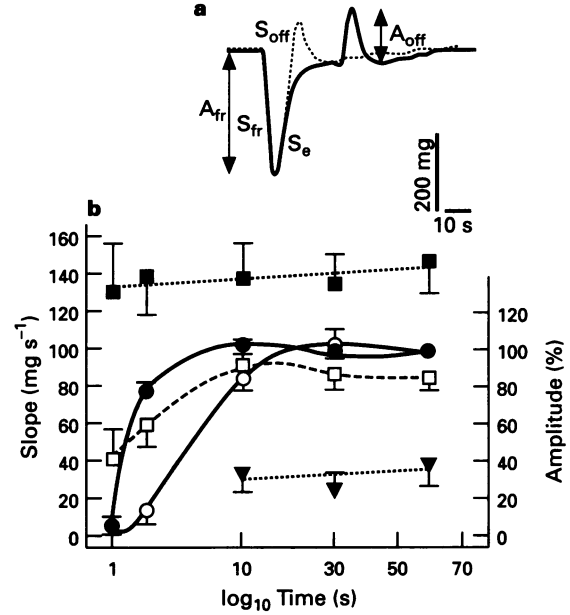

Figure 2 Kinetic variables of (+)-PN 202 791 photo-induced fast response components and their irradiation time-dependence. (a) (+)-PN 202 791 (1 μM) photo-induced response with 10 s (dotted line) and 30 s (continuous line) irradiation period. (b) Amplitude of the relaxation (A_{fr} (%), ●); amplitude of the off-contraction (A_{off} (%), ○); slope of the relaxation (S_{fr}, mg s^{-1}, ■); slope of the off-contraction (S_{off}, mg s^{-1}, □) and slope of the escape of the relaxation (S_e, mg s^{-1}, ▼). A_{fr} and A_{off} are expressed as % of the amplitude of the relaxation and the 'off-contraction', respectively, obtained at 60 s-irradiation (control). Experiments were carried out in 110 mM K^+, nominally calcium-free buffer in *cis*-dioxolane (0.3 μM)-precontracted preparations. Each point and vertical error bar represent the mean$\pm$s.e.mean of 4–6 experiments.

K 4407 (not illustrated) were also effective as photosensitizers. In 110 mM K^+ Tyrode solution at matched levels of CD-induced tonus, both antagonists induced comparable photosensitization. However, the photoresponse obtained in the presence of antagonists was qualitatively and quantitatively different from that of agonists as evidenced by its monophasic shape, slow speed (slope = 1.1$\pm$0.4 mg s^{-1}, n=4) and low amplitude.

The correlation between the amplitude of the photoresponse and the concentration of extracellular calcium concentration needed to maintain a predetermined level of contractile force was examined for agonist and antagonist photosensitizer DHPs (Figure 3). Linear regression analysis demonstrated that agonist-induced PhR, i.e. with Bay K 5417 and (+)-PN 202 791, was greater and required lower extracellular calcium as compared with PhR obtained in the presence of their enantiomer antagonists. No difference in the magnitude of the PhR was noted between racemic Bay K 8644 and the agonist enantiomer Bay K 5417.

A shift in photoresponse from agonist to antagonist pattern was obtained with the L-channel antagonist felodipine (1 μM) in (±)-Bay K 8644-sensitized tissues (Figure 4a). Tissues treated with felodipine alone (n=3) remained unresponsive to light (result not illustrated). Similarly, the sodium nitroprusside (30 μM)-induced fast biphasic photoresponse was transformed by the photosensitizing antagonist enantiomer Bay K 4407 (30–300 nM) into a slow monophasic PhR of small amplitude (Figure 4b).

The photoactivated response to both 3-NO_2-DHPs and NO-donors was concentration-dependent (Figure 5). PhR reached a maximum at 1 μM in the case of racemic Bay K 8644 or the agonist (+)-PN 202 791. At this concentration, PhR obtained with the antagonist (−)-PN 202 791 amounted to only 40% of the steady-state tonus. Streptozotocin was more

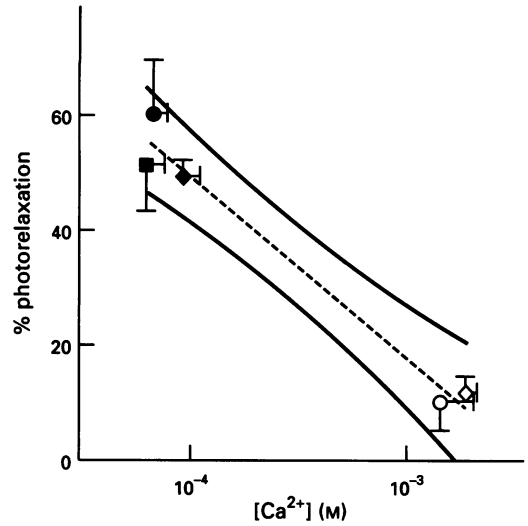

Figure 3 Correlation between equieffective extracellular calcium concentration and % of photorelaxation in the presence of 1 μM of the 3-NO_2-DHP agonists Bay K 5417 (●) and (+)-PN 202 791 (■) and their respective antagonist enantiomers Bay K 4407 (○) and (−)-PN 202 791 (◇) and racemic Bay K 8644 (◆). $[Ca^{2+}]$ and % of the photoresponse were determined at 500 mg active tension. Each data point represents the mean of four separate tissues with corresponding vertical and horizontal error bars (s.e.mean). Linear regression (correlation coefficient $r = 0.8$) and 95% confidence limits were determined by Sigmaplot curve fitting.

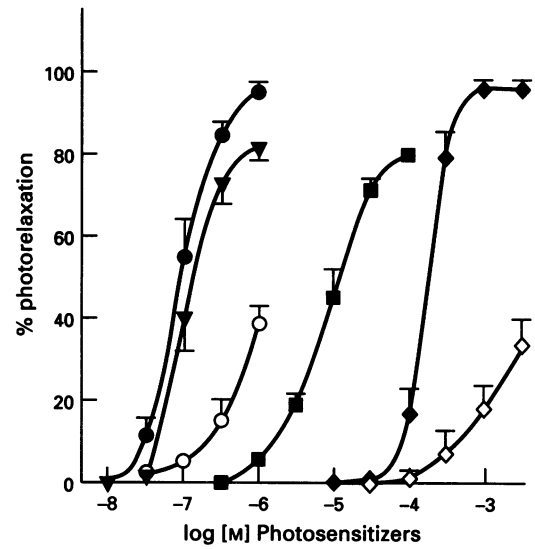

Figure 5 Concentration-response curves for the dihydropyridines, (±)-Bay K 8644 (▼), (+)-and (−)-PN 202 791 (● and ○, respectively) and the NO-donors, sodium nitroprusside (■), streptozotocin (◆), and sodium nitrite (◇) in *cis*-dioxolane (0.3 μM)-precontracted tissues in normal Tyrode solution. Photorelaxation is expressed as % of the existing level of tonus. Each point is the mean of 4 to 8 observations and vertical lines indicate s.e.mean.

efficacious, but less potent than SNP, whilst sodium nitrite was the weakest sensitizer. Relative to (+)-PN 202 791, streptozotocin had an equipotent molar ratio of 2.1×10^3. Within the range of concentrations tested, the NO-donors exhibited negligible effects on steady state tonus; however, SNP (100 μM) produced a transient partial (≤40%) relaxation that waned within 15 to 20 min.

Effect of extracellular calcium chelation and skinning of the plasmalemma on the DHP-induced photoresponse

Since regulation of calcium influx through L-channels seemed to be involved in the 3-NO_2-DHP photo-induced response, further experiments were done to determine the effect of eliminating plasmalemmal Ca^{2+} entry. First, extracellular free calcium was removed by chelation. Second, preparations were subjected to chemical skinning to eliminate any ionic gradients across the membrane.

In preparations maintained in 110 mM K^+, nominally calcium free buffer and precontracted with CD 0.3 μM, the calcium chelator BAPTA (100 μM) inhibited the (±)-Bay K 8644-induced photoresponse, leaving a residual slow relaxation (Figure 6a). In most tissues BAPTA lowered tonus (not illustrated) but in some instances steady state tonus was unaffected. When tonus was restored by raising CD bath concentration, inhibition of the photoresponse persisted with virtually no change in the steady state tonus. Analogous inhibitory effects ($n = 6$) were obtained with EGTA (up to 1 mM) in 110 mM K^+, nominally Ca^{2+}-free solution.

The slow PhR was enhanced by the cyclic GMP-dependent phosphodiesterase inhibitor, zaprinast (10 μM), and this effect could be reversed by the NO-inactivating agent, LY 83583 (10 μM; $n = 7$).

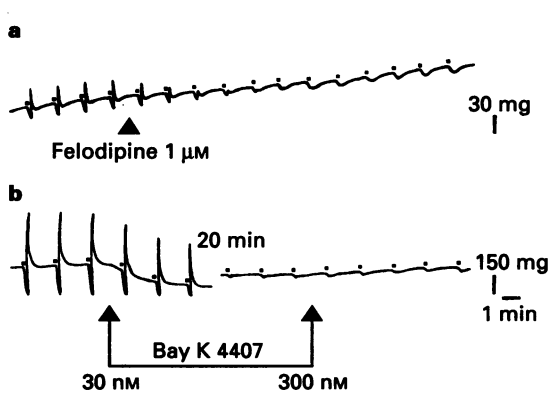

Figure 4 Dependence on L-channel activity of fast photoresponse components. (a) The nonphotosensitizing L-channel antagonist felodipine attenuates the (±)-Bay K 8644 (1 μM) photo-activated response. (b) Sodium nitroprusside (30 μM) photoactivated response is decreased by the L-channel antagonist Bay K 4407 (30–300 nM). Tracings showing response of individual preparations are representative of 4 to 6 independent experiments done in each group on separate tunica muscularis mucosae preparations. Experiments were carried out in 110 mM K^+, nominally calcium-free buffer in absence (a) or presence (b) of *cis*-dioxolane (0.3 μM).

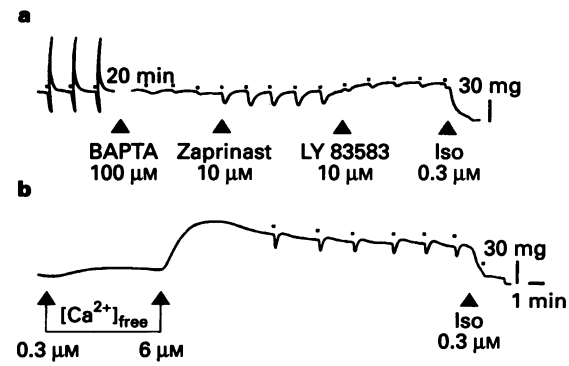

Figure 6 (a) Effect of the calcium chelator BAPTA on the (±)-Bay K 8644 (1 μM) photo-induced relaxation in 110 mM K^+, nominally calcium free Tyrode solution. Note the effect of zaprinast and LY 83583 on the residual slow relaxation. Tissue is precontracted with 0.3 μM *cis*-dioxolane and fully relaxes in response to isoprenaline (Iso). (b) (±)-Bay K 8644 (1 μM) photo-induced response in β-escin skinned preparation. Tissue develops active tonus at low extracellular calcium concentrations as indicated below the trace and is sensitive to isoprenaline. Tracings (a,b) showing response of individual tissues are representative of 6 independently conducted experiments on separate tunica muscularis mucosae preparations.

Attempts at skinning of the TMM were considered successful only in 1/3 of the experiments based on the following criteria: induction of tonus at extracellular Ca^{2+} concentrations in the low micromolar range and relaxation of active tonus by isoprenaline or by returning the preparations to a nominally calcium-free medium. Data obtained from viable preparations ($n=6$) demonstrated a marked change in photoresponsiveness as evidenced by a loss of the fast response, specifically the off-component, and the appearance of a slow PhR at an extracellular calcium concentration as low as 6 μM (Figure 6b).

Slow photorelaxation in calyculin A-induced tonus

In calcium-depleted TMM preparations, the specific protein phosphatase 2A inhibitor, calyculin A, gave rise to a gradually developing contraction (Figure 7). Upon addition of (+)-PN 202 791, concentration-dependent, photoactivated slow relaxations were evident that markedly increased in amplitude after treatment with zaprinast (10 μM).

Effect on (±)-Bay K 8644 photo-induced relaxation of cyclopiazonic acid and ryanodine

Since the SR plays a key role in intracellular calcium homeostasis, we next examined whether the DHP photo-induced response was affected by agents that alter the storage/release capacity of the SR, such as cyclopiazonic acid (CPA), an inhibitor of the sarcoplasmic Ca^{2+}-ATPase (Seidler *et al.*, 1989) and ryanodine, a modulator of the calcium-induced calcium release (Hwang & van Breemen, 1987). CPA inhibited the (±)-Bay K 8644 photo-induced response in normal Tyrode solution (Figure 8a) or in 110 mM K^+, nominally Ca^{2+}-free solution (Figure 8b), revealing a residual slow relaxation. In Ca^{2+}-containing buffer, the inhibition was of immediate onset and was accompanied by a rise in tonus. In nominally calcium-free Tyrode, the inhibition was delayed and coincided with a gradual reduction in CD-tonus. Inhibition of the relaxation and of the rebound contraction followed a similar time course. The residual slow relaxation persisted even after incubation with higher concentrations (up to 100 μM) of CPA. Analogous results were obtained with ryanodine (30 μM; $n=6$; not illustrated).

To determine whether the CPA effect on the SR was directly responsible for the inhibitory effect on PhR, tissues were repeatedly challenged with CD to deplete the SR calcium store (Figure 9a). Upon subsequent incubation with CPA (3 μM) and addition of 30 μM Ca^{2+}, a (±)-Bay K 8644-photoinduced fast response was obtained similar to that observed in tissues with an intact SR (Figure 9b). However, a further CD (0.3 μM) challenge demonstrated that the SR calcium store had not been refilled, as evidenced by the continued depression of the phasic (initial) component of the CD-evoked contraction (Figure 9c).

The possibility that CPA inhibition of PhR was secondary to calcium overflow from SR into the cytoplasm was further studied with the calcium-ionophore A 23187 (results not illustrated). In calcium-containing buffer, A 23187 (1 μM) completely inhibited the photoresponse and this effect was also accompanied by an increase in tonus ($n=3$). However, in nominally calcium free solution the calcium ionophore (1–30 μM) produced no effect ($n=3$).

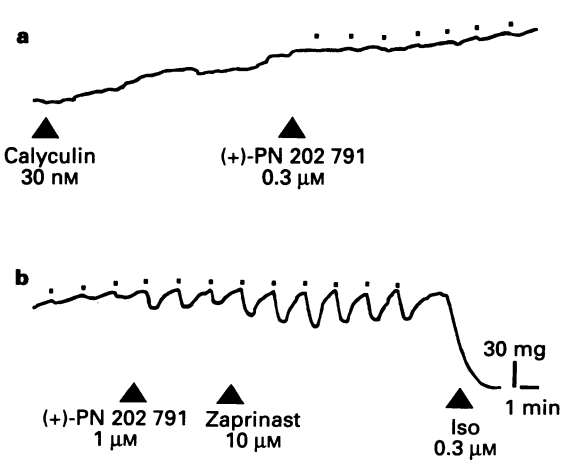

Figure 7 (+)-PN 202 791 photo-induced slow relaxations in calyculin A-precontracted tunica muscularis mucosae (TMM). Continuous tracing (a,b) from individual tissue is representative of five independent experiments replicated in separate TMM preparations. Experiments were carried out in calcium-depleted preparations, bathed in 110 mM K^+, nominally calcium-free buffer.

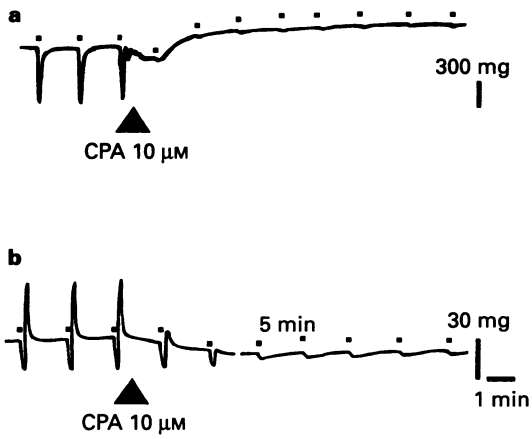

Figure 8 Effect of cyclopiazonic acid (CPA) on the (±)-Bay K 8644 (1 μM) photo-induced relaxation in normal Tyrode (a) and in 110 mM K^+, nominally calcium-free Tyrode solution. (b) Tissues were precontracted with 0.3 μM *cis*-dioxolane. Tracings showing response of an individual tunica muscularis mucosa preparation are representative of 5 independent replications on separate tissues in both groups.

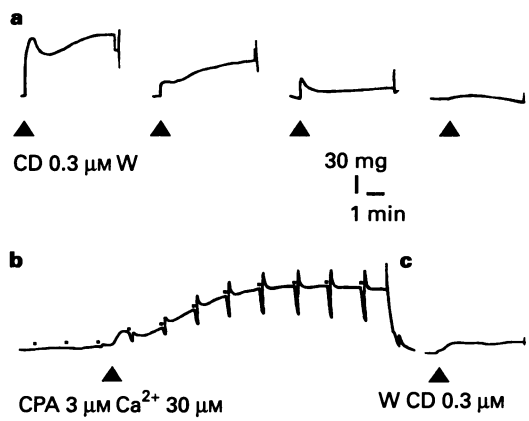

Figure 9 Persistence of Bay K 8644-photoactivated response in calcium depleted muscle. Tracings (a–c) from same tunica muscularis mucosa (TMM) preparation are representative of 4 independent experiments done on separate tissues. (a) Depletion of sarcoplasmic reticulum Ca^{2+} after repetitive stimulation with *cis*-dioxolane (CD, 0.3 μM) in nominally calcium-free depolarizing medium containing 30 μM BAPTA results in nearly complete loss of contractile response after fourth CD-challenge. (b) Subsequent incubation of same tissue with (±)-Bay K 8644 (1 μM), added to bath 10 min before start of trace, and CPA (3 μM) does not prevent photo-induced response seen after extracellular calcium is added to induce tonus. (c) Depression of the phasic component of the CD-induced contraction persists upon exposing the preparation to high K^+ nominally calcium-free medium.

Effect of cyclic GMP-modifying agents on the fast photoresponse

LY 83583 (10 μM) significantly diminished the fast relaxation at low, but not at maximal, concentrations of (+)-PN 202 791 (Figure 10). However, in the presence of the superoxide dismutase (SOD) inhibitor diethyldithiocarbamate (100 μM), LY 83583 produced a clear decrement of the photoresponse at all DHP concentrations tested. At the same concentration, the SOD inhibitor alone was ineffective in decreasing the photoresponse induced by (+)-PN 202 791 (1 μM; $n=4$).

Zaprinast (10 μM) increased the amplitude without changing the slope of the fast PhR (Table 1) or the off-contraction (S_{off}: 164.0±23.1 mg s^{-1} vs. 119.0±18.7 mg s^{-1}, control). However, its most conspicuous effect involved the escape response. As evidenced by its slope and the magnitude, zaprinast virtually eliminated this response component (Table 1).

Effect of other drugs on the DHP photo-induced response

Neither the biphasic fast response nor the slow relaxation induced by (±)-Bay K 8644 were affected by indomethacin (30 μM) or ouabain (100 μM) when tested in normal Tyrode ($n=3$) or 110 mM K^+, nominally calcium-free solution ($n=3$).

An extensive parasympathetic innervation has been demonstrated in the TMM (Bieger & Triggle, 1985) as well as a presumptive nitrergic innervation (Will *et al.*, 1990). To guard against a possible interference with the photoresponse due to depolarization-induced release of acetylcholine or NO from intramural nerve terminals, the muscarinic cholinoceptor antagonist methscopolamine and the nitric oxide synthase inhibitor N^G-nitro-L-arginine methylester were used. Incubation with either methscopolamine (10 nM) or the NO synthase inhibitor (0.1 or 1.0 mM) failed to alter the amplitude and shape of the (±)-Bay K 8644 photo-induced response in K^+-depolarized preparations ($n=4$ each).

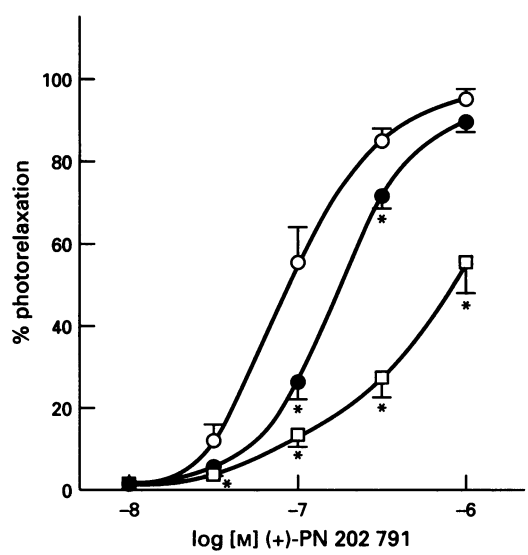

Figure 10 Effect of LY 83583 (10 μM) alone (●) or in the presence of the superoxide dismutase inhibitor diethyldithiocarbamate (100 μM; □) on the (+)-PN 202 791 (1 μM) photo-induced fast response as compared with the control (○). Experiments were carried out in *cis*-dioxolane (0.1 μM) precontracted preparations bathed in normal Tyrode solution. Each data point represents the mean (±s.e.mean) of 4 to 6 tissues treated with cumulative doses of the DHP agonist. Photorelaxation is expressed as % of steady-state tonus. *$P\leq0.05$ vs. control.

Discussion

As first shown by Ehrreich & Furchgott (1968), smooth muscle from various visceral organs, although not capable of an endogenous PhR, is rendered photosensitive by treatment with nitrite, now known to be a photolabile NO-donor (Matsunaga & Furchgott, 1991). More recently, Golenhofen *et al.* (1990) were able to demonstrate that smooth muscle responsive to another photo-degradable NO-donor, SNP, was similarly sensitized by 3-nitro substituted DHP compounds. As shown here, rat oesophageal smooth muscle, which also lacks intrinsic photosensitivity, is likewise susceptible to sensitization by these photolabile agents. Photosensitization with 3-NO_2-DHP may thus be equivalent to supplying a phototransducer substance, i.e., a photo-induced relaxing factor (PIRF), which is already present in vascular smooth muscle (Furchgott *et al.*, 1985). Since photodegradable NO-donors mimicked the 3-NO_2-DHP-induced photoresponse in the TMM, the mediator molecule could be NO or a NO-like molecule. At first glance, this assumption would seem to be implausible given that 3-NO_2-DHPs were effective at micromolar concentrations, 100 times lower than those required for the most effective NO-donor. However, as highly hydrophobic molecules, 3-NO_2-DHPs would be expected to accumulate in the lipid phase of the plasmalemma and sarcoplasmic membrane system with cell to medium ratios of 120:1 (Lüllmann & Mohr, 1987), permitting more effective access to the effector structure(s) than might be the case with hydrophilic NO donors. This would explain the higher potency of 3-NO_2-DHP agonists relative to the latter.

Our pharmacological data strengthen the hypothesis that photolytic NO release followed by activation of soluble guanylyl cyclase underlies the photoactivated response in TMM preparations. Thus, the NO scavenger LY 83583 inhibited the photoresponses whereas the cyclic GMP-dependent phosphodiesterase inhibitor, zaprinast, enhanced the photoinduced relaxations. These findings agree with previous results in vascular smooth muscle regarding the role of NO and cyclic GMP in the 3-NO_2-DHP-mediated photoresponse (Triggle *et al.*, 1991; Baik *et al.*, 1994).

Photo-induced cyclic GMP generation would appear to mediate three mechanical responses in TMM preparations consisting of a fast relaxation-contraction sequence and a slow relaxation. As evidenced by their functional properties and their differential sensitivity to calcium, the photoactivated ef-

Table 1 Effect of zaprinast on parameters of the (+)-PN 202 791 photo-induced fast relaxation

	Amplitude[2] A_{fr} (%)	*Slope* S_{fr} (mg s^{-1})	*Slope of escape* S_e (mg s^{-1})	*Amplitude of escape*[3] (%)	(n)
Control[1]	100	176.0±39.1	14.0±2.4	25.8±7.0	(5)
Zaprinast	138.0±11.6*	230±46	4.0±2.4*	3.9±1.9*	(5)

[1]Refer to Figure 2 for graphical illustration of parameters listed.
[2]Amplitude of the fast relaxation after zaprinast was expressed as % of the response to 10 s photostimulus before treatment with zaprinast (10 μM). Muscle preparations were photosensitized with (+)-PN 202 791 (1 μM) and maintained in nominally Ca^{2+}-free buffer containing K^+ 110 mM and *cis*-dioxolane 0.1 μM.
[3]% amplitude of the escape was calculated as $(R_{10}/R_{max})\times100$, where R_{max} and R_{10} represent the magnitude of the relaxation at its peak and at the end of the 10 s-irradiation interval.
*$P\leq0.05$ vs. control, paired *t* test. The number of individual experiments is given in parentheses.

fects of 3-NO_2-DHPs implicate two different effector mechanisms acting at the level of the plasmalemma and another substrate deep within the myoplasm.

Role of the plasmalemma

The present data clearly indicate a qualitative and quantitative difference between 3-NO_2-DHP agonist- and antagonist-induced photosensitization. With antagonists, the speed of PhR was diminished and the rebound ('off') contraction abolished, this difference being accentuated at extracellular Ca^{2+} concentrations below the physiological range. L-channels are therefore likely to mediate the expression of the fast photoresponse components. Our results contrast with previous reports showing the agonist-antagonist enantiomeric pairs of Bay K 8644 and PN 202 791 to be equieffective as photosensitizers in rat (Triggle & Bieger, 1990) and rabbit aorta or porcine stomach fundus (Golenhofen *et al.*, 1990). However, an explanation may be found in the experimental conditions used in this study and those carried out previously. Whereas the present results were obtained in preparations precontracted with a depolarizing solution containing low concentrations of Ca^{2+}, previous work was carried out in normal physiological solution (i.e. at a high $[Ca^{2+}]_o$). Due to the high-affinity binding of DHP antagonists to inactivated L-channels (Bean, 1984), the use of a depolarizing buffer may result in a higher L-channel binding rate of the DHP antagonist as compared with that in a normal physiological medium (Godfraind, 1992).

Previous work in TMM has also demonstrated that electrical field stimulation results in a relaxation response with a TTX-sensitive and -insensitive component (Akbarali *et al.*, 1986). The latter was mimicked by the channel openers BRL 34915 and pinacidil, blocked by the L-channel antagonists nifedipine, (+)-PN 200 110 and verapamil, and abolished by incubation in a depolarizing K^+ solution, suggesting the involvement of Ca^{2+}-activated K^+ channels (Akbarali *et al.*, 1988a,b). On the contrary, the 3-NO_2-DHP-induced photoresponse, albeit sensitive to L-channel antagonists, was unaffected by incubation in a K^+-depolarizing medium. Therefore, calcium entry followed by activation of K^+ channels is unlikely to mediate photo-induced relaxation in TMM.

Our findings suggest that photostimulation may cause a decrease in the calcium current through the L-channel. Consistent with this interpretation, extracellular calcium depletion as well as chemical skinning of TMM smooth muscle fibres significantly inhibited the fast response components. Provided that sufficient cholinoceptor stimulation was used, extracellular calcium chelation was effective in inhibiting the photoresponse with little or no alteration in steady-state tonus.

The proposed mechanism of photo-induced L-channel modulation by 3-NO_2-DHP agonists implies an increased probability of channel closure during fast relaxation. The present data point to cyclic GMP formation as the underlying event, followed by regulation of L-channel activity via a cyclic GMP-dependent protein kinase. Patch-clamp studies have provided evidence for a decrease in calcium current (I_{Ca}) through the L-channel in rat thoracic and rabbit pulmonary aorta treated with the NO-donor SNP (Magliola & Jones, 1990; Clapp & Gurney, 1991). A previous report regarding a quasi-irreversible inhibition of I_{Ca} after irradiation with polychromatic light of (±)-Bay K 8644-pretreated dorsal root ganglion cells (Scott & Dolphin, 1988), however, does not seem to explain our observations.

Two other pertinent features of the fast photoresponse are (i) the escape or fade of the initial relaxation and (ii) the 'off-contraction'. The former contrasts with the sustained relaxations of intrinsic (Furchgott *et al.*, 1961) or extrinsic (Ehrreich & Furchgott, 1968; Golenhofen *et al.*, 1990) origin in thoracic aorta and probably reflects a transient imbalance between cyclic GMP production and degradation as suggested by the ability of zaprinast to prevent fading. A noncholinergic 'post-irradiation stimulation' analogous to that described here has been observed previously in rabbit stomach smooth muscle (Ehrreich & Furchgott, 1968). Like the post-stimulation rebound that follows field stimulation-evoked, tetrodotoxin-insensitive relaxations in TMM preparations (Akbarali *et al.*, 1986), the 'off-contraction' following the photoactivated relaxation probably has a myogenic origin involving a regulatory process at the level of the L-channel. One possibility is that activation of a secondary excitatory mechanism via a cyclic GMP-dependent pathway could mediate the 'off-contraction'. As shown by Saha *et al.* (1993), SNP induces a post-relaxation contraction in opossum oesophageal longitudinal muscle. This contraction involves eicosanoids via a cyclic GMP-dependent pathway. A different mechanism has to be responsible for the 'off-contraction' in 3-NO_2-DHP-treated TMM preparations, since indomethacin did not modify this response.

Role of the sarcoplasmic reticulum

Two mechanisms acting at the level of the SR have been proposed to regulate myoplasmic calcium concentration, hence tonus generation in smooth muscle: the SR Ca^{2+}-ATPase and the caffeine channel (van Breemen & Saida, 1989; Quian *et al.*, 1992).

At first glance, the results with ryanodine and CPA suggest that the 3-NO_2-DHP-induced photoresponse requires a functional SR. However, further experiments demonstrated that it is possible to dissociate the CPA effect on the SR from that on the photoresponse. Thus, when the intracellular calcium store was depleted with CPA, a low concentration of extracellular calcium still supported the photoresponse even though SR refilling was prevented. This suggests that the SR Ca^{2+} pump is not responsible for the 3-NO_2-DHP photo-induced fast relaxation in the TMM. Notwithstanding the binding to SR Ca^{2+}-ATPase of DHPs (Zernig, 1990), a more plausible explanation would be that both drugs cause swamping of the myoplasm with calcium and thus mask any small change due to calcium movement across the plasmalemma. This interpretation agrees with the dependence of the time course of the action of CPA and ryanodine on the extracellular calcium concentration: rapid inhibition of the photoresponse, accompanied by tonus increase, occurred in calcium-containing buffer, delayed inhibition in nominally calcium-free solution. Similarly, at physiological levels of extracellular calcium, but not in nominally calcium-free medium, the calcium ionophore A 23187 produced inhibition of the 3-NO_2-DHP photo-induced response, probably because massive calcium entry induced by the ionophore overwhelms the buffer capacity of the SR.

Involvement of other intracellular mechanisms

In addition to the fast photoresponse, 3-NO_2-DHP photosensitization of TMM was also evident in the form of a slow, low amplitude relaxation. This response was Ca^{2+}-independent as evidenced by its resistance to extracellular calcium depletion or L-channel inhibition. Furthermore, the slow response appeared to be generated by a cyclic GMP-dependent pathway not involving the SR Ca^{2+}-ATPase, the ryanodine channel or the Na^+/K^+-ATPase, because it persisted in depolarizing buffer or in the presence of the Na^+/K^+-ATPase inhibitor, ouabain. Conceivably, this response involves cyclic GMP signalling at a substrate deep within the myoplasm, such as myosin light chain kinase, or a direct regulation of the calcium sensitivity of the contractile elements, probably by a cyclic GMP-dependent protein kinase (Nishimura & van Breemen, 1989). This hypothesis is supported by results obtained with the protein phosphatase 2A inhibitor calyculin A. Previous studies in smooth muscle (Shibata *et al.*, 1982; Hartshorne *et al.*, 1989) have demonstrated that calyculin A induces a slowly developing, calcium-independent contraction as a consequence of shifting the equilibrium between phosphorylation and dephosphorylation of the contractile proteins.

In terms of its Ca^{2+}-independence, the slow relaxation is similar to the 3-NO_2-DHP-augmented photorelaxation in rat

thoracic aorta (Triggle & Bieger, 1990). However, the 3-NO_2-DHP photo-induced slow relaxation in TMM differs from that of intrinsic (Raffa *et al.*, 1992) or extrinsic (Baik *et al.*, 1994) origin in aortic rings by virtue of its insensitivity to extracellular Na^+ depletion.

In conclusion, the present findings suggest that the 3-NO_2-DHP photo-induced response in rat oesophageal TMM involves three different mechanical components. Although all of these appear to be cyclic GMP-dependent, the initial fast relaxation/contraction sequence involves regulation of calcium L-channel activity whereas the slow relaxation may be mediated by phosphorylation of contractile proteins.

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