INVESTIGATION OF THE PHOTOSENSITIZING

ACTIVITY OF 1,4-DIHYDROPYRIDINE COMPOUNDS

IN RAT SMOOTH MUSCLE

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BY

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ABSTRACT

The general aim of this work was to investigate the photodynamic action of 1,4-dihydropyridines (DHPs) with respect to contractile force generation in rat smooth muscle. The preparations chosen possessed (thoracic aorta) or lacked (oesophageal tunica muscularis mucosae (TMM) and pyloric sphincter) intrinsic photoresponsiveness. Exposure to $3'$-NO_2-substituted DHP ($3'$-NO_2-DHP) photosensitized precontracted TMM preparations in a concentration-dependent fashion and this could be mimicked by photodegradable nitric oxide (NO) donors (streptozotocin, sodium nitroprussidem, sodium nitrite).

$3'$-NO_2-DHP- and NO donor-photoactivated responses in TMM, had at least three different components, consisting of (i) a transient fast relaxation, (ii) a fast "off-contraction", and (iii) a slow, delayed relaxation. Only the latter component persisted in calcium-depleted, calyculin A-precontracted preparations or following inhibition of the fast response by DHP L-type Ca^{++}-channel antagonists, skinning of the plasmalemma and extracellular Ca^{++} chelation.

Both fast and slow relaxations in the TMM were diminished by NO scavengers (LY 83583, carboxy-PTIO), whereas the fast relaxation was also diminished by pre-irradiation of the $3'$-NO_2-DHP solution. The selective cGMP-dependent phosphodiesterase inhibitor, zaprinast, enhanced the photorelaxation.

Unbuffering of the sarcoplasmic reticulum with either cyclopiazonic acid

or ryanodine inhibited the Bay K 8644-photoactivated fast response in TMM. This effect was accelerated in the presence of extracellular Ca^{++} and resembles that seen in tissues exposed to the calcium ionophore A23187.

In thoracic aorta, the endogenous photorelaxation was enhanced by all 3'-NO$_2$-DHPs tested. LY 83583 effectively inhibited both the endogenous and 3'-NO$_2$-DHP-augmented photorelaxation. In pyloric sphincter, photosensitization by the 3'-NO$_2$-DHP, (+)-PN 202 791, was evident as a transient inhibition of the muscarinic agonist-stimulated phasic contractions, followed by a post-irradiation contraction.

The present study supports the following conclusions: (1) 3'-NO$_2$-DHP-photoactivated responses are tissue-specific and mimic the endogenous or NO donor-photoactivated response in smooth muscles, (2) photoactivated release of NO, presumably followed by stimulation of soluble guanylate cyclase mediates both the fast and slow relaxations in the TMM, (3) functional L-type Ca^{++}-channels are required for the expression of the fast components of the photoactivated response, (4) the slow relaxation may involve direct regulation of contractile protein phosphorylation by a cGMP-dependent protein kinase.

Keywords: guanylate cyclase, 3'-NO$_2$-1,4-dihydropyridines, L-type Ca^{++}-channel, nitric oxide, tunica muscularis mucosae, photosensitization, pyloric sphincter, sarcoplasmic reticulum, thoracic aorta.

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cAMP, cyclic adenosine-3',5'-monophosphate
CD, cis-dioxolane
cGMP, cyclic guanosine-3',5'-monophosphate
DHP(s), 1,4-dihydropyridine(s)
EDRF, endothelium-derived relaxing factor
Hb, haemoglobin
IP_3, inositol triphosphate
MB, methylene blue
MLCK, myosin light chain kinase
NO, nitric oxide
NOS, NO synthase
PDE, phosphodiesterase
PKA, cAMP-dependent protein kinase
PKG, cGMP-dependent protein kinase
sGC, soluble guanylate cyclase
SNP, sodium nitroprusside
SR, sarcoplasmic reticulum
TMM, tunica muscularic mucosae
TTX, tetrodotoxin

CHAPTER ONE

INTRODUCTION

1.1 Photoactivated changes in contractility of smooth muscle

Visible or UV-irradiation of smooth muscle preparations may evoke relaxation or contraction. Furthermore, in certain preparations, irradiation induces a complex response consisting of a relaxation-contraction sequence. The following sections will describe the physical and pharmacological properties of the photoactivated responses in smooth muscle.

1.1.1 Photorelaxation

Photoactivated responsiveness in smooth muscle may be of intrinsic or extrinsic origin. Intrinsic photorelaxation does not require any added photosensitizing agent. On the contrary, certain compounds such as photodegradable NO-donors and 1,4-dihydropyridines (DHPs) have the property to enhance the intrinsic photorelaxation in vascular smooth muscle or to photosensitize de novo other preparations.

1.1.1.1 Endogenous photorelaxation

Forty years ago it was reported that visible or UV-irradiation evokes an oxygen-independent photorelaxation in norpinephrine-precontracted rabbit aorta (Furchgott et al., 1955). This response requiring no photosensitizing agent was

described as a reversible relaxation.

The action spectrum of the endogenous photorelaxation in aortic prepara-
tions revealed a peak at 310 nm, but photorelaxation of variable amplitude could
also be elicited in the visible range of the spectrum (Furchgott et al., 1961;
Chaudhry et al., 1993). However, since the action spectrum of the molecule
involved in the endogenous photorelaxation does not match the action spectrum
of most common chromophores (i.e., proteins, NADH, porphyrins, flavins) in
biological tissues, it has been suggested that more than one chromophore may
be involved in the photorelaxation (Chaudhry et al., 1993).

Other studies demonstrated that the amplitude of the endogenous
photorelaxation in rabbit aorta depends on the level of active tension of the
smooth muscle, but not on the stimulating drug used to produce contraction
(Furchgott et al., 1961). However, K^+-precontracted aortic preparations were
less responsive to the relaxant effect of UV-light. An explanation for this
observation may be found in the sensitivity of the photorelaxation to
extracellular Na^+ concentration, but not other ions, reported in vascular smooth
muscle (Raffa et al., 1991). Thus, Raffa et al. have described that the UV-
photoactivated relaxation in rabbit aorta is greatly inhibited by equimolar
replacement of the $[Na^+]_e$ with mannitol and Li^+, but not choline, in the
physiological buffer.

Preconstricted segments of rabbit aorta also relax when exposed to acetylcholine by an endothelium-dependent mechanism (Furchgott & Zawadski, 1980) (see section 1.4.2). Relaxation is mediated by an endothelium-derived relaxing factor (EDRF) with the ultimate mediator being nitric oxide (NO)(Palmer et al., 1987; Furchgott, 1988; Ignarro et al., 1987). The subsequent step in NO-mediated relaxation in smooth muscle involves activation of soluble guanylate cyclase (sGC) and increase in the levels of cyclic guanosine-3',5'-monophosphate (cGMP) (Rapoport & Murad, 1983). Pharmacological studies have suggested that the endogenous photorelaxation in vascular smooth muscle may be mediated by a photoactivated relaxing factor with similarities to NO (Furchgott et al., 1985). Recent evidence using a porphyrin sensor, further suggests that this factor is indeed NO (Kubaszewski et al., 1994). Similar to acetylcholine-induced relaxation, photorelaxation is accompanied by an increase in cGMP levels (Furchgott et al., 1984; Karlsson et al., 1984). The onset of the rise in cGMP precedes the onset of the photorelaxation. The NO-binding molecule haemoglobin (Hb) inhibits both the rise in cGMP and the photorelaxation resulting from UV-irradiation (Furchgott et al., 1984, 1985), whereas LY 83583, which generates superoxide radicals, also diminishes the photorelaxation (Furchgott & Jothianandan, 1991). Superoxide radicals react with NO to form peroxynitrite (Beckman et al., 1990) and thus represents one

means to diminish EDRF/NO vasodilation (Gryglewski et al., 1986). In rabbit thoracic aorta and bovine pulmonary artery, potentiation of the endogenous photorelaxation can be demonstrated with the selective inhibitor of cGMP-dependent phosphodiesterase, zaprinast (M&B 22948) (Furchgott & Jothianandan, 1991; Wolin et al., 1991).

The effect of the sGC inhibitor, methylene blue (MB), on the endogenous photorelaxation is presently unclear. Although MB significantly inhibits the UV irradiation-induced increase in cGMP in rabbit aortic preparations; pretreatment of aortic strips with MB results in a slowly developing, quasi-irreversible inhibition of the photorelaxation (Furchgott et al., 1984). Furthermore, recent data indicate that MB enhances the endogenous photorelaxation in several vascular smooth muscle preparations (Chen & Gillis, 1993).

The endogenous photorelaxation, however, also occurs in endothelium-denuded rabbit aortic preparations, suggesting that photoactivated release of NO from the vascular endothelium is not required for the photorelaxation (Furchgott et al., 1984). Since the superoxide scavenger, superoxide dismutase does not modify the intrinsic photorelaxation, it has been suggested that the source of NO may be an intracellular store in the smooth muscle cells (Matsunaga & Furchgott, 1989). Furthermore, this store could be depleted following repeated exposure to light stimulation (Venturini et al., 1993;

Kubaszewski et al., 1994).

Other pathways involving sGC activation are believed to mediate photorelaxation in smooth muscle as well. Wolin et al. (1991) have proposed that metabolic degradation of hydrogen peroxide by catalase, followed by activation of sGC, may mediate photorelaxation in bovine pulmonary artery. Direct photoactivation of sGC has also been inferred by Karlsson et al. (1985), which could underlie the increase in cGMP and photorelaxation in bovine mesenteric arteries (Karlsson et al., 1984).

Tallarida and colleagues have proposed two other mechanisms that mediate the endogenous photorelaxation in smooth muscle. The first mechanism involves a decrease in cytoplasmic free $[Ca^{++}]$ upon UV-irradiation (Jacob & Tallarida, 1977). They observed that UV irradiation of rabbit aortic microsomes enhances calcium binding which may explain the reduction in tonus shown in the intact smooth muscle after irradiation. Whether this effect is cGMP-dependent remains to be determined since an increase in cGMP could result in a decrease in intracellular $[Ca^{++}]$ via activation of a sarcolemmal Ca^{++}-ATPase (see section 1.4.2). The second mechanism supposedly involves a shift in the equilibrium binding constant for the tonus-inducing drug and its receptor (McGonigle & Tallarida, 1980) in such way that the drug is displaced from the receptor or the drug-receptor complex is inactivated, resulting in relaxation.

1.1.1.2 Exogenous photorelaxation

$NaNO_2$ (a non-acidified solution) enhances the endogenous photorelaxation in rabbit aortic strips (Matsunaga & Furchgott, 1989). Moreover, in smooth muscle preparations from rabbit stomach, duodenum and uterus which are insensitive to photostimulation, $NaNO_2$ is able to induce relaxation upon UV-irradiation (Ehrreich & Furchgott, 1968). Matsunaga and Furchgott (1991) demonstrated that $NaNO_2$ in solution is photoactivated by long wavelength UV-light to release a potent, but very labile relaxing substance with pharmacological properties similar to EDRF. This molecule is believed to be NO since photorelaxation can be stabilized by superoxide dismutase and completely inhibited by Hb, but not methemoglobin (Matsunaga & Furchgott, 1988, 1991). However, the peak of the action spectrum for the endogenous photorelaxation (310 nm) differs from that of the $NaNO_2^-$-potentiated photorelaxation (350 nm) (Matsunaga & Furchgott, 1989).

Other NO-donors are able to photosensitize smooth muscle as well. Thus, streptozotocin and N^ω-nitro-L-arginine enhance photorelaxation in rat aorta and induce photorelaxation in rat trachea (Chang et al., 1993). Significant release of NO following irradiation of a N^ω-nitro-L-arginine aqueous solution has been demonstrated by Bauer and Fung (1993). Sodium nitroprusside (SNP)-photoactivated relaxation has been described in porcine fundus (Golenhofen et

al., 1990). In contrast, phasic smooth muscle such as porcine antrum and pylorus are less sensitive to inhibition of the phasic contractions upon irradiation of SNP-treated preparations (Golenhofen et al., 1990).

The DHPs are chiefly known to modulate voltage-gated Ca^{++}-channels of the L-type. A less understood property of some members of this class of compounds is to sensitize smooth muscle to the relaxant effect of light (Mikkelsen et al., 1985a,b). Mikkelsen and colleagues proposed that, in rat thoracic aorta, UV irradiation induces a reversible shift of the DHP ligand, Bay K 8644, from an agonist to an antagonist binding site at the L-type Ca^{++}-channel, causing inhibition of smooth muscle tonus (Mikkelsen & Nyborg, 1986). However, subsequent studies of DHP L-type Ca^{++}-channel agonist-antagonist enantiomeric pairs have shown that both enantiomers produce photorelaxation in vascular smooth muscle regardless of their action on the Ca^{++}-channel (Golenhofen et al., 1990; Triggle et al., 1991). Based on the photosensitizing activity and modulation of L-type Ca^{++}-channel in rat thoracic aorta, DHPs have been divided into four groups: 1) L-type Ca^{++}-channel agonist-photosensitizers: Bay K 8644 and PN 202-791, 2) L-type Ca^{++}-channel agonist-nonphotosensitizers: CGP 28392 and YC-170, 3) L-type Ca^{++}-channel antagonist-photosensitizers: nifedipine, PN 200-110 and the antagonist enantiomers of Bay K 8644 and PN 202-791, and finally 4) L-type Ca^{++}-channel

antagonist-nonphotosensitizers: amlodipine, felodipine and PD 122860 (Triggle & Bieger, 1990). Subsequent investigations, however, have failed to corroborate photosensitizing activity in the case of nifedipine and PN 200-110 (D. Bieger, personal communication).

Preliminary structure-activity relationship (SAR) studies of DHPs suggest that all molecules with a $3'-NO_2$ substituent ($3'-NO_2$-DHP) are effective photosensitizers in smooth muscle (Golenhofen et al., 1990; Triggle et al., 1991). As determined by spectral analysis, both the Bay K 8644-photoactivated relaxation and absorbance in aqueous solution show a maximum around 410 nm (Golenhofen et al., 1990). These observations have led to the hypothesis that the DHP-photoactivated relaxation results from photolytic release of NO from the $3'-NO_2$-DHP molecule (Golenhofen et al., 1990) or from the formation of an intermediate nitrosyl radical capable of activating sGC (Triggle and Bieger, 1990).

Whether irradiation of the $3'-NO_2$-DHP molecule may release a photolabile relaxing substance similar to NO remains to be established since: 1) 2h-UV light irradiation of a Bay K 8644 solution does not decrease its photosensitizing activity in rat thoracic aorta. In addition, removal of the $3'-NO_2$-DHP from the incubation medium does not attenuate the photorelaxation (Mikkelsen et al., 1985a); 2) although the photoactivated response of Bay K 8644-treated

preparations is accompanied by an increase in cGMP content (Bieger & Triggle, 1991), no vasorelaxant product could be detected by bioassay during irradiation of vascular smooth muscle (Chen & Gillis, 1992); 3) 3'-NO_2-DHP-photoactivated relaxation is less sensitive to pharmacological compounds modifying endogenous photorelaxation as well as $NaNO_2$-augmented photorelaxation. Thus, superoxide dismutase has no effect on the Bay K 8644-mediated photorelaxation (Chen & Gillis, 1992) whereas LY 83583 only attenuates the DHP-augmented photorelaxation in aortic rings (Triggle & Bieger, 1990).

Other results, on the contrary, seem to support the hypothesis regarding photoactivated release of NO from the 3'-NO_2-DHP molecule. Recently, Bauer and Fung (1993) were able to demonstrate that irradiation of an ethanolic solution of Bay K 8644 results in a measurable release of NO as determined by chemiluminescence analysis. Moreover, an increase in cGMP production has been demonstrated in Bay K 8644-photosensitised rat aortic preparations (Triggle & Bieger, 1991) and (+)-PN 202 791-treated porcine coronary arteries (Baik et al., 1994). In the latter, MB inhibits both the cGMP increase and the photorelaxation. Whether the effect of 3'-NO_2-DHP on cGMP is a direct result of light stimulation remains to be determined since Bay K 8644 alone may also stimulate cGMP synthesis in pituitary tumour cells and rat aortic smooth muscle (Heisler, 1986; Triggle et al., 1991).

Changes in the chemical structure of the $3'$-NO_2-DHP molecule as a result of irradiation and its interaction with different biological structures remain to be studied, especially changes related to the $3'$-NO_2 group of the dihydropyridine ring. However, significant data regarding photoactivated decomposition of DHP antagonists have been described. Thus, irradiation of nimodipine and furnidipine results in aromatization of the dihydropyridine moiety, turning it into a pyridine ring (Nuñez-Vergara et al., 1994; Zanocco et al., 1992). If the DHP contains a -NO_2 group in the phenyl ring (e.g., nimodipine), it may undergo reduction to the nitroso derivate (Nuñez-Vergara et al., 1994) or transmutation of the nitro group within the phenyl ring (Zanocco et al., 1992). The irradiation wavelength seems to play a role in the rate of those transformations. Thus, UV-irradiation of furnidipine results in oxidation of the dihydropyridine, whereas artificial day light irradiation also includes reduction of the nitro group (Nuñez-Vergara et al., 1994).

1.1.2 Photocontraction

Light may evoke contraction in precontracted smooth muscle preparations as well as in preparations held at basal tonus. However, unlike photorelaxation which occurs mostly during photostimulation, contractions may take place during, as well as after, irradiation. Thus, in frog iris sphincter, polychromatic light irradiation induces photocontraction via release of calcium from an intracellular source (Kargacin & Detwiler, 1985). However, high intensity Ar-laser irradiation of rabbit aorta evokes photocontractions which are mediated by a thermal effect of the laser on the smooth muscle (Steg et al., 1988).

Although UV and polychromatic light-irradiation causes photorelaxation in smooth muscle, in certain preparations relaxation is followed by an "off-contraction", i.e., a contraction occurring at the offset of the irradiation. At the present time, the mechanism of this response remains unknown; however, it has mainly been described in non-vascular smooth muscle such as preparations from the gastrointestinal tract. Such post-irradiation stimulation was first reported by Ehrreich and Furchgott (1968) in acetylcholine-precontracted rabbit stomach smooth muscle, photosensitized with $NaNO_2$. This response was not cholinergically mediated since it was resistant to atropine and hexamethonium. When $NaNO_2$-pretreated preparations were stimulated electrically to induce phasic contractions, irradiation evoked a transient or long lasting inhibition of

the phasic activity, followed by an "off-contraction", the amplitude of the latter being significantly larger than the amplitude of the phasic contractions. In porcine longitudinal fundus, photostimulation of Bay K 8644-treated preparations also evokes a photorelaxation which is followed by an "off-contraction" after cessation of the photostimulus (Golenhofen et al., 1990).

While the foregoing review has focused only on the photoactivated changes in the mechanical activity in smooth muscle, it is well recognised that the cellular mechanisms of contraction and relaxation relate to the intrinsic physiological properties of smooth muscle. The next section deals with this aspect.

1.2 Smooth Muscle: structure-function relationship

Contraction of smooth muscle cells is thought to occur, as in skeletal muscle, through the interaction of myosin and actin filaments (Somlyo et al., 1976; Somlyo, 1980). Although not arranged in sarcomeres as in skeletal muscle, at least three distinct types of contractile filaments have been identified in smooth muscle. These are the thick filaments (15 nm in cross-sectional diameter), containing polymerized myosin monomers; the thin filaments (6-8 nm), composed mainly of actin and the intermediate filaments (10 nm), made up of desmin or vimentin. Another major component of the contractile apparatus in smooth muscle, called dense bodies, operate as attachment sites for actin filaments, analogous to the Z-line in striated muscles.

Myosin is composed of two high molecular weight (230 kDa) heavy chains and four low molecular weight (two 20 kDa and two 16 kDa or LC_{20} and LC_{16}, respectively) light chains (Craig et al., 1983; Yanagisawa et al., 1987; Messer & Kendric-Jones, 1988). The LC_{20} is the substrate for a specific calcium-dependent kinase named myosin light chain kinase (MLCK). MLCK is a high molecular weight protein ($\approx$105 kDa in chicken gizzard) that is activated after binding to the calcium-dependent protein calmodulin. Proteolysis of the purified enzyme has demonstrated that MLCK is composed of two domains: an active domain which binds to myosin, and a pseudosubstrate domain,

containing a calmodulin-binding site (Foyt et al., 1985; Walsh, 1985a). Under resting conditions, binding of the pseudosubstrate domain to the active site prevents myosin activation whereas calmodulin interaction with the pseudosubstrate domain releases this autoinhibition of the active site, allowing its activation of the myosin filaments.

Excitation-contraction coupling in smooth muscle involves the following sequence of events: 1) activation of $[Ca^{++}]_i$ by receptor-mediated events, 2) activation of the calcium-binding protein calmodulin as the result of $[Ca^{++}]_i$ increase; 3) stimulation of MLCK by the Ca^{+}-calmodulin complex (Lucas et al., 1986); 4) MLCK phosphorylation of the Ser 19 residue in the LC_{20} of the myosin head, exposing a binding site for actin (Jakes et al., 1976; Adelstein & Eisenber, 1980; Walsh, 1985b), 5) activation of myosin ATPase resulting in the generation of force through cycling of myosin cross-bridges with the actin filaments, and 6) activation of several phosphatases which dephosphorylate the LC_{20}, causing relaxation of smooth muscle (Morgan et al., 1976; Pato & Adelstein, 1981).

1.2.1 Regulation of contraction in smooth muscle

Although the details of molecular events mediating smooth muscle contraction have not been fully resolved, there is little doubt that calcium ions act as the second messenger regulating smooth muscle tension development.

Increases in intracellular calcium may occur as the result of receptor stimulation or/and membrane depolarization. Unlike skeletal muscle, where contraction is mainly supported by release of intracellular calcium, smooth muscle depends on extracellular calcium as well to maintain tension (Bolton, 1979; Daniel et al., 1979). Thus, it has been proposed that calcium entry in smooth muscle may occur through receptor-gated channels, activated by agonist receptor occupancy (Bolton, 1979), or through voltage-gated channels, activated by membrane depolarization (Triggle, 1972).

Another possible source of calcium is the sarcoplasmic reticulum (SR). Release of intracellular calcium from the SR may occur through the inositol triphosphate (IP_3)-activated channel and the caffeine modulated calcium-induced calcium release mechanism (Somlyo et al., 1985; Baron et al., 1989; Ilno, 1990). A variety of extracellular signals (neurotransmitters, hormones, etc.) interact with specific receptors located in the plasmalemma that are coupled via a GTP-binding protein to the enzyme phospholipase C. Activation of phospholipase C results in the hydrolysis of the membrane lipid,

phosphatidylinositol 4,5-bisphosphate, generating two second messengers: IP_3 and 1,2-diacylglycerol. The latter activates protein kinase C, whereas IP_3 diffuses into the cytoplasm and interacts with the IP_3-activated channel in the SR, allowing release of calcium into the myoplasm, down its concentration gradient. The calcium-induced calcium release mechanism allows the release of calcium ions from the SR as the result of an overall increase in myoplasmic calcium concentration.

The different time course of calcium entry from the extracellular space and calcium release from the SR is responsible for the biphasic components of the agonist-induced contraction (Bond et al., 1984; Daniel et al., 1979). This response contains an initial phasic contraction followed by a long lasting tonic contraction. The first phase is believed to involve SR calcium release by IP_3, whereas the tonic contraction is mediated by calcium entry from the extracellular space. Contraction induced by high K^+ entails mostly a tonic phase mediated by calcium entry through voltage-gated channels.

Based on this simplified scheme, it has been proposed that smooth muscle possesses two types of excitation-contraction coupling: electromechanical and pharmacomechanical coupling (Somlyo & Somlyo, 1968). The former is mediated by a change in resting membrane potential leading to Ca^{++} influx through voltage-gated channels. The latter involves: 1) Ca^{++} influx

through receptor-gated channels; 2) SR Ca^{++} release through IP_3-activated channel; and 3) regulation of kinase/phosphatase activity (see below).

Several lines of evidence, however, suggest that cytoplasmic $[Ca^{++}]$ and force generation are not rigidly coupled and that Ca^{++}-sensitivity of the contractile apparatus can be modified by physiological mechanisms. The tension/Ca^{++} relationship is higher during agonist than during high K^+-induced contractions (Bradley & Morgan, 1987; Himpens & Casteels, 1987; Rembold & Murphy, 1988). In staphylococcus α-toxin permeabilized smooth muscles, which still retaining their agonist-coupled responses, an increase in calcium sensitivity could be demonstrated by receptor stimulation (Kitazawa et al., 1989; Nishimura et al., 1988). Moreover, simultaneous measurements of tension and $[Ca^{++}]_i$ have shown that during continuous stimulation, although α-adrenergic agonist induced force is maintained at high constant levels, calcium concentration may decline to basal levels (Morgan & Morgan, 1982). In intact smooth muscle, it has been demonstrated that levels of both MLCK phosphorylation and shortening velocity decrease to control levels during the phase of force maintenance (Dillon et al., 1981). In order to account for these phenomena, a high calcium sensitivity state, the latch state, has been proposed to be involved in the development of force with significant decrease in calcium concentration and phosphorylation (Dillon et al., 1981). The latch bridge is a

dephosphorylated cross-bridge. Although both types of coupling are proposed to generate the same amount of force, the latch bridge has a fivefold slower detachment rate.

Several other studies have indicated the existence of regulatory mechanisms other than myosin phosphorylation that may affect the contractile tonus of smooth muscle but do not involve the formation of latch-bridges. Convincing evidence for this has emerged from studies with smooth muscle preparations permeabilized with staphylococcus α-toxin, indicating that a GTP-binding protein may be implicated in increasing the Ca^{++} sensitivity of the contractile proteins to various agonists (Kitazawa & Somlyo, 1991). Protein kinase C has also been implicated in the regulation of smooth muscle contraction. Thus, the tumour-promoting phorbol esters can induce slowly developing, sustained contractions. Moreover, gizzard myosin is a substrate of protein kinase C in vitro, with phosphorylation occurring at several sites (S1, S2, T9) of the LC$_{20}$ (Nishimura et al., 1990). However, it is unclear if the protein kinase C- induced tonus is the result of a direct effect on the contractile proteins or an increase in Ca^{++} influx through phosphorylation of voltage-gated channels (Yabu et al., 1992). Regulation of the phosphorylation/ dephosphorylation rate of MLCK through changes in the kinase/phosphatase activity may also play an important role in tonus generation in smooth muscles. Recent evidence demon-

strates that the phosphatase inhibitors, okadaic acid and calyculin A, induce tonic contractions in rabbit aorta and guinea-pig taenia caeci (Shibata et al., 1982; Hartshorne et al., 1989). Such contractions were independent of extracellular calcium and did not involve calcium mobilization from internal stores (Hartshorne et al., 1989).

Several investigators have also suggested that large increases in $[Ca^{++}]_i$ per se may decrease the sensitivity of the contractile apparatus to calcium (Stull et al., 1990; Kitazawa & Somlyo, 1990). The molecular mechanism of calcium-induced desensitization may be explained by phosphorylation of MLCK. In vitro MLCK activity is a function of both calcium and calmodulin concentration. However, increase in $[Ca^{++}]_i$ may activate a Ca^{++}-calmodulin-dependent protein kinase II which phosphorylates MLCK (Stull et al., 1990). Phosphorylated MLCK has a lower Ca^{++}-sensitivity than the dephosphorylated enzyme. This hypothesis may partially explain the phasic response of some smooth muscle (Kitazawa & Somlyo, 1990). Thus, exposure to high $[Ca^{++}]_i$ induces a transient contraction in staphylococcus α-toxin skinned smooth muscle. However, force declines to a significantly lower level despite a maintained high $[Ca^{++}]_i$, suggesting a time-dependent decrease in calcium-sensitivity of contraction.

Biochemical experiments also suggest the existence of contractile-filament linked regulatory mechanisms in smooth muscle. Although smooth

muscle lacks troponin, two other proteins called caldesmon and calponin may

be involved in the regulation of actin-myosin interaction in smooth muscle

(Sobue et al., 1981; Takahashi et al., 1986).

1.2.1.1 Voltage-gated channels.

As mentioned above, voltage-gated channels play a very important role in calcium-entry during force maintenance in smooth muscle. Electrophysiological studies indicate that there are at least two subtypes of voltage-gated channels in smooth muscles (Sturek & Hermsmeyer, 1986; Benham et al., 1987): i) L-type Ca^{++}-channels, characterized by activation at depolarized membrane potential and large conductance. Upon repolarization, L-type Ca^{++}-channels inactivate slowly; ii) T-type Ca^{++}-channels are activated at more polarized membrane potentials and have low conductance. Since the inactivation kinetics are very fast, T-type Ca^{++}-channels are characterized by a transient current.

The N-type Ca^{++}-channels appears to be confined only to neurons where it probably regulates neurotransmitter release (Miller, 1987).

Molecular analysis has demonstrated that the L-type Ca^{++}-channels is a complex of five protein subunits (α_1, α_2, ß, т, σ)(Glossmann & Ferry, 1983; Curtis & Catterall, 1984; Borsotto et al., 1985). The α_1-subunit forms the transmembrane channel pore and also contains the binding site for the three main ligands regulating channel activity: 1,4-dihydropyridines, benzothiazepines and phenylalkylamines.

The DHPs are modulators of the L-type Ca^{++}-channels, acting either as

activators (agonists) or inhibitors. The same binding site in the α_1 subunit seems to accommodate both types of ligands. DHP agonists and antagonists interact with a high enantiomeric specificity with the binding site (Franckowiak et al., 1985; Triggle et al., 1989). Patch clamp recordings have revealed that L-type Ca^{++}-channels exhibit three different gating states: closed, open and inactivated (Hess et al, 1984; Kokubun & Reuter, 1984). DHP agonists bind to the open state, whereas antagonists bind preferentially to the inactivated state (Bean, 1984).

The α_1 and the ß subunits of the L-type Ca^{+1}-channels contain at least one phosphorylation site (Curtis & Catterall, 1985; Hosey et al., 1986). Several observations suggest that phosphorylation of L-type Ca^{+1}-channels by second messengers regulates channel activity. Thus, calcium current increases as the result of phosphorylation by a calmodulin-dependent protein kinase II in stomach smooth muscle from the toad Bufo marinus (McCarron et al., 1992). Cyclic AMP also appears to stimulate L-type Ca^{++}-channels in cardiac myocytes and guinea pig ventricular cells (Kameyama et al., 1986). However, in rat ileum smooth muscle cAMP does not produce any effect in channel activity (Yabu et al., 1992). Modulation of L-type Ca^{++}-channels has also been associated with activation of a pertussis toxin-sensitive G-protein (Yatani et al., 1987).

1.2.2 Relaxation of smooth muscle

Relaxation of pre-contracted smooth muscle could be achieved either through a reduction in the free cytoplasmic calcium or by decreasing the rate of phosphorylation of the contractile proteins. $[Ca^{++}]_i$ can be reduced via (1) inhibition of calcium influx from the extracellular space and of calcium release from the SR, or (2) by enhancing the activity of mechanisms for Ca^{++} removal from the myoplasm. The latter may involve calcium-sequestration into the SR by a Ca^{++}-ATPase or extrusion into the extracellular space (Casteels et al., 1986) by the plasmalemmal Ca^{++}-ATPase and the Na^+/Ca^{++} exchange. Both the SR and the plasmalemmal calcium pump use ATP hydrolysis to transport calcium against its concentration gradient. However, the SR Ca^{++}-ATPase differs from the plasmalemmal pump in several properties, e.g. the SR pump is regulated by phospholamban, where the plasmalemmal pump is regulated by calmodulin (Raeymaekers & Jones, 1986).

Drug-induced relaxation of smooth muscle may occur through ß-adrenoceptor activation or nitric oxide release, leading to the stimulation of specific cAMP- and cGMP-dependent protein kinases, respectively.

1.2.2.1 ß-adrenoceptor stimulation and cAMP

Activation of the enzyme adenylate cyclase either by agonist binding to ß-adrenoceptors or by action of vasodilator prostaglandins induces relaxation of airway, gastrointestinal and other smooth muscle (reviewed by Bülbring & Tomita, 1987). Adenylate cyclase catalyses the production of cAMP from ATP. Increase in the intracellular level of cAMP stimulates a specific protein kinase, PKA (review by Langan, 1973).

Two potential sites for PKA action have been the focus of most studies: effects on intracellular Ca^{++} metabolism and phosphorylation of MLCK. Thus, the cAMP-mediated effects on intracellular calcium concentration, including stimulation of Ca^{++} efflux (Moore & Fay, 1993) and sequestration into the SR (Casteels & Raeymaekers, 1979; Mueller & van Breemen, 1979), have been demonstrated in several smooth muscles. Increase in Ca^{++} efflux could be mediated by activation of the Na^+/K^+-ATPase (Moore & Fay, 1993). The increase in the transmembrane Na^+-gradient will stimulate the Na^+/Ca^{++} exchange in the Ca^+-extrusion mode, therefore diminishing $[Ca^{++}]_i$ induced by excitatory stimuli.

It has also been demonstrated that PKA phosphorylates MLCK from gizzard smooth muscle at two sites (S815 and S828) (Conti & Adelstein, 1981; Payne et al., 1981). In the absence of bound calmodulin both sites are

phosphorylated; however, with calmodulin bound to MLCK, only the S828 site is phosphorylated. Phosphorylation of the S828 site alone has no effect on the kinase, but phosphorylation of both sites results in a decrease in the affinity of MLCK for calmodulin (Conti & Adelstein, 1981). Whether this mechanism is important for smooth muscle relaxation in vivo is not completely clear. However, in skinned guinea pig taenia coli and trachea, its significance has been demonstrated (Miesheri & Rüegg, 1983; Sparrow et al., 1984; Nishimura & van Breemen, 1989).

1.2.2.2 Nitric oxide and cGMP

In 1980 Furchgott & Zawadzki demonstrated that stimulation of intact vascular smooth muscle by acetylcholine lead to the release of EDRF. Further investigations determined that this factor is NO (Palmer et al., 1987; Ignarro et al., 1988). NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) with the formation of citrulline as a co-product (Moncada et al., 1989; Palmer & Moncada, 1989). Several cofactors such as NADPH, FMM, FAD and tetrahydrobiopterin are required for the catalytic activity of NOS. The NO-mediated mechanism of smooth muscle relaxation involves activation of sGC after binding to the haem iron of the enzyme (Ignarro, 1991), followed by an increase in intracellular cGMP. The same final pathway is involved in smooth muscle relaxation by nitrovasodilators and in endothelium-dependent relaxation stimulated by substance P, the calcium ionophore A23187 and bradykinin (Murad et al., 1978; Ignarro et al., 1987; Khan & Furchgott, 1987; Kelm & Schrader, 1988).

At least three different NOS have been described. These differ by their tissue localization and Ca^{++}-dependence. Thus, the activity of two constitutive NOS, i.e., the 135 kD endothelial NOS (Lamas et al., 1992) and the 168 kD neuronal NOS (Bredt et al., 1991), is stimulated by an increase in intracellular calcium. Conversely, an inducible, Ca^{++}-independent NOS is expressed in

macrophages following immunological stimuli (Lyons et al., 1992). This enzyme is believed to mediate the cytotoxic activity of NO in macrophages. The rate of NO synthesis from this enzyme is much higher than in endothelial and brain NOS. Moreover, the inducible NOS is bound to a calmodulin-regulatory sequence (Cho et al., 1992). This eliminates the requirement for calcium to stimulate the activity of the inducible NOS.

Degradation of cyclic nucleotides in vascular and non-vascular smooth muscle is mediated by at least five phosphodiesterase (PDE) isozymes, each encoded by a distinct gene or gene family (Torphy & Cielinski, 1990; Torphy et. al., 1993; Saeki & Saito, 1993). However, cGMP degradation may only involve two types known as cGMP-specific PDE (PDE V) and calmodulin-dependent PDE (PDE I). Although zaprinast inhibits both enzymes, the highest selectivity is shown toward PDE V (Saeki & Saito, 1993).

Although the mechanism of cGMP-mediated relaxation is not fully understood, several sites have been proposed as possible target for protein kinase G (PKG). It has been demonstrated that PKG activates Ca^{++}-ATPase activity in crude preparations. This result has been supported by studies demonstrating that PKG catalyses the phosphorylation of phospholamban, a regulator of SR Ca^{++}-ATPase (Raeymaekers et al., 1988; Twort & van Breemen, 1988). Moreover, agents which increase cGMP may induce relaxation of

vascular smooth muscle by activating K^+-channels which results in membrane repolarization and a decrease in calcium influx through voltage-gated channels (Nelson et al., 1990; Thornbury et al., 1991). Phosphorylation by PKG activates Ca^{++}-activated K^+-channels inducing hyperpolarization and relaxation of coronary artery (Taniguchi et al., 1993).

No direct effect of cGMP on channel regulation similar to that mediating phototransduction in retinal cells, has been found in smooth (reviewed by Kaupp & Koch, 1992). The role of phosphorylation by PKG on contractile tonus is presently unclear despite reports showing modulation of the calcium sensitivity of the contractile proteins by PKG (Pfitzer et al., 1982; Nishimura & van Breemen, 1989).

1.3 Objectives and rationales of this study

The aims of this study were the following:

1a) **to characterize and compare the DHP-photoactivated response in tonic and phasic smooth muscles devoid of any intrinsic responsiveness to light, and b) to contrast these responses with an endogenously mediated one.**

Several studies have demonstrated that the light-induced responses in vascular and non-vascular smooth muscle differ in their components. Whereas in vascular smooth muscle, DHPs or NO donors augment the intrinsic photorelaxation (Golenhofen et al., 1990; Triggle & Bieger, 1990; Chen & Gillis, 1992), the DHP- or NO donor-photoactivated response in rabbit stomach and porcine fundus consists of a relaxation followed by an "off-contraction" (Ehrreich & Furchgott, 1968; Golenhofen et al., 1990). On the contrary, phasic smooth muscle (porcine circular antrum) and smooth muscle with mixed tonic and phasic contractions (guinea-pig uterus) are insensitive to photostimulation even following treatment with $3'-NO_2$-DHP or NO-donors (Golenhofen et al., 1990).

In this study, the vascular and non-vascular smooth muscle preparations chosen to investigate the DHP-photoactivated response were the thoracic aorta and TMM, respectively. Furthermore, the TMM and pyloric sphincter were used to compare the DHP-photoactivated response in tonic and phasic smooth muscles, respectively.

2) **to determine whether the 3'-NO$_2$-DHP-photoactivated response is mediated by release of NO from the DHP molecule.**

Photoactivated release of NO from the DHP molecule has been implicated as the initial step in photorelaxation of smooth muscle. Thus, DHP-photoactivated relaxation in smooth muscle seems to involve only nitro-substituted DHP (Golenhofen et al., 1990, Triggle et al., 1990) and DHP photosensitization could be mimicked by photodegradable NO-donors (Golenhofen et al., 1990). Photoactivated release of NO has been demonstrated following irradiation of a 3'-NO$_2$-DHP compound (Bauer & Fung, 1993).

If photoactivated release of NO accounts for the 3'-NO$_2$-DHP-photoactivated response in the TMM, the following predictions can be made:

(i) established NO-donors should mimic the 3'-NO$_2$-DHP-photoactivated response;

(ii) the photoactivated response should be attenuated by the NO-inactivating agents LY 83583, haemoglobin and carboxy-PTIO;

(iii) the photosensitizing efficacy of a 3'-NO$_2$-DHP solution should be altered by prior polychromatic light irradiation of the solution.

3) to determine the effector mechanism(s) involved in the $3'$-NO_2-DHP-photoactivated response.

Since DHP-augmented photorelaxation in rat aorta are accompanied by an increase in cGMP production (Triggle at al., 1991, Baik et al., 1994), it has been proposed that activation of sGC following photoactivated release of NO, may lead to photorelaxation. If activation of sGC were to mediate the $3'$-NO_2-DHP-photoactivated response in the TMM, the selective cGMP-PDE inhibitor, zaprinast should enhance any NO-mediated effect occurring via stimulation of sGC.

Previous work in rat thoracic aorta has shown that the $3'$-NO_2-DHP-augmented photorelaxation does not require the presence of extracellular calcium (Triggle & Bieger, 1990) and it occurs independently of the effect of DHPs on the L-type Ca^{++}-channels (Golenhofen et al., 1990; Triggle et al., 1991). If the $3'$-NO_2-DHP-photoactivated response were dissociated from extracellular calcium, the following predictions could be made:

(i) the photoactivated response should be indistinguishable whether it is produced by photosensitizing DHPs with agonist or antagonist effects at the L-type Ca^{++}-channel;

(ii) chelation of extracellular calcium, inhibition of L-type Ca^{++}-channel with non-photosensitizing DHPs or skinning of the plasmalemma should not interfere with the photosensitizing activity of DHPs.

Microsomal Ca^{++}-binding has also been implicated as a possible mechanism for

photorelaxation (McGonigle & Tallarida, 1980). If the SR were to mediate the $3'$-NO_2-

DHP-photoactivated response, agents which interfere with the storage/release capacity

of the SR, such as cyclopiazonic acid and ryanodine, respectively, should modify the

photoactivated response.

CHAPTER TWO

METHODS AND MATERIALS

2.1 Animals

Male Sprague-Dawley rats, 150-300g, were purchased from Charles River Inc., Montreal, Quebec or from the vivarium at Memorial University of Newfoundland. The animals were housed in the animal quarters of the Faculty of Medicine, Memorial University of Newfoundland. Rats were kept on a 12 h light/dark schedule with controlled humidity and temperature. The animals had access to food and water ad libitum. Procedures performed on the animals were approved by the Animal Care Committee at Memorial University of Newfoundland in accordance with the guidelines of the Canadian Association on Animal Care.

2.2 Preparation of tissues for in vitro mechanical study

2.2.1 Isolated TMM

Rats were anaesthetized with urethane (1.2 g/kg, i.p.). The thorax was exposed with a midline incision, the rib cage cut open and the oesophagus dissected out from the pharynx to the stomach. A metal rod was slipped through the lumen and the oesophagus placed in a Sylgard (Dow Corning Corp., USA)-coated petri dish containing Tyrode's solution aerated with 95%O_2 /5%CO_2. Care was taken to maintain the length of the oesophagus during dissection equivalent to its original length. Dissection was carried out under white incandescent light (0.6 mW/cm^2). The tunica muscularis propria was split

lengthwise and dissected away leaving the smooth muscle tube, viz. the TMM. The TMM was divided into three or four segments. The segments corresponded to proximal (cervical), middle (supradiaphragmatic) and distal (infradiaphragmatic) portions, each 1-2 cm in length. Each segment was secured at one end via a loop of silk thread (5-0 silk, Ethicon) to a tissue holder. The other end was tied with the silk thread to a Grass FT03C force transducer. The preparation was bathed with Tyrode's solution in a 10 ml water-jacketed organ bath. Preparations were aerated with $95\%O_2$ /$5\%CO_2$ and maintained at $37°C$.

2.2.2 Isolated thoracic aorta

The rat thoracic aorta was excised and placed in a Sylgard-coated petri dish containing Tyrode's solution aerated with $95\%O_2$ /$5\%CO_2$. The vessel was cleaned of adherent fat and connective tissue and cut into rings of approximately 3-5 mm in length. Each ring was mounted between two triangular steel wires. One of the wires was tied to a tissue holder whereas the other wire was connected to the force tranducer. Each segment was maintained in water-jacketed organ bath containing Tyrode's buffer at $37°C$ and aerated with $95\%O_2$ /$5\%CO_2$. The endothelium was removed by saponin (100 $\mu g/ml$)-treatment for 5 minutes in Tyrode's buffer at $37°C$ (Gräser et al., 1988). After washing out saponin from the bath with fresh buffer, the absence of acetylcholine (1 μM)-induced relaxation was used to confirm endothelium removal.

2.2.3 Isolated pyloric sphincter

The pyloric sphincter was excised and placed in a Sylgard-coated petri dish containing Tyrode's solution aerated with 95%O_2 /5%CO_2. The pyloric sphincter was cleaned of adherent fat and connective tissue. To separate the inner circular layer of smooth muscle from the outer longitudinal layer, the pyloric sphincter was inverted. The longitudinal layer was removed and one or two strips were prepared from the circular layer of smooth muscle. Each strip was secured at one end via a loop of silk thread (5-0 silk, Ethicon) to a tissue holder. The other end was tied with the silk thread to a Grass FT03C force transducer. The preparation was bathed with Tyrode's solution at 37 °C in a 10 ml water-jacketed organ bath.

2.3 Mechanical recording

Isometric longitudinal tension was recorded via a Grass FT03C transducer on a Grass 7D polygraph. The transducers were mounted on adjustable clamps permitting fine adjustment of resting muscle tension without overstretching the tissues. TMM and pyloric sphincter preparations were set up at a preload of 0.3 g whereas aortic rings were set at 1 g preload.

To register and analyze fast changes in tension, force transducers were connected with a Digi-Med tension force analyzer (model 200), kindly supplied by Micro-Med (Louisville, KY). Data were stored as digital information using a PC 486DX2/50. Traces were analyzed using the software Acqknowledge for

Windows (Biopac System Inc.) or imported into SigmaPlot (Jandel Scientific) for graphic reproduction.

2.4 Experimental protocol

Tissues were allowed to equilibrate in Tyrode's solution for approximately 30 minutes. Muscle tissues were stimulated to generate active tonus by means of the following protocols: i) incubation with the muscarinic cholinoceptor agonist cis-dioxolane (CD) at the EC_{80-90} (0.1 μM or 0.3 μM) (Akbaraly, 1987) in Tyrode's solution; ii) incubation in 110 mM K^+-depolarizing solution containing a known calcium concentration; iii) incubation in 110 mM K^+, nominally calcium free solution, containing 0.3 μM CD. After a steady-state tonus was reached, photosensitization of smooth muscle was induced with DHP or NO-donors. Concentration-response curves for these compounds were obtained by cumulative additions to the organ bath. When drugs were examined for their ability to inhibit light-induced responses in TMM, precontracted preparations were first photosensitized with a fixed concentration of DHP or NO-donor and then, exposed to test concentrations of the drug. If tonus dropped as a result, it was titrated back to its original level with additional spasmogen. The relaxant effect of isoprenaline (INA, 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.

Tetrodotoxin (TTX)-sensitive, field stimulation-induced relaxations of

TMM were obtained in Tyrode's medium. Preparations were pre-treated with 1 nM methscopolamine, a muscarinic acetylcholine receptor antagonist lacking subtype selectivity . This concentration was sufficient to block field stimulation-induced cholinergic contractions followed by contraction with 1 μM CD. TTX-insensitive relaxations were obtained in TMM preparations treated with 0.5 μM TTX and precontracted with 1 μM CD.

The intrinsic or DHP-augmented photorelaxation in thoracic aorta was revealed using phenylephrine (EC_{80-90} = 0.1 μM)-precontracted preparations. The DHP-photoactivated response in pyloric sphincter was studied in CD (1 μM)-treated preparations. Activation of the muscarinic acetylcholine receptors induced phasic contractions in pyloric sphincter.

2.5 Skinning of muscle preparations

Skinning of the TMM was carried out with ß-escin (50 μM) for 20-30 minutes (Kobayashi et al., 1989) in the presence of a skinning solution (see section 2.7) at 22 °C and pH 7.0 ($pCa^{++} < 8$). Contraction was induced by increasing the Ca^{++}:ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) ratio, keeping the total EGTA concentration constant at 0.1 mM. The apparent binding constant of the Ca^{++}-EGTA complex was considered to be 2.52 x 10^6 M^{-1} at 22°C and pH 7.0, as calculated by the ancillary computer program STACONS (Fabiato & Fabiato, 1979). The concentrations of free calcium shown in the results were calculated by the

computer program ALEX, the updated version of SPECS (Fabiato, 1988). The software STACONS and SPECS were kindly supplied by Dr. Fabiato (Medical College of Virginia, USA).

2.6 SR calcium-depletion

Calcium-depletion of the SR was achieved by repetitive stimulation of the TMM preparation with CD in 110 mM K^+ nominally calcium-free solution containing 30 μM of the calcium chelator BAPTA. To prevent refilling of the SR after its depletion, preparations were subsequently incubated with the SR Ca^{++}-ATPase inhibitor cyclopiazonic acid (3-5 μM) (Seidler et al., 1989).

2.7 Buffers

The composition of the Tyrode's solution was (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. Prior to use the buffer was aerated with 95% O_2 + 5% CO_2. The 110 mM K^+-depolarizing, nominally Ca^{++}-free 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. The resulting isotonic solution was aerated with 95% O_2 + 5% CO_2.

The skinning solution had the following composition (in mM): KCl 126, NaCl 7, $MgCl_2$ 5, ATP 2.5, 3-[N-morpholino]propanesulfonic acid (MOPS) 10, EGTA 1.0.

2.8 Photostimultion

Photostimulation was carried out by means of a timer-controlled polychromatic halogen light source (EJL, 200W, General Electric) the intensity of which could be varied. Photostimuli (0.9 W/cm^2) were delivered for 10 s at intervals of 2-5 minutes through a fibre-optic light guide positioned 10 mm from the preparation. The change in the bath temperature as a result of 0.9 W/cm^2-irradiation was ≤ 0.5 °C. Intensity was determined by means of a radiometer (IL 1700, Ealing) and measured at the tip of the light guide .

2.9 Electrical field stimulation

Electrical field stimulation of isolated TMM preparations was delivered via a Grass S88 stimulator using two concentric platinum rings spaced 5 mm apart and surrounding the preparation. Rectangular pulses of 40V and 10 s duration were applied every 1.5-2 min. Pulse width and frequency were varied depending on the type of response studied.

2.10 Drugs and chemicals

The drugs used in this study and their sources were:

Anachemia Chemicals (Canada): sodium nitrite

BDH Chemicals (U.K.): methylene blue (MB)

Calbiochem(USA): carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-

oxide-1-oxyl (carboxy-PTIO), streptozotocin

Research Biochemicals International (USA): (+) cis-dioxolane (CD), ketanserin, L-N^G-nitroarginine methyl ester and ryanodine

Sigma (USA): calcium ionophore A23187, adenosine 5'-triphosphate (disodium salt, from equine muscle), atropine, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'- tetraacetic acid (BAPTA), calyculin A, cyclopiazonic acid, diethyldithiocarbamate, ß-escin, ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), guanethidine, bovine haemoglobin (Hb), indomethacin, isoprenaline, methscopolamine , nifedipine, 3-[N-morpholino]propanesulfonic acid (MOPS), ouabain, potassium cyanide, potassium fluoride, phenylephrine, propanolol, sodium nitroprusside (SNP), sodium dithionate, saponin, tetrodotoxin (TTX).

The following drugs were generously donated:

Bay 10 8495, Bay 0 9507 and Bay K 9073 (Dr. D.J. Triggle, School of Pharmacy, State University of New York at Buffalo., U.S.A)

CGP 28392 (Dr. G.B. Weiss, Ciba-Geigy Corp., USA)

Felodipine (Hässle, Sweden)

LY 83583 (Lilly Research Lab., USA)

racemic Bay K 8644 and its enantiomers Bay K 5714 and 4407, nicardipine, nimodipine and nitrendipine (Miles Inc., USA)

zaprinast (M&B 22948) (Rhône-Poulenc Inc., U.K.)

(+), (-)-PN 202 791 and (+)PN 200-110 (Sandoz Inc., Switzerland)

YC 170 (Yamanouchi Pharmaceuticals, Japan)

(±)-Bay K 8644 is the racemate of Bay K 5417 and Bay K 4407.

The mixture of reduced and oxidized forms of Hb supplied by Sigma Chemical was converted to the reduced form as described by Martin et al. (1986). A 10-fold molar excess of the reducing agent sodium dithionite was added to a 0.1 mM solution of Hb and then the sodium dithionite was removed by dialysis (Spectra/Por Membranes tubing (Fisher, USA)) against 100 volumes of distilled water for 2 h at 4°C. The resulting solution was frozen in 1 ml-aliquots at -20°C.

Stock solutions of DHPs, zaprinast, LY 83583 and ryanodine were prepared in 100% ethanol; corresponding volumes of the solvent were routinely tested to rule out a vehicle effect. The maximal amount of ethanol added into the 10 ml-organ bath did not exceed 50 μM. All the remaining drugs were dissolved in water. The aqueous solution of $NaNO_2$ had a pH of 8.1.

Polychromatic light-irradiation (0.9 W/cm^2) of SNP (1 M, aqueous solution) and (+)-PN 202 791 (1 mM, ethanol-dissolved) solution was carried for 60 minutes at 37°C.

2.11 Presentation of results and statistics

The polygraph traces shown in the figures are representative of data

replicated in 4-6 preparations. Traces were digitized with a Logitech scanner for graphic reproduction. The small squares above each trace represent the 10 second-photostimulation unless indicated otherwise. The vertical bar, indicating tension in each trace, is placed such that the botton of the line is at the level of basal tonus. Statistical results are expressed as mean ± standard error of the mean (SEM), n represents the number of preparations used for each mean. Where indicated, cumulative concentration-response curves were normalised and log concentrations interpolated for each predetermined response. Curve normalization involved expressing each response as a percentage of the maximal response.

Photorelaxation was expressed as a percent of the steady state tonus or as an absolute value in tension. The effect of a drug on the photorelaxation was examined by comparing the value of photorelaxation before and after the application of the drug. When two groups were compared only once, significant differences were analyzed using the Student's t-test for paired observations. When more than two groups were compared, one way analysis of variance (Student-Newman-Keuls test) was used to test for significant differences. Differences with a $p \leq 0.05$ as determined by either test were considered significant. The statistical software Sigmastat (Jandel Scientific) was used for all calculations. Data were plotted using SigmaPlot (Jandel Scientific). Curve fitting was carried out with TableCurve (Jandel Scientific).

CHAPTER THREE

RESULTS

3.1 Photoactivated responses in the rat tunica muscularis mucosae

Freshly dissected TMM preparations did not show a measurable intrinsic response to polychromatic light irradiation, irrespective of their contractile state. Photoresponsiveness was readily induced by several DHP such as Bay K 8644 and PN 202 791. Comparison of the chemical structures of DHPs (Appendix I) tested for photosensitizing activity (Table 1) revealed that only compounds containing a 3'-nitro group on the 1,4-dihydropyridine ring (3'-NO_2-DHPs) were able to induce photorelaxation, regardless of whether they were activators or inhibitors of L-type Ca^{++}-channels. However, agonist and antagonist photosensitizers each induced a characteristic photoresponse pattern consisting of a respectively complex relaxation-contraction sequence and relaxation. The photosensitizing activity of 3'-NO_2-DHPs persisted for at least 5 h after wash out from the tissue without any apparent change in the photoactivated response.

Table 1. DHP-mediated photoactivity in oesophageal TMM at equieffective extracellular calcium concentrations which induce 500 mg steady-state tension.

DHPs	Light-induced activity (%)[1]	[Ca^{++}] (μM)[2]
Control	$\emptyset$[3]	410 ± 71
(±)-Bay K 8644	- 51.0 ± 7.8	60 ± 12
Bay K 5417	- 60.0 ± 9.5	66 ± 9
Bay K 4407	- 10.3 ± 5.0	1427 ± 58
(+)-PN 202 791	- 49.3 ± 3.0	90 ± 15
(-)-PN 202 791	- 12.0 ± 3.1	1867 ± 23
Bay 10 8495	- 34.3 ± 7.0	567 ± 27
Bay 0 9507	- 46.6 ± 13.3	203 ± 29
CGP 28392	$\emptyset$	507 ± 12
YC-170	$\emptyset$	607 ± 19
Felodipine	$\emptyset$	1633 ± 130
Nicardipine	$\emptyset$	1760 ± 44
Nimodipine	$\emptyset$	2000 ± 82
Nitrendipine	$\emptyset$	1533 ± 55
(+)-PN 200-110	$\emptyset$	1733 ± 56
4-(p-nitrophenyl)-1,4-DHP	$\emptyset$	600 ± 100
Nifedipine	+ 13.8 ± 3.0	1900 ± 53
Bay K 9073	+ 6.0 ± 0.6	53 ± 17

[1] light-induced activity expressed as percent of 500 mg-active tonus (n = 4-6). (+) represents photocontraction whereas (-) represents photorelaxation.

[2] concentration of extracellular calcium required for obtaining 500 mg of tonus in the presence of 1 μM of the DHP (except for nifedipine where 10 nM was used). Experiments were carried out in 110 mM K$^+$ buffer plus 0.3 μM CD.

[3] $\emptyset$ represents compounds devoid of any photoactivity.

3.1.1 1,4-dihydropyridine-photoactivated responses

When exposed in a K^+-depolarizing solution to the $3'$-NO_2-DHP agonist (+)-PN 202 791, CD-precontracted tissues displayed a characteristic photoactivated response consisting of an initial fast relaxation followed by an off-contraction (Fig.1A). In some preparations, a low amplitude, late relaxation occurred before the pre-existing tonus level recovered (Fig 1A, Fig 2 (inset)). A virtually identical photoactivated response was obtained with racemic Bay K 8644 and its enantiomer the L-type Ca^{++}-channel agonist Bay K 5417. The amplitude of the fast relaxation as well as the amplitude and slope of the off-contraction increased with stimulus duration, whereas the slope of the fast relaxation was constant (Fig.2). The fast relaxation reached its nadir within 2.6 ± 0.2s and then faded rapidly (escape). The escape response was characterized as the decrement of the fast relaxation while photostimulation was still applied. The escape of the fast relaxation was more evident at irradiation periods > 10s (Fig. 2, inset) and the slope did not show any irradiation time-dependence.

The "off-contraction" was seen only under two conditions: at submaximal levels of CD (<0.1 μM)-induced tonus in Tyrode's buffer or at $[Ca^{++}]$<1 mM in K^+-depolarizing medium, indicative of mechanical saturation when tissues were fully contracted. However, the "off-contraction" was evident at steady-state active tension in tissues pretreated with (+)-PN 202 791 (1 μM) bathed

Fig.1   Photoactivated response by: A) the DHP L-type Ca^{++}-channel agonist (+)-PN 202 791 (1 μM); B) its antagonist enantiomer (-)-PN 202 791 (1 μM); and C) the NO-donor streptozotocin (1 mM) in TMM preparations. In all cases, experiments were carried out at similar CD (0.3 μM)-induced tonus levels ($\approx$180 mg) in 110mM K$^+$ depolarizing buffer.
The horizontal bar above each trace represents the 10 s-irradiation period.

A (+) - PN 202 791

60 mg

B (-) - PN 202 791

15 mg

C Streptozotocin

60 mg

10 sec
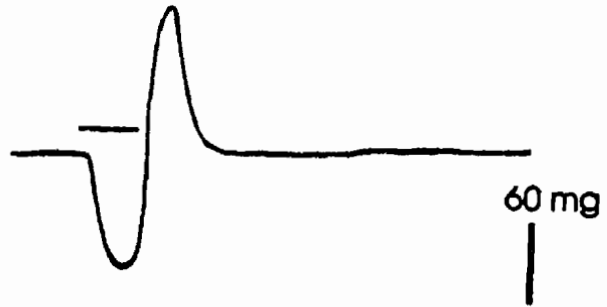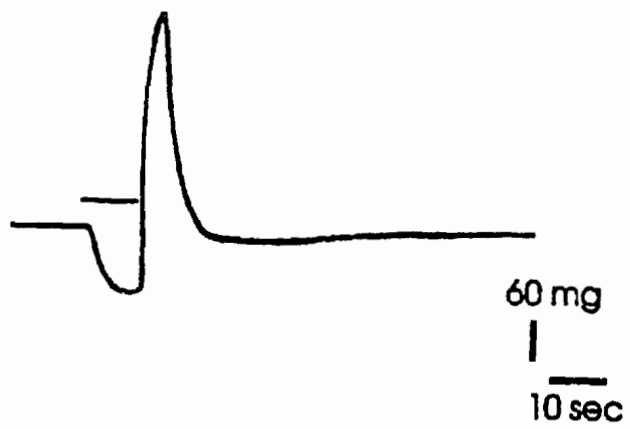
Fig.2 Irradiation time dependence of the amplitude of the relaxation (A_{fr} (%), filled circle); the amplitude of the off-contraction (A_{off} (%), open circle); the slope of the relaxation (S_{fr} (mg/s), filled square); the slope of the off-contraction after turning off the light source, (S_{off} (mg/s), open square) and the slope of the escape of the relaxation (S_e (mg/s), filled triangle) during the fast (+)-PN 202 791-photoactivated response in TMM.
Inset: (+)-PN 202 791 (1 μM)-photoactivated response after 10 s- and 30 s-irradiation period.
A_{fr} and A_{off} are expressed as percent of the amplitude of the relaxation and the "off-contraction, respectively, at 60 s-irradiation (control).
Note that the units for the ordinate are in percent or mg/s as referred to the amplitude or slope of the photoactivated responses, respectively.
Experiments were carried out in 110 mM K^+, nominally calcium-free buffer in CD (0.3 μM)-precontracted preparations (n = 4).
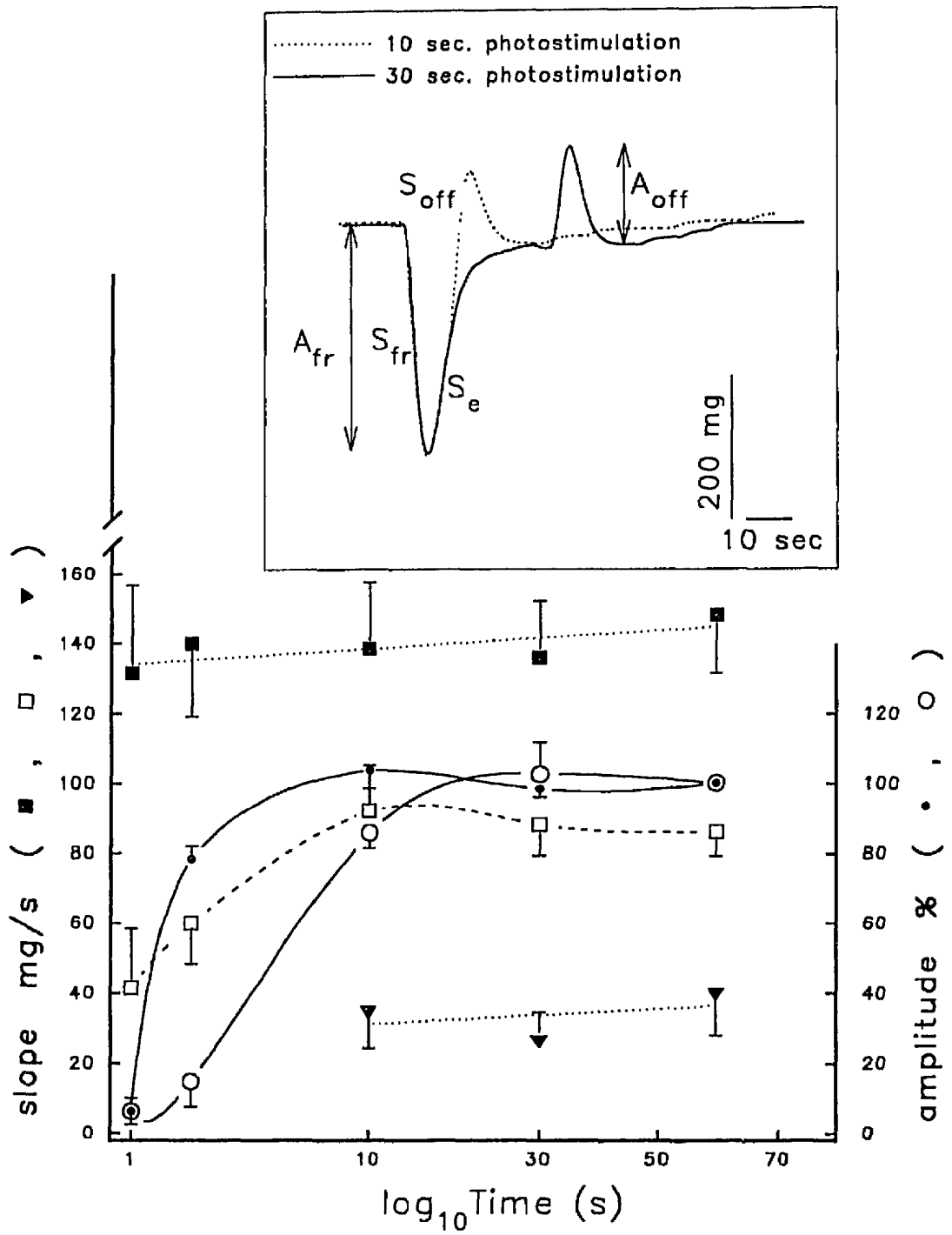
in a K^+-depolarizing, nominally calcium-free medium.

The 3'-NO_2-DHP antagonists (-)-PN 202 791 (Fig. 1B) and Bay K 4407 (not illustrated) were also effective as photosensitizers. In 110 mM K^+- depolarizing solution at matched levels of CD-induced tonus, both antagonists induced comparable photosensitization. However, the photoactivated response obtained in the presence of L-type Ca^{++}-channel antagonists differed from that to agonists in that the amplitude of the relaxation was smaller, the kinetics of the response was slower (slope of relaxation = 1.1 ± 0.4 mg/s, n = 4) and there was no off-contraction.

The correlation between the amplitude of the photorelaxation and the concentration of extracellular calcium needed to maintain a predetermined level of contractile force was estimated for both agonist and antagonist photosensitizing DHPs (Fig.3). The linear regression demonstrated that the photoactivated relaxation by the 3'-NO_2-DHP agonists, Bay K 5417 and (+)-PN 202 791, was greater and required lower extracellular calcium as compared with the photorelaxation obtained in the presence of their enantiomeric antagonist, Bay K 4407 and (-)-PN 202 791, respectively. No difference in the value of the photorelaxation was noted between racemic Bay K 8644 and the agonist enantiomer Bay K 5417. The equieffective calcium concentrations for the other DHPs are also presented in Table 1.

Fig. 3 Correlation (r = 0.81, n = 4) between equieffective extracellular calcium concentration and percent of photorelaxation of 3'-NO$_2$-DHP L-type Ca''-channel agonist and antagonist enantiomers and racemic Bay K 8644 in TMM. [Ca''] and percent of the photorelaxation were calculated at 500 mg active tension. Concentration of all the 3'-NO$_2$-DHPs shown was 1 μM.
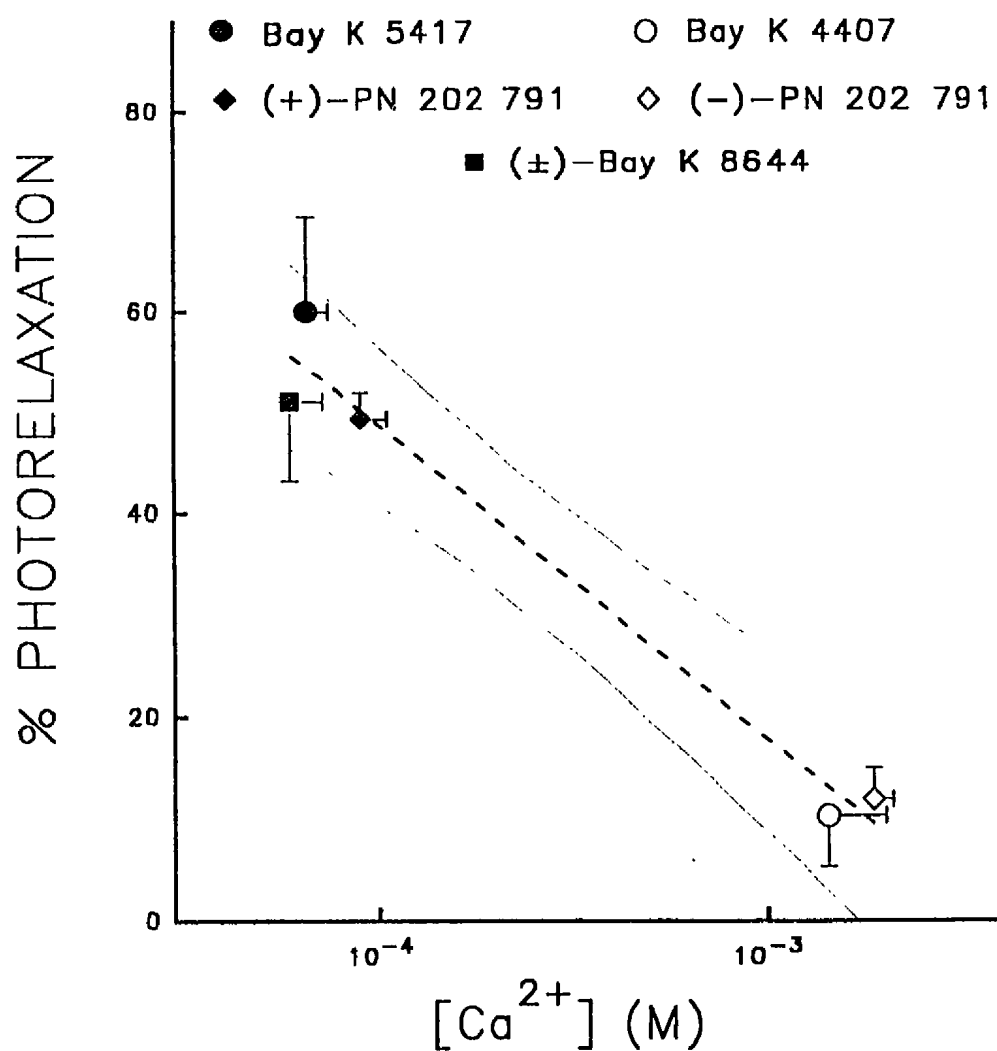
Fig.4 Effect of DHP L-type Ca^{++}-channel antagonists on the $3'-NO_2$-DHP- and NO-donor-photoactivated fast response in TMM.

A-Effect of the nonphotosensitizing DHP felodipine on the ($\pm$)-Bay K 8644 (1 μM)-photoactivated response in 110 mM K^+, nominally calcium-free solution.

B-Photoactivated response of the NO-donor sodium nitroprusside (30 μM) is attenuated by the photosensitizing $3'-NO_2$-DHP Bay K 4407 (30-300 nM) in 110 mM K^+, nominally calcium-free solution. TMM preparations were contracted with CD 0.3 μM.

A Felodipine 1 μM   30 mg

B Bay K 4407   30 nM   300 nM   20 min   150 mg   1 min
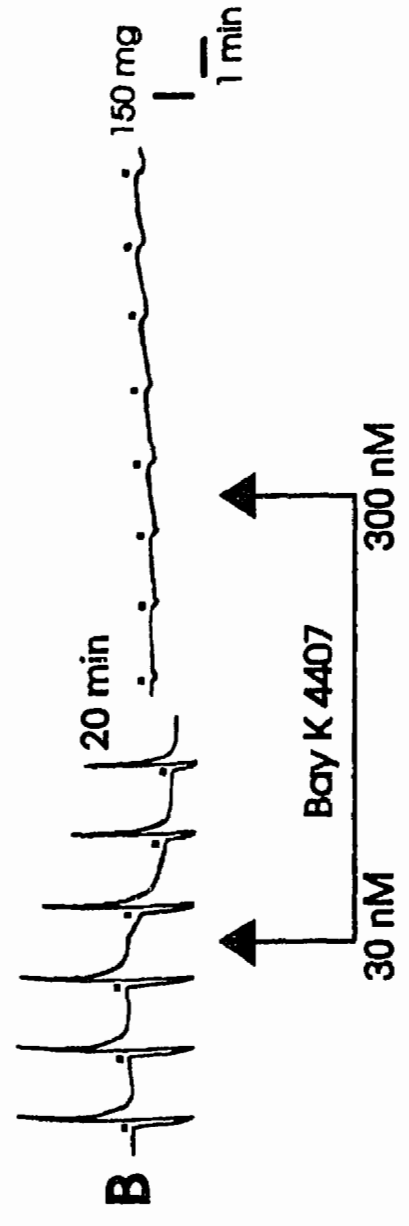
A shift in the $3'$-NO_2-DHP-photoactivated response from agonist to antagonist pattern was obtained with the DHP L-type Ca^{++}-channel antagonist felodipine (1 μM) in ($\pm$)-Bay K 8644-sensitized tissues (Fig. 4A). Tissues treated with felodipine alone remained unresponsive to light (Table 1).

The photoactivated relaxation for $3'$-NO_2-DHPs was concentration-dependent (Fig.5). Photorelaxations reached a maximum at 1 μM in the case of racemic Bay K 8644 or the agonist (+)-PN 202 791. At that concentration, photorelaxation obtained with the antagonist (-)-PN 202 791 amounted to only 40% of the active tension.

DHP compounds containing an *o-nitro* group in the phenyl ring such as nifedipine and Bay O 9073 induced a concentration-independent, long lasting photocontraction in TMM (Fig. 6A, B). The duration and the slope of the nifedipine-photoactivated contractions were 180 $\pm$ 4 s and 2.14 $\pm$ 0.5 mg/s (n = 4), respectively. Bay O 9073-photoactivated contraction was preceded by a small relaxation (Fig. 6B). As demonstrated by the Ca^{++}-sensitivity of the contraction, nifedipine had an antagonistic effect at L-type Ca^{++}-channels, whereas Bay O 9073 had the oppositive effect, i.e., an agonistic effect at the Ca^{++}-channels (Table 1). In 6 out of 12 experiments, there was a slow increase in tension after intermittent irradiation of nifedipine-treated preparations whereas in 5 out of 7 Bay O 9073-treated preparations, the tonus tended to decrease.

Fig.5 Concentration-response curves for the DHPs: (±)-Bay K 8644, (+)- and ( )-PN 202 791 and the NO-donors: streptozotocin, sodium nitroprusside (SNP) and sodium nitrite in TMM preparations bathed in Tyrode's buffer.
Photorelaxation is expressed as percent of the CD (0.3 μM)-induced tension (n = 4-8).
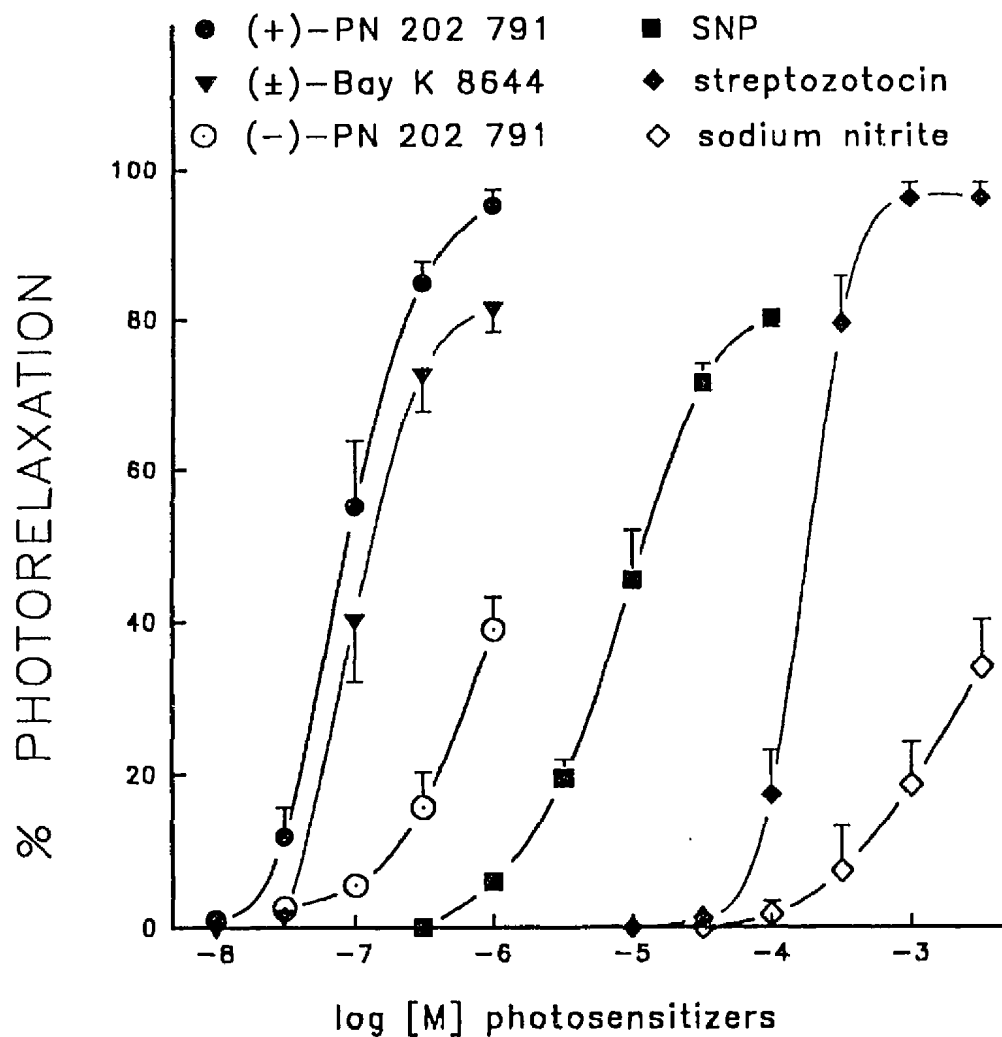
Fig.6 Photoactivated response by o-nitrophenyl-substituted DHPs in TMM.

A-Nifedipine (10 nM)-photoactivated contractions in CD-precontracted TMM. Preparations were bathed in 110 mM K$^+$, depolarizing buffer, containing 1 mM Ca^{++}.

B-Bay O 9073 (1 μM)-photoactivated response in CD-precontracted TMM. Experiments were carried out in 110 mM K$^+$, nominally Ca^{++}-free buffer.

The 10 s-irradiation period is represented by the horizontal bar below each trace.

A

75 mg

B

50 mg

1 min
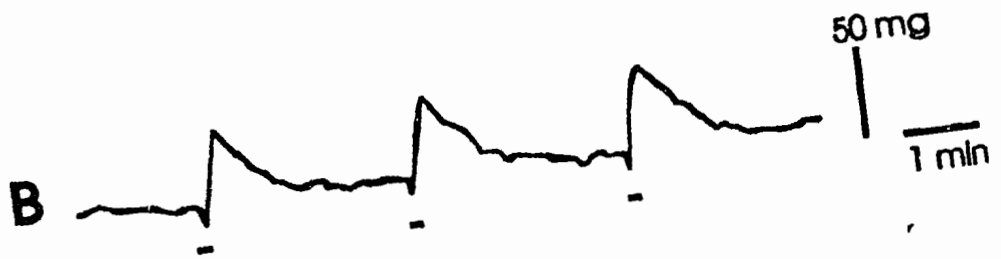
3.1.2 Comparison between the 3'-NO₂-DHP- and NO donor-photoactivated response

Precontracted TMM preparations also became responsive to photostimulation after exposure to NO-donors such as streptozotocin, SNP and sodium nitrite. The NO-donor-mediated photosensitization in rat TMM mimicked that obtained with the 3'-NO₂-DHP in displaying: (i) an initial fast relaxation, (ii) a fast "off-contraction" and (iii) a late relaxation before the pre-existing tonus level recovered (Fig. 1C). The SNP (30 μM)-photoactivated fast relaxation-contraction sequence was transformed by the photosensitizing L-type Ca' ' -channel antagonist Bay K 4407 (30-300nM) into a slow monophasic photorelaxation of small amplitude (Fig. 4B). In addition, the NO-donor-photoactivated response was concentration-dependent. However, all three NO-donors were less potent photosensitizers than 3'-NO₂-DHPs (Fig. 5) and differed in terms of efficacy. Streptozotocin was more efficacious, but less potent than SNP. Sodium nitrite was the least potent sensitizer. Relative to (+)-PN 202 791, streptozotocin had an equipotent molar ratio of 2.1 x 10³.

3.1.3 Comparison between the NO-donor-photoactivated response and the neurogenic NO-mediated relaxation

As described above, photosensitization of the TMM with $3'-NO_2$-DHP agonists and NO-donors produced an off-contraction. It thus appeared of interest to compare, in the same preparation, the NO donor-photoactivated response with the response induced by electrical stimulation of intramural NO-producing (nitroxidergic) nerves (Will et al., 1990). As shown in Fig. 7A, the field stimulation-evoked, TTX-sensitive relaxation was devoid of any post-stimulation contraction contrary to that resulting from photostimulation of SNP-pretreated TMM preparations. However, a post-stimulation contraction was obtained following the electrically evoked, TTX-insensitive relaxations (Fig 7B). The TTX-sensitive relaxation (Fig. 7A) but not the SNP-photoactivated response or the TTX-insensitive relaxation (not illustrated) was blocked by the NOS-inhibitor, L-N^G-nitroarginine methyl ester (L-NAME, 1 mM).

Fig.7 SNP-photoactivated response and field stimulation (FS)-evoked TTX-sensitive (A) and -insensitive relaxations (B) in CD (1 μM)-precontracted TMM preparations, bathed in Tyrode's buffer.

A- Preparation was pretreated with 10 nM methscopolamine to block FS-induced cholinergic contractions. Photoactivated response was induced with 50 μM SNP. Electrical field stimulation parameters: 8 Hz, 0.5 ms pulse width, 40 V, 10 s trains of stimulation.

B- Preparation was pretreated with TTX (0.5 μM) to inhibit FS-evoked nitroxidergic relaxations. Photoactivated response was induced with 30 μM SNP. Electrical field stimulation parameters: 4 Hz, 2 ms pulse width, 40 V, 10 s trains of stimulation.

Squares represent 10 s-photostimulation.

A

FS   FS   FS   FS   60 mg

5 min

L-NAME 1 mM

B

FS   60 mg

1 min
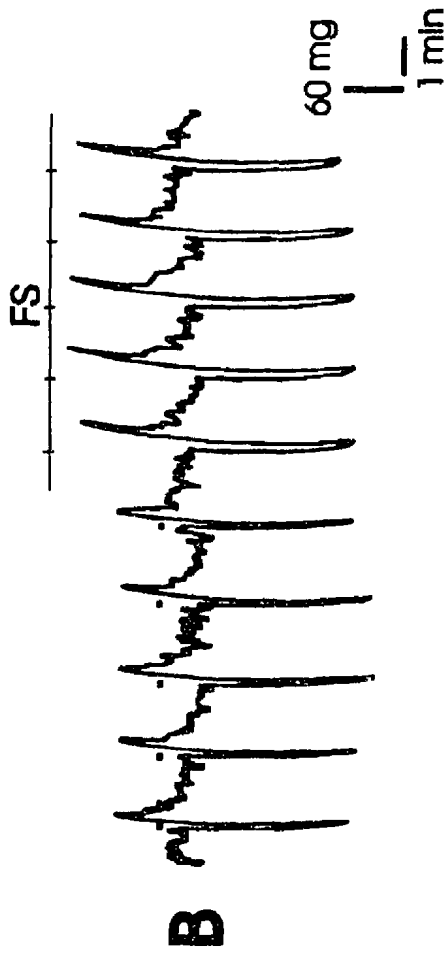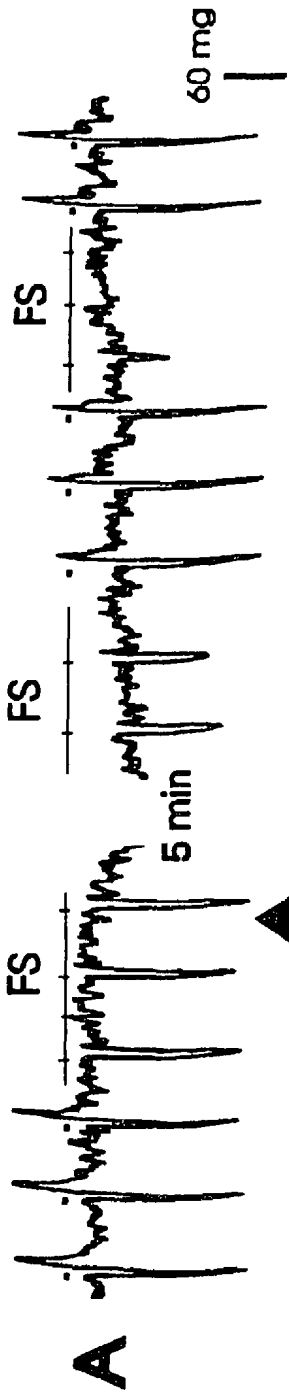
3.1.4 Effect of skinning of the plasmalemma and extracellular calcium chelation on the (±)-Bay K 8644-photoactivated response

Since regulation of calcium influx through L-type Ca^{++}-channel seemed to be involved in the $3'-NO_2$-DHP-photoactivated response in the TMM, further experiments were done to determine the effect of eliminating plasmalemmal Ca^{++} entry. First, preparations were subjected to chemical skinning with β-escin to eliminate any ionic fluxes across the membrane. Second, extracellular free calcium was removed by chelation.

Attempts at skinning of the TMM were considered successful only in 1/3 of the experiments based in the following criteria: high calcium sensitivity of the contraction and production of a tonus that was reversible either with isoprenaline or by transferring the preparations to a calcium-free medium. Data obtained from viable preparations (n = 6) revealed the depenaence of the fast photoactivated response on an intact plasmalemma. The skinning procedure employed thus resulted in the loss of the fast relaxation-contraction sequence, specifically the off-contraction, unmasking a slow photorelaxation at a calcium concentration as low as 6 μM (Fig. 8).

In preparations maintained in 110 mM K^+, nominally free calcium buffer and precontracted with CD 0.3 μM, the calcium chelator BAPTA (3 to 100 μM) inhibited the (±)-Bay K 8644-photoactivated fast response, leaving a residual slow photorelaxation (Fig. 9). In most tissues BAPTA lowered tonus (Fig. 9A),

but in some instances steady state tonus was unaffected (Fig. 9B). When tonus

was restored by raising the CD concentration, inhibition of the photoactivated

response persisted with virtually no change in the steady state tonus.

Analogous inhibitory effects on the fast photoactivated response and steady-

state active tension (n = 6) were obtained with EGTA (maximal concentration 1

mM, not illustrated) in CD-(0.3 μM)-precontracted TMM preparations bathed in

110 mM K$^+$, nominally calcium-free buffer.

Fig.8 (±)-Bay K 8644 (1 μM)-photoactivated response in ß-escin skinned TMM. Contraction was induced with calcium as indicated below the trace. Note relaxant response to isoprenaline (INA).

30 mg

1 min

INA 0.3 μM

$[Ca^{++}]_{free}$

6 μM

0.3 μM
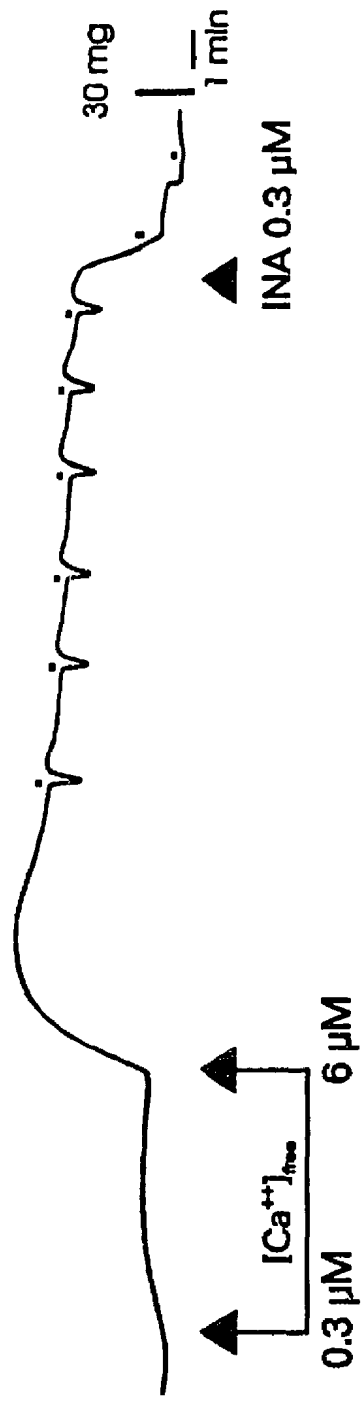
Fig.9 Effect of the calcium chelator BAPTA on the ($\pm$)-Bay K 8644 (1 μM)-photoactivated response in TMM preparations bathed in 110 mM K$^+$, nominally calcium-free buffer. TMM preparations were precontracted with 0.3 μM CD. A and B represent the variability in the contractile tonus after BAPTA treatment in two different preparations.
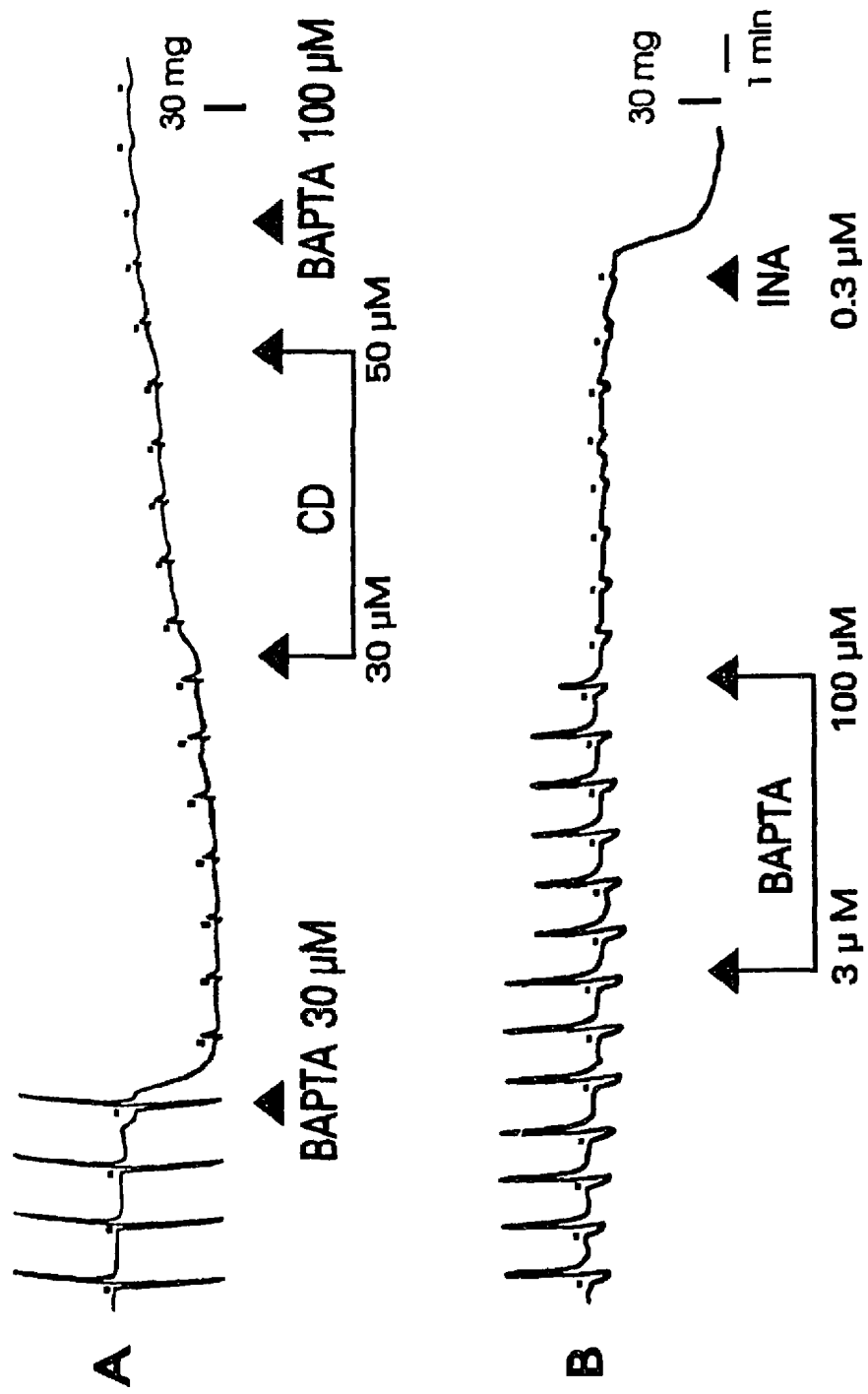
3.1.5 Effect of cyclopiazonic acid and ryanodine on the (±)-Bay K 8644-photoactivated response

The effect on the photoactivated response of the Ca^{++}-ATPase inhibitor, cyclopiazonic acid, and the modulator of calcium-induced calcium release, ryanodine, were examined in tissues precontracted with CD (0.3 μM) in Tyrode's buffer (Fig. 10 A,C) or in 110 mM K^+, nominally calcium-free buffer (Fig. 10 B,D). Under both experimental conditions, cyclopiazonic acid (10 μm) and ryanodine (30 μM) inhibited the Bay K 8644-photoactivated fast response. In Ca^{++}-containing buffer, the inhibition was of immediate onset and accompanied by a rise in tonus. In 110 mM K^+, nominally calcium-free buffer, the inhibition was delayed and coincided with a gradual reduction in CD-tonus. Inhibition of the fast photorelaxation and of the off-contraction followed a similar time course. A residual slow photorelaxation of small amplitude persisted even after incubation with higher concentrations (up to 100 μM) of cyclopiazonic acid or ryanodine.

In order to test the possibility that the cyclopiazonic acid effect on the 3'-NO_2-DHP- photoactivated response resulted from a calcium "overflow" into the myoplasm, tissue were depleted of the intracellular calcium by repetitive stimulation of the TMM preparation with CD in 110 mM K^+, nomi..ally calcium-free solution containing 30 μM of the calcium chelator BAPTA (Fig. 11A). However, in the presence of cyclopiazonic acid (3 μM), a fast (±)-Bay K 8644-

photoactivated response was obtained similar to that observed in tissues with an intact SR (Fig. 11B). In this case, a small amount of calcium (30 μM) was added for tonus generation. 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 (Fig. 11C).

Fig.10 Effect of ryanodine (RYA, 30 μM) and cyclopiazonic acid (CPA, 10 μM) on the ($\pm$)-Bay K 8644 (1μM)-photoactivated response in TMM preparations bathed in Tyrode's (A,C) or nominally calcium-free, depolarizing buffer (B,D). In all cases, tonus was induced with CD 0.3 μM.

A

150 mg

▲
RYA 30 μM

B

5 min

30 mg

▲   ▲
RYA 30 μM   CD 1 μM

C

300 mg

▲
CPA 10 μM

D

5 min

30 mg

▲
CPA 10 μM

1 min
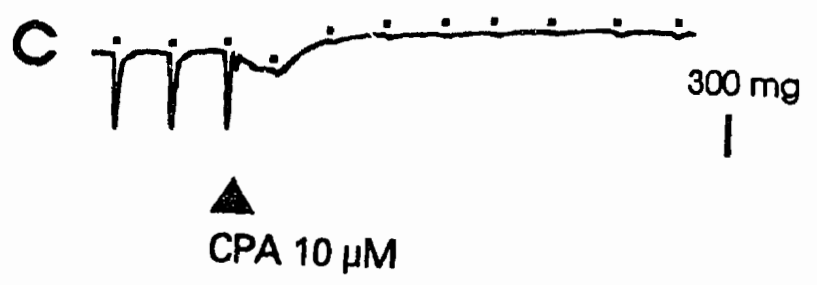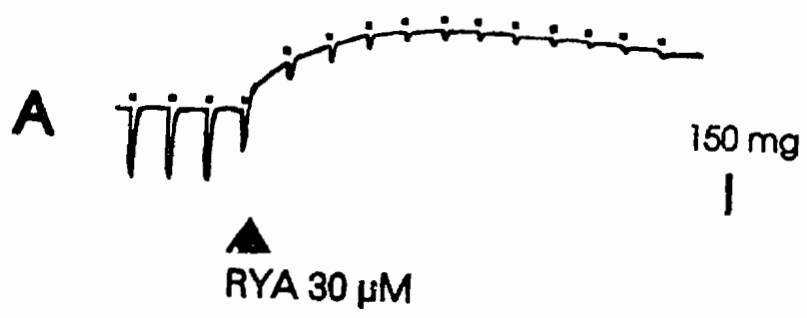
Fig.11 Depletion of the sarcoplasmic reticulum Ca^{++} prevents inhibition of the $(\pm)$-Bay K 8644-photoactivated response by cyclopiazonic acid in TMM.

A-Depletion of SR Ca^{++} after repetitive stimulation with CD $(0.3\ \mu M)$ in 110 mM K^+, nominally calcium-free solution containing 30 μM BAPTA, as demonstrated by nearly complete loss of contractile response after the fourth CD-challenge.

B-In a preparation previously depleted of the SR calcium, incubation with cyclopiazonic acid (CPA,3 μM) did not prevent the $(\pm)$-Bay K 8644-photoactivated response evident when low extracellular calcium was added to induce tonus. Experiment was carried out in 110 mM K^+, nominally calcium-free solution.

C-Incubation with CPA (3 μM) of SR Ca^{++}-depleted preparation prevents the refilling of the SR as evidenced by the depression in the phasic component of the CD-induced contraction.

A

CD 0.3 µM   W   W   W   W

30 mg
|
1 min

B

CPA 3 µM   Ca⁺⁺ 30 µM

C

W   CD 0.3 M
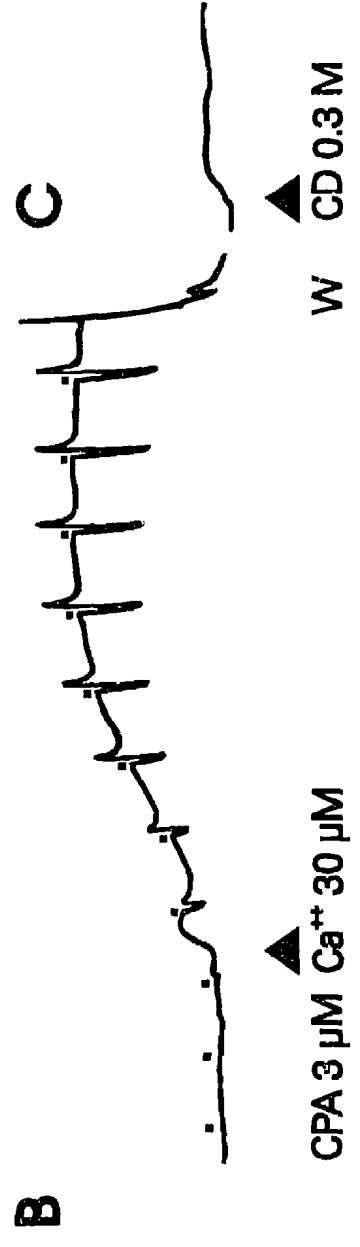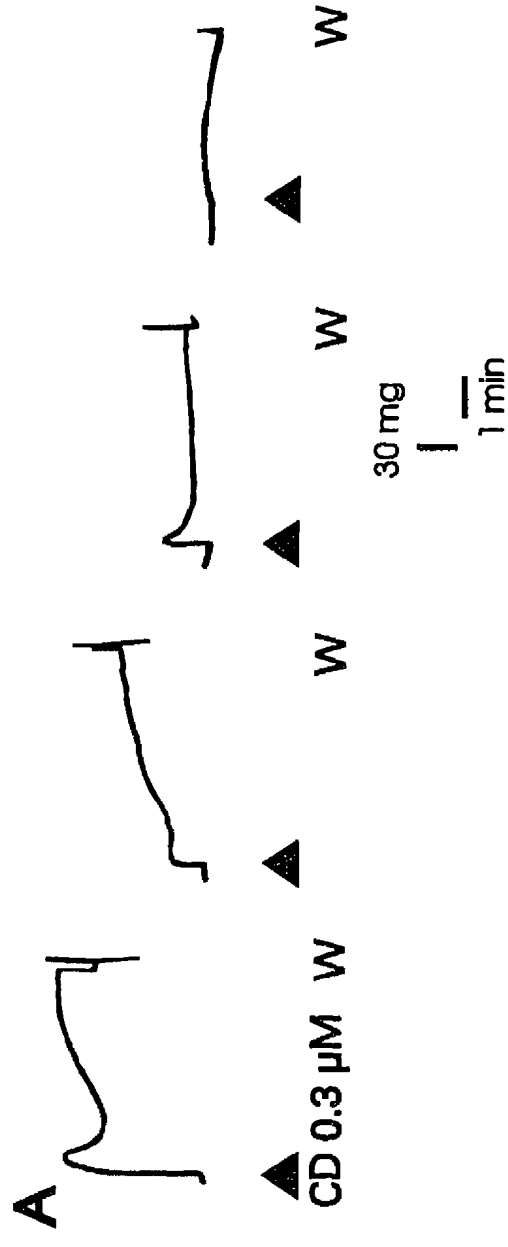
3.1.6 Effect of the calcium ionophore A 23187 on the (±)-Bay K 8644-photoactivated response

Inhibition of the (±)-Bay K 8644-photoactivated response by calcium overflow from SR into the cytoplasm was further examined with the calcium-ionophore A 23187 (Fig. 12A). In calcium-containing buffer, A 23187 (1 μM) inhibited the fast photoactivated response. However, in nominally-free calcium solution the same concentration of the calcium ionophore did not cause a significant inhibition of the photoactivated response (Fig. 12B).

Fig.12 Effect of the calcium ionophore A23187 on the (±)-Bay K 8644 (1 μM)-photoactivated response in TMM preparations bathed in Tyrode's (A) and in 110 mM K^{+}, nominally calcium-free buffer (B).

A

300 mg

A 23187 1 μM

B

30 mg

1 min
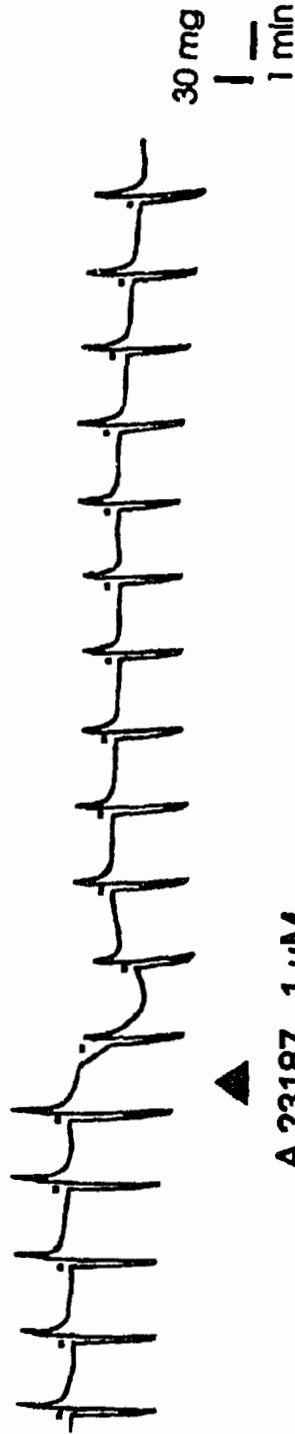
A 23187 1 μM

3.1.7 Effect of NO-inactivating procedures on the fast photoactivated response

In CD- or K^+-precontracted TMM preparations, LY 83583 (Fig. 13), carboxy-PTIO and Hb (not illustrated) failed to produce any significant change in the fast photorelaxation when a near-maximal photoactivated response was induced with 1 μM (+)-PN 202 791. In CD (0.3 μM)-precontracted preparations bathed in a 110 mM K^+-depolarizing solution, LY 83583 (10 μM) failed to diminish the ($\pm$)-Bay K 8644 (1 μM)-photoactivated fast relaxation whereas the amplitude of the "off-contraction" was significantly decreased. In this case, the amplitude of the relaxation and the "off-contraction" after LY 83583-treatment were expressed as percent of the pre-treatment rf'=xation or contraction, respectively. The photorelaxation was not altered following LY 83583-treatment (pre-LY 83583: 100 % vs. post-LY 83583: 86.5 $\pm$ 7.1 % (n = 10, p > 0.05)). On the contrary, the amplitude of the "off-contraction" was significantly decreased (pre-LY 83583: 100 % vs. post-LY 83583: 9.1 $\pm$ 2.6 % (n = 10, p < 0.05)).

At lower concentrations of (+)-PN 202 791 or in the presence of the superoxide dismutase inhibitor, diethyldithiocarbamate (DDC, 100 μM), a 10 μM concentration of LY 83583 significantly diminished the (+)-PN 202 791-photoactivated fast relaxation (Fig. 13). Diethyldithiocarbamate (100 μM) alone did not cause a significant inhibition of the (+)-PN 202 791-photoactivated fast

Fig. 13 Effect of LY 83583 (10 μM) alone or in combination with the superoxide dismutase inhibitor diethyldithiocarbamate (DDC, 100 μM) on the (+) PN 202 791 (1 μM)-photoactivated response in TMM.

Experiments were carried out in CD (0.1 μM)-precontracted preparations bathed in Tyrode's buffer, n = 4-6 preparations per group. Photorelaxation was expressed as percent of steady-state tonus. * p< 0.05 vs. control.
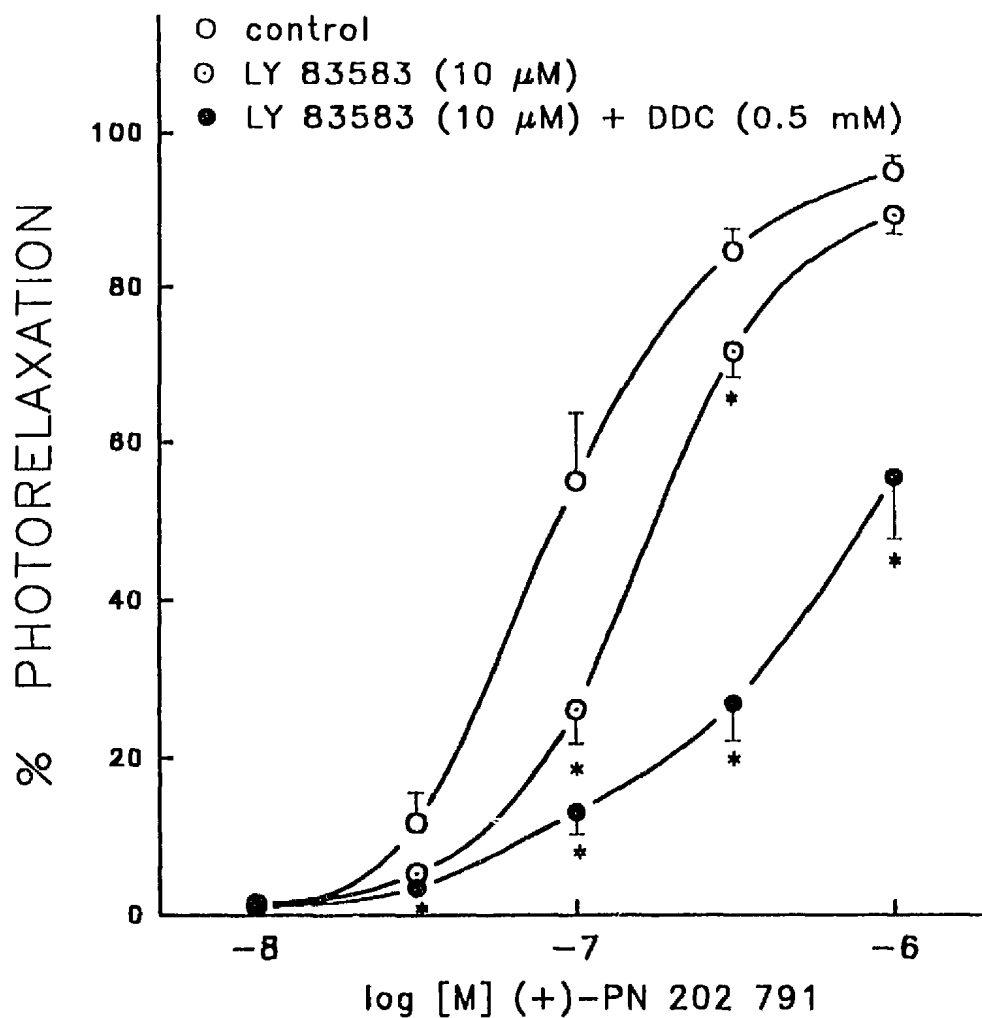
Table 2. Effect of haemoglobin (Hb) and carboxy-PTIO on the (+)-PN 202 791-photoactivated fast relaxation in TMM.

Drug	Amplitude of the photorelaxation (mg)	% inhibition vs. control
Control[1] Hb (10 μM) Hb (30 μM)	253 ± 25 214 ± 26 155 ± 35	- 14 ± 6 40 ± 7
Control[2] carboxy-PTIO (100 μM)	64 ± 7 36 ± 8*	- 59 ± 9

[1] experiment was carried out in CD (0.1 μM)-precontracted preparations bathed in Tyrode's buffer and photosensitized with 0.1 μM (+)-PN 202 791.

[2] experiment was carried out in CD (0.1 μM)-precontracted preparations bathed in 110 mM K^+, nominally calcium-free buffer and photosensitized with 30 nM (+)-PN 202 791.

* $p < 0.05$ vs. control, n = 4 preparations per group.

relaxation. The fast photorelaxation induced by low concentrations of (+)-PN 202 791 was also sensitive to the NO-trapping agent carboxy-PTIO (Table 2). The decrease in the (+)-PN 202 791-photoactivated fast relaxation by Hb (10 or 30 μM) was not statistically different from the control (Table 2).

Irradiation of the (+)-PN 202 791 stock solution caused a rightward shift in the photorelaxation concentration response curve as compared with the control (a non-irradiated solution of (+)-PN 202 791)(Fig. 14A). The concentration-response curve for the irradiated solution also showed a decrease in the maximal response to light-stimulation. As compared with the (+)-PN 202 791 concentration-response curve, a right- and downward shift was also obtained with non-irradiated racemic PN 202 791. The slopes of the sigmoid curves for irradiated (+)-PN 202 791 and ($\pm$)-PN 202 791 were statistically different from the control. In contrast, irradiation of an SNP solution induced a rightward shift in the concentration response curve (Fig. 14B) with no decrease in the maximal photorelaxation.

Fig.14 Effect of (+)-PN 202 791 (A) and SNP (B) irradiated solutions on the photorelaxation concentration response curve in TMM. (+)-PN 202 791 (1 μM) and SNP (1mM) stock solutions were irradiated for 60 min at 37 °C. *p<0.05 vs. control (non-irradiated solution). After 10 min irradiation, the SNP solution became blue probably because of the formation of $Na_2[Fe(CN)_5(NOH)]$.

The respective slope values as determined by curve fitting analysis were: 4.06 ± 0.34 for (+)-PN 202 791, 2.9 ± 0.34ʼ for irradiated (+)-PN 202 791 and 2.14 ± 0.44ʼ for racemic PN 202 791,ʼ p<0.05 vs (+)-PN 202 791 (n=4 preparations per group).

A

- ● (+)-PN 202 791
- ○ (+)-PN 202 791 (irradiated sol.)
- ◉ (±)-PN 202 791

% PHOTORELAXATION

log [M] PHOTOSENSITIZER

B

- ● control
- ○ irradiated blue solution

% PHOTORELAXATION

log [M] SNP
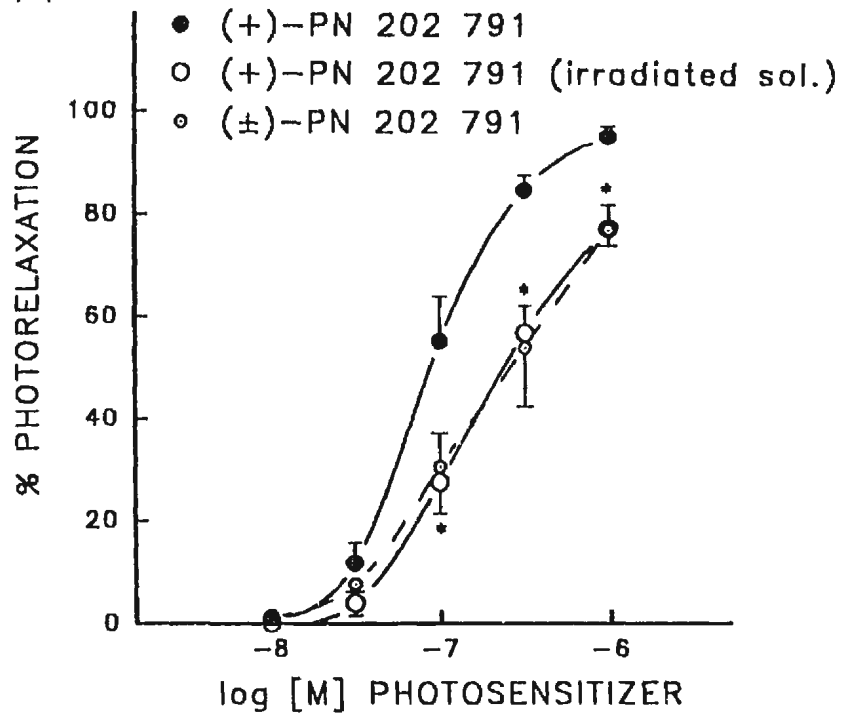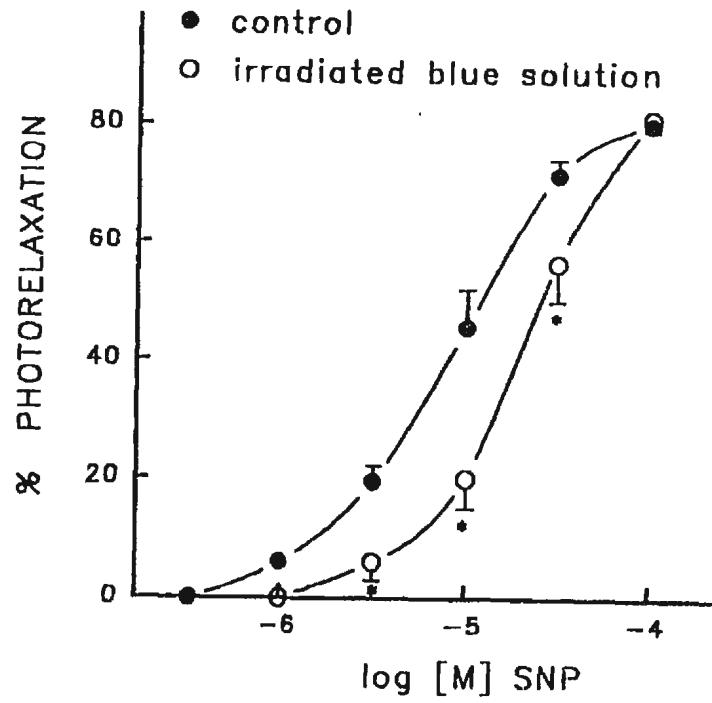
3.1.8 Effect of zaprinast on the fast and the slow residual photorelaxations

Two second-irradiation of (+)-PN 202 791 pretreated preparations displayed a relaxation followed by an recovery from irradiation. Both the amplitude of the relaxation and the slope of the offset response were significantly increased by the cGMP-dependent PDE inhibitor, zaprinast, whereas the slope of the relaxation remained unchanged.

At 10 s-irradiation, zaprinast enhanced the amplitude of the fast photorelaxation (Fig. 15). However, neither the slope of the fast photorelaxation nor the slope of the off-contraction were affected by zaprinast, whereas the slope and the percent decrement of the escape were diminished.

Incubation of the DHP agonist or NO-donor photosensitized TMM with DHP antagonists (Fig. 4), BAPTA (Fig. 9), cyclopiazonic acid and ryanodine (Fig. 10) unmasked a low amplitude slow residual photorelaxation as described above (section 3.1.5). Zaprinast enhanced the slow photorelaxation under all conditions (Fig. 16, 17) and this effect was readily reversed by LY 83583 (Fig. 16).

Fig.15 Effect of zaprinast (filled bars) on the parameters of the fast photoresponse at two (2) and ten (10) s irradiation in TMM.

The parameters of the relaxation were as follows: amplitude of the fast relaxation (A_{fr} (%)), and slope of the fast relaxation (S_{fr} (mg/s)), slope of the offset response at 2 s-irradiation and at 10 s-irradiation (S_{off} (mg/s)), slope of the escape response (S_e (mg/s)), and percent decrement of the fast relaxation ($\%D_e$ (%)).

A_{fr} was expressed as percent of the amplitude of the relaxation at 10 s-irradiation (control).

$\%D_e$ was calculated as $(R_{10}/R_{max})*100$, where R_{max} and R_{10} represent the value of the relaxation at its peak and at the end of the 10 s-irradiation period.

Experiments were carried out in Tyrode's buffer. CD (0.1 μM)-precontracted preparations were photosensitized with 1 μM (+)-PN 202 791 (n=4 preparations per group). Control values are represented as open bars.

A 2 sec. photostimulation
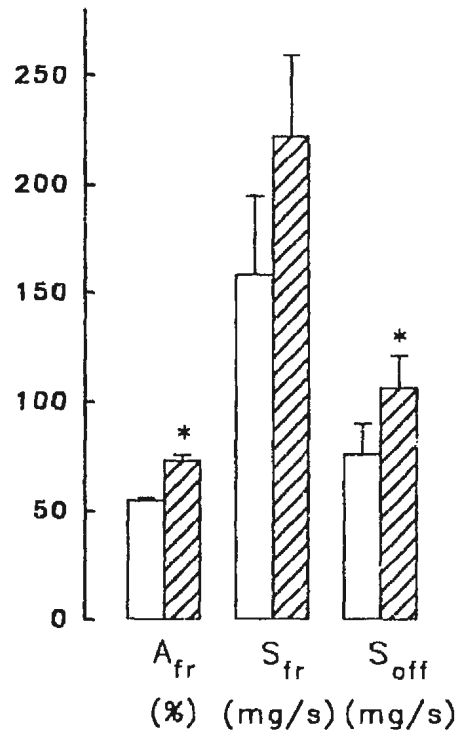
B 10 sec. photostimulation
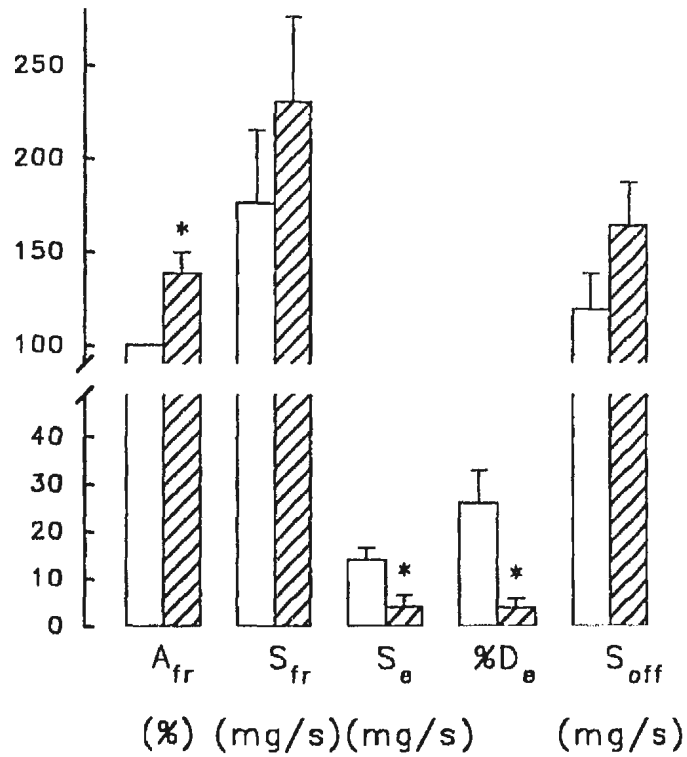
Fig.16 Effects of zaprinast and LY 83583 on the slow photorelaxation in TMM bathed in 110 mM K$^+$, nominally calcium free buffer. Tissues were precontracted with 0.3 μM CD and the slow photorelaxation was obtained after treatment with BAPTA (100 μM). Photosensitization was induced with 1 μM of (±)-Bay K 8644.

30 mg

1 min

BAPTA
100 µM

Zaprinast
10 µM

LY 83583
10 µM

INA
0.3 µM
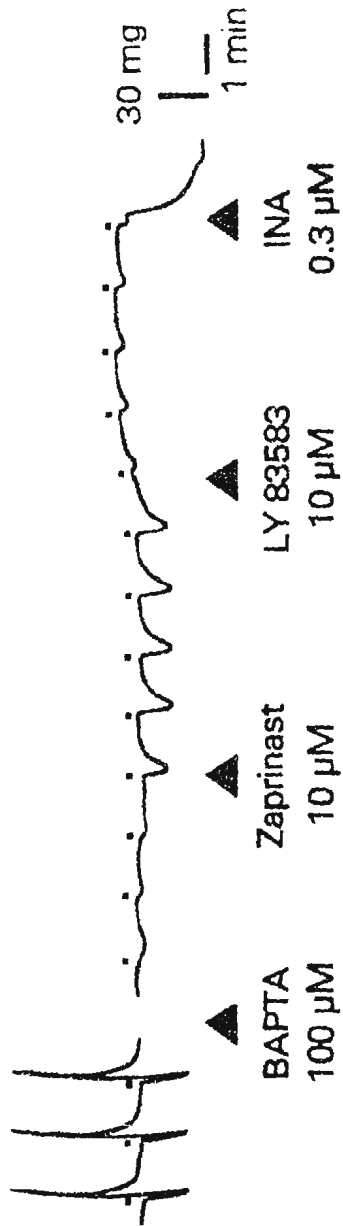
Fig.17 Amplitude of the (+) Bay K 8644 photoactivated slow relaxation before (open columns) and after zaprinast (right hatching) in a depolarizing nominally calcium free medium (A) and before (solid columns) and after (left hatching) zaprinast (10 μM) in Tyrode's buffer (B) (*p<0.05 vs. control, (n=4-5 preparations per group)). The slow relaxations were obtained following treatment of TMM preparations with BAPTA (100 μM), felodipine (1 μM), ryanodine (RYA, 30 μM) and cyclopiazonic acid (CPA, 10 μM).
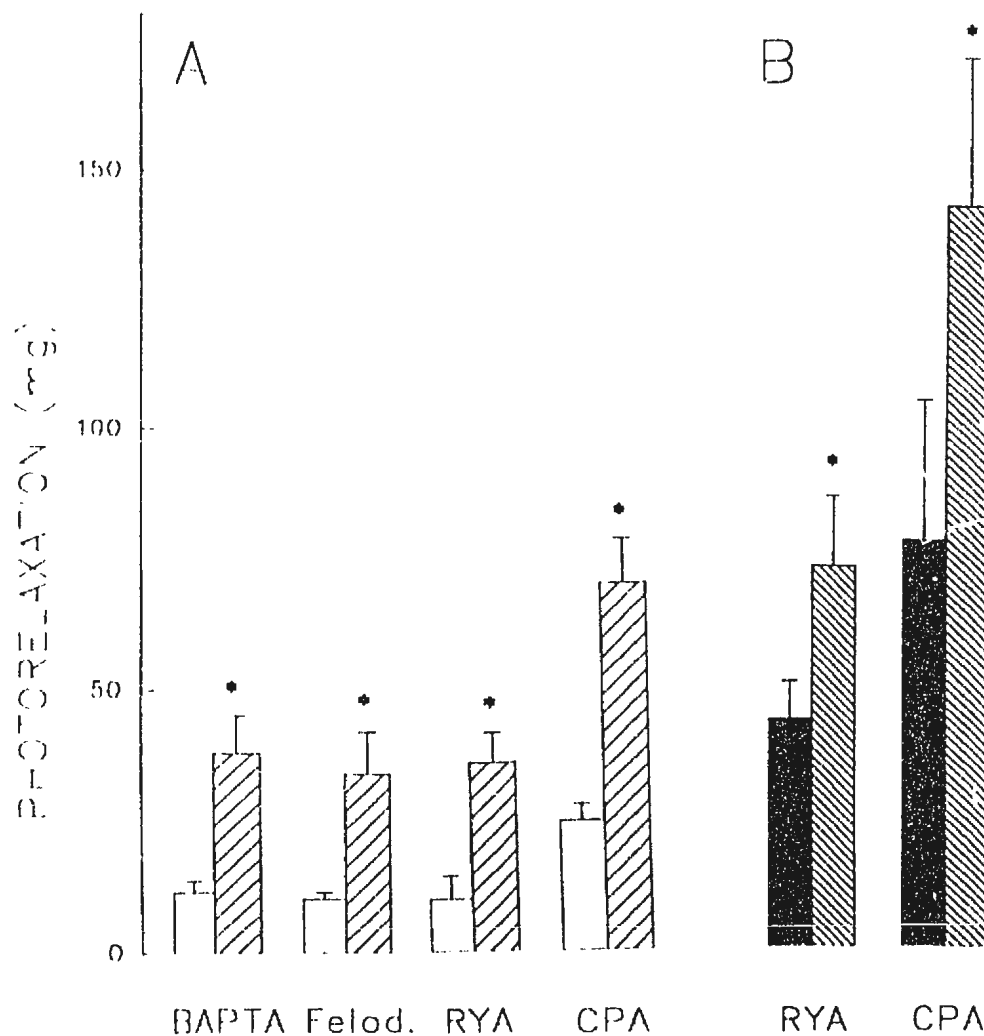
3.1.9 Dependence of the 3'-NO$_2$-DHP-photoactivated relaxation on contractile stimuli and the active tension level

In other to determine how different mechanisms of tonus generation may affect the photoactivated response in the TMM, the fast photorelaxation was examined under various contractile states induced with such pharmacological stimuli as the muscarinic agonist CD, a depolarizing solution and the phosphatase 1, 2A inhibitor, calyculin A. The magnitude of the 3' NO$_2$ DHP photoactivated relaxation varied with both the pharmacological stimulus used and with the level of contractile tension.

Pretreatment of TMM preparations with the phosphatase 1,2A inhibitor calyculin A induced a slowly developing contraction in calcium-depleted tissues (as described in section 2.6). In the presence of calyculin A-induced tonus, the DHP photosensitizer (+)-PN 202 791 gave rise to slow photorelaxations in a concentration-dependent fashion (Fig. 18). A similar concentration of calyculin A did not affect tonus-generation or the fast photorelaxation in calcium replenished preparations (not illustrated). In calyculin A precontracted preparations, the slow photorelaxation was enhanced by zaprinast (Fig. 8), similar to the slow photorelaxation obtained after calcium depletion and inhibition of L-type Ca^{++}-channels (described above).

The fast photorelaxation was evident either in CD- or K$^+$-precontracted preparations. The 3'-NO$_2$-DHP-photoactivated response showed identical

features under either contractile stimulus. However, irregular fluctuations in tonus occurred when CD (<0.1 μM)-precontracted preparations were exposed to DHP agonists in Tyrode's solution. In contrast, CD- or calcium-induced tonus in the depolarizing solution was maintained at a steady level for several hours (not illustrated).

The amplitude of the fast photorelaxation varied with the level of active tonus. Thus, in 110 mM K^+, nominally Ca^{++}-free buffer and the absence of active tonus, a photoactivated response was undetectable. With graded increments in $[Ca^{++}]_o$ over the range of 10 to 1000 μM, the amplitude of the photorelaxation measured in absolute terms increased in parallel with contractile tonus, reaching a maximum at 300 μM, i.e. the $[Ca^{++}]_o$ causing a near maximal increase in tonus (Fig. 19A). With a further increase in $[Ca^{++}]_o$, the absolute value of both tonus and photorelaxation declined. However, over the range of $[Ca^{++}]_o$ tested, the photorelaxation expressed as percent of the steady state tonus was not significantly different (Fig 19A).

A different relationship between the ($\pm$)-Bay K 8644-photoactivated fast relaxation and the level tonus was obtained in sarcoplasmic Ca^{++}-depleted tissue contracted with CD. Fig. 19B shows the bell-shaped relationship between the amplitude of the photorelaxation and the level of CD-induced tonus in 110 mM K^+ solution containing 10 μM calcium. The amplitude of the photorelaxation

reached a maximum at 0.1 μM CD, corresponding to the EC_{80} of this agonist. With further increases in the CD concentration, the amplitude of the photorelaxation fell to near zero. Expressed as percentage of CD-induced tonus, the relative photorelaxation amplitude declined from a maximal value of 60% to 5%.

Fig.18 (+)-PN 202 791-photoactivated slow relaxation in calyculin A-precontracted TMM. Experiment was carried out in extracellular calcium-depleted preparations (treated with 30 μM BAPTA), bathed in 110 mM K', nominally calcium-free medium.

Calyculin A
30 nM

(+)-PN 202 791
0.3 µM   1 µM

Zaprinast
10 µM

INA
0.3 µM

30 mg
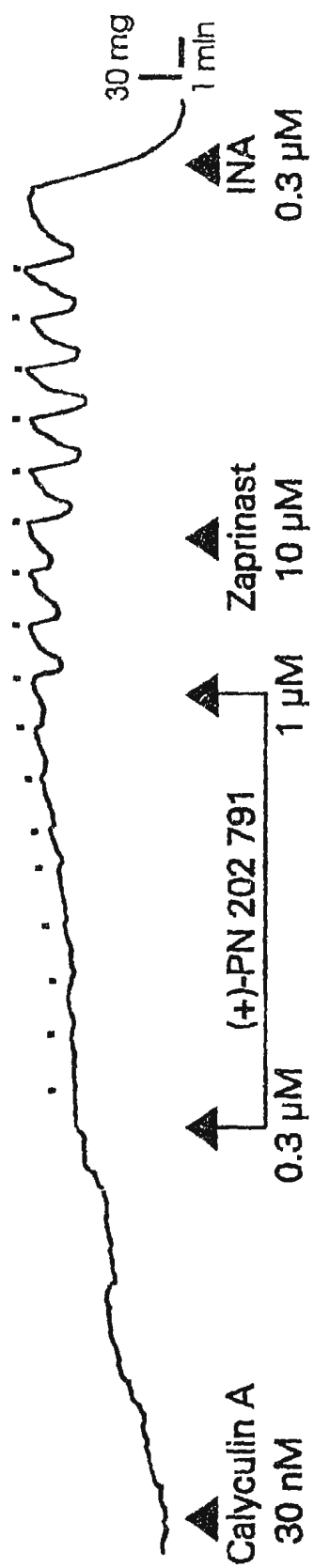
1 min

Fig.19 Dependence of the (±)-Bay K 8644-photoactivated fast relaxation on the active tonus in TMM.

A-Calcium dependence of tonus (top panel) and (±)-Bay K 8644 (1 μM)-induced photorelaxation (middle and lower panels). Experiments were carried out in 110 mM K⁺ depolarizing solution, tonus was induced with cumulative addition of calcium chloride solution.
The tonus response was standardized with reference to the maximal response of the TMM in 110 mM K⁺ solution to calcium (753 ± 95 mg, n = 4). The photorelaxation is shown in its absolute value (lower panel) and as percent of the induced tonus (middle panel). In the latter, the dashed line corresponds to the linear regression for the plotted values (r = 0.65).

B-Dependence of the (±)-Bay K 8644 (1 μM)-photoactivated fast relaxation (middle and lower panels) on the CD-induced tonus of sarcoplasmic calcium-depleted tissues. Top panel shows the change in the level of tonus as a function of CD concentration. Experiments were carried out in the presence of cyclopiazonic acid 5 μM in 110 mM K⁺, depolarizing solution containing 10 μM calcium.
The tonus response was standardized with reference to the maximal response of the TMM to CD (204 ± 45 mg, n = 4). Photorelaxation is shown in the same manner as in panel A.
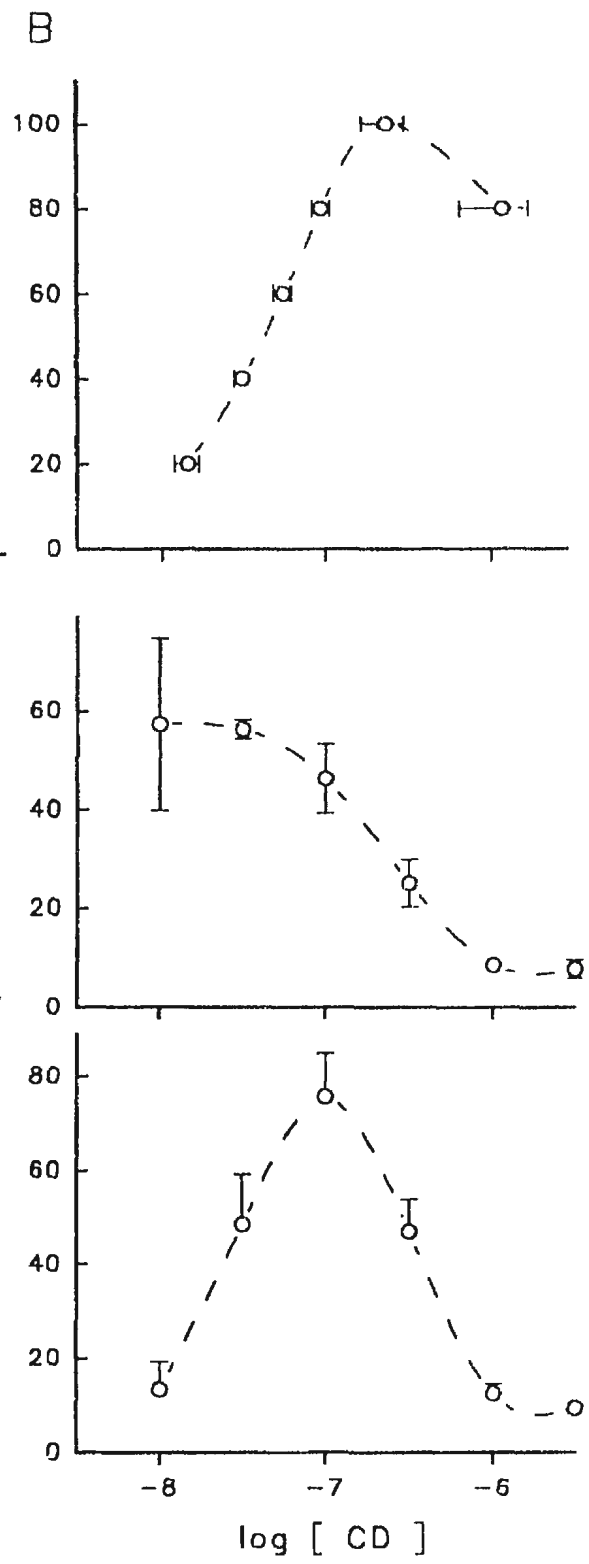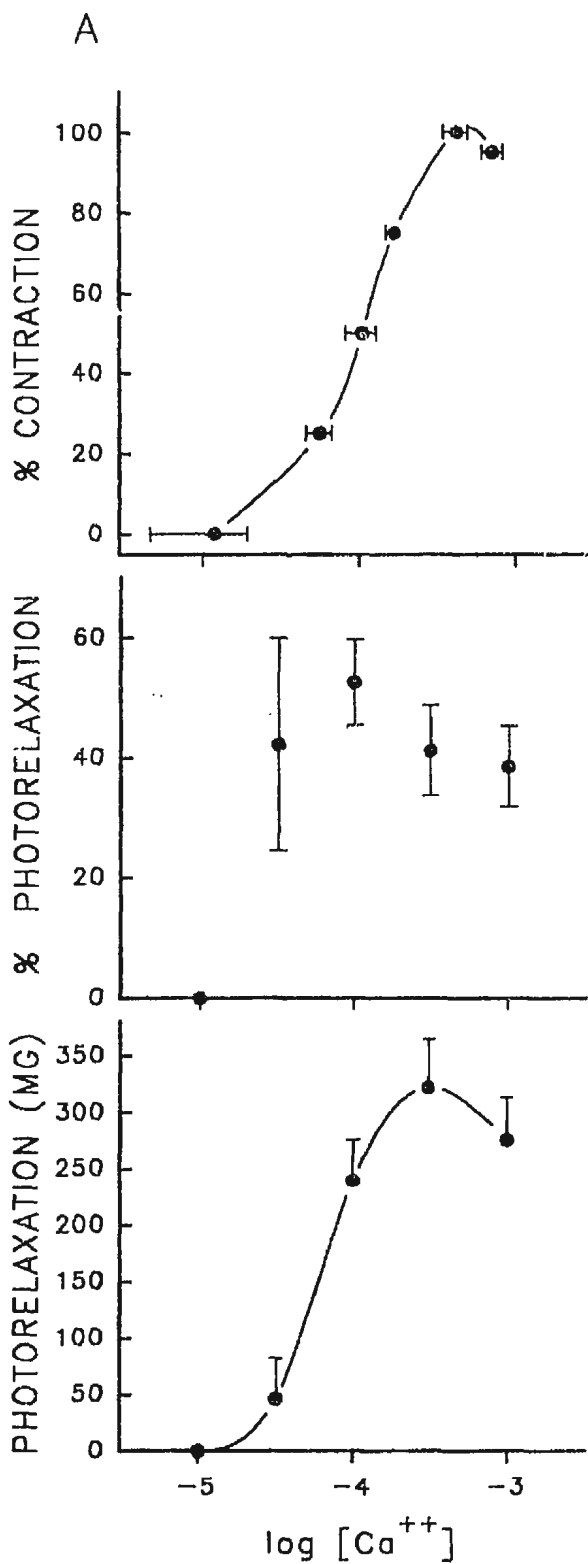
3.1.10 Effect of pharmacological agents on the 3'-NO$_2$-DHP-photoactivated response in the TMM

Several pharmacological agents were examined as potential modifiers of the 3' NO$_2$-DHP-photoactivated response. In CD (0.3 μM)-precontracted TMM preparations bathed in a depolarizing buffer, the ($\pm$)-Bay K 8644 (1 μM)-induced fast photorelaxation was significantly inhibited by sodium fluoride (NaF, 30 mM). Expressed as percent of the existing tonus, the amplitude of the photorelaxation before and after NaF was 85.2 $\pm$ 7.7 vs. 21.0 $\pm$ 3.2 (n = 5, p < 0.05), respectively. The NaF-mediated effect on the photorelaxation was preceded by an increase in contractile tonus.

Other pharmacological agents tested during this study were ineffective against the 3'-NO$_2$-DHP-photoactivated response (Table 3). Neither the biphasic fast response not the slow photorelaxation induced by ($\pm$)-Bay K 8644 or (+)-PN 202 791 were affected by these drugs when tested in Tyrode's or 110 mM K', nominally calcium-free solution (n = 3-4).

Table 3. Pharmacological agents devoid of any effect on the 3' NO. DHP photoactivated response.

Agent	Pharmacological action	max. concentration
TTX	Na^+ channel blocker	10 nM
methscopolamine	mAChR antagonist	100 nM
propanolol	ß-adrenoceptor antagonist	10 μM
guanethedine	adrenergic neuron blocker	10 μM
indomethacin	cyclo-oxygenase inhibitor	100 μM
ouabain	Na^+/K^+ ATPase inhibitor	100 μM
ICS 205-930	$5HT_3$ receptor antagonist	10 μM
ketanserin	$5HT_{1c}/5HT_2$ receptor antagonist	1 μM
L-arginine	NOS substrate	1 mM
L-N^G-nitroarginine methyl ester	NOS inhibitor	1 mM
KCN	inhibitor of oxidative phosphorylation	0.1 mM

3.2 Photoactivated response in the rat pyloric sphincter and thoracic aorta

The rat pyloric sphincter responded with phasic contractions to CD (1 μM)-stimulation. Similar to the TMM, pyloric sphincter was devoid of any intrinsic responsiveness to light irradiation. Photostimulation of CD-pretreated pyloric sphincter did not produce any change in the frequency of the phasic contractions or in the basal tonus (Fig 20 A, upper trace). 30 s-irradiation of (+) PN 202 791-treated preparations revealed a transient inhibition (duration $\approx$ 10 s) of the phasic contractions followed by an escape or resumption of the phasic activity (Fig 20 A, lower trace). Termination of the photostimulation resulted in an "off-contraction" of larger amplitude than the phasic contractions.

Contrary to the TMM and pyloric sphincter, precontracted rat thoracic aorta showed an endogenous photorelaxation after polychomatic light irradiation (Fig. 20B, upper trace). Following the relaxation there was a delayed recovery to the prestimulation tension. The effects of the $3'$-NO_2-DHP photosensitizer (+)-PN 202 791 on the endogenous photorelaxation, under different conditions of irradiation intensity and photostimulation interval, are summarised in Fig. 21. Moreover, a sample trace of the (+)-PN 202 791-augmented relaxation in rat thoracic aorta is shown in Fig. 20 B (lower trace). Both the relaxation and the recovery components of the endogenous and the (+)-PN 202 791-augmented responses were described by a logistic function (Fig. 20B). Each component

Fig.20 Sample traces of the photoactivated response in rat pyloric sphincter and thoracic aorta.

A- 30 s-photostimulation of CD-pretreated pyloric sphincter before (upper trace) and after (+)-PN 202 791 (1 μM) treatment (lower trace). The horizontal bar represents 30 s irradiation.

B- 10 s-photostimulation of PE (0.1 μM)-precontracted rat thoracic aorta before (upper trace) and after (+)-PN 202 791 (1 μM)-treatment (lower trace). The 10 s-irradiation interval is represented by the horizontal bar.
The logistic equations for the relaxation and recovery of the endogenous relaxation are $y = 2.77 + 0.93(1 + (x/5.13)^{3.52})$ and $y = 2.57 + 1.22(1 + (x/5.53)^{0.77})$ (coefficient of determination $r^2 > 0.97$, F-statistic > 1103), whereas the equations describing the relaxation and recovery of the (+)-PN 202 791 augmented response are $y = 2.50 + 1.14(1 + (x/6.26)^{2.54})$ and $y = 2.46 + 1.43(1 + (x/35.55)^{1.16})$ ($r^2 > 0.99$, F-statistic > 2834).

A

B

2 g

0.5 g
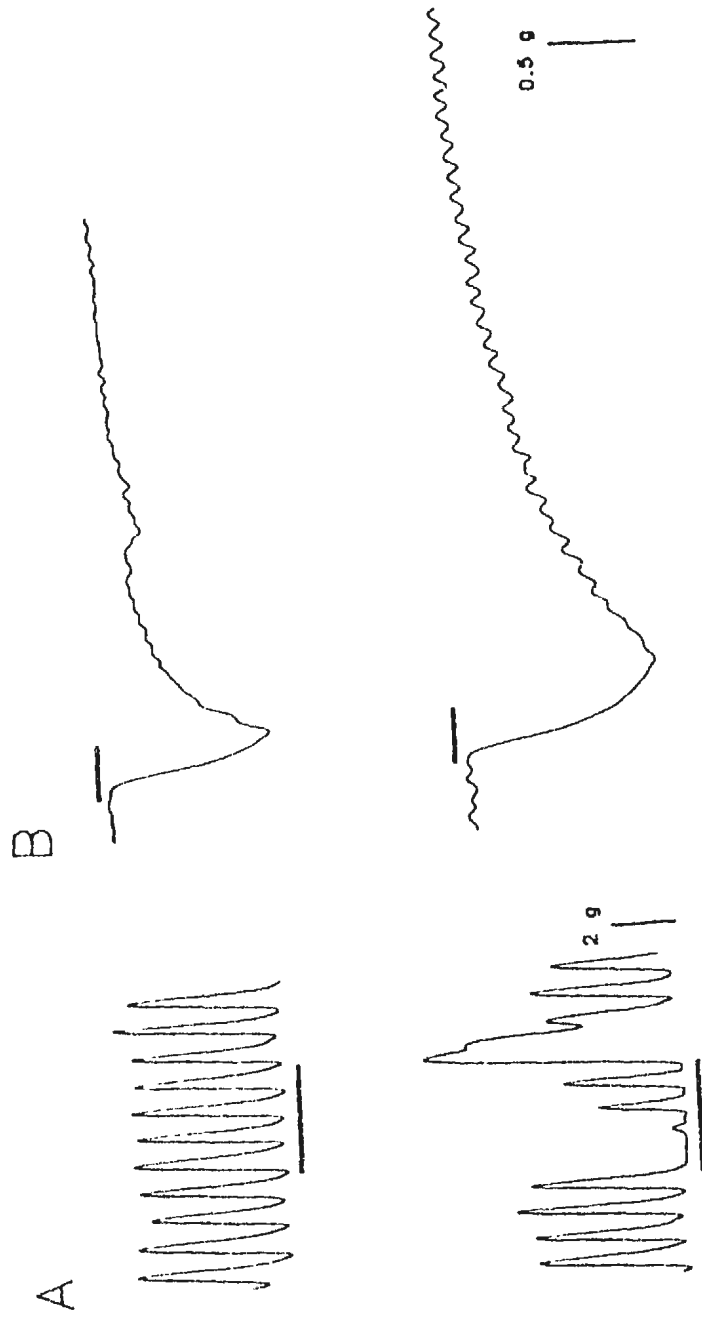
contained three phases: 1) an initial slow phase, 2) an intermediate fast phase and 3) a late slow phase. The duration of the initial slow phase was negligible as compared with the duration of the following two phases. The effect of (+) PN 202 791 on the endogenous photorelaxation was characterized by the slope of the fast phase. Moreover, the photorelaxation and recovery were described by the time required for the relaxation to reach its maximal value ($T_{peak\ relax}$) or the time necessary for the recovery to reach 50% of the prephotostimulation tension (T_{50}), respectively (Fig. 21).

The results in thoracic aorta can be summarised as follows:

1) the amplitude of the endogenous photorelaxation was increased following (+)-PN 202 791-treatment. This effect was observed both at different intensities (0.08 W/cm^2 or 0.9 W/cm^2) or durations (10 s or 30 s at 0.9 W/cm^2) of light-irradiation;

2) the slope of the fast phase of the (+)-PN 202 791-augmented photorelaxation at 0.08 W/cm^2 was similar to the slope of the intrinsic photorelaxation at a higher intensity (0.9 W/cm^2). No further increase in the slope of (+)-PN 202 791-augmented photorelaxation was noticed at 0.9 W/cm^2 irradiation or at 30 s photostimulation;

3) at 0.9 W/cm^2, $T_{peak\ relax}$ increased following sensitization by (+)-PN 202 791, as the irradiation period increased;

4) no change in the slope of the recovery for the endogenous or the (+)-PN 202 791-enhanced photorelaxation was found at different intensities or intervals of irradiation. The long duration of the recovery resulted from the slope of the recovery (fast phase) being at least 1.5 times smaller than the slope of the relaxation.

5) (+)-PN 202 791 was without effect on T_{50} at 0.08 W/cm^2. However, an increase was noticed at 0.9 W/cm^2 which seemed to be maximal since a longer irradiation interval (30 s) did not produce any further change.

Fig.21 Parameters of the endogenous and the (+)-PN 202 791 (1 μM) augmented photorelaxation (PhR) in rat thoracic aorta under 0.08 W/cm^2 ($I_{0.08}$) (10 s-irradiation) or 0.9 W/cm^2 ($I_{0.9}$)(10 s-, 30 s-irradiation) photostimulation. Experiments were carried out in Tyrode's buffer. Preparation were precontracted with phenylephrine (0.1 μM).

$T_{peak\ relax}$ represents the interval for the relaxation to reach its maximal value, whereas T_{50} represents the time required to recover to 50% of the pre photostimulation tension level.

[1] amplitude of the relaxation was expressed as percent of the endogenous photorelaxation obtained after 10 s-irradiation at 0.9 W/cm^2.

* $p \leq 0.05$ vs. same parameter of the endogenous relaxation (n - 4 10).

AMPLITUDE (%)[1] SLOPE RELAXATION (mg/sec) $T_{peak\ relax.}$ (sec) SLOPE RECOVERY (mg/sec) T_{50} (sec)

Legend:
- □ endogenous PhR, $I_{0.08}$, 10s
- ▨ endogenous PhR, $I_{0.9}$, 10s
- ▥ endogenous PhR, $I_{0.9}$, 30s
- ■ (+)-PN 202791 PhR, $I_{0.08}$, 10s
- ▨ (+)-PN 202 791 PhR, $I_{0.9}$, 10s
- ▤ (+)-PN 202 791 PhR, $I_{0.9}$, 30s
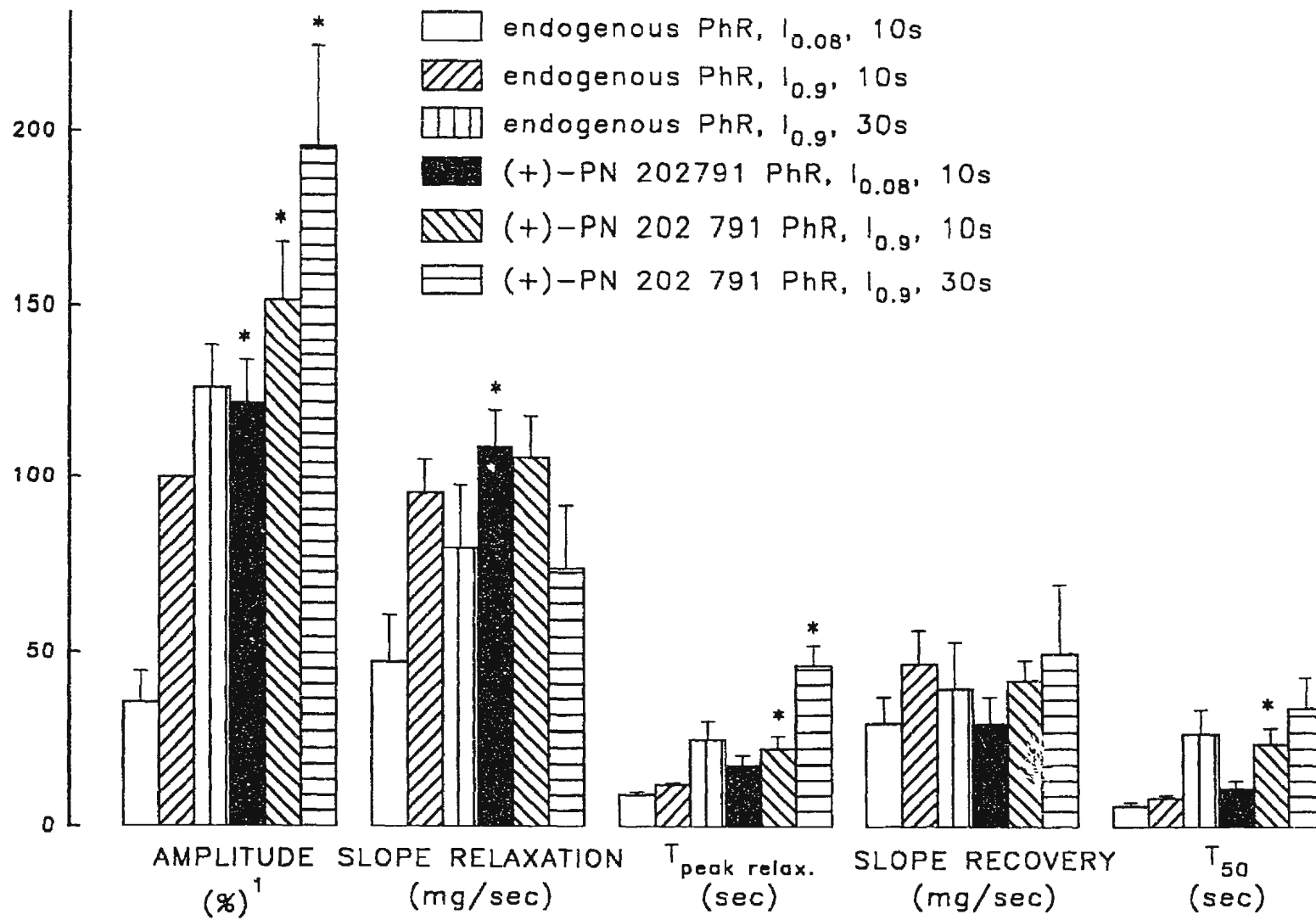
3.2.1 Effect of methylene blue and LY 83583 on the photorelaxation in thoracic aorta

In rat thoracic aorta, LY 83583 inhibited the endogenous photorelaxation at 1 μM concentration (Table 3). On the contrary, a ten-fold larger concentration of LY 83583 was ineffective against the ($\pm$)-Bay K 8644 (1 μM) augmented photorelaxation. After removal of ($\pm$)-Bay K 8644 from the incubation buffer, LY 83583 significantly reduced the photorelaxation. The removal of ($\pm$) Bay K 8644 did not affect the amplitude of the photorelaxation. As in the TMM, LY 83583 was also an effective inhibitor of the photorelaxation augmented by a low concentration of the 3'-NO_2-DHP, Bay K 8643 (3 nM). The inhibitor of sGC, methylene blue, effectively blocked the Bay K 8643-augmented photorelaxation in rat thoracic aorta.

Table 4. Effect of LY 83583 and methylene blue (MB) on the endogenous and the 3'-NO_2-DHP-augmented photorelaxation in phenylephrine (0.1 μM)-precontracted rat thoracic aorta.

Treatment	Amplitude of the photorelaxation (%)
Endogenous photorelaxation (control)	40.0 ± 4.0
+ LY 83583 (1 μM)	2.2 ± 1.5*
(±)-Bay K 8644 (1 μM)(control)[1]	65.5 ± 7.1
+ LY 83583 (10 μM)	61.4 ± 5.9
Photorelaxation after (±)-Bay K 8644	
removal[2]	67.6 ± 2.7
+ LY 83583 (10 μM)	14.4 ± 5.0*
Bay K 8643 (3 nM)-augmented	
photorelaxation (control)	47.5 ± 3.5
+ LY 83583 (1 μM)	14.8 ± 4.3*
Bay K 8643 (30 nM)-augmented	
photorelaxation (control)	83.0 ± 5.8
+ MB (3 μM)	5.8 ± 2.3*

[1] (±)-Bay K 8466-augmented photorelaxation after the endogenous photoresponse was eliminated with 1 μM LY 83583.

[2] Rat aorta preparations were treated with 1 μM (±)-Bay K 8644 for at least 15 min. After that Bay K was removed from the bath by washing with fresh buffer. After that tissues were precontracted again with phenylephrine (0.1 μM).

* $p < 0.05$ vs. control (n = 4-5 preparations per group).

3.2.2 Effect of zaprinast on the 3'-NO_2-DHP-photoactivated response in rat pyloric sphincter

In pyloric sphincter, the phasic contractions observed in the presence of zaprinast during the 30 s-irradiation (similar to an escape response during photostimulation) were decreased by 78% as measured by the area of the phasic contractions (pre-zaprinast: 66.5 ± 2.5 vs. post-zaprinast: 14.3 ± 0.4, ($p < 0.05$, n = 3)). No significant change was observed in the amplitude of the "off-contraction".

CHAPTER FOUR

DISCUSSION

4.1 General properties of the DHP-photoactivated response

The DHPs examined in this investigation mediated multiple photoactivated response patterns in the three smooth muscle preparations studied. These photodynamic effects were confined to compounds possessing nitro substituents and encompassed simple relaxation or contraction events and complex relaxation-contraction sequences. Photoactivated responses involving relaxation with or without a contraction component were mediated by $3'\text{-}NO_2$-DHPs, whereas pure photocontractions were obtained solely with o-nitrophenyl-substituted DHPs. Since the former but not the latter were concentration-dependent, it is suggested that the photocontraction by o-nitrophenyl-substituted DHPs is a passive process not involving release of a photodynamic derivative. Non nitro-substituted DHPs, specially those with antagonistic action at L-type Ca^{++}-channels were able to alter the complex relaxation-contraction response in TMM preparations.

Exposure to $3'\text{-}NO_2$-DHPs augmented the intrinsic photorelaxation in rat thoracic aorta or photosensitized de novo preparations devoid of any intrinsic photoresponsiveness, i.e., rat TMM and pyloric sphincter. In preparations lacking endogenous photoresponsiveness, photodegradable NO-donors mimicked the $3'\text{-}NO_2$-DHPs in conferring photoactivity. In TMM preparations, however, the

NO donor-photoactivated response differed qualitatively from the neurally evoked NO-mediated response.

4.1.1 Tissue selectivity of the photoactivated response components

The present study demonstrates that the $3'-NO_2$-DHP-photoinduced effect on smooth muscle contractility is expressed in a tissue-selective manner. In TMM preparations, L-type Ca^{++}-channel agonist $3'-NO_2$-DHPs produced a complex response, consisting of a fast relaxation-contraction sequence, followed by a late slow relaxation. In pyloric sphincter, the same compounds mediated a transient inhibition of the phasic contractions followed by an "off contraction". In rat thoracic aorta, however, sensitization by $3'$ NO_2 DHPs augmented the intrinsic photorelaxation.

These findings are consistent with the diversity of the photoactivated response previously described in other smooth muscle preparations. Thus, the $3'-NO_2$-DHP-photoactivated biphasic relaxation-contraction sequence in the TMM is similar to the Bay K 8644-photoactivated response in porcine fundus (Golenhofen et al., 1990) or the $NaNO_2$-photoactivated response in rabbit stomach smooth muscle (Ehrreich & Furchgott, 1968). However, a distinctive feature of the biphasic photoactivated response in the TMM was the transient duration of the relaxation as compared with the sustained relaxations described

by others (Furchgott et al., 1961; Golenhofen et al., 1990). The possible basis for this difference will be discussed in a later section. The $3'$-NO_2-DHP-photoactivated response in pyloric sphincter is similar to the transient photoactivated inhibition of the electrically stimulated phasic contractions in the $NaNO_2$-pretreated rabbit stomach (Ehrreich & Furchgott, 1968).

As first shown by Furchgott (1961) in rabbit thoracic aorta, the intrinsic response consists of a photorelaxation followed by a long lasting recovery. Both the relaxation and recovery contain an initial slow phase of short duration, an intermediate fast phase and a late slow phase. $3'$-NO_2-DHPs have been shown to enhance the intrinsic photorelaxation in vascular smooth muscle (Golenhofen et al., 1990; Triggle et al., 1990; Chen & Gillis, 1992); however, the effect of $3'$ NO_2-DHP on the photorelaxation-recovery sequence has not been described before. As shown here, photosensitization of rat thoracic aorta by $3'$-NO_2-DHP does not alter the pattern of the intrinsic relaxation; it only modifies the quantitative properties of the intrinsic photorelaxation (i.e., the amplitude and slope of the endogenous photorelaxation).

4.1.2 Structure-activity relationship

The TMM preparation was specially selected for the investigation of the structure-activity relationship between DHPs. Although several DHPs were tested in this study, only nitro-substituted DHPs were reported as possessing a photo-induced activity in TMM preparations. $3'$-NO_2-DHPs induced a photoactivated response involving relaxation whereas nitrophenyl-substituted DHPs either mediated a long-lasting photocontraction (o-nitrophenyl-substituted DHPs) or lacked any photosensitizing activity (m- and p-nitrophenyl-substituted DHPs). Non nitro-substituted DHPs with L-type Ca^{++}-channel antagonistic action, albeit devoid of any photodynamic activity, were able to modify the components of the $3'$-NO_2-DHP-photoactivated response.

Previous studies carried out in vascular smooth muscle (Golenhofen et al., 1990; Triggle et al., 1991) have demonstrated that $3'$-NO_2-DHPs augment the endogenous photorelaxation. However, the presence of an intrinsic photoresponsiveness in this type of smooth muscle may have obscured any other photo-induced activity. This drawback was avoided in the present study by using a preparation lacking any endogenous photoresponsiveness, the oesophageal TMM.

The present results demonstrate that the nitro group in the dihydropyridine ring is absolutely required for photoactivated responses

involving relaxation. If photoactivated release of NO from the 3'-NO$_2$-DHP molecule is to account for the photosensitizing activity of these compounds (Golenhofen et al., 1990), the present results are consistent with this possibility. By contrast, m- and p-nitrophenyl-substituted DHPs (nicardipine, nimodipine, nitrendipine and 4-(p-nitrophenyl)-1,4-DHP) did not photosensitize TMM preparations, suggesting that the nitro group in the phenyl ring may have a less favourable energetic configuration to allow for the release of NO by polychromatic light irradiation.

The o-nitrophenyl-substituted DHP, nifedipine, mediated a concentration-independent, long lasting photocontraction which could be differentiated by its duration from the 3'-NO$_2$-DHP-induced "off-contraction" in the TMM. Because only one such compound was tested, a definitive conclusion regarding o-nitrophenyl-substituted DHPs as a mediator of photocontractions is preliminary. However, it is noteworthy that Bay O 9073 which contains a nitro group on both the dihydropyridine and the phenyl rings, had a mixed photoactivated response intermediate between that of 3'-NO$_2$-DHP and nifedipine.

The concentration-independence of the nifedipine- and the Bay O 9073-photoactivated response suggests that photocontractions are most likely mediated by the photodecomposition of these molecules. In heart cell membranes, it has been shown that irradiation results in the rapid removal of

calcium channel blockade by nifedipine (Gurney et al., 1985) or nisoldipine, whereas the m-nitrophenyl DHP, nimodipine, is less photosensitive (Sanguinetti & Kass, 1984). This may also explain the effect of irradiation on tonus. Thus, in several cases, irradiation of nifedipine-sensitized preparations produced an increase in steady state tonus, whereas a decrement in tension was observed after Bay K 9073. This is consistent with the antagonistic effect of nifedipine on the TMM and the agonist effect of Bay K 9073.

4.2 Role of photoactivated release of NO in the 3'-NO$_2$-DHP-photoactivated response

Unlike vascular smooth muscle (Furchgott et al., 1961), the TMM and pyloric sphincter are devoid of any intrinsic photoactivated response to light-irradiation. Photosensitization with 3'-NO$_2$-DHP may be equivalent to supplying a phototransducer substance, i.e., a photo-induced relaxing factor, which is already present in vascular smooth muscle (Furchgott et al., 1985). Since photodegradable NO-donors mimicked the 3'-NO$_2$-DHP-photoactivated response 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$_2$-DHP would be expected to accumulate in the lipid phase of the plasmalemma and sarcoplasmic membrane system with a cell to medium ratio of 120:1 (Lüllmann & Mohr, 1987), permitting the effector structure(s) to be accessed more effectively than with hydrophilic NO donors. This would explain the high potency of 3'-NO$_2$-DHP agonists in comparison with the latter.

Pharmacological evidence also suggests that NO mediates the 3'-NO$_2$-DHP-photoactivated response in the TMM. Thus, agents that interfere with NO and cGMP production/degradation affected both the fast and slow components of the photoactivated response in the TMM. In particular, the NO scavenger LY

83583 and carboxy-PTIO inhibited the fast photorelaxation following photosensitization with low concentrations of the 3'-NO$_2$-DHP. However, at a maximal concentration of the 3'-NO$_2$-DHP, photorelaxations were minimally affected by LY 83583 and carboxy-PTIO. The fast photorelaxation was reduced following treatment with LY 83583 only after pre-incubation with the superoxide dismutase inhibitor diethyldithiocarbamate. To explain these results, the following factors should be considered: 1) the 3'-NO$_2$-DHP-photoactivated fast relaxation may be the result of a very sensitive photo-inducible step requiring few molecules of the phototransducer (NO) to produce relaxation, 2) the close proximity of the photoinducible NO-source to its target may preempt any effect of the NO-scavenger on the photoactivated response. Accordingly, NO scavengers are only effective at low concentrations of the 3' NO$_2$ DHP or when the superoxide production is presumably enhanced by inhibition of endogenous superoxide dismutase.

Although Hb inhibits the endothelium-dependent, acetylcholine mediated relaxation as well as the NO donor-augmented photorelaxation in vascular smooth muscle (Furchgott et al., 1984, 1985), in this study Hb was ineffective against the 3'-NO$_2$-DHP-photoactivated response. Since Hb does not cross the plasmalemmal barrier, one possible explanation is that Hb only affects NO released extracellularly. Therefore, Hb will not affect photoactivated release of

NO from 3'-NO_2-DHP which takes place at the plasmalemmal and sarcoplasmic membrane level.

Irradiation of the 3'-NO_2-DHP solution, similar to the NO-donor SNP, resulted in a decrease in the photosensitizing activity of the compound as indicated by the rightward shift in the dose-response curve. This is consistent with the hypothesis that the irradiated molecule will loose its photoactivity due to release of the active moiety mediating photorelaxation. As demonstrated by chemiluminescence analysis, this active moiety appears to be NO (Bauer & Fung, 1994). Irradiation of the 3'-NO_2-DHP solution was also accompanied by a depression of the maximal response. This effect would be consistent with racemization of the 3'-NO_2-DHP molecule and the formation of the L-type Ca' ' - channel antagonist analog. The similarity between the concentration-response curves for irradiated (+)-PN 202 791 and racemic PN 202 791 seems to corroborate this conclusion.

The results in rat thoracic aorta are also consistent with the hypothesis of photoactivated release of NO from 3'-NO_2-DHPs. Exposure to 3'-NO_2-DHP resulted in an increase in the slope of the fast and the final slow phases of the intrinsic photorelaxation. Thus, following low intensity irradiation, the slope of the fast phase of the 3'-NO_2-DHP-augmented photorelaxation was equivalent to the slope of the intrinsic photorelaxation at a higher irradiation intensity. This

would be expected if more molecules of the mediator were formed by irradiation of $3'-NO_2$-DHP-treated preparations. However, the slow phase only was augmented with increased duration of photostimulation. This suggests that the fast and the following slow phase may be regulated by a different NO-mediated mechanism. It is unlikely, however, that the final slow phase represents a change in the visco-elastic properties of the smooth muscle as proposed by Furchgott et al. (1961) since this phase was augmented by $3'$ NO, DHP treatment.

Pharmacological evidence in rat thoracic aorta further supports the conclusion that the $3'-NO_2$-DHP-augmented photorelaxation is mediated by photoactivated release of NO. As in the TMM, LY 83583 was an effective blocker of the photorelaxation enhanced by a low concentration of the $3'$ NO, DHP, Bay K 8643, but not when a high concentration of the $3'-NO_2$-DHP was used. Furthermore, after removal of the $3'-NO_2$-DHP from the bath, the augmented photorelaxation was greatly diminished by LY 83583. As previously shown by Mikkelsen et al. (1985a) and supported by the present results, removal of the $3'-NO_2$-DHP did not result in the loss of the photoactivated response following intermittent photostimulation for at least 5 h, probably reflecting the accumulation of these drugs in cell phospholipid membranes (Lüllmann & Mohr, 1987).

4.3 Effector mechanisms involved in the photoactivated response

The physiological action of NO in smooth muscle has been mainly related to the activation of sGC. However, NO release in living cells may mediate other effects apart from cGMP formation. NO has been implicated in the cytotoxic effects mediated by macrophage activation (Hibbs et al., 1988) or glutamate receptor stimulation in neurons (Dawson et al., 1993). Several pathways mediate this cytotoxicity via the formation of highly reactive radicals, alteration in the genetic material or binding to iron-sulphur-containing enzymes. Thus, formation of peroxynitrite radicals as the result of NO reaction with superoxide ion could mediate NO-induced neurotoxicity (Lipton et al., 1993). NO formation may also alter the genetic material in cellular organisms as a result of deamination of DNA (Wink et al., 1991). NO binding to iron-sulphur-containing enzymes may block oxidative phosphorylation. Thus, activation of peritoneal macrophages inhibits oxidative phosphorylation by a L-arginine-dependent mechanism (Drapier & Hibbs, 1988). However, these NO-mediated cytotoxic effects are unlikely to be involved in the photoactivated response by $3'-NO_2$-DHP since: 1) intermittent photostimulation for long periods did not lead to decreases in the photoactivated response and smooth muscle contractility; 2) the fast photorelaxation was insensitive to 0.1 mM cyanide. A similar concentration of cyanide completely inhibits mitochondrial cytochrome oxidase

(Griffiths & Wharton, 1961).

4.3.1 Role of cGMP in the 3'-NO₂-DHP-photoactivated response

Photolytic release of NO from the 3'-NO$_2$-DHP should lead to activation

of sGC and formation of cGMP since previous work has already revealed an

increase in cGMP as the result of polychromatic (Triggle & Bieger, 1991) or UV

light irradiation of 3'-NO$_2$-DHP-treated smooth muscle preparations (Baik et al.,

1994). In keeping with this hypothesis the cGMP-dependent PDE inhibitor,

zaprinast, enhanced both the fast and slow components of the photoactivated

response in the TMM, whereas LY 83583 still reversed the zaprinast mediated

enhancement of the slow photorelaxation. In rat thoracic aorta, the sGC

inhibitor, MB, significantly inhibited the photorelaxation supported by Bay K

8643, confirming recent results in porcine coronary artery (Baik et al., 1994)

and gastric fundus (Golenhofen et al., 1990).

Two additional cGMP-dependent pathways have been implicated in the

mechanism of intrinsic photoresponsiveness of smooth muscle. Whereas a

direct activation of guanylate cyclase by light has been inferred by Karlsson et

al. (1985) from the study of the endogenous photorelaxation in bovine

mesenteric artery, such a mechanism can be ruled out in the case of the TMM

since the photoactivated response is only observed in tissues sensitized with 3'

NO_2-DHPs. Wolin et al. (1991) have proposed that photorelaxation in pulmonary artery is associated with an increase in cGMP levels via a catalase-dependent activation of sGC. However, it is unlikely that a similar pathway plays any role in the 3'-NO_2-DHP-photoactivated response in oesophageal TMM since the photorelaxation was insensitive to cyanide. As a Fe^{3+}-containing enzyme, catalase would be inhibited by CN (Schonbaun & Chance, 1976).

This investigation also revealed a novel feature of the 3'-NO_2-DHP-induced response in smooth muscle: the escape or fade of the fast photorelaxation in the TMM, i.e., the decrement in the fast photorelaxation while irradiation was being applied. This phenomenon probably reflects a shift in the balance between cGMP production and degradation as suggested by the ability of zaprinast to prevent the escape of the fast photorelaxation in the TMM and to delay the appearance of phasic contractions in pyloric sphincter within the irradiation interval. Another possibility is that irradiation will deplete most of the available NO-releasing molecules in the vicinity of the target enzyme. However, the fade of the fast photorelaxation was still present in tissues photosensitized with a maximal concentration of the 3'-NO_2-DHP and remained sensitive to zaprinast, suggesting that NO availability may not be the limiting factor.

The escape of the photorelaxation in the TMM contrasts with the

photorelaxation (endogenous and 3'-NO$_2$-DHP-augmented) in rat aorta in which the relaxation remained for as long as irradiation was applied. Moreover, after 10 s irradiation, the intrinsic photorelaxation was still progressing and this was enhanced by 3'-NO$_2$-DHP. This suggests that the cGMP effect may continue after irradiation even when supposedly photoactivated release of NO should have declined. Despite conflicting evidence regarding the temporal correlation between post-stimulation intracellular cGMP levels and contractile force generation (Nakatsu & Diamond, 1989), the recovery of the relaxation could be related to the degradation of cGMP.

The pharmacological characteristics of the 3'-NO$_2$-DHP-photoactivated response in smooth muscle are consistent with NO as the primary step leading to photorelaxation. As inferred from the study of the photoactivated mechanical response in the TMM, however, the following cGMP-dependent steps could generate two kinetically different responses, i.e., a fast and a slow photorelaxation. As described below these two responses may point to the existent of different effector mechanisms.

4.3.2 Role of the plasmalemma in the 3'-NO$_2$-DHP-photoactivated fast response

3'-NO$_2$-DHPs have a dual character, combining activity as L-type Ca^{++}-channel modulators and as photosensitizing agents. The present data clearly indicate a qualitative and quantitative difference between 3'-NO$_2$-DHP agonist and antagonist-induced photosensitizing activity. With antagonists, the rate of photorelaxation was diminished and the "off"-contraction abolished. This difference was most evident at extracellular Ca^{++} concentrations below the physiological range. L-type Ca^{++}-channels are, therefore, likely to mediate the expression of the fast components of the photoactivated response.

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 rabbit aorta and porcine stomach fundus (Golenhofen et al., 1990), rat thoracic aorta (Triggle & Bieger, 1990) and porcine coronary artery (Baik et al., 1994). However, the explanation for these contrasting results may be found in the experimental conditions used in this study and those carried out previously:

1) whereas the present results were obtained in preparations precontracted with a depolarizing solution containing low concentrations of Ca^{++}, previous work was carried out in normal physiological solution, hence at high extracellular Ca^{++} concentration. Due to the high-affinity binding of DHP antagonists to

inactivated L-type Ca^{++}-channels (Bean, 1984), the use of a depolarizing solution may result in a higher binding rate of DHP antagonists to L type Ca^{++} channels as compared with that in a normal physiological buffer (Godfraind, 1992);

2) the use of L-type Ca^{++}-channel antagonists results in a decrease in tension output. Whereas this is without effect in preparations devoid of any endogenous photoresponsiveness (e.g., TMM), in vascular smooth muscle preparations this suggests an increase in photosensitizing activity. Thus, Triggle & Bieger (1990) found that the L-type Ca^{++}-channel antagonists PN 202-110 and nifedipine enhanced the endogenous relaxation in rat thoracic aorta. However, further studies with more rigorous controls for tonus dependence have failed to confirm these results (Bieger, personal communication). On the contrary, the present results revealed a different type of response in the case of nifedipine, which probably results from photolytic inactivation of this L-type Ca^{++} channel antagonist. Therefore, previous results in vascular smooth muscle describing the photosensitizing effect of L-type Ca^{++}-channel antagonists may have to be re examined, with proper controls for the endogenous and the $3'\text{-}NO_2$ DHP antagonist-augmented photorelaxation.

Past work in the 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 K^+-channel openers, BRL 34915 and pinacidil, and blocked by the L-type Ca^{++}-channel antagonists, nifedipine, (+)-PN 200-110 and verapamil, as well as by incubation in a depolarizing K^+ solution, suggesting the involvement of Ca^{++}-activated K^+ channels (Akbarali et al., 1988a,b). On the contrary, the 3'-NO_2-DHP-photoactivated fast response, albeit sensitive to L-type Ca^{++}-channel antagonists, was unaffected by incubation in a K^+-depolarizing medium. Therefore, calcium entry followed by activation of K^+-channels is unlikely to mediate the photorelaxation in the TMM.

These findings suggest that photostimulation may cause a decrease in the calcium current through the L-type Ca^{++}-channel. Consistent with this interpretation, extracellular calcium depletion as well as chemical skinning of TMM smooth muscle fibers significantly inhibited the fast photoresponse components. At appropriate levels of cholinoceptor stimulation, extracellular calcium chelation was effective in inhibiting the photoactivated response in K^+-depolarized TMM with little or no alteration in steady-state tonus. By contrast, the 3'-NO_2-DHP-augmented photorelaxation in rat aorta is not affected by L-type Ca^{++}- channel antagonists and is independent of extracellular [Ca^{++}] (Bieger & Triggle, 1990). Therefore, the 3'-NO_2-DHP-photoactivated response in rat aorta, unlike the fast photorelaxation in the TMM, does not appear to involve

plasmalemmal Ca^{++} channels.

The proposed mechanism of photoactivated L-type Ca^{++}-channel modulation by $3'$-NO_2-DHP agonists implies an increased probability of channel closure during relaxation. As argued above, NO acting through sGC is the primary step in this process. Activation of sGC will lead to cGMP formation and stimulation of cGMP-dependent protein kinase. Patch-clamp studies have pointed to an inhibition of calcium current (I_{Ca}) through L-type Ca^{++}-channels in rat thoracic aorta and rabbit pulmonary artery treated with the NO-donor SNP (Magliola & Jones, 1990, Clapp & Gurney, 1991). Moreover, polychromatic light irradiation of ($\pm$)-Bay K 8644-pretreated rat dorsal root and hippocampal neurons resulted in an inhibition of L-type Ca^{++}-channel current (Scott & Dolphin, 1988; O'Regan et al., 1990). However, these results cannot be extrapolated to explain the $3'$-NO_2-DHP-photoactivated response in the TMM. The inhibition of I_{Ca} following irradiation of neuronal preparations is partially or fully irreversible whereas in the TMM the photosensitized state persists for a long period (at least 5 h) following washout of the $3'$-NO_2-DHP from the bathing medium. Recently, it has been demonstrated that the effect of NO on L type Ca^{++}-channel activity plays an important role in vascular tonus regulation (Omote & Mizusawa, 1994). Thus, inhibition of NO release by endothelium removal or L-N^G-nitroarginine methyl ester-treatment of rabbit coronary arteries

results in spontaneous contractions sensitive to L-type Ca^{++}-channel blockade.

4.3.2.1 Regulation of the "off-contraction"

The fast photoactivated response in the oesophageal TMM consists of a biphasic relaxation-contraction sequence with the contraction occurring as a post-irradiation response. This "off-contraction" was also present in pyloric sphincter following photosensitization by $3'-NO_2$-DHPs. Similar post-irradiation contractions have been described in Bay K 8644-photosensitized fundic preparations (Golenhofen et al., 1990) and $NaNO_2$-treated rabbit stomach (Ehrreich & Furchgott, 1968). The post-irradiation stimulation in the TMM is not cholinergically mediated since methscopolamine failed to block it. This observation agrees with that of Ehrreich and Furchgott (1968) who first demonstrated the atropine-resistance of the "post-irradiation stimulation" in rabbit stomach.

Although the "off-contraction" showed a linear-dependence on the amplitude of the preceeding relaxation at irradiation periods $\leq$ 10 s, the "off-contraction" was still evident when tonus level did not allow for expression of the relaxation, i.e., at baseline level. In other words, the preceding events occurring during photostimulation triggered the "off-contraction" even when the photorelaxation was not manifested. The "off-contraction" was also inhibited

by the same procedures that inhibited the fast relaxation. It seems therefore that the sequence of relaxation-contraction is coupled and may reflect a regulatory process occurring at the level of the L-type Ca^{++}-channel.

An "off-contraction" following electrical stimulation-induced relaxation has been described in several gastrointestinal smooth muscles, including guinea pig sphincter of Oddi (Pauletzki et al., 1993), rat duodenum (Mule et al., 1990), guinea pig colon (Maggi & Giuliani, 1993), opossum and human lower esophageal sphincter and lower oesophagus (Preiksaitis et al., 1994). In most cases the relaxation seems to be mediated by a non-cholinergic, non-adrenergic mechanism. NO, prostaglandins and neuropeptides have been implicated as the neurotransmitters mediating the electrical stimulation-induced relaxation. Activation of non-cholinergic, non-adrenergic nerves also mediates hyperpolarization of the canine colon smooth muscle via NO release. This response also known as an inhibitory junction potential is followed by a post stimulus excitatory response (depolarization) (Ward et al., 1992).

Contrary to the nerve-mediated "off-contraction" in other tissues (Maggi & Giuliani, 1993; Pauletzki et al., 1993, Preiksaitis et al., 1994), the light induced "off-contraction" was TTX-insensitive and persisted in K^+-depolarized preparations maintained at subnormal extracellular calcium concentrations, suggesting that the photoactivated "off-contraction" is of myogenic origin.

Consistent with this conclusion, the present results show that the electrically evoked TTX-insensitive relaxation was also followed by a rebound contraction. By contrast, the nerve-mediated nitroxidergic relaxations lacked any post-stimulation contraction, suggesting that the preceding NO-mediated event is not required for the expression of the photoactivated "off-contraction". Whether this should be attributed to different actions of NO released intracellularly or extracellularly by photo- or electrical stimulation, respectively, requires further studies with photosensitizing compounds that are incapable of permeating the cell membrane. Therefore, it would be premature to rule out the involvement of the preceding photoactivated release of NO in the "off-contraction" considering that the post-irradiation response was not only evident with photodegradable NO donors but also inhibited by the NO scavenger LY 83583. Moreover, in smooth muscle preparations other than TMM, the TTX-sensitive, nitroxidergic relaxations are followed by a post-simulation contraction (Pauletzki et al., 1993, Preiksaitis et al., 1994). Furhtermore, in canine colon, L-N^G-nitroarginine methyl ester inhibits both the electrical stimulation evoked inhibitory junction potential and the following rebound (Ward et al., 1992).

Another conceivable explanation is that activation of a secondary mechanism via a cGMP-dependent pathway could be involved in the "off-contraction". As demonstrated in opossum oesophageal longitudinal muscle,

SNP produces a biphasic response, i.e, an initial transient relaxation followed by a indomethacin-sensitive, long lasting contraction (Saha et al., 1993). However, the $3'-NO_2$-DHP-photoactivated "off-contraction", albeit sensitive to LY 83583, does not seem to involve prostaglandins or thromboxane as demonstrated by the failure of indomethacin to modify this response. In addition, it seems that the cGMP sensitivity of this pathway may differ from that mediating photorelaxation since LY 83583 was more effective at inhibiting the contraction than the fast relaxation induced by a maximal concentration of the $3'$ NO_2 DHP.

4.3.3 Role of the sarcoplasmic reticulum in the 3'-NO$_2$-DHP-photoactivated response

Along with the plasmalemma, the SR is another primary structure controlling tonus generation in smooth muscle (van Breemen & Saida, 1989; Quian et al., 1992). Previously, McGonigle and Tallarida (1980) have demonstrated that UV irradiation of rabbit aortic microsomes is accompanied by increased calcium binding. This effect could underlie the decrease in tension elicited in precontracted rabbit aorta by photostimulation. In other words, photorelaxation in smooth muscle may involve Ca^{++}-sequestration by the SR.

Two mechanisms acting at the level of the SR are thought to regulate myoplasmic calcium concentration, and hence, tonus generation in smooth muscle: the SR Ca^{++}-ATPase and the ryanodine channel. The former controls calcium sequestration into the SR, whereas the ryanodine channel is responsible for calcium-induced calcium release from the SR. Ryanodine and cyclopiazonic acid are primary tools in the study of regulation of Ca^{++} by the SR. The natural alkaloid, ryanodine, locks the ryanodine channel in a subconductance state (Hwang & van Breemen, 1987), whereas cyclopiazonic acid inhibits the SR Ca^{++}-ATPase (Seidler et al., 1989). Both agents give rise to calcium depletion of the SR.

At first glance, the results with ryanodine and cyclopiazonic acid suggest that the 3'-NO$_2$-DHP-photoactivated fast response requires a functional SR.

However, since cyclopiazonic acid and ryanodine interfere with different calcium mechanisms, it is unlikely that the photoactivated response observed in TMM is the result of a direct interaction of the 3'-NO$_2$-DHP molecule with either the SR Ca^{++}-ATPase or the ryanodine channel. This conclusion was further confirmed in cyclopiazonic acid-pretreated TMM preparations where the (±) Bay K 8644-photoactivated response persisted while the SR function, as assessed by the phasic response to muscarinic acetylcholine receptor agonist challenge, was blocked. Nonetheless, at least for the SR Ca^{++}-ATPase there have been reports concerning the binding of DHPs (Zerning, 1990). A simpler explanation of the cyclopiazonic acid- and ryanodine-mediated inhibitory effect on the 3' NO$_2$-DHP-photoactivated response would be that swamping of the myoplasm with calcium mobilized from the SR masks any small change due to calcium influx/efflux across the plasmalemma. However, when the intracellular calcium store is depleted, a low concentration of extracellular calcium is sufficient to support photorelaxation even in the continued presence of cyclopiazonic acid.

The above hypothesis is consistent with observations on the dependence of the time course of the action of cyclopiazonic acid and ryanodine on the extracellular Ca^{++} concentration. In calcium-containing buffer both compounds produced a rapid inhibition of the photoactivated response, accompanied by an increase of tonus. The opposite situation occurred in nominally calcium free

solution, i.e., inhibition of the photoactivated response took significantly longer in the presence of either drug. Similarly, at physiological levels of extracellular Ca^{++} concentration, the calcium ionophore A 23187 inhibited the $3'-NO_2$-DHP-photoactivated response, probably because the massive calcium entry induced by the ionophore overwhelms the Ca^{++} buffering capacity of the SR. However, in nominally calcium-free medium the photoactivated response was barely affected by A 23187.

Consistent with previous results in rat thoracic aorta (Triggle and Bieger, 1991), sodium fluoride inhibited the ($\pm$)-Bay K 8644-photoactivated response in the TMM. Since fluoride ions inhibit the sarcoplasmic Ca^{++}-ATPase (Utegalieva et al., 1990), fluoride could mimic the cyclopiazonic acid-mediated effect on the photoactivated response. However, in TMM preparations bathed in a depolarizing, nominally calcium-free buffer, the cyclopiazonic acid-mediated effect on the photoactivated response was accompanied by a decline in tension, whereas under similar conditions fluoride mediated contraction. This could be the resulting effect of fluoride on several mechanisms mediating contraction in smooth muscle. As demonstrated by several studies, the fluoride-mediated contraction could involve G protein-mediated activation of the phospholipase C cascade and (Fermum et al., 1991; Murthy et al., 1992; Watson et al., 1988; Zeng et al., 1989) or an increase in the calcium sensitivity of the contractile

proteins (Adeagbo & Triggle, 1991).

Summarizing the above results, Ca^{++} sequestration in the SR is not directly involved in the $3'-NO_2$-DHP-photoactivated response in the TMM. However, drugs which alter the buffering capacity of the SR could indirectly interfere with the expression of the photoactivated response.

4.3.4 Involvement of other intracellular mechanisms in the 3'-NO$_2$-DHP-photoactivated slow relaxation

While the mechanisms of cGMP-induced relaxation in smooth muscle remain incompletely understood (for review see Lincoln, 1989), the present results show that the cGMP mediated slow photorelaxation is resistant to such factors as L-type Ca''-channel blockade, depolarization of the cell membrane or inhibition of the SR Ca''-ATPase and the ryanodine channel. Conceivably this response involves cGMP signalling at a substrate such as myosin light chain kinase or a direct regulation of the calcium sensitivity of the contractile elements, probably by a cGMP-dependent protein kinase (Nishimura & van Breemen (1989). This hypothesis is supported by the results obtained with the phosphatase inhibitor calyculin A. Several reports have demonstrated that calyculin A induces a slowly developing contraction in smooth muscle. This response is independent of extracellular calcium and occurs even in SR calcium-depleted preparations (Shibata et al., 1982; Hartshorne et al, 1989). It has been suggested that inhibition of the phosphatase shifts the equilibrium between phosphorylation and dephosphorylation of the contractile proteins towards phosphorylation, resulting in contraction. The calyculin A-induced contraction in calcium-depleted TMM was photosensitized by (+)-PN 202 791 and the resulting slow photorelaxation was enhanced by zaprinast. Therefore, the slow photorelaxation probably involves regulation of the contractile proteins via a

cGMP-dependent protein kinase.

In comparison with the fast photorelaxation, the lower amplitude, more shallow slope and longer duration of the slow photorelaxation suggest that regulation of the contractile proteins by a cGMP-dependent mechanism occurs at a lower velocity than does the modulation of L-type Ca^{++}-channel. This could be due to the different cellular structures where the slow photorelaxation and fast response take place, i.e., the myoplasma and the plasmalemma, respectively. If photoactivated release of NO occurs in the sarcolemmal membrane where $3'-NO_2$-DHPs accumulate, it is likely that diffusion into the myoplasma will take longer.

As revealed by the present study, several similarities exist between the slow photorelaxation in TMM and the $3'-NO_2$-DHP-augmented photorelaxation in rat thoracic aorta: 1) both types of relaxations are insensitive to extracellular calcium depletion (Triggle & Bieger, 1991), and 2) in both cases the relaxations also seem to be mediated by photoactivated release of NO followed by activation of sGC (Golenhofen et al., 1990; Triggle et al., 1991; Baik et al., 1994). Whether the intracellular mechanism of the intrinsic or the $3'-NO_2$ DHP augmented relaxation in rat thoracic aorta occurs via regulation of contractile protein phosphorylation remains to be determined.

4.3.5 Dependence of the 3'-NO$_2$-DHP-photoactivated response on the contractile stimuli and the active tonus

Mechanical activity of smooth muscle represents the final physiological output resulting from various membrane-mediated and intracellular events. Therefore, tension recording is an indirect measure of earlier biophysical and/or biochemical processes including change in membrane conductance, synthesis of intracellular messengers and activation of specific protein substrates. To study how different components of tonus generation are involved in the 3'-NO$_2$-DHP-photoactivated response, several stimuli were used to induce contraction. Each stimulus activated specific intracellular steps of the contractile processes allowing discrimination between those events which support photorelaxation and those which do not. Thus, K$^+$-induced tension is mediated by Ca^{++}-entry through voltage-gated channels, leading to stimulation of the contractile proteins, whereas muscarinic agonist-induced tonus may also involve activation of DHP-insensitive receptor-gated channels, IP$_3$-induced calcium release and secondary regulation of the contractile proteins by phosphorylation. Moreover, muscarinic acetylcholine receptor-induced tonus in TMM is partially sensitive to DHP antagonists suggesting that voltage-gated channels are also involved in tonus generation (Akbarali, 1987). On the contrary, in calcium-depleted preparations calyculin A-induced tonus may involve a shift in the phosphorylation/dephosphorylation state of the contractile proteins (Hartshorne

et al., 1989). The present results thus demonstrate that the fast and slow photorelaxations in the TMM depend on the contractile stimuli applied which could be related to the different components involved in tonus generation, i.e., L-type Ca^{++}-channels as well as other membrane-related and intracellular structures.

The proportion of the L-type Ca^{++}-channel component in the overall tension output may define the dependence of the 3'-NO_2-DHP photoactivated fast relaxation on active tonus. Thus, striking differences between high K^+ and CD-induced tonus are evident after expressing photorelaxations as a percent of active tonus. In the former condition, the magnitude of the photorelaxation remains virtually unchanged as tonus increases in a Ca^{++} concentration dependent-manner. On the contrary, the relative value of the photorelaxation is diminished as the CD-induced tonus increases. It is likely that differences in the intracellular mechanisms leading to regulation of the contractile proteins under either condition are responsible for this behaviour. Thus, several components could contribute to agonist-induced tonus and not all of them are subject to photorelaxation. Conceivably the fast photorelaxation is susceptible to that component of the contraction that depends on calcium influx though voltage gated channels. Therefore, the higher the CD-induced tonus, the higher the non photorelaxable components of contraction and the lower the photorelaxation as

expressed as a percent of tonus.

When expressed in absolute values of tension, the $3'$-NO_2-DHP-photoactivated fast relaxation shows a biphasic dependence on the level of active tonus with no apparent differences between high K^+- and muscarinic agonist-induced tension. The absolute amount of the photorelaxation increases almost in parallel as the tonus increases. However, after contraction reaches the maximal value, a further increase in muscarinic agonist or calcium concentration induces a decrease in both tonus and photorelaxation. One possible explanation for this observation could be a decrease in the sensitivity of the contractile proteins to calcium (Stull et all., 1990; Himpens & Casteel, 1987; Kitazawa & Somlyo, 1990). This mechanism termed Ca^{++}-induced desensitization may act to prevent adverse effects resulting from a prolonged increase in $[Ca^{++}]_i$. Although the molecular mechanism responsible for the Ca^{++}-induced desensitization of contraction in smooth muscle is not fully understood, one possible target is phosphorylation of MLCK via activation of calmodulin-dependent protein kinase. Thus, in rabbit renal arteries, a high level of α-adrenoceptor occupancy decreases the relaxant effect of nifedipine by increasing MLCK phophorylation (Ratz, 1993).

In contrast to smooth muscle preparations from either rat duodenum (Dave et al.,1979) or fundus (D. Bieger, personal communication), the $3'$-NO_2-

DHP-photoactivated response in the TMM persisted undiminished under depolarizing conditions and did not show dependence on extracellular Na^+ as reported for the endogenous relaxation in rabbit aortic smooth muscle (Raffa et al. , 1992). Thus, light still produced a response in tissue maintained in a high K^+-depolarizing solution and high concentrations of the Na^+/K^+-ATPase inhibitor ouabain did not affect the light-induced response in Tyrode's solution. Although the rat Na^+/K^+-ATPase is relatively insensitive to ouabain (Allen & Schwartz, 1969; Toda, 1974), a tenfold lower concentration than that used in this study was able to block the K^+-induced relaxation in rat tail artery (Webb & Bohr, 1978) and TMM (Akbarali et al., 1987).

4.4 Photoactivated response and neurogenic control of the TMM

One question which arose during the study of the photoactivated response in the TMM was the possibility that an endogenous transmitter was released following sensitisation with $3'-NO_2$-DHP. However, since the photoactivated response was also evident in a depolarizing medium and in the presence of TTX, a neurogenic component did not seem to be involved. As discussed below, the ineffectiveness of pharmacological drugs interfering with neurotransmitter action further corroborates this conclusion.

Several studies regarding neurogenic control of the TMM have demonstrated an extensive innervation by the parasympathetic nervous system (Bieger & Triggle, 1985). The cholinergic two-neuron pathway mediates contraction of oesophageal smooth muscle via activation of muscarinic receptors. Jacob and Tallarida (1977) proposed that UV irradiation interferes with the agonist-receptor complex resulting in relaxation of precontracted aortic strips from rabbit. However, muscarinic receptor-induced contractile activity per se does not appear to be a prerequisite for $3'-NO_2$-DHP-induced photoresponsiveness. Since photosensitization was observed in the presence of methscopolamine in TMM preparations bathed in Tyrode's or K^+-depolarizing buffer at appropriate concentrations of extracellular calcium to support a tonic contraction (> 1 μM), involvement of the agonist-receptor complex as a possible

target for the light-induced effect in TMM is unlikely.

Although histological studies reveal the presence of adrenergic fibers in the TMM, their physiological role is unknown (Akbarali, 1987). Stimulation of ß-adrenoceptors in TMM induces relaxation of the smooth muscle. However, incubation of precontracted preparations with guanethidine and the ß adrenoceptor antagonist propranolol do not produce any effect on the photoactivated response. Moreover, the adrenoceptor agonist isoprenaline was routinely tested in this study to determine the tissue's capability for relaxation. ß-adrenoceptor-mediated relaxation in smooth muscle could involve stimulation of Ca^{++}-efflux (Moore & Fay, 1993), stimulation of intracellular Ca^{++} sequestration (Casteels & Raeymaekers, 1979; Mueller & van Breemen, 1979), or decrease in the Ca^{++}-sensitivity of the contractile proteins by PKA phosphor ylation (Conti & Aldestein, 1981). In the TMM, none of these mechanisms seems to be involved in the fast photorelaxation since the isoprenaline-induced relaxation was preserved after blockade of the $3'-NO_2$-DHP-photoactivated fast response. With regard to the slow relaxation, it was still evident in ouabain treated or K^+-depolarized tissues, as well as, after inhibition of SR Ca^{++} ATPase, indicating that stimulation of Ca^{++} extrusion or sequestration is not involved in the slow photorelaxation. Therefore, the slow photorelaxation and the ß-adrenoceptor-mediated relaxation may share a similar mechanism of

relaxation based on a decrease in the Ca^{++}-sensitivity of the contractile proteins by phosphorylation via PKG and PKA, respectively.

5-HT release from mast cells may also regulate contractile tonus in TMM (Akbarali, 1987). Where 5-HT induces a hexamethonium-resistant, ketanserin-sensitive increase in tension in the distal portion of the TMM, relaxation of the TMM is most evident after a muscarinic-agonist-induced contractile response (Akbarali, 1987). 5-HT-induced relaxation in the TMM is ketanserin insensitive but it is blocked by the $5-HT_3$ receptor antagonist ICS 205-930 (Ohia et al., 1992). However, ketanserin or ICS 205-930 treatment of CD-precontracted TMM preparations does not prevent the ($\pm$)-Bay K 8644-photoactivated response.

4.5 Future experiments

The results and the hypotheses regarding the 3'-NO_2-DHP photoactivated response in the TMM raise several issues for future consideration.

1) In view of the postulated mechanism for the fast photorelaxation, regulation of the L-type Ca^{++}-channel current by cGMP in the TMM merits further study by electrophysiological methods.

2) Although photoactivated release of NO has been demonstrated with racemic Bay K 8644, corresponding evidence regarding other 3'-NO_2-DHP is lacking. Chemiluminescence analysis may help to determine whether photoactivated release of NO is a general property of the DHPs.

3) Nifedipine was the only authentic o-nitro-substituted DHP mediating photocontraction. It would be interesting to determine if other DHPs with an *o nitro* in the phenyl ring are able to evoke photocontractions in smooth muscle.

4) One important observation from this work is that the "off-contraction", widely observed in other gastrointestinal smooth muscles, is present after light stimulation. The nature of this response requires further exploration.

5) The presence of a 3'-nitro group on the dihydropyridine ring, but not on the phenyl ring is necessary to induce photorelaxation. It remains to be determined if other compounds containing a nitro group on other positions of the dihydropyridine ring are photosensitizers as well.

4.8 Summary

1) Exposure to DHP compounds give rise to different photoactivated responses in smooth muscle that depend on the chemical structure of the DHP molecule and differ from organ to organ.

2) DHP-induced photodynamic activity was observed only in compounds containing nitro-substituent. Photoactivated responses involving relaxation are mediated by $3'$-NO_2-DHPs whereas photosensitization by o-nitrophenyl-substituted DHPs cause contractions. As evidenced by contractile force generation, $3'$-NO_2-DHP-induced photorelaxations, but not o-nitrophenyl-substituted DHP-mediated photocontractions, are concentration-dependent. Compounds lacking any photodynamic activity but possessing L-type Ca^{++}-channel affinity can modify the $3'$-NO_2-DHP-photoactivated response in TMM.

3) The photoactivated response by $3'$-NO_2-DHP L-type Ca^{++}-channel agonists in TMM is mimicked by photodegradable NO-donors and consists of a fast relaxation-"off-contraction" sequence that is superimposed on a normally concealed slow relaxation.

4) In rat thoracic aorta, $3'$-NO_2-DHPs enhance the intrinsic photorelaxation.

5) An "off-contraction" is evident following photoinhibition of the muscarinic agonist-stimulated phasic contractions in $3'$-NO_2-DHP-treated pyloric

sphincter or following field stimulation-evoked TTX-insensitive relaxations, but not by electrical stimulation of intramural nitroxidergic nerves in TMM preparations.

6) The $3'-NO_2$-DHP-photoactivated response is sensitive to NO scavengers and cGMP-modulators, suggesting that photoactivated release of NO from the DHP molecule, followed by stimulation of sGC, is involved in the photoactivated response.

7) Cyclic GMP generation may lead to two kinetically different mechanical responses in the TMM: 1) a fast relaxation, highly sensitive to 1,4- DHP L-type Ca^{++}-channel antagonists, chelation of extracellular calcium, or skinning of the plasmalemma, suggesting a mechanism of calcium influx regulation via L-type Ca^{++}-channels; and 2) a slow relaxation probably mediated by regulation of contractile protein phosphorylation by a cGMP-dependent protein kinase.

8) Depleting the SR of calcium with ryanodine or cyclopiazonic acid as well as treatment with the calcium ionophore A 23187 inhibits the fast photoactivated response in the TMM.
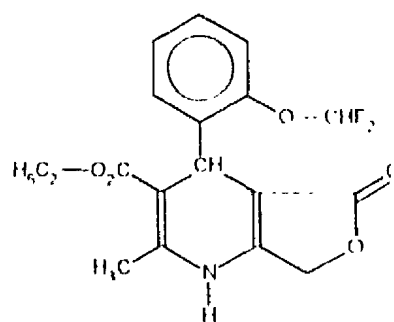
9) The amplitude of the fast relaxations varies with the contractile stimuli used and the level of contractile tension.

Appendix I
CHEMICAL STRUCTURES OF 1,4-DIHYDROPYRIDINES
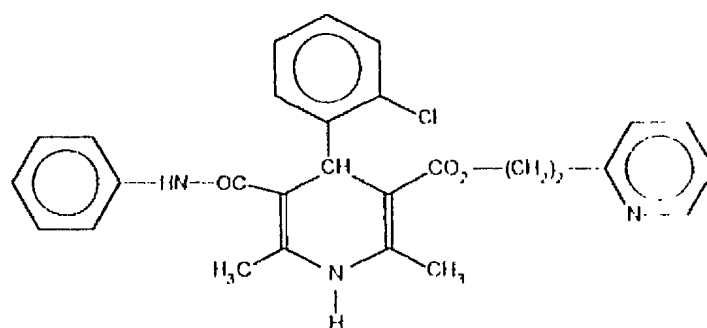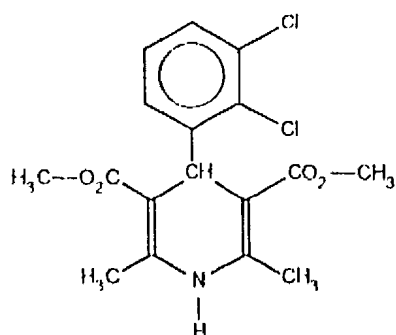

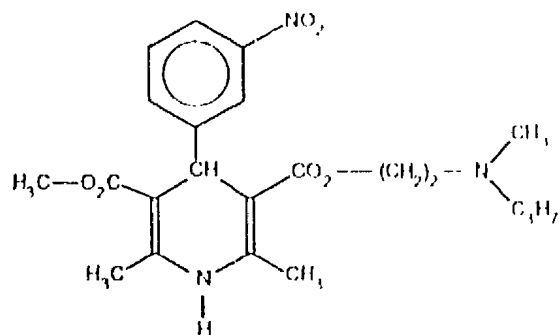Bay K 5417

Bay K 4407

Bay K 8643

(+) PN 202 791

(-)-PN 202 791

Bay O 9507
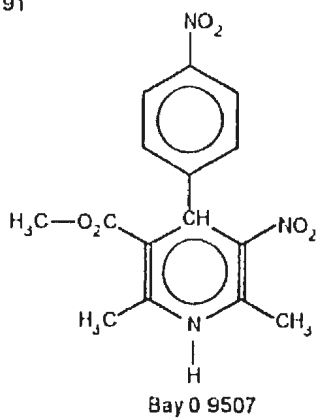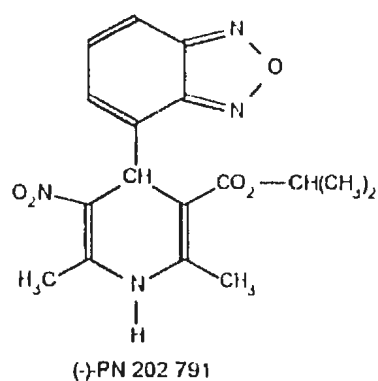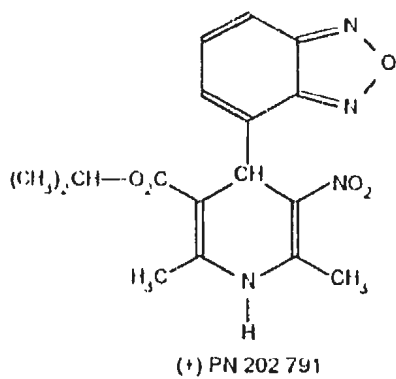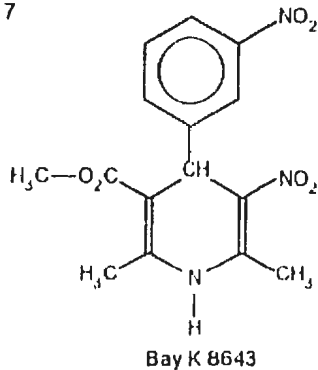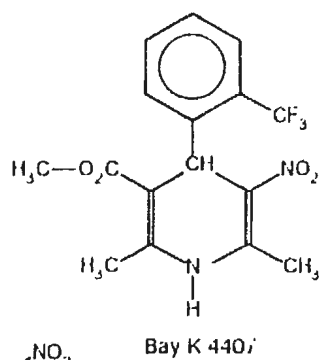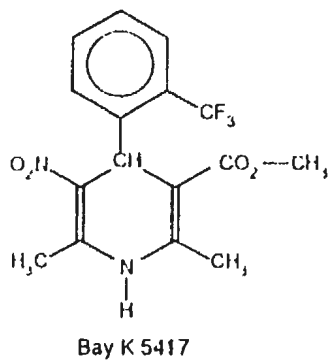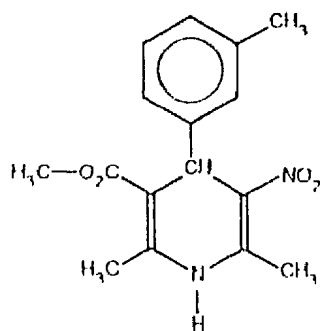
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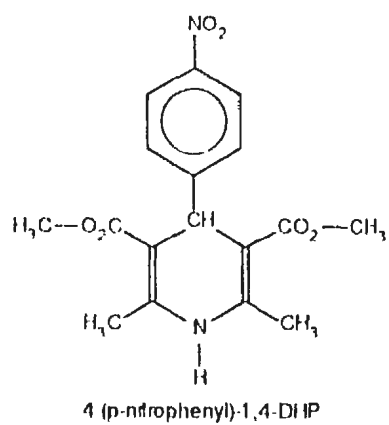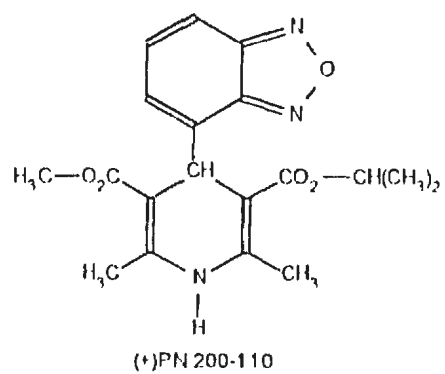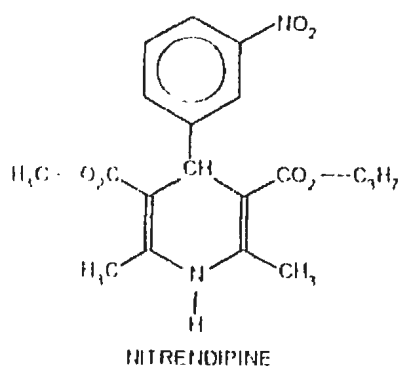
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(+)PN 200-110

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NIFEDIPINE

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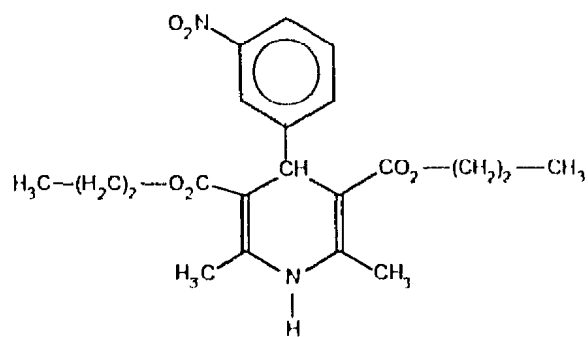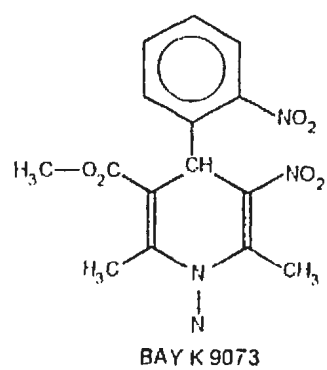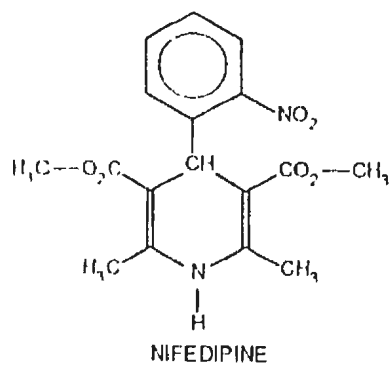
LIST OF REFERENCES

Adeagbo, A.S.O., Triggle, C.R (1991) Mechanism of vascular smooth muscle contraction by sodium fluoride in the isolated aorta of rat and rabbit. J. Pharmacol. Exp. Ther. 258 (1): 66-73.

Adelstein, R.S., Eisenber, E. (1980) Regulation and kinetics of the actin-myosin ATP interaction. Annu. Rev. Biochem. 49: 921-956.

Akbarali, H.I., Bieger, D., Ohia, S.E., Triggle, C.R. (1988a) Similarity of relaxations evoked by BRL 34915, pinacidil and field-stimulation in rat oesophageal tunica muscularis mucosae. Br. J. Pharmacol. 95: 519-525.

Akbarali, H.I., Bieger, D., Triggle, C.R. (1988b) Inhibition of field stimulation evoked relaxations in rat oesophageal smooth muscle by the calcium antagonist PN 200-110. Br. J. Pharmacol. 95: 512-518.

Akbarali, H.I. (1987) A pharmacological study of some relaxation processes in the rat oesophageal tunica muscularis mucosae. (Ph.D. Thesis) Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland.

Akbarali, H.I., Bieger, D., Triggle, C.R. (1986) Tetrodotoxin-sensitive and -insensitive relaxations in rat oesophageal tunica muscularis mucosea. J Physiol. 381: 49-63.

Allen, J.C., Schwartz, A. (1969) A possible biochemical explanation for the insensitivity of the rat to cardiac glycoside. J. Pharmacol. Exp. Ther. 168: 42-46.

Baik, Y.H., French, J.F., Schwartz, A., Rapoport, R.M. (1994) Dihydropyridine Ca^{2+} channel agonists and antagonists potentiate ultraviolet light induced relaxation through cyclic GMP formation in porcine coronary artery. J Cardiovasc. Pharmacol. 23: 785-791.

Baron, C.B., Pring M., Coburn, R.F. (1989) Inositol lipid turnover and compartmentation in canine trachealis smooth muscle. Am. J. Physiol. 256: C375-C383.

Bauer, J.A., Fung, H.L. (1994) Photochemical generation of nitric oxide from nitro-containing compounds: possible relation to vascular photorelaxation phenomena. Life Sci. 54: PL1-PL4.

Bean, B.P. (1984) Nitrendipine blocks the cardiac calcium channels: high affinity binding to the inactivated state. Proc. Natl. Acad. Sc. USA 81: 6388-6392.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freemar, B.A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA 87: 1620-1624

Benham C.D., Hess, P., Tsien, R.W. (1987) Two types of calcium channels in single smooth muscle cells from rabbit ear artery studies with whole-cell and single-channel recordings. Circ. Res. 61 (supl.1): I10-I16.

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R., Snyder, S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351: 714-718.

Bieger, D., Triggle, C.R. (1985) Pharmacological properties of mechanical responses of the rat oesophageal muscularis mucosae to vagal and field stimulation. Br. J. Pharmacol. 84: 93-103.

Bolton, T.B. (1979) Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59: 606-718.

Bond, M., Kitazawa, T., Somlyo, A.P., Somlyo, A.V. (1984) Release and recycling of Ca^{2+} by the sarcoplasmic reticulum in guinea-pig portal vein smooth muscle. J. Physiol. (London) 355: 677-695.

Borsotto, M., Barhanin, J., Fosct, M., Lazdunski, M. (1985) The 1,4-dihydropyridine receptor associated with the skeletal muscle voltage dependent Ca channel. Purification and subunit composition. J. Biol. Chem. 260: 14255-14263.

Bradley, A.B., Morgan, K.G. (1987) Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. J. Physiol. (London) 385: 437-448.

Bülbring, E., Tomita, T. (1987) Catecholamine action on smooth muscle. Pharmacol. Rev. 39: 49-69.

Casteels, R., Wuytack, F., Himpens, B., Raeymaekers, L. (1986) Regulatory systems for the cytoplasmic calcium concentration in smoooth muscle.

Biomed. Biochim. Acta 45: S147-S152.

Casteels, R., Raeymaekers, L. (1979) The action of acetylcholine and catecholamines on the intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. J. Physiol. 294: 51-68.

Chang, K.C., Chong, W.S., Park, B.W., Seung, B.W., Chun, G.W., Lee, I.J., Park, P.S. (1993) NO- and -NO_2 carrying molecules potentiate photorelaxation in rat trachea and aorta. Biochem. Biophys. Res. Commun. 191: 509-514.

Chaudhry, H., Lynch, M., Schomacker, K., Birngruber, R., Gregory, K., Kochevar, I. (1993) Relaxation of vascular smooth muscle induced by low-power laser irradiation. Photochem. Photobiol. 58 (5): 661-669.

Chen, K, Gillis, C.N. (1993) Methylene blue enhanced photorelaxation in aorta, pulmonary artery and corpus cavernosum. Biochem Biophys. Res. Commun. 190 (2): 559-563.

Chen, X., Gillis, C.N. (1992) Enhanced photorelaxation in aorta, pulmonary artery and corpus cavernosum produced by Bay K 8644 or N-nitro-L-arginine. Biomed. Biophys. Res. Commun. 186 (3): 1522-1527.

Cho, H.J., Xie, Q., Calaycay, J., Mumford, R.A., Swiderek, K.M. Lee, T.D., Nathan, C. (1992) Calmodulin is a subunit of nitric oxide synthase from macrophages. J. Exp. Med. 176: 599-604.

Clapp, L.H., Gurney, A.M. (1991) Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. Pflügers Arch. 418: 462-470.

Conti, M.A., Adelstein, R.S. (1981) The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3',5' cAMP-dependent protein kinase. J. Biol. Chem. 256: 3178-3181.

Craig, R., Smith, R., Kendrick-Jones, J. (1983) Light-chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. Nature 302: 436-439.

Curtis, B.M., Catterall, W.A. (1985) Phosphorylation of the calcium antagonist receptor of the voltage-sensitive Ca channel by cAMP-dependent protein

kinase. Proc. Natl. Acad. Sci. USA 82: 2528-2532.

Curtis, B.M., Catterall, W.A. (1984) Reconstitution of the calcium-antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. Biochem. 23: 2113-2118.

Daniel, E.E., Crankshaw, D.J., Kwan, C.Y. (1979) Intracellular sources of Ca^{2+} for activation of smooth muscle. In: Trends in Autonomic Pharmacology, ed. by S. Kalsner, vol. 1, pp. 443-484. Urban & Schwanzerberg, Baltimore.

Dave, K.C., Jindal, M.N., Kelkar, V.V., Trivedi, H.D. (1979) Characterization of ultraviolet light-induced relaxation of isolated duodenum of the rat. Br. J. Pharmac. 66: 197-201.

Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhl, G.R., Snyder, S.H. (1993) Mechanism of nitric oxide-mediated neurotoxicity in primary brain cultures. J. Neuroscience 13 (6): 2651-2661.

Dillon, P.F., Aksoy, M.O., Driska, S.P., Murphy, R.A. (1981) Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science 211: 495-497.

Drapier, J-C., Hibbs, J.B (1988) Differentiation of murine macrophages to express nonspecific cytotoxicity for tumour cells results in L-arginine-dependent inhibition of mitochondrial iron-sulphur enzymes in the macrophage effector cells. J. Immun. 140: 2829-2838.

Ehrreich, S.J., Furchgott, R.F. (1968) Relaxation of mammalian smooth muscle by visible and ultraviolet radiation. Nature 218: 682-684.

Fabiato, A. (1988) Computer programs for calculating total from specified free total ionic concentrations in aqueous solutions containing multiples metals and ligands. In: Methods in Enzymology, ed. S. Fleischer and B. Fleischer, vol. 157, pp. 378-417. Academic Press, San Diego.

Fabiato, A., Fabiato, F. (1979) Calculator programs for computing the composition of the solutions containinn multiple metals and ligands used for experiments in skinned muscle cells. J. Physiol. (Paris) 75: 463-505.

Fermum, R., Kosche, D., Moritz, K.U. (1991) Membrane-associated signal transduction modulates contractile responses to Ca^{2+} in saponin-skinned coronary smooth muscle. Naunyn-Schmiedebergs Arch. Pharmacol. 343

(2): 209-216.

Foyt, H.L., Guerriero, V., Means, A.R. (1985) Functional domains of chicken gizzard myosin light chain kinase. J. Biol. Chem. 260: 7765-7774.

Franckowiak, G., Bechen, M., Schramm, M., Thomas, G. (1985) The optical isomers of the 1,4-dihydropyridine Bay K 8644 show oppositive effects on the Ca channels. Eur. J. Pharmacol. 114: 223-226.

Furchgott, R.F., Jothianandan, D. (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 28: 52-61.

Furchgott, R.F. (1988) Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activable inhibitory factor from the retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Mechanisms of Vasodilation, pp. 401-414, ed. P.M. Vanhoutte. Raven Press, New York.

Furchgott, R.F., Martin, W., Cherry, P.D., Jothianandian, D., Villani, G.M. (1985) Endothelium-dependent relaxation, photorelaxation and cyclic GMP. In: 5th International Symposium, Vascular Neuroeffector Mechanisms, ed. J. Bevan, T. Godfraind, R. Maxwell, J.C. Stoclet & M. Worcel, pp. 105-114. Elsevier, Amsterdam.

Furchgott, R.F., Martin, W., Jothianandian, D., Villani, G.M. (1984) Comparison of endothelium-dependent relaxation by acetylcholine and endothelium-independent relaxation by light in rabbit aorta. In: Proceedings of the IUPHAP 9th Int. Congress of Pharmacology, ed. W. Paton, J. Mitchell & P. Turner, vol. 1, pp. 149-156. MacMillan Press, London.

Furchgott, R.F., Zawadski, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-376.

Furchgott, R.F., Ehrreich, S.J., Greenblat, E. (1961) The photoactivated relaxation of smooth muscle of rabbit aorta. J. Gen. Pharmacol. 44: 499-519.

Furchgott, R.F., Sleator, W., McCaman, M.W., Elchlepp, J. (1955) Relaxation of arterial strips by light, and the influence of drugs on this photodynamic effect. J. Pharmacol. Exp. Ther. 113: 22-23.

Glossmann, H., Ferry, D.R. (1983) Solubilization and partial purification of putative calcium channels labelled with [³H]-nimodipine. Naunyn-Schmeideberg's Arch. Pharmacol. 323: 279-291.

Godfraind, T. (1992) Analysis of factors involved in the tissue selectivity of calcium antagonist. In: Excitation-Contraction Coupling in Skeletal, Cardiac and Smooth Muscle, ed. G.B Frank, pp. 103-121. Plenun Press, New York.

Golenhofen, K. Finger, K.,Förster, B., Mandrek, K., Noak, T. (1990) Light-induced relaxation of smooth muscle after treatment with Bay K 8644 is related to release of nitric oxide. In: Frontiers in Smooth Muscle Research. p.597-604. Alan R. Liss Inc, New York.

Gräser, T., Hanschuk, L., Glusa, E. (1988) A new method for removal of vascular endothelium by saponin in isolated porcine coronary artery and art aorta. Biomed. Biochim. Acta 47: 79-82.

Griffiths, D.E., Wharton, D.C. (1961) Purification and properties of cytochrome oxidase. J. Biol. Chem. 236 (6): 1850-1856.

Gryglewski, R.J., Palmer, R.M.J., Moncada, S. (1986) Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320: 454-456.

Gurney, A.M., Nerbonne, J.M., Lester, H.A. (1985) Photoinduced removal of nifedipine reveals mechanisms of calcium antagonist action on single heart cells. J. Gen. Physiol. 86: 353-379.

Hartshorne, D.J., Ishihara, H., Karaki, H., Ozaki, H., Sato, K., Hori, M., Watabe, S. (1989) Okadaic acid and calyculin A: effects on smooth muscle system. Adv. Protein Phosphatases 5: 219-231.

Heisler, S. (1986) Calmodulin antagonists inhibit dihydropyridine calcium channel activator (Bay K 8644) induced cGMP synthesis in pituitary tumour cells. Can. J. Physiol. Pharmacol. 64: 760-763.

Hess, P., Lansman, J.B., Tsien, R.W. (1984) Different modes of Ca channel gating behaviour favoured by Ca agonist and antagonist. Nature 311: 538-544.

Hibbs, J.B., Taintor. R.R., Vavrin, Z, Rachlin, E.M. (1988) Nitric oxide: a cytotoxic

activated macrophage effector molecule. Biochem. Biophys. Res. Commun. 157: 87-94.

Himpens, B., Casteels, R. (1987) Measurement by Quin2 of changes of the intracellular calcium concentration in strips of the rabbit ear artery and of the guinea-pig ileum. Pflügers Arch. 408: 32-37.

Hosey, M.M., Borsotto, M., Lazdunski, M. (1986) Phosphorylation and dephosphorylation of the major component of the voltage dependent Ca channel in skeletal muscle membranes by cAMP and Ca-dependent processes. Proc. Natl. Acad. Sci. USA 83: 3733-3737.

Hwang, K.S., van Breemen, C. (1987) Ryanodine modulation of ^{45}Ca efflux and tension in rabbit aortic smooth muscle. Pflügers Arch. 408: 343-350.

Ignarro, L.J. (1991) Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. Blood Vessels 28: 67-73.

Ignarro, L.J., Byrns, R.E., Wood, K,S. (1988) Biochemical and pharmacological properties of endothelium-derived relaxant factor and its similarity to nitric oxide radical. In: Mechanisms of Vasodilation, pp. 427-435. ed. P.M. Vanhoutte. Raven Press, New York.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and veins is nitric oxide. Proc. Natl. Acad. Sci. USA. 84: 9265-9269.

Iino, M. (1990) Calcium release mechanism in smooth muscle. Japan. J. Pharmacol. 54: 345-354.

Jacob, L.S., Tallarida, R.J. (1977) Further studies on the action of ultraviolet light on vascular smooth muscle: effect of partial reversible receptor blockade. Arch. Int. Pharmacodyn. 225: 166-176.

Jakes, R., Northrop, F., Kendric-Jones, J. (1976) Calcium binding regions of myosin 'regulatory' light chains. FEBBS Lett. 70: 229-234.

Kameyama, M., Heschler, J., Hofmann, F., Trautwein, W. (1986) Modulation of Ca current during phosphorylation cycle in guinea pig heart. Pflügers Arch. 407: 123-128.

Kameyama, M., Hoffmann, F., Trautwein, W. (1985) On the mechanism of ß-

adrenergic regulation of the Ca channel in the guinea-pig heart. Pflüegers Arch. 405: 285-293.

Kargacin, G.J., Detwiler, P.B. (1985) Light-evoked contraction of the photosensitive iris of the frog. J. Neuroscience 5 (11): 3081-3087.

Karlsson, J.O.G.,Axelsson, K.L., Andersson, R.G.G.(1985) Effects of hydroxyl radical scavengers KCN and CO on ultraviolet light-induced activation of crude soluble guanylate cyclase. J. Cycl. Nucleot. Protein Phosphoryl. 10 (4): 309-315.

Karlsson, J.O.G.,Axelsson, K.L., Andersson, R.G.G.(1984) Effects of ultraviolet radiation on the tension and the cyclic GMP levels of bovine mesenteric arteries. Life Sci. 34: 1555-1563.

Kaupp, U.B., Koch, K.W. (1992) Role of cGMP and Ca^{2+} in vertebrate photoreceptor excitation and adaptation. Annu. Rev. Physiol. 54: 153-175.

Kelm, M., Schrader, J. (1988) Nitric oxide release from the isolated guinea-pig heart. Eur. J. Pharmacol. 155: 313-316.

Kitazawa, T., Somlyo, A.P. (1991) Modulation of Ca^{2+} sensitivity by agonists in smooth muscle. In: Regulation of Smooth Muscle Contraction, ed. by R.S. Moreland, pp 97-109. Plenum Press, New York.

Kitazawa, T., Somlyo, A.P. (1990) Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca^{2+} in smooth muscle. Biochem. Biophys. Res. Commun. 172: 1291-1297.

Kitazawa, T., Kobayashi, S., Horiuti, K., Somlyo, A.V., Somlyo, A.P. (1989) Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca^{2+}. J. Biol. Chem. 264: 5339-5342.

Khan, M.T., Furchgott, R.T. (1987) Additional evidence that endothelium-derived relaxing factor is nitric oxide. In: Pharmacology, ed. M.J. Rand & C. Roper, pp. 341-344. Elsevier, Amsterdam.

Kobayashi, S., Kitazawa, T., Somlyo, A.V., Somlyo, A.P. (1989) Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. J. Biol. Chem. 264: 17997-18004.

Kokubun, S., Reuter, H. (1984) Dihydropyridine derivates prolong the open state of the Ca channels in cultured cardiac cells. Proc. Natl. Acad. Sci. USA 81: 4824-4827.

Kubaszewski, E., Peters, A., McClain, S., Bohr, D., Malinski, T. (1994) Light activated release of nitric oxide from vascular smooth muscle of normotensive and hypertensive rats. Biochem. Biophys. Res. Commun. 200 (1): 213-218.

Lamas, S., Marsden, P.A., Li, G.K., Tempst, P., Michel, T. (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc. Natl. Acad. Sci. USA 89: 6345 6352.

Langan T.A. (1973) Protein kinases and protein kinases substrates. Adv. Cycl. Nucleot. Res. 3: 99-153.

Lincoln, T.M (1989) Cyclic GMP and mechanisms of vasodilation. Pharmac. Ther. 41: 479-502.

Lipton, S.A., Choi, Y.B, Pan, Z.H., Lei, S.Z., Chen, H.S.V., Sucher, N.J., Loscalzo, J., Singel, D.J., Stamler, J.S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature, 364: 626-632.

Lukas, T.J., Burges, W.H., Prendergast, F.g., Lau, W., Watterson, D.M. (1986) Calmodulin-binding domains: characterization of a phosphorylation and calmodulin binding site from myosin light chain kinase. Biochemistry 25: 1458-1464.

Lüllmann, H., Mohr, K. (1987) High and concentration-proportional accumulation of [^{3}H]-nitrendipine by intact cardiac tissue. Br. J. Pharmacol. 90: 567 573.

Lyons, C.R., Orloff, G.J., Cunningham, J.M. (1992) Molecular cloning and functional expression of an inducible nitric oxide synthase from murine macrophage cell line. J. Biol. Chem. 267: 6360-6374.

Magliola, L., Jones, A.W. (1990) Sodium nitroprusside alters Ca^{2+} flux components and Ca^{2+}-dependent fluxes of K' and Cl in rat aorta. J. Physiol. (London) 421:411-424.

Maggi, C.A., Giuliani, S. (1993) Multiple inhibitory mechanisms mediate non

adrenergic non-cholinergic relaxation in the circular muscle of the guinea-pig colon. Naunyn-Schmiedeberg's Arch. Pharmacol. 347: 630-634.

Martin, W., Furchgott, R.F., Villani, G.M., Jothianandam, D. (1986) Depression of the contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor (EDRF). J. Pharmacol. Exp. Ther. 237: 529-538.

Matsunaga K., Furchgott, R.F. (1991) Responses of rabbit aorta to nitric oxide and superoxide generated by ultraviolet irradiation of solutions containing inorganic nitrate. J. Pharmacol. Exp. Ther. 259 (3): 1140-1146.

Matsunaga, K., Furchgott, R.F (1989) Interactions of light and sodium nitrite in producing relaxation of rabbit aorta. J. Pharmacol. Exp. Ther. 248:687-695.

McCarron, J.G., McGeown, J.G., Reardon, S., Ikebe, M., Fay, F.S., Walsh, J.V. (1992) Calcium-dependent enhancement of calcium current in smooth muscle by calmodulin-dependent protein kinase II. Nature 357: 74-77.

McGonigle P., Tallarida, R.J. (1980) The effect of ultraviolet radiation and angiotensin on calcium binding in rabbit aorta microsomes. Arch. Int. Pharmacodyn. Ther. 248: 26-32.

Messer, N.G., Kendric-Jones, J. (1988) Molecular cloning and sequencing of the chicken smooth muscle myosin regulatory light chain. FEBS Lett. 234: 49-52.

Miesheri, K.D., Rüegg, J.C. (1983) Dependence of cAMP induced relaxation on Ca'' and calmodulin in skinned muscle of guinea pig taenia coli. Plüegers Arch. 399: 315-320.

Mikkelsen, E.O., Nyborg, N.G.B., (1986) Comparison of the effects of the vasoconstrictive dihydropyridines Bay K 8644 and CGP 28392 on isolated rat aorta: different sensitivity to ultraviolet radiation. J. Card. Pharmacol. 8: 476-486.

Mikkelsen, E.O., Kazda, S., Nyborg, N.C.B. (1985a) Effects of light and Bay K 8644, a new 1,4-dihydropyridine on mechanical response of rat thoracic aorta. Acta Pharmacol. Toxicol. 56: 126-132.

Mikkelsen, E.O., Nyborg, N.C.B., Jacobsen, P. (1985b) A comparison of effects

of Bay K 8644 and CGP 28392, 1,4-dihydropyridines with calcium agonist properties on spontaneous mechanical activity in rat portal vein: modification of effect by UV radiation. Acta Pharmacol. Toxicol. 57: 340-344.

Miller, R.J. (1987) Multiple calcium channels and neuronal function. Science 235: 46-52.

Moncada, S., Palmer, R.M., Higgs, E.A. (1989) Biosynthesis of nitric oxide from L-arginine. Biochem. Pharmacol. 38 (11): 1709-1715.

Moore, E.D.W, Fay, F.S. (1993) Isoproterenol stimulates rapid extrusion of sodium from isolated smooth muscle. Proc. Natl. Acad. Sci. USA 90: 8058-8062.

Morgan, J.P., Morgan, K.G. (1982) Vascular smooth muscle: the first recorded Ca' ' transients. Pflügers Arch. 2395: 75-77.

Morgan, M., Perry, V.S., Ottaway, J. (1976) Myosin-light-chain phosphatase. Biochem. J. 157: 687-697.

Mueller, E., van Breemen, C. (1979) Role of intracellular Ca²' sequestration in ß-adrenergic relaxation in smooth muscle. Nature 281: 682-683.

Mule, P., Serio, R., Postorino, A. (1990) Evidence that adenosine is not involved in the non-adrenergic, non-cholinergic relaxation in the rat duodenum. Arch. Int. Physiol. Biochem. 98 (4): 149-154.

Murad, F., Mittal, C.K., Arnold, W.P., Katsuki, S., Kimura, H. (1978) Guanylate cyclase: activation by azide, nitro compounds, nitric oxide and hydroxyl radical and inhibition by haemoglobin and myoglobin. Adv. Cyclic Nucleotides Res. 9: 145-158.

Murthy, K.S., Grider, J.R., Makhlouf, G.M. (1992) Receptor-coupled G proteins mediate contraction and Ca' ' mobilization in isolated intestinal muscle cells. J. Pharmacol. Exp. Ther. 260 (1): 90-97.

Nakatsu, K., Diamond, J. (1989) Role of cGMP in relaxation of vascular and other smooth muscle. Can. J. Physiol. Pharmacol. 67: 251-262.

Nelson, M.T., Patlak, J.B., Worley, J.F., Standen, N.B. (1990) Calcium channels, potassium channels and voltage dependence of arterial smooth muscle tone.

Am. J. Physiol. 259: C3-C18.

Nishimura, J., Khali, R.A., Drenth, J.P., van Breemen, C. (1990) Evidence for increased myofilament Ca⁺⁺ sensitivity in norepinephrine-activated vascular smooth muscle. Am. J. Physiol. 259: H2-H8.

Nishimura, J., van Breemen, C., (1989) Direct regulation of smooth muscle contractile elements by second messenger. Biochem. Biophis. Res. Commun. 163: 929-935.

Nishimura, J., Kolber, M., van Breemen, C. (1988) Norepinephrine and GTP-γ-S increase myofilament Ca⁺⁺ sensitivity in α-toxin permeabilized arterial smooth muscle. Biochem. Biophys. Res. Commun. 157: 677-683.

Nuñez-Vergara, L.J., Sunkel, C., Squella, J.A. (1994) Photodecomposition of the new 1,4-dihydropyridine: furnidipine. J. Pharmac. Sc. 83 (4): 502-507.

Ohia, S.E., Cheung, Y.D., Bieger, D., Triggle, C.R. (1992) Pharmacological profile of the 5-hydroxytryptamine receptor that mediates relaxation of rat oesophageal smooth muscle. Gen. Pharmacol. 23 (4): 649-658.

Omote, M., Mizusawa, H. (1994) Endothelium-derived nitric oxide protection of endogenous coronary contraction in the rabbit by countering Ca²⁺ influx via voltage-sensitive Ca²⁺ channel. Life Sci. 55 (8): 157-161.

O'Regan, M.H., Kocsis, J.D., Waxman, S.G. (1990) Depolarization-dependent actions of dihydropyridines on synaptic transmission in the in vitro rat hippocampus. Brain Res. 527: 181-191.

Palmer, R.M.J., Moncada, S. (1989) A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cell. Biochem. Biophys. Res. Commun. 158: 348-352.

Palmer, R.M, Ferrige, A.G., Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-relaxing factor. Nature 327: 524-526.

Pato, M.D., Adelstein, R.S. (1981) Characterization of myosin phosphatase. Biophys. J. 33: 278a.

Pauletzki, J.G., Sharkey, K.A., Davison, J.S., Bomzon, A., Shaffer, E.A. (1993)

Involvement of L-arginine-nitric oxide pathways in neuronal relaxation of the sphincter of Oddi. Eur. J. Pharmacol. 232 (2-3): 263-270.

Payne, M.E., Elzinga, M., Adelstein, R.S. (1981) Smooth muscle myosin light chain kinase. Amino acid sequence at the site of phosphorylated by adenosine cyclic 3',5'-phosphate- dependent protein kinase whether or not calmodulin is bound. J. Biol. Chem. 261: 16346-16350.

Pfitzer, G., Rüegg, J.C., Flockerzi, V., Hofmann, F. (1982) cGMP-dependent protein kinase decreases calcium sensitivity of skinned cardiac fibers. FEBS Lett. 149: 171-175.

Preiksaitis, H.G., Tremblay, L., Diamant, N.E. (1994) Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. Digestive Diseases & Sc. 39 (4): 770-775.

Quian, C., Cannell, M., van Breemen, C. (1992) The superficial buffer barrier in vascular smooth muscle. Can. J. Physiol. Pharmacol. 70: 509-514.

Raeymaekers, L., Hofmann, F., Casteels, R. (1988) Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. Biochem. J. 252: 269 273.

Raeymaekers, L., Jones, L.R. (1986) Evidence for the presence of phospholamban in the endoplasmic reticulum of smooth muscle. Biochim. Biophys. Acta 882: 258-265.

Raffa, R.B., Aceto, J.F., Goldschmidt, J.E., Tallarida, R.J. (1991) The effect of lowered extracellular Na^+ concentration on ultraviolet light-induced relaxation of vasoconstricted rabbit isolated thoracic aorta. Life Sci. 51: PL 249-254.

Rapoport, R.M., Mured, F. (1983) Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be madiated through cGMP. Circ. Res. 52 (3): 352-357.

Ratz, P.H. (1993) High α_1-adrenoceptor occupancy decreases relaxing potency of nifedipine by increasing myosin light chain phosphorylation. Circ. Res. 72 (6): 1308-1316.

Rembold, C., Murphy, R.A. (1988) Myoplasmic [Ca^{++}] determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. Circ.

Res. 63: 593-603.

Saeki, T., Saito, I. (1993) Isolation of cyclic nucleotide phosphodiesterase isozymes from pig aorta. Biochem. Pharmacol. 46 (5): 833-839.

Saha, J.K., Hirano, I., Goyal, R.K. (1993) Biphasic effect of SNP on opossum esophageal longitudinal muscle: involvement of cGMP and eicosanoids. Am. J. Physiol. 265 (2 Part 1): G403-G407.

Sanguinetti, M.C., Kass, R.S. (1984) Photoalteration of calcium channel blockade in cardiad Purkinje fiber. Biophys. J. 45: 873-879.

Schonbaun, G.R., Chance, B. (1976) Catalase. In: The Enzymes. ed. P.D Bayer (3r. edition) pp. 363-408. Academic Press, New York.

Scott, R.H., Dolphin, A.C. (1988) The agonist effect of Bay K 8644 on neuronal calcium channel is promoted by G-protein activation. Nueroscience Lett. 89: 170-175.

Seidler, N.W., Jona, I., Vegh, M., Martonosi, A. (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of the sarcoplasmis reticulum. J. Biol. Chem. 264: 17816-17823.

Shibata, S., Ishida, Y., Kitano, H., Ohizumi, Y., Habon, J., Tsukitani, Y., Kikuchi, H. (1982) Contractile effects of okadaic acid, a novel ionophore-like substance from black sponge, on isolated smooth muscles under conditions of Ca^{++} deficiency. J. Pharmacol. Exp. Ther. 223: 135-143.

Somlyo, A.V., Bond, M., Somlyo, A.P., Scarpa, A. (1985) Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. Proc. Natl. Acad. Sci. USA. 82: 5231-5235.

Somlyo, A.V. (1980) Ultrastructure of vascular smooth muscle. In: The Handbook of Physiology: Vascular Smooth Muscle. ed. D.F. Bohr, A.P. Somlyo, H.V. Sparks, pp. 33-67. Am. Physiol. Soc., Williams & Wilkins Co, Baltimore.

Somlyo, A.P., Somlyo, A.V., Ashton, F.A., Vallieres, I. (1976) Vertebrate smooth muscle: ultrastructure and function. Cold Spring Harbor Conf. Cell Proliferation, vol. 3, Cell Motility, 165-183.

Somlyo, A.P., Somlyo, A.V. (1968) Electromechanical and pharmacomechanical coupling in vascular smooth muscle. J. Pharmacol. Exp. Ther. 59: 129-145.

Sobue, K., Muramoto, Y., Fujita, M., Kakiuchi, S. (1981) Purification of a calmodulin-binding protein from chicken gizzard that interacts with F actin. Proc. Natl. Acad. Sci. USA 78: 5652-5655.

Sparrow, M.P., Pfitzer, G., Gagelmann, M., Rüegg, J.C. (1984) Effect of calmodulin, calcium and cAMP protein kinase on skinned tracheal smooth muscle. Am. J. Physiol. 246: C308-C314.

Steg, P.G., Gal, D., Rongione, A.J., DeJesus, S.R., Clarke, R.H., Levine, H.J., Isner, J.M. (1988) Effect of argon laser irradiation on rabbit aortic smooth muscle: evidence for endothelium independent contraction and relaxation. Cardiovasc. Res. 22: 747-753.

Stull, J.T., Hsu, Tansey, M.G., Kamm, K.E. (1990) Myosin light chain kinase phosphorylation in tracheal smooth muscle. J. Biol. Chem. 265: 16683 16690.

Sturek, M., Hermsmeyer, K. (1986) Calcium and sodium channels in spontaneously contracting vascular smooth muscle cells. Science 233: 475-478.

Takahashi, K., Hiwada, K., Kokubu, T. (1986) Isolation and characterization of a 34 kDa calmodulin- and F-actin binding protein from chicken gizzard. Biochem. Biophys. Res. Commun. 141: 20-26.

Taniguchi, J., Furukawa, K.I., Shigekawa, M. (1993) Maxi K' channels are stimulated by cyclic guanosine monophosphate-dependent protein kinase in canine coronary artery smooth muscle cells. Pflügers Arch. 423; 167 172.

Thornbury, K.D., Ward, S.M., Dalziel, H.H., Carl, A., Westfall, D.P., Sanders, K.M. (1991) Nitric oxide and nitrosocysteine mimic nonadrenergic, noncholinergic hyperpolarization in canine proximal colon. Am. J. Physiol. 261: G553-G557.

Toda, N. (1974) Responsiveness to potassium and calcium ions of isolated cerebral arteries. Am. J. Phyiol. 227: 1206-1211.

Torphy, T.J., Undem, B.J., Cieslinski, L.B., Luttmann, M.A., Reeves, M.L., Hay, D.W.P. (1993) Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. J. Pharmacol. Exp. Ther. 265 (3): 1213-1223.

Torphy, T.J., Cieslinski, L.B. (1990) Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. Mol. Pharmacol. 37 (2): 206-214.

Triggle, C.R., O'Neill, S.K., Bieger, D. (1991) NO: role in dihydropyridine-enhance photorelaxation of vascular smooth muscle. In: Resistance Arteries, Structure and Function. ed. M.J. Mulvaney, pp. 2333-237. Elsevier, Amsterdam.

Triggle, C.R., Bieger, D. (1990) Can dihydropyridines enhance photorelaxation of smooth muscle by a calcium-independent mechanism? Proc. West. Pharmacol. Soc. 33: 227-233.

Triggle, D.J., Langs, D.A., Janis, R.A. (1989) Ca'' channel ligands: structure-function relationships of the 1,4-dihydropyridines. Med. Res. Rev. 9: 123-180.

Triggle, D.J. (1972) Effects of calcium on excitable membranes and neurotransmitter function. Prog. Mem. Surface Sci. 5: 267-325.

Twort, C.H.C., van Breemen C. (1988) Cyclic guanosine monophosphate-enhanced sequestration of Ca'' by sarcoplasmic reticulum in vascular smooth muscle. Circ. Res. 62: 961-964.

Utegalieva, R.S., Ivashchenko, V.I., Esyrev, O.V. (1990) Competitive inhibition of Ca-ATPase in the sarcoplasmic reticulum by sodium fluoride. Ukr. Biokhim. Zh. 62 (2): 43-48.

van Breemen, C., Saida, K., (1989) Cellular mechanism regulating |Ca''|, in smooth muscle. Ann. Rev. Physiol. 51: 315-329.

Venturini, C.M., Palmer, R.M.J., Moncada, S. (1993) Vascular smooth muscle contains a depletable store of a vasodilator which is light-activated and restored by donors of nitric oxide. J. Pharmacol. Exp. Ther. 266 (3): 1497-1500.

Watson, S.P., Stanley, A.F., Sasaguri, T. (1988) Does the hydrolysis of inositol phospholipids lead to the opening of voltage operated Ca^{2+} channels in guinea- pig ileum? Studies with fluoride ions and caffeine. Biochem Biophys. Res. Commun. 153 (1): 14-20.

Walsh, M.P. (1985a) Limited proteolysis of smooth muscle myosin light chain

kinase. Biochemestry 24: 3724-3730.

Walsh, M.P. (1985b) Calcium regulation of smooth muscle contraction. In: Calcium and Cell Physiology, ed. D. Marme, pp. 170-203. Springer Varlag, Berlin.

Ward, S.M., Dalsier, H.H., Thornbury, K.D., Westfall, D.P., Sanders, K.M. (1992) Nonadrenergic, noncholinergic inhibition and rebound in canine colon depend on nitric oxide. Am. J. Physiol. 262: G237-G243.

Webb, R.C., Bohr, D.R. (1978) Potassium-induced relaxation as an indicator of the Na/K-ATPase activity in vascular smooth muscle. Blood Vessels 15: 198-201.

Will , S., Bieger, D., Triggle, C.R. (1990) NO: possible role in TTX-sensitive field stimulated relaxations of the rat oesophageal tunica muscularis mucosae? Eur. J. Pharmacol. 183 (6): 2419-2420.

Wink, D.A., Kasprzak, K.S., Maragos, C.M. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254: 1001 1003.

Wolin, M.S., Omar, H.A., Mortelliti, M.P., Cherry, P.D. (1991) Association of pulmonary photorelaxation with H_2O_2 metabolism by catalase. Am. J. Physiol. 261 (4 part 2): H1141-H1147.

Yabu, H., Obara, K., Usuki, T. (1992) Calcium and potassium regulation by protein phosphorylation in smooth muscle cells of guinea pig taenia coli In: Excitation-Contraction Coupling in Skeletal, Cardiac and Smooth Muscle, ed. G.B. Frank, pp. 41-52. Plenum Press, New York.

Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T. (1987) Complete primary structure of vertebrate smooth muscle myosin heavy chain deduced from its complementary DNA sequence. Implications on topography and function of myosin. J. Mol. Biol. 198: 143-157.

Yatani, A., Codina, J., Imoto, Y., Reeves, J., Birnhaumer, L., Brown, A.M. (1987) A G protein directly regulates mammalian cardiac calcium channels. Science 238: 1288-1292.

Zerning, G. (1990) Widening potential for Ca' ' antagonists: non L type Ca' ' channel interaction. Trends Pharmacol. Sc. 11: 38-44.

Zanocco, A.L., Diaz, L., Lopez, M., Nuñez-Vergara, L.J., Squella, J.A. (1992) Polarographic study of the photodecomposition of nimodipine. J. Pharmac. Sc. 81 (9): 920-924.

Zeng, Y.Y., Benishin, C.G., Pang, P.K. (1989) Guanine nucleotide binding proteins may modulate gating of calcium channels in vascular smooth muscle. I. Studies with fluoride. J. Pharmacol. Exp. Ther. 250 (1): 343-351.