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AN EVALUATION OF THE POSSIBLE BIOCHEMICAL MECHANISMS OF
NITROGLYCERIN TOLERANCE IN SMOOTH MUSCLE: SULFHYDRYL
OXIDATION AND REDUCTION OF CYCLIC GMP GENERATION

The Ohio State University

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AN EVALUATION OF THE POSSIBLE BIOCHEMICAL MECHANISMS OF NITROGLYCERIN TOLERANCE IN SMOOTH MUSCLE: SULFHYDRL OXIDATION AND REDUCTION OF CYCLIC GMP GENERATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Richard Alan Keith, B.S.

*****

The Ohio State University

1981

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Division of Pharmacology
College of Pharmacy
This is dedicated to my wife, Jill,

whose love and understanding

made this work possible.
ACKNOWLEDGMENTS

My thanks and appreciation go to:

Dr. Allan M. Burkman
for his understanding and friendship throughout this project.

Dr. Richard H. Fertel
for his technical assistance and insightful suggestions.

Drs. Dennis R. Feller, Norman J. Uretsky, Popat N. Patil, and Theodore D. Sokoloski
for their critical evaluation of the merits of this work.
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CHAPTER I

INTRODUCTION

Clinical status of nitroglycerin tolerance.

Vascular tolerance to nitroglycerin has been observed since the first clinical reports of nitroglycerin therapy for hypertension in Bright's disease (nonsuppurative nephritis with albuminuria and edema). Stewart (1888) reported a case in which one man required 20 grains (1.3 g) of pure nitroglycerin to produce a hypotensive effect equivalent to that initially produced by 1/100 grain (0.65 mg). More commonly, however, nitroglycerin tolerance results in the loss of headache that occurs in early nitroglycerin therapy, without losing the clinically beneficial effects of the drug (Stewart, 1905; Crandall et al., 1931; Horwitz et al., 1972).

Although early clinical experience demonstrated only the phenomenon of nitroglycerin tolerance (i.e., a larger dose is required to obtain a given therapeutic effect), physical dependence (characterized by withdrawal symptoms upon cessation of nitroglycerin exposure) was demonstrated in workers who were exposed to extremely high levels of nitroglycerin in the course of manufacturing dynamite.
(Bright, 1914; Schwartz, 1946). Withdrawal symptoms normally consisted of mild to severe headaches ("powder headaches") in exposed workers when they were away from work for more than 48 hours. However, more serious problems, including chest pain and even sudden death, have been reported following withdrawal from industrial nitroglycerin exposure (Munch et al., 1965; Lund et al., 1968; Klock, 1975). The withdrawal symptoms are thought to be due to unopposed coronary and systemic vasoconstriction with subsequent coronary ischemia (Lange et al., 1972). Therefore, it appears that in extreme cases, both tolerance to and dependence on nitroglycerin may develop, which could result in serious complications upon withdrawal from the drug.

While reviews of nitrate therapy have routinely cautioned physicians about the problems that may be associated with nitroglycerin tolerance (Nickerson, 1975; Schelling and Lasagna, 1966), very few clinicians have ever observed a case of nitroglycerin tolerance resulting from therapeutic administration of the drug (Abrams, 1980). In addition, prospective clinical studies have demonstrated that long-term oral administration of nitroglycerin does not result in loss of clinical efficacy in either anti-anginal therapy (Winsor and Berger, 1975; Cole and Kaye, 1975; Davidov and Mrozek, 1977), or in the treatment of congestive heart failure (Chandraratna et al., 1978; Mehta et al., 1978). In the clinical environment,
therefore, it appears that nitroglycerin tolerance is not a serious problem when nitroglycerin is given orally over a relatively long period of time.

It should be noted that nitroglycerin is being administered intravenously to produce a sustained hypotension for a variety of pathologic conditions, e.g., intraoperative hypertension, unstable angina, and acute myocardial infarction (Cottrell and Turndorf, 1978; Hill et al., 1981). One study reported that intravenous nitroglycerin administration was unsuccessful in lowering blood pressure in approximately 10% of the patients tested (Albin et al., 1978). Although the inability of nitroglycerin to decrease blood pressure in these cases was attributed to the loss of nitroglycerin to plastic intravenous bags (Crouthamel et al., 1978), another possibility may be the development of tachyphylaxis and/or tolerance to nitroglycerin. Therefore nitroglycerin tolerance may indeed be present in the clinical setting, although its existence may not be fully recognized by the clinicians.

Experimental studies examining the role of sulfhydryl oxidation in nitroglycerin tolerance.

Early studies on the mechanism of nitroglycerin tolerance ruled out the possibilities of increased metabolic transformation (Clark and Litchfield, 1969; Needleman et al., 1971) and increased sympathetic compensatory activity (Rush et al., 1971; Johnson et al., 1972).
The observation that aortic strips from nitroglycerin tolerant rats were less sensitive to challenge doses of nitroglycerin in vitro, suggested that the site of alteration in tolerance was at the level of the tissue (Needleman, 1970). The subsequent demonstration of in vitro-induced nitroglycerin tolerance supported this suggestion (Needleman and Johnson, 1973).

The sulfhydryl oxidation hypothesis of nitroglycerin tolerance is based primarily on experimentation which was performed on the in vitro model of nitroglycerin tolerance (Needleman and Johnson, 1973). In this model, isolated rabbit aortic strips were preincubated with $4.4 \times 10^{-4}$ M nitroglycerin under alkaline conditions (pH 9.1) for 2 hr. Nitroglycerin's ability to oxidize free sulfhydryl groups is enhanced under alkaline conditions, therefore, it was proposed that nitroglycerin tolerance results from the oxidation of a critical sulfhydryl group at the reactive site for nitroglycerin. The observations that tolerant tissues exhibited a decreased tissue sulfhydryl content, and that nitroglycerin tolerance was reversed by dithiothreitol, a disulfide reducing agent, supported the sulfhydryl hypothesis (Needleman and Johnson, 1973).
Experimental studies examining the role of cGMP generation in nitroglycerin tolerance.

Although the role of guanosine 3'':5''-monophosphate (cGMP) in the regulation of smooth muscle tone is not clearly understood, Schultz et al. (1977) observed that several smooth muscle relaxants, including nitroglycerin, increase tissue levels of cGMP. This suggested a possible role for cGMP in drug-induced smooth muscle relaxation. Observations consistent with the hypothesis that cGMP is involved in smooth muscle relaxation include: 1) many smooth muscle relaxants increase levels of cGMP in various tissues (Katsuki and Murad, 1977; Katsuki et al., 1977; Bohme et al., 1978; Janis and Diamond, 1979); 2) a lipophilic derivative of cGMP, 8-bromoguanosine-3'':5''-monophosphoric acid (8-Br-cGMP), is capable of relaxing smooth muscle (Katsuki and Murad, 1977; Schultz et al., 1979; Napoli et al., 1980); 3) time course studies have demonstrated a close correlation between the degree of smooth muscle relaxation and the extent of cGMP formation by nitroglycerin, nitroprusside, and other smooth muscle relaxing agents (Katsuki and Murad, 1977; Axelsson et al., 1979; Janis and Diamond, 1979; Kukovetz et al., 1979); and 4) recent reports have shown that methylene blue inhibits relaxation induced by nitroglycerin and other vasodilators, and reduces drug-induced activation of bovine
coronary soluble guanylate cyclase (Gruetter et al., 1980; Gruetter et al., 1981).

There is substantial evidence supporting the hypothesis that cGMP is mediating smooth muscle relaxation, however, there are still some areas of controversy regarding the cause-effect relationship. For example, nitroprusside was shown to increase tissue levels of cGMP in the rat vas deferens without promoting relaxation (Diamond and Janis, 1978), and nitroglycerin increased cGMP formation in the guinea pig vas deferens at doses which did not elicit relaxation (Wikberg et al., 1980). In addition, Janis and Diamond (1979) have demonstrated that under certain conditions, the relaxant effects of several drugs can be dissociated from an elevation of tissue levels of cGMP.

The effect of nitroglycerin tolerance on cGMP generation has recently been studied by Wikberg et al. (1980) and Braughler (1981). If a cGMP is mediating the relaxant effects of nitroglycerin, then one would expect a reduction in cGMP generation during the nitroglycerin tolerant state. However, the studies to date have yielded conflicting results. Wikberg et al. (1980) demonstrated that the induction of nitroglycerin tolerance in vitro was associated with a reduction of nitroglycerin-induced cGMP generation in bovine mesenteric arteries. Braughler (1981), on the other hand, was unable to demonstrate such an inhibition in aortic strips from rats made tolerant in vivo.
Assuming that species differences do not account for the opposing results, these observations suggest the possibility that the biochemical correlate for nitroglycerin tolerance induced in vitro may be different from that induced in vivo.

Nitroglycerin tolerance may be a phenomenon unique to vascular smooth muscle.

The effect of nitroglycerin tolerance on nonvascular smooth muscle was examined by Needleman (1970). He found no tolerance development in the isolated rat ileum or uterus which were taken from animals made tolerant to nitroglycerin in vivo. In these animals vascular tolerance to nitroglycerin was maximal, therefore, nitroglycerin tolerance may be unique to vascular smooth muscle.

Objectives of this study.

The general perception that nitroglycerin tolerance is not a serious clinical problem may be partly responsible for the relative paucity of studies directed at determining the mechanism of nitroglycerin tolerance. Regardless of the extent of tolerance in clinical practice, a better understanding of this basic mechanism would reveal greater knowledge of the regulatory processes of vascular smooth muscle tone which should assist physicians in the management of cardiovascular diseases in general.
Based on the results in one laboratory, it has been suggested that sulfhydryl oxidation is responsible for nitroglycerin tolerance (Needleman and Johnson, 1973). This hypothesis has not been confirmed by other investigators. A second hypothesis states that nitroglycerin tolerance may result from a reduction of cGMP generation (Wikberg et al., 1980; Braughler, 1981). These studies, however, have yielded conflicting results. Therefore, it is the objective of this study to re-examine the sulfhydryl hypothesis, and attempt to clarify the role of cGMP generation in nitroglycerin tolerance in vascular and nonvascular smooth muscle.
CHAPTER II

METHODS AND MATERIALS

Experimental animals.

Male albino rats (Sprague-Dawley, 225-350 g) were supplied by Harlan. Male albino rabbits (New Zealand White, 11-13.2 kg) were supplied by King's Wheel. Male and female guinea pigs (English Short Hair, 325-550 g) were supplied by Carr.

Drugs and Chemicals.

All drugs were prepared daily and dissolved in saline, except nitroglycerin for subcutaneous injection, which was dissolved in polyethylene glycol 400 (PEG 400) and refrigerated for use within 2 weeks. Test tubes containing freshly prepared solutions of nitroprusside were wrapped with aluminum foil to prevent degradation caused by fluorescent light (Vesey and Batistoni, 1977).

Drugs and chemicals were obtained from the following sources: nitroglycerin, as a 1:10 lactose triturate from ICI Americas, Inc., Wilmington, DE; sodium nitroprusside dihydrate, Roche, Nutley, NJ; 8-bromoguanosine 3',5'-monophosphoric acid, sodium salt, Sigma Chemical Co., St. Louis, MO; methylene blue, MCB Manufacturing Chemists,
Norwood, OH; \textit{\textsuperscript{1}\textdagger{-}\textdagger{-}}-norepinephrine bitartrate, Sigma Chemical Co., St. Louis, MO; carbachol (carbamylcholine chloride), Aldrich Chemical Co., Inc., Milwaukee, WI; and 5,5'-dithiobis(2-nitrobenzoic acid), Aldrich Chemical Co., Inc., Milwaukee, WI.

Preparing and handling of isolated tissues.

Male albino rats and rabbits were killed by a sharp blow to the head. The thoracic aortae were removed and dissected free of fat and connective tissue while immersed in modified Kreb's solution at room temperature. Helically cut strips, approximately 2 mm wide and 25-30 mm long, were prepared as described by Furchgott and Bhadrakom (1953). One strip was obtained per rat and four strips per rabbit. Aortic strips were suspended in 10-ml organ baths containing modified Kreb's solution at 37°C and aerated with a 5% carbon dioxide-95% oxygen mixture. The composition of the modified Kreb's solution was (millimolar concentrations): NaCl, 118; KCl 4.6; MgCl\textsubscript{2}·6H\textsubscript{2}O, 0.54; CaCl\textsubscript{2}·2H\textsubscript{2}O, 2.5; NaH\textsubscript{2}PO\textsubscript{4}, 1.0; NaHCO\textsubscript{3}, 2.5; glucose, 11; and ethylenediamine tetra-acetic acid (EDTA), 0.027. The aortic strips were attached to Grass FT-03 isometric transducers connected to a Grass model 7 polygraph and allowed to equilibrate under a resting tension of 1 g (rat) or 2 g (rabbit). Concentration-response curves for relaxant drugs were constructed on strips contracted with 10^{-7} M norepinephrine (rat) or 10^{-6} M
norepinephrine (rabbit), concentrations which provoke a near maximal contraction.

For guinea pig ileum experiments, 24 hr fasted guinea pigs were killed by a sharp blow to the head. The longitudinal smooth muscle was prepared as described by Rang (1964). The terminal 10 cm of the ileum was discarded and an adjacent 16 cm segment removed and threaded on a glass rod. Using the sharp point of a pair of scissors, the longitudinal smooth muscle was split on either side of the mesenteric connections and teased from the underlying circular muscle using a cotton swab soaked in modified Kreb's solution. Four longitudinal muscle strips were prepared per animal. Mechanical recordings were determined in the same manner as described for the aortic strips, and the modified Kreb's solution was identical, except that EDTA was not included. After equilibration under 500 mg initial resting tension, concentration-response curves for relaxant drugs were constructed on ileal longitudinal muscle strips contracted with $3 \times 10^{-7}$ M carbachol, which provokes a near maximal contraction.

In experiments measuring cyclic nucleotides or sulfhydryl content, all conditions were identical to those just described except that one end of each strip was tied to a wire for easy handling (Katsuki and Murad, 1977), and 3 or 4 strips were suspended in each bath without applying resting tension. Furthermore, in cyclic nucleotide...
experiments using the rat aorta, two strips per animal were utilized instead of one, and strips from the same animal were placed in different treatment groups.

**Blood pressure measurement in rats.**

Mean blood pressure measurements were taken on rats which were anesthetized with a total dose of 1.75 g/kg urethane (50% of the dose given intraperitoneally and 50% of the dose given subcutaneously). After tracheal cannulation, the left carotid artery was cannulated with PE 10 tubing which was connected to a Gould Statham model p23 pressure transducer. Blood pressure and heart rate were monitored on a model 7 Grass polygraph. Challenge doses of nitroglycerin and other drugs were administered intravenously through a cannula (PE 10 tubing which had been previously drawn down over a flame) in the left jugular vein. No drugs were administered until blood pressure had stabilized for 30 min after surgery.

**Induction of in vivo nitroglycerin tolerance in rats.**

The method of in vivo tolerance induction described by Needleman (1970) was slightly modified in this study. Rats were administered various doses of nitroglycerin subcutaneously 1 or 2 times a day for up to 3 days. Concentrated solutions of nitroglycerin for subcutaneous injection were prepared by dissolving pure nitroglycerin in an appropriate volume of PEG 400 such that the volume
of injected solution was less than 0.2 ml. Control rats received only PEG 400. From 16 to 18 hr after the last dose of nitroglycerin, rats were either prepared for blood pressure recording, or thoracic aortae were removed for experimentation as previously described. Aortae removed for isolated tissue experiments were equilibrated under 1 g tension for 2 hr, at which time concentration-response curves for nitroglycerin were constructed.

**Induction of in vitro nitroglycerin tolerance at pH 7.4 and methylene blue inhibition of relaxation in aortic strips.**

After 1 hr equilibration under tension, in vitro nitroglycerin tolerance was induced by incubating aortic strips in $5.5 \times 10^{-4} \text{ M}$ nitroglycerin for varying periods of time. Tissues were then washed every 15 min for 1 hr, at which time concentration-response curves for relaxant drugs were constructed on strips contracted with norepinephrine.

A concentration and an incubation period for methylene blue were chosen which would produce a level of inhibition of nitroglycerin relaxation comparable to a 1 hr induction of in vitro nitroglycerin tolerance. In preliminary studies, a 1 hr incubation with $10^{-5} \text{ M}$ methylene blue gave appropriate results. In vitro nitroglycerin tolerance produced a 2.6-fold reduction in norepinephrine sensitivity, while methylene blue pretreatment had no significant effect (figure 1).
The effects of in vitro nitroglycerin tolerance (Δ—Δ—Δ) and methylene blue (□—□—□) on norepinephrine contraction compared to control (○——○), in rat aortic strips. In vitro nitroglycerin tolerant and methylene blue tissues were incubated with $5.5 \times 10^{-4}$ M nitroglycerin and $10^{-5}$ M methylene blue, respectively, for 1 hr as described in Methods. Each curve represents the mean ± S.E. for 5 to 8 rat aortic strips.
Figure 1
Induction of in vitro nitroglycerin tolerance at pH 9.0.

In vitro tolerance to nitroglycerin at pH 9.0 was induced by slightly modifying the method described by Needleman and Johnson (1973). Helically cut rat or rabbit aortic strips were incubated with $5.5 \times 10^{-4}$ M nitroglycerin for varying periods of time in a modified Kreb's solution adjusted to pH 9.0. At the end of the pH 9.0 incubation period, strips were washed in pH 7.4 modified Kreb's solution every 15 min for 1 hr. At that time concentration-response curves for nitroglycerin were constructed on strips contracted with norepinephrine.

The pH 7.4 and 9.0 modified Kreb's solutions were identical except that the pH 9.0 solution (1) contained 35 mM Tris base, adjusted to pH with HCl, instead of bicarbonate, (2) was bubbled with 100% oxygen instead of a 95% oxygen-5% carbon dioxide mixture, and (3) contained 10% of the normal Ca$^{++}$ concentration to prevent precipitation of Ca(OH)$_2$. This lowered Ca$^{++}$ concentration had no effect on tolerance induction at pH 7.4 (data not shown).

Induction of in vitro nitroglycerin tolerance at pH 7.4 and methylene blue inhibition of relaxation in the longitudinal smooth muscle of the guinea pig ileum.

This study was designed to compare the effects of in vitro nitroglycerin tolerance and methylene blue preincubation between the nonvascular longitudinal smooth muscle
of the guinea pig ileum and the vascular smooth muscle of
the rat aorta. Therefore, times of incubations and concentra-
tions of nitroglycerin and methylene blue were identical
to those previously described for the isolated aorta.

Measurements of tissue sulfhydryl groups.

Tissue and sulfhydryl content was measured using
5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the
method described by Ellman (1959). Briefly, aortic strips
were homogenized by grinding in a mortar with washed sand
in 3.5% KCl, 0.1 M sodium phosphate (pH 7.4). The suspen-
sion was filtered through a #1 qualitative filter (Whatman)
and adjusted to pH 8.0. The pH-adjusted filtrate was then
reacted with DTNB for 20 min, at which time, the absorbance
was determined at 420 nm in a model 240 Gilford Spectro-
photometer. The sulfhydryl concentration was determined
using the molar extinction coefficient of 13,600/M/cm, and
expressed as moles sulfhydryl groups per 100 g wet weight
(w.w.) tissue.

Determinations of cyclic nucleotide levels.

Frozen rat aortic or guinea pig longitudinal muscle
strips were homogenized in 6% trichloroacetic acid (TCA)
using a glass pestle tissue homogenizer powered by a
"Caframo" stirrer, type RZRL-64, on speed #7 for 45 sec
(rat aorta) or 30 sec (longitudinal muscle of the guinea
pig ileum). Homogenates were centrifuged at 1500 x g for
15 min to separate precipitated protein from soluble extract which contained the cyclic nucleotides. The TCA was removed from the solution with three extractions of three volumes of water-saturated ethyl ether. The remaining solution was brought to pH 6.0 by the addition of 0.1 ml of 1 M sodium acetate (pH 6.5) per ml of remaining solution. Cyclic nucleotides, cGMP and adenosine 3':5'-monophosphate (cAMP), were assayed by a radioimmunoassay technique which has been previously described (Unverferth et al., 1981).

Time course studies for a given dose of nitroglycerin and nitroprusside were undertaken in order to determine the time of peak effect on cGMP generation. The time of incubation refers to the time between addition of drug to the bath and removal of tissue from the bath for freezing. Tissues were frozen by quickly removing muscle strips from the bath and placing them between blocks of dry ice. Tissues were frozen solid within 5 to 10 sec after removal from the bath.

**Statistical analysis.**

The data are expressed as the means ±S.E.'s. Statistical differences between two means (\( P < 0.05 \)) were determined by the Student's \( t \) test for unpaired observations (Sokal and Rohlf, 1969a). Where two or more treatment means were compared to one control mean, statistical differences (\( P < 0.05 \)) were determined by Dunnett's \( t \) test.
(Dunnett, 1955). Cyclic nucleotide data were converted to log values for analysis in order to statistically equalize variances between groups (Sokal and Rohlf, 1969b).
CHAPTER III

RESULTS

Models of in vivo and in vitro nitroglycerin tolerance.

In vivo induction of nitroglycerin tolerance in rats.

The extent of in vivo induction of nitroglycerin tolerance was dependent on the total tolerance-inducing dose of nitroglycerin administered (figure 2). The dose-response curve for the hypotensive effects of nitroglycerin was shifted approximately 300-fold to the right when rats were pre-treated with 300 mg/kg (1.32 moles/kg) nitroglycerin subcutaneously 2 times a day for 1 day for a total dose of 600 mg/kg. A typical blood pressure tracing of the hypotensive effects of nitroglycerin in this level of tolerance is presented in figure 3. The 1 mg/kg dose of nitroglycerine (figure 2) was administered 1 hr prior to injecting challenge doses of nitroglycerin since this level of tolerance is totally reversed within 16 hr of subcutaneous injection (data not shown).

The effects of in vivo nitroglycerin tolerance on basal levels of blood pressure and heart rate were monitored by taking measurements 30 min after completion of surgery (table 1). The greatest magnitude of in vivo tolerance
Figure 2

Dose dependence of in vivo nitroglycerin tolerance. Total tolerance-inducing subcutaneous doses of nitroglycerin are indicated on the figure. 1 mg/kg was administered at 1 hr, 100 mg/kg at approximately 18 hr, and 600 mg/kg in two equal doses at approximately 26 and 18 hr prior to the experiment. Control animals (Con) received PEG 400. Each curve represents the mean ± S.E. for 3 to 6 rats.
Figure 2
TABLE 1

The effect of *in vivo* nitroglycerin tolerance on initial blood pressure and heart rate in the rat.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Tolerant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blood pressure&lt;sup&gt;a&lt;/sup&gt;</td>
<td>heart rate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 mg/kg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114 ± 3.5</td>
<td>377 ± 11</td>
</tr>
<tr>
<td>600 mg/kg&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115 ± 3.1</td>
<td>375 ± 10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean blood pressure (mm Hg) and heart rate (beats per min) were measured 30 min after cannulation of the carotid artery. Each value represents a mean±S.E. of 3 (100 mg/kg) or 10 (600 mg/kg) measurements. Doses administered as in figure 1.

<sup>b</sup>Total tolerance-inducing doses of nitroglycerin.

<sup>c</sup>Significantly different from control (P < 0.05).
resulted in an 8% decrease in resting blood pressure compared to control animals.

The specificity of in vivo nitroglycerin tolerance was examined by testing the hypotensive responses of acetylcholine, verapamil and papaverine, and the hypertensive response of norepinephrine (figure 4). There were no significant differences in the effects of these drugs between control and nitroglycerin tolerant rats. In addition, tolerance is totally reversed 6 days after the last subcutaneous injection of a tolerance-inducing dose of nitroglycerin (figure 5).

The effects of in vivo nitroglycerin tolerance on nitroglycerin sensitivity in isolated aortae taken from tolerant rats is presented in figure 6. A subcutaneous dose of 300 mg/kg 2 times a day for 3 days was required to produce an approximate 200-fold shift to the right of the nitroglycerin concentration-response curve. As compared to the effects on hypotensive responses, a larger total tolerance-inducing dose of nitroglycerin was required to produce a comparable shift to the right of nitroglycerin concentration-response curves for isolated aortae. This is illustrated by comparing the total tolerance-inducing dose of nitroglycerin with the resultant shift to the right of the hypotensive (ED 20's) and aortic relaxant (EC 50's) effects of nitroglycerin (figure 7). This suggests that the blood vessels mediating the hypotensive
Figure 3

Typical hypotensive effects of nitroglycerin (NTG) in control and tolerant rats. Total tolerance-inducing dose of 600 mg/kg nitroglycerin was administered as described in figure 1.
Figure 3

Control

100 mm Hg

100 mm Hg

600 mg/Kg

1 min

NTG (moles/kg) 1x10^{-7} 1x10^{-6} 3x10^{-6}

Figure 3
Figure 4

Specificity of in vivo nitroglycerin tolerance. Nitroglycerin (NTG) tolerant rats were pretreated with a total tolerance-inducing dose of 600 mg/kg nitroglycerin as described in figure 1. X’s mark the ED 20’s for nitroglycerin in control (6.7 x 10^{-8} moles/kg) and tolerant (5.6 x 10^{-5} moles/kg) rats as computed from figure 1. Ach = acetylcholine; NE = norepinephrine; Ver = verapamil; and Ppv = papaverine. Each curve represents a mean for 3 rats. All S.E.’s were less than 5%.
Reversal of *in vivo* nitroglycerin tolerance. A total tolerance-inducing dose of 600 mg/kg nitroglycerin was administered in equal doses approximately 26 and 18 hr prior to time 0. Hypotensive effects of nitroglycerin were tested at time 0, and 1.3 and 6 days subsequently. X marks the ED 20 for control animals \(6.7 \times 10^{-8}\) moles/kg as computed from figure 1. Each curve represents the mean ± S.E. for 3 rats.
Figure 5
Figure 6

The effects of in vivo nitroglycerin tolerance on nitroglycerin relaxation in isolated rat aortae. Total tolerance-inducing subcutaneous doses of nitroglycerin are indicated on the figure. 100 mg/kg and 600 mg/kg doses were administered as in figure 1. 1800 mg/kg was administered by giving 300 mg/kg 2 times a day for 3 days prior to experimentation. Control animals (Con) received PEG 400. Each curve represents the mean ± S.E. for 4 (tolerant) or 12 (control) rat aortic strips.
Figure 6

% RELAXATION

NITROGLYCERIN (LOG MOLAR CONC.)
response of nitroglycerin are more sensitive to tolerance induction than the aorta, which is not involved significantly in blood pressure regulation.

**In vitro induction of nitroglycerin tolerance in isolated rat and rabbit aortae.** Needleman and Johnson (1973) reported that for the isolated rabbit aorta, significant *in vitro* induction of nitroglycerin tolerance required a 2 hr incubation with $4.4 \times 10^{-4}$ M nitroglycerin under alkaline conditions (pH 9.1). Using similar incubations, the present study found no difference in *in vitro* nitroglycerin tolerance induced at pH 7.4 or 9.0 (figure 8). In an attempt to explain the conflicting data, a time course study of the *in vitro* induction of nitroglycerin tolerance at pH 7.4 and 9.0 was undertaken in order to examine the possibility that a difference in kinetic parameters was involved (figure 8). Alkaline conditions enhanced nitroglycerin tolerance at 15 and 30 min incubations, while at 60 and 120 min there was no difference between tolerance induced at pH 7.4 and 9.0. It should be noted that the level of *in vitro* tolerance at pH 9.0 was less than that at pH 7.4 at the 5 min incubation period.

In contrast to the rabbit aorta, alkaline incubation conditions did not enhance *in vitro* induction of nitroglycerin tolerance in the rat aorta at any time period (figure 9). Near maximal tolerance could be demonstrated at either pH after a 60 min incubation with $5.5 \times 10^{-4}$ M
Comparative effects of in vivo nitroglycerin tolerance on blood pressure (BP) reduction and relaxation of isolated aortae by nitroglycerin. The effects of the total tolerance-inducing subcutaneous (S.C.) dose of nitroglycerin on the -fold shifts of nitroglycerin blood pressure responses (figure 1) and isolated aortae relaxation (figure 5) are examined. Shifts in response curves were computed by comparing the shifts of ED 20's for blood pressure and EC 50's for relaxation.
Figure 7

SHIFT IN BP OR RELAXATION

1000
FOLD

100
FOLD

10
FOLD

10^0  10^1  10^2  10^3  10^4

MG/KG

NITROGLYCERIN (TOTAL S.C. DOSE)

BP

AORTA

Figure 7
nitroglycerin. Control tissues were less sensitive to nitroglycerin after 120 min incubation under alkaline conditions (figure 9). Therefore, this duration of exposure to alkaline pH may be toxic to the rat aorta. Figure 10 summarizes the results from figures 8 and 9 by comparing the shifts of the EC 50's between tolerance induced at physiological and alkaline pH conditions.

The role of sulfhydryl oxidation in nitroglycerin tolerance.

The effect of nitroglycerin tolerance on tissue sulfhydryl content. Needleman and Johnson (1973) demonstrated that in vitro nitroglycerin tolerance at pH 9.1 was associated with a decreased tissue sulfhydryl content. Since in the present study it was found that maximal induction of in vitro tolerance could be induced under physiological conditions (pH 7.4) (figures 8, 9 and 10), it was decided to examine the sulfhydryl content of these tissues (figure 11). For both rat and rabbit aortae, tissue sulfhydryl content was decreased only in tissues incubated with $5.5 \times 10^{-4}$ M nitroglycerin at pH 9.0 for 120 min, even though all treated tissues exhibited maximal or near maximal levels of nitroglycerin tolerance.

Attempted reversal of nitroglycerin tolerance with dithiothreitol. A critical observation in support of the sulfhydryl oxidation hypothesis of nitroglycerin tolerance was the demonstration that $1.13 \times 10^{-3}$ M dithiothreitol
Time course of induction of *in vitro* nitroglycerin tolerance in rabbit aortae. Control (solid circles) and nitroglycerin treated (open circles) tissues were incubated at pH 7.4 (solid lines) or pH 9.0 (dashed lines) for indicated time periods. Tissues were then washed in pH 7.4 modified Kreb's solution for 1 hr, at which time nitroglycerin concentration-response curves were constructed on strips contracted with norepinephrine. Each curve represents the mean ± S.E. of 4 strips.
RABBIT AORTA

pH 7.4  pH 9.0

CON  TOL

% RELAXATION

5 MIN  15 MIN

30 MIN  60 MIN  120 MIN

% RELAXATION

-log molar nitroglycerin

Figure 8
Figure 9

Time course of induction of \textit{in vitro} nitroglycerin tolerance in rat aortae. Control (solid circles) and nitroglycerin treated (open circles) tissues were incubated at pH 7.4 (solid lines) or pH 9.0 (dashed lines) for indicated time periods. Tissues were then washed in pH 7.4 modified Kreb's solution for 1 hr, at which time nitroglycerin concentration-response curves were constructed on strips contracted with norepinephrine. Each curve represents the mean ± S.E. for 4 strips.
Figure 9

RAT AORTA

pH 7.4  pH 9.0

CON
TOL

% RELAXATION

5 MIN  15 MIN

30 MIN  60 MIN  120 MIN

- LOG MOLAR NITROGLYCERIN

Figure 9
Figure 10

Time course of the shift of nitroglycerin concentration-response curves in in vivo nitroglycerin tolerance in rabbit (A) and rat (B) aortae induced at pH 7.4 (solid lines) and pH 9.0 (dashed lines). This figure summarizes data presented in figures 8 and 9. *P < 0.05.
could reverse tolerance (Needleman and Johnson, 1973). All attempts at repeating dithiothreitol-reversal of tolerance in the present study were unsuccessful. In addition, it was found that dithiothreitol exhibits vascular smooth muscle relaxant properties, which are enhanced in tolerant aortae (figure 12).

The effects of nitroglycerin tolerance and methylene blue preincubation on cGMP generation in the rat aorta.

Control values for cyclic nucleotides in rat aortic strips were $5.7 \pm 0.78$ pmoles cGMP/g w.w (n=9) and $191 \pm 27$ pmoles cAMP/g w.w (n=9). These values are in general agreement with values reported in the literature for bovine vasculature (Axelsson et al., 1979; Kukovetz et al., 1979). The concentration of norepinephrine used in these studies had no significant effect either on tissue levels of cyclic nucleotides, or on the ability of nitroglycerin to generate cGMP.

Time course studies. Near maximal relaxant concentrations for nitroglycerin and nitroprusside were used in this study to compare time courses of relaxation to those of increases in cGMP levels (figure 13). For given doses of each compound, maximal increases in cGMP occurred prior to maximal relaxation. Although the maximal increase in cGMP by nitroprusside was somewhat sustained (from 30 sec to 3 min), that of nitroglycerin was transient and dropped from
Figure 11

The effect of *in vitro* nitroglycerin tolerance induced at pH 7.4 and 9.0 on tissue sulfhydryl (SH) content of rabbit (A) and rat (B) aortae. Control (C) and tolerant (T) aortae were treated in an identical manner as described in figures 7 and 9. Each bar represents the mean ± S.E. of 4 aortic strips. *P < 0.05.*
Figure 11

A) RABBIT AORTA

B) RAT AORTA

umoles SH/100 g. ww.
Figure 12

The effect of *in vitro* nitroglycerin tolerance on dithiothreitol relaxation of isolated rat aortae.
Tolerant (Tol) strips were preincubated with $5.5 \times 10^{-4}$ M nitroglycerin for 60 min. Con = control strips. Each curve represents the mean ± S.E. for 6 aortic strips. The EC 50's for dithiothreitol in control and tolerant aortic strips are significantly different ($P < 0.05$).
Figure 12
Figure 13

Time courses of the relaxant and cGMP-increasing effects of $10^{-5}$ M nitroglycerin (A) and $10^{-6}$ M nitroprusside (B) in the isolated rat aorta. Each value represents the mean ± S.E. for 3 to 12 rat aortic strips. All increases in cGMP above controls were significant ($P < 0.05$).
Figure 13

Graph A: NITROGLYCERIN, $10^{-5}$ M
Graph B: NITROPRUSSIDE, $10^{-6}$ M

% RELAXATION (W)

TIME, minutes
a level of 265 times control at 15 sec to only 12 times control at 2 min. In spite of this rapid decline in cGMP levels, relaxation was only minimally decreased at 10 min.

There were no time dependent changes in cAMP levels that correlated with relaxation. All values of cAMP ranged between 0.6 and 1.6 times control (data not shown).

Concentration-response studies. Based on the time course studies, the times of peak generation of cGMP by nitroglycerin (15 sec) and nitroprusside (30 sec) were used to examine concentration-dependency of the increases in cGMP (figure 14). A good correlation was found between the extent of relaxation and log of the -fold increase in cGMP levels. There were no concentration-dependent changes of cAMP levels within functionally effective ranges of the drugs. All values of cAMP ranged between 0.7 and 1.6 times control (data not shown).

The effects of in vitro nitroglycerin tolerance at pH 7.4 and methylene blue preincubation on cGMP generation. The time course studies of in vitro induction of nitroglycerin tolerance in the rat aorta (figures 8 and 9) demonstrated that a 1 hr incubation with 5.5 x 10^{-4} M nitroglycerin produced a near maximal level of tolerance. A 1 hr incubation with 10^{-5} M methylene blue caused a comparable 200-fold shift to the right of the nitroglycerin concentration-response curve (figure 15). However, nitroprusside was not significantly affected by the presence of
Figure 14

Concentration-response of percent relaxation and log of the -fold increase in cGMP by nitroglycerin (A) and nitroprusside (B) in the isolated rat aorta. Tissue levels represent cGMP generated during 15 sec and 30 sec incubation periods for nitroglycerin and nitroprusside, respectively. Each value represents a mean ± S.E. for 3 to 12 rat aortic strips. All increases in cGMP above control were significant (P < 0.05), except for 10^{-8} M nitroglycerin.
Figure 14
The effects of *in vitro* nitroglycerin tolerance \( \triangle \) and methylene blue \( \square \) on the relaxant effects of nitroglycerin (A) and nitroprusside (B) compared to control (O), in rat aortic strips. *In vitro* nitroglycerin tolerant and methylene blue tissues were incubated with \( 5.5 \times 10^{-4} \) M nitroglycerin and \( 10^{-5} \) M methylene blue, respectively, for 1 hr as described in Methods. Concentration-response curves were constructed on aortic strips contracted with norepinephrine. Each curve represents the mean ± S.E. for 4 to 6 rat aortic strips.
Figure 15

![Graph A](#)

**% RELAXATION**

**NITROGLYCERIN (log molar conc.)**

-8  -7  -6  -5  -4

![Graph B](#)

**% RELAXATION**

**NITROPRUSSIDE (log molar conc.)**

-9  -8  -7  -6  -5
in vitro nitroglycerin tolerance, while methylene blue produced a 6-fold shift to the right of the nitroprusside concentration-response curve (figure 15). The ability of nitroglycerin and nitroprusside to generate cGMP under conditions of in vitro nitroglycerin tolerance and methylene blue pretreatment is presented in table 2. Whenever there was a reduction of relaxant effects (figure 15) there was a corresponding inability of these agents to generate cGMP. It should be noted that in vitro nitroglycerin tolerance and methylene blue pretreatment significantly increased basal levels of cGMP.

The specificity of the inhibitory effects of in vitro nitroglycerin tolerance and methylene blue was examined by measuring the relaxant effects of 8-Br-cGMP (figure 16), an agent that acts like cGMP upon entering the cell (Schultz et al., 1979). In vitro nitroglycerin tolerance produced a 2.5-fold shift to the left of the 8-Br-cGMP concentration-response curve. Preincubation with methylene blue did not significantly alter the sensitivity of tissues to 8-Br-cGMP.

The effect of in vivo nitroglycerin tolerance on cGMP generation in the rat aorta. This series of experiments was designed to determine if nitroglycerin tolerance induced in vivo resembles nitroglycerin tolerance induced in vitro. Nitroglycerin-induced cGMP generation was examined (table 3) in aortae taken from tolerant rats in
TABLE 2

The effects of in vitro nitroglycerin tolerance and methylene blue pretreatment on cGMP generation in the isolated rat aorta.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>NTG Tol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MB&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>5.7 ± 0.8</td>
<td>24 ± 5.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16 ± 3.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; NTG&lt;sup&gt;e&lt;/sup&gt;</td>
<td>596 ± 117</td>
<td>25 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>79 ± 25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt; NTG&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1738 ± 369</td>
<td>73 ± 23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>373 ± 25&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3x10&lt;sup&gt;-8&lt;/sup&gt; NP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>68 ± 6.1</td>
<td>55 ± 3.5</td>
<td>27 ± 6.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>3x10&lt;sup&gt;-7&lt;/sup&gt; NP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>382 ± 40</td>
<td>316 ± 62</td>
<td>104 ± 5.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each value represents a mean±S.E. for 3 to 9 rat aortic strips.

<sup>b</sup>In vitro nitroglycerin tolerance (1 hr incubation with 5.7 x 10<sup>-4</sup> M nitroglycerin).

<sup>c</sup>Methylene blue preincubation.

<sup>d</sup>Significantly different from respective control (P < 0.05).

<sup>e</sup>Molar concentration of nitroglycerin incubated for 15 sec.

<sup>f</sup>Molar concentration of nitroprusside incubated for 30 sec.
The effects of \textit{in vitro} nitroglycerin tolerance (\(\triangle \cdots \triangle\)) and methylene blue (\(\square \cdots \square\)) on the relaxant effects of 8-Br-cGMP compared to control (\(\bigcirc \cdots \bigcirc\)), in rat aortic strips. Incubations were identical to those described in figure 15. Each curve represents the mean \(\pm\) S.E. for 4 to 5 rat aortic strips. In all cases, the S.E. did not exceed the length of the symbol.
Figure 16
### TABLE 3

The effects of *in vivo* nitroglycerin tolerance on cGMP generation in the isolated rat aorta.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>NTG Tol&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14 ± 4.8</td>
<td>21 ± 3.2</td>
</tr>
<tr>
<td>$10^{-6}$ NTG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>409 ± 20</td>
<td>18 ± 9.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>$10^{-4}$ NTG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5560 ± 340</td>
<td>204 ± 16&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each value represents a mean ± S.E. for 3 rat aortic strips.

<sup>b</sup>*In vivo* nitroglycerin tolerance.

<sup>c</sup>Molar concentrations of nitroglycerin incubated for 15 sec.

<sup>d</sup>Significantly different from control (P < 0.05).
which nitroglycerin relaxation curves exhibited an approximate 180-fold shift to the right (figure 17). As with the \textit{in vitro} nitroglycerin tolerance model (table 2), there was a significant reduction in the ability of nitroglycerin to generate cGMP. However, the basal levels of cGMP were not significantly affected.

The effects of \textit{in vitro} nitroglycerin tolerance at pH 7.4 and methylene blue preincubation on relaxation and cGMP generation in the longitudinal muscle of the guinea pig ileum.

Control values for cyclic nucleotides were $8.8 \pm 1.8$ pmoles cGMP/g w.w (n=9) and $418 \pm 19.5$ pmoles cAMP/g w.w. (n=9). The contractile dose of carbachol ($3 \times 10^{-7}$ M) caused a 2.4-fold increase in cGMP levels and had no effect on cAMP levels. Since nitroglycerin- or nitroprusside-induced increases in cGMP were measured in tissues contracted with carbachol, the increase in cGMP levels due to carbachol (12.5 pmoles/g w.w.) was subtracted from the cGMP increases produced by smooth muscle relaxant plus carbachol. This gave the value of the increase in cGMP due only to the smooth muscle relaxant, and this value was divided by the noncarbachol control value of $8.8$ pmoles cGMP/g w.w. to compute the "-fold increase" values. The ability of carbachol to promote contraction of the longitudinal smooth muscle of the ileum was not affected by
The effect of in vivo nitroglycerin tolerance on the relaxant effects of nitroglycerin on isolated aortic strips. Rats were administered 300 mg/kg nitroglycerin (□□□□) 2 times a day for 3 days, or PEG 400 (○○○○) as a diluent control. Thoracic aortae were removed 16-18 hr after the last dose of nitroglycerin. Each curve represents the mean ± S.E. for 4 rat aortic strips. (Data taken from figure 5).
Figure 17
either \textit{in vitro} nitroglycerin tolerance or methylene blue preincubation (data not shown).

\textbf{Time course studies.} Near maximal relaxant concentrations for nitroglycerin and nitroprusside were used in this study to compare time courses of relaxation to those of increases in cGMP levels (figure 18). The increase in tissue levels of cGMP by nitroglycerin preceded relaxation and reached a maximum value of 110 times control at 15 sec. The nitroglycerin-induced increase in cGMP was transient and fell to a level of 3.3 times control at 2 min. Nitroprusside relaxation, however, preceded significant increases in tissue levels of cGMP, and maximal relaxation occurred at 20 sec after the addition of the drug, while the maximal increase in cGMP did not occur until 1 min.

There were no time dependent changes in tissue levels of cAMP that correlated with relaxation. All values of cAMP ranged between 0.7 and 1.3 times control.

\textbf{Concentration-response studies.} Based on the time course studies, the times of peak generation of cGMP by nitroglycerin (15 sec) and nitroprusside (1 min) were used to examine concentration-dependency of the increases in cGMP (figure 19). A good correlation was found between the extent of relaxation and the log of the \(-\)fold increase in cGMP levels. There were no concentration-dependent changes of cAMP levels within functionally effective ranges of
Figure 18

Time courses of the relaxant and cGMP-increasing effects of $10^{-5}$ M nitroglycerin (A) and $10^{-5}$ M nitroprusside (B) in the longitudinal muscle of the guinea pig ileum. Each value represents the mean ± S.E. for 4 to 7 longitudinal muscle strips. All increases in cGMP above controls were significant ($P < 0.05$), except for 2 min for nitroglycerin and 5 sec for nitroprusside.
Figure 18

**A**

- **cGMP**
  - PMOLES/G WET WT. (O-O)
  - Time (Min)
  - Nitroglycerin, 10^-5 M
  - % Relaxation (ΔΔ)

- **B**
  - Nitroprusside, 10^-3 M
  - % Relaxation (ΔΔ)
Figure 19

Concentration-response of percent relaxation and log of the -fold increase in cGMP by nitroglycerin (A) and nitroprusside (B) in the longitudinal muscle of the guinea pig ileum. Tissue levels represent cGMP generated during 15 sec and 1 min incubation periods for nitroglycerin and nitroprusside, respectively. Each value represents a mean ± S.E. of 4 to 7 longitudinal muscle strips. All increases in cGMP above controls were significant (P < 0.05), except for $10^{-8}$ M nitroglycerin, and $10^{-8}$ M and $10^{-7}$ M nitroprusside.
Figure 19
the drugs. All values of cAMP ranged between 0.7 and 1.1 times control.

The effects of nitroglycerin tolerance and methylene blue preincubation on relaxation and cGMP generation. The effects of in vitro nitroglycerin tolerance (1 hr incubation with $5.5 \times 10^{-4}$ M nitroglycerin) and methylene blue (1 hr incubation with $10^{-5}$ M methylene blue) on nitroglycerin and nitroprusside-induced relaxation are presented in figure 20. Nitroglycerin relaxation curves are shifted to the right approximately 200-fold and 6-fold by in vitro tolerance and methylene blue preincubation, respectively. Both in vitro nitroglycerin tolerance and methylene blue preincubation produced comparable shifts to the right of nitroprusside concentration-response curves (approximately 5-fold). The ability of nitroglycerin and nitroprusside to generate cGMP under conditions of in vitro nitroglycerin tolerance and methylene blue preincubation is presented in table 4. Whenever there was a reduction of the relaxant effects (figure 20), there was a corresponding inability of these agents to generate cGMP.

The specificity of the inhibitory effects of in vitro nitroglycerin tolerance and methylene blue was examined by measuring the relaxant effects of 8-Br-cGMP (figure 21). While in vitro nitroglycerin tolerance had no significant effect, methylene blue produced a 2-fold shift to the right of the 8-Br-cGMP concentration-response curve.
Figure 20

The effects of in vitro nitroglycerin tolerance (Δ---Δ) and methylene blue (□---□) on the relaxant effects of nitroglycerin (A) and nitroprusside (B) compared to control (○---○), in guinea pig ileum longitudinal muscle strips. In vitro nitroglycerin tolerant and methylene blue tissues were incubated with 5.5 x 10^{-4} M nitroglycerin and 10^{-5} M methylene blue, respectively, as described in Methods. Concentration-response curves were constructed on longitudinal muscle strips contracted with carbachol. Each curve represents the mean ± S.E. for 4 to 6 longitudinal muscle strips.
Figure 20

(A) Nitroglycerin (Log Molar Conc.)

(B) Nitroprusside (Log Molar Conc.)
TABLE 4

The effects of *in vitro* nitroglycerin tolerance and methylene blue pretreatment on cGMP generation in the longitudinal smooth muscle of the guinea pig ileum.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control</th>
<th>NTG Tol(^{\text{b}})</th>
<th>MB(^{\text{c}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>none(^{\text{d}})</td>
<td>21 ± 3.3</td>
<td>21 ± 3.7</td>
<td>20 ± 5.1</td>
</tr>
<tr>
<td>10(^{-6}) NTG(^{\text{f}})</td>
<td>390 ± 28</td>
<td>26 ± 2.0(^{\text{e}})</td>
<td>248 ± 61(^{\text{e}})</td>
</tr>
<tr>
<td>10(^{-4}) NTG(^{\text{f}})</td>
<td>1571 ± 290</td>
<td>44 ± 8.1(^{\text{e}})</td>
<td>599 ± 50(^{\text{e}})</td>
</tr>
<tr>
<td>10(^{-6}) NPG(^{\text{g}})</td>
<td>126 ± 26</td>
<td>44 ± 11(^{\text{e}})</td>
<td>52 ± 4.9(^{\text{e}})</td>
</tr>
<tr>
<td>10(^{-5}) NPG(^{\text{g}})</td>
<td>245 ± 29</td>
<td>139 ± 22(^{\text{e}})</td>
<td>162 ± 20(^{\text{e}})</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Each value represents a mean ± S.E. for 3 to 7 longitudinal muscle strips.

\(^{\text{b}}\) In *vitro* nitroglycerin tolerance (1 hr incubation with 5.5 x 10\(^{-4}\) M nitroglycerin).

\(^{\text{c}}\) Methylene blue preincubation.

\(^{\text{d}}\) Incubated with 3 x 10\(^{-7}\) M carbachol only.

\(^{\text{e}}\) Significantly different from respective control (P < 0.05).

\(^{\text{f}}\) Molar concentration of nitroglycerin incubated for 15 sec.

\(^{\text{g}}\) Molar concentration of nitroprusside incubated for 1 min.
The effects of \textit{in vitro} nitroglycerin tolerance (Δ——Δ) and methylene blue (□——□) on the relaxant effects of 8-Br-cGMP compared to control (○——○), in guinea pig ileum longitudinal muscle strips. Incubations were identical to those described in figure 20. Each curve represents the mean ± S.E. of 4 longitudinal muscle strips.
Figure 21
CHAPTER IV
DISCUSSION

Possible role of sulfhydryl oxidation in nitroglycerin tolerance.

The sulfhydryl hypothesis of nitroglycerin tolerance states that tolerance results from the oxidation of critical sulfhydryl groups by the nitroglycerin molecule at the nitroglycerin "receptor" (Needleman and Johnson, 1973). This modification presumably results in decreased receptor affinity, therefore, given doses of nitroglycerin will be less effective in producing vasodilation.

The observations supporting the sulfhydryl oxidation hypothesis are (1) significant induction of in vitro tolerance occurs only at alkaline pH, a condition that enhances oxidation of sulfhydryl groups by nitroglycerin, (2) a decreased level of tissue sulfhydryl content is associated with the tolerant state, and (3) dithiothreitol, a disulfide reducing agent that restores free sulfhydryl groups, reverses tolerance exhibited in isolated aortae (Needleman and Johnson, 1973).

In contrast to what was previously reported, the present investigation found that for isolated rat and
rabbit aortae, maximal *in vitro* induction of vascular tolerance to nitroglycerin occurred at the physiological pH of 7.4 (figures 8, 9, and 10). Although alkaline conditions enhanced the initial rate of tolerance induction in the rabbit aorta, there was no significant enhancement of tolerance induced under alkaline conditions in the rat aorta at any time period.

A decreased tissue sulfhydryl content was not associated with tolerance at all times (figure 11). Tissues incubated with $5.5 \times 10^{-4}$ M nitroglycerin for 60 or 120 min at pH 7.4 or 9.0 exhibited maximal or near maximal magnitudes of tolerance. The sulfhydryl content was decreased only in those tissues incubated for 120 min at pH 9.0. Therefore, the appearance of nitroglycerin tolerance did not correlate in time with a detectable decrease in tissue sulfhydryl content.

According to Needleman and Johnson (1973), reversal of tolerance in isolated rat aortae could be effected by incubating the tissues with $1.13 \times 10^{-3}$ M dithiothreitol while constructing the concentration-response curve for nitroglycerin. This is not a true reversal, however, since tolerance returned when dithiothreitol was washed from the bath. All attempts at repeating dithiothreitol reversal of nitroglycerin tolerance in this investigation were unsuccessful. Indeed, the dose of dithiothreitol used by Needleman and Johnson (1973) inhibited the norepinephrine
contraction, and the relaxant effects of dithiothreitol were more potent in tolerant aortae (figure 12). This suggests the possibility that the dithiothreitol reversal seen by Needleman and Johnson (1973) could be due to additive relaxant effects of dithiothreitol and nitroglycerin.

The results of the present study are inconsistent with the sulfhydryl oxidation hypothesis of nitroglycerin tolerance. However, the possibility can not be ruled out that the oxidation of a small, and therefore, undetectable population of sulfhydryl groups results in nitroglycerin tolerance. In addition, if this small population of sulfhydryl groups exists in an environment relatively resistant to pH changes of the external medium, a high external pH would not be expected to enhance tolerance development.

**The role of cGMP in vascular smooth muscle relaxation and nitroglycerin tolerance.**

In the rat aorta tissue levels of cGMP were increased by nitroglycerin and nitroprusside prior to the onset of relaxation (figure 13), and were concentration-dependent within the pharmacological range of drug-induced relaxation (figure 14). The time course of cGMP generation is interesting since tissue levels of cGMP fall rapidly after an initial peak, while a sustained relaxation is maintained. This is qualitatively very similar to previous time course
studies on bovine coronary and mesenteric vasculature (Axelsson et al., 1979; Kukovetz et al., 1979), and may imply that cGMP performs an intermediary role in promoting relaxation. Since cGMP-dependent protein kinases have been demonstrated in vascular smooth muscle (Shoji et al., 1977; Ives et al., 1980), cGMP-induced kinase activation may be responsible for vascular relaxation. However, further studies are required to clarify this point.

Previous studies on the effects of nitroglycerin tolerance on cGMP generation by nitroglycerin have yielded conflicting results. Wikberg et al. (1980) found an inhibition of nitroglycerin-induced generation of cGMP using the in vitro method of nitroglycerin tolerance described by Needleman and Johnson (1973). As previously discussed, this method requires a preincubation with 4.4 x 10^{-4} M nitroglycerin for 2 hr under alkaline conditions to demonstrate significant tolerance. Experiments in the present study have shown that in the isolated rat aorta, maximal in vitro nitroglycerin tolerance was induced at physiological pH, and alkaline conditions had no enhancing effect (figures 9 and 10). In vitro nitroglycerin tolerance induced under physiological conditions was associated with an inhibition of nitroglycerin relaxation and cGMP generation without significantly affecting nitroprusside's profile of activity (figure 15 and table 2). These results
are similar to those reported by Wikberg et al. (1980), in which in vitro nitroglycerin tolerance was induced in bovine mesenteric arteries under alkaline pH.

In contrast to the results found in the in vitro studies, Braughler (1981) found no inhibition of nitroglycerin-induced cGMP generation in aortic strips from rats made tolerant to nitroglycerin by the in vivo method described by Needleman (1970). This finding suggests the possibility that the in vivo and in vitro mechanisms of nitroglycerin tolerance were different. The present study, however, strongly suggests that the in vitro and in vivo models of nitroglycerin tolerance are quite similar. In both models, the inhibition of nitroglycerin-induced relaxation (figures 15 and 17) and cGMP generation (tables 2 and 3) exhibited a similar profile.

Braughler (1981) measured cGMP in aortic strips after a 1 min incubation period with nitroglycerin, as opposed to 15 sec in this study. From figure 13, it can be seen that at 1 min, cGMP levels are relatively low after an initial peak. Therefore, a difference in cGMP generation between tolerant and nontolerant tissues may have been obscured due to the use of a less than optimal incubation period.

Methylene blue has been shown to inhibit coronary arterial relaxation elicited by nitroglycerin, nitroprusside and several other vasodilators (Gruetter et al.,
1979; Gruetter et al., 1980; Gruetter et al., 1981). In addition, activation of bovine coronary soluble guanylate cyclase by nitroglycerin and nitroprusside was inhibited by methylene blue (Gruetter et al., 1979; Gruetter et al., 1981). Therefore, it was suggested that methylene blue inhibition of vasodilator-induced relaxation may be due to an inhibition of guanylate cyclase activation. Our results support this hypothesis, since methylene blue-induced inhibition of relaxation (figure 15) was associated with an inhibition of cGMP generation by nitroglycerin and nitroprusside (table 2). Although methylene blue inhibition resembled nitroglycerin tolerance with regard to its effects on nitroglycerin (figure 15 and table 2), methylene blue apparently is less specific since nitroprusside-induced relaxation and cGMP generation are also impaired.

The observation that the relaxant effect of 8-Br-cGMP was not inhibited by in vitro nitroglycerin tolerance or methylene blue (figure 16) suggests that once cGMP is formed, its subsequent action is not being adversely affected. It is not known why in vitro nitroglycerin tolerance enhanced the sensitivity of 8-Br-cGMP (figure 16) and decreased norepinephrine sensitivity (figure 12). This could possibly be due to the increased basal levels of cGMP in in vitro nitroglycerin tolerant tissues (table 2). Thus, the endogenous cGMP could have an additive effect
with 8-Br-cGMP, and an inhibitory effect on the contractile action of norepinephrine. The enhancement of dithiothreitol-induced relaxation in in vitro nitroglycerin tolerance (figure 11) and the decreased resting blood pressure in in vivo nitroglycerin tolerance (table 1) could possibly result from a similar mechanism. Methylene blue, however, increased basal levels of cGMP (table 2) yet had no significant effect on the sensitivities of 8-Br-cGMP or norepinephrine (figures 12 and 16). Further investigation is clearly needed in order to resolve this discrepancy.

The role of cGMP in nonvascular smooth muscle relaxation and nitroglycerin tolerance.

Needleman (1970) examined the effects of in vivo nitroglycerin tolerance on nonvascular tissues and found no tolerance development in rat uterus or ileum strips from animals in which maximal vascular tolerance to nitroglycerin could be demonstrated. Therefore, the induction of nitroglycerin tolerance may be unique to vascular tissue. To further investigate this possibility, the effects of in vitro nitroglycerin tolerance and methylene blue pre-incubation on relaxation and cGMP generation in the longitudinal muscle of the guinea pig ileum were examined.

In contrast to the earlier study by Needleman (1970), it was found that the effects of nitroglycerin and nitroglycerin tolerance on the longitudinal smooth muscle of the
guinea pig ileum were similar to those in the isolated rat aorta. Nitroglycerin produced a concentration-dependent (figure 19), but transient increase in tissue levels of cGMP which preceded the onset of relaxation (figure 18). The potency of nitroglycerin with regard to relaxation and cGMP generation in the nonvascular and vascular tissues were comparable (compare figures 14 and 19). In addition, the inhibitory effects of in vitro nitroglycerin tolerance on nitroglycerin-induced relaxation (figure 20) and cGMP generation (table 4) were similar to those in the rat aorta (figure 16 and table 2). This suggests that the mechanisms of nitroglycerin relaxation and tolerance are similar in both the vascular smooth muscle of the rat aorta and the nonvascular smooth muscle of the guinea pig ileum longitudinal muscle.

The effects on vascular and nonvascular smooth muscle differ, however, when methylene blue preincubation and nitroprusside relaxation are examined. Methylene blue was relatively ineffective in inhibiting nitroglycerin relaxation (figure 20) and cGMP generation (table 4) in the longitudinal muscle of the guinea pig ileum, when compared to the rat aorta (figure 15 and table 2). There also appears to be a nonspecific component of methylene blue’s inhibitory effect on relaxation, since 8-Br-cGMP relaxation is inhibited (figure 21). This is the first demonstration
of the effects of methylene blue in nonvascular tissue. The reason for the different profile of activity between the vascular and nonvascular tissues is not known.

The profile of nitroprusside's effects in vascular and nonvascular tissue were also different. The potency of nitroprusside-induced relaxation and cGMP generation was approximately 100 times less in the nonvascular tissue (compare figures 14 and 19). Although nitroprusside produced a concentration-dependent increase in tissue levels of cGMP (figure 19), relaxation preceded the appearance of a significant increase in cGMP levels (figure 18) in the longitudinal muscle of the guinea pig ileum. Therefore, a cause-effect relationship between cGMP and relaxation with regard to nitroprusside in the nonvascular smooth muscle was not clearly demonstrated. Furthermore, nitroglycerin tolerance inhibited nitroprusside relaxation (figure 20) and cGMP generation (table 4), whereas these effects were not significantly impaired by tolerance in the rat aorta (figure 15 and table 2).

This study demonstrates a difference between the effects of nitroprusside in vascular and nonvascular smooth muscle. Similar findings have been demonstrated in separate studies by other investigators. Axelsson et al. (1979) and Kukovitz et al. (1979) have shown that nitroprusside produced a concentration-dependent increase in cGMP in
bovine vascular smooth muscle that preceded relaxation. Janis and Diamond (1978) found that nitroprusside increased tissue levels of cGMP in the rat vas deferens without promoting relaxation. Based on the results of the present study and recent investigations, it appears that the mechanism of nitroprusside relaxation in vascular and nonvascular smooth muscle may be different. In addition, the observation that nitroglycerin tolerance inhibits nitroprusside relaxation and cGMP generation in the longitudinal muscle of the guinea pig ileum may suggest a common site of action for nitroglycerin and nitroprusside in this tissue.

Other possible mediators of nitroglycerin-induced relaxation.

There is substantial evidence linking cAMP and relaxation in vascular and nonvascular smooth muscle (reviewed by Bar, 1974). Specifically, relaxation of vascular strips by either beta adrenergic stimulation or various phosphodiesterase inhibitors was correlated with increases in intracellular cAMP levels (Triner et al., 1972). Relaxation produced by cAMP is thought to be due to the activation of protein kinases which may result in either the removal of Ca++ from the cytoplasm (Andersson et al., 1975; Bhalla et al., 1978), or an inhibition of phosphorylation of the myosin light chain, which may be the
process responsible for smooth muscle contraction (Stull et al., 1980).

Andersson (1973) found that relaxation induced by nitroglycerin was associated with increased tissue levels of cAMP in the rabbit colon and bovine mesenteric arteries. Recent studies, however, have not supported this finding. Axelsson et al. (1979) and Kukovetz et al. (1979) found that nitroglycerin had no significant effect on cAMP content in bovine coronary and mesenteric arteries, respectively. The present study supports the latter investigations, since nitroglycerin had no time or concentration dependent effect on the cAMP content of the rat aorta or the longitudinal muscle of the guinea pig ileum.

Prostacyclin is a potent vasodilating and platelet aggregation inhibiting agent synthesized from arachidonic acid in endothelial cells of vascular tissue (Moncada et al., 1976). Levin et al. (1981) have recently reported that nitroglycerin stimulates the synthesis of prostacyclin in cultured endothelial cells. This suggests that nitroglycerin may be producing relaxation by promoting the synthesis of prostacyclin. This possibility was tested in preliminary studies in the present investigation, by examining the effects of aspirin and indomethacin on vascular relaxation by nitroglycerin. Aspirin and indomethacin have been shown to inhibit the synthesis of prostacyclin.
(Vane, 1971), therefore nitroglycerin relaxation should be inhibited by these agents if prostacyclin is acting as the mediator of nitroglycerin-induced relaxation. Aspirin (40 mg/kg, intraperitoneally) administration to rats had no effect on the hypotensive responses of nitroglycerin, and the relaxant and tolerance-inducing effects of nitroglycerin were not affected in isolated aortic strips pretreated with aspirin (10^{-3} M) or indomethacin (10^{-5} M) (data not shown). Although nitroglycerin may be promoting the synthesis of prostacyclin, the results of the present investigation suggest that a role for prostacyclin in nitroglycerin-induced relaxation is at this time inconclusive.

Since the intracellular concentration of free Ca^{++} is thought to play an important role in the regulation of vascular tone (Fleckenstein, 1977), it is possible that nitroglycerin may induce relaxation by either preventing the entry, or promoting the exit of Ca^{++} from intracellular contractile proteins. For example, verapamil is thought to inhibit contraction by preventing the transmembrane influx of Ca^{++} induced by contractile agonists (Rosenberg and Trigger, 1978). It was shown in the present study that the hypotensive responses of verapamil are not affected by nitroglycerin tolerance (figure 4), which suggests that verapamil and nitroglycerin act by different mechanisms.
In addition, Kreye and Schlicker (1980) found that nitroglycerin had no effect on Ca\(^{++}\) uptake by microsomes derived from vascular smooth muscle. Vascular microsomal uptake of Ca\(^{++}\) may represent a mechanism for removal of Ca\(^{++}\) from contractile proteins, which would result in relaxation. Therefore, studies to date do not support the possibility that changes in intracellular Ca\(^{++}\) mediate relaxation induced by nitroglycerin.
SUMMARY AND CONCLUSIONS

The purpose of this study was to examine the role of sulfhydryl oxidation and cGMP generation in nitroglycerin tolerance. The results indicate that sulfhydryl oxidation occurs subsequent to the development of tolerance (figure 10), therefore an etiological role of sulfhydryl oxidation in tolerance is equivocal, but can not be entirely ruled out.

Nitroglycerin tolerance was shown to be associated with an inability of nitroglycerin to promote cGMP generation in both the rat aorta (tables 2 and 3) and the longitudinal muscle of the guinea pig ileum (table 4). This lends support to the hypothesis that cGMP mediates nitroglycerin-induced smooth muscle relaxation, since a close correlation between effective smooth muscle relaxation and cGMP generation was evident.

The effects of nitroprusside in vascular and non-vascular smooth muscle were different. In the rat aorta nitroprusside increased tissue levels of cGMP prior to the onset of relaxation (figure 13), and vascular tolerance to nitroglycerin had no significant effect on either nitroprusside relaxation (figure 15) or its ability to increase tissue levels of cGMP (table 2). This suggests that in the
rat aorta, nitroprusside and nitroglycerin act via different mechanisms to promote cGMP generation. These sites could be separate from, but coupled to the guanylate cyclase enzyme, or specific sites on the enzyme molecule. Nitroglycerin tolerance could conceivably alter the nitroglycerin "receptor" whereas methylene blue, which inhibits both nitroglycerin and nitroprusside, apparently affects a site required by both drugs to promote cGMP generation.

In the longitudinal smooth muscle of the guinea pig ileum, nitroprusside was approximately 100 times less potent than in the rat aorta (compare figures 14 and 19). Since cGMP was increased subsequent to the onset of relaxation (figure 18), a causal role for cGMP in nitroprusside-induced relaxation in this tissue was not clearly demonstrated. These results suggest that the specific nitroprusside "receptor" site which apparently exists in the rat aorta, is not present in the longitudinal muscle of the guinea pig ileum. This would explain the high degree of potency and specificity for the drug in the rat aorta.

The mechanism(s) of nitroglycerin tolerance and methylene blue inhibition of relaxation and cGMP generation is (are) not clearly understood. Needleman and Johnson (1973) have proposed that nitroglycerin tolerance results from the oxidation of a critical sulfhydryl group at the nitroglycerin "receptor". The fact that methylene blue is
also an oxidizing agent, prompted Gruetter et al. (1981) to suggest that the inhibitory effects of methylene blue may be due to an oxidative process. In addition, a recent study has shown that the oxidative formation of mixed disulfides in partially purified preparations of guanylate cyclase result in inhibition of cGMP generation (Brandwein et al., 1981). The inhibition was reversed by the disulfide reducing agent, dithiothreitol. Therefore, although it appears that an oxidative event may result in inhibition of guanylate cyclase activity, and consequently relaxation, further study is required to determine the precise involvement of oxidation in nitroglycerin tolerance and methylene blue inhibition.

Although nitroglycerin tolerance is not considered a serious clinical problem, experimental studies can utilize the phenomenon to examine the regulatory processes of smooth muscle tone. In this study in vitro and in vivo models of nitroglycerin tolerance provided support for the hypothesis that cGMP mediates smooth muscle relaxation in some instances. In addition, the different effects of methylene blue and nitroprusside between vascular and nonvascular smooth muscle indicates there may be some basic differences in the way muscle tone is regulated in these tissues. Future investigations can use these models to clarify the mechanism of cGMP in promoting relaxation in vascular and nonvascular smooth muscle.
LIST OF REFERENCES


