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DESENSITIZATION TO NITROGLYCERIN IN VASCULAR SMOOTH MUSCLE FROM RAT AND HUMAN

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Abstract—Guanylate cyclase in high speed supernatant fractions obtained from rat thoracic aorta or human coronary arteries pretreated with nitroglycerin exhibited a marked desensitization to activation by nitroglycerin, nitroprusside, and nitric oxide. However, activation of soluble guanylate cyclase by arachidonic acid was unaffected by pretreatment of vessels with nitroglycerin. Furthermore, activation of soluble guanylate cyclase by protoporphyrin IX was increased 4-fold when vessels were pretreated with nitroglycerin. Soluble guanylate cyclase partially purified from nitroglycerin-pretreated rat thoractic aorta by immunoprecipitation with a specific monoclonal antibody exhibited persistent desensitization to nitrate-induced activation. These data suggest that nitroglycerin-induced desensitization of guanylate cyclase to activation by nitrovasodilators represents a stable alteration of the enzyme. In contrast, activation by protoporphyrin IX of guanylate cyclase immunoprecipitated from nitroglycerin-pretreated or control vessels was not significantly different. This suggests that the mechanism of protoporphyrin activation of guanylate cyclase is different than the mechanism with nitrovasodilators. Activation of particulate guanylate cyclase by Lubrol-PX, hemin, or atrial natriuretic factor was not significantly different with enzyme prepared from nitroglycerin-pretreated or control vessels from rat and human. Thus, nitroglycerin-induced desensitization of rat thoracic aorta or human coronary artery results in a relatively stable molecular alteration of soluble guanylate cyclase such that the enzyme is specifically less sensitive to activation by nitrovasodilators whereas the effects of other activators of the enzyme are either unchanged or increased.

Organic nitrates are important therapeutic agents for treating syndromes of myocardial ischemia and ventricular dysfunction [1–4]. However, tolerance to the pharmacological effects of these agents has been described in animals and humans upon chronic exposure [3–5]. Chronic therapy with organic nitrates has been associated with attenuation of the ability of these agents to produce headache, flushing, a decrease in blood pressure, and an increased exercise tolerance [3]. Similarly, prior exposure of blood vessels to organic nitrates *in vitro* results in a decreased relaxation response upon subsequent exposure to these agents [3, 5]. There is considerable evidence supporting the suggestion that relaxation of vascular smooth muscle by nitrovasodilators is mediated by activation of guanylate cyclase and accumulation of cyclic GMP‡ [6, 7]. It has been reported that rat blood vessels exposed to organic nitrates demonstrate desensitization to both the relaxant effects and the elevation in cyclic GMP levels stimulated by nitrovasodilators [8–10]. Guanylate cyclase in crude low-speed supernatant fractions obtained from nitroglycerin (GTN)-desensitized rat blood vessels exhibits a decreased ability to be activated by sodium nitroprusside [9]. In this report we demonstrate that treatment of rat thoracic aorta or human coronary arteries with nitroglycerin *in vitro* results in a relatively stable molecular alteration of the partially purified soluble form of guanylate cyclase that is associated with a decreased ability of this enzyme to be activated by nitrovasodilators. In contrast, the particulate form of the enzyme remains unaffected. The effects of GTN pretreatment are specific only for the effects of nitrovasodilators on guanylate cyclase; the effects of other agents that activate this enzyme remain unaltered or are increased by GTN pretreatment of blood vessels.

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‡ Abbreviations: cyclic GMP, guanosine 3′,5′-monophosphate; GTN, nitroglycerin; SNP, sodium nitroprusside; PPIX, protoporphyrin IX; and AA, arachidonic acid.

METHODS

Preparation of vessel segments and desensitization to nitroglycerin. Aortas were obtained from Sprague–Dawley rats (225–300 g) after decapitation. Human coronary arteries were provided by the Stanford Heart–Lung Transplantation Program. Segments of rat aortas or human coronary arteries were prepared without their endothelium as previously described [11, 12], and were placed in Krebs–Ringer bicarbonate buffer which was gassed with 95% O_2–5% CO_2 and had the following composition (mM): NaCl, 118.5; KCl, 4.74; $MgSO_4$, 1.18; KH_2PO_4, 1.8; $CaCl_2$, 2.5; $NaHCO_3$, 24.9; and glucose, 10. Some tissues were desensitized by exposure to either 0.55 mM (rat aorta) or 0.1 mM (human coronary

arteries) GTN for 1 hr and then washed every 15 min over the following hour [10]. Some tissues remained unexposed to GTN. Control and desensitized vascular segments were frozen in liquid nitrogen, homogenized in buffer containing 50 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, and 250 mM sucrose, and centrifuged at 105,000 *g* for 60 min. The resulting supernatant and particulate fractions were used immediately for guanylate cyclase determinations. Guanylate cyclate was assayed as described previously [13, 14]. Briefly, incubations (100 μl for 20 min at 37°) contained 50 mM Tris–HCl, pH 7.6, 15 mM creatine phosphate, 20 μg creatine phosphokinase (135 units/mg protein), 10 mM theophylline, 1–200 μg protein, and other agents where indicated. Incubations containing GTN received 5 mM cysteine prior to initiating the reaction. Although cysteine has no effect on basal enzyme activity, it is required for GTN activation of soluble guanylate cyclase [15]. Reactions were initiated by the addition of substrate (4 mM $MgCl_2$, 1 mM GTP) and terminated by the addition of 50 mM sodium acetate, pH 4.0, followed by immersion in a boiling water bath for 3 min. Samples were acetylated [16], and cyclic GMP was determined by radioimmunoassay [17]. Enzyme activity was linear over the protein concentrations and time course of the experiments. Pretreatment of blood vessels with nitroglycerin had no effect on basal guanylate cyclase activities. We have reported previously that activation of guanylate cyclase with nitrovasodilators is a reversible phenomenon [18, 19].

Immunoprecipitation of soluble guanylate cyclase. Immunoprecipitation with monoclonal antibodies was performed as previously described [20, 21]. Briefly, 200 μl of purified monoclonal antibody directed against soluble guanylate cyclase (about 50 μg/ml) was incubated with 400 μl of crude soluble guanylate cyclase obtained from homogenates of control and GTN-pretreated rat thoracic aorta. Incubations were performed at 4° for 30 min after which 200 μl of crude mouse serum and 200 μl of rabbit anti-mouse IgG antisera were added. Incubations were continued for 30 min, and then mixtures were centrifuged at 2000 *g* for 30 min. Immunoprecipitates were washed with 1 ml of 20 mM Tris–HCl, pH 7.6, resuspended in homogenization buffer, and used immediately for guanylate cyclase assays. Immunoprecipitation resulted in recoveries in the pellet of 70 ± 21 and 80 ± 25% of the total activities added for control and nitroglycerin-pretreated rat aorta respectively. Total recoveries were 109 ± 24 and 127 ± 30% of the activities added for control and nitroglycerin-pretreated aortas respectively. Similar experiments could not be performed on particulate guanylate cyclase from rat aorta or the soluble or particulate enzymes from human coronary artery because of the lack of cross-reactivity of these isoenzymes with the monoclonal antibodies [20, 21].

Other techniques. Protein was determined by the method of Lowry *et al.* [22] using bovine serum albumin as standard. Statistical significance was accepted at the 0.05 level of probability using paired Student's *t*-test.

Materials. Sodium nitroprusside was obtained from Sigma, atrial natriuretic factor (atriopeptin II) from Peninsula Laboratories, and nitroglycerin (as a 1:10 powder in lactose) from ICI Americas, Inc. Other materials were obtained as previously described [23, 24].

RESULTS

GTN, sodium nitroprusside, and nitric oxide gas (NO) gave a dose-dependent activation of crude soluble guanylate cyclase prepared from rat aorta (Fig. 1); these effects were similar to our earlier observations. The activation of soluble guanylate cyclase by these agents was decreased markedly when rat aortas were pretreated with GTN (Fig. 1). The effects of GTN pretreatment were predominately on the maximal activation of soluble guanylate cyclase observed with these agents, although inhibitory effects at low concentrations also were observed. Maximal activation of soluble guanylate cyclase by these agents in preparations from GTN-pretreated tissues was about one-third to one-half of that observed in preparations from control tissues. The concentrations required for half-maximal activation of GTN-pretreated tissues were variable with GTN, nitroprusside and nitric oxide. Basal activities of soluble guanylate cyclase in preparations from control or GTN-pretreated tissues were not significantly different (data not shown).

The ability of GTN pretreatment to decrease guanylate cyclase activation with other classes of activators was also examined. The effects of arachidonic acid and protoporphyrin IX on the activation of soluble guanylate cyclase prepared from control and GTN-pretreated rat aortas are summarized (Fig. 2). Activation of soluble guanylate cyclase with arachidonic acid yielded a bell-shaped dose–response curve with maximal activation at 10 μM arachidonate. The maximal activation and configuration of the dose–response curve to arachidonate were not significantly different in control tissues and those pretreated with GTN. In contrast, the activation of soluble guanylate cyclase with protoporhyrin IX was enhanced markedly in tissues pretreated with GTN (Fig. 2); maximal activation was increased about 4-fold with GTN pretreatment.

Qualitatively similar results with respect to desensitization were observed with preparations of soluble guanylate cyclase from human coronary arteries. When human coronary artery segments were pretreated with GTN, activation of soluble guanylate cyclase from these preparations by GTN or nitroprusside was decreased markedly compared to the effects on control tissues (Fig. 3). The activation of soluble enzyme with arachidonate from GTN-pretreated coronary vessels was unaltered, whereas the activation with protoporphyrin IX was enhanced markedly. The magnitude of the changes in maximal activation with these agents of soluble guanylate cylcase from human coronary arteries was similar to the effects in preparations from rat aorta. GTN pretreatment did not alter basal enzyme activity (data not shown).

The effects of GTN pretreatment on the ability to activate particulate guanylate cyclase in vascular preparations were also examined. As reported pre-

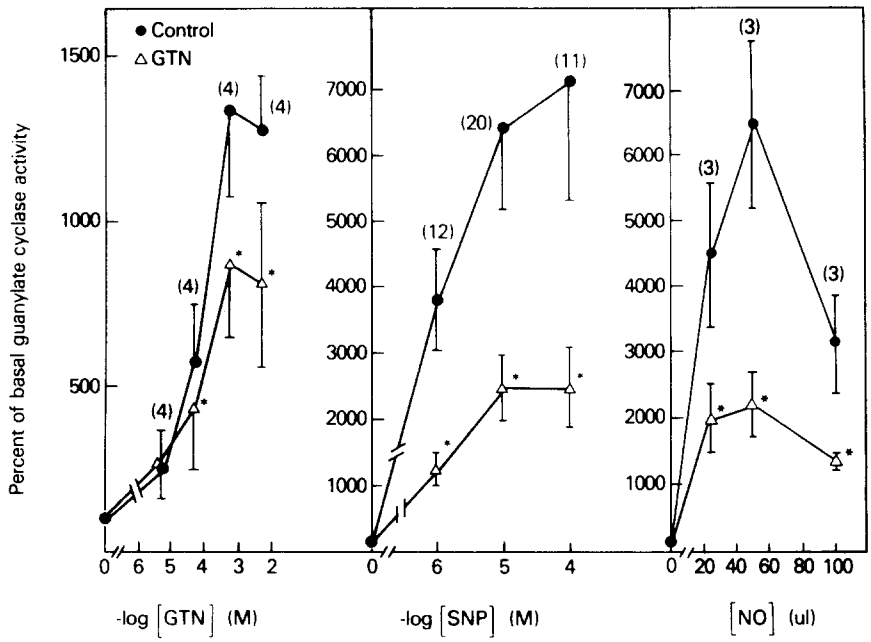

Fig. 1. Dose–response of soluble guanylate cyclase to activation by nitrovasodilators in control (●) and GTN-pretreated (△) rat aorta. Rat aortas were obtained and pretreated with GTN, and supernatant fractions were obtained as described in Methods. Guanylate cyclase activity was assayed as described. Some incubations contained the indicated concentrations of GTN, sodium nitroprusside (SNP), or nitric oxide (NO). Data points represent the mean ± S.E.; the number of experiments is indicated in parentheses. An asterisk indicates values that are significantly different, $P < 0.05$.

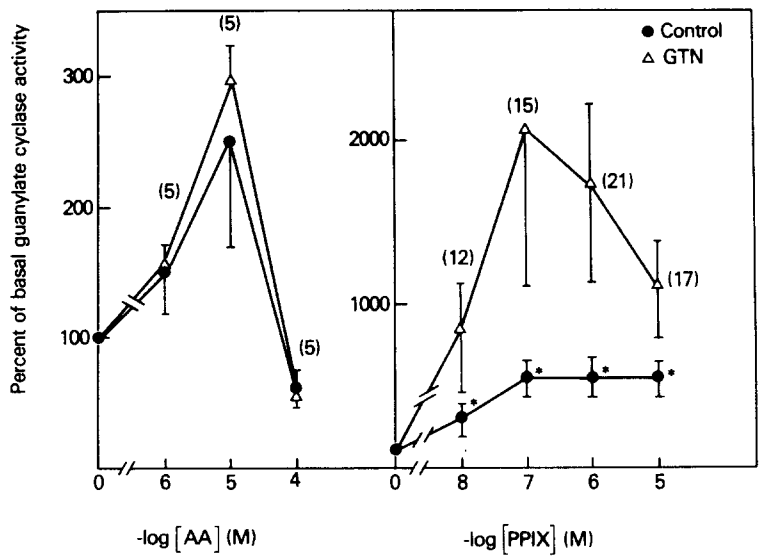

Fig. 2. Dose–response of soluble guanylate cyclase to activation by arachidonic acid (AA) or protoporphyrin IX (PPIX) in control (●) and GTN-pretreated (△) rat aorta. Tissue preparation, experimental conditions, and data presentation are similar to those described in Fig. 1. An asterisk indicates values that are significantly different, $P < 0.05$.

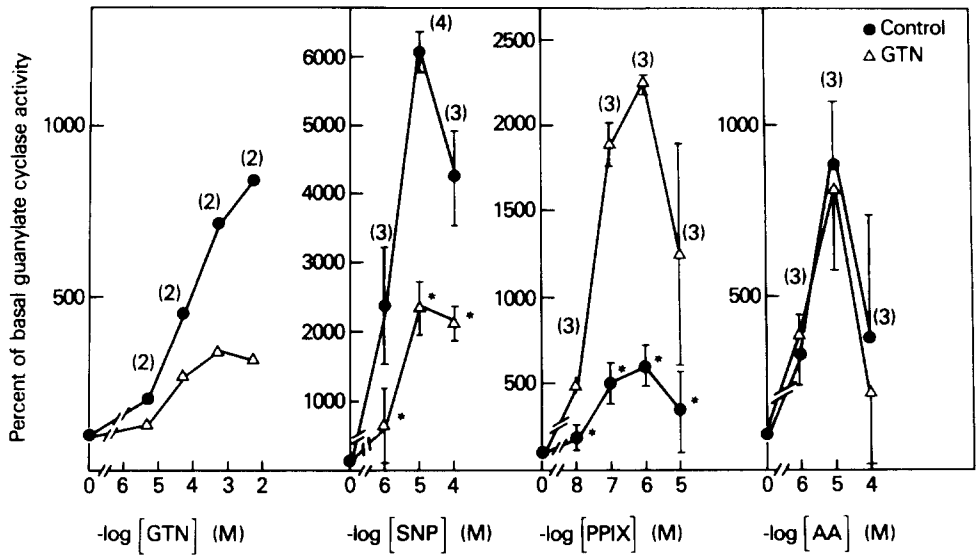

Fig. 3. Dose–response of soluble guanylate cyclase to activation by various agents from control (●) and GTN-pretreated (△) human coronary artery. Human coronary artery was obtained and pretreated, and supernatant fractions were obtained as described in Methods. Experimental conditions and data presentation are similar to those described in Fig. 1. An asterisk indicates values that are significantly different, $P < 0.05$.

viously, Lubrol-PX [21], hemin [25] and atrial natriuretic factor [26, 27] activated particulate guanylate cyclase from rat aorta preparations (Table 1). The effects of these activators on particulate guanylate cyclase at maximally effective concentrations were not altered in preparations from vessels pretreated with GTN.

Although activation of crude or purified soluble guanylate cyclase with nitrovasodilators such as azide, nitroprusside and nitric oxide is reversible [18, 19], the desensitization of guanylate cyclase in the above studies suggested that a relatively stable alteration of the preparation was induced with GTN pretreatment since desensitization of the enzyme was observed after tissue homogenization and centrifugation. This question was examined further with partially purified soluble guanylate cyclase from control and GTN-pretreated tissues. Purification of soluble enzyme from rat tissues could be rapidly accomplished with immunoprecipitation from soluble fractions of homogenates with monoclonal antibodies that specifically reacted with the soluble isoenzyme [20]. The effects of GTN, nitroprusside and nitric oxide on immunoprecipitated soluble guanylate cyclase were quantitatively similar to the effects observed with crude supernatant fractions (compare Figs. 1 and 4). Furthermore, the desensitization of soluble guanylate cyclase to these activators with GTN pretreatment was also observed after immunoprecipitation of enzyme. As we have observed previously [14, 18, 28], the dose–response curves to these activating agents were shifted to the left with purification of the enzyme (compare Figs. 1 and 4). These results strongly suggest that GTN pretreatment produces a relatively stable alteration of soluble guanylate cyclase that survives partial purification under the conditions used.

In striking contrast, the enhanced activation of soluble guanylate cyclase with protoporphyrin IX observed after GTN pretreatment was lost with immunoprecipitation of soluble enzyme (compare Figs. 2 and 4). These observations suggest that the enhanced protoporphyrin IX activation of guanylate cyclase after GTN pretreatment of tissues may be due to some soluble factor that is removed during immunoprecipitation of enzyme. The effects of arachidonate were not modified with immunoprecipitation of enzyme (data not shown).

Table 1. Effects of activators on guanylate cyclase in high-speed pellets prepared from GTN-pretreated and control rat aorta

Pretreatment	Addition	Percent of basal activity
Control	Hemin (100 μM)	711 ± 153
GTN	Hemin (100 μM)	690 ± 151
Control	Lubrol (1.0%)	522 ± 218
GTN	Lubrol (1.0%)	574 ± 76
Control	ANF (1 μM)	371 ± 79
GTN	ANF (1 μM)	309 ± 102

Rat thoracic aortas were exposed to 0.55 mM nitroglycerin for 60 min and then washed as described in Methods. Control vessels remained unexposed. Segments were homogenized, particulate fractions were prepared, and guanylate cyclase activity was determined as described in Methods. Some assays included the indicated concentrations of hemin, lubrol, or atrial natriuretic factor (ANF). Results are expressed as the mean of at least three experiments ± S.E. Basal activities for control and GTN-pretreated enzyme were 2.38 ± 0.54 and 2.07 ± 0.33 pmoles/min/mg protein respectively.

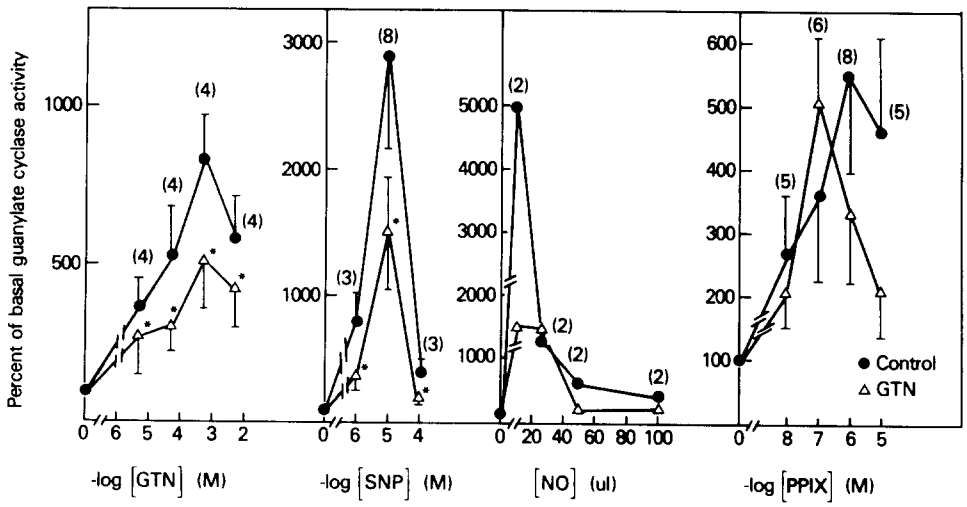

Fig. 4. Dose–response to activation by various agents of soluble guanylate cyclase immunoprecipitated with monoclonal antibodies from supernatant fractions of control (●) and GTN-pretreated (△) rat aorta. Immunoprecipitation was conducted as described in Methods. Tissue preparation, experimental conditions, and data presentation are similar to those described in Fig. 1. An asterisk indicates values that are significantly different, $P < 0.05$.

DISCUSSION

Many studies have suggested that the effects of nitroglycerin, sodium nitroprusside and related nitrovasodilators on smooth muscle relaxation are related to guanylate cyclase activation and cyclic GMP accumulation (see Refs. 6 and 7 for recent reviews of this topic). This laboratory has proposed that these agents lead to the formation of nitric oxide that can activate guanylate cyclase [6, 7, 13, 14, 18, 24, 28–30]. The increase in cyclic GMP levels in vascular smooth muscle induced with the nitrovasodilators which act directly on the smooth muscle, or vasodilators which act on the endothelium to induce relaxation, is associated with activation of cyclic GMP-dependent protein kinase [6, 7, 31], decreased phosphorylation of myosin light chain [6, 7, 32, 33], and relaxation [6, 7, 12, 32, 33].

Although these agents can alter the properties of soluble guanylate cyclase, the precise mechanism of activation is unknown. It has been suggested by some investigators that thiol groups are important for both basal enzyme activity and activation by nitrovasodilators [19, 28, 34–37]. Heme can also facilitate activation of guanylate cyclase by this class of agents [18, 36, 38, 39]. Some investigators have found heme in purified preparations of enzyme [36], suggesting that heme may be a prosthetic group on the protein. Other agents can also activate the soluble form of guanylate cyclase, and these include protoporphyrin IX [40, 41] and unsaturated fatty acids such as arachidonate [42, 43]. The particulate form of guanylate cyclase can be activated by detergents [21], hemin [25] and in some tissues by peptides such as *Escherichia coli* heat-stable enterotoxin [44–46] and atrial natriuretic factor [26, 27]. The molecular mechanisms of activation of soluble or particulate guanylate cyclase by this diverse group of agents have not been determined. The present study suggests that the mechanisms of activation of soluble guanylate cyclase by nitrovasodilators, arachidonate and protoporphyrin IX are different.

Tolerance or desensitization to organic nitrates can develop with their repeated administration. Recent reports of desensitization with GTN have described decreased cyclic GMP accumulation in vessels and decreased activation of crude guanylate cyclase [8, 9]. These studies did not report the effects of other activators of guanylate cyclase. In earlier studies it was reported that the relaxant effects of GTN in vessels could be restored by treating tolerant tissues with thiols [5].

The present studies utilized this model of GTN desensitization to examine the properties and mechanisms of regulation of guanylate cyclase from rat thoracic aorta and human coronary artery preparations. In preliminary experiments we established that the relaxant effects of GTN were diminished markedly when these tissues were pretreated with GTN *in vitro* (data not shown). These observations are similar to the reports by others using segments of bovine mesenteric artery or rat aorta [8–10]. Basal guanylate cyclase activity of soluble or particulate fractions was not altered by pretreatment of tissues with GTN (data not shown). However, the ability to activate soluble but not particulate guanylate cyclase was altered markedly when tissues were pretreated with GTN. Qualitatively similar results were obtained with rat thoracic aorta and human coronary artery. Crude soluble guanylate cyclase from control tissues which were not pretreated with GTN could be markedly activated with GTN, sodium nitroprusside or nitric oxide (Figs. 1 and 3). After pretreatment of intact tissues with GTN, the maximal activation of soluble guanylate cyclase by these agents was decreased to about one-third to one-half of that observed in tissues without GTN pretreatment. The concentration of activator required for

half-maximal activation of soluble guanylate cyclase from control or GTN-pretreated tissues was usually similar. The desensitization of soluble guanylate cyclase was specific for the nitrovasodilator class of agents (GTN, nitroprusside and NO). The effects of arachidonate on activation of soluble guanylate cyclase were similar in control versus GTN-pretreated tissue, while the maximal activation with protoporphyrin IX was enhanced markedly in GTN-pretreated tissue (Figs. 2 and 3), suggesting that the three classes of agents activate soluble guanylate cyclase through different mechanisms.

The desensitization of soluble guanylate cyclase to nitrovasodilators after pretreatment of vessels with GTN was also observed after immunoprecipitation of soluble guanylate with specific monoclonal antibodies. These observations further support a relatively stable modification of soluble guanylate cyclase after GTN pretreatment of tissue to explain the desensitization. The effects of protoporphyrin IX on immunoprecipitated enzyme were similar in control and GTN-pretreated tissue (Fig. 4). The loss of the enhanced protoporphyrin IX effect of GTN-pretreated tissue observed with crude soluble enzyme (Fig. 2) suggests that the potentiation of the protoporphyrin IX effect was not due to a modification of the enzyme but rather to some factor in the supernatant fraction that was removed with immunoprecipitation of enzyme. The properties of the factor generated by nitroglycerin pretreatment of blood vessels are being investigated currently in this laboratory. It is of interest that a soluble protein with a molecular weight of about 120,000 daltons has been described that increases protoporphyrin IX activation of guanylate cyclase [47].

The changes in guanylate cyclase after GTN pretreatment were confined to the soluble isoenzyme. Activation of the particulate enzyme with detergent, hemin and atrial natriuretic factor was not modified with GTN pretreatment.

These studies support the hypothesis that nitroglycerin-induced tolerance or desensitization may, in part, be mediated by a modification of soluble guanylate cyclase [9]. The conditions and requirements to reverse the desensitization and alterations of guanylate cyclase are currently under investigation in this laboratory. In preliminary experiments the addition of dithiothreitol to GTN-treated tissue failed to reverse the desensitization to GTN-induced relaxation or the desensitization of soluble guanylate cyclase to activation by nitrovasodilators. The effects of GTN desensitization on the activities of other vasodilators whose mechanism of action may be mediated through cyclic GMP accumulation are currently unknown and are also being examined.

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