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Nitroglycerine-induced biphasic relaxation in vascular smooth muscle of rat aorta

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1 Nitroglycerine induced biphasic relaxation in the rat aorta, previously contracted by noradrenaline; a rapid decrease in tension was followed by a gradual increase reaching a steady level below the control contractile tension. No initial transient relaxation was induced by nitroglycerine in high K-stimulated muscle.

2 The initial transient relaxation, but not the sustained relaxation, was dependent on the concentration of external K; maximum relaxation was observed in the presence of 2.7 mM K solution and only a slight relaxation was observed in 0 mM or 10.8 mM K solution. The initial transient relaxation was also inhibited by ouabain or low Na solution.

3 On an appropriate increase in the concentration of external K, noradrenaline-induced contraction was transiently relaxed. Previous application of nitroglycerine potentiated this K-induced relaxation.

4 Pretreatment of the muscle with methylene blue, an inhibitor of guanylate cyclase, inhibited both the initial transient and the sustained relaxations induced by nitroglycerine, but not the K-induced transient relaxation.

5 It is suggested that the nitroglycerine-induced initial transient relaxation, but not the sustained relaxation, may be due to a stimulation of an electrogenic Na pump. Both relaxation phases may be mediated by cyclic GMP.

Introduction

Nitroglycerine has been widely used for relief of the acute anginal attack, and its effect is attributed mainly to its vasodilator action. It has been reported to stimulate guanylate cyclase, and the increase in cellular cyclic guanosine-3′, 5′-monophosphate (GMP) may lead to a relaxation of vascular smooth muscle (Katsuki *et al.*, 1977; Kukovetz *et al.*, 1981). However, little is known of the mechanism by which cellular cyclic GMP induces vasodilatation. In the present paper, we found that part of the effect of nitroglycerine may be attributable to stimulation of an electrogenic Na pump in the vascular smooth muscle of rat aorta.

Methods

Tissue preparation

Male Wistar rats, weighing about 200 g, were stunned and bled. The thoracic aorta was dissected out and spiral strips, 2–3 mm wide and 5–8 mm long, were prepared. The muscle strips were equilibrated in the bathing solution for 60 to 90 min before experiments were started.

Solutions

The normal bathing solution contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5 (Karaki *et al.*, 1981). The concentration of KCl was changed in some experiments. High K solution was made by substituting 60 mM Na in the normal solution with equimolar K (isosmotic 65.4 mM K). Low Na solution was made by replacing NaCl with equimolar LiCl. These solutions were aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C (pH 7.4).

Tension

Muscle tension was recorded isometrically with a force-displacement transducer connected to a Nihon Kohden polygraph (Japan). After equilibration, 65.4

mM K solution was repeatedly applied until the same size of contractions were obtained. Concentration-inhibition curves for nitroglycerine were constructed by the following two procedures. The first curve was obtained by cumulative application of nitroglycerine during a sustained contraction induced by noradrenaline; a higher concentration of nitroglycerine was added when previously added nitroglycerine induced a maximum relaxation. The second curve was constructed from cumulative applications of noradrenaline to a muscle pretreated with nitroglycerine for 20 min. The effect of a single application of nitroglycerine was examined in the following way: noradrenaline was added to the muscle and 20 min later nitroglycerine was added. After 30 min, the muscle was washed with normal solution and a 20 min rest was allowed; then 65.4 mM K was added for 10 min. This was followed by a 30 min resting period, noradrenaline was added again and the above procedure was repeated. With this time schedule, it was found that although the response to the first application of nitroglycerine was sometimes less than that induced by the following applications, constant responses were obtained at the second and the third applications of nitroglycerine when the concentration of nitroglycerine was below 10^{-5} M. We used the second application of nitroglycerine as a control and the third as a test.

Drugs and statistics

Nitroglycerine (donated by Nippon Kayaku Co., Tokyo), noradrenaline bitartrate (Tokyo Kasei Co.) and methylene blue (Tokyo Kasei Co.) were used. All data are expressed as the mean ± s.e.mean. Statistical significance was determined by Student's *t* test.

Results

Concentration-inhibition curves obtained by cumulative application of nitroglycerine during a sustained contraction induced by noradrenaline are shown in Figure 1a. The effect of nitroglycerine was dependent on the concentration of added nitroglycerine as well as on the concentration of noradrenaline. In the presence of 10^{-6} M noradrenaline, the concentration of nitroglycerine inducing 50% relaxation (IC_{50}) was 8×10^{-8} M; in the presence of 10^{-7} M noradrenaline, the IC_{50} was 3×10^{-8} M, and in the presence of 10^{-8} M noradrenaline, the IC_{50} was 2×10^{-9} M. In Figure 1b, concentration-inhibition curves constructed from the data obtained by cumulative application of noradrenaline to the muscle pretreated with nitroglycerine are shown. Although the result of this experiment was qualitatively similar to that in Figure 1a, the IC_{50} values were much higher; in the presence of 10^{-6} M noradrenaline the IC_{50} was higher than 10^{-6} M, in the presence of 10^{-7} M noradrenaline the IC_{50} was approximately 1×10^{-6} M, and in the presence of 10^{-8} M noradrenaline, the IC_{50} was 3×10^{-8} M.

From these results, it seemed possible that although nitroglycerine relaxes vascular smooth muscle soon after the application, the relaxant effect decreases. Therefore, the time course of the relaxant effect of nitroglycerine was examined and the result is shown in Figure 2. Nitroglycerine, 10^{-7} M, added in the presence of 10^{-6} M noradrenaline, rapidly relaxed the muscle by 73.4 ± 3.3% (mean ± s.e.mean, $n = 12$) in approximately 2 min. Muscle tension then

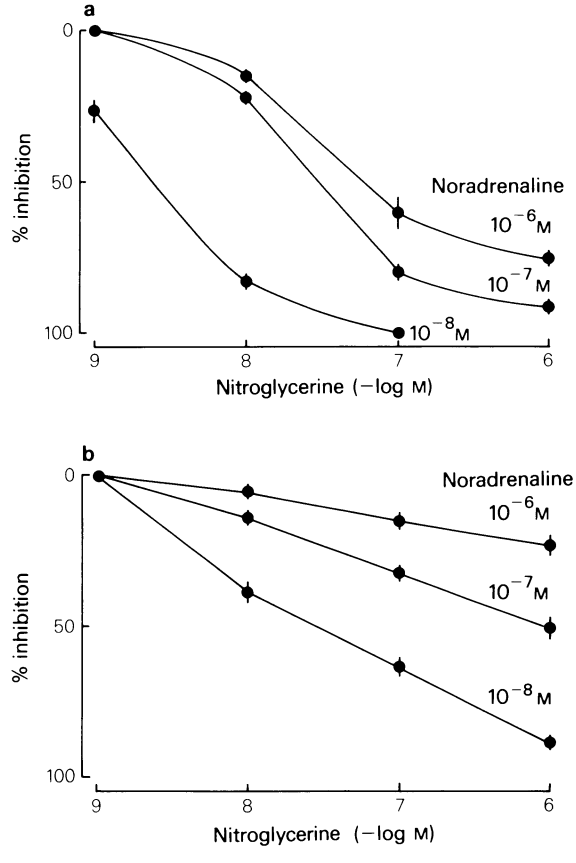

Figure 1 Inhibitory effect of nitroglycerine on noradrenaline-induced contraction in rat aorta. (a) Nitroglycerine was applied cumulatively during the sustained contraction induced by noradrenaline. (b) Noradrenaline was applied cumulatively to the aorta pretreated with nitroglycerine for 20 min. Mean of 4 to 6 experiments are shown; s.e.mean indicated by vertical lines.

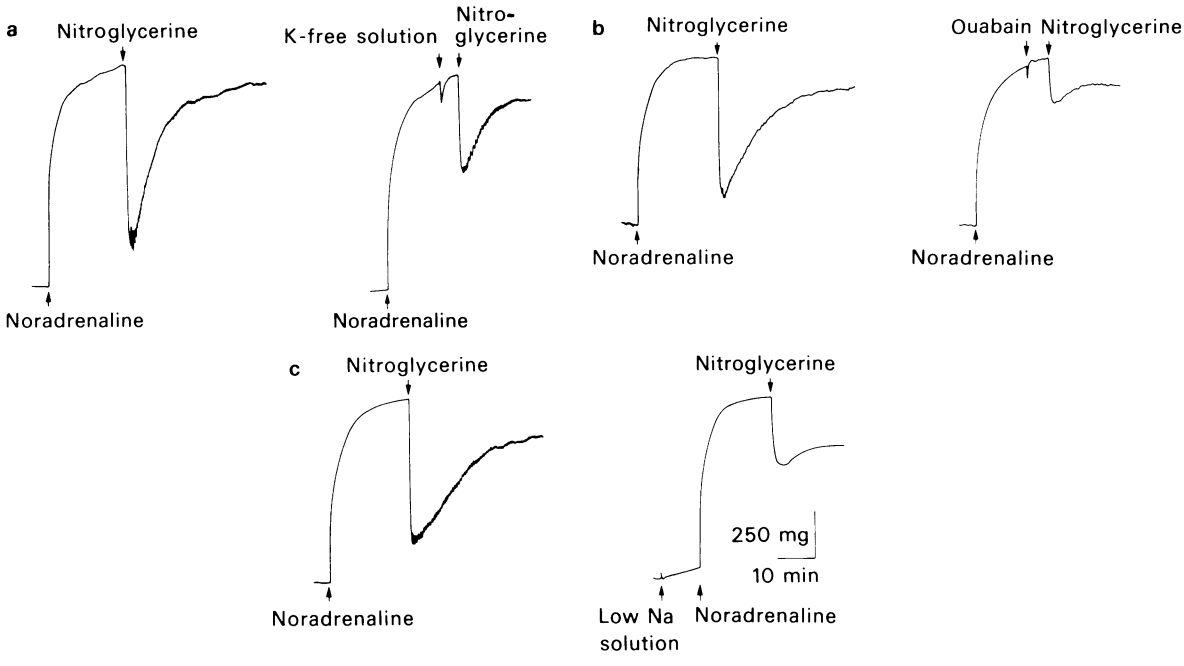

Figure 2 Biphasic relaxant effect of 10^{-7} M nitroglycerine on 10^{-6} M noradrenaline-induced contraction in rat aorta (left), and modification of the biphasic relaxation by K-free solution (a, right), 5×10^{-4} M ouabain (b, right) and low Na, Li solution (c, right). K-free solution or ouabain was added 5 min before the application of nitroglycerine and low Na solution was added 20 min before the application of noradrenaline.

gradually increased and reached a steady level in 20 to 30 min which was $18.8 \pm 1.2\%$ ($n = 12$) lower than the control level. Thus, nitroglycerine showed biphasic relaxant effects; an initial transient relaxation and a subsequent sustained relaxation. Such a biphasic relaxant effect was observed with concentrations of nitroglycerine ranging from 10^{-8} M to 10^{-6} M in the presence of noradrenaline 10^{-8} M to 10^{-6} M, although the respective magnitude of the phases of relaxation was different depending on the concentration of nitroglycerine and noradrenaline. In contrast, in the presence of 65.4 mM K, only a sustained relaxation but not an initial transient relaxation was induced by nitroglycerine. To test the possibility that added nitroglycerine is rapidly decomposed allowing muscle tension to increase gradually, 10^{-7} M nitroglycerine was added to the 10^{-6} M noradrenaline-stimulated muscle. The bath solution was removed 30 min later and another muscle which was already contracted by 10^{-6} M noradrenaline was immersed in it. This procedure induced biphasic relaxation of the muscle with the same magnitude as that induced by an application of 10^{-7} M nitroglycerine, suggesting that nitroglycerine is not decomposed during a 30 min incubation.

In Figure 2, the effects of a K-free solution, ouabain 5×10^{-4} M, and a low Na solution on the biphasic effect of nitroglycerine are shown. The K-free solution and ouabain were added 5 min before the addition of nitroglycerine. Low Na solution was added 20 min before the addition of noradrenaline. These procedures significantly inhibited the initial transient relaxation induced by nitroglycerine; control $73.4 \pm 3.3\%$ (mean $\pm$ s.e.mean, $n = 12$); K-free solution, $34.3 \pm 3.1\%$ ($n = 4$); in the presence of ouabain, $26.2 \pm 1.8\%$; and in low Na solution, $36.9 \pm 3.5\%$ ($n = 4$). In contrast, the sustained relaxation was not affected; control 18.8 ± 1.2 ($n = 12$); K-free solution, $17.9 \pm 3.4\%$ ($n = 4$); ouabain, $16.3 \pm 2.8\%$ ($n = 4$); low Na solution, $21.3 \pm 3.0\%$ ($n = 4$). In the aorta stimulated with 65.4 mM K, the sustained relaxation induced by 10^{-7} M nitroglycerine ($21.3 \pm 2.1\%$, $n = 4$) was not affected by 5×10^{-4} M ouabain ($19.2 \pm 2.0\%$, $n = 4$).

The effects of changes in external K concentration on the biphasic relaxation were examined further. The concentration of K was changed 20 min before the addition of noradrenaline. As show in Figure 3, the initial transient relaxation induced by nitroglycerine was affected by external K. Maximum relaxation was obtained when the K concentration was 2.7mM to 5.4mM and only a slight relaxation was

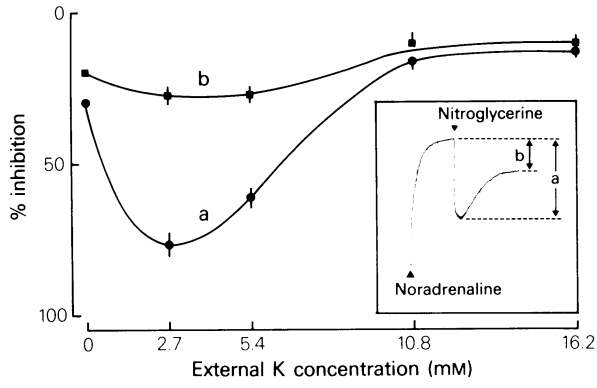

Figure 3 Effect of changes in the concentration of external K on the nitroglycerine-induced biphasic relaxation of noradrenaline-induced contraction in rat aorta. Experiments were performed as shown in the inset. The K concentration was changed 20 min before the addition of 10^{-6} M noradrenaline and 10^{-7} M nitroglycerine was added 20 min after the addition of noradrenaline. (a) Initial transient relaxation, (b) sustained relaxation. Mean of 4 to 6 experiments are shown; vertical lines indicate s.e.mean.

obtained when the K concentration was 0, 10.8 or 16.2 mM. On the other hand, sustained relaxation was only slightly affected by external K.

The effect of nitroglycerine on K-induced relaxation is shown in Figure 4. This experiment was done in the presence of either 2.7 mM K or 1.4mM K. During the contraction induced in 2.7 mM K solution by 10^{-6} M noradrenaline, addition of 10 mM K (total K concentration 12.7 mM) slightly relaxed the muscle ($3.0 \pm 0.2\%$, mean ± s.e.mean, $n = 4$). When 10 mM K was added 10 min after the addition of 10^{-7} M nitroglycerine the K-induced relaxation was significantly increased ($29.3 \pm 3.2\%$, $n = 4$) (Figure 4a). In 1.4 mM K solution, addition of 10 mM K (total K concentration 11.4 mM) relaxed the noradrenaline-induced contraction by $41.4 \pm 1.1\%$ ($n = 6$). When 10 mM K was added 20 min after the addition of nitroglycerine, the magnitude of the K-induced relaxation did not change ($40.5 \pm 1.5\%$, $n = 6$) but the duration of the relaxation (time interval between the onset of K-induced relaxation and the time when muscle tension recovered the level before the K-application) increased from 5.6 ± 0.4 min ($n = 6$) to 7.7 ± 0.4 min ($n = 6$) (Figure 4b).

In Figure 5a, the effects of nitroglycerine on the noradrenaline-induced contraction in muscle pretreated for 60 min with methylene blue are shown. Such pretreatment did not change the noradrenaline-induced contraction. However, both the initial transient and the sustained relaxation induced by nitroglycerine were significantly inhibited by methylene blue pretreatment. As shown in Figure 5b, addition of 10 mM K transiently relaxed the 10^{-6} M noradrenaline-stimulated muscle pretreated with K-free solution by $48.6 \pm 3.7\%$ (mean ± s.e.mean, $n = 6$) for a period of 8.9 ± 0.2 min ($n = 6$). Methylene blue pretreatment did not modify this K-induced relaxation (relaxation, $49.4 \pm 3.5\%$; duration, 9.5 ± 0.5 min, $n = 6$).

Discussion

Nitroglycerine induced biphasic relaxation in the noradrenaline-contracted rat aorta. The initial relaxation was more pronounced than the sustained relax-

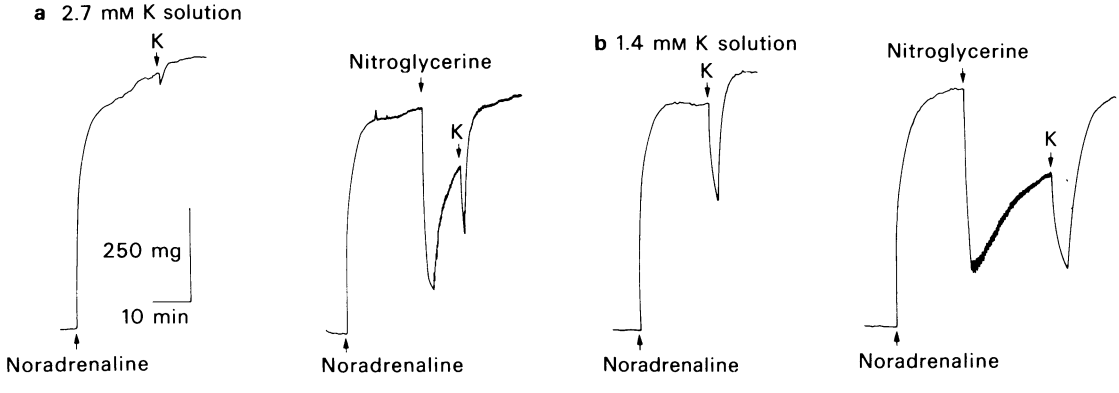

Figure 4 Potentiation by nitroglycerine of the K-induced transient relaxation of noradrenaline-induced contraction in rat aorta. (a) In 2.7 mM K solution, muscle contracted by 10^{-6} M noradrenaline was slightly and transiently relaxed by the addition of 10 mM K (left). When 10^{-7} M nitroglycerine was added 10 min before the addition of 10 mM K, the K-induced relaxation was potentiated (right). (b) In 1.4 mM K solution, duration but not magnitude of the 10 mM K-induced transient relaxation was prolonged by a nitroglycerine-pretreatment.

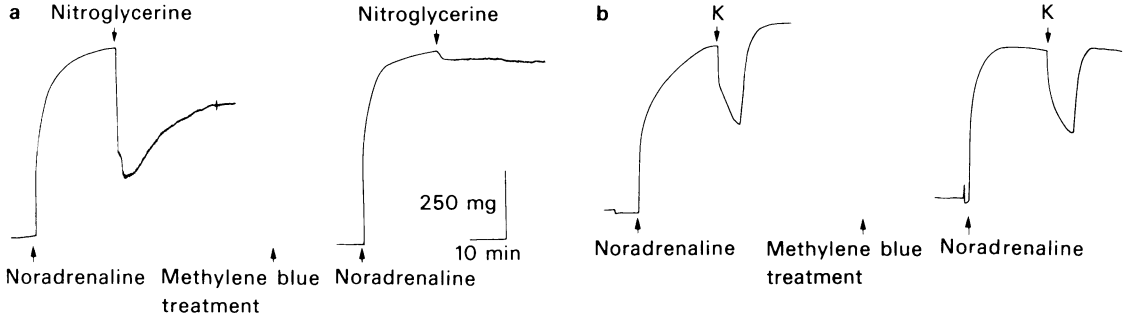

Figure 5 Effect of pretreatment of the muscle with methylene blue on the nitroglycerine- (a) and 10 mM K-induced relaxation (b) in rat aorta. (a) After the control response to 10^{-7} M nitroglycerine was obtained (left), the muscle strip was exposed to 10^{-6} M methylene blue for 60 min. The muscle was then washed with normal solution for 20 min and 10^{-6} M noradrenaline was added. Nitroglycerine had little effect on this contraction (right). (b) Muscle contraction induced by 10^{-6} M noradrenaline in 0 mM K solution was transiently relaxed by 10 mM K (left). Methylene blue pretreatment did not change the K-induced relaxation (right).

ation. Thus, the IC_{50} for nitroglycerine calculated from the cumulative application of nitroglycerine to the noradrenaline-stimulated muscle (which mainly represents the initial effect of nitroglycerine) was much less than that calculated from the cumulative applications of noradrenaline to muscle pretreated with nitroglycerine (which mainly represents the sustained effect of nitroglycerine).

The initial, but not the sustained, relaxation was affected by the inhibitors of Na, K-ATPase, i.e., K-free solution, ouabain and low Na solution. Maximum relaxation was induced by nitroglycerine when external K concentration was 2.7 mM. In the guinea-pig portal vein, Karashima (1980) found that nitroglycerine in concentrations above 2.8×10^{-8} M produced an initial transient hyperpolarization followed by a depolarization. Further, we observed that nitroglycerine induced only a sustained relaxation in high K-depolarized aorta, and ouabain had no effect on it. These results suggest that the initial transient relaxation may be due to stimulation of electrogenic Na pumping by Na, K-ATPase, resulting in a membrane hyperpolarization.

An appropriate increase in the concentration of external K induces smooth muscle relaxation, and this effect of K is the result of stimulated Na, K-ATPase activity producing membrane hyperpolarization (Anderson, 1976; Bonaccorsi *et al.*, 1977; Haddy, 1978; Karaki & Weiss, 1981). In the present experiments, it was found that addition of 10 mM K relaxed the noradrenaline-induced contraction by 3% in 2.7 mM K solution and by 41% in 1.4 mM K solution (Figure 3). In 0 mM K solution in which the effect of readdition of K could be fully seen, although the magnitude of relaxation (49%) was similar to that in 1.4 mM K solution, the duration of relaxation was longer in this solution (8.9 min) than in 1.4 mM K solution (5.6 min) (Figures 4b and 5b). Pretreatment of the muscle with nitroglycerine increased the magnitude (in 2.7 mM K solution) or the duration (in 1.4 mM K solution) of the relaxation induced by 10 mM K. This finding supports the above suggestion that nitroglycerine potentiates the activity of Na, K-ATPase in cooperation with K.

It has been reported that nitroglycerine-induced stimulation of guanylate cyclase activity in vascular smooth muscle is inhibited by methylene blue (Ignarro *et al.*, 1981). In the present experiments, both the transient and the sustained relaxation phases induced by nitroglycerine were inhibited by methylene blue but the K-induced relaxation was not. These results suggest that both phases of nitroglycerine-induced relaxation may be due to an increase in cellular cyclic GMP level, although the K-induced transient relaxation is not.

Recently, Rapoport & Murad (1983) showed that relaxation of the rat aorta induced by sodium nitroprusside was inhibited by exposure to K-free solution or ouabain, and that the K-induced relaxation was increased by sodium nitroprusside. From these and other results, they concluded that sodium nitroprusside may induce relaxation through cyclic GMP formation, effects on the Na, K-pump and/or hyperpolarization of the smooth muscle cell membrane. Since they applied sodium nitroprusside cumulatively to the muscle stimulated by noradrenaline, the effect of sodium nitroprusside observed by them could be an initial relaxant effect of this agent. In preliminary experiments, we found that the various nitrogen oxide-containing vasodilator compounds (i.e., sodium nitroprusside, isosorbide dinitrate and nicorandil (2-nicotinamidoethyl nitrate)), induced biphasic relaxation in rat aorta stimulated by noradrenaline; only the initial transient relaxation ap-

peared due to stimulation of Na, K-ATPase. These compounds induced only a sustained relaxation in the high K-stimulated rat aorta which was not inhibited by ouabain and suggest that the nitrogen oxide-containing vasodilator compounds share a common mechanism of action. It may also be noteworthy that the relaxant effect of isoprenaline in vascular smooth muscle, mediated by cyclic AMP, may be due to augmentation of Na, K-ATPase (Somylo *et al.*, 1970) and Na, K-ATPase may also play a role in the vasodilatation induced by prostaglandins (Lockette *et al.*, 1980), acetylcholine (De Mey & Vanhoutte, 1980) and desoxycorticosterone (Koehler *et al.*, 1979).

In conclusion, it is suggested that the initial transient relaxation induced by nitroglycerine is the result of stimulation of electrogenic Na pumping whereas the sustained inhibitory effect of nitroglycerine is not dependent on Na, K-ATPase activity. Both relaxation phases may be mediated by cyclic GMP.

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