

Changes in Cytosolic Ca^{2+} but not in cGMP Contents May be more Important to Nitric Oxide-Mediated Relaxation in Depolarized Vascular Smooth Muscle

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Nitric oxide (NO)-mediated relaxation in vascular smooth muscle involves not only activation of guanylate cyclase but also hyperpolarization of the membrane. It has been shown that depolarization decreases the $[\text{Ca}^{2+}]$ sensitivity of myosin light chain kinase in arterial smooth muscle, and nitric oxide (NO)-mediated relaxation was attenuated in this situation. However, why potassium inhibits or attenuates the action of EDRF/NO is not clear. Therefore, we investigated the magnitude of relaxation and cGMP contents using measures known to release NO, such as photorelaxation, photo activated NO-mediated relaxation, and NO-donor (SNP)-mediated relaxation in porcine coronary arterial rings in which contractile conditions were made by different degree of depolarization, i.e., contraction in response to U46619 or U46619 plus KCl. In all cases, the magnitude of relaxation was significantly greater ($P < 0.05$) in U46619-contracted rings than in U46619+KCl-contracted ones. Although accumulation of cGMP was evident with three measures employed in the present study, no difference was found in cGMP contents between U46619 and U46619+KCl conditions, indicating that the diminished relaxation in KCl containing solution is cGMP-independent mechanism(s). To understand this further, cytosolic Ca^{2+} changes due to NO were compared in rat thoracic aorta by exploiting photoactivated NO using streptozotocin (STZ) that was contracted with either NE or KCl. Fura-3 $[\text{Ca}]_{\text{cyt}}$ signal caused by NO was small and transient in high K^{+} , but large and sustained in NE-contracted aorta. The inhibitory potency of STZ expressed in terms of IC_{50} was 5.14 and 3.88 μM in NE and in high K^{+} , respectively. These results suggest that modification of the cellular mobilization of Ca^{2+} rather than cGMP levels may be an important mechanism for the NO-mediated relaxation when vascular membrane is depolarized, such as atherosclerosis and hypertension.

Key Words: Nitric oxide, Ca^{2+} sensitivity, cGMP, Vascular smooth muscle, Depolarization

INTRODUCTION

UV light irradiation of vascular smooth muscle (photorelaxation) produces a liable photo-induced

relaxing factor which acts in a manner similar to EDRF/NO (Furchgott et al, 1984; Matsunaga & Furchgott, 1991; Chang et al, 1993; 1994; 1997). Vascular smooth muscle contains a store of depletable NO which is light activated and restored by donors of NO (Ventrini et al, 1993). Chang et al, (1993, 1994) reported that so called photo-induced adequate nitric oxide (PIANO), in which a NO- or NO_2 -carrying molecule is photoactivated to release a

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potent relaxing substance, NO. Thus, PIANO can be exploited to investigate the role of NO in various physiological processes in which NO is a mediator. (Chang et al, 1993; 1994; Chung & Chang, 1994; Lee & Chang, 1995, Chang, 1995, Chung et al, 1996). It has been shown that precontraction of arterial rings with potassium is known to inhibit or attenuate endothelium-dependent relaxation (Luckhoff and Busse, 1990; Laskey et al, 1990; Ishii et al, 1997). Depolarization decreases the $[Ca^{2+}]$ sensitivity of myosin light-chain kinase in arterial smooth muscle (Gilbert et al, 1991). The ability of ACh or NO to hyperpolarize vascular smooth muscle appears to be dependent upon the level of the membrane potential (Tare et al, 1990). However, why potassium inhibits the release or action of EDRF/NO is not known. Although NO-mediated relaxation in vascular smooth muscle involves not only activation of guanylate cyclase but also hyperpolarization of the membrane, it is still not clear whether cGMP increment or membrane hyperpolarization or both are equally responsible for the NO-mediated relaxation in membrane depolarized state. It has been reported that hyperosmolarity may be a factor for impaired nitroergic neurotransmission in diabetic animals and in certain diseases. Therefore, in the present study, the magnitude of relaxation and cGMP contents using measures known to release NO, such as UV light (photorelaxation), UV light exposure in the presence of NO-carrying molecules (photoactivated NO) and sodium nitroprusside (SNP, NO donor) in both U46619- and U46619 + KCl-contracted porcine coronary arteries were compared. Finally, the effect of photo-activation of caged NO on cytosolic Ca^{2+} change and muscle tension in agonist- and in high K^+ -contracted rat thoracic aorta were investigated.

METHODS

Materials

Streptozotocin (STZ), phenylephrine HCl (PE), indomethacin, sodium nitroprusside (SNP) and N^w -nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, USA). U46619 (9- α ,11 α -methanoepoxy-PGF_{2 α}) was obtained from Biomol (Biomol Research Laboratory Inc., PA, USA).

Tissue preparations

Male Wistar rats of either sex (250~300 g) were sacrificed by stunning and the thoracic aorta was removed. The tissues were cleaned of adhering fat and connective tissue, and thoracic aortic rings were (3~4 mm wide) prepared. For measurement of Ca^{2+} signal, aortic strips were prepared instead of rings. The vascular endothelium was mechanically removed by rubbing gently with wooden stick as described (Chang et al, 1992). For photorelaxation experiments, endothelium-denuded porcine coronary artery rings (3~4 mm wide) were prepared as the same as described above in rat aorta. Pig heart was obtained from local slaughter.

Isometric tension study

Each tissue was placed in 5 ml water jacketed muscle chamber containing Krebs-Ringer bicarbonate solution which was gassed with 95% O₂ - 5% CO₂ and had the following composition (mM): NaCl, 136.9; KCl, 5.4; MgCl₂, 1.0; NaHCO₃, 23.8; CaCl₂, 1.5; glucose, 5.5; and EDTA, 0.03. The rings were equilibrated at 1 g (rat aorta) and 8 g (porcine artery) for 60 min, with washing at 20 min intervals, prior to drug addition. Indomethacin (10 μ M) was included through the entire of experiment and isometric tension was recorded on a Grass physiograph (Model 7E) using a force displacement transducer (FT-03). To understand whether NO-mediated response is diminished in KCl-containing solutions, photorelaxation, photoactivated NO-mediated relaxation using different type of photosensitizers (STZ, L-NAME) and NO donor (SNP)-mediated relaxation were compared in either U46619- or U46619 + KCl- contracted PCA rings. To obtain more accurate result, the maximum contractile force of both cases were similarly adjusted by the concentration of U46619. After reaching a plateau of contraction by administration of U46619 or U46619 + KCl, PCA rings were subjected to expose to UV light (60 sec) 3 to 6 times using a long wavelength UV lamp (366 nm, Minealight UV GL 58, San Gabriel, CA, USA) in the absence (photorelaxation) of presence (photoactivated NO-mediated relaxation) of photosensitizers such as STZ and L-NAME. To negate the functional antagonism, nifedipine (300 nM) was added 20 min before addition of U46619 and U46619 + KCl. In separate experiments, rat thoracic aorta was contracted with either

NE (0.1 μM) or KCl (65.4 mM), and concentration-relaxation response curves of STZ was constructed. Photoactivated NO-related experiments were done in dark conditions to avoid releasing NO from the photosensitizers.

Cyclic GMP measurement

Cyclic GMP levels were measured using the same protocol as in tension study, in which the PCA rings contracted with either U46619 or U46619+KCl. Aortic rings were quickly frozen with the aid of brass clamps precooled in liquid nitrogen. Samples were extracted and assayed for cGMP by radioimmunoassay as described (Lee & Chang, 1995).

Intracellular Ca²⁺ change and muscle tension in rat aorta

Cytosolic Ca²⁺ levels was measured simultaneously with muscle contraction as described by Ozaki

et al (1987) using a fluorescent indicator, fura-3. The muscle was loaded with 5 mM acetoxymethyl ester of fura-3 for 3 hr in the presence of 0.02 % cremophore EL at room temperature (23°C) and then placed in a tissue bath to measure Ca²⁺ signal and tension at 35°C. The muscle was illuminated alternatively (48 Hz) with 340 nm and 380 nm light after administration of either 1 μM NE or 65.4 mM KCl, in which the contraction reached plateau, STZ was applied and 500 nm emission was detected with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). The amounts of the 500 nm fluorescence induced by the 340 nm excitation (F380) was measured and the ratio of these two fluorescence (R340/380) was calculated.

Statistics

Data are expressed as mean ± SEM. Differences between two groups were determined by Student's t test and were considered significantly different if P < 0.05.

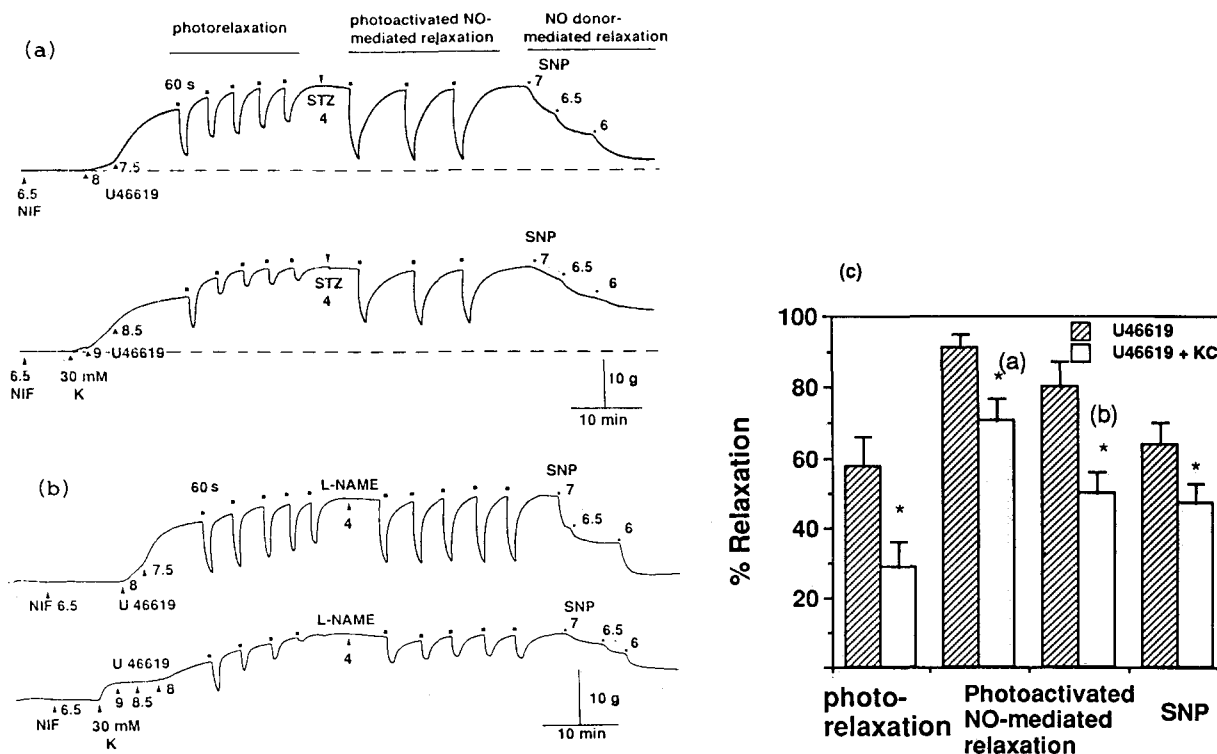


Fig. 1. Typical tracings of photorelaxation, photoactivated NO-mediated relaxation [UV irradiation in the presence of NO-releasing compound, streptozotocin, STZ(a) and L-NAME (b)], and NO-donor-mediated relaxation (SNP) in porcine coronary artery rings contracted with U46619 (upper tracing) or with U46619 in the presence of KCl (lower tracing). Data represent mean ± SEM of 3 different experiments (c). * indicates P < 0.05.

RESULTS

Comparison of magnitude of relaxation and cGMP contents by UV light, photoactivated NO, and SNP response in U46619-contracted and U46619+KCl-contracted porcine coronary artery

As shown in Fig. 1a and b, UV light exposure for 60 s alone relaxed the aorta, but repeated exposure gradually decreased the relaxation response. After addition of STZ, UV light exposure for 60 s greatly potentiated relaxation without diminishing the response to NO by repeated exposure. Likewise, using L-NAME as photosensitizer, similar result to STZ was obtained. SNP also concentration- dependently relaxed the PCA rings in both contractile conditions. However, the magnitude of relaxation was significantly greater in U46619-contracted rings than in U46619+KCl- contracted ones (Fig. 1c). To know whether cGMP increment may be a sole factor responsible for the differences between the two conditions, we measured cGMP contents separately using the same protocols as described in Method section. As shown in Fig. 2, cGMP contents were increased irrespective of measures used. The amount of cGMP accumulation was the greatest in photoactivated NO, which was well correlated with the magnitude of relaxation. However, there were no

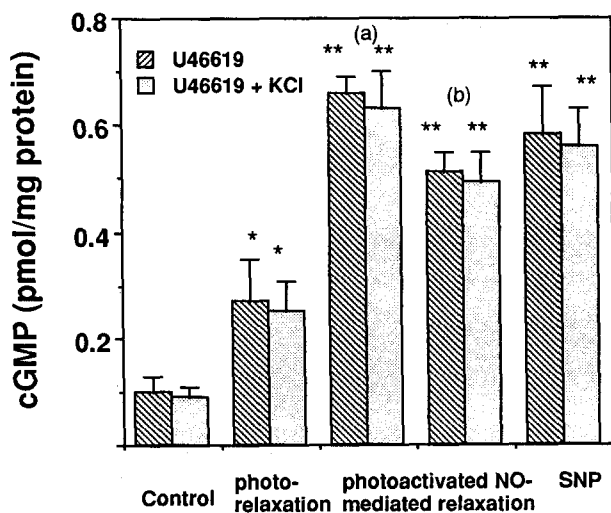


Fig. 2. Effects of cGMP on photorelaxation, photoactivated NO-mediated relaxation [STZ (a), L-NAME (b)] and SNP (0.3 μ M) in porcine coronary artery rings contracted with U46619 or U46619+KCl. * and ** indicate significantly different from the corresponding controls at $P < 0.05$ and $P < 0.01$, respectively.

differences in the increment of cGMP in conditions with or without KCl.

Effects of photoactivated NO by STZ on cytosolic Ca^{2+} change and muscle tension in NE and high K^+ -contraction in rat aorta

To understand the underlying mechanism of action for the differential response to NO, we investigated the effect of NO (fluorescent light in the presence of STZ) on cytosolic Ca^{2+} and muscle tension simultaneously in agonist [1 μ M norepinephrine (NE)]- and 65.4 mM K^+ -contracted rat aorta. Fig. 3 shows cytosolic Ca^{2+} was decreased transiently and restored the original level in high K^+ contracted aorta, in contrast, cytosolic Ca^{2+} was decreased sharply and sustained in NE-contracted aorta. As a result, the magnitude of relaxation is also smaller in case of KCl compare to NE.

STZ-mediated relaxation in NE-and KCl-contracted rat aorta

STZ is known to relax vascular smooth muscle by releasing NO (Thomas & Rasmwell, 1989). In rat endothelium denuded aorta, STZ relaxed both NE and KCl-contracted aorta concentration-dependently. From the dose-response curves, the inhibitory potency of

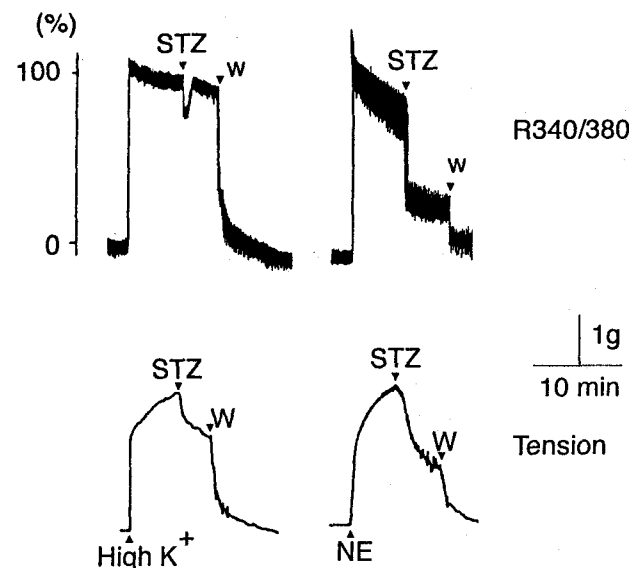


Fig. 3. Comparison of fura-3 calcium signal and muscle tension to photoactivated NO-induced relaxation (STZ, 0.1 M) in high K^+ and norepinephrine (NE)-contracted rat endothelium denuded thoracic aorta.

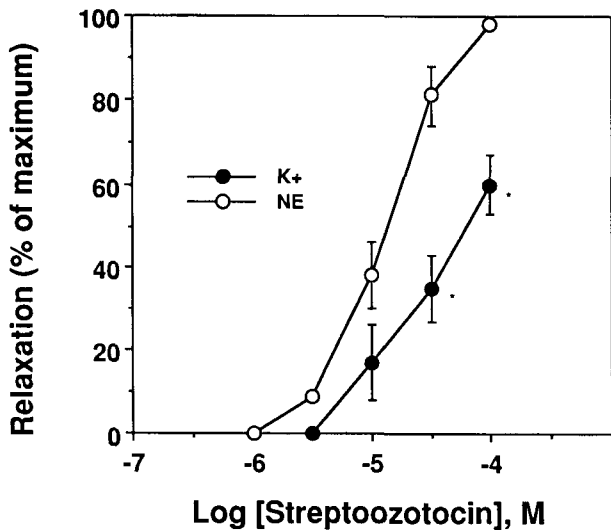


Fig. 4. Concentration-response curves of streptozotocin in norepinephrine (NE) and high K⁺-contracted rat endothelium-denude thoracic aorta. Each data represent mean \pm SEM of 3 experiments. * indicates significantly different ($P < 0.05$) (NE vs. K⁺).

50% (pD₂) of NE and KCl was 3.88 and 5.14 μ M, respectively (Fig. 4).

DISCUSSION

The results of the present investigation clearly demonstrate that in PCA rings, photorelaxation (UV light alone), photoactivated NO-mediated relaxation (in the presence of STZ or L-NAME with UV light) and NO-donor mediated relaxation (SNP) were always greater in U46619-contracted aortas than U46619+KCl (30 mM)-contracted ones even though the magnitude of contraction between the two were almost similar. Photorelaxation (UV light alone) resulted in approximately 60~80% decline in tone on repeated exposure to light, which confirms that vascular smooth muscle contains a store depletable of NO that is light activated (Ventrini et al, 1993). The decline of relaxation on repeated exposure to light was more sensitive in U46619+KCl-contracted tissues than U46619-contracted ones. We believe that the amount of NO depleted at each stimulation of UV light may be the same in both aortas either containing KCl or not. The diminished relaxation in KCl-containing tissues may be attributed to hyperpolarizing action of NO, since membrane hyperpolarizing factor has been suggested to contribute the NO response in

vascular smooth muscle (Gilbert et al, 1991). Furthermore, Chang et al. (1997) reported that UV light-induced relaxation involves in hyperpolarizing action in vascular smooth muscle. In contrast, photoactivated caged NO-mediated relaxation does not decline on repeated exposure to light, indicating that certain amounts of NO can be released from the caged molecules by photolysis not from the muscle with each stimulation (4). In this condition, the NO-mediated relaxation in U46619 tissues was also greater than U46619+KCl-contracted ones. We (Chang et al, 1997, Chang, 1996) reported that photoactivated NO-mediated relaxation of rat aorta involves hyperpolarization. It has also been established that photorelaxation is accompanied by an increase in cGMP (Furchgott et al, 1984). Therefore, it is likely that different amounts of cGMP increase may be responsible for the reduction of magnitude of relaxation in KCl-containing medium. However, this is not the case, since when measured the cGMP contents by each measure employed, there were no differences in cGMP contents whether KCl was included or not in the medium. Thus, it is unlikely that this potassium concentration has a significant inhibitory action on the formation or the cellular action of cGMP. This result is similar to our previous study that photoactivated NO-induced cGMP increase was not different between PE- and KCl-contracted rabbit corpus cavernosum (Chang, 1996). It seems to be certain from the present study that cGMP is not critical factor for the diminished NO-mediated relaxation in the presence of KCl. Functional antagonism was found to be an important factor in determining the maximum response to endothelium-dependent and independent vasorelaxants. Because we used nifedipine, a Ca²⁺-channel blocker, it can be ruled out the possibility that functional antagonism attributes the diminished relaxation in KCl-containing solutions. We reasoned that modulation of cytosolic Ca²⁺ by NO during membrane depolarized states may be responsible for the diminished relaxation even though the exact mechanism of action is not known yet, which, in turn, may influence hyperpolarizing action of NO. It is evident from the present experiment that cytosolic Ca²⁺ change by STZ, which releases NO by fluorescent light, during KCl contraction was small and transient. On the other hand, in NE-contracted aorta, change of cytosolic Ca²⁺ was huge and sustained. Thus, modification of the cellular mobilization of Ca²⁺ may be an important mecha-

nism for the NO-mediated relaxation. It should be warranted that in cardiovascular diseases, such as atherosclerosis, diabetes mellitus, hypertension etc., if the diminished NO-mediated relaxation including nitrenergic relaxation is due to defect of modification of the cellular Ca^{2+} mobilization or membrane depolarization. It seems possible, therefore, that in depolarization states of the smooth muscle, membrane hyperpolarization is much more important factor than guanylate cyclase activation to NO-mediated relaxation. The present data strongly suggest that membrane depolarization by itself makes less sensitive to change in cytosolic Ca^{2+} but not cGMP increase with yet unknown mechanism, which is responsible for the diminished relaxation in potassium containing solutions.

ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support of the Korea Science and Engineering Foundation (96-0403-1701-3).

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