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Role of PKC in the attenuation of the cGMP-mediated relaxation of skinned resistance artery smooth muscle seen in glyceryl-trinitrate-tolerant rabbit

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1 We examined whether 10 days' *in vivo* treatment with glyceryl trinitrate (GTN) might reduce cGMP-induced relaxation in the smooth muscle of rabbit mesenteric resistance arteries and, if so, whether protein kinase C (PKC) plays a role in this downregulation.

2 The relaxation responses to GTN and the nitric oxide donor NOC-7 were significantly reduced in endothelium-denuded strips from GTN-treated rabbits. In β-escin-skinned smooth muscle, the ability of 8-bromoguanosine 3′,5′ cyclic monophosphate (8-Br-cGMP, a phosphodiesterase-resistant cGMP analogue) to relax the contraction induced by 0.3 μM Ca^{2+} was significantly reduced in GTN-treated rabbits.

3 In β-escin-skinned smooth muscle, an inhibitor of conventional and/or novel PKCs, GF109203X (0.6 μM), inhibited the Ca^{2+}-induced contraction and enhanced the 8-Br-cGMP-induced relaxation. However, since the relaxing ability of 8-Br-cGMP was found to be unchanged by GF109203X when contractions were amplitude-matched (0.2 μM Ca^{2+} alone vs 0.3 μM Ca^{2+} + GF109203X), the increase in the 8-Br-cGMP-response seen with GF109203X was probably due to its inhibitory action on the Ca^{2+}-induced contraction. Furthermore, although the PKC activator phorbol 12,13-dibutyrate (PDBu, 0.1 μM) decreased the 8-Br-cGMP-induced relaxation of the Ca^{2+} (0.3 μM) contraction, this was probably due to its enhancement of the Ca^{2+}-induced contraction since no such effect of PDBu was seen when the Ca^{2+}-induced contractions were amplitude-matched (0.2 μM Ca^{2+} + PDBu vs 0.3 μM Ca^{2+} alone).

4 These results suggest that the relaxing response to cGMP is reduced in the smooth muscle of mesenteric resistance arteries in GTN-treated rabbits but that conventional and/or novel PKCs do not play a major role in maintaining this downregulation.
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Keywords: Glyceryl trinitrate, cGMP, protein kinase C, vascular smooth muscle, tolerance, phorbol ester, nitric oxide

Abbreviations: CPI-17, protein-kinase-C-potentiated inhibitory protein for heterotrimeric myosin light-chain phosphatase of 17 kDa; E_{max}, maximal response; 8-Br-cGMP, 8-bromoguanosine 3′, 5′ cyclic monophosphate; GTN, glyceryl trinitrate; NOC-7, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene; PDBu, phorbol 12, 13-dibutyrate

Introduction

Glyceryl trinitrate (GTN) releases nitric oxide and increases the cellular concentration of cGMP through an activation of soluble guanylyl cyclase (sGC) which, in turn, leads to an activation of PKG and the production of a smooth muscle relaxation (Kuriyama *et al*., 1998). GTN is widely used in the management of cardiovascular diseases such as angina pectoris, acute myocardial infarction and congestive heart failure. Despite the beneficial haemodynamic and anti-ischaemic profile of GTN, its usefulness is limited by the development of tolerance during continuous therapy (Gori & Parker, 2002a, b). The mechanisms responsible for GTN-tolerance have been suggested to be multifactorial and the existing hypotheses can be subsumed into two different categories (Gori & Parker, 2002a, b): (1) decreased biotransformation of GTN to nitric oxide or decreased activity of the nitric oxide itself ('end-organ tolerance'), (2) important counter-regulatory mechanisms involving stimulation of the sympathetic nervous system and renin–angiotensin–aldosterone axis ('pseudo-tolerance').

In the setting of GTN-tolerance, PKC, following its activation by angiotensin II or endothelin, has been suggested to play a permissive or causal role in the increase in sensitivity to vasoconstrictors (phenylephrine, serotonin, angiotensin II and KCl) (Münzel *et al*., 1995a) and/or in the decrease in the vascular response to GTN (Zierhut & Ball, 1996). PKC stimulates the production of superoxide *via* an activation of

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NAD(P)H oxidase in both endothelial cells and smooth muscle cells (Griendling *et al*., 1994; Münzel *et al*., 1995b; Heitzer *et al*., 1999). Once produced, the superoxide may reduce the concentration of nitric oxide derived from GTN through the formation of peroxynitrite (Harrison, 1997), decrease sGC activity (Weber *et al*., 2001) or increase the activity of phosphodiesterases (PDEs, the enzymes responsible for the catabolism of cGMP, Kim *et al*., 2001), thus leading to a reduction in the bioavailability of cGMP and thereby impairing GTN-induced relaxation (Parker & Parker, 1998). In addition, PKC may directly or indirectly (through an action mediated by superoxide) decrease the activity of PKG and downregulate the GTN-induced response, as suggested recently (Soff *et al*., 1997; Mülsch *et al*., 2001). However, it has not yet been fully clarified whether, *in vivo*, GTN tolerance develops upstream or downstream of cGMP in the smooth muscle of resistance arteries and whether PKC mediates such tolerance if it develops downstream of cGMP.

To attempt to clarify these issues, we studied the effect of 8-bromoguanosine 3′,5′ cyclic monophosphate (8-Br-cGMP, a PDE-resistant cGMP analogue) during the Ca^{2+}-induced contraction in β-escin-skinned smooth muscle strips from mesenteric resistance arteries obtained from rabbits treated for 10 days *in vivo* with GTN (the effects being compared with those seen in tissues obtained from GTN-untreated rabbits). The possible involvement of PKC in the on-going downregulation of the cGMP-response was studied by observing the actions of the PKC inhibitor GF109203X and the PKC activator phorbol 12,13-dibutyrate (PDBu) on the 8-Br-cGMP-induced relaxation in such strips.

Methods

Animals

All experiments performed in this study conformed to guidelines on the conduct of animal experiments issued by Nagoya City University Medical School and were approved by the Committee on the Ethics of Animal Experiments of Nagoya City University Medical School. To induce nitrate tolerance, male Japan White albino rabbits (supplied by Kitayama Labes, Ina, Japan), weighing 2.0–3.0 kg were treated by applying transdermal glyceryl trinitrate (GTN) patches (Nitroderm TTS; Novartis Pharma, Tokyo, Japan) to a shaved dorsal thoracic area of the body. Such patches were present continuously for a period of 10 days (each patch being replaced daily with a new one). The theoretical delivery of GTN was 5 mg per 24 h. Male rabbits of a similar size served as controls ('GTN-untreated rabbits').

Tissue preparation

Rabbits treated or untreated with GTN were anaesthetized by injection of pentobarbitone sodium (40 mg kg^{-1} given i.v.), then killed by exsanguination. The third and fourth branches of the mesenteric artery distributing to the region of the ileum (diameter, approximately 80–120 μM) were immediately excised and placed in Krebs solution, then cleaned by removal of connective tissue. After each artery had been cut open along its long axis using small scissors, circumferential strips were carefully prepared. The endothelium was carefully removed by gentle rubbing of the internal surface of the vessel using small pieces of razor blade, satisfactory ablation of the endothelium being pharmacologically verified by the absence of a relaxing effect when acetylcholine (3 μM) was applied during the contraction induced by noradrenaline (10 μM), as described elsewhere (Itoh *et al*., 2003).

Recording of mechanical responses

Circumferential strips (0.2–0.3 mm long, 0.07–0.10 mm wide, 0.02–0.03 mm thick) were mounted horizontally in a small chamber and attached to a strain gauge (AE801; SensoNor a.s, Horten, Norway), allowing us to record isometric tension. The transducer was connected to a carrier amplifier (AS 2101; NEC-San-ei Instruments Ltd, Tokyo, Japan) and the output signal was fed into a Macintosh computer (Apple Co., Tokyo, Japan) through an analog-digital converter (MacLab; ADInstruments Pty Ltd, Castle Hill, Australia). Drift was less than 3 μN h^{-1}. The resting tension was adjusted to obtain maximum contraction in high-K^+ solution (128 mM) and was not greater than 15 μN.

To observe the concentration-dependent effects of GTN or NOC-7 in endothelium-denuded strips from GTN-treated and -untreated rabbits, noradrenaline (10 μM) and propranolol (a nonselective β-receptor antagonist, 3 μM) were coapplied. After the steady state of the noradrenaline-induced contraction had been attained, various concentrations of GTN (0.01–10 μM) or NOC-7 (0.001−1 μM) were cumulatively applied from low to high. The experiments using endothelium-denuded unskinned strips were performed at 37°C.

Permeabilized smooth muscle

To prepare skinned smooth muscle strips, muscle strips were permeabilized by a 30-min application of β-escin (30 μM). This was applied in a 'relaxing solution' containing the calcium ionophore A23187 (3 μM, to avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle; Itoh *et al*., 1986). To prevent deterioration of the Ca^{2+}-induced contraction, 0.1 μM calmodulin was present throughout the experiments, which were performed at 25°C. Furthermore, all experiments on skinned strips were performed in the presence of 0.1 mM GTP.

In each skinned strip, the contraction induced by 10 μM Ca^{2+} was first recorded (to obtain the maximum Ca^{2+}-induced contraction in each strip) and the amplitude of each subsequent Ca^{2+}-induced contraction was normalized with respect to that induced by 10 μM Ca^{2+}. In preliminary experiments, we found that the magnitude of the relaxing response to 8-Br-cGMP was inversely related to the concentration of Ca^{2+} used to produce the contraction: 8-Br-cGMP (1 μM) produced ~70% relaxation on the contraction induced by 0.3 μM Ca^{2+} but less than 15% relaxation on that induced by 1 μM Ca^{2+}. Therefore, in the present experiments, we studied the effect of 8-Br-cGMP on the contraction induced by 0.3 μM Ca^{2+} in strips from both GTN-treated and -untreated rabbits. To observe the concentration-dependent effect of 8-Br-cGMP in β-escin-skinned strips from GTN-treated and -untreated rabbits, the strips were first contracted with 0.3 μM Ca^{2+}. After the steady state of the Ca^{2+}-induced contraction had been attained, various concentrations of 8-Br-cGMP (0.01–10 μM) were cumulatively applied from low to high.

When the effects of the PKC inhibitor GF109203X and those of the PKC activator PDBu on the concentration-dependent relaxation induced by 8-Br-cGMP were to be observed, strips were first contracted with 0.3 μM Ca^{2+}. After the peak of the Ca^{2+}-induced contraction had been obtained, 0.6 μM GF109203X or 0.1 μM PDBu was then added. Subsequently, various concentrations of 8-Br-cGMP (0.01–10 μM) were cumulatively applied from low to high during the steady state of the Ca^{2+}-induced contraction obtained in the presence of GF109203X or PDBu. Since GF109203X attenuated, while PDBu enhanced, the Ca^{2+}-induced contraction, the experiment was repeated using amplitude matching. That is to say, in the GF109203X experiment the concentration of Ca^{2+} alone used to induce contraction was reduced to 0.2 μM (to produce a contraction of the same amplitude as that induced by 0.3 μM Ca^{2+} in the presence of 0.6 μM GF109203X). Further, the concentration of Ca^{2+} used to induce contraction in the presence of 0.1 μM PDBu was reduced to 0.2 μM (to produce the same amplitude of contraction as that induced by 0.3 μM Ca^{2+} alone).

Solutions

The ionic composition of the Krebs solution was as follows (mM): 137.4 Na^+, 5.9 K^+, 1.2 Mg^{2+}, 2.6 Ca^{2+}, 15.5 HCO_3^-, 1.2 $H_2PO_4^-$, 134 Cl^-, 11.5 glucose. High-K^+ solution (128 mM) was prepared by replacing sodium chloride with potassium chloride isosmotically. The solutions were bubbled with 95% oxygen and 5% carbon dioxide.

The relaxing solution used in the skinned-muscle experiments contained (mM): 4 EGTA, 87 potassium methanesulphonate (KMS), 5.1 magnesium methanesulphonate, 5.2 ATP, 5 creatine phosphate and 20 Pipes together with 3 μM A23187, 0.1 μM calmodulin and 0.1 mM GTP. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of calcium methanesulphonate to 4 mM EGTA, the amounts being calculated using the equations reported previously (Itoh *et al*., 1986). The pH was adjusted to 7.1 at 25°C using KOH and the ionic strength was standardized at 0.18 M by changing the amount of KMS added.

Drugs

The drugs used in the current experiments were as follows: 8-bromoguanosine 3′,5′ cyclic monophosphate (8-Br-cGMP), β-escin, calmodulin, noradrenaline (all Sigma Chemical Co., St Louis, MO, U.S.A.), A23187 (free acid; Calbiochem, La Jolla, CA, U.S.A.), GTP (Bohringer Mannheim, Mannheim, Germany), phorbol 12,13-dibutyrate (PDBu), GF109203X (Wako Pure Chemical, Tokyo, Japan), 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7), EGTA (Dojindo Laboratories, Kumamoto, Japan) and acetylcholine-HCl (Daiichi Pharmaceutical, Tokyo, Japan). Glyceryl trinitrate (GTN) was kindly provided by Nippon Kayaku Co. (Tokyo, Japan).

NOC-7 was dissolved in 0.1 N NaOH (as a 10 mM stock solution), stored at −80°C and used within a week. The stock solution was diluted in Krebs solution to the required final concentrations immediately before use. A23187 was dissolved in DMSO (as a 10 mM stock solution) and diluted in relaxing solution (see above). All other drugs were dissolved in ultrapure Milli-Q water (Japan Millipore Corp., Tokyo, Japan). At their final concentration in Krebs solution or relaxing solution (less than 0.1%), none of the solvents had any noticeable effect on muscle contraction or relaxation.

Statistical analysis

The slope of the concentration–response relationship for the effect of GTN, NOC-7 or 8-Br-cGMP was calculated as the Hill coefficient (n) and mid-point position ($pK = -\log K$, where K is the dissociation constant). These were obtained by fitting the data points for each curve to the equation below by a nonlinear least-squares method using software (Kaleida graph; Synergy Software, PA, U.S.A.) written for the Macintosh computer (Apple Co.):

$$F/F_0 = (C/K)^n/[1 + (C/K)^n]$$

where C represents the concentration of the agent, F is the amplitude of contraction in the presence of any given concentration of the agent and F_0 is the response before application of the agent (as a relative tension of 1.0). All results are expressed as the mean ± s.e.m. The n values represent the number of rabbits used. A two-way repeated-measures ANOVA (followed by Scheffé's F-test for *post hoc* analysis) or a Student's paired or unpaired t-test with an F-test were used for statistical analysis. The level of significance was set at $P < 0.05$.

Results

Decrease in the response to 8-Br-cGMP in glyceryl trinitrate (GTN)-treated rabbits

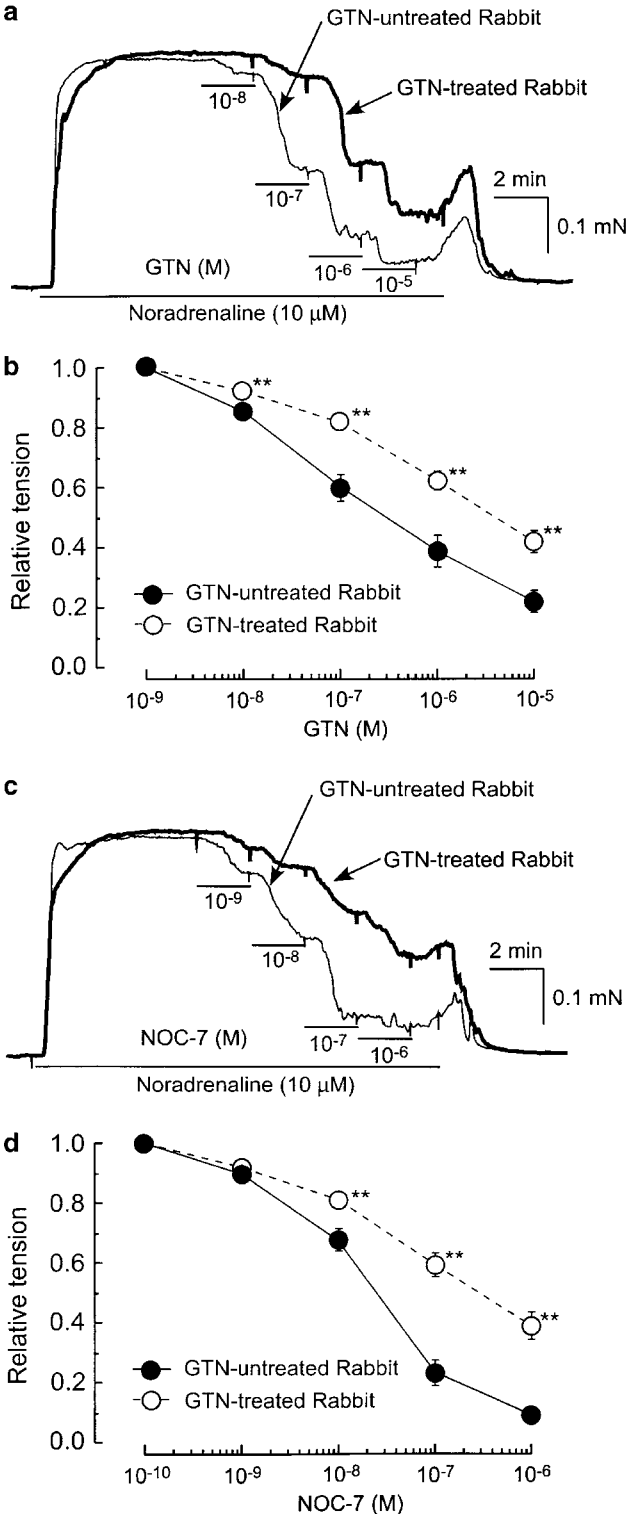
In endothelium-denuded strips, the absolute tension induced by 10 μM noradrenaline was not significantly different between GTN-treated rabbits (271.7 ± 19.1 μN, $n = 9$) and GTN-untreated rabbits (250.3 ± 22.9 μN, $n = 9$; $P > 0.5$). GTN (0.01–10 μM) produced a concentration-dependent relaxation during the contraction induced by 10 μM noradrenaline in both groups of rabbits (Figure 1, upper panels). However, the relationship was significantly shifted to the right ($EC_{50} = 1.6 \pm 0.5$ μM, $n = 9$) with a reduction in the maximal response ($E_{max} = 0.42 \pm 0.04$) in GTN-treated rabbits *versus* GTN-untreated rabbits ($EC_{50} = 0.27 \pm 0.10$ μM, $E_{max} = 0.23 \pm 0.04$, $n = 9$; $P < 0.05$ for each parameter).

The relaxation response to the nitric oxide donor NOC-7 in endothelium-denuded strips was also downregulated in GTN-treated rabbits (compared to that in GTN-untreated rabbits; Figure 1, lower panels). The EC_{50} and E_{max} values for NOC-7 were 159.3 ± 47.5 nM and 0.39 ± 0.05, respectively, in GTN-treated rabbits ($n = 9$) and 34.9 ± 15.4 nM ($P < 0.05$) and 0.09 ± 0.02 ($P < 0.01$) in GTN-untreated rabbits ($n = 9$). In the above experiments, the application time for GTN or NOC-7 at each concentration was standardised at 2 min to facilitate comparison between their effects on the NA-induced maintained contraction. Therefore, the EC_{50} values given for GTN and/or NOC-7 may be slight underestimates.

In β-escin-skinned strips, Ca^{2+} concentrations over 0.1 μM produced a contraction, with the maximum being obtained at 10 μM Ca^{2+} (Itoh *et al*., 1986). The relative amplitude of contraction induced by 0.3 μM Ca^{2+} was 0.14 ± 0.01 times that induced by 10 μM Ca^{2+} in strips from GTN-treated rabbits

($n=12$) and this was not significantly different from the value obtained for GTN-untreated rabbits (0.15 ± 0.01 times, $n=9$; $P>0.1$). The absolute tension induced by $0.3\,\mu$M Ca^{2+} in skinned strips from GTN-treated rabbits ($40.9\pm4.6\,\mu$N) was also not significantly different from the value obtained for GTN-untreated rabbits ($43.0\pm6.7\,\mu$N, $P>0.05$).

In time-matched control experiments, we found that without addition of cGMP, the amplitude of contraction induced by $0.3\,\mu$M Ca^{2+} was well maintained over a 60-min period (at 60 min, 0.94 ± 0.07 times and 0.96 ± 0.05 times the initial value for GTN-treated and -untreated rabbits, respectively, $n=4$ in each case). During the Ca^{2+}-induced contraction, 8-Br-cGMP (0.01-1 μM) produced a concentration-dependent relaxation in skinned strips from both groups of rabbits (Figure 2) but the relationship obtained for GTN-treated rabbits was significantly shifted to the right ($EC_{50}=0.52\pm0.15\,\mu$M, $n=12$) with a reduction in the maximal response ($E_{max}=0.20\pm0.03$) *versus* that for GTN-untreated rabbits ($EC_{50}=0.11\pm0.02\,\mu$M, $E_{max}=0.06\pm0.01$, $n=9$; $P<0.05$ for each parameter).



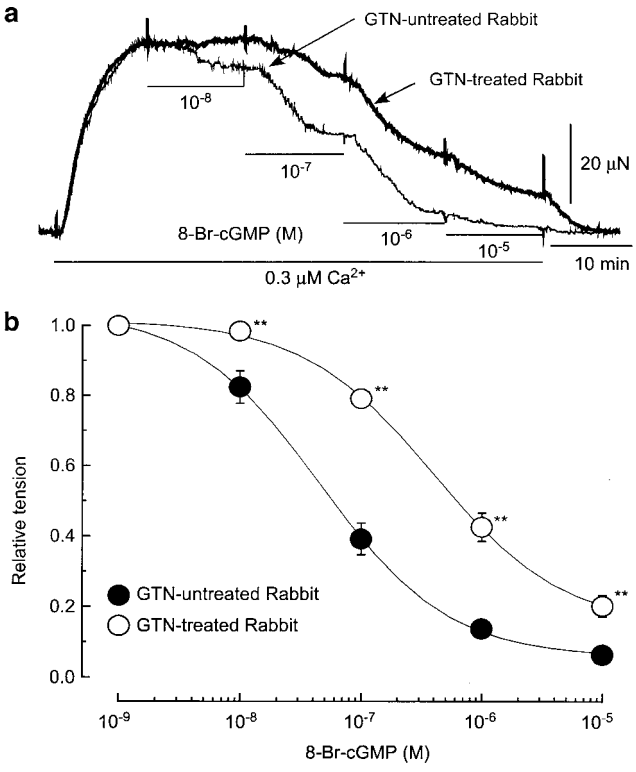
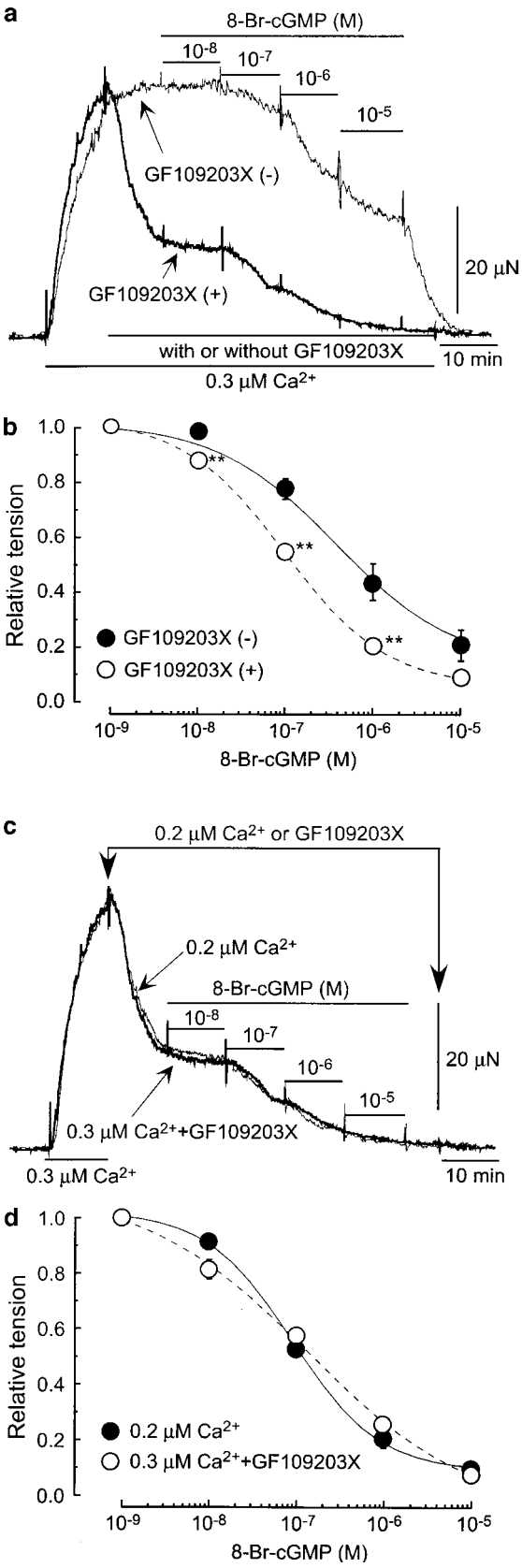


Figure 2 Effects of 8-Br-cGMP on Ca^{2+}-induced contraction in skinned strips. (a) Actual tracing of the concentration-dependent effect of 8-Br-cGMP on the contraction induced by $0.3\,\mu$M Ca^{2+} in skinned strips from GTN-treated and -untreated rabbits. (b) Summary of the concentration-dependent effects of 8-Br-cGMP on the contraction induced by $0.3\,\mu$M Ca^{2+} in skinned strips from GTN-treated and -untreated rabbits. Number of strips used was nine (GTN-untreated rabbits) or 12 (GTN-treated rabbits). Data are shown as mean$\pm$s.e.m. $^{**}P<0.01$ vs 'GTN-untreated Rabbit' (two-way repeated-measures ANOVA and Scheffé's F-test).

Figure 1 Effects of glyceryl trinitrate (GTN) and NOC-7 on the noradrenaline-induced contraction in endothelium-denuded mesenteric resistance arteries from GTN-treated and -untreated rabbits. (a) and (c) show actual tracings of the concentration-dependent effects of GTN (a) and NOC-7 (c) on the contraction induced by $10\,\mu$M noradrenaline in intact strips from GTN-treated and -untreated rabbits. (b) and (d) show summaries of the concentration-dependent effects of GTN (b) and NOC-7 (d) on the contraction induced by $10\,\mu$M noradrenaline in strips from GTN-treated and -untreated rabbits. Number of strips used was nine for each group (GTN-untreated rabbits and GTN-treated rabbits). Data are shown as mean$\pm$s.e.m. $^{**}P<0.01$ vs 'GTN-untreated Rabbit' (two-way repeated-measures ANOVA and Scheffé's F-test).



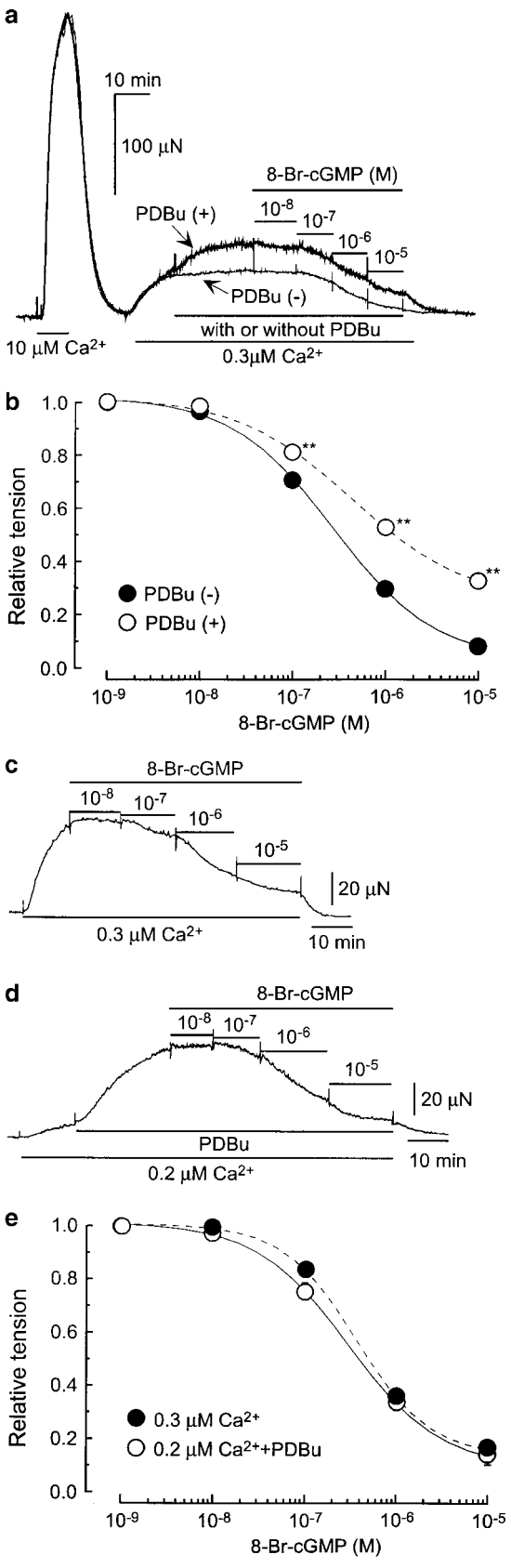
Effects of a PKC inhibitor and a PKC activator on 8-Br-cGMP-induced relaxation in GTN-treated rabbits

The PKC inhibitor GF109203X (0.6 μM) attenuated the contraction induced by 0.3 μM Ca^{2+} in skinned strips from both GTN-treated and GTN-untreated rabbits but its effect was more potent in the former than in the latter. In the absence of GF109203X, the relative tensions induced by 0.3 μM Ca^{2+} (normalized with respect to the 10 μM Ca^{2+}-induced tension) were 0.17 ± 0.01 ($n=6$) and 0.17 ± 0.01 ($n=5$, $P>0.5$) in GTN-treated and -untreated rabbits, respectively. In the presence of GF109203X, the corresponding values were 0.06 ± 0.01 ($n=6$) and 0.10 ± 0.01 ($n=5$, $P<0.01$) in GTN-treated and -untreated rabbits, respectively.

In skinned strips from GTN-treated rabbits, GF109203X enhanced the relaxation induced by 8-Br-cGMP. The EC_{50} values for 8-Br-cGMP were 0.38 ± 0.09 and 0.11 ± 0.02 μM ($P<0.05$) and the E_{max} values 0.203 ± 0.06 and 0.08 ± 0.02 ($P<0.05$) in the absence and presence of GF109203X, respectively (Figure 3, upper panels). To examine whether GF109203X enhances the 8-Br-cGMP-induced response through an inhibitory action on the Ca^{2+}-induced contraction, the effect of 8-Br-cGMP was examined on the contraction induced by 0.2 μM Ca^{2+} (which produced an amplitude of contraction (relative tension $= 0.08 \pm 0.01$, $n=4$) the same as that induced by 0.3 μM Ca^{2+} in the presence of GF109203X ($P>0.1$)). The relaxing response to 8-Br-cGMP on the contraction induced by 0.2 μM Ca^{2+} ($EC_{50} = 0.11 \pm 0.01$ μM, $n=4$) was not significantly different from that seen on the 0.3 μM Ca^{2+} + GF109203X-induced contraction ($P>0.5$; Figure 3, lower panels).

In strips from GTN-treated rabbits, the PKC activator PDBu (0.1 μM) enhanced the contraction induced by 0.3 μM Ca^{2+} (1.43 ± 0.04 times that obtained before application of PDBu, $n=4$) and attenuated the 8-Br-cGMP-induced relaxation. The EC_{50} values for the 8-Br-cGMP-induced relaxation were 0.29 ± 0.03 μM and 0.49 ± 0.10 μM and the E_{max} values 0.08 ± 0.01 and 0.33 ± 0.02 in the absence and presence of PDBu, respectively (each, $n=4$; $P<0.05$; Figure 4, upper panels). To examine whether PDBu attenuates the 8-Br-cGMP-induced relaxation through its enhancing action on the Ca^{2+}-induced contraction, the effect of 8-Br-cGMP was examined using amplitude-matched contractions (that induced by 0.2 μM Ca^{2+} + PDBu (relative tension $= 0.17 \pm 0.02$, $n=6$) being of the same amplitude as that induced by 0.3 μM Ca^{2+} alone (0.15 ± 0.01, $n=6$; $P>0.1$)). The relaxing response to 8-Br-cGMP on the contraction induced by 0.2 μM Ca^{2+} + PDBu ($EC_{50} = 0.30 \pm 0.04$ μM, $n=6$) was not significantly different from that seen on the contraction induced by 0.3 μM Ca^{2+} alone ($P>0.5$; Figure 4, lower panels).

Figure 3 Effects of the PKC inhibitor GF109203X on the relaxation induced by 8-Br-cGMP in skinned strips from GTN-treated rabbits. Upper panels show actual tracings (a) and a summary (b) of effects of 8-Br-cGMP on the contraction induced by 0.3 μM Ca^{2+} in the presence and absence of 0.6 μM GF109203X. Lower panels show actual tracings (c) and a summary (d) of effects of 8-Br-cGMP on the contraction induced by 0.3 μM Ca^{2+} in the presence of 0.6 μM GF109203X and on that induced by 0.2 μM Ca^{2+} alone. Number of strips used was 4–6 (each from a different GTN-treated rabbit). Data are shown as mean ± s.e.m. $^{**}P<0.01$ vs 'GF109203X (−)' (two-way repeated-measures ANOVA and Scheffé's F-test).



Discussion

In the present study on smooth muscle strips from rabbit mesenteric resistance arteries, we found that the relaxing response to GTN, as well as that to the nitric oxide donor NOC-7, was significantly reduced following *in vivo* treatment with GTN (for 10 days). These results suggest that such treatment downregulates the relaxing response to nitric oxide in the smooth muscle not only of the conduit aorta (Molina *et al*., 1987; Münzel *et al*., 1995b) but also of resistance arteries.

Decrease in cGMP-induced relaxation in GTN-treated rabbits

PKG mediates many of the effects of NO/cGMP in vascular smooth muscle cells (Pfeifer *et al*., 1998; Lincoln *et al*., 2001). Some years ago, it was found that *in vivo* treatment with GTN attenuates the increases in the vascular cGMP level in the rat aorta that occur in response to endothelium-derived nitric oxide and other nitrovasodilators, whereas the relaxation response to the direct PKG activator 8-Br-cGMP is not altered (Molina *et al*., 1987). Furthermore, in homogenized-aorta preparations, a high concentration of the nitrovasodilator sodium nitroprusside (10 μM) increased the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at a PKG-sensitive site to a similar extent whether the aortae were from rats treated with GTN *in vivo* or from GTN-untreated ones (Mülsch *et al*., 2001). These results suggested that the cGMP-signalling pathway upstream of cGMP might be impaired in conduit arteries in GTN-treated animals. In addition, it has been found that *in vivo* treatment with the organic nitrate isosorbide dinitrate downregulates the expression of PKG in aortic smooth muscle cells (Soff *et al*., 1997), suggesting that the action of PKG within the cell may be reduced in the smooth muscle of nitrovasodilator-treated animals. In the present experiments, the relaxing response to 8-Br-cGMP on the Ca^{2+}-induced contraction was reduced in the β-escin-skinned smooth muscle of mesenteric resistance arteries from rabbits treated *in vivo* with GTN. This was not due to an increase in the activity of phosphodiesterase (PDE), an effect previously observed in the rat aorta (Kim *et al*., 2001), because 8-Br-cGMP is a PDE-resistant cGMP analogue. Since cGMP-PKG inhibits Ca^{2+}-induced contraction through an activation of myosin light chain phosphatase (MLCP; Lee *et al*., 1997; Kuriyama *et al*., 1998), the above results, taken together, suggest that in addition to the down-regulation of NO-sGC pathways, *in vivo* treatment with GTN may downregulate the downstream signal after cGMP (possibly the cGMP-PKG-MLCP system) in the smooth muscle of the rabbit mesenteric resistance artery. The difference between previous reports (Molina *et al*., 1987; Mülsch

Figure 4 Effects of the PKC activator phorbol 12,13-dibutyrate (PDBu) on the relaxation induced by 8-Br-cGMP in skinned strips from GTN-treated rabbits. Upper panels show actual tracings (a) and a summary (b) of effects of 8-Br-cGMP on the contraction induced by 0.3 μM Ca^{2+} in the presence and absence of 0.1 μM PDBu. Lower panels show actual tracings of concentration-dependent effects of 8-Br-cGMP on the contraction induced by 0.3 μM Ca^{2+} alone (c) and on that induced by 0.2 μM Ca^{2+} in the presence of 0.1 μM PDBu (d) and a summary of these effects (e). Number of strips used was four to six (each from a different GTN-treated rabbit). Data are shown as mean$\pm$s.e.m. $^{**}P<0.01$ vs 'PDBu (−)' (two-way repeated-measures ANOVA and Scheffé's *F*-test).

et al., 2001) and the present study in the modulations of the effect of PKG activity in vascular smooth muscle following *in vivo* treatment with GTN may be due to the difference in the duration of the GTN treatment (3 vs 10 days), to the vessels chosen for the studies (conduit aorta vs mesenteric resistance arteries) or to the difference in species (rat vs rabbit).

Role of PKC in the attenuation of the cGMP-induced relaxation

PKC is activated by diacylglycerol, which is produced by vasospasmogenic-agent-induced hydrolysis of phosphatidylinositol-4,5-bisphospate (*via* activation of phospholipase C) and of phosphatidylcholine or phosphatidylethanolamine (*via* activation of phospholipase D) (Touyz & Schiffrin, 2000). It has recently been reported that PKC phosphorylates CPI-17 (full name, PKC-potentiated inhibitory protein for heterotrimeric myosin light-chain phosphatase of 17 kDa) and thereby enhances Ca^{2+}-induced contraction through an inhibition of MLCP in vascular smooth muscle (Eto *et al*., 2001; Kitazawa *et al*., 2003). It has been found that *in vivo* treatment with GTN increases the vascular concentrations of both angiotensin II and endothelin and it has been suggested that these peptides may, *via* activation of PKC, enhance the contraction induced by vasospasmogenic agents (such as angiotensin II, serotonin, noradrenaline and KCl) and produce a desensitization to nitric oxide (Münzel *et al*., 1995a, b; Kurz *et al*., 1999). Furthermore, treatment with the PKC inhibitor N-benzoyl-staurosporine *in vivo* prevents the development of GTN tolerance and the development of hypersensitivity to vasoconstrictors in rat aortic rings (Zierhut & Ball, 1996). In the present experiments, we found that the absolute tensions induced by 10 μM noradrenaline in endothelium-denuded strips and by 0.3 μM Ca^{2+} in β-escin-skinned smooth muscle were not significantly modified by *in vivo* GTN treatment. This is in contrast to previous suggestions of enhanced contractile responsiveness (Zierhut & Ball, 1996). However, since NA (10 μM) produces a maximal contraction our result does not exclude the possibility that GTN-treated strips might show greater contractile responsiveness at submaximal contractions of NA. We found that in β-escin skinned smooth muscle the PKC inhibitor GF109203X (0.6 μM) inhibited the 0.3 μM Ca^{2+}-induced contraction in strips from GTN-treated rabbits. Furthermore, the inhibitory action of GF109203X (0.6 μM) on the Ca^{2+}-induced contraction was more potent in skinned strips from GTN-treated rabbits than in those from GTN-untreated ones. In permeabilized vascular preparations, GF109203X apparently inhibits both conventional PKCs and novel PKCs (but not atypical PKCs) and also abolishes the myofilament Ca^{2+}-sensitization induced by phorbol 12,13-dibutyrate (Gailly *et al*., 1997; Eto *et al*., 2001; Kitazawa *et al*., 2003). These results are consistent with the previous findings of Molina *et al*. (1987) and Münzel *et al*. (1995a) and suggest that conventional and/or novel PKCs within smooth muscle cells are more strongly activated in GTN-treated rabbits than in GTN-untreated ones, and that this may underlie the different modulations of the Ca^{2+}-induced force seen in these two groups of rabbits.

In the present experiments, the PKC inhibitor GF109203X significantly enhanced, while the PKC activator phorbol 12,13-dibutyrate (PDBu) significantly decreased, the relaxing responsiveness to 8-Br-cGMP shown by β-escin skinned smooth muscle from GTN-treated rabbits. However, since the responsiveness to 8-Br-cGMP was unaltered by either GF109203X or PDBu when amplitude-matching was employed, the changes in the response to 8-Br-cGMP seen with these PKC modulators were probably due to their actions on the Ca^{2+}-induced contraction itself. On the basis of these results (obtained by acutely modifying PKC activity *in vitro*), we suggest that in GTN-tolerant rabbits, conventional and/or novel PKCs may not play a significant role in maintaining the on-going downregulation of cGMP-induced relaxation in the smooth muscle of mesenteric resistance arteries. However, it remains to be clarified whether these PKCs play a significant role in the process involved in the initiation and/or development of the downregulation of cGMP-induced relaxation.

In conclusion, 10 days' treatment with GTN *in vivo* produces a down-regulation of cGMP-induced relaxation in the smooth muscle of the rabbit mesenteric resistance artery. It is suggested that conventional and/or novel PKCs do not play a major role in the maintenance of this downregulation.

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