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Evidence that additional mechanisms to cyclic GMP mediate the decrease in intracellular calcium and relaxation of rabbit aortic smooth muscle to nitric oxide

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1 The role of cyclic GMP in the ability of nitric oxide (NO) to decrease intracellular free calcium concentration $[Ca^{2+}]_i$ and divalent cation influx was studied in rabbit aortic smooth muscle cells in primary culture. In cells stimulated with angiotensin II (AII, 10^{-7} M), NO ($10^{-10}-10^{-6}$ M) increased cyclic GMP levels measured by radioimmunoassay and decreased $[Ca^{2+}]_i$ and cation influx as indicated by fura-2 fluorimetry.

2 Zaprinast (10^{-4} M), increased NO-stimulated levels of cyclic GMP by 3–20 fold. Although the phosphodiesterase inhibitor lowered the level of $[Ca^{2+}]_i$ reached after administration of NO, the initial decreases in $[Ca^{2+}]_i$ initiated by NO were not significantly different in magnitude or duration from those that occurred in the absence of zaprinast.

3 The guanylyl cyclase inhibitor, H-(1,2,4) oxadiazolo(4,3-a) quinoxallin-1-one (ODQ, 10^{-5} M), blocked cyclic GMP accumulation and activation of protein kinase G, as measured by back phosphorylation of the inositol trisphosphate receptor. ODQ and Rp-8-Br-cyclic GMPS, a protein kinase G inhibitor, decreased the effects of NO, $10^{-10}-10^{-8}$ M, but the decrease in $[Ca^{2+}]_i$ or cation influx caused by higher concentrations of NO ($10^{-7}-10^{-6}$ M) were unaffected. Relaxation of intact rabbit aorta rings to NO ($10^{-7}-10^{-5}$ M) also persisted in the presence of ODQ without a significant increase in cyclic GMP. Rp-8-Br-cyclic GMPS blocked the decreases in cation influx caused by a cell permeable cyclic GMP analog, but ODQ and/or the protein kinase G inhibitor had no significant effect on the decrease caused by NO.

4 Although inhibitors of cyclic GMP, protein kinase G and phosphodiesterase can be shown to affect the decrease in $[Ca^{2+}]_i$ and cation influx *via* protein kinase G, these studies indicate that when these mechanisms are blocked, cyclic GMP-independent mechanisms also contribute significantly to the decrease in $[Ca^{2+}]_i$ and smooth muscle relaxation to NO.

Keywords: Nitric oxide; smooth muscle; calcium; cyclic GMP; protein kinase G

Introduction

Endothelium-dependent vasodilation is mediated by nitric oxide (NO), which after being synthesized by endothelial cell NO synthase diffuses to smooth muscle cells. NO is thought to mediate relaxation primarily by activating guanylyl cyclase to produce cyclic GMP from GTP (Lincoln *et al*., 1996; Waldman & Murad, 1987; Ignarro & Kadowitz, 1985). Cyclic GMP activates cyclic GMP-dependent protein kinase (protein kinase G) which is thought to have several actions which could mediate relaxation (Lincoln *et al*., 1996). These actions include a decrease in intracellular calcium concentration $[Ca^{2+}]_i$ mediated by (1) inhibition of phospholipase C (Hirata *et al*., 1990; Rapoport, 1986), (2) inhibition of inositol trisphosphate (IP_3) receptor mediated Ca^{2+} release from intracellular Ca^{2+} stores (Koga *et al*., 1994; Komalavilas & Lincoln, 1994), (3) inhibition of Ca^{2+} influx through ion channels (Quignard *et al*., 1997; Blatter & Wier, 1994), and (4) stimulation of Ca^{2+} removal from the cytoplasm by Na^+/Ca^{2+} exchange (Furukawa *et al*., 1991), or plasma membrane (Furukawa *et al*., 1988) and sarcoplasmic reticulum Ca^{2+} ATPase transporters (Cornwell *et al*., 1991; Raeymaekers *et al*., 1988). Although contractile tone of blood vessels is largely dependent on $[Ca^{2+}]_i$, there may be other means by which NO mediates relaxation. These include cyclic GMP-dependent decrease in smooth muscle contractile protein sensitivity to Ca^{2+} (McDaniel *et al*., 1992), as well as cyclic GMP-dependent (Robertson *et al*., 1993; Taniguchi *et al*., 1993; Archer *et al*., 1994) and cyclic GMP-independent activation of K^+ ion channels (Bolotina *et al*., 1994) or Na^+/K^+ ATPase (Gupta *et al*., 1994), which could mediate hyperpolarization and relaxation by inhibiting voltage-dependent Ca^{2+} influx. Although each of these cyclic GMP-independent mechanisms has been proposed, their relative contribution to NO-induced vasodilation with respect to cyclic GMP-dependent mechanisms has been controversial (Moro *et al*., 1996).

The purpose of the current studies was to assess the role of cyclic GMP in mediating the Ca^{2+} inhibitory and relaxant effects of NO in vascular smooth muscle. In these studies the increases in cyclic GMP stimulated by NO were manipulated by (1) inhibiting the breakdown of cyclic GMP with a phosphodiesterase inhibitor, zaprinast (Schoeffter *et al*., 1988), (2) inhibiting guanylyl cyclase with H-(1,2,4) oxadiazolo(4,3-a) quinoxallin-1-one (ODQ) (Olson *et al*., 1997; Brunner *et al*., 1996; Garthwaite *et al*., 1995), and (3) inhibiting protein kinase G activation with a cell permeable, non-hydrolyzable,

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inactive cyclic GMP analogue, Rp-8-Br-cyclic GMPS (Butt *et al*., 1990; Corbin *et al*., 1986). Because of the possibility that cyclic GMP levels may not accurately reflect protein kinase G activation, protein kinase G activity stimulated by NO was assayed by back phosphorylation of the IP_3 receptor (Komalavilas & Lincoln, 1994, 1996). These findings provide evidence for mechanisms additional to those *via* cyclic GMP and protein kinase G by which NO inhibits increases in $[Ca^{2+}]_i$ in vascular smooth muscle.

Methods

Cell culture

Strips were aseptically removed from the medial section of rabbit thoracic aorta, dispersed with collagenase (4 mg ml^{-1}) and elastase (1 mg ml^{-1}), and grown in media M199 with 20% heat-inactivated fetal bovine serum as previously described (Weisbrod *et al*., 1997). Cells were seeded into 24-well plates for cyclic GMP studies, and onto 9×22 mm #1 glass coverslips for $[Ca^{2+}]_i$ studies. Upon reaching confluence, cells were growth arrested by placing them in M199 with 1% serum for 3–4 days before experiments. All experiments were done on primary cultures which stain uniformly for smooth muscle α-actin (Weisbrod *et al*., 1997).

Cyclic GMP measurements

Cells were equilibrated for 60 min in HEPES buffered (pH 7.4) physiological salt solution (PSS) of the following composition (mM): NaCl 119, NaHEPES 20, KCl 4.6, $CaCl_2$ 1.2, $MgSO_4$ 1.0, Na_2HPO_4 0.15, KH_2PO_4 0.4 and $NaHCO_3$ 5.0, supplemented with 0.1% albumin. At the appropriate time, PSS from unstimulated cells (basal) or from NO-stimulated cells was immediately removed, and 200 μl of 0.1 N HCl was added to the cells and allowed to remain for 1 h to extract cyclic GMP. Cyclic GMP in the HCl was assayed with a radioimmunoassay kit (Research Biochemicals Inc., Stoughton, MA, U.S.A.). After removing HCl, cells were solubilized in 1 N NaOH and protein content determined by the bicinchoninic acid assay. Cyclic GMP levels are expressed as fmol μg^{-1} protein. The lowest value of cyclic GMP measured in one sample of cells exposed to ODQ in the absence of NO contained 1 fmol. The detection limit of cyclic GMP assay was 0.5 fmol, indicating that all changes in cyclic GMP were within the detection limits of the assay.

$[Ca^{2+}]_i$ measurement

Cells on coverslips were loaded for 45 min with the fluorescent $[Ca^{2+}]_i$ indicator, fura-2 acetoxymethyl ester (5 μM) in PSS at 37°C, and rinsed for 15 min to allow ester cleavage. Fluorescence was measured in an IonOptix (Milton, MA, U.S.A.) fura-2 fluorimeter. Light from a xenon lamp was passed through a chopper with alternating filters at 340 nm and 380 nm, and then passed through a liquid light guide to the coverslip mounted in a cuvette. Emission was collected through the same light guide, passed through a 510 nm filter, and measured with a photomultiplier tube. Data was continuously collected on IonOptix software. $[Ca^{2+}]_i$ is calculated from the ratio of the 340/380 nm signals by using the following formula (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K_d((R_{max} - R)/(R - R_{min}))(S_{f2}/S_{b2})$$

where K_d for fura-2 is 225 nM at 37°C, R_{max} and R_{min} are the ratio values for external standards with either saturating Ca^{2+} levels (maximum) or without Ca^{2+} (minimum), respectively, R is the sample ratio of 340/380 nm fluorescence, and S_{f2} and S_{b2} are the raw 380 nm fluorescence values for minimum and maximum standards, respectively. R_{min}, R_{max}, S_{f2}, and S_{b2} values were determined using external standard solutions consisting of PSS containing 50 nM of the pentapotassium salt of fura-2 with either no $[Ca^{2+}]_i$ (EGTA-buffered, 'minimum') or 1.2 mM $[Ca^{2+}]_i$ ('maximum'). Background autofluorescence was subtracted at the end of each experimental run by adding 1 μM ionomycin and 20 mM $MnCl_2$ to quench all fura-2 fluorescence. $[Ca^{2+}]_i$ is expressed as the concentration (nM) estimated by the above equation.

The duration of each experimental run was 20 min. Cells were allowed to equilibrate for 6 min before 0.1 μM angiotensin II (AII) was added to increase $[Ca^{2+}]_i$. Other pharmacological compounds were present for at least 30 min before AII was added. In analysing the response to AII, the maximal rise in $[Ca^{2+}]_i$ during the first 10 s (peak), and the level achieved 30 s after the addition of AII (plateau) were quantified. Logarithmic increases in NO or SNAP concentration were added beginning 30 s following the increase in $[Ca^{2+}]_i$ caused by AII, and each subsequent concentration was added after $[Ca^{2+}]_i$ reached a new stable level. Data from these experiments were analysed by (1) subtracting basal $[Ca^{2+}]_i$ levels and calculating the maximal percent decrease in $[Ca^{2+}]_i$ caused by each concentration of NO or SNAP, and (2) measuring the duration(s) of the transient reduction in $[Ca^{2+}]_i$ to each concentration of NO starting from the initial decrease until the $[Ca^{2+}]_i$ level rose to reach a value that equalled the mean of the trough $[Ca^{2+}]_i$ value and the new stable level which followed the response. In addition, because $[Ca^{2+}]_i$ was not constant after AII, in some experiments (Figures 5 and 7) the average value of $[Ca^{2+}]_i$ over a 10 min period following the addition of NO (10^{-6} M) was compared to values in experiments in which NO was not added. These average values were obtained with an integration algorithm and are reported as a percentage decrease in the $[Ca^{2+}]_i$ measured at the time at which NO was added.

Measurement of divalent cation influx

Manganese (Mn^{2+})-induced quenching of fura-2 fluorescence was used to estimate rates of divalent cation influx, which reflects Ca^{2+} influx (Chen & Rembold, 1992). Fluorescence of fura-2 loaded cells was measured at the isosbestic wavelength of 360 nm. Cells were placed in nominally free Ca^{2+} PSS for 8 min before $MnCl_2$ (100 μM) was added for basal measurements. To determine agonist-induced cation influx, AII was added 3 min before $MnCl_2$. To determine the effect of NO, NO or SNAP was added 1 min before AII, and to determine the effect of cyclic GMP, 8-pCPT-cyclic GMP was added 4 min before AII. Mn^{2+} influx rates were calculated from the slope of the decline in fura-2 fluorescence during the first minute after addition of Mn^{2+} and was normalized to the level of fluorescence at the time of Mn^{2+} addition. Data are expressed as the initial rate of Mn^{2+} influx which was linear during the first minute following addition of Mn^{2+}.

Isometric force and cyclic GMP measurement in intact rabbit aorta

Rings of proximal rabbit thoracic aorta were suspended in organ chambers for tension measurements as previously described (Cowan *et al*., 1993). Prior to mounting, the endothelium was removed by rubbing the intimal surface. After equilibration at the optimal resting tension (6 g), the

smooth muscle was contracted with phenylephrine hydrochloride, and after achieving a stable contraction, NO was added in increasing logarithmic concentrations.

Additional rings were freeze clamped with tongs cooled in liquid nitrogen for measurements of cyclic GMP as previously described (Cowan *et al*., 1993). The rings were frozen either after reaching a stable contraction caused by phenylephrine for basal levels of cyclic GMP, or 60 s after adding NO (10^{-6} M). Some rings were pretreated with ODQ for at least 30 min. As previously described (Cowan *et al*., 1993) the cyclic GMP was extracted from the rings in trichloroacetic acid and assayed by the same radioimmunoassay as was used for the cultured cells.

Protein kinase G assay

Protein kinase G was assayed by the back phosphorylation of the IP_3 receptor as described earlier (Komalavilas & Lincoln, 1996). Briefly the treated cells were solubilized in a lysis buffer containing 50 mM Tris-Cl, pH 7.7, 1 mM EDTA, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 50 mM tetrasodium pyrophosphate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} pepstatin A, 10 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin A, 10 nM calyculin A, and incubated for 1 h at 4°C. The samples were centrifuged at $14{,}000 \times g$ for 15 min. The supernatants were removed and protein was estimated. The IP_3 receptor was immunoprecipitated from 250 μg total protein using an antibody to the IP_3 receptor (antisera raised in rabbit to an N-terminal peptide of the type I IP_3 receptor). The immunoprecipitated samples were phosphorylated using purified protein kinase G by adding 30 μl of phosphorylation buffer containing 40 mM Tris-Cl, pH 7.4, 0.1% Triton X-100, 10 mM magnesium acetate, 100 μM [γ-^{32}P]ATP (5 μci per sample), and 1 μM cyclic GMP to the agarose beads with the receptor. Phosphorylation was initiated by adding 5 μl of purified protein kinase G (40 nM final concentration) diluted in 20 mM potassium phosphate, pH 7.0, 2 mM EDTA, 150 mM NaCl, 15 mM 2-mercaptoethanol, 1 mg ml^{-1} bovine serum albumin and 1 μM cyclic GMP. The samples were incubated at 30°C for 10 min and the reaction was stopped by adding 10 μl of stopping buffer (312 mM Tris-Cl, pH 6.9, 0.5 M sucrose, 15% SDS, 10 mM EDTA, 2.5 M 2-mercaptoethanol, and 0.1% bromophenol blue). The samples were heated at 95°C for 5 min, and the denatured proteins were resolved on 7.5% SDS–polyacrilamide gel, and analysed by autoradiography at −80°C. The band corresponding to the IP_3 receptor was cut out of the gel and radioactivity determined by Cerenkov counting. The autoradiogram was analysed by densitometry and the amount of ^{32}P incorporated into the IP_3 receptor band was quantitated. The amount of ^{32}P incorporated into the IP_3 receptor band from the control cells with AII alone was taken as control. Decreases (or increases) in the amount of ^{32}P incorporated into the IP_3 receptor band in the treated samples was calculated as a percent of the respective control value and reflected an increase (or decrease) in endogenous phosphorylation by protein kinase G. Changes in endogenous phosphorylation by protein kinase G are expressed as per cent of the control.

Materials

Cell culture media was from Gibco (Grand Island, NY, U.S.A.). Dissociation enzymes for cell culture were from Worthington (Freehold, NJ, U.S.A.). Rp-8-Br-cyclic GMPS and 8-pCPT-cyclic GMP were obtained from Biolog (Bremen, Germany). NO gas was obtained from Matheson Gas (Gloucester, MA, U.S.A.). Saturated NO solution was prepared at 4°C and diluted in deoxygenated sealed tubes of distilled water as described (Weisbrod *et al*., 1997). Responses of intact rings and cultured cells to NO (10^{-10}–10^{-6} M) were entirely prevented by deoxyhemoglobin (10^{-5} M). Fura-2 was from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals were dissolved in water except for IBMX and ionomycin which were dissolved in DMSO, and zaprinast which was dissolved in 0.1 N NaOH.

Statistical analysis

Concentration-responses were evaluated by ANOVA for repeated measures. Differences between means ± s.e.mean were determined by Student's *t*-test, with $P<0.05$ accepted as significant.

Results

Effect of phosphodiesterase inhibition

NO (10^{-10}–10^{-6} M) caused a time and concentration-dependent increase in cyclic GMP levels in smooth muscle cells (Figure 1). The peak increase in cyclic GMP occurred at 10 s after addition of NO. Increasing $[Ca^{2+}]_i$ with either AII (10^{-7} M) or ionomycin (10^{-6} M) for 1 min prior to addition of NO, caused a significant decrease in the rise in cyclic GMP (Table 1). IBMX (10^{-4} M) or zaprinast (10^{-4} M) attenuated the decrease in NO-stimulated cyclic GMP levels which occurred in the presence of AII or ionomycin alone (Table 1). In the presence of AII, zaprinast significantly increased basal cyclic GMP from 0.8 ± 0.4 to 18 ± 8 fmol μg^{-1} ($P<0.026$, Figure 1). Zaprinast also significantly increased NO stimulated cyclic GMP levels from 3–20 fold higher than in cells exposed to AII alone (Figure 1).

Under control conditions, AII caused a rapid rise in $[Ca^{2+}]_i$ from a basal value of 65 ± 4 to 975 ± 57 nM, which was not significantly affected by zaprinast (10^{-4} M, 30 min; Figure 2A and Table 2). Zaprinast also did not significantly affect the plateau level of $[Ca^{2+}]_i$ attained 30 s after AII (Table 2) or during the subsequent 10 min (Figure 2A). NO (10^{-10}–10^{-6} M) caused transient decreases in $[Ca^{2+}]_i$ with the maximal decrease in $[Ca^{2+}]_i$ occurring within 4 s (Figure 2B). With increasing concentrations of NO, its predominant effect was to increase the duration, rather than the magnitude of the decrease in $[Ca^{2+}]_i$ (Figure 2D and E). Zaprinast had no significant effect on the initial magnitude or duration of the decrease in $[Ca^{2+}]_i$ caused by NO (Figure 2C, D and E). However, compared with the untreated control cells, zaprinast did significantly decrease the level of $[Ca^{2+}]_i$ reached after the effect of each dose of NO had subsided (Figure 2F).

Effect of guanylyl cyclase inhibition with ODQ

ODQ (10^{-8}–10^{-5} M) caused concentration-dependent decreases in cyclic GMP levels stimulated by NO (10^{-10}–10^{-6} M, Figure 3A and B). Under control conditions in the absence of AII or zaprinast, NO (10^{-6} M) increased cyclic GMP approximately 50 fold over the basal level from 0.9 ± 0.2 to 48 ± 5 fmol μg^{-1} protein. By comparison, the change in cyclic GMP caused by NO (10^{-6} M) was insignificant in the presence of ODQ (10^{-5} M, 0.08 ± 0.02 to 0.39 ± 0.11 fmol μg^{-1} protein).

ODQ caused a small significant rise in basal $[Ca^{2+}]_i$, but it had no significant effect on the AII-induced peak or plateau

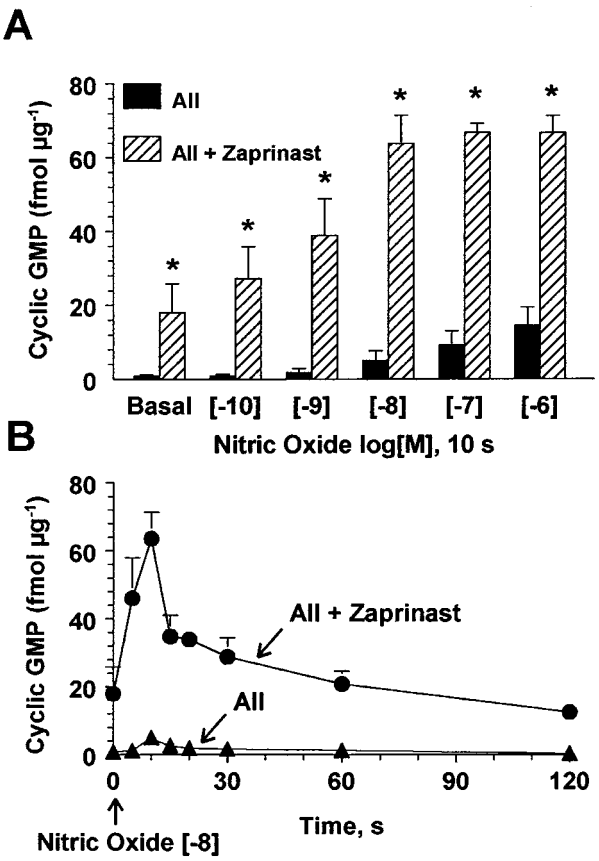

Figure 1 Effect of zaprinast on NO-stimulated cyclic GMP levels in smooth muscle cells stimulated with AII. Cyclic GMP levels were determined in cells exposed to AII to mimic conditions under which $[Ca^{2+}]_i$ was measured in Figure 2. Cells were exposed to AII (0.1 μM) alone for 1 min (AII) or to zaprinast (100 μM) for 30 min plus AII for 1 min (AII + zaprinast) before the addition of NO (10^{-10}–10^{-6} M) for 10 s (A) or the addition of NO (10^{-8} M) for 5–120 s (B). The asterisks indicate that zaprinast significantly increased cyclic GMP levels by 3–20 fold (A, $P<0.05$, ANOVA, $n=4$). These conditions are identical to those used for measurement of $[Ca^{2+}]_i$ in Figure 2.

Table 1 Effect of IBMX, zaprinast, AII, and ionomycin on cyclic GMP

	Cyclic GMP (fmol μg^{-1} protein)		
	Control	*IBMX*	*Zaprinast*
None	63±7.6	99±24	95±11
+AII (10^{-7} M)	14±4.9*	89±19	67±4.6#
+Ionomycin (10^{-6} M)	7.3±0.3*	94±15	N.D.

Levels of cyclic GMP (fmol μg^{-1} protein) measured in cells stimulated with NO (10^{-6} M) for 10 s, either under control conditions or pretreated with AII or ionomycin for 1 min. Additional comparisons are made to cells pretreated with IBMX (10^{-4} M) or zaprinast (10^{-4} M) for 30 min. AII or ionomycin significantly decreased the level of cyclic GMP stimulated by NO compared with control (*$P<0.05$). In the presence of IBMX or zaprinast, cyclic GMP levels in the presence of either AII or ionomycin were not significantly different from control ($n=4$). NO-stimulated cyclic GMP levels were significantly less (#$P<0.05$) in cells treated with zaprinast and AII than in those treated with zaprinast alone. However, the levels were significantly greater ($P<0.001$) in cells treated with zaprinast and AII than in cells treated with AII alone. N.D. = not determined.

$[Ca^{2+}]_i$ value (Table 2). ODQ caused concentration-dependent inhibition of the decreases in $[Ca^{2+}]_i$ caused by NO (10^{-10}–10^{-7} M, Figure 3). Both the magnitude and the duration of the decrease in $[Ca^{2+}]_i$ were affected (Figure 3C and D). In contrast, at concentrations of NO above 10^{-7} M, ODQ had no significant effect on the magnitude or duration of the decrease in $[Ca^{2+}]_i$.

The effect of ODQ (10^{-5} M) was further studied in the presence of the phosphodiesterase inhibitor, zaprinast (10^{-4} M), so that even low levels of guanylyl cyclase activity could be detected by increases in cyclic GMP. In the presence of zaprinast, ODQ inhibited both the magnitude and the duration of the decrease in $[Ca^{2+}]_i$ caused by NO at concentrations below 10^{-7} M (Figure 4B and C and D). With concentrations at or above 10^{-7} M, the effect of NO persisted. In the presence of AII and zaprinast, ODQ (10^{-5} M) blocked the peak increase in cyclic GMP that occurred 10 s after adding NO (10^{-10}–10^{-6} M, Figure 4E).

The actions of the highest concentration of NO (10^{-6} M) were examined further in the presence of zaprinast (Figure 5). NO (10^{-6} M) decreased $[Ca^{2+}]_i$ in the presence or absence of ODQ (10^{-5} M, Figure 5A–C). Although the response to NO was significantly reduced by ODQ 5 s after adding NO, the per cent decrease in $[Ca^{2+}]_i$ caused by NO over 10 min was not significantly different in the presence or absence of ODQ (81±2% vs 85±3%, Figure 5). Under the same conditions in which $[Ca^{2+}]_i$ was measured in the presence of AII and zaprinast, cyclic GMP levels did not rise significantly in response to NO in the presence of ODQ. In contrast, in the absence of ODQ, cyclic GMP levels during the first 20 s rose to levels that were 33 fold greater than basal levels (Figure 5D).

In order to determine whether protein kinase G was activated due to a low level of cyclic GMP in the presence of ODQ, cells were treated with zaprinast and stimulated with AII in the absence or presence of ODQ, and the phosphorylation of the IP_3 receptor was determined following exposure to NO (10^{-6} M, Figure 6). In the absence of ODQ, a significant increase in endogenous phosphorylation did not occur until 30 s after adding NO. In the presence of ODQ (10^{-5} M), there was no significant endogenous phosphorylation of the IP_3 receptor by protein kinase G at all time points following NO.

Effect of protein kinase G inhibition

Rp-8-Br-cyclic GMPS (3×10^{-4} M) had no significant effect on basal or AII-induced increase in $[Ca^{2+}]_i$ (Table 2). The competitive inhibitor of protein kinase G significantly decreased the magnitude and the duration of the decrease in $[Ca^{2+}]_i$ caused by NO (10^{-10}–10^{-8} M), but had no significant effect on the response to NO (10^{-7}–10^{-6} M, Figure 7A, B, C and D). The same concentration of Rp-8-Br-cyclic GMPS prevented the inhibition by the cyclic GMP analogue, 8-p-chlorophenylthio-cyclic GMP (10^{-4} M), on the response to AII (Figure 7E and F).

To examine the possibility that the rise in cyclic GMP caused by NO could overcome the blockade of protein kinase G by Rp-8-bromo-cyclic GMPS, the inhibitor of protein kinase G was combined with ODQ. The time course over 10 min of the decrease in $[Ca^{2+}]_i$ caused by NO (10^{-6} M) is shown in Figure 7G. Over 10 min, NO decreased $[Ca^{2+}]_i$ by 71±4% compared with cells treated with ODQ (10^{-5} M, 61±8%) alone or with ODQ in combination with Rp-8-bromo-cyclic GMPS (3×10^{-4} M, 57±3%). There was no significant difference in the per cent reduction in $[Ca^{2+}]_i$ caused by NO in cells treated with ODQ compared with control, and there was no significant further inhibition by the combination of inhibitors compared with ODQ alone (Figure 7G).

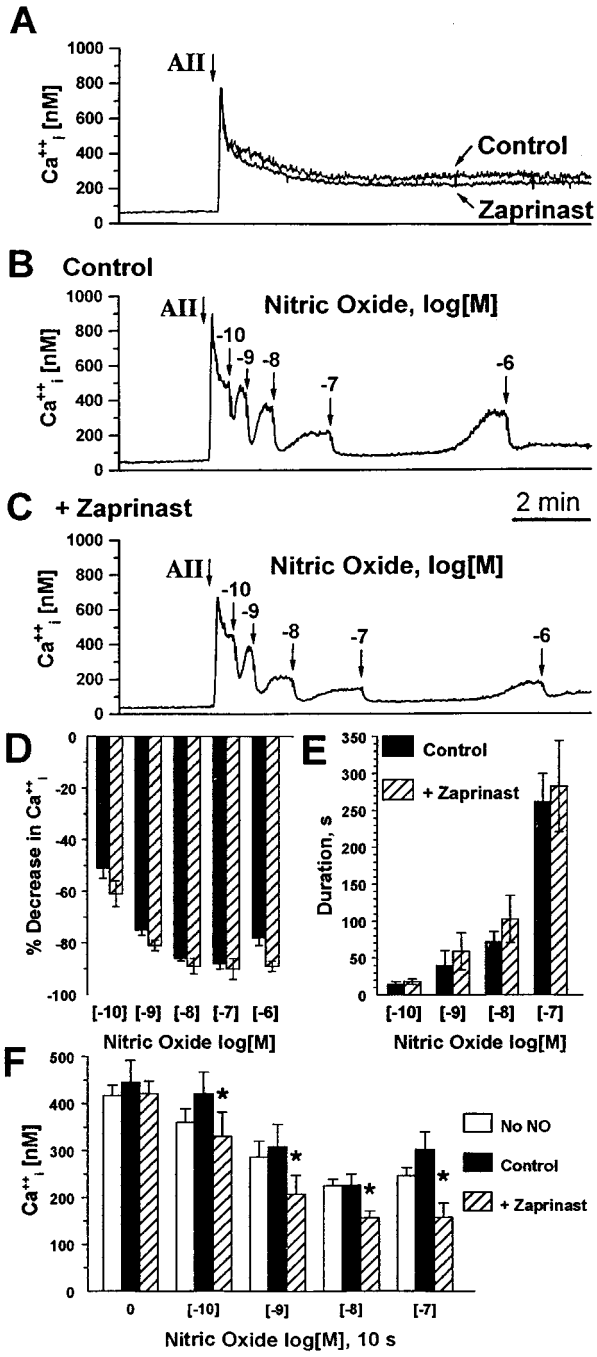

Figure 2 Effect of zaprinast on NO-stimulated decreases in $[Ca^{2+}]_i$. Representative recordings in A show that cells treated with or without zaprinast (10^{-4} M, 30 min) respond similarly to AII. NO ($10^{-10}-10^{-6}$ M) decreased $[Ca^{2+}]_i$ in cells stimulated by AII (AII, 10^{-7} M) under control conditions (B) and similarly after treatment with zaprinast (C). Data ($n=4$) summarizing the effect of zaprinast on the magnitude (D) and duration (E) of NO-induced decreases in $[Ca^{2+}]_i$. Because the response to NO (10^{-6} M) lasted for over 10 min, the duration of the response to this concentration and the plateau value following it were not quantified. The asterisks indicate that zaprinast significantly lowered the level of $[Ca^{2+}]_i$ reached following the response to NO, however it had no significant effect on the magnitude or duration of the NO-induced decrease in $[Ca^{2+}]_i$. Shown in F are the plateau levels of $[Ca^{2+}]_i$ reached following each concentration of NO. Also shown in F are the values of $[Ca^{2+}]_i$ measured in experiments in which NO was not added for comparison to those in which NO was added (No NO). In D–F, filled bars indicate control values and hatched bars indicate values for cells treated with zaprinast.

Rp-8-Br-cyclic GMPS (3×10^{-4} M) had no significant effect on the concentration-dependent reductions in $[Ca^{2+}]_i$ caused by the NO donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP, $10^{-9}-10^{-5}$ M, Figure 8A–C).

Effect of guanylyl cyclase and/or protein kinase G inhibition on the inhibition by NO of the AII-induced increase in intracellular Ca^{2+}, and Ca^{2+} or Mn^{2+} influx

In the absence of extracellular Ca^{2+}, AII causes the transient release of intracellular Ca^{2+}, as well as the influx of extracellular Ca^{2+}. In order to determine the role of cyclic GMP on each phase of the AII response, $[Ca^{2+}]_i$ was measured in cells in nominally free Ca^{2+} buffer supplemented with EGTA (5×10^{-5} M) in the absence of zaprinast (Figure 9A). Both the intracellular $[Ca^{2+}]_i$ increase caused by AII in the absence of extracellular Ca^{2+}, and the increase in $[Ca^{2+}]_i$ upon addition of extracellular Ca^{2+} (1.2 mM) were significantly reduced by NO (10^{-6} M) added 1 min prior to AII (Figure 9B). Although the transient intracellular Ca^{2+}response to AII remained significantly reduced by NO (Figure 9C and D), ODQ (10^{-5} M) significantly diminished the effect of NO. When extracellular Ca^{2+} was added to cells treated with ODQ, $[Ca^{2+}]_i$ increased to levels similar to those in cells not treated with the guanylyl cyclase inhibitor until approximately 11 min after adding NO, and then returned to levels attained in cells in which NO was not added (Figure 9C and D).

In order to study the role of cyclic GMP in the effect of NO on cation influx without interference from effects on efflux and intracellular uptake mechanisms, Mn^{2+}influx was measured by its ability to quench fura-2 fluorescence. AII caused a 4 fold increase over the basal rate of Mn^{2+} influx (Figure 10A and B). NO (10^{-6} M, Figure 10A), SNAP (10^{-5} M), or 8-p-chlorophenylthio-cyclic GMP (3×10^{-5} M) significantly and similarly inhibited AII-stimulated Mn^{2+} influx (Figure 10B). Rp-8-Br-cyclic GMPS (3×10^{-4} M) inhibited the decrease in Mn^{2+} influx caused by 8-p-chlorophenylthio-cyclic GMP, but had no effect on the decrease caused by NO or SNAP. ODQ (10^{-5} M) also had no effect on the decrease in Mn^{2+} influx caused by NO. Furthermore, the combination of ODQ and Rp-8-Br-cyclic GMPS also had no significant effect on the decrease in Mn^{2+} influx caused by NO.

Table 2 Effect of zaprinast, ODQ, and Rp-8-Br-cyclic GMPS on basal and AII-stimulated $[Ca^{2+}]_i$ levels in smooth muscle cells

	$[Ca]_i$ nM		
		AII (0.1 μM)	
	Basal	*Peak*	*Plateau*
Control	65±4	975±57	432±19
Zaprinast (10^{-4} M)	64±8	1138±121	438±20
ODQ (10^{-5} M)	92±8*	1254±214	465±39
ODQ+Zaprinast	76±16	1066±116	425±21
Control	63±6	822±128	431±60
Rp-8-Br-cyclic GMPS (3×10^{-4} M)	69±7	793±89	453±55

Two series of experiments are shown, each with its own control data. Values of $[Ca^{2+}]_i$ in nM are expressed as means±s.e.mean. Cells were exposed to zaprinast (10^{-4} M, $n=4$) and/or ODQ (10^{-5} M, $n=4$) for 60 min, or Rp-8-Br-cyclic GMPS (3×10^{-4} M, $n=5$) for 25 min. Basal values were obtained just prior to addition of AII. Peak values represent the initial rise in $[Ca^{2+}]_i$ caused by AII and the plateau value represents the $[Ca^{2+}]_i$ level 30 s after the addition of AII (cf. Figure 2A). The asterisk indicates significant differences from the control data ($P<0.05$).

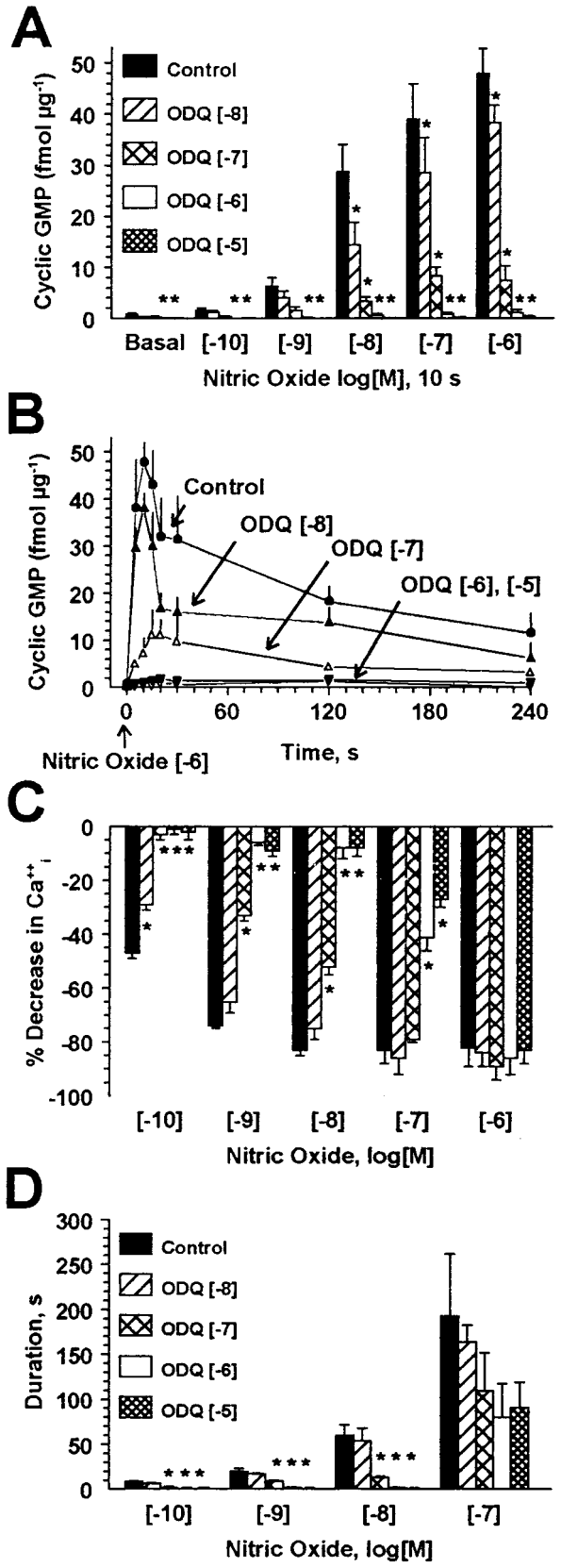

Figure 3 Effect of ODQ on NO-stimulated cyclic GMP levels and decreases in $[Ca^{2+}]_i$. Cells were exposed to ODQ ($10^{-8}-10^{-5}$ M) for 60 min before addition of NO ($10^{-10}-10^{-6}$ M) for 10 s (A) or NO (10^{-6} M) for 5–240 s (B). ODQ significantly decreased cyclic GMP levels as indicated by the asterisks ($P<0.05$, $n=3$). Data summarizing the effect of ODQ ($10^{-8}-10^{-5}$ M) on the magnitude (C) and duration (D) of the decreases in the AII-stimulated $[Ca^{2+}]_i$ level caused by NO ($10^{-10}-10^{-6}$ M, $^*P<0.05$, $n=3$). At lower concentrations of NO, the inhibition of the decrease in $[Ca^{2+}]_i$ caused by ODQ correlated with the decrease in cyclic GMP levels. At concentrations greater than 10^{-7} M, NO persisted in decreasing $[Ca^{2+}]_i$ in the presence of ODQ, despite preventing the rise in cyclic GMP. The data in A and B were obtained without adding AII or zaprinast, those in C and D also were obtained without zaprinast in cells stimulated with AII.

Effect of guanylyl cyclase inhibition on relaxation of rabbit aorta to NO

In rabbit aorta rings denuded of endothelium and contracted with phenylephrine, basal levels of cyclic GMP (2.5 ± 0.3 fmol μg^{-1}) were significantly increased by NO (10^{-6} M, 18 ± 2.2, $P<0.05$, $n=4$). ODQ (10^{-6} and 10^{-5} M), while having no significant effect on basal cyclic GMP (2.3 ± 0.1 and 1.9 ± 0.2, respectively), prevented the rise in cyclic GMP caused by NO (3.6 ± 0.9 and 3.0 ± 0.9, respectively).

NO ($10^{-10}-10^{-5}$ M) caused concentration-dependent, transient relaxations (Figure 10). ODQ ($10^{-6}-10^{-4}$ M) significantly inhibited the relaxations to NO, but significant relaxations to NO ($10^{-7}-10^{-5}$ M) persisted (Figure 11B, C, D and E). Pretreatments with IBMX (10^{-4} M) had no significant effect on relaxation caused by NO, nor did the phosphodiesterase inhibitor affect the NO-induced relaxation in the presence of ODQ (10^{-4} M, Figure 11E).

Discussion

The association of cyclic GMP with the actions of NO has been based primarily on studies which showed that NO stimulates guanylyl cyclase and increases the levels of cyclic GMP, as well as by the fact that exogenous cyclic GMP analogues have similar smooth muscle relaxant effects as NO (Waldman & Murad, 1987; Ignarro & Kadowitz, 1985). In the present study, the levels of cyclic GMP stimulated by NO were manipulated by either inhibiting its production (ODQ) or its degradation (zaprinast), or by blocking its actions mediated through protein kinase G. In doing so, it became evident that there were significant additional mechanisms to cyclic GMP and protein kinase G by which NO could decrease $[Ca^{2+}]_i$ and cause relaxation of vascular smooth muscle.

The effects of NO were evaluated in rabbit aortic smooth muscle cells in primary culture in which the decrease in $[Ca^{2+}]_i$ caused by NO is similar in time course and potency as in the intact aorta. Indeed, a dissociation between the elevation of cyclic GMP and the NO-induced relaxation was demonstrated also in aortic rings treated with the guanylyl cyclase inhibitor, ODQ. Although the dissociation of cyclic GMP from the actions of NO was most apparent at concentrations of NO above 10^{-7} M, these concentrations are physiological, being reached in close proximity to native endothelial cells when stimulated by agonists such as acetylcholine (Cohen *et al.*, 1997; Malinski *et al.*, 1993). For example, acetylcholine fully relaxes and hyperpolarizes the rabbit carotid artery while releasing concentrations of NO up to 2.5×10^{-7} M from the endothelium (Cohen *et al.*, 1997). The smooth muscle responses to these endogenous levels of endothelium-derived NO are only partially inhibited by ODQ (Plane *et al.*, 1998).

Role of phosphodiesterase in the response to NO

In the concentrations used in this study, IBMX non-specifically inhibits all phosphodiesterase isoenzymes, and

zaprinast inhibits cyclic GMP-activated and Ca^{2+}-activated isoforms (Beltman *et al*., 1993). In cells stimulated with AII or ionomycin, phosphodiesterases were active in metabolizing cyclic GMP, because zaprinast or IBMX significantly increased both basal and NO-stimulated cyclic GMP levels in the presence of these agonists (Table 1 and Figure 1). The decrease in cyclic GMP levels in agonist-stimulated cells likely occurs *via* Ca^{2+}-dependent phosphodiesterases (Smith & Lincoln, 1987). This is borne out by the fact that either IBMX or zaprinast attenuated the decrease in cyclic GMP levels in cells stimulated with AII or ionomycin (Table 1).

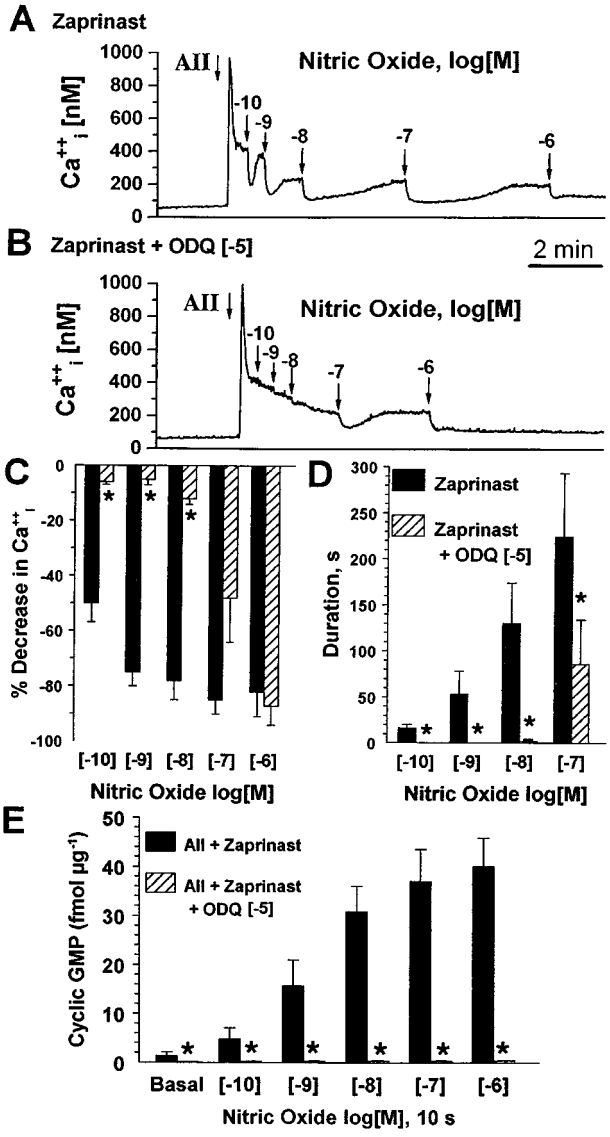

Figure 4 Effect of ODQ on NO-stimulated cyclic GMP levels and decreases in $[Ca^{2+}]_i$. Cells were treated with zaprinast (10^{-4} M) for 60 min before the addition of AII. Representative recordings of decreases in AII-stimulated $[Ca^{2+}]_i$ caused by NO (10^{-10}–10^{-6} M) under control conditions (A) and in cells treated with ODQ (10^{-5} M) for 60 min (B). Data summarizing the effect of ODQ (10^{-5} M) on the magnitude (C) and duration (D) of NO-induced decreases in $[Ca^{2+}]_i$ ($^*P<0.05$, $n=4$), showing that ODQ significantly inhibits the effect of lower concentrations of NO, but that NO (10^{-7}–10^{-6} M) decreased $[Ca^{2+}]_i$ despite the fact that ODQ abolished increases in cyclic GMP (E). Cyclic GMP data in E were obtained in cells treated with both AII and zaprinast to mimic the conditions of the $[Ca^{2+}]_i$ measurements. Filled bars indicate control values; hatched bars indicate values for cells treated in addition with ODQ.

Although it was not possible to study the influence of IBMX on $[Ca^{2+}]_i$ changes caused by NO because of interference by the phosphodiesterase inhibitor in measure-

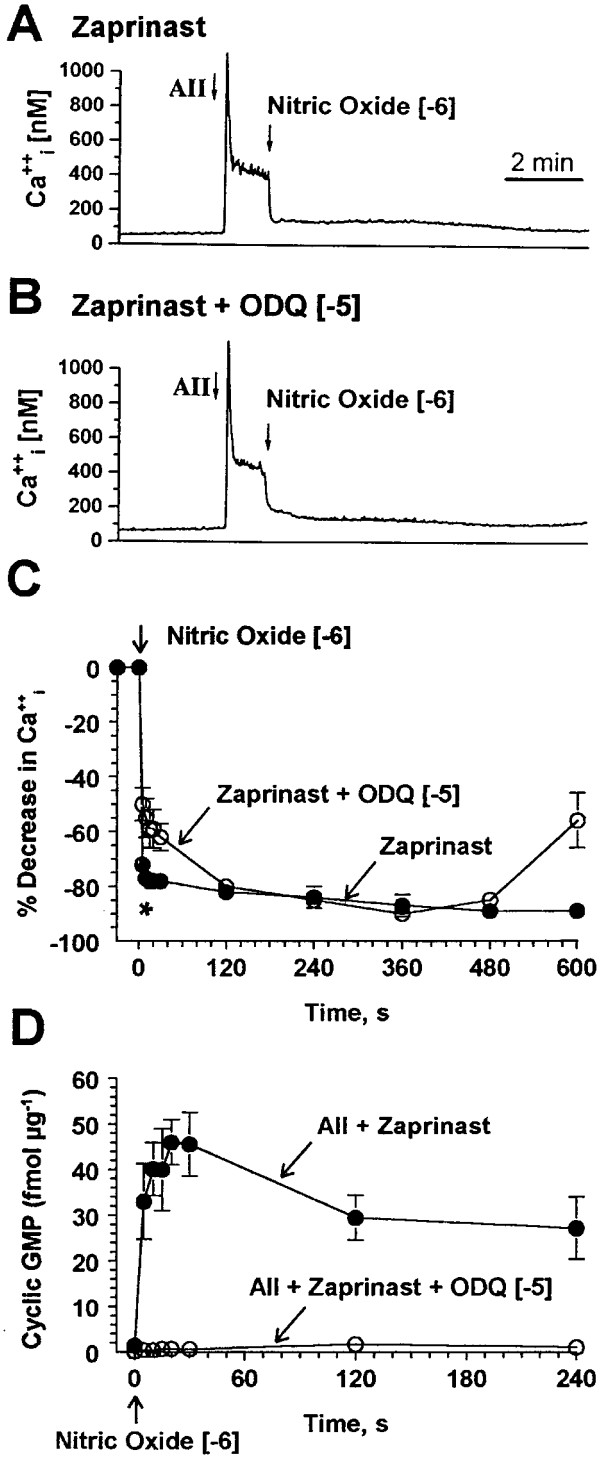

Figure 5 Effect of ODQ on NO-stimulated cyclic GMP levels and decrease in $[Ca^{2+}]_i$ in smooth muscle cells. All cells were treated with zaprinast (10^{-4} M) for 60 min before the addition of AII (10^{-7} M), and NO (10^{-6} M) was added 1 min later. Representative recordings of the decrease in $[Ca^{2+}]_i$ caused by NO (10^{-6} M) under control conditions (A) or in cells treated with ODQ (10^{-5} M) for 60 min (B). Summary data (C, $n=4$) showing the decrease in $[Ca^{2+}]_i$ caused by NO in control and ODQ-treated cells over 600 s. Only at 5 s, the decrease in $[Ca^{2+}]_i$ was significantly less in cells treated with ODQ (asterisk), but the mean per cent reduction in $[Ca^{2+}]_i$ over 600 s was not significantly different. The rise in cyclic GMP caused by NO (10^{-6} M, D) was blocked by ODQ (10^{-5} M) when measured in the presence of both AII and zaprinast under the same conditions as $[Ca^{2+}]_i$ in A–C.

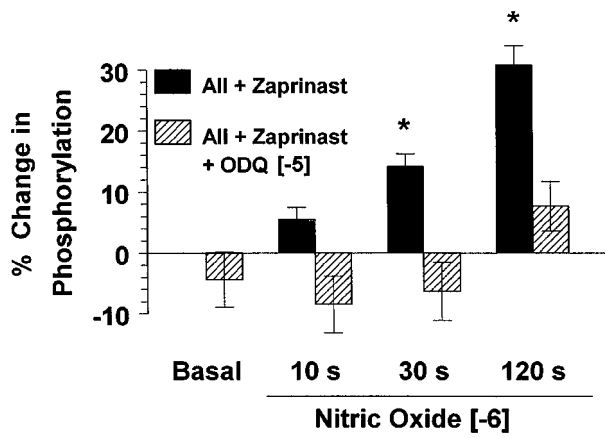

Figure 6 Phosphorylation of the inositol trisphosphate receptor caused by NO. Cells were treated with zaprinast (10^{-4} M, 60 min) and AII (10^{-7} M, 1 min) prior to adding NO (10^{-6} M). A significant increase in endogenous phosphorylation of the IP_3 receptor was evident from a decrease in ^{32}P label back phosphorylation that occurred 30 and 120 s after NO (*$P<0.05$, $n=4$). In cells treated with ODQ (10^{-5} M, 60 min), NO had no significant effect.

ment of the $[Ca^{2+}]_i$ response to AII, the effects of inhibiting cyclic GMP breakdown were examined with zaprinast. Despite the 3–20 fold higher levels of cyclic GMP stimulated by NO in the presence of zaprinast, the initial decreases in AII-stimulated $[Ca^{2+}]_i$ caused by NO were not significantly different from those that occurred in the absence of the phosphodiesterase inhibitor. Cyclic GMP levels in Figure 1 were measured 10 s after addition of NO in cells stimulated by AII in the same protocol and under the same conditions used for the measurements of $[Ca^{2+}]_i$. A study of the time course of the increase in cyclic GMP caused by NO (10^{-8} M) indicated that even during the initial 5 s in which $[Ca^{2+}]_i$ reached a minimum following addition of NO, cyclic GMP levels were far greater in the presence of zaprinast. Indeed, in the absence of the phosphodiesterase inhibitor, cyclic GMP levels were not increased by NO during the first 5 s when $[Ca^{2+}]_i$ reached a minimum. The detection limits of the cyclic GMP assay were sufficient to make unlikely our inability to measure the basal levels of cyclic GMP in cells treated with the phophodiesterase inhibitor. Zaprinast did significantly lower $[Ca^{2+}]_i$ following each dose of NO, suggesting an effect of the higher concentrations of cyclic GMP in the presence of the phosphodiesterase inhibitor, but one which occurred later in the time course of the decrease in $[Ca^{2+}]_i$ caused by NO.

Although the effect of zaprinast suggests a large dissociation between the levels of cyclic GMP and the initial effects of NO, these data also indicate that phosphodiesterases rapidly breakdown cyclic GMP after stimulation of guanylyl cyclase by NO. This breakdown is exaggerated in cells stimulated with AII in which Ca^{2+}-dependent phosphodiesterase is activated. The dissociation observed between cyclic GMP levels and the physiological action of NO in the presence and absence of zaprinast might therefore be explained by rapid turnover of cyclic GMP in the absence of the phosphodiesterase inhibitor, the possibility that cyclic GMP or protein kinase G is compartmentalized within the cell (Cornwell *et al*., 1991; Eckly-Michel *et al*., 1997), or the possibility that the low levels of cyclic GMP can accomplish all the effects of the higher levels measured in the presence of the phosphodiesterase inhibitor. We also found that IBMX had no significant effect on the relaxation of the rabbit aorta to NO. A similar dissociation in cyclic GMP levels and relaxation in the presence and absence of zaprinast has been noted in respiratory smooth muscle (Sadeghi-hashjin *et al*., 1996).

Role of guanylyl cyclase in the response to NO

When used in concentrations above 10^{-6} M, ODQ effectively prevented any increase in cyclic GMP levels caused by NO even when the vasodilator was administered in concentrations up to 10^{-6} M. Despite its effectiveness in preventing the increase in cyclic GMP, ODQ prevented only the effects of lower, but not those of higher concentrations of NO to decrease $[Ca^{2+}]_i$. An assessment of the time course of cyclic GMP levels and the fall in $[Ca^{2+}]_i$ caused by NO (Figure 5) showed that even within the first 5 s when $[Ca^{2+}]_i$ fell to nearly the same level in cells treated with ODQ as in untreated cells, cyclic GMP levels were not stimulated by NO in the presence of ODQ, whereas they reached near maximal levels in untreated cells. Because these studies were performed in cells treated with zaprinast, it is unlikely that an increase in cyclic GMP production was missed due to its breakdown by phosphodiestereases. Indeed, ODQ (10^{-5} M) had similar effects on the decrease in $[Ca^{2+}]_i$ caused by NO (10^{-6} M) whether zaprinast was present (Figures 4 and 5) or not (Figures 3 and 7). Thus, the majority of the response to the higher concentrations of NO can apparently occur without cyclic GMP elevation, and this finding does not depend upon the breakdown of cyclic GMP. Cyclic GMP-independent responses were also apparent in NO-induced relaxation of the intact artery treated with ODQ in which the persistent response was also not influenced by inhibiting phosphodiesterase.

ODQ is a non-competitive inhibitor of guanylyl cyclase (Garthwaite *et al*., 1995) and has been reported to completely block the effects of some NO donors, such as 3-morpholino-sydnonimine or SNAP, in isolated arteries (Plane *et al*., 1998; Brunner *et al*., 1996). However, these donors may be poor analogues for endothelium-derived NO, due to the fact that they release lower concentrations of NO or release it more slowly than NO is released from endothelium (Cohen *et al*., 1997). In other studies, authentic NO has been shown to be a better mimic of the endothelium-derived relaxing factor released by acetylcholine, and that relaxations to acetylcholine and NO, unlike some NO donors, are largely resistant to ODQ (Plane *et al*., 1998). This indicates that cyclic GMP-independent mechanisms of vasodilation caused by NO are physiologically important.

Role of protein kinase G in the response to NO

Cyclic GMP is thought to mediate relaxation primarily through activation of a specific kinase which has multiple phosphorylation targets in smooth muscle cells which include myosin, ion channels, and Ca^{2+} transporters in the plasma membrane and sarcoplasmic reticulum (Lincoln *et al*., 1996). The active analogue of cyclic GMP, 8-p-CPT-cyclic GMP, inhibited AII-induced $[Ca^{2+}]_i$ increases by a protein kinase G-dependent mechanism, being blocked by the inactive analogue of cyclic GMP and competitive inhibitor of protein kinase G, Rp-8-Br-cyclic GMPS (Butt *et al*., 1990; Corbin *et al*., 1986). Nevertheless, the protein kinase G inhibitor reduced only the transient decreases in $[Ca^{2+}]_i$ caused by lower concentrations of NO, whereas there was little effect on the decreases in $[Ca^{2+}]_i$ caused by higher concentrations of NO or those caused by SNAP. Even submaximal responses to low concentrations of the NO donor were unaffected by the protein kinase G inhibitor. When ODQ was combined with the inhibitor of

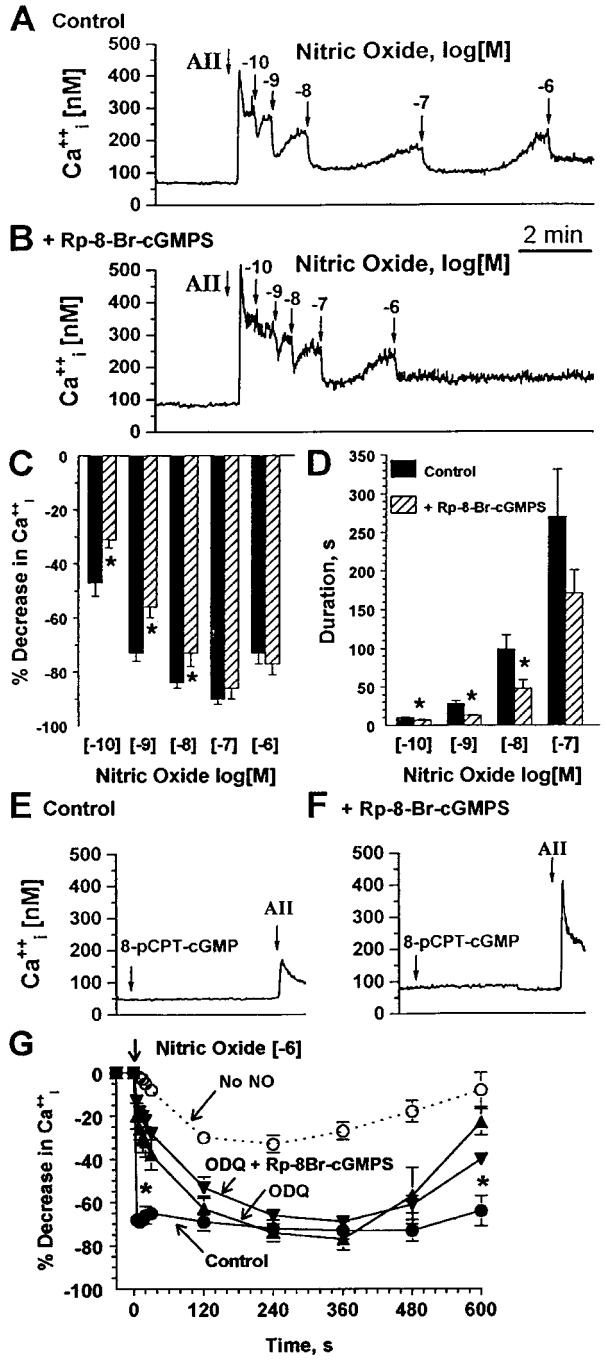

Figure 7 Effect of Rp-8-Br-cyclic GMPS on NO- and 8-pCPT-cyclic GMP-induced decreases in $[Ca^{2+}]_i$ in smooth muscle cells. The effect of NO ($10^{-10}-10^{-6}$ M, A–D and G) and 10^{-4} M 8-pCPT cyclic GMP (E and F) on AII-stimulated $[Ca^{2+}]_i$ in control and Rp-8-Br-cyclic GMPS (3×10^{-4} M, 25 min) treated cells. Zaprinast was not included. Rp-8-Br-cyclic GMPS significantly decreased ($^*P<0.05$, $n=5$) the magnitude (C) and the duration (D) of the response to NO ($10^{-10}-10^{-8}$ M). Filled bars indicate control values; hatched bars indicate values for cells treated with Rp-8-Br-cyclic GMPS. At lower concentrations of NO, decreases in $[Ca^{2+}]_i$ persisted in the presence of Rp-8-Br-cyclic GMPS, and the response to NO ($10^{-7}-10^{-6}$ M) was unaffected by the inhibitor of protein kinase G. 8-pCPT-cyclic GMP (10^{-4} M) decreased the peak increase in $[Ca^{2+}]_i$ caused by AII (compare A and E), and the effect of the cyclic GMP analogue was prevented by Rp-8-Br-cyclic GMPS (3×10^{-4} M, F). In panel G are shown the responses to NO (10^{-6} M, $n=4$) in untreated cells and in cells treated with ODQ (10^{-5} M) or ODQ combined with Rp-8-bromo-cyclic GMPS (3×10^{-4} M). For comparison, the $[Ca^{2+}]_i$ is shown in cells stimulated with AII without adding NO (No NO, dotted line). Over 10 min the average $[Ca^{2+}]_i$ decreased by $24\pm4\%$ without NO. Compared to this value, NO caused a significantly greater reduction in $[Ca^{2+}]_i$ under control conditions or in the presence of ODQ, or ODQ combined with Rp-8-bromo-cyclic GMPS (71 ± 4, 61 ± 8, and $57\pm3\%$, respectively). ODQ alone did not significantly decrease the average per cent decrease in $[Ca^{2+}]_i$ after NO, nor was there a significant difference in the response to NO in the presence of both inhibitors compared with ODQ alone. Only the decrease in $[Ca^{2+}]_i$ at 5–30 s and at 600 s was significantly less in cells treated with the inhibitors compared with control ($^*P<0.05$).

protein kinase G to further exclude the possibility that protein kinase G was stimulated despite the antagonist by increases in cyclic GMP caused by NO (10^{-6} M, Figure 7G) there was no further effect on the decrease in $[Ca^{2+}]_i$. Thus, while a role for protein kinase G could be postulated in mediating responses to lower concentrations of NO, protein kinase G-independent mechanisms need to be implicated to fully explain responses to NO and SNAP.

NO was capable of stimulating protein kinase G in these cells as demonstrated by phosphorylation of the IP_3 receptor (Komalavilas & Lincoln, 1994, 1996). However, significant activation of protein kinase G-dependent phosphorylation required more than 30 s. This in itself suggests that the initial rapid decrease in $[Ca^{2+}]_i$ caused by NO occurs independently of protein kinase G. The stimulation of protein kinase G was blocked by ODQ, giving an additional indication of the efficacy of ODQ in blocking cyclic GMP and protein kinase G-dependent effects of NO. This also excludes the possibility that the failure of ODQ to block the rapid response to higher concentrations of NO was due to overcoming the effect of the inhibitor.

Potential mechanisms mediating the effect of NO on $[Ca^{2+}]_i$

NO inhibited both the intracellular Ca^{2+} release and the Ca^{2+} influx phase of the response to AII (Weisbrod *et al.*, 1997). However, ODQ only partly inhibited the ability of NO to decrease the intracellular response to AII. In addition, the initial decrease caused by NO in $[Ca^{2+}]_i$ during Ca^{2+} influx was unchanged by ODQ, although the guanylyl cyclase inhibitor did shorten the duration of the effect of NO on $[Ca^{2+}]_i$. These observations suggest that cyclic GMP-independent mechanisms contribute to the effect of NO both on intracellular Ca^{2+} release and Ca^{2+} influx mechanisms.

The inhibitory effects of NO on the ion channels which conduct agonist-induced Ca^{2+} influx in these rabbit aortic smooth muscle cells was assessed indirectly by the rate of influx of Mn^{2+} following stimulation of the cells with AII. This method assesses cation influx directly by excluding effects of cation efflux and intracellular uptake mechanisms which can effect $[Ca^{2+}]_i$, 8-pCPT-cyclic GMP inhibited AII-induced Mn^{2+} influx, and Rp-8-Br-cyclic GMPS completely prevented the inhibition caused by the active cyclic GMP analogue. This indicates the ability of activated protein kinase G to inhibit cation influx in these cells. Although NO and SNAP caused similar decreases in AII-induced cation influx, their effect was not prevented by Rp-8-Br-cyclic GMPS, suggesting protein kinase G-independent actions. Even when the increases in cyclic GMP caused by NO were reduced with ODQ, the combined inhibitors of protein kinase G and guanylyl cyclase failed to decrease the ability of NO to inhibit cation influx. This demonstrates that although activated protein kinase G can inhibit cation influx in these cells, and would be expected to do so when cyclic GMP is normally stimulated by NO, the action of NO does not rely exclusively on cyclic GMP or protein kinase G. Furthermore, the results of studies combining the

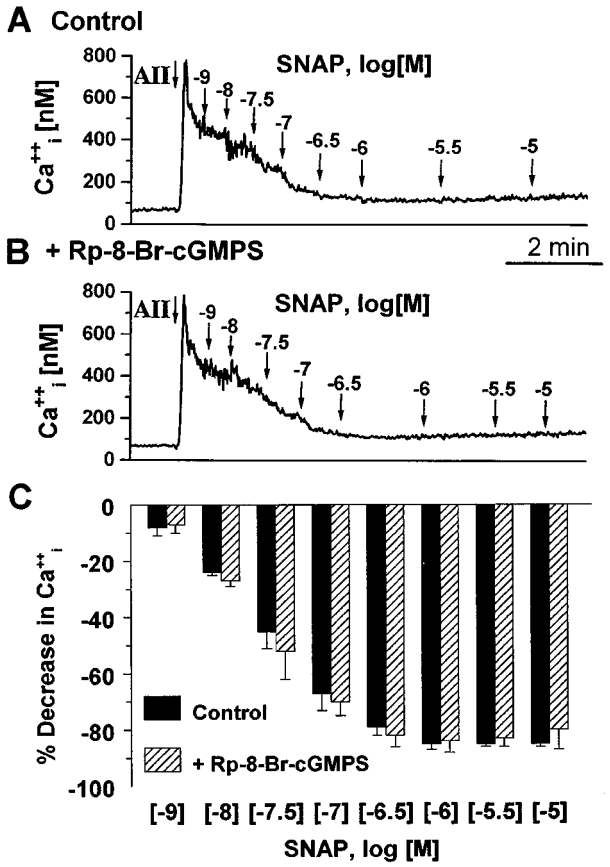

Figure 8 Effect of Rp-8-Br-cyclic GMPS on SNAP-induced decreases in $[Ca^{2+}]_i$ in smooth muscle cells. Representative recordings of the effect of SNAP (10^{-10}–10^{-5} M) on AII (10^{-7} M)-stimulated $[Ca^{2+}]_i$ in control (A) and Rp-8-Br-cyclic GMPS treated (3×10^{-4} M, 25 min (B) cells. Rp-8-Br-cyclic GMPS had no significant effect on the magnitude (C) of the SNAP-induced $[Ca^{2+}]_i$ decrease ($n=3$).

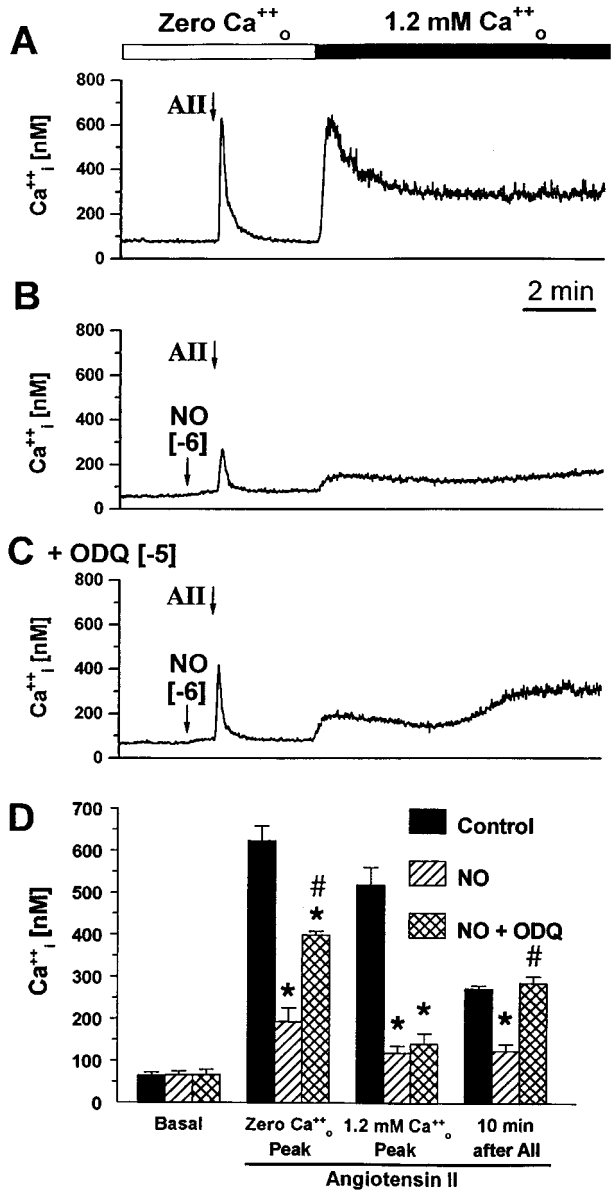

Figure 9 Effect of ODQ on the NO-induced inhibition of the AII-induced increase in $[Ca^{2+}]_i$ in the absence and presence of extracellular Ca^{2+}. The response to AII was determined in the nominal absence of extracellular Ca^{2+} ($Ca^{2+}{}_o$) and after the addition of extracellular Ca^{2+} (1.2 mM, 3 min after AII). NO (10^{-6} M, 1 min prior to AII) inhibited the transient increase in $[Ca^{2+}]_i$ caused by AII as well as the rise in $[Ca^{2+}]_i$ following the addition of extracellular Ca^{2+} (B and summarized in D). In cells treated with ODQ (10^{-5} M, 1 h; C and D) the reduction by NO of the peak transient release of Ca^{2+} caused by AII remained statistically significant, but was significantly less than in the absence of ODQ. The effect of NO on the peak rise in $[Ca^{2+}]_i$ which occurred upon extracellular Ca^{2+} addition was unaffected by ODQ, but approximately 10 min after AII (or 11 min after NO was added), $[Ca^{2+}]_i$ returned to levels not significantly different from those observed in cells not treated with NO. *Indicates values significantly less than control; #Indicates that values in the presence of ODQ are significantly greater than NO alone ($P<0.05$).

protein kinase G inhibitor with ODQ to eliminate increases in cyclic GMP (Figures 7 and 10) would indicate that the response to NO is not mediated by potential protein kinase G-independent actions of cyclic GMP which have been described on ion channels, protein kinase A, or phosphodiesterases (Schoeffter *et al.*, 1988; Williams *et al.*, 1988; Biel *et al.*, 1994). The effect of NO on Mn^{2+} influx was assessed 3 min after adding NO, indicating that protein kinase G- and cyclic GMP-independent mechanisms are not only restricted to the initial few seconds of the response to NO, but extend for as long as $[Ca^{2+}]_i$ is decreased. This conclusion is in agreement with that based on the prolonged NO-induced decrease in $[Ca^{2+}]_i$ which occurred in the presence of ODQ (Figures 5, 7, and 9).

NO strongly and rapidly stimulates the production of cyclic GMP by guanylyl cyclase. Having shown that in the cells studied here that NO can activate protein kinase G, it is clear that the increase in cyclic GMP levels caused by NO can contribute to mediating the decrease in $[Ca^{2+}]_i$ and relaxation of smooth muscle. However, it is also apparent from the present studies that activation of protein kinase G is too slow to account for the initial rapid response to higher concentrations of NO, and that after inhibiting either the production of cyclic GMP or the activation of protein kinase G, significant effects of NO persist. Of course, the role of guanylyl cyclase may differ in different tissues or species, as indicated by the report that a protein kinase G inhibitor abolished the effect of sodium nitroprusside on gastric smooth muscle of the guinea-pig and rabbit (Murthy & Makhlouf, 1995).

The data presented here raise the important question as to how NO could decrease $[Ca^{2+}]_i$ without the involvement of cyclic GMP. Cyclic GMP and protein kinase G are thought to have multiple protein targets which regulate $[Ca^{2+}]_i$. These include L-type Ca^{2+} channels (Quignard *et al.*, 1997), Ca^{2+} dependent K^+ channels (Taniguchi *et al.*, 1993; Archer *et al.*, 1994), sarcoplasmic (Cornwell *et al.*, 1991) and plasma

membrane (Furukawa *et al*., 1988) Ca^{2+} ATPases, the Na^+/Ca^{2+} exchanger (Furukawa *et al*., 1991), and the inositol trisphosphate receptor (Ruth *et al*., 1993). The function of any or all of these proteins might also be affected independently of cyclic GMP. NO activates Ca^{2+}-dependent K^+ channels through a process dependent on protein thiols (Bolotina *et al*., 1994) and this mechanism has been implicated in cyclic GMP-independent relaxation (Li *et al*., 1998; Cohen *et al*., 1997; Bolotina *et al*., 1994) and hyperpolarization (Plane *et al*., 1998; Cohen *et al*., 1997) to both endothelium-derived and exogenous NO. However, under normal conditions blockade of K^+ channels with charybdotoxin alone does not prevent NO-mediated relaxation (Plane *et al*., 1998; Li *et al*., 1998; Bolotina *et al*., 1994) or the decrease in $[Ca^{2+}]_i$ caused by NO observed in this study (unpublished observations), suggesting

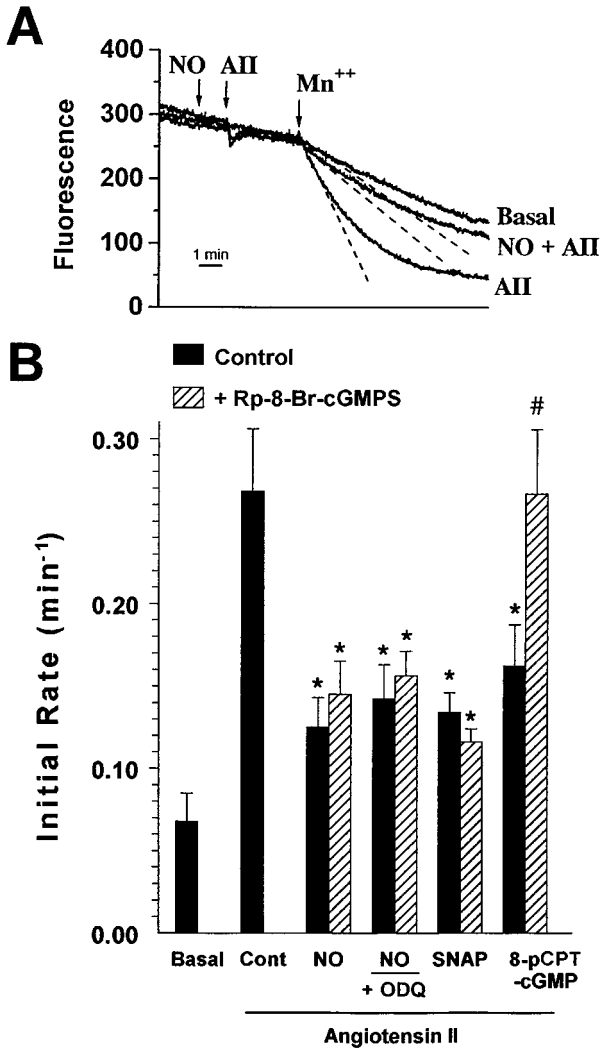

Figure 10 Effect of NO on cation influx in smooth muscle cells. (A) Shows an example, summarized in (B), of the effect of AII (10^{-7} M) and NO on the rate of quenching of fura-2 caused by Mn^{2+} influx. The fura-2 fluorescence is normalized to 100% at the time Mn^{2+} is added, and the initial rate of quenching (min^{-1}) is estimated from the slope of the quenching during the first min after adding Mn^{2+} (shown as dotted lines). AII increased the rate of Mn^{2+} influx by 4 fold over basal conditions. NO (10^{-6} M) or SNAP (10^{-5} M) added 1 min before AII significantly reduced AII-stimulated influx by 70 ± 9 and 54 ± 2%, respectively. Rp-8-Br-cyclic GMPS (3×10^{-4} M) did not significantly affect the reduction of influx by NO or SNAP (61 ± 4 and 64 ± 2%, respectively). ODQ (10^{-5} M), either alone or in combination with Rp-8-Br-cyclic GMPS, failed to significantly affect the reduction caused by NO in Mn^{2+} influx. 8-pCPT-cyclic GMP (3×10^{-5} M) decreased Mn^{2+} influx by 52 ± 7%, and this effect was completely blocked by Rp-8-Br-cyclic GMPS (-1 ± 7%). $^*P<0.05$ when compared to AII alone; $\#P<0.05$ when compared to 8-pCPT-cyclic GMP alone ($n=4$).

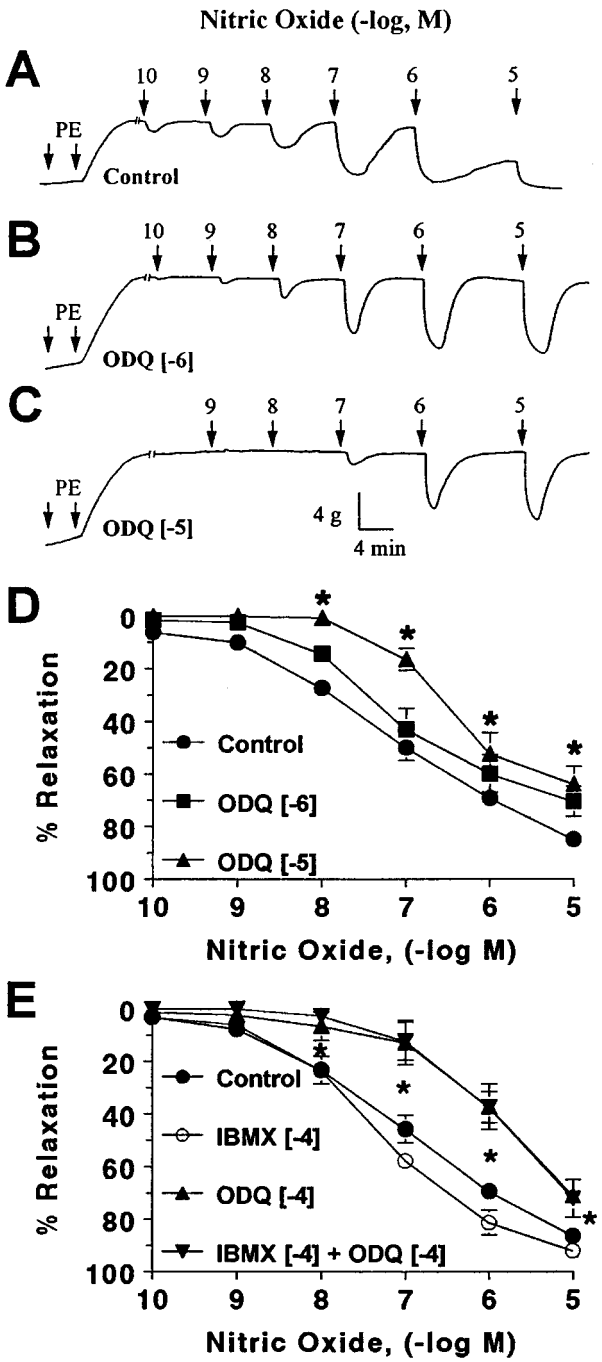

Figure 11 Effect of ODQ on NO-induced relaxation in rabbit aortic rings contracted with phenylephrine. Representative recordings of changes in tension in aortic rings denuded of endothelium (A–C). Rings were contracted with an average concentration of phenylephrine of 6.6×10^{-8} M to achieve a contraction which averaged 7.9 ± 0.6 g. These values were not significantly different in rings treated with ODQ. NO (10^{-10}–10^{-5} M) caused concentration-dependent relaxations under control conditions (A), and after 60 min incubation with 10^{-6}–10^{-4} M ODQ (B–E). Data summarizing the maximal relaxation caused by each concentration of NO (D and E) demonstrate that ODQ (10^{-5} M, D; 10^{-4} M, E) significantly inhibited the relaxations ($^*P<0.05$, ANOVA, $n=4$–10). However, significant relaxations to NO (10^{-7} and 10^{-5} M) persisted in the presence of ODQ (10^{-6}–10^{-5} M). Data in E show the effect of IBMX (10^{-4} M) on relaxations of rabbit aorta rings to NO. IBMX had no significant effect on the response to NO in the presence or absence of ODQ (10^{-4} M).

that other mechanisms which decrease $[Ca^{2+}]_i$ are involved. Furthermore, the increase in $[Ca^{2+}]_i$ and Mn^{2+} influx in rabbit aortic smooth muscle cells caused by AII or contraction of the intact rabbit aorta caused by phenylephrine resists block of L-type Ca^{2+} channels by nifedipine indicating that Ca^{2+} entry occurs importantly by voltage-independent Ca^{2+} entry pathways which would not be expected to be inhibited by hyperpolarization in response to K^+ channel activation (Bolotina *et al*., 1997). The Ca^{2+} regulatory mechanisms affected by NO may therefore include one or more voltage-dependent and independent ion channels or ion transporters which have not yet been fully defined.

In summary, the studies presented here demonstrate that mechanisms that do not require cyclic GMP and protein kinase G are important mediators of the reduction of $[Ca^{2+}]_i$ and relaxation caused by NO in vascular smooth muscle. Just as with cyclic GMP, the Ca^{2+} regulatory protein targets which respond to NO in a cyclic GMP-independent manner may be multiple and redundant. These studies also provide a rationale to study the role of cyclic GMP-independent regulation of $[Ca^{2+}]_i$ in vascular disease states in which nitric oxide function is decreased (Weisbrod *et al*., 1997).

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