British Journal of Pharmacology (1996) 119, 737-743 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

Cyclic GMP-independent effects of nitric oxide on guinea-pig uterine contractility

Karri A. Kuenzli, Michael E. Bradley & [1]Iain L.O. Buxton

Department of Pharmacology, University of Nevada, Reno, Reno, NV 89557, U.S.A.

1 The role of nitric oxide (NO) in the regulation of uterine contractility has yet to be clearly defined. We evaluated the effect of NO (in the form of *S*-nitroso-L-cysteine, CysNO) upon uterine contractility and guanosine 3',5'-cyclic monophosphate (cyclic GMP) accumulation in pregnant and nonpregnant guinea-pig myometrium.

2 While CysNO had no effect upon spontaneous contractile activity in either pregnant or nonpregnant uterine tissues, addition of CysNO resulted in an immediate and reversible relaxation of oxytocin- or acetylcholine (ACh)-evoked contractions.

3 Relaxation of agonist-evoked contractions in response to CysNO was associated with significant elevations in intracellular cyclic GMP concentrations ([cyclic GMP]$_i$).

4 Elevations in [cyclic GMP]$_i$ were not required for relaxation, as inhibition of guanylyl cyclase by methylene blue prevented [cyclic GMP]$_i$ accumulation while having no effect upon the ability of CysNO to relax agonist-evoked contractions.

5 Addition of the cyclic GMP-analogues, 8-Br-cyclic GMP and PET-cyclic GMP, only at high concentrations, produced partial relaxation of agonist-contracted tissues, suggesting the possibility that cyclic GMP may be sufficient but not necessary for myometrial relaxation.

6 Our studies not only provide evidence for a functional role for NO-modulation of agonist-evoked contractions in the pregnant and nonpregnant guinea-pig uterus, but also that these occur by a mechanism which is not dependent upon guanylyl cyclase activity.

Keywords: Nitric oxide; guanylyl cyclase; cyclic GMP; oxytocin; acetylcholine; guinea-pig uterus

Introduction

Although nitric oxide (NO) has been found to play a role in the regulation of vascular (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987) and nonvascular (Ward *et al.*, 1992; Kannan & Johnson, 1992; Thornbury *et al.*, 1992) smooth muscle tone, inhibition of platelet aggregation (Azuma *et al.*, 1986), and the mediation of some immunological responses (Moncada *et al.*, 1991), its role in the control of uterine smooth muscle contractility has yet to be fully characterized. Studies have been performed in the rat (Papka & McNeill, 1992; Shew *et al.*, 1993; Natuzzi *et al.*, 1993; Izumi *et al.*, 1993; Conrad *et al.*, 1993; Yallampalli *et al.*, 1993; 1994; Franchi *et al.*, 1994), guinea-pig (Weiner *et al.*, 1994), human subjects (Izumi *et al.*, 1993; Telfer *et al.*, 1995; Buhimschi *et al.*, 1995) and rabbit (Sladek *et al.*, 1993) which suggest that the uterus is an NO-producing organ. Despite the number of morphological and biochemical analyses evaluating the presence of the enzyme necessary to produce NO in the uterus, only a few investigators have explored the possible mechanism of action of NO in the uterus (Izumi *et al.*, 1993; Yallampalli *et al.*, 1993; 1994; Buhimschi *et al.*, 1995). These latter studies offered evidence consistent with current dogma, that NO affects uterine smooth muscle tone via elevations in [cyclic GMP]$_i$ and concluded that an NO-guanylyl cyclase relaxation pathway exists in uterine smooth muscle. While a role for cyclic GMP in the actions of NO in many smooth muscles is now widely accepted, such a notion is at odds with previous studies demonstrating that intracellular cyclic GMP is incapable of altering uterine smooth muscle tension (Diamond, 1983; Word *et al.*, 1991). The goals of the present study were to examine the effects of exogenous NO upon uterine contractility in both pregnant and nonpregnant guinea-pig uteri, and to elucidate the role of guanylyl cyclase in any effects observed. We find that NO can indeed alter agonist-evoked uterine contractility, but that it does so by means of a mechanism which does not require the activation of guanylyl cyclase.

Methods

Source and preparation of uterine samples

Contractile studies were performed on full-thickness uterine tissue obtained from nonpregnant (mid-cycle) and pregnant (35-45 days of gestation) guinea-pigs (Simonsen, Gilroy, CA); the start of gestation was determined from the date of caging of females with proven males and midcycle nonpregnant animals were timed-based on the condition of the vaginal epithelium. Animals were killed by CO_2 asphyxiation according to approved protocols and uterine horns were removed. One horn was stored overnight (4°C) in tissue buffer consisting of the following (in mM): NaCl 120, KCl 5, KH_2PO_4 0.59, Na_2HPO_4 0.6, $MgCl_2$ 2.5, α,D-glucose 20, and tris[hydroxymethyl]amino-methane 25; $CaCl_2$ was present at a final concentration of 0.36 mM. Control studies demonstrated that uterine contractility in tissues stored for 24 h was identical to that observed in fresh uterine tissues and that 0.36 mM Ca^{2+} yielded reproducible responses to agonists during the course of the experiments. Uterine horns were opened by sharp dissection along the mesometrial border; in pregnant animals, foetuses and adherent placental tissues were carefully removed. Strips (~1 cm × 2 mm × 2 mm) were cut from the centre of the horn with the longitudinal axis oriented with the long axis of the horn *in situ*, mounted into organ baths, and attached to isometric force transducers (Kent Scientific, Litchfield, CT, U.S.A.) by silk thread. Transducer voltages were amplified and converted to digital signals by an ACJr A/D board mounted within a computer system running the Workbench data acquisition system (Strawberry Tree, Inc., Sunnyvale, CA, U.S.A.). Strips were maintained at 37°C, aerated with 100%

[1] Author for correspondence.

O_2, loaded with initial tensions of 0.25 g, and challenged with 10 μM ACh several times during the course of a 1 h equilibration period; in pregnant animals, tissues were conditioned by equimolar replacement of sodium with 60 mM KCl.

Preparation of the nitric oxide donor, CysNO

CysNO was prepared by the method of Gibson *et al.* (1992). In essence, L-cysteine (100 mM) and sodium nitrite (100 mM) were mixed in equal proportions and placed on ice for 30 min. The resulting solution was neutralized and kept on ice in an airtight container for the remainder of the day; solutions were prepared daily. Control experiments employing addition of vehicle or direct addition of NO (by addition of NO saturated water; see Ward *et al.*, 1992) verified that the effect of CysNO was due to the delivery of NO.

Agonist concentration-response relationships and effect of relaxing agents

Noncumulative concentration-response curves to ACh and oxytocin were obtained in a manner which allowed each tissue strip to serve as its own control: each agonist was present in the organ bath for 5 min, and tissues were stimulated twice with the desired agonist before proceeding with the evaluation of the effects of CysNO. CysNO (100 μM) or equivalent concentrations of vehicle (L-cysteine or 'spent' CysNO which had been allowed to react with oxygen) were added to agonist-evoked contractions which had become maximal (~1 min). Addition of the cyclic GMP analogues, 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cyclic GMP) and β-phenyl-1,N^2-ethenoguanosine-3',5'-cyclic monophosphate (PET-cyclic GMP) was performed in the same manner except that they were allowed 10 min of contact with tissue strips pre-contracted with 1 nM oxytocin. Tissues were allowed a 15 min 'rest' period between all treatments, during which time tensions returned to a constant baseline. The effect of CysNO on ACh-induced contractions in pregnant uterine tissue was not evaluated, since a consistent response to ACh was not obtained in these tissues.

To inhibit guanylyl cyclase activities, tissue strips were pretreated with methylene blue (10 μM) for 30 min, after which control responses to oxytocin (1 nM) and ACh (3 μM) were obtained. Tissues were then re-challenged with either oxytocin (1 nM) or ACh (3 μM) followed by CysNO addition at maximum (100 μM) concentrations.

Assay of cyclic GMP

Tissue strips were flash-frozen at various time points during the contractile cycle (basal tension, maximum agonist-evoked contraction, or maximum CysNO-induced relaxation in the presence and absence of methylene blue); maximum relaxation always occurred within 30 s after CysNO addition. Frozen samples were homogenized in 6% trichloroacetic acid in acetone while immersed in a dry ice : methanol slurry. Acetone was removed by lyophilization, samples were resuspended in water, and protein was removed by microcentrifugation. Acid was removed by triplicate extraction with 3 volumes of diethyl ether and residual ether was evaporated by heating at 70°C for 10 min. Aqueous lyophilates were resuspended in 1 ml phosphate-buffered saline and assayed for cyclic GMP in duplicate by enzyme-linked immunoassay (Cayman Chemical Co.).

Data analysis

Contractions were quantified by integration of the area under each contractile record for periods of 5 min following addition of agonists, or for identical times during spontaneous activity. For experiments evaluating the effects of CysNO on spontaneous or agonist-evoked contractions, integration periods began at the point of CysNO addition and ended upon timed washout. To control for time, all agonist-evoked contractions were integrated and were not to differ significantly from one another (i.e. responses to agonists did not diminish over the course of the experiment). The effects of CysNO were thus determined from integration for identical periods (2 min) in the absence or presence of CysNO following addition at the peak of agonist-evoked tension. Tissue responses were evaluated by one-way or two-way analysis of variance (ANOVA), while differences in cyclic GMP concentrations were determined by Student's *t* test. Unless otherwise stated, values for force of contraction are expressed as average mg tension generated per mm^2 cross-sectional area, ±one standard error of the mean (s.e.mean).

Materials

PET-cyclic GMP was obtained from Ruth Langhorst Biolog International (La Jolla, CA, U.S.A.), and cyclic GMP EIA assay materials were obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All other compounds were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Spontaneous and agonist-evoked uterine contractility

Strips of uterine tissue from both pregnant and nonpregnant guinea-pigs exhibited a high degree of spontaneous contractile activity at the beginning of each experiment even in the presence of reduced (0.36 mM) extracellular calcium (Figure 1a); spontaneous activity persisted for approximately 60 min and thereafter the frequency gradually diminished. Reduction of extracellular calcium in this fashion does not alter agonist-mediated signalling and is an effective way of studying regulation of contraction in guinea-pig myometrium by reducing spontaneous activity (Moritoki *et al.*, 1979; Smith *et al.*, 1988; 1989; Schiemann & Buxton, 1991). Addition of agonists such as oxytocin (Figure 1a) or ACh (Figure 1c) elicited an immediate increase in tissue tension with an amplitude greater than that of maximal spontaneous activity observed at the beginning of each experiment; however the duration of this increased tension, persisted for a period significantly longer than that associated with a spontaneous contraction. Phasic contractions with a constant frequency and increasing amplitudes were superimposed upon the increased tonic tension seen in response to agonist addition; a return to baseline tensions and basal contractile activity was seen only following washout of the agonist, or after an average of 10 min for oxytocin and 5 min for ACh. Contractile responses to agonists (in terms of amplitude, duration, or phasic character) did not change significantly with time during the course of experiments, which lasted up to 8 h.

Effects of CysNO on spontaneous and agonist-evoked contractions

The effect of CysNO on spontaneous contractile activity was evaluated within the first 60 min of each experiment, while the effects of CysNO on agonist-evoked contractions was determined in the presence of minimal spontaneous contractile activity (after a 90 min equilibration period). Addition of CysNO at final concentrations of 0.1 – 100 μM had no discernible effect upon the frequency or amplitude of spontaneous contractions in either nonpregnant (Figure 1a) or pregnant (not shown) tissues. Results were identical when experiments were carried out in the presence of 1.8 mM and 2.5 mM extracellular calcium, verifying that low extracellular Ca^{2+} concentrations were not responsible for the failure of CysNO to affect spontaneous activity. The ability of CysNO to relax the uterus was also evaluated in tissue strips precontracted with the uterotomics oxytocin and ACh at concentrations determined from preliminary concentration-response experiments to be near maximal; similar experiments

demonstrated CysNO to be maximally effective at a concentration of 100 μM. The responses shown in Figure 1b and c are typical of the responses to oxytocin and ACh, respectively. Addition of CysNO following agonist addition immediately reversed the increase in tonic contractile response to both oxytocin (Figure 1b) and ACh (Figure 1c). While CysNO treatment usually resulted in a complete abolition of both tonic and phasic components of the response to agonists, on occasion the phasic component persisted, even in the continued presence of the NO donor (Figure 1b). The typical amount of spontaneous activity present during the evaluation of CysNO effects on oxytocin- and ACh-evoked contractions is shown in Figure 1b and c, respectively. Addition of vehicle (L-cysteine or 'spent' CysNO) at volumes equivalent to those necessary to deliver CysNO, was never observed to affect tissue tension; gaseous NO which was bubbled into organ bath media was also found to be capable of relaxing ACh and oxytocin-evoked contractions in a manner similar to that observed with CysNO (not shown).

Addition of 100 μM CysNO to uterine strips pre-contracted with various concentrations of oxytocin resulted in statistically significant reductions in tissue tension in both nonpregnant (Figure 2; $P<0.001$) and pregnant (Figure 3; $P<0.05$) animals. Significant effects of CysNO on nonpregnant uterine tissue tension were also observed when ACh was employed as the contractile agent (Figure 4; $P<0.05$).

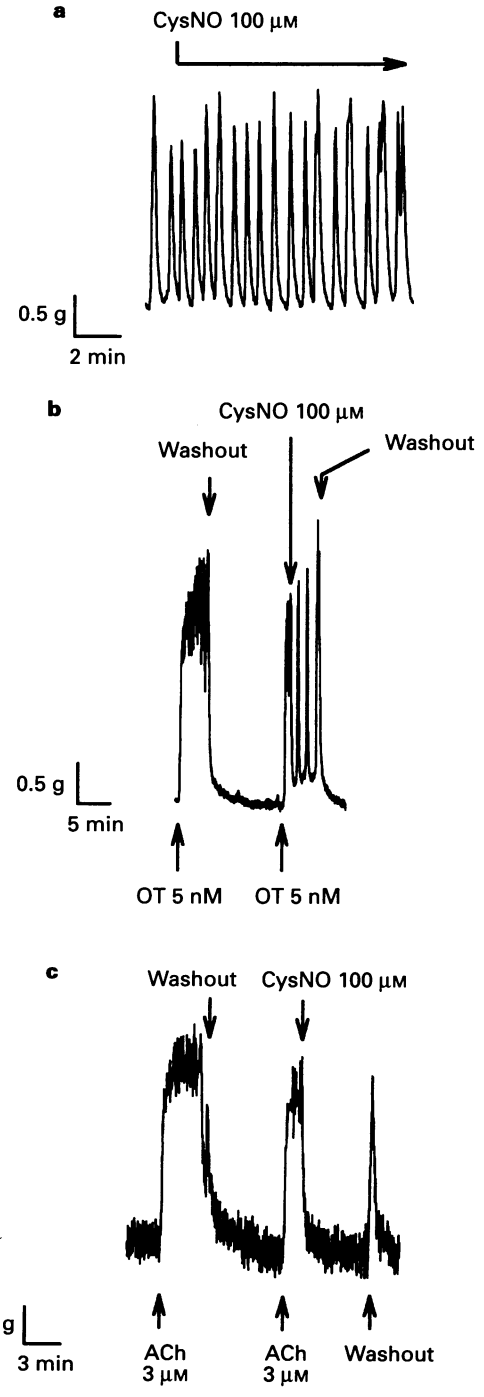

Figure 1 Effect of CysNO on spontaneous (a) or agonist-evoked contractions (b and c) in full-thickness uterine tissues. Contractile recordings are representative of results obtained in numerous pregnant and nonpregnant tissues in the presence and absence of agonist. Although CysNO-addition had no effect upon spontaneous contractile activity (a) in either pregnant (not shown) or nonpregnant tissues, CysNO (100 μM) had significant inhibitory effects on tissues pre-contracted with 1 nM oxytocin (OT, b) that resulted in the return of the force trace to baseline followed by a phasic contraction in the continued presence of oxytocin. Similarly, in (c), tonic contraction to 3 μM ACh was immediately abolished by addition of CysNO (100 μM). Contractile tracings in (b) and (c) are representative of results from experiments summarized in Figures 2 and 3, respectively.

Effects of guanylyl cyclase inhibition

Correlation between the effects of CysNO upon uterine tissue tension and tissue cyclic GMP content were simultaneously evaluated by flash-freezing tissue samples at precise points during CysNO-induced relaxations of agonist-evoked contractions. Inhibition of oxytocin- or ACh-evoked contractions by CysNO was found to be associated with statistically-significant elevations in $[\text{cyclic GMP}]_i$ (Figure 5a and b, respectively). Pretreatment of tissues with the guanylyl cyclase inhibitors methylene blue (10 μM) or 6-(phenylamino)-5,8-quinolinedione (LY83,583) (1 μM, data not shown) for 30 min was found to have no significant effect upon the ability of CysNO to inhibit

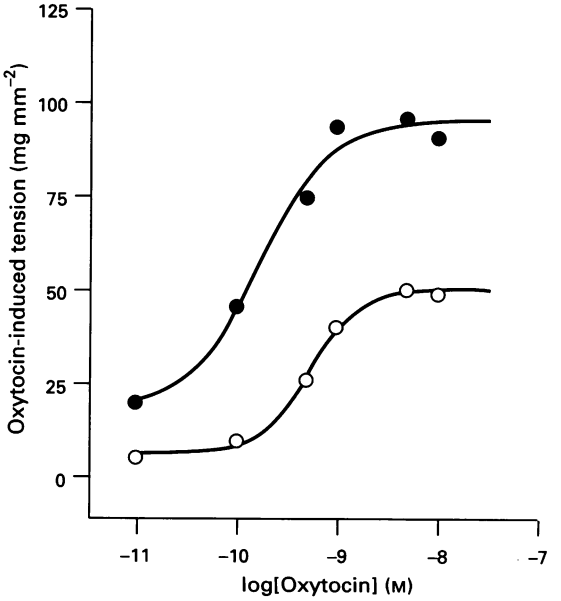

Figure 2 Concentration-response relationship for oxytocin in the presence and absence of 100 μM CysNO in nonpregnant guinea-pig uterus. Responses to oxytocin alone (●) and oxytocin followed by CysNO (○) are expressed as average mg tension per mm^2 cross sectional area ($mg\ mm^{-2}$); for clarity, s.e.mean bars have been omitted, but the s.e.mean was less than ±15% of the averaged response in all cases. Two way ANOVA indicated that CysNO caused significant reductions in oxytocin-evoked contractions at all concentrations of oxytocin tested ($P<0.001$). Data are the mean of quadruplicate determinations in each of six animals.

tissue tension elicited by either oxytocin (Figure 6a) or ACh (Figure 6c), despite its ability to completely block CysNO-dependent increases in [cylic GMP]$_i$ (Figure 6b and d). Methylene blue at 10 μM had no effect upon the frequency or amplitude of spontaneous activity.

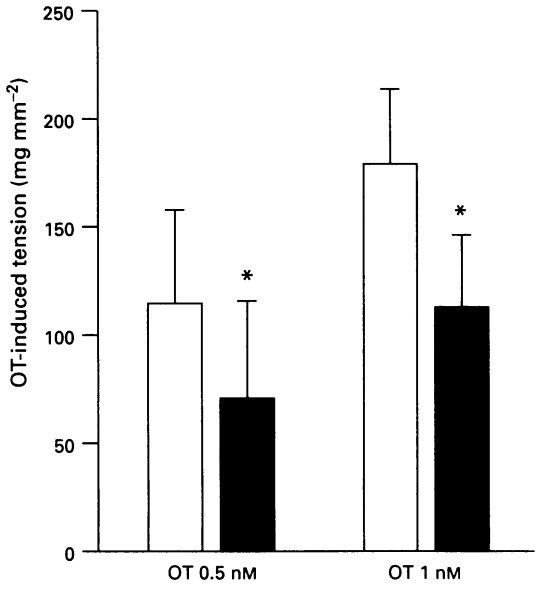

Figure 3 Effect of CysNO on oxytocin-evoked contractions in pregnant guinea-pig uterus. Responses to oxytocin alone (open columns) and oxytocin in the presence of CysNO (solid columns) are expressed as average mg tension per mm^2 cross sectional area, ±s.e.mean. CysNO (100 μM) caused significant reductions in submaximal (0.5 nM) as well as maximal (1.0 nM) oxytocin-evoked contractions in full-thickness pregnant guinea-pig uterine tissue strips as indicated by two way ANOVA ($P=0.012$; $n=3$ for 0.5 nM and $n=4$ for 1 nM oxytocin).

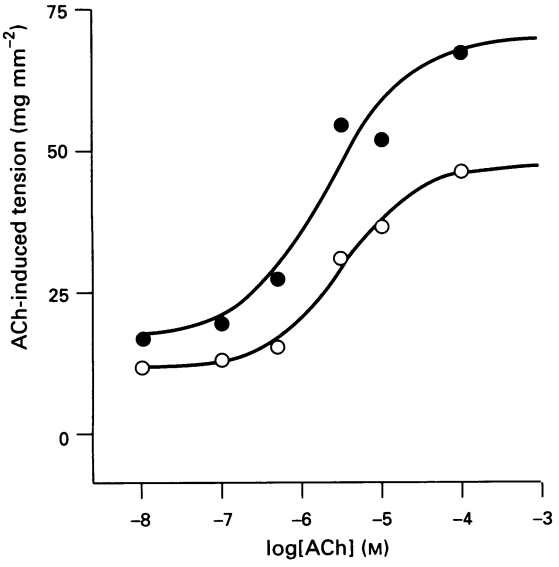

Figure 4 Concentration-response relationship for acetylcholine (ACh) in the presence and absence of 100 μM CysNO in nonpregnant guinea-pig uterus. Responses to ACh alone (●) and ACh followed by addition of 100 μM CysNO (○) are expressed as average mg tension per mm^2 cross sectional area. For clarity, the s.e.mean bars have been omitted, but the s.e.mean was less than ±15% of the averaged response. Results were analysed by two way ANOVA which indicated that tensions in tissues treated with CysNO were significantly lower than those treated with ACh alone ($P<0.05$). Data are the mean of quadruplicate determinations in tissues from four animals.

Effect of cyclic GMP analogues on agonist-evoked contractions

The effects of two relatively permeant and non-hydrolysable cyclic-GMP analogues on oxytocin-evoked contractions in nonpregnant uterine tissue were assessed by establishing non-cumulative concentration-response relationships for 8-Br-cyclic GMP (Figure 7a) and PET-cyclic GMP (Figure 7b). At higher concentrations, both analogues (50, 100 or 500 μM 8-Br-cyclic GMP; 10 or 100 μM PET-cyclic GMP) had significant relaxing effects on tissue contractions elicited by 1 nM oxytocin.

Discussion

Our study was designed to determine the ability of exogenous NO to affect guinea-pig uterine contractile activity and to assess the role of guanylyl cyclase:cyclic GMP signal transduction pathway in the observed relaxations. Nitric oxide (donated by CysNO) was determined to have no significant

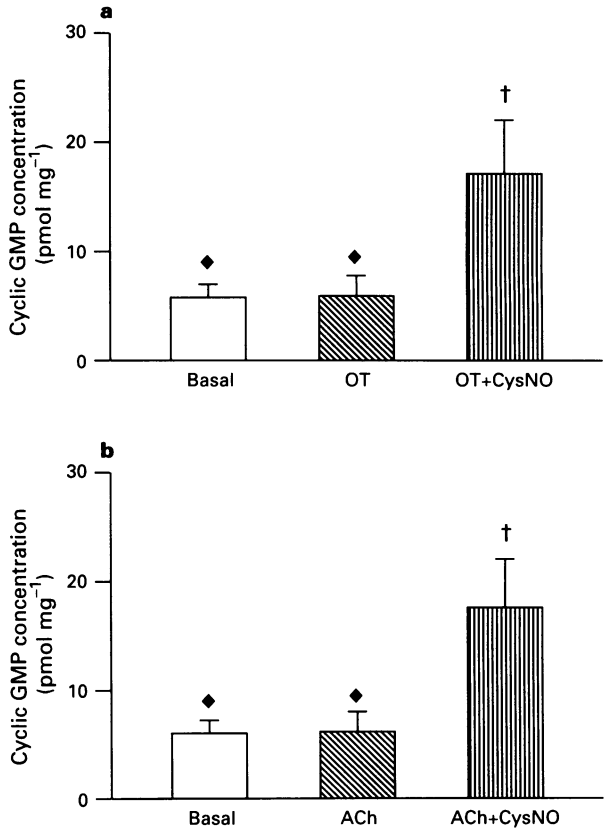

Figure 5 Simultaneous determination of the effect of CysNO on tissue tension and intracellular cyclic GMP concentrations in nonpregnant guinea-pig uterine tissues. Tissues were frozen in liquid nitrogen at precise times, while at resting tension (Basal), following addition of 1 nM oxytocin (OT) or 3 μM acetylcholine (ACh), and after addition of 100 μM CysNO to tissues pre-contracted with oxytocin (OT + CysNO) or ACh (ACh + CysNO). Intracellular cyclic GMP concentrations were measured in uterine samples taken from 11 animals in studies evaluating the effect of CysNO on oxytocin-evoked contractions (a), and 8 animals when ACh was used as the agonist (b). Cyclic GMP concentrations were determined by enzyme immunoassay and compared by Student's t test. Statistical analysis indicated that CysNO addition to either oxytocin or ACh-induced contractions was associated with significant elevations in [cyclic GMP]$_i$ ($^{\dagger}P<0.05$). Cyclic GMP levels under basal conditions did not significantly differ from those detected after the addition of either oxytocin or ACh ($P=0.948$ and $P=0.954$, respectively; ◆).

effect upon spontaneous contractile activity in either pregnant or nonpregnant guinea-pig uterine tissue strips. However, CysNO was capable of relaxing contractions elicited by two contractile agents (oxytocin and ACh) over a range of agonist concentrations (Figures 2–4). The lack of effect of NO on spontaneous contractions is noteworthy, since it suggests that a mechanism which is not involved in the regulation of spontaneous contractility is stimulated by agonists, and that this agonist-dependent mechanism of contraction is reversible by NO. Thus, the target for NO in the guinea-pig uterus is regulated by contractile agonists.

Our findings of a functional role for NO in the regulation of uterine contraction join those of others to make up a relatively small body of literature. While a number of studies have examined the possible type and distribution of nitric oxide synthase(s) present in uterine tissues obtained from the rat (Papka & McNeill, 1992; Shew *et al.*, 1993; Natuzzi *et al.*, 1993; Izumi *et al.*, 1993; Conrad *et al.*, 1993; Yallampalli *et al.*, 1993; 1994; Franchi *et al.*, 1994), guinea-pig (Weiner *et al.*, 1994) rabbit (Sladek *et al.*, 1993) and human subjects (Izumi *et al.*, 1993; Telfer *et al.*, 1995; Buhimschi *et al.*, 1995), there have been only a few reports which have evaluated the effects of exogenous NO upon uterine tissue tension (Izumi *et al.*, 1993; Yallampalli *et al.*, 1993; 1994; Buhimschi *et al.*, 1995). The effects of NO upon guinea-pig uterine contractility have not previously been reported.

Given the well-known ability of NO to stimulate soluble guanylyl cyclase in smooth muscle cells, we have evaluated the ability of NO to do so in nonpregnant guinea-pig uterine tissue. We find that CysNO-induced relaxation of agonist-evoked contractions in the guinea-pig is associated with significant increases in $[\text{cyclic GMP}]_i$. However, we also find that inhibition of cyclic GMP production by methylene blue treatment does not prevent NO-induced relaxation. That methylene blue treatment does not result in a shift in the time course of cyclic GMP generation in response to NO is confirmed by our sampling tissues at a variety of points during the course of contraction; at no point did we see significant elevations in $[\text{cyclic GMP}]_i$ when tissues were treated with the guanylyl cyclase inhibitor (Figure 6). It therefore appears that while guanylyl cyclase may indeed serve as a target for NO in the guinea-pig uterus, this activity may be ancillary to the actual mechanism of relaxation in this tissue. These findings raise the possibility that there exists in guinea-pig uterine tissues parallel pathways which mediate NO effects upon smooth muscle tension – one pathway being the often described effect of NO upon a soluble guanylyl cyclase in target cells, and the other a guanylyl cyclase: cyclic GMP-independent effect which also acts to reduce uterine tissue tension. The specific mechanisms of this latter pathway remains to be determined, but would appear to be agonist-dependent since NO has no effect on spontaneous contraction.

According to the criteria established by Sutherland and coworkers (Robison *et al.*, 1971), support for a role for cyclic GMP in NO-induced relaxation for smooth muscle would be found in the demonstration of an effect of exogenous cyclic GMP analogues. We find that stable cyclic GMP analogues (including PET-cyclic GMP, an analogue specific for the cyclic GMP-dependent protein kinase isoform present in uterine tissues, Sekhar *et al.*, 1992), when employed at sufficiently high concentrations, can produce relaxation of oxytocin-induced contractions in guinea-pig uteri. Our finding of a lack of obligatory involvement of guanylyl cyclase in NO-dependent relaxations does not rule out the possibility that another pathway exists such that NO-induced $[\text{cyclic GMP}]_i$ elevation is sufficient, but not necessary, for relaxation.

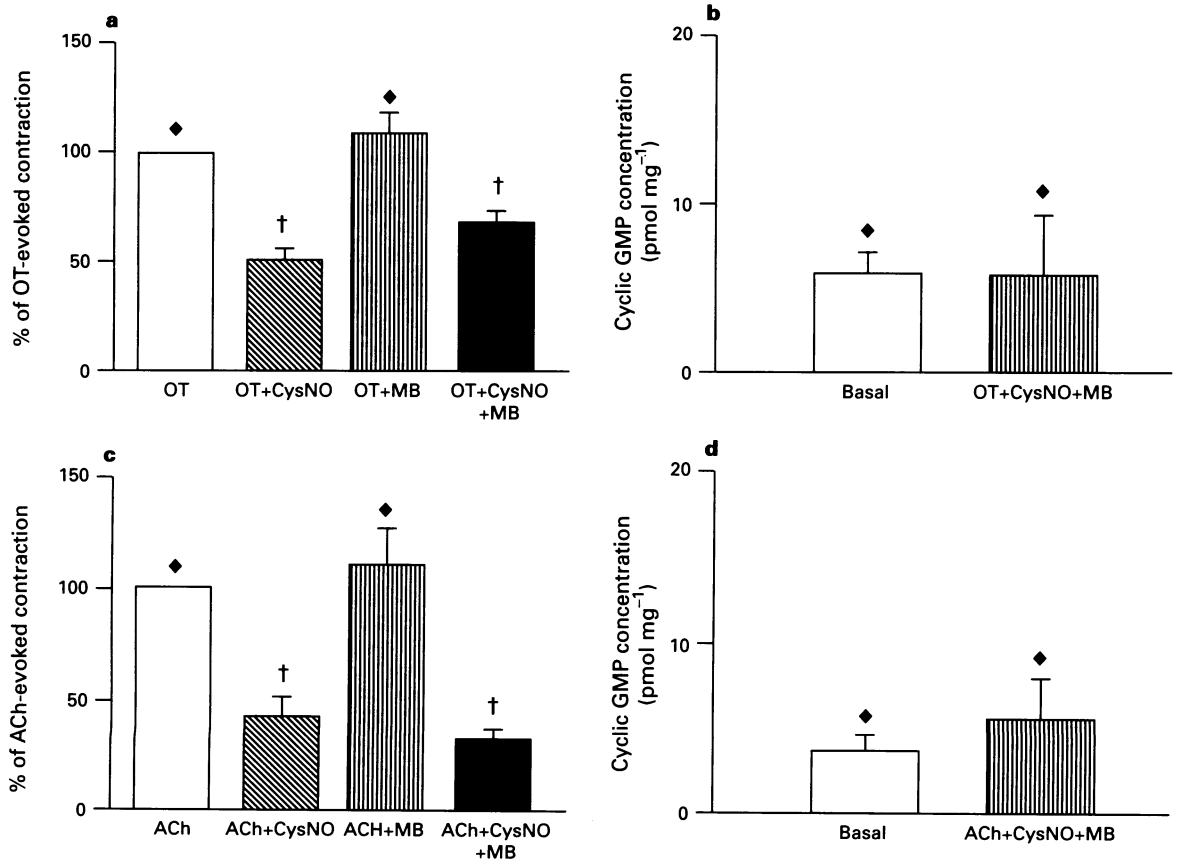

Figure 6 CysNO inhibition of agonist-evoked contractions in the presence of methylene blue. One way ANOVA followed by Student-Newman-Keuls multiple comparison test indicated that addition of 100 μM CysNO caused significant reductions in tissue tension generated by either 1 nM oxytocin (OT; a) or 3 μM acetylcholine (ACh; c) even in the presence of 10 μM methylene blue (MB) ($^{\dagger}P<0.05$). Pre-treatment of tissues with methylene blue (10 μM, 30 min) resulted in cyclic GMP concentrations which were not significantly different from basal (b and d; $P=0.965$ and $P=0.404$, respectively).

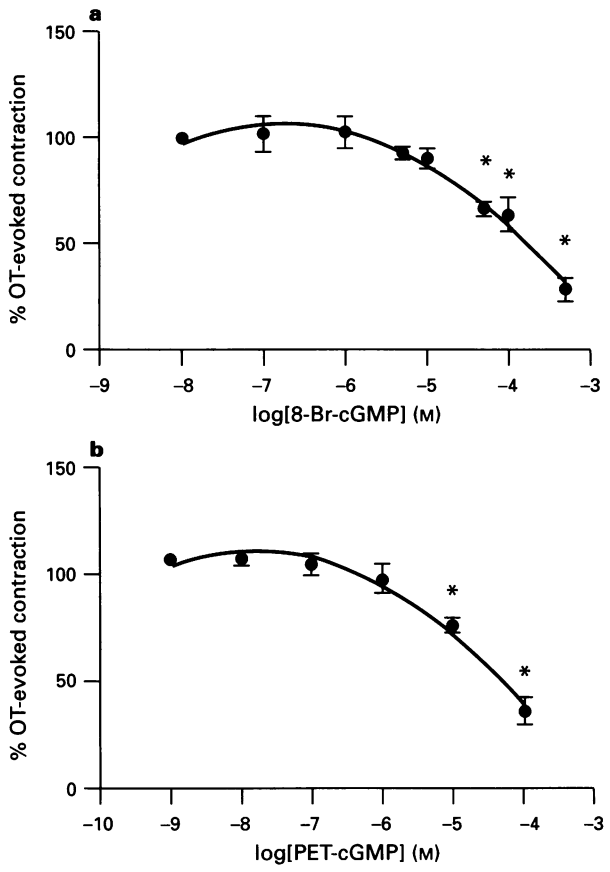

Figure 7 Effect of cyclic GMP analogues on oxytocin (OT)-evoked contractions in the nonpregnant guinea-pig uterus. Responses to noncumulative increases in 8-Br-cyclic GMP concentrations (a) or PET-cyclic GMP (b) on 1 nM oxytocin-induced contractions were quantified in 12 strips of tissue obtained from 3 nonpregnant animals. Concentration-response relationships were fit by second order polynominal regression. One-way ANOVA followed by Student-Newman-Keuls multiple comparison test indicated that tension generated in the presence of 50, 100, or 500 μM 8-Br-cyclic GMP was significantly different from that of oxytocin plus 10^{-8} M, 10^{-7} M, or 10^{-6} M 8-Br-cyclic GMP; tissue tension in the presence of 10 or 100 μM PET-cyclic GMP was significantly ($^{*}P<0.05$) different from that measured in the presence of oxytocin plus 10^{-9} M, 10^{-8} M, 10^{-7} M or 10^{-6} M PET-cyclic GMP.

The significance of our findings lies in the observation that CysNO can relax pre-contracted guinea-pig uterine tissues by an agonist-stimulated mechanism, and that this relaxation does not require guanylyl cyclase activation. Our results, in addition to those obtained by others in non-uterine tissues (Garg & Hassid, 1991; Bolotina *et al.*, 1994; Kannan & Johnson, 1995; Bialecki & Stinson-Fisher, 1995), demonstrate that NO need not exclusively utilize the guanylyl cyclase:cyclic GMP signal transduction pathway, and that while guanylyl cyclase may indeed serve as an intracellular target for NO in the guinea-pig uterus, its activity is not necessary for relaxation of agonist-evoked contractions. There are now a number of reports of cyclic GMP-independent actions of NO in a variety of (non-uterine) tissues. Bolotina *et al.* (1994) have found a guanylyl cyclase-independent relaxing effect of NO upon vascular smooth muscle, and furthermore that NO is capable of interacting directly with a calcium-dependent potassium channel in these tissues; Bialecki & Stinson-Fisher (1995) have found a similar direct effect of NO in tracheal smooth muscle cells. In a number of cases the effects of NO have been shown to be mediated by intracellular mechanisms which do not require the formation of cyclic GMP, e.g. alterations in intracellular Ca^{2+} concentrations (Garg & Hassid, 1991) or poly-ADP ribosylation (Szabó *et al.*, 1996). These findings support our suggestion that changes in intracellular cyclic GMP concentrations are not necessarily required for NO-induced relaxations in uterine smooth muscle.

Our findings support those of Diamond (1983; 1989) and Word *et al.* (1991), which have suggested that $[\text{cyclic GMP}]_i$ may not be important in the regulation of uterine smooth muscle tension. Indeed, in studies we have performed upon human uterine smooth muscle we find a similar guanylyl cyclase-independent effect of NO (unpublished observations). In the human subject however, cyclic GMP-analogues have no effect upon uterine tension (even at the highest concentrations reported here), and it appears NO affects human uterine contractile activity *via* activation of a calcium-dependent potassium channel; whether such a mechanism mediates the effects of NO in either the pregnant or nonpregnant guinea-pig uterus is unknown.

This work was supported by NIH Grants HL35416 and HD26227 and a grant from the Foundation for Research. M.E.B. was supported by a National Research Service Award (HD07448). We are indebted to Mr Kirk Korver for the development of the integration software employed in these studies. We thank Dr David Bradley for aiding in the performance of the statistical analyses.

References

AZUMA, H., ISHIKAWA, M. & SEKIZAKI, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.*, **88**, 411–415.

BIALECKI, R.A. & STINSON-FISHER, C. (1995). K_{Ca} channel antagonists reduce NO donor-mediated relaxation of vascular and tracheal smooth muscle. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **268**, L152–L159.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368**, 850–853.

BUHIMSCHI I, YALLAMPALLI, C., DONG, Y. & GARFIELD, R.E. (1995). Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. *Am. J. Obstet. Gynecol.*, **172**, 1577–1584.

CONRAD, K.P., JOFFE, G.M., KRUSZYNA, H., KRUSZYNA, R., ROCHELLE, L.G., SMITH, R.P., CHAVEZ, J.E. & MOSHER, M.D. (1993). Identification of increased nitric oxide biosynthesis during pregnancy in rats. *FASEB J.*, **7**, 566–571.

DIAMOND, J. (1983). Lack of correlation between cyclic GMP elevation and relaxation of nonvascular smooth muscle by nitroglycerin, nitroprusside, hydroxylamine and sodium azide. *J. Pharmacol. Exp. Ther.*, **225**, 422–426.

DIAMOND, J. (1989). β-Adrenoceptors, cyclic AMP, and cyclic GMP in control of uterine motility. In *Uterine Function*, pp. 249–275. New York and London: Plenum Press.

FRANCHI, A.M., CHAUD, M., RETTORI, V., SUBURO, A., MCCANN, S.M. & GIMENO, M. (1994). Role of nitric oxide in eicosanoid synthesis and uterine motility in estrogen-treated rat uteri. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 539–543.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARG, U., & HASSID, A. (1991). Nitric oxide decreases cytosolic free calcium in Balb/c 3T3 fibroblasts by a cyclic GMP-independent mechanism. *J. Biol. Chem.*, **266**, 9–12.

GIBSON, A., BABBEDGE, R., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107**, 715–721.

IZUMI, H., YALLAMPALLI, C. & GARFIELD, R.E. (1993). Gestational changes in L-arginine-induced relaxation of pregnant rat and human myometrial smooth muscle. *Am. J. Obstet. Gynecol.*, **169**, 1327–1337.

KANNAN, M.S. & JOHNSON, D.E. (1992). Nitric oxide mediates the neural nonadrenergic, noncholingeric relaxation of pig tracheal smooth muscle. *Am. J. Physiol.*, **262,** L511–L514.

KANNAN, M.S. & JOHNSON, D.E. (1995). Modulation of nitric oxide-dependent relaxation of pig tracheal smooth muscle by inhibitors of guanylyl cyclase and calcium activated potassium channels. *Life Sci.*, **56,** 2229–2238.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43,** 109–142.

MORITOKI, H., TAKEI, M., KASAI, T., MATSUMURA, Y. & ISHIDA, Y. (1979). Possible involvement of prostaglandins in the action of ATP on guinea-pig uterus. *J. Pharmacol. Exp. Ther.*, **211,** 104–111.

NATUZZI, E.S., URSELL, P.C., HARRISON, M., BUSCHER, C. & RIEMER, R.K. (1993). Nitric oxide synthase activity in the pregnant uterus decreases at parturition. *Biochem. Biophys. Res. Commun.*, **194,** 1–8.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524–526.

PAPKA, R.E. & MCNEILL, D.L. (1992). Distribution of NADPH-diaphorase-positive nerves in the uterine cervix and neurons in dorsal root and paracervical ganglia of the female rat. *Neurosci. Lett.*, **147,** 224–228.

ROBISON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1971). In *Cyclic AMP*. pp. 72–90. New York: Academic Press.

SCHIEMANN, W.P. & BUXTON, I.L.O. (1991). Adenosine A_1 receptor coupling to phosphoinositide metabolism in guinea pig myometrium. *Am. J. Physiol.*, **261,** E665–E672.

SEKHAR, K.R., HATCHETT, R.J., SHABB, J.B., WOLFE, L., FRANCIS, S.H., WELLS, J.N., JASTROFF, B., BUTT, E., CHAKINALA, M.M. & CORBIN, J.D. (1992). Relaxation of pig coronary arteries by new and potent cGMP analogs that selectively activate type Iα, compared with type Iβ, cGMP-dependent protein kinase. *Mol. Pharmacol.*, **42,** 103–108.

SHEW, R.L., PAPKA, R.E., MCNEILL, D.L. & YEE, J.A. (1993). NADPH-diaphorase-positive nerves and the role of nitric oxide in CGRP relaxation of uterine contraction. *Peptides*, **14,** 637–641.

SLADEK, S.M., REGENSTEIN, A.C., LYKINS, D. & ROBERTS, J.M. (1993). Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Obstet. Gynecol.*, **169,** 1285–1291.

SMITH, M.A., BUXTON, I.L.O. & WESTFALL, D.P. (1988). Pharmacological classification of receptors for adenyl purines in guinea pig myometrium. *J. Pharmacol. Exp. Ther.*, **247,** 1059–1063.

SMITH, M.A., SILVERSTEIN, J.L., WESTFALL, D.P. & BUXTON, I.L.O. (1989). Dissociation between adenosine receptors and adenylate cyclase in the smooth muscle of guinea pig myometrium. *Cell. Signal.*, **1,** 357–365.

SZABO, C., ZINGARELLI, B. & SALZMAN, A.L. (1996). Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous and endogenous nitric oxide and peroxynitrite. *Circ. Res.*, **78,** 1051–1063.

TELFER, J.F., LYALL, F., NORMAN, J.E. & CAMERON, I.T. (1995). Identification of nitric oxide synthase in human uterus. *Hum. Reprod.*, **10,** 19–23.

THORNBURY, K.D., HOLLYWOOD, M.A. & MCHALE, N.G. (1992). Mediation by nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in sheep. *J. Physiol.*, **451,** 133–144.

WARD, S.M., DALZIEL, H.H., BRADLEY, M.E., BUXTON, I.L.O., KEEF, K.D., WESTFALL, D.P. & SANDERS, K.M. (1992). Involvement of cyclic GMP in non-adrenergic, non-cholinergic inhibitory neurotransmission in dog proximal colon. *Br. J. Pharmacol.*, **107,** 1075–1082.

WEINER, C.P., LIZASOAIN, I., BAYLIS, S.A., KNOWLES, R.G., CHARLES, I.G. & MONCADA, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 5212–5216.

WORD, R.A., CASEY, M.L., KAMM, K.E. & STULL, J.T. (1991). Effects of cGMP on $[Ca^{2+}]_i$, myosin light chain phosphorylation, and contraction in human myometrium. *Am. J. Physiol. Cell Physiol.*, **260,** C861–C867.

YALLAMPALLI, C., GARFIELD, R.E. & BYAM-SMITH, M. (1993). Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. *Endocrinology*, **133,** 1899–1902.

YALLAMPALLI, C., IZUMI, H., BYAM-SMITH, M. & GARFIELD, R.E. (1994). An L-arginine-nitric-oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. *Am. J. Obstet. Gynecol.*, **170,** 175–185.

(Received February 20, 1996
Revised June 13, 1996
Accepted June 27, 1996)