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Evidence that spontaneous contractile activity in the rat myometrium is not inhibited by NO-mediated increases in tissue levels of cyclic GMP

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1 There is conflicting evidence in the literature concerning the role of cyclic GMP in the regulation of myometrial contractility and the importance of hormonal status on the uterine response to cyclic GMP-elevating agents. The objective of the present study was to investigate further the importance of cyclic GMP in the control of uterine contractility, by monitoring the effects of cyclic GMP-elevating agents on spontaneous contractions and cyclic GMP levels in myometrial strips from pregnant rats and from ovariectomized rats under the influence of oestrogen and/or progesterone.

2 Sodium nitroprusside (SNP) 5 mM, atrial natriuretic peptide (ANP) 100 nM, L-arginine 1 mM and 8-bromo-cyclic GMP 100 mM had no relaxant effect on the spontaneous contractions of myometria from pregnant rats or from ovariectomized rats under the influence of oestrogen or progesterone.

3 Tissue levels of cyclic GMP were significantly elevated by SNP in all treatment groups, including pregnant animals. For example, in ovariectomized, progesterone-treated rats, SNP raised cyclic GMP levels approximately 8 fold from a basal level of 2.9 ± 0.4 pmol mg^{-1} protein to 24.8 ± 4.0 pmol mg^{-1} protein. ANP increased cyclic GMP levels approximately 2 fold in all treatment groups, except in the pregnant animals. L-Arginine elevated cyclic GMP significantly only in ovariectomized, vehicle-treated myometria.

4 The activity of cyclic GMP-dependent protein kinase (PKG) was significantly increased (3 fold) in myometria exposed to SNP (5 mM). Thus, the inability of SNP to relax uterine preparations was not due to a failure of SNP-elevated cyclic GMP to activate PKG.

5 The more potent NO donor, S-nitroso-N-acetylpenicillamine (SNAP), at a concentration of 100 μM was able to inhibit spontaneous contractions significantly in myometrial preparations from both non-ovariectomized and ovariectomized rats treated with oestrogen or progesterone.

6 Tissue levels of cyclic GMP were markedly increased by SNAP at concentrations of 10, 30 and 100 μM. At 100 μM, cyclic GMP levels increased from 1.9 ± 0.2 pmol mg^{-1} protein to 74.0 ± 18.0 pmol mg^{-1} protein. However, complete or partial blockade of SNAP-induced increases in cyclic GMP levels by the soluble guanylyl cyclase inhibitor, ODQ (25 μM), had no effect on the relaxant response to SNAP. Thus, the relaxant effect of SNAP in this tissue appears to be mediated via a mechanism independent of cyclic GMP.

7 Taken as a whole, the results of the present study indicate that cyclic GMP does not play an important role in the control of contractility in the rat uterus.

Keywords: Sodium nitroprusside; atrial natriuretic peptide; L-arginine; S-nitroso-N-acetylpenicillamine; cyclic GMP; cyclic GMP-dependent protein kinase; myometrium; spontaneous contractility; pregnancy; nitric oxide

Introduction

It is generally well accepted that the vascular smooth muscle relaxing effects of drugs such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), L-arginine and atrial natriuretic peptide (ANP) are mediated via increases in tissue levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (see Waldman & Murad, 1987; Warner *et al*., 1994 for review). The nitrovasodilators, which include compounds such as SNP, SNAP and nitroglycerin, have been shown to act through a common moiety, nitric oxide (NO), which is generated by either spontaneous or enzymatic breakdown of the original agent. Nitric oxide, and not the parent compound, is believed to be responsible for the activation of soluble guanylyl cyclase, the increased production of cyclic GMP and the vascular smooth muscle relaxation induced by these agents (Schmidt *et al*., 1993). Despite good evidence for a role for the NO/cyclic GMP pathway in relaxation of vascular smooth muscle, its role in regulating uterine smooth muscle contractility remains controversial. In rat myometrial preparations, SNP has been shown to increase markedly tissue levels of cyclic GMP without relaxing the preparations (Diamond, 1983). High concentrations of SNP produced elevations of cyclic GMP as high as 6 fold in these muscles, but no relaxation or inhibition of spontaneous contractions was observed. More recently, Kuenzli *et al*. (1996) demonstrated that elevations in cyclic GMP induced by another NO donor, S-nitroso-L-cysteine, were not accompanied by inhibition of spontaneous contractions in pregnant or non-pregnant guinea-pig myometrium, although drug-induced contractions were inhibited by the compound. Blockade of cyclic GMP elevation by methylene blue did not block this relaxant effect of S-nitroso-L-cysteine, indicating that cyclic GMP elevation was not required for relaxation of drug-induced contractions. In contrast to these results, a number of recent studies have suggested that cyclic GMP may play an important role in the control of uterine motility. For example, ANP has been shown to inhibit

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completely spontaneous contractions of myometrial strips from oestrogen-treated virgin rats and from sterile horns of 10–14 day pregnant rats (Potvin & Varma, 1990). This tocolytic effect of ANP was abolished in myometria exposed to exogenous or placentally-produced progesterone and this loss of effect was concluded to be the result of decreases in particulate guanylyl cyclase activity and cyclic GMP elevation (Potvin & Varmer, 1990; Potvin *et al*., 1991).

In another series of studies, Yallampalli *et al*. (1993a,b), Izumi *et al*. (1993) and Buhimschi *et al*. (1995) demonstrated that a NO/cyclic GMP relaxation system is present in human and rat pregnant uteri, and that it plays an important role in inhibiting spontaneous contractions. These studies also concluded that the NO/cyclic GMP pathway may be responsible for maintaining a quiescent uterus during pregnancy and that a decrease in uterine responsiveness to the relaxant effects of NO at term may lead to the initiation of labour. The latter conclusion is in direct contrast to the results of Potvin & Varma (1990) and Potvin *et al*. (1991); they showed that progesterone administration and/or late stage pregnancy were accompanied by decreases in particulate guanylyl cyclase activity and cyclic GMP elevation.

Thus, there is conflicting evidence in the literature concerning the role of cyclic GMP in the control of uterine motility and the effects of changes in hormonal status on this process. The objective of the present study was to investigate further the importance of cyclic GMP in the uterus, by monitoring the effects of cyclic GMP-elevating agents on spontaneous contractions and cyclic GMP levels in myometrial strips from pregnant rats and from ovariectomized rats under the influence of oestrogen and/or progesterone. The effect of a cyclic GMP analogue, 8-bromo-cyclic GMP, and a guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), were also studied. Elevation of cyclic GMP was not well correlated with the inhibition of contractions in any of these studies. Therefore, our results do not provide support for the putative role of cyclic GMP as a mediator of uterine relaxation, at least in the rat uterus.

Methods

Source, preparation and tension measurements of isolated tissues

Myometrial strips were obtained from female Sprague Dawley rats weighing 250–300 g for all experiments. Groups A–D were ovariectomized under halothane anaesthesia, accompanied by 80% N_2O and 20% O_2. A small incision was made on the hind quarter directly above the ovary, the ovary and ovarian artery were tied off and the ovary was removed. Wound clips were used to seal the openings and rats were given 7 days to recover before subcutaneous hormone injections were initiated. Group A received one injection of 200 μl of vehicle (peanut oil) 48 h before death. Group B received one injection of 100 μg β-oestradiol 48 h before death. Group C received one injection a day for three days of progesterone (3 mg kg^{-1}). Group C animals were killed 24 h after the third injection. Group D received the same initial treatment as group B, and after the first 48 h each rat received one injection a day for three days of progesterone (3 mg kg^{-1}). Timed pregnant rats were received on the 17th day of gestation and killed on the 18th day. All animals were killed in a CO_2 inhalation chamber. Uteri were removed, trimmed free of loosely adhering connective tissue and fat, and cut longitudinally along the vascularized side to expose the endometrium. Next the endometrium and circular smooth muscle were peeled away from the longitudinal smooth muscle of the myometrium. Myometrial strips of approximately 6 mm by 3 mm were suspended in isolated organ baths at 37°C with a preload of 0.5 g in a physiological salt solution of the following composition (mM): KCl 4.7, NaCl 118, $MgSO_4$ 1.19, KH_2PO_4 1.19, $CaCl_2$ 2.5, glucose 11 and $NaHCO_3$ 25. Tissue baths were aerated with 95% O_2, 5% CO_2 which maintained a pH of approximately 7.4. Isometric tension was recorded with force displacement transducers (Grass Model FT03C) which were connected to a Grass Model 7D polygraph recorder. Tissues were given 20 min to equilibrate and achieve steady state contractions before the addition of agents. At predetermined times following the addition of drug, tissues were frozen with liquid nitrogen-cooled clamps and stored at −80°C until assessment of enzyme activity. % inhibition of spontaneous contractions was calculated by measuring the change in the amplitudes of the contractions following the addition of the drug.

Cyclic GMP estimation

Frozen myometrial strips (10–15 mg) were placed in liquid nitrogen-cooled teflon capsules (1 ml capacity) (Hansen Industries Ltd., Richmond, B.C., Canada) with a chilled metal pestle and pulverized in a ProMix, Dentsply dental amalgam mixer (20 s at high speed). Next, 0.75 ml of ice-cold trichloroacetic acid (TCA) (6% w/v) was added to the capsule and the tissue was homogenized for another 20 s at high speed. The homogenate was removed and the capsule was washed with an additional 0.25 ml of TCA. The total homogenate (1 ml) was then centrifuged at 2000 *g* for 15 min at 4°C. The supernatant was removed and the TCA was extracted with 4 washes of ice-cold water-saturated ether (5 ml per wash). Cyclic GMP levels were measured by use of a commercially available scintillation proximity radioimmunoassay kit (Amersham). Tissue cyclic GMP levels were calculated as pmol cyclic GMP mg^{-1} protein. The remaining pellet was stored at −80°C for protein estimation.

Cyclic GMP-dependent protein kinase assay

Frozen myometrial strips weighing approximately 40 mg were pulverized in teflon capsules similar to the method used in the cyclic GMP extraction above, except a homogenization buffer was substituted for TCA. The buffer contained the following (mM): HEPES 10, EDTA 1, DTT 10, IBMX 1, KCl 125, benzamidine 1 plus leupeptin 10 μg ml^{-1} and pepstatin 10 μg ml^{-1}. The homogenate was centrifuged at 30,000 *g* for 5 min and the supernatant was assayed for soluble protein kinase G (PKG) activity. A phosphocellulose paper assay was used to measure the phosphotransferase activity of PKG by utilizing a method modified from that described by Jiang *et al*. (1992). PKG activity was determined by measuring the transfer of the [γ-^{32}P]-phosphoryl group of ATP to BPDEtide (RKISASEFDRPLR), which is relatively specific substrate for PKG (Colbran *et al*., 1992). The assay was carried out in a total volume of 70 μl containing 150 μM BPDEtide, 10 mM HEPES, 35 mM β-glycerophosphate, 4 mM magnesium acetate, 200 μM [γ-^{32}P]-ATP (2.5 μCi per tube), 5 μM synthetic PKA inhibitor (PKI), 0.5 mM EGTA, and in the absence or presence of 5 μM cyclic GMP. The reaction was initiated by adding 20 μl of the sample supernatant. The reaction was allowed to proceed for 10 min at 4°C and was stopped by spotting 50 μl of the reaction mixture onto 2 cm × 2 cm squares of phosphocellulose paper (Whatman P81). The paper was then washed 4 times in 0.5% *o*-phosphoric acid for 10 min each. The papers were allowed to dry

and then transferred to scintillation vials containing 2.5 ml liquid scintillant. Radioactivity was counted in a Beckman LS 6000TA liquid scintillation counter. PKG activity was expressed as pmol phosphate incorporated into substrate min^{-1} mg^{-1} protein. PKG activation was assessed by calculating the activity ratio, which is a measure of the PKG activity in the absence of exogenously added cyclic GMP (endogenous cyclic GMP only) divided by the PKG activity in the presence of enough exogenous cyclic GMP (5 μM) to activate maximally the kinase.

Protein estimation

Frozen pellets from the cyclic GMP extraction were assayed for protein content by the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1981). Pellets were resuspended in 1 ml of buffer containing (mM): Na_2CO_3 1.9, NaOH 600, sodium tartrate 0.07, sodium lauryl sulphate 0.35 and vortexed to distribute evenly the protein contents. Thirty microlitres of this suspension was used in the protein determination. Protein in the PKG soluble fraction was estimated by use of a commercially available assay (Bio-Rad), based on the method of Bradford (1976).

Statistical analysis

% inhibition of spontaneous contractions was calculated by measuring the change in the amplitude of the contractions after drug or vehicle addition. In Figure 1 and Table 1, each drug-treated tissue had its own respective control which allowed treatments to be compared to controls by use of a paired t test. PKG activity ratios in tissues treated with SNP were compared to untreated control ratios by use of Student's t test. Effects of SNAP, ODQ and ODQ with SNAP on myometrial contractility and cyclic GMP levels were compared to controls (vehicle treated) at the same time by a 1-way ANOVA followed by Student Newman Keuls test. The effect of charybdotoxin (CTX) on the SNAP-induced relaxation was compared by Student's t test. All results were considered significant when $P<0.05$. SigmaStat for Windows Version 1.0 (Jandel Scientific, San Mateo, California, U.S.A.) software was used for statistical analyses. All expressed results are presented as mean $\pm$ s.e.mean.

Materials

BIOTRAK cyclic GMP scintillation proximity assay kits were obtained from Amersham International (Little Chalfont, Buckinghamshire, U.K.). PKG substrate (BPDEtide) and ANP (rat, 1-28 amino acids) were obtained from Bachem California (Torrence, CA, U.S.A.). [γ-^{32}P]-adenosine 5′-triphosphate was obtained from DuPont NEN Research Products (Boston, MA, U.S.A.). Scintiverse scintillation fluid was obtained from Fischer Scientific Co. (Fair Lawn, NJ, U.S.A.). 1H-[1,2,4]oxadiazolo[4,3-a]quinonxalin-1-one (ODQ) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Effect of hormonal status and SNP, ANP, L-arginine and 8-bromo-cyclic GMP on spontaneous myometrial contractility

Spontaneous contractile activity varied slightly among the five groups of animals tested. Figure 1 illustrates representative tracings from each group. In the untreated ovariectomized rats (group A), spontaneous contractile activity lasted approximately 60 min and was characterized by rapid, small amplitude contractions. In the oestrogen-treated, ovariectomized animals (group B), the spontaneous activity was inconsistent and often tapered off within the first 20 min of the experiment. When contractions did occur they were of greater amplitude than those observed in group A. Spontaneous contractile activity was similar in the ovariectomized progesterone-treated (group C) and oestrogen-primed progesterone-treated (group D) animals. In both groups there was a high degree of spontaneous activity with strong and regular contractions lasting up to 60 min. Treatments with progesterone alone and with progesterone following oestrogen-priming were performed, because it has previously been shown that pretreatment with oestrogen increases the number of available receptors for progesterone (Toft & O'Malley, 1972). Thus, group D may be under a stronger influence of progesterone than group C. In the 18-day pregnant rats (group E), contractions were markedly reduced in amplitude compared to groups B–D and spontaneous activity was often irregular. Despite these differences in the spontaneous contractile activity of the myometrial tissues from the groups above, they were consistent in their lack of response to the cyclic GMP-elevating agents and cyclic GMP analogue used (Figure 1).

The effects of SNP (5 mM), ANP (100 nM), L-arginine (1 mM) and 8-bromo-cyclic GMP (100 μM) on spontaneous myometrial activity are shown in Figure 1. In all five groups (A–E) there was no measurable effect observed with any of these agents. Although Figure 1 shows only the first five minutes following drug addition, in several instances, tissues were allowed to remain in the presence of the agent for longer periods but no detectable change was ever observed. In some strips from the oestrogen-treated group (group B), 8-bromo-cyclic GMP appeared to exert a relaxing effect as shown in Figure 1. However, this response was not seen consistently and in many cases the control muscles in this group exhibited similar diminished spontaneous contractions during the same period of time. No responses to 8-bromo-cyclic GMP were observed in any of the other groups of animals. In preliminary experiments, we could not demonstrate any relaxation of drug-induced (oxytocin-induced) contractions by any of these agents (data not shown).

Effect of SNP, ANP and L-arginine on cyclic GMP levels in the rat myometrium

Cyclic GMP levels were determined in experiments identical to those illustrated in Figure 1 and the results are presented in Table 1. In a comparison of the control levels of cyclic GMP between the different hormone treatment groups it was found that basal levels of cyclic GMP were significantly lower in the ovariectomized, untreated group than in all other groups. None of the remaining groups were found to have significantly different control cyclic GMP levels. SNP produced a significant elevation of cyclic GMP above control in all five treatment groups (A–E). The magnitude of the cyclic GMP elevation varied between groups, ranging from 3.4 fold in the pregnant group to 8.8 fold in the ovariectomized progesterone-treated group. However, as shown in Figure 1, SNP did not inhibit spontaneous contractions in any of the groups. ANP significantly increased cyclic GMP levels in groups A, B, C and D, while L-arginine elevated cyclic GMP significantly only in group A. As was the case with SNP, no relaxant effects were seen with

these agents in any of the groups. Thus, there did not appear to be a correlation between cyclic GMP elevation and inhibition of tension development in rat myometria.

Effect of SNP on PKG activity in the rat myometrium

The possibility was considered that the lack of relaxation seen with SNP-treated tissues, in spite of a marked elevation of cyclic GMP, was due to a failure of cyclic GMP to activate cyclic GMP-dependent protein kinase (PKG). In order to test this possibility, the effect of SNP on PKG activity ratios was measured in myometrial preparations from ovariectomized, oestrogen-treated animals. Since cyclic GMP levels were significantly increased by SNP in all hormonal treatment groups, and since no relaxation was seen in any of the groups, only one representative group was used in the PKG study. Myometrial strips were suspended in tissue baths, allowed to equilibrate and either left untreated or exposed to 5 mM SNP for 5 min. As shown in Table 2, SNP produced a significant increase in the PKG activity ratio in rat myometrium. No

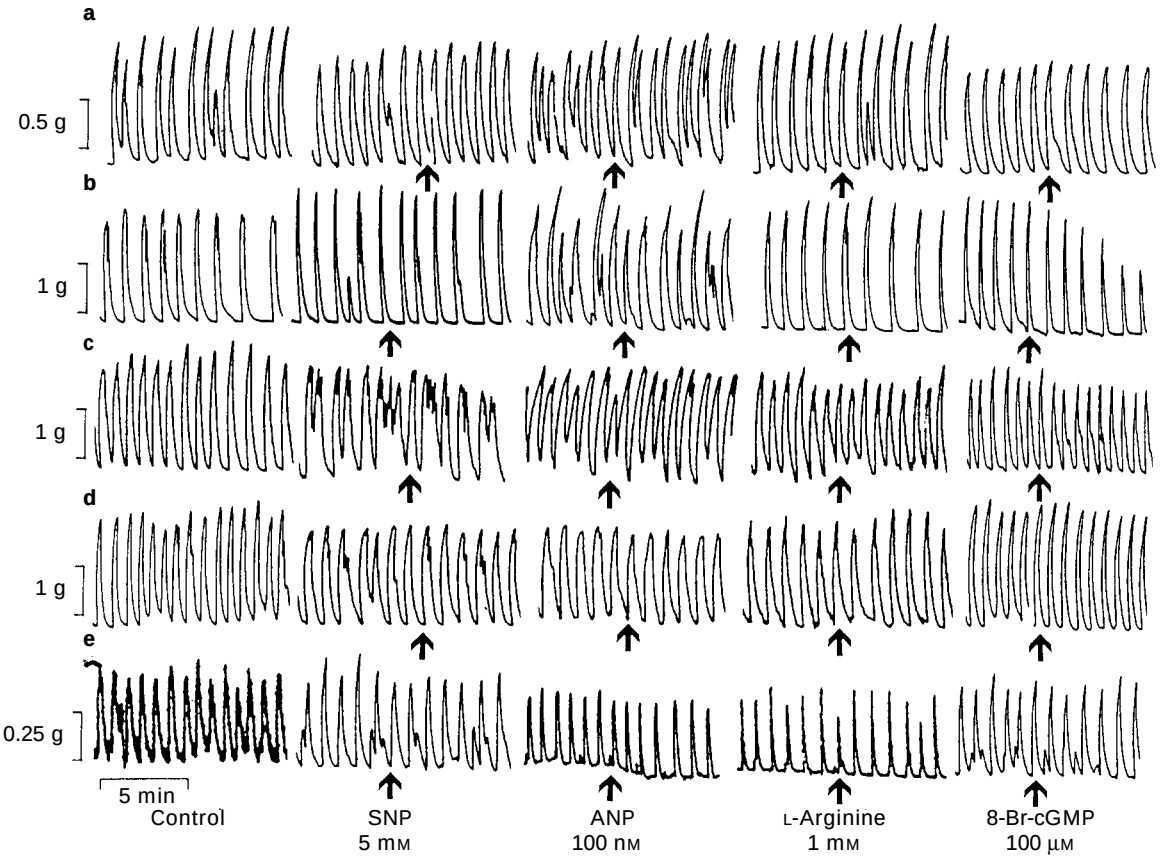

Figure 1 Representative tracings illustrating the effects of SNP (5 mM), ANP (100 nM), L-arginine (1 mM) and 8-bromo-cyclic GMP (100 μM) on spontaneous myometrial contractility from (a) ovariectomized, vehicle-treated (peanut oil), (b) ovariectomized, oestrogen-treated, (c) ovariectomized, progesterone-treated, (d) ovariectomized, oestrogen-primed, progesterone-treated, and (e) 18-day timed pregnant rats. Addition of drug is indicated by (↑) and results are typical of 6 experiments in groups A–D (a–d, respectively) and 4 experiments in group E (e).

Table 1 Effect of hormonal status, and SNP, ANF and L-arginine on cyclic GMP levels in spontaneously-contracting rat myometrium

	Ovariectomized, untreated (*A*)	*Ovariectomized, oestrogen-treated* (*B*)	*Ovariectomized, progesterone-treated* (*C*)	*Ovariectomized, oestrogen-primed, progesterone-treated* (*D*)	*Pregnant* (*E*)
Control	0.7±0.3	2.3±0.4	2.9±0.4	2.3±0.4	2.2±0.6
SNP (5 mM)	4.6±1.2*	11.5±1.1*	24.8±4.0*	16.2±1.3*	7.6±1.3*
Control	0.6±0.2	3.1±0.2	2.9±0.7	2.3±0.4	2.2±0.4
ANF (100 nM)	1.2±0.2*	4.6±0.8*	4.8±0.6*	3.7±0.4*	2.7±0.4
Control	1.2±0.4	4.7±0.8	1.6±0.5	2.9±0.3	2.2±0.4
L-Arginine (1 mM)	3.0±0.5*	3.6±0.5	2.1±0.4	2.8±1.0	2.1±0.6

All values were calculated as pmol cyclic GMP mg^{-1} protein and results are expressed as mean±s.e.mean for $n=5$ or 6. Treated tissues were compared to their respective controls by paired Student's t test and basal levels of cyclic GMP between the different hormone groups were compared by one-way ANOVA on ranks followed by Dunn's multiple comparison test. Group A control levels of cyclic GMP differed significantly from control levels in all other treatment groups ($P<0.05$). Asterisks indicate a significant drug-induced elevation of cyclic GMP compared to the corresponding control ($P<0.05$).

significant change in total PKG activity was observed. Thus, the increase in the activity ratio demonstrated with SNP was due to activation of specific, cyclic GMP-dependent protein kinase by increases in endogenous cyclic GMP, and not to an increase in non-specific (cyclic nucleotide-independent) protein kinase activity.

Effect of SNAP on spontaneous myometrial contractility

S-nitroso-N-acetylpenicillamine (SNAP) has been shown to be a better NO donor than SNP (Marks *et al*., 1995). The effects of this compound on myometrial contractions were studied in a separate set of experiments. In a preliminary study, SNAP (100 μM) induced consistent inhibitions of spontaneous contractions in uteri from hormonal treatment groups B, C and D. Since there was no difference in the responses with the different hormonal treatments, subsequent experiments were carried out in uteri from non-ovariectomized, oestrogen-primed progesterone-treated animals which exhibited a high degree of strong, regular spontaneous activity. Figure 2 illustrates representative tracings from these experiments. As shown in Figure 2c, 100 μM SNAP produced a consistent inhibition of spontaneous contractions (average of 5 experiments: $59.0 \pm 9.7\%$ inhibition). DMSO, the vehicle used for SNAP, had no effect (Figure 2a). Since our earlier experiments had shown that cyclic GMP elevation by SNP was not accompanied by relaxation, we used ODQ, a specific inhibitor of guanylyl cyclase (Schrammel *et al*., 1996), to determine the importance of cyclic GMP in the relaxant responses to SNAP. Figure 2b illustrates an ODQ control preparation treated with DMSO and (d) illustrates the effect of SNAP (100 μM) in the presence of ODQ. ODQ had no effect on the relaxant response produced by SNAP (average inhibition $67.6 \pm 6.7\%$). In many instances ODQ appeared to potentiate inhibition of spontaneous contractions induced by 100 μM SNAP, but this was not statistically significant. In order to demonstrate further the lack of effect of ODQ on SNAP-induced inhibition of spontaneous contractions in rat myometrium, cumulative dose-response curves to SNAP were determined in the absence and presence of ODQ. As shown in Figure 3, 25 μM ODQ produced no measurable shift in the relaxant responses induced by SNAP over a concentration range of 1 μM to 440 μM.

Table 2 Effect of sodium nitroprusside on cyclic GMP-dependent protein kinase activity in spontaneously-contracting rat myometrium

	PKG activity (pmol PO_4 min^{-1} mg^{-1})		
	(−cGMP)	*(+cGMP)*	*Activity ratio*
Control	3.9 ± 0.1	41.5 ± 0.6	0.09 ± 0.002
SNP (5 mM)	10.5 ± 0.3*	38.3 ± 2.1	0.27 ± 0.02*

Values are mean $\pm$ s.e.mean for $n=3$. Control and SNP-treated tissues were compared by Student's t test. Asterisks indicate a significant difference from control ($P<0.05$).

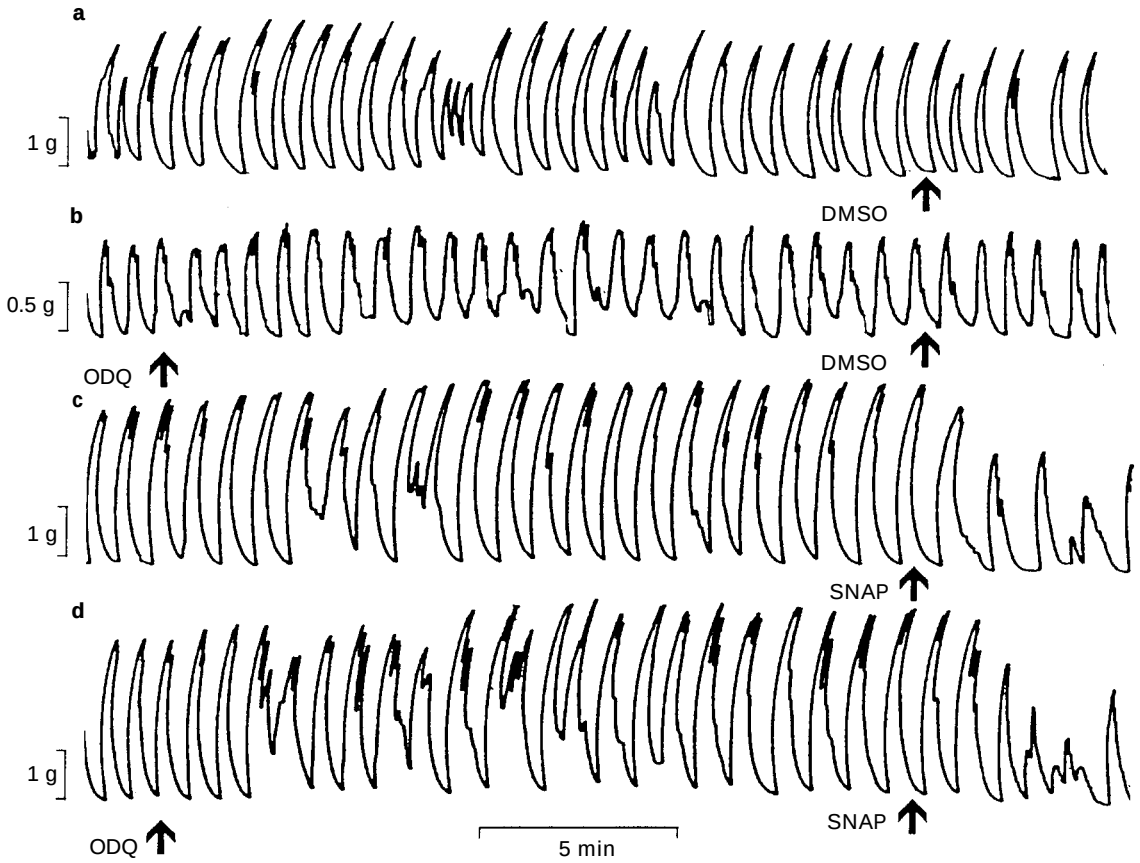

Figure 2 Representative tracings illustrating the effects of SNAP, in the presence and absence of ODQ, on spontaneous myometrial activity from oestrogen-primed, progesterone-treated rats. (a) Vehicle (DMSO), (b) ODQ (25 μM) for 20 min + vehicle, (c) SNAP (100 μM), (d) ODQ (25 μM) for 20 min + SNAP (100 μM). Drug additions are indicated by (↑) and results are typical of 5 experiments.

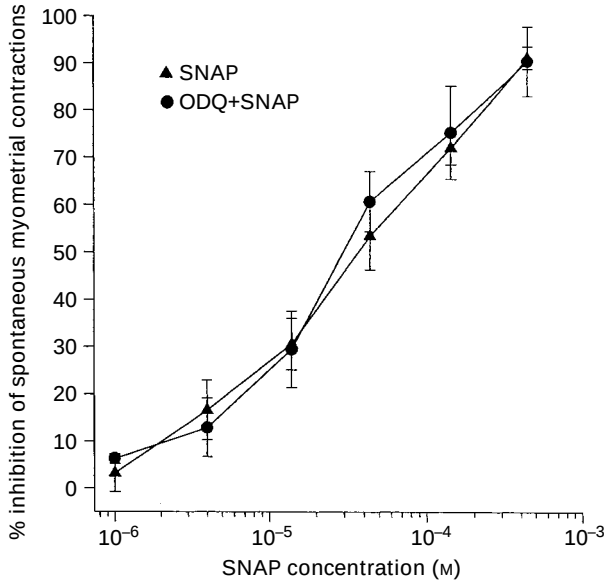

Figure 3 Effects of SNAP on spontaneous contractions from oestrogen-primed, progesterone-treated rats in the absence and presence of 25 μM ODQ. % inhibition of spontaneous contractions was determined by measuring the change in the amplitudes of the contractions over a five minute period following the addition of drug. Data are expressed as mean, with vertical lines showing s.e.mean, for 6–7 myometrial strips from 3 different rats.

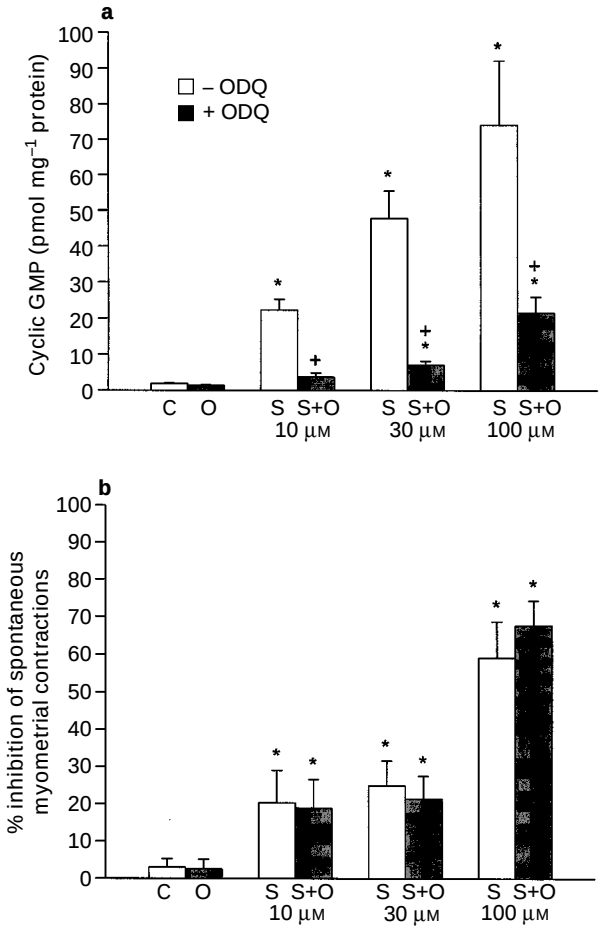

Figure 4 Effect of ODQ on SNAP-induced elevation of cyclic GMP and inhibition of spontaneous contractions in myometrial strips from oestrogen-primed, progesterone-treated rats. (a) The effects of 10, 30 and 100 μM SNAP (S) on cyclic GMP levels in the absence and presence of ODQ (O; 25 μM). ODQ had no significant effect on cyclic GMP levels in untreated control muscles (C). (b) The contractility for the same muscles used in the cyclic GMP studies shown in (a). % inhibition of spontaneous contractions was determined by measuring the change in the amplitudes of the contractions following the addition of drug. Results are expressed as mean $\pm$ s.e.mean for 5 experiments. Asterisks indicate a significant difference from control ($P<0.05$) and plus signs indicate a significant difference from SNAP alone ($P<0.05$). All comparisons were made by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test.

Effect of SNAP and ODQ on cyclic GMP levels in spontaneously-contracting rat myometrium

The effects of 10, 30 and 100 μM SNAP on cyclic GMP levels in myometrial strips from oestrogen-primed, progesterone-treated rats are shown in Figure 4a. All three concentrations of SNAP generated a significant increase in the level of cyclic GMP. In addition, each concentration caused a significant inhibition of spontaneous myometrial contractions (Figure 4b). The effects of SNAP at 10, 30 and 100 μM were also measured in the presence of 25 μM ODQ. At each concentration of SNAP, ODQ produced a significant reduction in the ability of SNAP to increase cyclic GMP levels. However, this decrease in cyclic GMP elevation had no effect on the relaxant response elicited by SNAP. In the experiments with 10 μM SNAP, the cyclic GMP elevation was reduced such that cyclic GMP levels were not significantly different from control values, yet an $18.7\pm7.7\%$ inhibition of spontaneous contractions still occurred. With 30 and 100 μM SNAP, ODQ was unable to produce a complete blockade of the cyclic GMP elevation, although the levels were markedly reduced. The cyclic GMP levels obtained with these concentrations of SNAP in the presence of ODQ were similar to the levels generated by 5 mM SNP which had no relaxant effect, as shown in Figure 1 and Table 1. Once again, there appears to be a poor correlation between increases in cyclic GMP and decreases in myometrial tension development. An interesting aspect of the SNAP relaxant response was the finding that the inhibition of spontaneous contractions lasted for less than 10 min and a full recovery was common even in the continued presence of SNAP. To investigate the role of cyclic GMP in the transient nature of this response, cyclic GMP levels were measured at 12 min following the addition of 100 μM SNAP, with and without pretreatment with ODQ (25 μM). The cyclic GMP levels found in these experiments (in pmol mg^{-1} protein) were as follows ($n=4$): control, 2.4 ± 0.2; ODQ control, 2.0 ± 0.4; SNAP, 17.0 ± 4.0; ODQ + SNAP, 3.0 ± 0.8. Although spontaneous contractions had completely recovered within 12 min, cyclic GMP was still significantly elevated in the SNAP-treated tissues. In the ODQ + SNAP treated muscles, tissue levels of cyclic GMP were not significantly different from control levels. The ability of myometrial strips to recover completely in the presence of elevated cyclic GMP, and the lack of effect of ODQ on rate of recovery, provides further evidence against a role for cyclic GMP in the relaxant effects induced by SNAP.

To ensure that the relaxant effects of SNAP were indeed due to a NO-mediated event, the compound acetyl penicillamine was used. At concentrations of 100 μM and 200 μM, this derivative of SNAP had no effect on spontaneous myometrial contractions (data not shown). Therefore, it would appear that the S-nitroso, or NO donating portion, of SNAP is required for its relaxant effect.

Further investigation of the relaxant effects induced by SNAP

Recently, several studies have suggested that NO may cause vascular smooth muscle relaxation by a direct effect on calcium-activated potassium channels in the sarcolemma (Bolotina *et al*., 1994; Dong *et al*., 1997). Since SNAP appears to relax the uterus independently of cyclic GMP, we investigated the possibility that NO may be acting directly on such a potassium channel to produce inhibition of spontaneous contractions in our preparations. Myometrial strips were relaxed with 100 μM SNAP, washed twice, allowed to re-equilibrate and pretreated with 50 nM charybdotoxin (CTX), a specific inhibitor of calcium-activated potassium channels. After a 20 min incubation with the inhibitor, SNAP was once again added to the bath. CTX produced no measurable decrease in the inhibition of spontaneous contractions induced by SNAP (relaxation before CTX was $41.8 \pm 7.92\%$, relaxation after CTX was $55.7 \pm 8.65\%$, $n=4$). In preliminary experiments, concentrations of CTX as high as 200 nM had no measurable effect on the relaxant responses to SNAP. Therefore, direct actions of NO on calcium-activated potassium channels cannot explain the relaxant effects of SNAP in the rat myometrium.

Nitric oxide has also been suggested to mediate some of its effects through changes in pH (Wyeth *et al*., 1996). Therefore, a preliminary study was carried out to investigate whether SNAP could alter pH levels following its addition to the tissue bath. In this study, 100 μM SNAP was added to a bath containing a myometrial strip and the pH was monitored with a pH probe. The pH did not vary by more than 0.02 pH units over a ten minute period following the addition of the drug (data not shown).

Effect of SNAP on myometrial contractility and cyclic GMP levels in 18-day timed pregnant rats

In a preliminary experiment with pregnant myometrial strips it was found that SNAP (100 μM) did not cause inhibition of spontaneous contractions (data not shown). This is in contrast to the results in non-pregnant animals shown in Figure 2 and 3 above. Cyclic GMP levels were found to be significantly elevated by SNAP in the pregnant myometria (SNAP-treated tissues, 12.6 ± 1.2 pmol cyclic GMP mg^{-1} protein; control tissues, 2.2 ± 0.5 ($n=4$)). However, the fold increase was much lower than that previously observed in the non-pregnant animals (Figure 4a). In the pregnant myometrial preparations, SNAP increased cyclic GMP levels by only 5.7 fold, whereas in the non-pregnant, oestrogen-primed progesterone-treated rats, tissue levels of cyclic GMP increased 38.6 fold above control values. Thus, the hormonal state of the uterus during pregnancy appears to affect the generation of cyclic GMP by SNAP, but the significance of this effect is not yet understood.

Discussion

As noted in the introduction, it is generally well accepted that the vascular smooth muscle relaxing effects of a variety of agents including SNP, ANP and L-arginine are mediated via elevations of cyclic GMP and activation of PKG (see Waldman & Murad, 1987; Warner *et al*., 1994 for review). However, the role of cyclic GMP in regulation of uterine contractility remains controversial and several recent studies have presented conflicting evidence concerning this point. The main objective of the present study was to repeat some of these recent experiments in an attempt to resolve some of the apparent inconsistencies. In many of the present experiments, an effort was made to duplicate the experimental conditions and protocols used in the original studies.

In the studies of Potvin and Varma (1990) and Potvin *et al*. (1991) it was found that the particulate guanylyl cyclase activator, ANP, relaxed myometrial strips from ovariectomized, oestrogen-treated rats and that this relaxant effect was abolished by progesterone treatment. It was suggested that the relaxant effect was due to cyclic GMP elevation and that the absence of relaxation in the progesterone-treated muscles was due to the failure of ANP to elevate cyclic GMP under conditions of progesterone dominance. In the present study, we were unable to demonstrate any relaxant effect of ANP in myometrial strips from ovariectomized oestrogen-treated rats or from any of the other hormonal treatment groups studied. Cyclic GMP levels were significantly elevated by ANP in our studies, but there was no significant difference in the degree of elevation seen in the different treatment groups, irrespective of whether they were under the influence of oestrogen or progesterone. At the present time, we have no explanation for the difference between our results and those of Potvin and Varma. Although obtained from different sources, the same type of ANP (rat, amino acids 1–28) was used in both studies. One difference in the experimental approach was that in the studies of Potvin and Varma, oestrogen and progesterone were dissolved in sesame oil and injected intraperitoneally, whereas in the present study these hormones were dissolved in peanut oil and injected subcutaneously. In preliminary experiments, no differences were found in responses to drugs in animals treated by either method. Therefore, subcutaneous hormone injections were utilized in all subsequent experiments. With subcutaneous injections in peanut oil, the hormones will gradually enter the blood stream and be delivered to the uterus continuously over a period of several days. This approach is the same as that used previously to demonstrate the effects of ovarian hormones on the response of rat myometrium to α- and β-adrenoceptor agonists, and marked differences were found between hormonal treatment groups in that study (Diamond & Brody, 1966). In the present experiments, oestrogen administration had the expected dramatic effects on the size and vascularity of the uterus in ovariectomized animals, indicating that they were under the influence of oestrogen. It is interesting to note that Potvin and Varma, while concluding that ANP could relax uterine preparations by elevating cyclic GMP, also found that a soluble guanylyl cyclase activator, SNP, did not relax any of their myometrial preparations. This agrees with our results, where concentrations of SNP as high as 5 mM, which elevated myometrial cyclic GMP levels by as much as 9 fold, had no demonstrable effect on spontaneous contractions in any of our preparations.

In contrast to our results, and those of Potvin and Varma, Yallampalli *et al*. (1993a,b) and Izumi *et al*. (1993) showed that several cyclic GMP-elevating agents, including SNP, were able to inhibit spontaneous contractions of myometrial strips from 18-day pregnant rats. Although no cyclic GMP measurements were made in these studies, it was concluded that a NO/cyclic GMP pathway is present in the uterus and is responsible for regulation of uterine contractility. As shown by Yallampalli *et al*. (1993a,b), spontaneous contractions of 18-day pregnant myometria were not inhibited by 5 mM SNP until 15–20 min of exposure, at which time the contractions ceased abruptly. Since cyclic GMP levels are maximally elevated by SNP in smooth muscle preparations in less than one minute (see e.g. Janis & Diamond, 1979), this does not appear to be a cyclic GMP-mediated process. It is possible that long exposure to

high concentrations of SNP might have a non-specific toxic effect due, for example, to release of cyanide on breakdown of SNP. Surprisingly, L-arginine, a substrate for nitric oxide synthase and therefore a precursor of nitric oxide, was shown in the same study to cause an instantaneous and complete inhibition of spontaneous contractions (Yallampalli *et al*., 1993a,b). Since no cyclic GMP levels were obtained in these studies, it is difficult to decide what role the cyclic nucleotide plays in this process. As noted above, L-arginine, at the same concentration as that used by Yallampalli (1 mM), produced little or no cyclic GMP elevation and failed to relax any of our myometrial preparations. Thus, our results fail to provide support for a role for cyclic GMP in the control of uterine motility.

In the present experiments, the possibility was considered that the lack of relaxation seen in SNP-treated tissues, in spite of a marked elevation of cyclic GMP, was due to a failure of cyclic GMP to activate PKG. As shown in the results, SNP produced a significant elevation in PKG activity compared to a non-treated control. Therefore, a failure to activate PKG cannot be the cause of the inability of cyclic GMP-elevating agents, such as SNP, to inhibit spontaneous contractions of rat myometrium. To our knowledge these results are the first demonstration of activation of PKG, by cyclic GMP-elevating agents, in the rat myometrium. Since elevation of cyclic GMP and activation of PKG by SNP were not accompanied by inhibition of spontaneous contractions in these studies, the results provide strong evidence that cyclic GMP does not play a direct role in the regulation of myometrial contractility.

In the present study, we also investigated the effects of SNAP, which has been shown to be a better NO donor than SNP (Marks *et al*., 1995). Unlike SNP, SNAP produced significant inhibition of spontaneous contractions in myometrial strips from oestrogen-primed, progesterone-treated rats. Initially, it appeared that SNAP might be capable of relaxing the uterine preparations simply because it produced much larger elevations in cyclic GMP than did SNP, a result which would be consistent with a role for cyclic GMP in the control of uterine motility. However, the ability of SNAP to inhibit spontaneous contractions was unaffected by a blockade of soluble guanylyl cyclase with ODQ. The cyclic GMP elevation caused by 10 μM SNAP was completely blocked by ODQ, and the cyclic GMP elevations caused by 30 and 100 μM SNAP were markedly decreased, but there was no change in the inhibition of spontaneous contractions produced by any concentration of SNAP. These results suggest that the relaxant effect of SNAP is exerted by a mechanism independent of cyclic GMP elevation. The finding that acetylpenicillamine (a derivative of SNAP without the S-nitroso group) did not have a relaxant effect, further suggests that the effect of SNAP may be due to the direct action of high concentrations of NO released from this compound. These results are in agreement with those of Kuenzli *et al*. (1996) who found that cyclic GMP elevation was not required for the relaxation of drug-induced contractions of guinea-pig myometrium caused by another NO donor, S-nitroso-L-cysteine. Possible roles of NO in control of uterine function have been thoroughly reviewed by Sladek *et al*. (1997).

It has recently been shown that NO may exert a direct relaxant effect in vascular smooth muscle preparations through the activation of a calcium-activated potassium channel (Bolotina *et al*., 1994; Dong *et al*., 1997). However, the administration of an inhibitor of this channel, charybdotoxin, to our smooth muscles had no effect on the relaxant response to SNAP. As noted in the results, a change in pH was also ruled out as a possible mechanism of SNAP-induced relaxation. Thus, at the present time, the underlying mechanism of the relaxation produced by SNAP is still unclear. The fact that SNAP did not cause relaxation in preliminary experiments in pregnant myometrial tissues warrants further investigation. In any case, it is unlikely that a NO/cyclic GMP relaxation pathway is responsible for maintaining a quiescent uterus during pregnancy, since none of the cyclic GMP-elevating agents used in this study, including SNAP, caused any inhibition of spontaneous contractility during late stage pregnancy.

Previous findings have suggested that, in contrast to vascular smooth muscle, some smooth muscle tissues can be classified as 'non-responsive' to changes in cyclic GMP levels. These tissues, which include the rat vas deferens (Diamond & Janis, 1978; Diamond, 1983) and the rat distal colon (Suthamnatpong *et al*., 1993; Patel *et al*., 1997) are not relaxed by drug-induced increases in cyclic GMP levels. The present study confirms the previous suggestion (Diamond, 1983) that the rat myometrium constitutes another cyclic GMP non-responsive smooth muscle. The results provide strong support for the conclusion that cyclic GMP does not play an important role in the control of contractility in the rat myometrium. It is possible that high concentrations of NO itself may directly influence contractility in this tissue. However, the physiological significance of this remains unclear.

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