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MECHANISMS UNDERLYING UTERINE QUIESCENCE DURING PREGNANCY

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Dedicate to
Pamela, Diego and Cristóbal…
this work would have not been possible
without their support

Index

Preface

The Medical Sciences Ph.D. program at the Pontificia Universidad Católica de Chile is a three years program. After completing my first year in Chile, I was fortunate to receive funding from the Fogarty International Center, a division of the National Institute of Health (NIH) of United States. Coupled with support from our School of Medicine, I was able to perform my Thesis work at the University of Maryland.

With the approval of my proposal by the Thesis Committee, I started research work in the Department of Obstetrics, Gynecology and Reproductive Sciences at the University of Maryland Baltimore in September 1998.

This text is the result of a two years work in the laboratory of Perinatal Research of Dr. Carl P. Weiner M.D. at the University of Maryland Baltimore. The Laboratory is located in the Bressler Research Building in the University Neighborhood in Downtown Baltimore, State of Maryland.

Acknowledgments

I was fortunate to receive a strong humanistic education from my parents Jahel and Jorge. They, as well as my brother Sergio, the cornerstone in my medical education, my grandmother Laura and my loved wife Pamela strongly support my decision to pursue a career in academic medicine. My gratitude to all of them.

I would like to give special thanks to the School of Medicine of the Pontificia Universidad Católica de Chile, the Department of Obstetrics and Gynecology and the Medical Sciences Ph.D. program for all the assistance provided me during training for my M.D., residency in Obstetrics and Gynecology, and now the Ph.D. degree.

I would like to state my appreciation for Dr. Alfredo M. Germain, who as served as my mentor since the last year of my residency. He encouraged me to become a researcher and provided important advice along the way. The success of my work reflects his collaboration.

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I also wish to acknowledge all the support I received from my colleagues in the Perinatal Research Laboratory at the University of Maryland Baltimore. I make special mention of Drs. Loren P. Thompson, Ph.D.; Irina A. Buhimschi, M.D.; and Kripamoy Aguan, Ph.D. Last but not least, I wish to offer special thanks to our research assistants in Maryland: Gerard Pinkas, Michele Huber and Renee Bilkas and for Rossana Vidal L. from our laboratory in Chile.

Abstract

We hypothesize that the chorion releases a substance or substances necessary for the maintenance of myometrial quiescence during pregnancy. A decrease in the release of this quiescent factor at the end of the pregnancy is required for normal myometrial activation. The objective of this thesis research is to test portions of the working hypothesis. Our experimental protocols use both the guinea pig as an animal model and human tissues, coupled with state of the art techniques in physiology, biochemistry and molecular biology. We demonstrate that the chorion releases a substance or substances that reduces oxytocin-stimulated myometrial contractility and may be involved in the maintenance of uterine quiescence. While definitive identification awaits ongoing study, we have discarded a role for the myometrial cGMP-PKG pathway, and for chorion derived NO, CO and prostaglandins. We found that the inhibitory action of the chorion is mediated in great part by the opening of myometrial large conductance Ca^{2+} activated K^{+} channels. We suggest that the chorion releases a natriuretic peptide, possibly brain natriuretic peptide or a similar compound, which may be one of the chorion-derived relaxing substances responsible for the maintenance of myometrial quiescence. We demonstrate the ability of atrial and brain natriuretic peptides to inhibit oxytocin-stimulated contraction of isolated myometrium obtained from the pregnant guinea pig. Most important, we demonstrate that natriuretic peptide-induced relaxation involves neither guanylate cyclase nor natriuretic peptide clearance receptor activation, and is not mediated by the cGMP pathway. This novel mechanism is reported here for the first time. Finally, we discard cGMP as a key mediator of myometrial quiescence despite demonstrating the ability of chorion to increase myometrial cGMP content. We conclude that chorion releases several factors that act on the myometrium in a paracrine fashion to generate and maintain uterine quiescence during pregnancy. The identification and/or characterization of the mechanism of action of these substances will provide important insights on the physiology of pregnancy and constitute valuable tools to develop new strategies for the management of preterm labor.

Introduction

The length of mammalian pregnancy is tightly regulated to assure the delivery of a newborn mature enough to survive the extra-uterine environment. A successful pregnancy requires near complete relaxation of the uterus for more than ninety-five percent of gestation, overcoming the inherent tendency of the myometrium to contract with stretch. This active and highly regulated process is called myometrial quiescence. It requires not only the near absence of myometrial contractions, but also its refractoriness to contractile agents. Myometrial quiescence is mediated by complex molecular mechanisms that remain poorly understood despite much research (Cunningham et al., 1993).

Myometrial quiescence precedes myometrial activation, the process of myometrial awakening that occurs late in pregnancy prior to labor (Norwitz et al., 1999). During myometrial activation, the molecular mechanisms responsible for quiescence are nullified and the ability of the myometrium to contract to uterotonins restored (Cunningham et al., 1993).

The balance between substances that generate myometrial quiescence and activation no doubt impacts greatly on the timing of parturition. Numerous studies in several animal models confirm that a balance between the bioaction of progesterone (pro quiescence) and estrogen (pro activation) is central to the initiation of parturition (Olson et al., 1995). However, such a relationship has been difficult to confirm in all mammals including the human and guinea pig.

Several recent studies focused on the paracrine rather than the endocrine regulation of myometrial contractility. The amnion and chorion are of fetal origin, metabolically active and strategically located adjacent to the decidua. The latter is a maternal tissue in direct contact with the myometrium. Thus, the amnion, chorion and decidua are each potential sources of paracrine regulators of myometrial contractility (Bryant-Greenwood and Greenwood, 1998).

Several investigators studying guinea pigs, rats, and rabbits have documented a time dependent increase in total myometrial cGMP content during pregnancy, which decreases shortly prior to the onset of labor (Weiner et al., 1994; Yallampalli et al., 1994). Considering this

temporal profile and the known smooth muscle relaxing capability of cGMP (Carvajal et al., 2000; Lincoln and Cornwell, 1993; Vaandrager and de Jonge, 1996), these investigators proposed that this second intracellular messenger had a central role in the maintenance of uterine quiescence though disagreeing on the source of the cGMP (Weiner et al., 1994; Yallampalli et al., 1994).

Nitric oxide (NO) is a potent, endogenous relaxing agent of smooth muscle, and its action is predominantly mediated by an increase in intracellular cGMP (Moncada et al., 1991). Several groups pursued the possibility that NO, by its activation of soluble guanylate cyclase, was responsible for the increase in myometrial cGMP during pregnancy and consequently was central to uterine quiescence (Bansal et al., 1997; Natuzzi et al., 1993; Sladek et al., 1993). However, Weiner demonstrated that the increase in myometrial cGMP of pregnant guinea pig was independent of NO production and proposed that it was secondary to particulate rather than soluble guanylate cyclase activation (Weiner et al., 1994).

The natriuretic peptide system is present in gestational tissues. Particulate guanylate cyclase, a membrane-bound receptor molecule, is found in the rat (Dos Reis et al., 1995) and human uterus (Itoh et al., 1994). The natural activators of these receptors are members of a family of natriuretic peptides (Rosenzweig and Seidman, 1991) whose presence has been demonstrated in gestational tissues. The human placenta produces atrial natriuretic peptide (ANP) (Graham et al., 1996). Brain natriuretic peptide (BNP) is present in amniotic fluid (Itoh et al., 1993) and synthesized by cultured amniotic cells (Itoh et al., 1993). Further, humans express particulate guanylate receptors type A and B (GC-A and GC-B) in the myometrium (Itoh et al., 1994).

Hypothesis

Based on preliminary data from our laboratory, we hypothesize that fetal membranes, specifically the chorion, release a substance essential for uterine quiescence. We also propose that a reduction in the release of the chorion-derived quiescent substance must occur late in gestation for normal myometrial activation. We further hypothesize that a locally produced natriuretic peptide, by activation of a particulate guanylate cyclase receptor, is the stimulus for the increased myometrial cGMP content. Finally, we hypothesize that this natriuretic peptide is central to the maintenance of myometrial quiescence during pregnancy via the cGMP pathway.

Objectives

General Objectives

Test portions of the working hypothesis by:

1. Measuring and characterizing the effect of fetal membranes on oxytocin-stimulated myometrial contractility.
2. Investigate the ability of natriuretic peptides to inhibit oxytocin-stimulated contractility and elucidate their mechanism of action.
3. Investigate the source and the role of myometrial cGMP during pregnancy.

Specific Objectives

1. Investigate the ability of fetal membranes to inhibit, in a paracrine fashion, oxytocin-stimulated contraction of myometrium obtained from the pregnant guinea pig.
2. Elucidate the mechanism of action for the chorion-derived inhibitory substance.
3. Confirm the presence of a chorion-derived inhibitory substance in human chorion.
4. Demonstrate the ability of natriuretic peptides to inhibit oxytocin-stimulated contraction of myometrium obtained from the pregnant guinea pig.
5. Elucidate the mechanism of action for natriuretic peptide-induced myometrial relaxation.
6. Investigate the presence of the natriuretic peptide system in gestational tissues.
7. Determine the source of myometrial cGMP during gestation.
8. Determine the contribution of myometrial cGMP to the maintenance of uterine quiescence.

Material and Methods

Animal Model and Tissue Preparation

Timed pregnant, Duncan-Hartley guinea pigs (term pregnancy = 65-68d) were purchased from a commercial breeder (Harlan Sprague Dawley, Indianapolis, IN). Myometrium was obtained from pre-term (43 + 2 days gestation), near-term (60 + 2 days gestation) and near labor (67+2 days gestation and pelvis opened), anesthetized (xylazine 4-mg/kg i.m., ketamine hydrochloride 100-200 mg/kg i.p.) guinea pigs respiring spontaneously. A hysterotomy was performed and the pups delivered. The fetal membranes (chorion and amnion) were carefully separated from the myometrium and from each other by gentle traction, immediately frozen in liquid nitrogen, and stored at -80 °C until used. Full thickness myometrial strips opposite the placental implantation site were excised including decidua and endometrium but excluding fetal membranes. The myometrial strips were placed in Krebs buffer and stored on wet ice until used for isometric tension recording performed within one hour of removal from the animal. The Committee on Animal Care at the University of Maryland Baltimore approved the protocol.

Human Tissues

Human fetal membranes were obtained at the time of cesarean section performed for medical reasons. The amnion and chorion were separated from each other by gentle traction, frozen in liquid nitrogen and stored at -80 °C until studied. The clinical histories of the women were analyzed and recorded for the presence of labor (term or preterm) as defined by gestational age and the presence of spontaneous regular uterine contractions and cervical dilatation. The Institutional Review Board approved the study, and informed consent was obtained from each patient. One human brain and two myometrial samples were graciously donated by another researcher obtained for a different protocol and used in preliminary studies contained herein.

Isometric Tension Studies

Longitudinal strips (10 + 2 mm length x 2 mm width) of full thickness myometrium were placed in organ chambers and attached to a force transducer (Grass Instruments, Quincy, MA) for isometric tension recording. The chambers contained Krebs buffer composed of: 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11.1 mM D-glucose, 0.016 mM EDTA, 2.2 mM $CaCl_2$, 15.8 mM $NaHCO_3$, pH=7.35-7.45; maintained at 37°C and continuously bubbled with carbon dioxide (95% O_2 / 5% CO_2). The myometrial strips were equilibrated under 1-g of passive tension until a stable baseline was achieved (typically requiring 30 min). The bath buffer solution was changed every 5 minutes during the equilibration period. After stabilization, myometrial contractions were stimulated by 10^{-8} M oxytocin. This concentration approximates the EC_{50} obtained during prior concentration/response curves. The experiment was begun after a pattern of regular contractions was reproducibly achieved (typically 15-20 min following the addition of oxytocin).

The myometrial contractions were recorded and analyzed using a combination of PowerLab/800 hardware and Chart v3.4 software (AD Instruments, Mountain View, CA). To quantify contractile activity for analysis, the integrated area under the curve over 5 or 10-min intervals was measured and normalized for the cross sectional area of the myometrial strip. The cross sectional area was calculated as W/(L x D), where W = weight (gr), L = length (cm) and D (density)=1.05 g/cm^3. The oxytocin-stimulated, basal activity was calculated as the integral activity of the stable contractions over 5 or 10-min before the addition of fetal membranes or drugs to the tissue bath and designated as basal activity.

cGMP Measurement by Radio Immuno-Assay (RIA)

cGMP was measured as previously described (Weiner et al., 1994). At the appropriate time, myometrial samples (about 25 mm^2) were placed directly into boiling 50 mM Tris with 4 mM EDTA at a pH of 7.5 for 10 minutes. The tissue was then minced, homogenized, centrifuged at 14,000g (-4°C) for 20 minutes and the supernatant snap frozen until assayed for cGMP content. Total protein content of the pellet was measured spectrophoto-metrically using the bicinchoninic acid

method (BCA kit, Pierce). The frozen supernatants were thawed and diluted (1:75) in 25mM TRIS/ 4mM EDTA, pH 7.5. cGMP content was measured by radioimmunoassay using a commercially available kit (Amersham, Piscataway, NJ). All cGMP measurements were corrected for total protein content in the sample. Duplicate values were averaged and the measurements expressed as ρmol/mg protein. To represent the changes in cGMP content, we compared the cGMP levels induced by natriuretic peptides with the basal level and expressed the results as times basal. Intra and inter-assay variations were each less than 5%.

Reverse Transcription -Polymerase Chain Reaction (RT-PCR)

RNA extraction: Total RNA was extracted from human samples (brain, myometrium, chorion and amnion) using a commercially available kit (RNeasy-Midi kit; QIAGEN, Bothell, Washington). A portion of previously stored frozen tissue (200-250 mg) was homogenized in 4 ml guanidine isothiocyanate containing buffer and centrifuged for 10 min at 5000 x g. The pellet was discarded, the supernatant transferred to a new tube, and 70% ethanol was added. After vigorous shaking, the lysate was purred into a RNaeasy spin column and centrifuged for 5 min at 5000 x g. The flow-through was discarded and the column washed 3 times. Finally, RNA was eluted in 250 μl RNase-free water by 5 min centrifugation at 5000 x g.

cDNA Synthesis: cDNA was synthesized from total RNA using the SUPERSCRIPT First-Strand Synthesis System (Life Technologies, Rockville, MD). In a total volume of 10 μl, total RNA (5 μg) was incubated at 65 °C for 5 min with 5 μl random hexamers (50 ng/ml) and 1 μl dNTP mix (10 mM), then place on ice for at least 1 min. For annealing 2 μl RT buffer (10 x), 4 μl MgCl (25 mM), 2 μl DTT (0.1 M) and 1 μl RNase were added and incubated at 25 °C for 2 min. Reverse transcription was performed by addition of 1 μl (50 units) of SuperScript II Reverse Transcriptase and 10 min incubation at 25 °C. Tubes were transferred to 42 °C (50 min), the reaction was terminated at 70 °C (15 min) and then chilled on ice. RNA template was removed by digestion with RNase H at 37 °C for 20 min.

Polymerase Chain Reaction: a reaction mixture was prepared as instructed by the supplier using human cDNA and self-designed primers for human BNP and β-actin. Briefly, 2 μl of cDNA were subjected to a round of 36 cycles of PCR

amplification (94 °C for 20 s, 60 °C for 60 s, 72 °C for 30 s). The BNP sequence was amplified using 5'-TTCTTGCAT-CTGGCTTTCCT-3' as the forward primer and 5'-GCAGGGTGTAGAGGACCATT-3' as the reverse primer. The β-actin sequence was amplified in a separate assay using 5'-GCTGTGCTATC-CCTGTACGC-3' (forward) and 5'-GCCATGGTGATGACCGGC-3' (reverse) primers. The resulting PCR product was run in a 2% agarose gel and the bands identified by UV transillumination. A DNA ladder was run for identification of the desired band. The band was cut and the gene product was eluted using the Gel Extraction Kit QIAEX II® (QIAGEN, Bothell, Washington). The product was then submitted for sequencing to prove it was BNP or β-actin.

Ribonuclease Protection Assay (RPA)

Synthesis of ^{32}P labeled RNA probes: T7 Ligation: The BNP and β-actin PCR products provided the templates for generating the RNA probes necessary for the RPA assay. Each individual PCR-amplified DNA fragment was ligated to a T7-adapter using Lig'n-Scribe™ (Ambion, Austin, TX). The reaction was setup at room temperature for 30 min by mixing 1 μl ligation buffer (10x), 1 μl T7 promoter, 2 μl PCR product (10-25 ng DNA), and 1 μl T4 DNA ligase (final volume 10 μl with DEPC water).

T7 PCR: The ligated DNA/T7 adapter complex was further PCR amplified using the T7 primer and forward primers for the specific human BNP or β-actin genes, respectively (the same used for RT-PCR). A 25 μl reaction mixture (2 μl from ligation, 5 μl of 10x PCR buffer, 3 μl of 25 mM Mg Cl, 1 μl of 10 mM dNTP, 2 μl of 10 mM T7 primer adapter, 2 μl of specific forward primer and 0.5 μl of Taq Polymerase) underwent 30 cycles of PCR amplification (94 °C for 20 s, 58 °C for 60 s, 72 °C for 30 s).

The PCR product (DNA/T7) was then run in a 2% agarose gel, the band cut from the wet gel, and the DNA/T7 product eluted using the Gel Extraction Kit QIAEX II® (QIAGEN, Bothell, Washington).

Transcription: The DNA purified from the gel was converted into RNA probes in the presence ^{32}P-[α-UTP] and T7 RNA polymerase using the In vitro-Transcription Kit ™ (Ambion, Austin, TX). The transcription was performed for 1 hour at 37 °C by mixing 5 μl DNA/T7, 3 μl nucleotides, 2 μl reaction buffer, 6 μl ^{32}P-[α-UTP], 2 μl UTP and 2 μl RNA Polymerase. After transcription, the reaction was boiled for 5 min and chilled

on ice for at least 1 minute. The template DNA/T7 was extracted by digestion with Dnase I at 37 °C for 30 min.

Probe elution: The transcribed ^{32}P-labeled RNA probe was run onto a 3.7 % urea-PAGE gel, and exposed to a radiographic film for 15 min. The wet gel, was aligned with the exposed film and the corresponding gel band, containing the RNA probe was cutout and placed into an Eppendorf tube. The RNA was eluted during a 3 hours incubation at 37 °C in 1 ml of RNA elution buffer (RPA III Kit, Ambion, Austin, TX). In general, 70% of the RNA will be eluted within this time period.

Hybridization: About 5 μl of the eluted buffer (containing RNA probe) was counted in scintillation counter. About 20,000 cpm of the BNP-RNA probe and 30,000 cpm of the labeled β-actin RNA probe (for normalization of gene expression levels) were hybridized with 15 μg sample RNA. Hybridization was carried out in an Eppendorf tube containing 20 μl hybridization buffer and incubated at 47^{0}C for 18 hrs.

Digestion: The hybridization mixture was digested with RNAseA/T1 (to remove nonhybridizing, single stranded RNA), precipitated and dissolved in loading buffer. Positive and negative controls for digestion were prepared using yeast RNA. The product was loaded onto a urea-PAGE gel and run slowly at 250 V for over 3 hours. The gel was dried and autoradiographed. Expression levels of the each gene were directly analyzed by Phospho-imaging machine (GS 525; BioRad, San Francisco, CA) and normalized against the expression levels of β-actin gene.

Immunohistochemistry (IHC)

Guinea pig tissues were fixed in 4% paraformaldehyde solution for 10-15 min, and then incubated at 4°C over night in 0.1 M phosphate buffered saline (PBS) with $CaCl_2$ (pH 7.5) . The tissue was then dehydrated by increasing concentrations of ethanol up to 100%, immersed sequentially into solutions of 50:50 paraffin and xylene followed by 100% paraffin. The paraffin embedded tissue was sectioned cut into 6-7 mm slices and mounted on microscope slides. The sections were deparaffinized in xylene, hydrated to water in a series of decreasing ethanol solutions before incubating in PBS (pH 7.5) with 0.1% Triton X-100 and 3% hydrogen peroxide followed by non-specific blocking with normal donkey serum (Jackson Lab, Bar Harbor, Maine). Due to the high avidin concentration in the chorion, the slides were also blocked using the Avidine-Biotin

Blocking Kit (Vector Laboratories Inc., Burlingame, CA). The slides were then incubated overnight at 37°C with sheep polyclonal anti-cGMP primary antibody (Transduction Laboratories, Lexington, KY), which is highly specific for cGMP and does not cross react with the cAMP. The next day, the primary antibody was washed and the slides incubated for 1 hour at room temperature with a biotin conjugated secondary antibody (donkey anti-sheep IgG, Jackson Lab, Bar Harbor, Maine). The slides were developed with the avidin biotinilated enzyme complex method (Vectastin®-ABC Kit, Vector Laboratories Inc., Burlingame, CA) and using Ni-diaminobenzidine, as instructed by the manufacturer. A black reaction product marked the location of cGMP. Negative control slides were generated by replacing the primary antibody with non-immune sheep serum. Some sections were lightly counterstained with hematoxylin-eosin and photographed with a microscope model Nikon Microphot-FXA (Nikon, Tokyo, Japan).

Experimental Protocols

a. Effect of Fetal Membranes on Oxytocin-Stimulated Contractility

To determine the effect of the fetal membranes on oxytocin-stimulated myometrial contractility, either chorion or amnion was added directly to the organ bath containing the oxytocin-stimulated myometrial strips and changes in contractile activity measured.

Previously stored frozen membranes (chorion or amnion) from a single near-term animal were weighed and thawed for 5 min in 37 °C Krebs solution.

Approximately 1000 mg was added directly to the 10-ml organ bath (100 mg membranes/ml buffer) after the establishment of regular contractions by oxytocin. The isometric tension was recorded an additional 30 min. The contractile activity after the addition of the fetal membranes was measured in 10-min intervals, represented in the text and figures by midpoint of the interval (i.e. 5, 15 and 25 min). Contractility (defined as the integrated area under the curve) of each interval was compared to the basal activity and expressed as its percentage. Thus, 100% indicates no change from baseline, while less than 100% denotes a decrease in contractile activity (i.e. myometrial relaxation). The pH of the muscle bath was measured at the end of several experiments and always found to be unchanged (7.45 + 0.05).

Three experiments were done using fresh chorion membranes. The chorion was obtained as indicated, weighed and placed

into buffer for 30 minutes at 37°C, approximately 100 mg membranes/ml of buffer. Both the fresh chorion conditioned media and the chorion membranes were directly added to the organ bath after a regular pattern of myometrial contractions was produced. The effect of fresh chorion and fresh chorion conditioned media was evaluated as described above for thawed chorion.

To determine whether the effect of chorion on oxytocin-stimulated myometrial contraction was gestational age dependent, the membranes of pre-term and near-term animals were thawed as previously described and added to myometrial strips obtained from one animal per experiment. To test whether the response of the myometrium to the factor (s) released by chorion changed with the gestational age of the myometrium, near-term membranes from the same animal were added to myometrium from pre-term and near-term guinea pigs. To establish whether the effect of chorion was dose-dependent, chorion-induced relaxation of oxytocin-stimulated myometrium in the presence of either ~50-mg tissue/ml or ~100-mg tissue/ml in separate experiments was compared using chorion from the same animal.

b. Effect of Fetal Membranes on Spontaneous Contractility

While the myometrial strips of pregnant guinea pig do not generally exhibit a regular pattern of spontaneous contractility, some 3-5% of strips will do so. To determine the effect of the fetal membranes on spontaneous myometrial contractility, either chorion or amnion was added directly to the organ bath containing the spontaneous contracting myometrial strips and the change in contractile activity measured.

c. Effect of Chorion-Conditioned Medium on Oxytocin-Stimulated Contractility

To determine whether chorion-induced relaxation of oxytocin-stimulated myometrium was the product of a substance released into the media, chorion-conditioned media was prepared as described below and its effect on myometrial contractility evaluated.

A sample of chorion (1500 mg) from an animal was thawed for 5 min in 37°C Krebs buffer. The membranes were incubated an additional 30 min in a glass container with 5-ml of 37 °C Krebs buffer. The membranes were then discarded and the freshly prepared supernatant added directly to the 10-ml organ bath (final ~100 mg chorion/ml buffer). The effect of chorion-conditioned media on oxytocin-stimulated myometrial contraction was measured as described for the chorion membranes.

d. Effect of Human Chorion on Oxytocin-Stimulated Contractility

To determine whether the chorion of humans had a similar impact on oxytocin-stimulated contraction of guinea pig myometrium, chorion from a single, near term pregnancy delivered by cesarean section prior to labor was weighed and thawed for 5 min in 37 °C Krebs solution. Approximately 1000 mg was directly added to the 10-ml organ bath (100 mg membranes/ml buffer) after the establishment of regular contractions stimulated by oxytocin, and the isometric tension recorded an additional 30 min. This is the same protocol used in our studies of the guinea pig chorion.

e. Evaluation of the Mechanism of Action of Chorion-Induced Relaxation

e.1. Myometrial cGMP Pathway Blocking: To determine whether the chorion relaxes the myometrium by increasing cGMP, the pathway of cGMP action was pharmacologically dissected in additional series of experiments. After stabilization and prior to the addition of oxytocin, the myometrial strips were incubated for 30 min with 30 μM Rp-8Br-cGMP, a membrane permeable cGMP analog that specifically block cGMP-dependent protein kinase (PKG). Vehicle incubation was run in parallel. A sample of chorion from the same animal was added (as described above) to treated or control strips and the changes in contractile activity compared.

The incubation time and concentration used for the Rp-8Br-cGMP was previously shown to inhibit both PKG activation and the relaxation induced by the activation of the cGMP pathway in vascular tissues (Dhanakoti et al., 2000). We also tested whether this drug concentration and incubation period inhibited PKG in our myometrial samples by measuring PKG activity in a series of experiments. PKG activity was quantitated as previously described by measuring the incorporation of $^{32}P_i$ into a PKG specific substrate (Diwan et al., 1994; Patel and Diamond, 1997). Basal myometrial PKG activity was 16% of the maximal activity induced by 5mM cGMP. A 30 min incubation with Rp-8Br-cGMP alone generated a small increase in PKG activity (26% of maximum). In contrast, PKG activity increased to 40% of maximum 5 min after the addition of a single dose (10^{-7}M) of BNP. This effect of BNP on PKG was completely blocked by the 30 min incubation with 30μM Rp-8-Br-cGMP; the enzymatic activity was only 10% of maximum under this condition.

To determine whether the chorion relaxes oxytocin-stimulated contraction by activating myometrial soluble guanylate cyclase and increasing cGMP, myometrial

strips were incubated for 30 min with 30 μM 1H-[1,2,4] oxadiazolo [4,3-α] quinoxalime-1-one (ODQ), a specific blocker of soluble guanylate cyclase prior to the addition of oxytocin. This concentration of ODQ was previously shown to be effective for the inhibition of soluble guanylate cyclase in myometrial strips (Hennan and Diamond, 1998). A sample of chorion from one guinea pig was added to treated or control strips to determine the effect of soluble guanylate cyclase antagonism.

e.2. Chorion Synthesis of Putative Relaxing Agonists Inhibition:

To test the potential role for chorion-derived NO in the provision of myometrial quiescence, potential production of NO by the chorion was blocked by N (w)-Nitro-L-Arginine (L-NNA), a nitric oxide synthase (NOS) inhibitor. After thawing the chorion membranes, they were incubated for 30 minutes with 10^{-4} M L-NNA, or vehicle, and then added to the myometrial organ bath. We have used L-NNA at this concentration and for this incubation period to successfully inhibit endothelial NOS activity (Kim et al., 1994).

To test the contribution of chorion-derived CO, its production was blocked with Tin-protoporphyrin, a hemoxygenase inhibitor. The thawed chorion was incubated for 30 minutes with 30 μM tin-protoporphyrin and compared to vehicle-incubated chorion studied in parallel. This concentration of Tin-protoporphyrin effectively blocks hemin-stimulated CO production in myometrial strips (Acevedo and Ahmed, 1998).

To evaluate whether chorion-induced relaxation was mediated by a chorion-derived prostaglandin, the synthesis of prostaglandins in the chorion was blocked with indomethacin, a cycloxygenase inhibitor. The chorion was thawed as described, incubated by 30 minutes with 10μM indomethacin before its addition to the organ bath. This dose was previously shown to inhibit prostaglandin synthesis in guinea pig tissues (Naderali and Poyser, 1996). Chorion from the same animal, incubated in vehicle solution was studied in parallel as a control.

e.3. Myometrial K^+ Channels Blocking:

As an alternative mechanism for chorion-induced relaxation, we investigated the effect of myometrial K^+ channel antagonism. Chorion-induced relaxation was measured in the presence and absence of different K^+ channel antagonists: 2 mM Tetraethylamonium (TEA, non-specific K^+ channel blocker), 100 nM Iberiotoxin (IbTx, large conductance Ca^{2+} activated K^+ channel blocker), 10 μM Glibenclamide (Glib, ATP-sensitive K^+ channel blocker) and 2 mM

4-Aminoperidine (4-AP, voltage gated K^+ channel blocker). The agent under study was added to the tissue bath 10 minutes before the addition of chorion; vehicle only was added to the other strips to provide a parallel control.

f. Effect of Natriuretic Peptides on Oxytocin-Stimulated Contractility

The effect of natriuretic peptides on myometrial contractility was investigated by generating concentration / response curves to the cumulative addition of each peptide. Contractility was induced by oxytocin (10^{-8} M) and relaxation to ANP, BNP and c-type natriuretic peptide (CNP) determined at concentrations ranging from 10^{-9} to 10^{-6} M (final concentration in the organ bath). Contractility was measured during the 5-minutes interval after the addition of the natriuretic peptide and compared to the basal contractile activity. The difference between basal activity and that after the natriuretic peptide was calculated and expressed as the percentage of basal activity, and identified as percentage relaxation.

The temporal course of the natriuretic peptide-induced relaxation in response to a single concentration of each natriuretic peptide (10^{-7} M) was investigated by recording myometrial contractility for 30 minutes after peptide addition. The effect was measured in 5 minutes-intervals, represented in the text and figures by their time at the midpoint (e.g. 2.5 minutes, 7.5 minutes, etc.), and expressed as percentage relaxation.

g. Evaluation of the Mechanism of Natriuretic Peptide-Induced Relaxation

g.1. Myometrial GC-A blocking: To determine whether natriuretic peptide-induced relaxation occurred by the activation of particulate guanylate cyclase A (GC-A), myometrial strips were pre-incubated for 5 minutes with anantin (10^{-6} M), a competitive antagonist of the GC-A receptor, prior to the addition of a single dose of natriuretic peptide. As the relaxation induced by natriuretic peptides is maximal during the first 5 minutes after exposure, this time period was selected to compare the effect of anantin alone, and the effect of anantin on natriuretic peptide induced relaxation.

g.2. Myometrial PKG blocking with Rp-8Br-cGMP: To determine whether natriuretic peptides relax myometrium by increasing cGMP, the cGMP pathway was blocked before the addition of a single dose of BNP by a 30 minutes preincubation with 30 μM Rp-8-Br-cGMP, a membrane permeable cGMP analog that specifically inhibits cGMP-dependent protein kinase (PKG). As discussed earlier, we demonstrated that this concentration and incubation period were

adequate to inhibit PKG in our myometrial samples.

g.3. Natriuretic Peptide Clearance Receptor Agonist/Blocker: Some of the biological actions of natriuretic peptides are mediated by activation of a natriuretic peptide clearance receptor (Anand-Srivastava and Trachte, 1993; Hempel et al., 1998). To determine whether clearance receptor activation was part of the biological action of the natriuretic peptides, the effect of a single dose of atrial natriuretic peptide des-(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})-fragment 4-23 amide (cANP), a specific agonist of the natriuretic peptide clearance receptor, was tested on oxytocin-induced contractility. It was reported that natriuretic peptide action by the clearance receptor involves a heterotrimeric G protein coupled mechanism (Anand-Srivastava and Trachte, 1993; Murthy and Makhlouf, 1999) and results in an increase in K^+ outward conductance (Anand-Srivastava and Trachte, 1993; Kanwal and Trachte, 1994). To determine whether the mechanism of natriuretic peptide mediated relaxation of oxytocin-stimulated contraction involved a pertussis toxin (PTX) sensitive G protein, myometrial strips were preincubated for 3 hours prior to the addition of BNP to oxytocin-stimulated myometrium, with PTX (5mg/ml), a specific blocker of heterotrimeric G protein α_i subunit. This concentration and incubation time were shown to effectively block PTX sensitive G protein of porcine myometrial strips (Kitazawa et al., 2000). The effect of 2mM tetraethylammonium (TEA), a non-specific K^+ channel blocker, on BNP-induced relaxation was investigated by adding the drug to the organ bath 5 minutes before the addition of BNP. At this concentration and incubation time, TEA effectively blocks myometrial K^+ channels (Khan et al., 1997; Perez et al., 1993).

h. Effect of Natriuretic Peptides on Myometrial cGMP

To determine the effect of natriuretic peptides on particulate guanylate cyclase activation, we measured cGMP content of guinea pig myometrial samples under basal conditions and after the stimulus of a single dose of 10^{-7}M ANP, BNP and CNP; either in the presence or absence of anantin (10^{-6} M). To determine whether contractility altered the effect of natriuretic peptides on cGMP content, three additional experiments were performed in duplicate in the presence of 10^{-8} M oxytocin (the same concentration used to induce contraction of the myometrial strips). To clarify the temporal profile of natriuretic peptide-induced cGMP content, cGMP was measured in duplicate tissue samples every 5 minutes beginning 2.5

minutes after the addition of the peptide (up to 27.5 minutes total).

i. Evaluation of BNP expression on Human Gestational Tissues

To further test the possibility of a role for BNP in the mechanism of chorion induced relaxation, preliminary data of BNP expression was obtained from human tissues, by RT-PCR and RPA.

The presence of BNP mRNA was studied by RT-PCR in a sample of chorion obtained from a woman at term but not in labor. The product of this RT-PCR was sequence to prove it was BNP, and used to generate the RNA probe for the RPA.

The presence of BNP mRNA in human gestational tissues was studied by RPA comparing one sample of human brain, non-pregnant myometrium, pregnant myometrium, chorion and amnion (obtained from a woman at term but not in labor). Additionally, changes in BNP expression during gestation were studied by comparing the BNP expression between preterm (one sample 30 weeks gestation) and term not in labor (2 samples) human chorion.

j. Effect of 8-Br-cGMP on Oxytocin-Stimulated Myometrial Contractility

To test the ability of cGMP to modulate oxytocin-induced contractile activity of myometrium from pregnant guinea pig, a concentration response curve was prepared to 8-Br-cGMP, a membrane permeable cGMP analog.

After the presence of regular contractions was documented, the drug was added directly to the organ chamber in 0.5 log-unit increments and in volumes of 20 – 40 μl as appropriate. A new dose was added each 10 minutes. Vehicle controls were run in parallel. The amount of drug used was calculated as its final concentration in the organ bath. The effect of 8-Br-cGMP was expressed as the contractile activity during the 10-minutes interval after the addition of the drug, and compared to basal activity. The results are expressed as the percent change from basal.

k. Effect of Fetal Membranes on Myometrial cGMP Content

To further elucidate the mechanism of chorion-induced relaxation of guinea pig myometrium, we determined the effect of chorion on myometrial cGMP in vitro, using the standard laboratory protocol.

For each experiment, several strips (25 mm^2) of full thickness guinea pig myometrium (decidua attached) were cut from a single uterus and incubated in oxygenated Krebs buffer for 40 minutes at 37 °C before exposure to the fetal membranes. Previously stored frozen

membranes of the desired gestational age were pooled, crushed to generate a powder, weighed and aliquoted into separate tubes. Pieces of myometrium were then incubated in a 2-ml bath of Krebs solution, with 1 mM IBMX at 37°C under agitation by 15 minutes, in the presence of 400-mg of crushed chorion (200 mg tissue/ml bath) or control (amnion or myometrium alone). After the incubation, two pieces of myometrium from each intervention were taken for cGMP assay.

To correlate the relaxing effect of chorion and the cGMP generation induced by chorion, a protocol similar to the one used for chorion-induced myometrial relaxation was employed to study alterations in myometrial cGMP content over time. Previously stored frozen membranes (chorion or amnion) from a single near-term animal were weighed and thawed for 5 minutes in 37 °C Krebs solution. Pieces of myometrium (25 mm^2) were incubated with thawed chorion membranes (100 mg chorion/ml bath) for 30 minutes. Two pieces of myometrium were removed at regular intervals (basal, 5, 15 and 25 minutes) for the measurement of cGMP.

l. Evaluation of the Mechanism of Chorion-Induced cGMP Increase

Inhibitors of soluble and particulate guanylate cyclase were employed to further elucidate the mechanism of chorion-induced myometrial cGMP generation. Before the addition of chorion to the bath, the myometrium was incubated with one of the following drugs: 10^{-4} M Nitro L-arginine (LNA; a NO synthase inhibitor), 10^{-6} M 1H-[1,2,4] oxadiazolo [4,3, -α] quinoxalin -1-one (ODQ; a highly specific soluble guanylate cyclase antagonist), 1 mg/ml HS 142-1 (a microbial polysaccharide that inhibits all particulate guanylate cyclase receptors) and 10^{-6} M anantin (a microbial peptide that specifically inhibits the particulate guanylate cyclase type A receptor).

The generation of cGMP and its release into the medium by the chorion was also investigated by incubating 400-mg of crushed chorion, or 400 mg of thawed chorion, in 2 ml of Krebs buffer for 15 minutes at 37°C in the presence of 1mM IBMX. The chorion was removed and the cGMP in the solution measured by RIA and normalized to its protein content.

To further clarify the mechanism by which chorion increased myometrial cGMP, myometrium was incubated in Krebs solution with increasing concentrations of exogenous cGMP (range from 1 to 5000 nM). For each experiment, several strips (25 mm^2) of full thickness guinea pig myometrium (with decidua attached) were cut from a single uterus and incubated in

oxygenated Krebs buffer for 40 minutes at 37 °C before exposure to the fetal membranes. Next, the myometrial samples were incubated in a 2-ml bath of Krebs solution with 1 mM IBMX at 37 °C under agitation for 15 minutes in the presence or absence of synthetic cGMP. After incubation, two pieces of myometrium from each intervention were taken for the measurement of cGMP.

m. Evaluation of the source of cGMP in gestational tissues

To identify the main source of cGMP in gestational tissues, samples (biopsy sample) of myometrium, chorion and amnion were taken from preterm, near-term and near-labor animals as described previously, and immediately assayed for cGMP. The cGMP in situ was semi-quantitated in paired samples using immunohistochemistry by immediately fixing the biopsy samples in 4% paraformaldehyde.

The location of cGMP in the myometrial preparations was determined after incubating the sample with a known concentration of exogenous cGMP. Myometrial samples, including decidua, but not fetal membranes were incubated in Krebs solution bubbled continuously with CO_2 at 37°C for 40 minutes. The samples were then incubated for an additional 15 minutes with 1-5000 nM cGMP in the presence of 1mM IBMX. The myometrium was then fixed in 4% paraformaldehyde and the cGMP location determined by immunohistochemistry.

Drugs and Solutions

The cGMP assay kit (BIOTRAK) was purchased from Amersham International (Piscataway, NJ). Oxytocin, human ANP, BNP, CNP, Anantin, IBMX, LNA, 8-Br-cGMP, cANP, PTX, TEA, IbTx, Glibenclamide, 4-AP, indomethacin, tin protoporphyrin, were obtained from SIGMA Chemical Co. (St. Louis, MI). ODQ and Rp-8-Br-cGMP were purchased from Alexis Laboratory (Alexis Corp., San Diego, CA). All drugs were dissolved in double deionized water unless otherwise specified. Glibenclamide and ODQ were solved in 0.01 N NaOH.

Statistical Analysis

All data sets (mean ± standard error of the mean) were subjected to a test of normalcy (Shapiro-Wilk test) and parametric or non-parametric tests performed where appropriate. For normally distributed data, statistical comparisons between two groups were conducted using Student 't'-test and among multiple groups, a one-way blocked Analysis of Variance (ANOVA) followed by Dunnett multiple comparison test (parametric). With non-normal sets, comparisons between two groups were performed by Wilcoxon Signed Rank test and among multiple groups by a Friedman's test (nonparametric). A two-tailed $p < 0.05$ was considered indicative of statistically significant difference.

RESULTS

Guinea Pig Myometrium as a Model for Isometric Tension Studies

The spontaneous contractile activity of isolated guinea pig myometrial strips was characterized by irregular contractions of long duration (approximately 3 minutes for each contraction), high amplitude and variable frequency (from 0 to 4 per 10 minutes). While most of the strips have no spontaneous contractions; 3-5% have a regular pattern of spontaneous activity. Oxytocin initially produces a tetanic contraction lasting 3-5 minutes. Tetany is followed by regular contractions of short duration (30-60 sec), low amplitude compared to the spontaneous contractions, with a frequency of 10-15 in 10 minutes (Figure 1). This pattern of activity continues with little variation for about 30 minutes. Thereafter, the contractile activity declines somewhat by 60 minutes after initiating the experiment to a 80% of the oxytocin-stimulated basal activity (p=NS). In preliminary studies, we determined that 10^{-8} M oxytocin was the optimal concentration to produce a regular pattern of contractions.

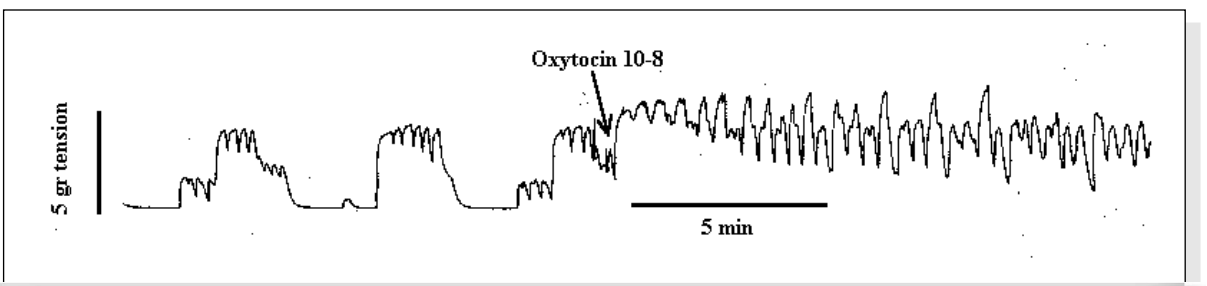

Figure 1. Representative tracing of spontaneous and oxytocin induced contractile activity of the myometrium from near-term pregnant guinea pig.

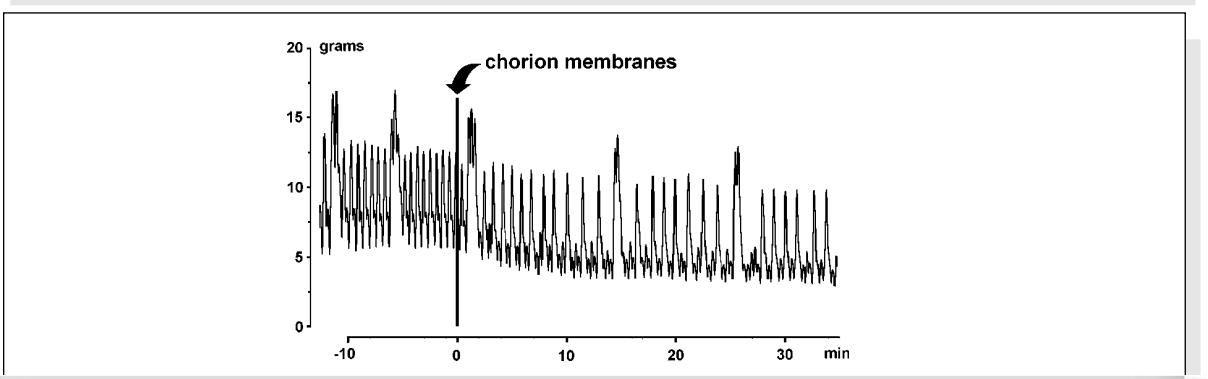

Figure 2. Representative tracing of the effect of chorion on oxytocin- stimulated contractility of the myometrium from the pregnant guinea pig.

Effect of Chorion and Amnion on Myometrial Contractile Activity

Chorion but not amnion produced a time dependent decrease in the oxytocin-stimulated contractile activity of myometrium obtained from near-term pregnant guinea pigs (Figure 2). As illustrated in Figure 3, chorion significantly ($p<0.05$ vs. temporal control) reduced the contractile activity to 58 % and 39 % of the basal level at 15 and 25 minutes, respectively. There was no significant change in myometrial contractile activity without the addition of chorion (temporal control) (Figure 3). Contractility returned to near basal levels within 30 minutes of removing the membranes from the bath and replacing the existing solution with fresh buffer.

Fresh chorion inhibited oxytocin-stimulated contraction to the same magnitude and with the temporal profile as thawed chorion. The average of three experiments conducted with fresh chorion revealed a decrease in contractile activity to 85%, 60% and 42% of basal at 5, 15 and 25 minutes respectively.

In contrast to the chorion, the addition of amnion, either from pre-term or near-term animals, had no significant effect on the oxytocin-stimulated myometrial contraction (Figure 4).

Similar to oxytocin-stimulated myometrial contractility, the chorion, but not the amnion decreased spontaneous myometrial contractility in the four strips

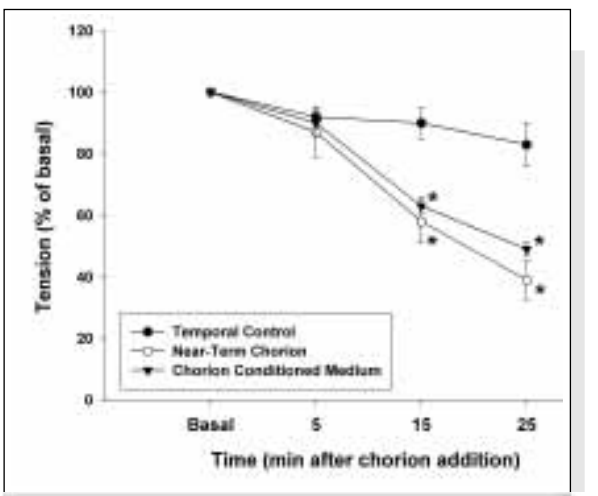

Figure 3. Effect of near-term chorion membranes and chorion-conditioned medium on oxytocin-stimulated myometrial contractility. Values are mean + SEM of 8 experiments (chorion from 8 different guinea pigs). * = $p < 0.05$ compared to temporal control.

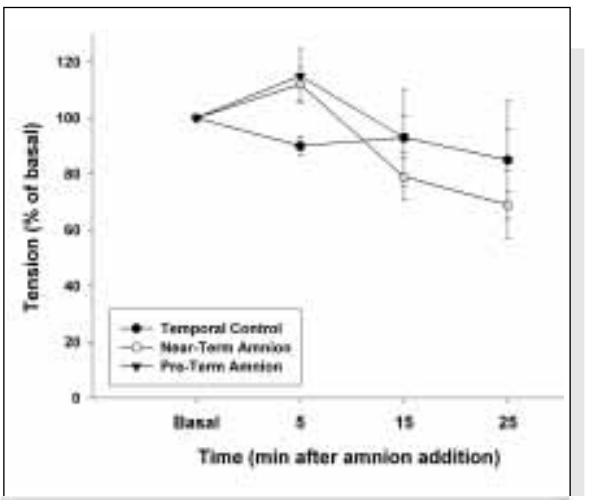

Figure 4. Effect of amniotic membranes from pre-term and near-term animals on oxytocin-stimulated contractility of near-term myometrium. Values are mean + SEM of 5 experiments (amnion from 5 near-term and 5 pre-term guinea pigs).

evaluated. After the addition of chorion (obtained from two different animals), contractility decreased to 78%, 68% and 56% of basal at 5, 15 and 25 minutes respectively, while after amnion, contractility was 95%, 89% and 86% at the same times, respectively.

The inhibitory effect of chorion on oxytocin-stimulated myometrial contraction was gestational age dependent (Figure 5). Pre-term chorion produced a significantly greater ($p < 0.05$) reduction in contractile activity (41 % and 23 % of the basal activity at 15 and 25 minutes respectively) compared to near-term chorion (60 % and 41 % from baseline).

Chorion induced-relaxation was independent of the gestational age of the myometrium. There were no significant differences in the ability of near-term chorion to inhibit oxytocin-stimulated contraction of myometrium obtained from pre-term and near- term animals (Figure 6).

Relaxation induced by chorion was dose-dependent (Figure 7). Oxytocin-stimulated contractility was reduced to 75 % and 61 % of baseline at 15 and 25 minutes, respectively, after adding ~50-mg of chorion membranes per ml. The reduction in contractile activity was significantly greater after the addition of ~100 mg membranes/ml, declining to 58% and 39% of baseline, respectively, at 15 and 25 minutes ($p<0.05$).

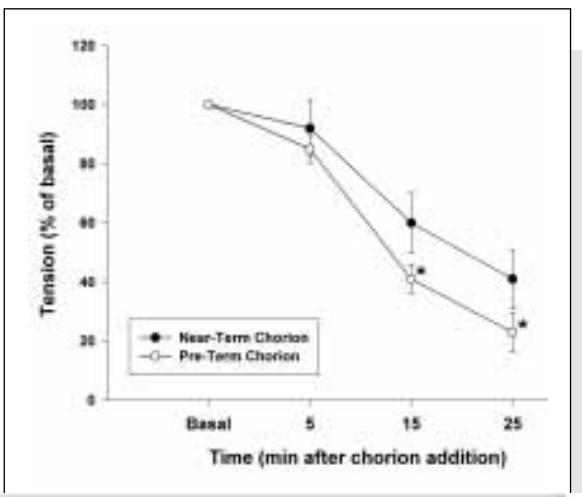

Figure 5. Effect of chorion membranes from near-term and preterm animals on oxytocin-stimulated contractility of near-term myometrium. Values are mean + SEM of 5 experiments (chorion from 5 near-term and 5 pre-term guinea pigs). * = $p < 0.05$ compared to near-term membranes.

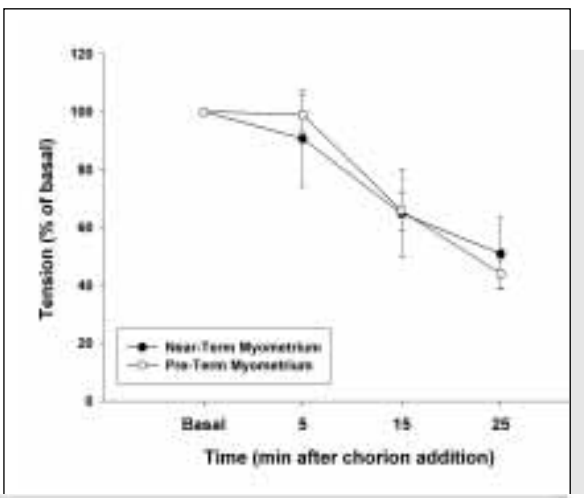

Figure 6. Effect of near-term chorion on oxytocin-stimulated contractile activity of myometrium from near-term and preterm animals. Values are mean + SEM of 8 experiments (myometrium from 8 near-term and 8 pre-term guinea pigs; chorion from 8 guinea pigs).

Effect of Chorion Conditioned Medium on Myometrial Contractile Activity

Chorion-conditioned media had the same effect as whole chorion on oxytocin-stimulated myometrial contraction (Figure 3). The addition of chorion-conditioned media reduced contractility to 63 % and 49 % of the basal level at 15 and 25 minutes ($p < 0.05$ vs. temporal control). Similar to whole chorion, the effect of chorion-conditioned media was reversed by rinsing the organ bath and replacing the conditioned media with fresh buffer.

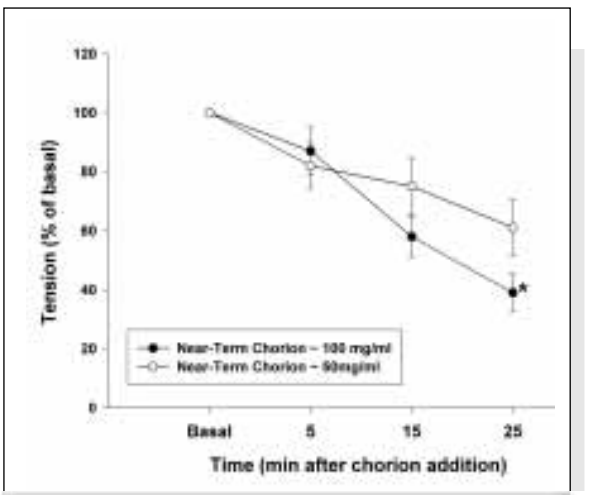

Figure 7. Effect of variable amounts of chorion on oxytocin-stimulated myometrial contractility. Values are mean + SEM of 8 experiments (chorion from 8 different guinea pigs). * = $p < 0.05$ compared to low concentration.

Conditioned media prepared from fresh chorion also decreased myometrial contractile activity. The average contractility of three experiments using conditioned media from fresh chorion was 90%, 64% and 50% of basal at 5, 15 and 25 minutes after the addition of conditioned media to the tissue bath.

Effect of Human Chorion on Myometrial Contractile Activity

Figure 8 illustrates an experiment performed in duplicate testing the effect of human chorion on oxytocin-stimulated

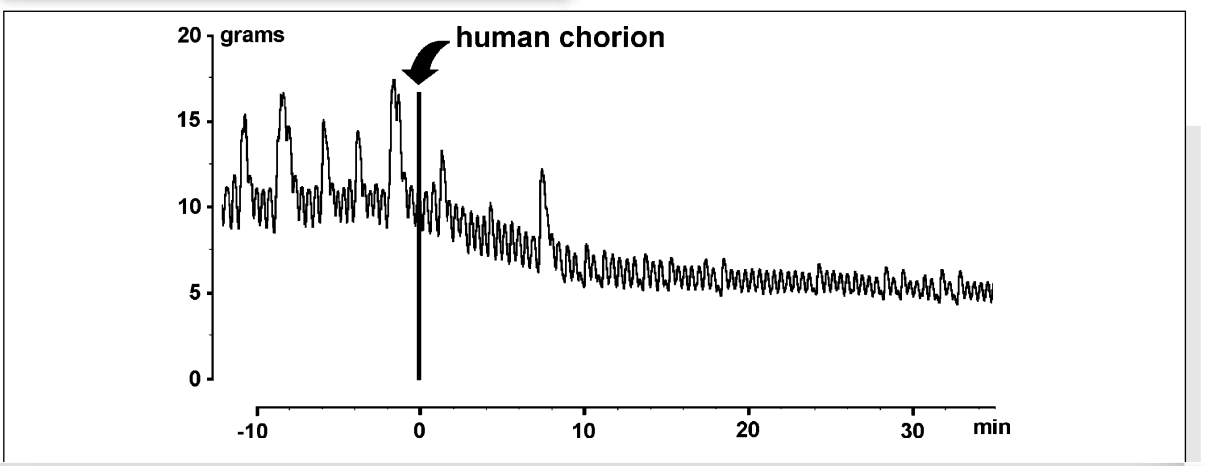

Figure 8. Representative tracing of the effect of human chorion on oxytocin-stimulated contractility of the myometrium obtained from a near-term pregnant guinea pig.

contraction of myometrium obtained from the pregnant guinea pig. Chorion decreased contractility to 69%, 47% and 39% of basal activity during the 5, 15 and 25 minutes period after chorion addition. This effect was reversed by removing the chorion and replacing the solution with fresh buffer.

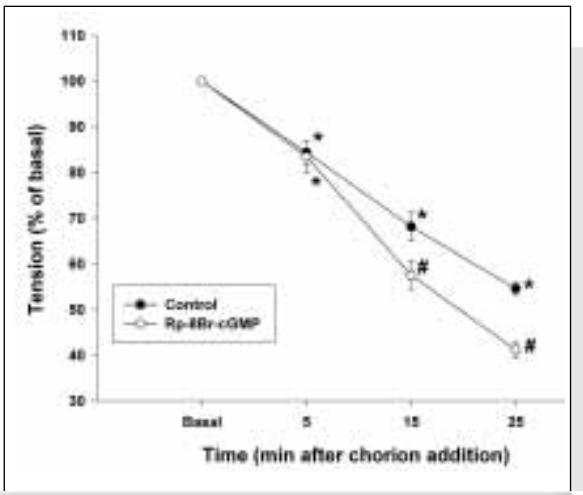

Figure 9. Effect of myometrial incubation with Rp-8-Br-cGMP (30 μM) on chorion induced relaxation. Values are mean + SE from 5 experiments (chorion from 5 different guinea pigs). * = $p < 0.05$ vs. basal; # = $p < 0.05$ vs. basal and control.

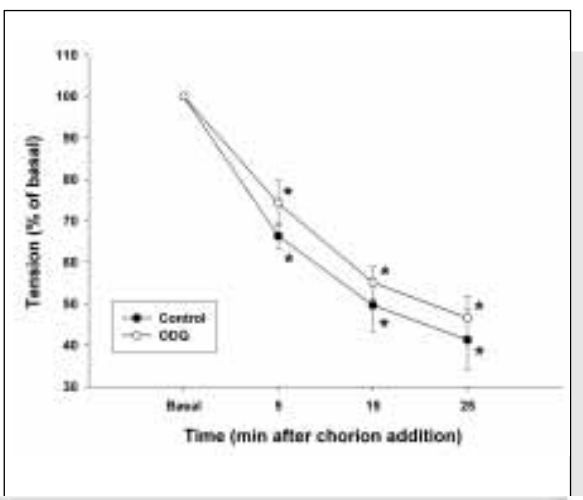

Figure 10. Effect of myometrial incubation with ODQ (30μM) on chorion induced relaxation. Values are mean + SE from 5 experiments (chorion from 5 different guinea pigs). * = $p < 0.05$ vs. basal.

Thus, human chorion has virtually an identical effect as did the guinea pig chorion on oxytocin-stimulated contraction of guinea pig myometrium.

Effect of Blocking Myometrial cGMP Pathway on Chorion-Induced Relaxation

The protein kinase G (PKG) inhibitor Rp-8-Br-cGMP (30 μM) failed to block the relaxing effect of chorion. On the contrary, the inhibitory activity of chorion was slightly but significantly increased by the drug. As illustrated in Figure 9, the relaxation induced by chorion at 15 and 25 minutes in those strips pre-incubated with Rp-8Br-cGMP was significantly greater than in the controls ($p < 0.05$).

Figure 10 illustrates that the inhibition of soluble guanylate cyclase activity in myometrial strips by ODQ did not alter the response of oxytocin-stimulated myometrium to chorion. Chorion significantly decreased oxytocin-induced contractility at 5, 15 and 25 minutes ($p < 0.01$) in both control and treated strips.

Effect of Inhibition of Putative Relaxing Agonist Synthesis in the Chorion

Blocking NO synthesis in the chorion by L-NNA failed to alter chorion-induced relaxation. As demonstrated in Figure 11, at 5, 15 and 25 minutes after chorion addition, there was a significant reduction in contractile activity in both control and treated myometrial strips ($p < 0.01$)

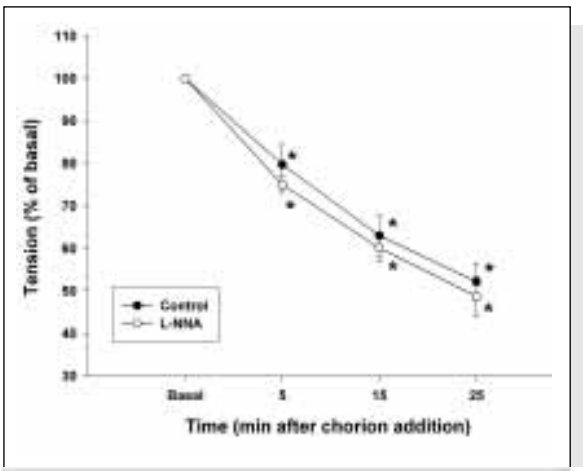

Figure 11. Effect of chorion incubation with L-NNA (10^{-4} M) on chorion induced relaxation. Values are mean + SE from 5 experiments (chorion from 5 different guinea pigs). * = $p < 0.01$ vs. basal.

The inhibition of any potential CO production in the chorion by blocking hemoxygenase with tin-protoporphyrin failed to impact on chorion-induced relaxation, as illustrated in Figure 12. Both tin-protoporphyrin and vehicle incubated chorion significantly reduced oxytocin-stimulated contraction compared to baseline at all the studied periods ($p < 0.05$ at 5 minutes; $p < 0.01$ at 15 and 15 minutes).

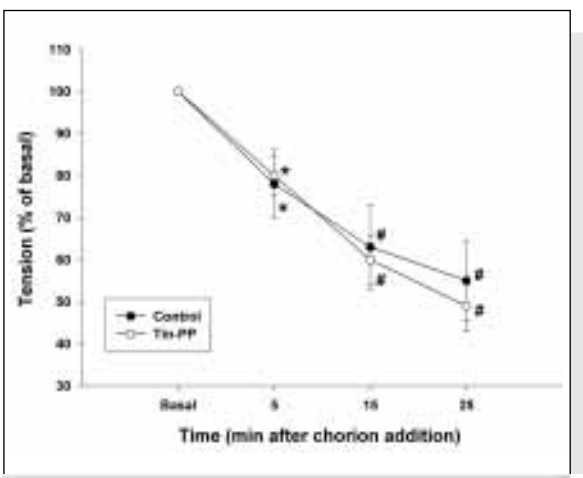

Figure 12. Effect of chorion incubation with Tin-Protoporphyrin (30 μM) on chorion induced relaxation. Values are mean + SE from 5 experiments (chorion from 5 different guinea pigs). # = $p < 0.05$ vs. basal; # = $p < 0.01$ vs. basal.

Inhibition of prostaglandin synthase with indomethacin failed to alter the chorion-induced relaxation of the myometrium. In

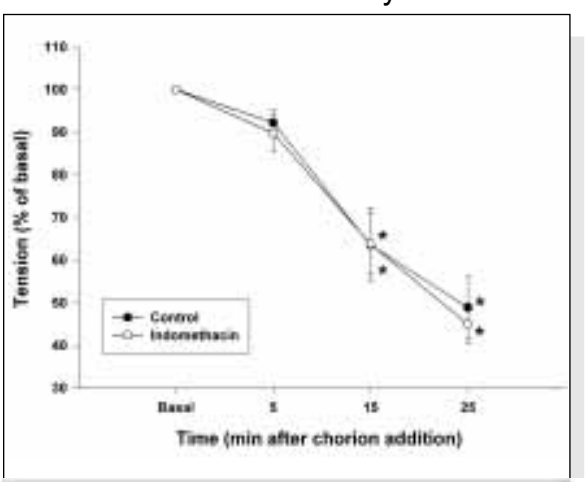

Figure 13. Effect of chorion incubation with Indomethacin (10 μM) on chorion induced relaxation. Values are mean + SE from 5 experiments (chorion from 5 different guinea pigs). * = $p < 0.01$ vs. basal.

this series, both treated and control chorion produced a significant reduction in contractility at 15 and 25 minutes ($p < 0.01$) as showed in Figure 13.

Effect of Blocking Myometrial K^+ Channels on Chorion-Induced Relaxation

The addition of K^+ channel antagonists to the organ bath produced an immediate but small increase in oxytocin-stimulated contractile activity that was statistically significant only for 4-aminoperidin (120% of basal contractility, $p< 0.05$). As illustrated in Figure 14, only tetraethylammonium (TEA) and iberiotoxin (IbTx) among K^+ channel blockers significantly ($p<0.05$) inhibited chorion-induced relaxation. TEA and IbTx added to the tissue bath, prior to the addition of chorion, produced a 40% reduction in chorion relaxing capability compared to control (chorion from the same animal added to myometrium in the absence of K^+ channel blocker). Neither glibenclamide nor 4-AP had any significant effect on chorion-induced relaxation. In addition, higher dose of TEA (20 mM) failed to increase its inhibition of the relaxing activity of chorion.

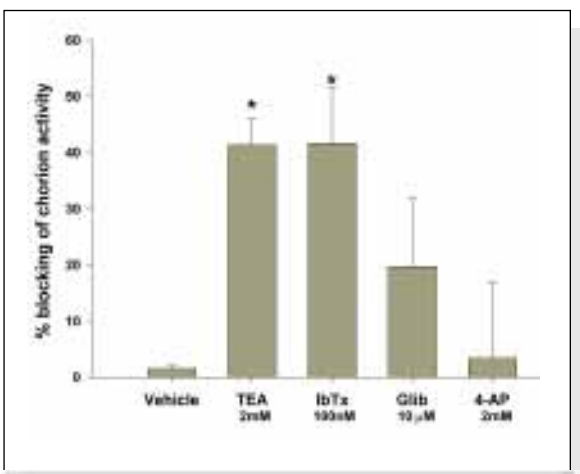

Figure 14. Effect of myometrial incubation with K^+ channel blockers on chorion induced relaxation. Values are mean + SE from 8 experiments for each drug used (chorion from 8 different guinea pigs for each drug used). * = $p < 0.05$ vs. vehicle.

Effect of Natriuretic Peptides on Oxytocin-Stimulated Contractility

Both ANP and BNP, but not CNP produced dose dependent relaxation of oxytocin-stimulated myometrial contractility (Figure 15). The effect of BNP was greater than ANP ($p<0.05$). This result was unexpected considering that ANP, compared to BNP, has greater affinity for the guanylate cyclase type A receptor (GC-A). The threshold concentration for both BNP and ANP was 10^{-8} M; relaxation induced at 10^{-6} M (the maximal dose used), was 52% and 32% of basal respectively ($p<0.05$).

Temporal Course of Natriuretic Peptide-Induced Relaxation

Figure 16 illustrates the effect of a single concentration (10^{-7} M) of ANP and BNP on oxytocin-stimulated contractility. During

the first 5 minutes, the magnitude of the relaxation induced by BNP was greater than ANP (31% vs. 12% relaxation respectively; $p < 0.05$).

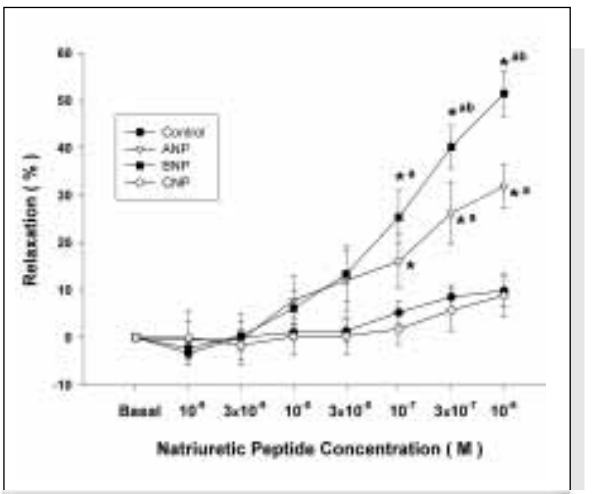

Figure 15. Effect of Natriuretic Peptides on oxytocin-induced contractility of pregnant guinea pig myometrium. Values are mean + SEM from 5 experiments in duplicate. * = $p < 0.05$ vs. basal; a = $p < 0.05$ vs. control; b = $p < 0.05$ vs. ANP.

The relaxation induced by BNP but not ANP decreased significantly over time, though relaxation to both peptides remained greater than baseline for the entire 30 minutes study period ($p<0.05$, Figure 16) and from the temporal control at each time up to 12.5 minutes for BNP and 22.5 minutes for ANP ($p<0.05$, Figure 16).

Effect of Anantin on Natriuretic Peptide-Induced Relaxation

Figure 17 illustrates the effect of anantin (10^{-6} M) on the natriuretic peptide-induced relaxation (during the first 5 minute interval). There was no effect of anantin alone on oxytocin-stimulated contractility.

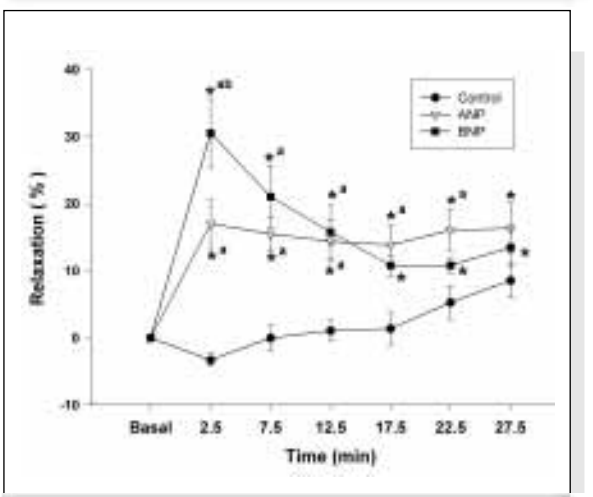

Figure 16. Temporal course of a single dose natriuretic peptide (10^{-7} M) induced relaxation of oxytocin-induced contractility. Times in minutes are 5-min intervals represented by the midpoint of the interval. Values are mean + SEM from 5 experiments in duplicate. * = $p < 0.05$ vs. basal; a = $p < 0.05$ vs. control; b = $p < 0.05$ vs. ANP.

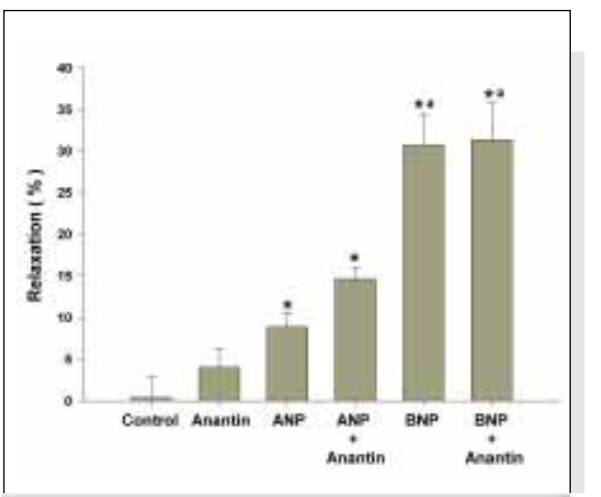

Figure 17. Effect of Anantin (10^{-6}M) on single dose natriuretic peptide (10^{-7} M) induced relaxation of oxytocin-induced contractility. Anantin was added to the bath 5 min before natriuretic peptide. Contractility was measured in the 5-min interval after drug addition. Values are mean + SEM from 5 experiments in duplicate. * = $p < 0.05$ vs. control; a = $p < 0.05$ vs. ANP.

Both ANP and BNP alone relaxed the oxytocin-stimulated myometrium by 9% and 31%, respectively ($p< 0.05$). Anantin did not alter the relaxation produced by a single concentration (10^{-7} M) of either ANP or BNP.

Effect of Rp-8-Br-cGMP on BNP-induced relaxation

The effect of Rp-8-Br-cGMP on BNP-induced relaxation of oxytocin-stimulated myometrium is shown in Figure 18. Under control conditions, a single dose of 10^{-7} M BNP produced a 30% relaxation. The 30 minutes preincubation with 30 μM Rp-8-Br-cGMP had no effect on this BNP-induced relaxation (32% relaxation, Figure 18).

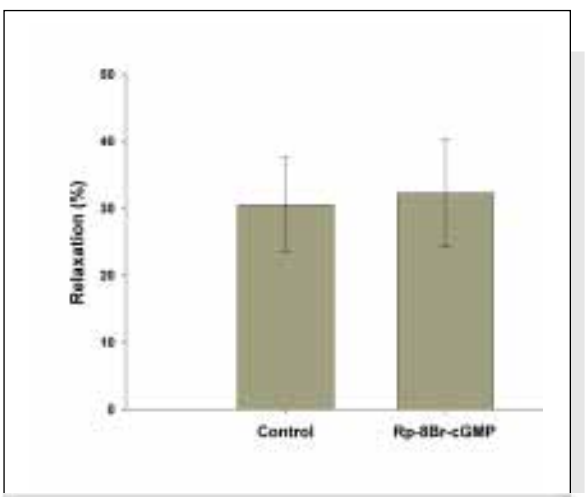

Figure 18. Effect of Rp-8-Br-cGMP (30 μM) on single dose BNP (10^{-7} M) induced relaxation of oxytocin-induced contractility. Strips were pre-incubated with Rp-8-Br-cGMP by 30 min. Contractility was measured in the 5-min interval after BNP addition. Values are mean + SEM from 5 experiments in duplicate.

Effect of cANP on Oxytocin Induced Contractility

Atrial natriuretic peptide-fragment 4-23 amide (cANP), a specific agonist of the natriuretic peptide clearance receptor, had no significant effect on oxytocin-stimulated myometrial contractions at either 10^{-7} or 10^{-6} M (Figure 19).

Effect of PTX and TEA on BNP-induced relaxation

The effects of PTX and TEA preincubation on BNP-induced relaxation are illustrated in Figure 20. Under control conditions, BNP produced 26% relaxation at 10^{-7} M. Neither PTX (5 mg/ml) nor TEA (2mM) had any effect on this BNP-induced relaxation

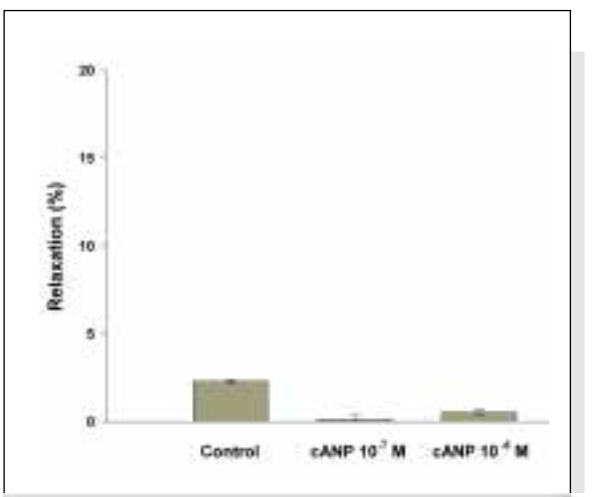

Figure 19. Effect of a single dose of cANP on oxytocin-induced contractility of pregnant guinea pig myometrium. Values are mean + SEM from 5 experiments in duplicate.

of oxytocin-stimulated myometrial contractions (Figure 20).

Natriuretic Peptide-Induced Myometrial cGMP Generation

Both ANP and BNP, but not CNP increased myometrial cGMP content (Figure 21). The myometrial cGMP content under basal conditions was 7.41 + 1.6 ρmol/mg protein (average + SE). After the addition of 10^{-7} M ANP or BNP, the content rose to 45.14 + 9.5 ρmol/mg protein and 28.87 + 4.58 ρmol/mg protein, respectively ($p < 0.05$).

As illustrated in Figure 21, a single concentration (10^{-7} M) of ANP, compared to BNP, generated a larger increase in cGMP levels at 2.5 minutes (7 vs. 4.5 fold increase from the basal level respectively; $p<0.05$). This finding is consistent with the higher GC-A receptor affinity reported for ANP compared to BNP. The results of this experiment did not change when conducted in the presence of 10^{-8} M oxytocin. In this situation, the basal levels of cGMP were increased 6.5 fold by 10^{-7}M ANP and 4.5 fold by 10^{-7} M BNP ($p< 0.05$).

The temporal course of the cGMP increase (Figure 21) paralleled the relaxation profile induced by both ANP and BNP (Figure 16). cGMP content decreased over time, but remain significantly elevated over the basal level throughout the experiment ($p < 0.05$).

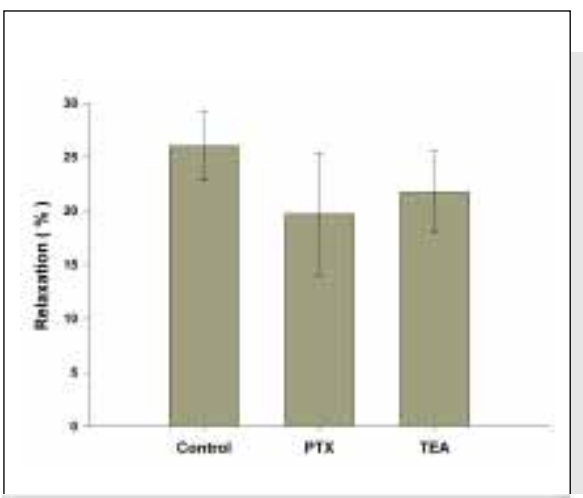

Figure 20. Effect of pertussis toxin (PTX, 5mg/ml) and tetraethylammonium (TEA, 2mM) on single dose BNP (10^{-7} M) induced relaxation of oxytocin-induced contractility. Strips were pre-incubated with PTX by 3 hours. TEA was added to the bath 5 min before BNP. Contractility was measured in the 5-min interval after BNP addition. Values are mean + SEM from 5 experiments in duplicate.

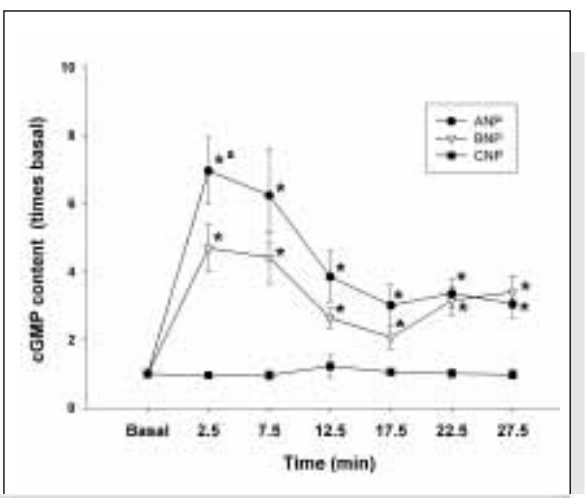

Figure 21. Temporal course of myometrial cGMP generation induced by a single dose (10^{-7} M) of natriuretic peptides. cGMP was measured at basal time and every 5 min starting at 2.5 min after natriuretic peptide addition. Values are mean + SEM from 5 experiments in duplicate. * = $p < 0.05$ vs. basal; a = $p < 0.05$ vs. BNP.

Effect of Anantin on Natriuretic Peptide-Induced cGMP Generation

Figure 22 illustrates the effect of anantin (10^{-6} M) on myometrial cGMP content at 2.5 minutes after a single concentration (10^{-7}M) of either ANP or BNP. Anantin alone had no effect on cGMP content (6.57 + 2.0 ρmol/mg protein), compared to the basal myometrial cGMP level of 5.26 + 1.1 ρmol/mg protein.

ANP alone increased cGMP to 34.95 + 6.7 ρmol/mg. In the presence of anantin, however, ANP increased only to 21.77 + 4.1 ρmol/mg ($p < 0.05$ compared to no anantin). BNP produced a cGMP increase to 22.38 + 3.4 and 14.17 + 2.64 in the absence and presence of anantin respectively ($p < 0.05$). Therefore, as shown in Figure 22, anantin significantly reduced the rise in cGMP stimulated by both ANP and BNP approximately 40%.

BNP Expression in Human Gestational Tissues

The presence of BNP mRNA in a sample of human term not in labor chorion was demonstrated by RT-PCR (Figure 23). The primers were designed to generate a 250 bp product, corresponding to the band identified in the gel. The gene sequence of the product eluted from this band was 100% concordant with a portion of the reported coding sequence of human BNP.

An RPA of human gestational tissues revealed BNP mRNA only in the chorion (Figure 24). BNP was also present at high

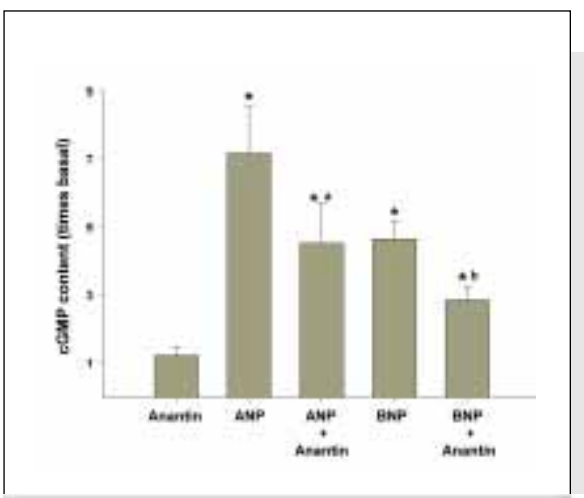

Figure 22. Effect of Anantin (10^{-6} M) on myometrial cGMP levels induced by a single dose (10^{-7} M) of natriuretic peptides. Anantin was added to the bath 5 min before natriuretic peptide. cGMP was measured 2.5 min after natriuretic peptide addition. Values are mean + SEM from 5 experiments in duplicate. * = $p < 0.05$ vs. basal; a = $p < 0.05$ vs. ANP; b = $p < 0.05$ vs. BNP.

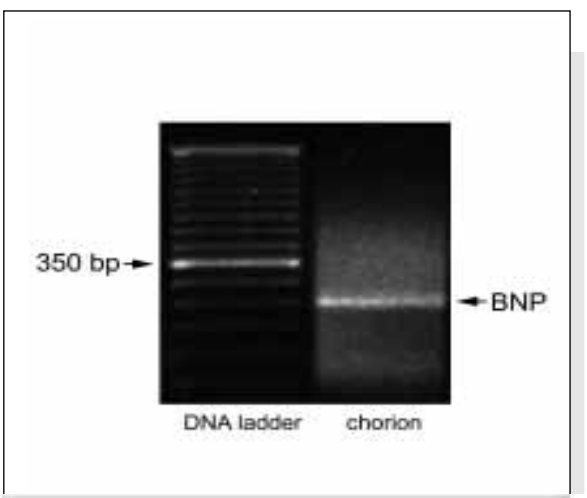

Figure 23. RT-PCR for BNP in a human chorion sample, obtained from a woman at term, not in labor. The right arrow indicates the 250 bp product amplified by PCR. The band was cut, the product was eluted and sequenced to prove it was BNP. The left lane shows the 50 bp DNA ladder used to identify the size of the products

levels in the positive control (human brain), confirming the effectiveness of our probe. We could not demonstrate BNP mRNA in human myometrium (either non pregnant or pregnant at term) or amnion at term. The expression of BNP mRNA in human term chorion was in a similar magnitude to the human brain (Figure 24).

BNP mRNA was present in both preterm and term chorion. However, the expression level of BNP mRNA, normalized to β-actin, was 4 times greater in the chorion obtained from preterm pregnancy compared to that near term but not in labor (Figure 25).

Effect of 8-Br-cGMP on Oxytocin-Stimulated Myometrial Contractility

8-Br-cGMP produced a dose dependent decrease in oxytocin-stimulated contractile activity in myometrium of the pregnant guinea pig. Relaxation was apparent at 10^{-5} M ultimately reaching 38% of the agonist-stimulated baseline at $3x10^{-4}$ M (the maximal concentration used) as illustrated in Figure 26. The relaxing capability of 8-Br-cGMP was independent of myometrial gestational age and similar between myometrium from pregnant and non-pregnant guinea pig.

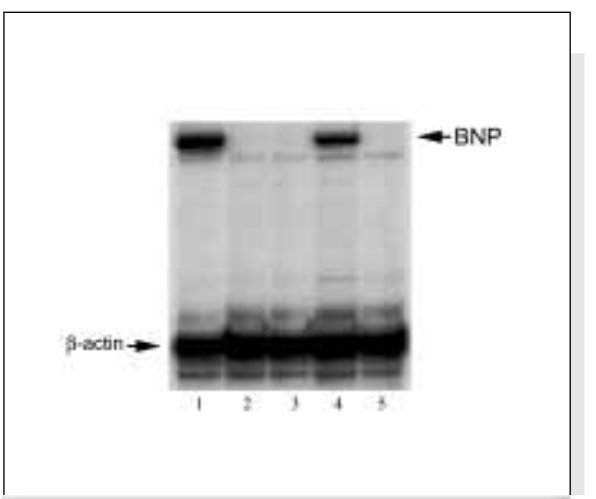

Figure 24. RPA for BNP in human gestational tissues. The presence of BNP mRNA was demonstrated only in the brain (positive control) and in the chorion sample. Lanes represent: 1 brain control; 2 non pregnant myometrium; 3 pregnant myometrium at term not in labor; 4 chorion at term; 5 amnion at term.

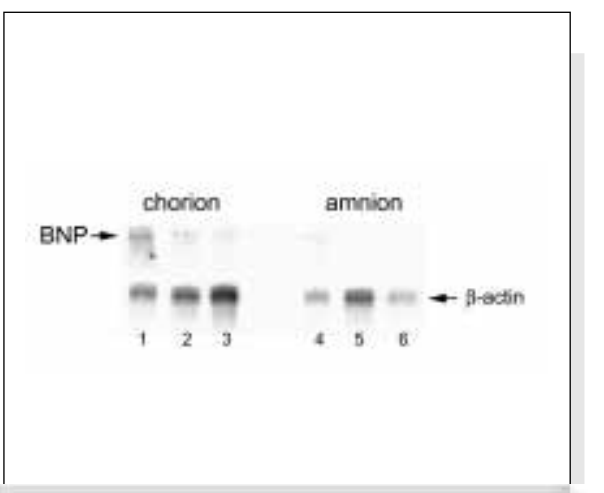

Figure 25. RPA for BNP in human chorion and amnion. The presence of BNP was demonstrated in all chorion samples, but not in the amnion. BNP expression levels were 4 times higher in the preterm chorion compared to term chorion. Lanes represent: 1 and 4 chorion and amnion at preterm not in labor (30 weeks); 2 and 5 chorion and amnion at term not in labor (38 weeks); 3 and 6 chorion and amnion at term not in labor (39 weeks).

Effect of Fetal Membranes on Myometrial cGMP Content

We investigated the fetal membranes as potential source of an endogenous inducer of myometrial cGMP production. The incubation of strips of myometrium with chorion increased dramatically myometrial cGMP content (more than 3 times) (Figure 27). In contrast, the incubation of myometrium with amnion had no effect. Furthermore, the stimulatory effect of the chorion on myometrial cGMP production was gestational age dependent: chorion from pre-term gestation elicited a greater effect compared to chorion from near-term gestation (Figure 28).

Opposite to the afore noted results, incubation of the myometrium with thawed chorion in the absence of IBMX (similar protocol to the chorion-induced relaxation protocol) did not alter myometrial cGMP content at any time point studied. Therefore, it was not possible to correlate the degree of relaxation and the magnitude of the cGMP increase produced by chorion. The explanation for this observation became clear subsequently.

Effect of Guanylate Cyclase Blockers on Chorion-Induced cGMP Increase

The inhibitors of the NO-cGMP pathway failed to alter the basal cGMP content of myometrial strips. Further, neither the NO synthase inhibitor, LNA (Figure 29), nor the specific soluble guanylate cyclase antagonist ODQ (Figure 29) inhibited the

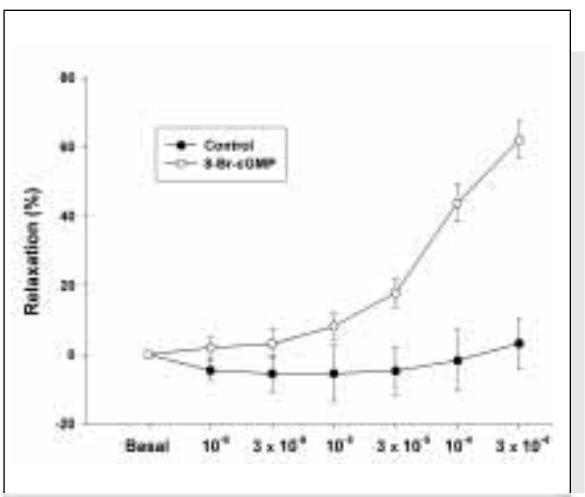

Figure 26. Effect of cumulative doses 8-Br-cGMP on oxytocin induced contractility in myometrial strips from term pregnant guinea pig. Values are mean + SE from 6 experiments in duplicate.

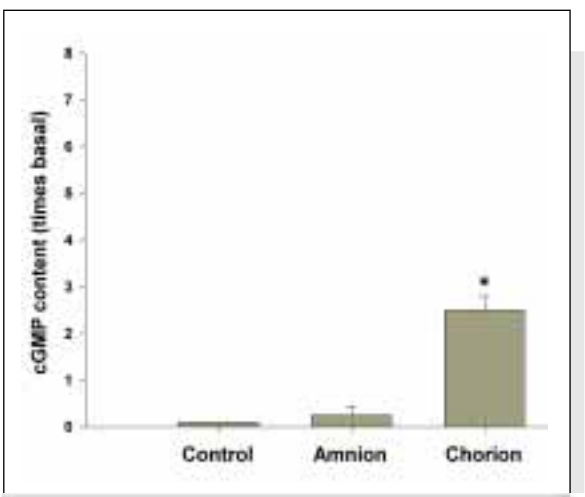

Figure 27. Myometrial cGMP levels in strips from near-term pregnant guinea pig after in-vitro incubation with fetal membranes. Values are mean + SE from 10 experiments in duplicate. * = p< 0.05 vs. control and amnion.

increase in myometrial cGMP produced by co-incubation with chorion.

Inhibitors of the natriuretic peptide-cGMP pathway also failed to produce any change in the basal cGMP. Further, the antagonists of particulate guanylate cyclase (HS 142-1 and Anantin) failed to inhibit the increase in cGMP content of the myometrial strips incubated with chorion (Figure 30). As shown in the Figure 30, HS 142-1 had a paradoxical effect further enhancing the chorion-induced increase in cGMP.

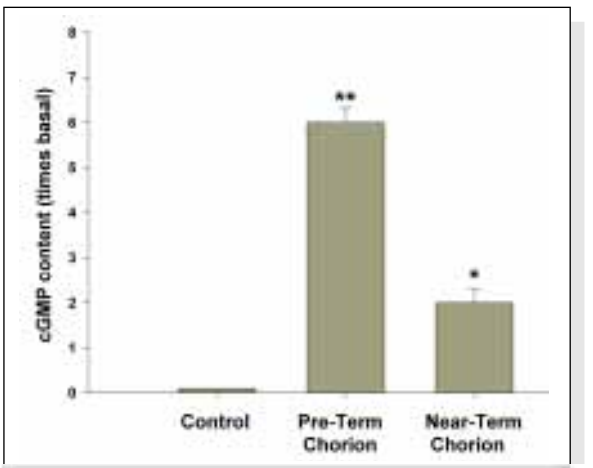

Figure 28. Myometrial cGMP levels in strips from near-term guinea pig after in-vitro incubation with pre-term and near-term chorion. Values are + SE from 10 experiments in duplicate. ** = $p < 0.05$ vs. near-term chorion and control; * = $p < 0.05$ vs. control.

cGMP Production by the Chorion

Chorion released large amounts of cGMP into the media. After a 15-minutes incubation in the presence of IBMX, the concentration of cGMP reached on average 2.53 μM (range 0.26 - 7.5 μM)

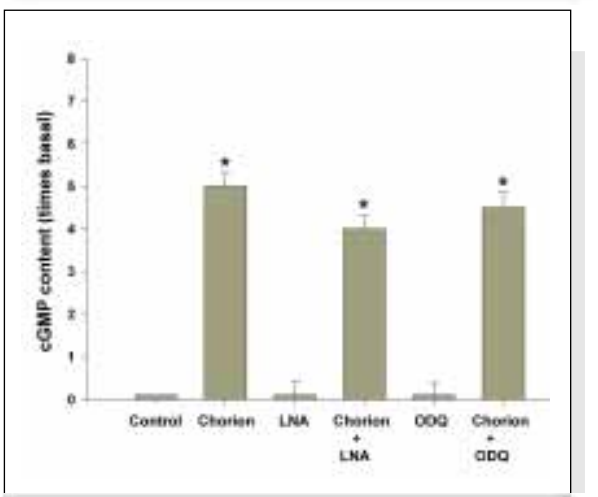

Figure 29. Effect of NOS inhibition (LNA, 10^{-4} M) and soluble guanylate cyclase blocking (ODQ, 30μM) on chorion-induced cGMP levels of myometrium from near-term pregnant guinea pig. Values are mean + SE from 5 experiments in duplicate. * = $p < 0.05$ vs. control.

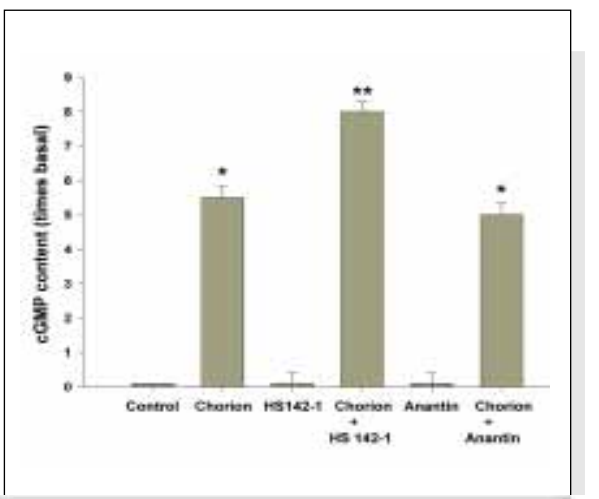

Figure 30. Effect of Particulate Guanylate Cyclase Inhibition with HS 142-1 and Anantin on chorion-induced cGMP levels of myometrium from near-term pregnant guinea pigs. Values are mean + SE from 5 experiments in duplicate. * = $p < 0.05$ vs. control; ** = $p < 0.05$ vs. chorion alone and control.

for crushed chorion, and 0.36 μM (range 0.15 - 0.68 μM) for thawed chorion.

Effect of Myometrial Incubation with cGMP

As illustrated in Figure 31, incubation of myometrial strips with exogenous cGMP produced a significant increase in the cGMP content as measured by RIA. In the range studied (1 to 5000 nM), the cGMP content of the myometrium increased linearly from 2.8 to 35.6 ρmols/mg protein between 100 nM to 5000 nM (0.1 μM - 5 μM).

Tissue Distribution of cGMP

Among gestational tissues, chorion contained significantly more cGMP (by RIA) compared to the amnion and myometrium ($p < 0.01$). As illustrated in Figure 32, the amount of cGMP per mg of protein was more than 30 times greater in the chorion compared to the myometrium. The mean + SE was 11.54 + 3.22 ρmol/mg protein; 373.11 + 83.35 ρmol/mg protein; and 16.66 + 6.26 ρmol/mg protein in the amnion, chorion and myometrium, respectively.

The cGMP content of all those tissues studied changed with advancing

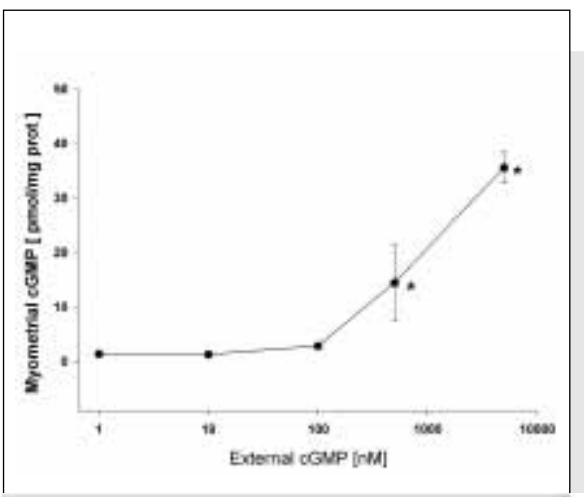

Figure 31. Effect of myometrial incubation with external cGMP on myometrial cGMP content. Values are mean + SE from 5 experiments in triplicate. * = $p < 0.05$ vs. basal level.

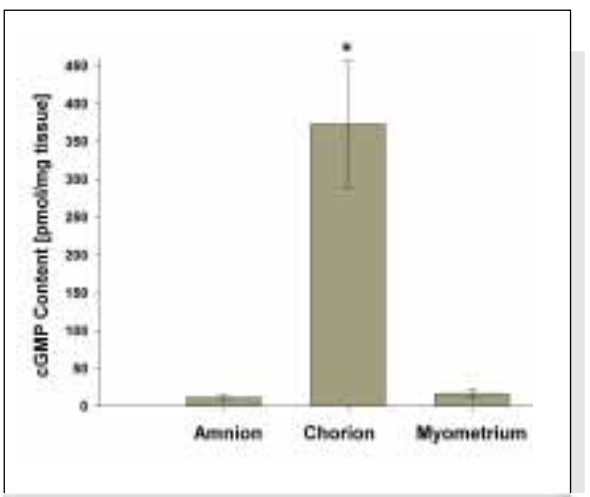

Figure 32. cGMP content of gestational tissues from guinea pig. Values are mean + SE from 15 animals. * = $p < 0.01$ vs. myometrium and amnion.

gestational age. As previously described, myometrial cGMP content rose significantly ($p<0.05$) during pregnancy before declining significantly ($p<0.05$) immediately prior to labor (Figure 33). Those samples classified as near-labor belonged to guinea pigs at 67 + 2 d whose symphysis pubis was separated >0.5 cm, a sign that precedes delivery by approximately 24h.

The cGMP content of chorion also increased significantly ($p<0.05$) with advancing gestational age, but a decrease prior to labor was not seen (Figure 34). Amnion cGMP followed the same temporal pattern as the chorion (Figure 35).

The immunohistochemistry studies were consistent with the measurements of cGMP by RIA. As illustrated in Figure 36,

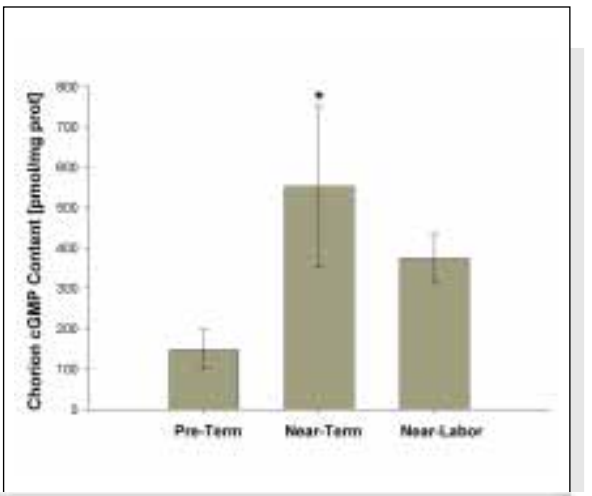

Figure 34. Chorion cGMP content across gestation. Values are mean + SE from 5 animals. * = $p < 0.01$ vs. pre-term.

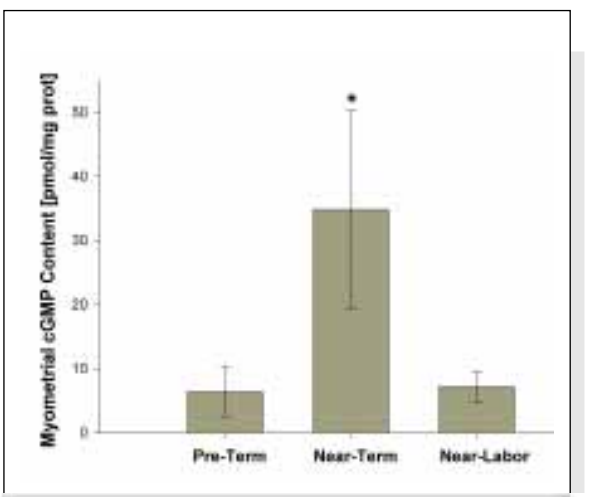

Figure 33. Myometrial cGMP content across gestation. Values are mean + SE from 5 animals. * = $p < 0.01$ vs. pre-term and near-labor.

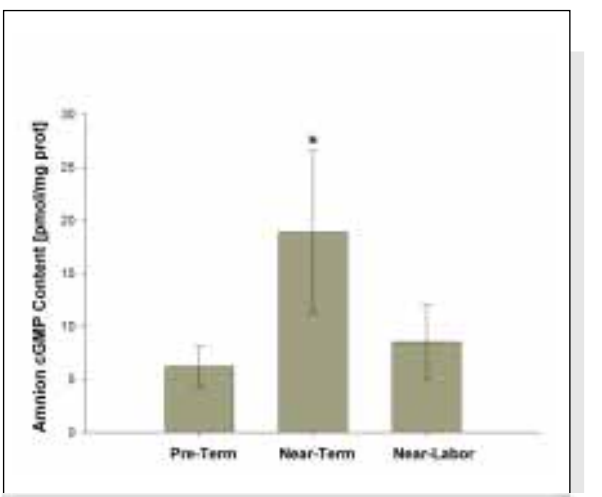

Figure 35. Amnion cGMP content across gestation. Values are mean + SE from 5 animals. * = $p < 0.01$ vs. pre-term.

cGMP immunoreactivity was very strong in the chorion. cGMP was undetectable by this method in the myometrium and decidua; amnion was not studied. All tissues were negative in the control slides (absence of primary antibody) confirming the specificity of the technique.

After incubation of the myometrial strips with exogenous cGMP, the immunohistochemistry slides were positive in the decidua, but in low amount. No cGMP immunoreactivity was detected in the myometrial cells after incubation with exogenous cGMP (Figure 37).

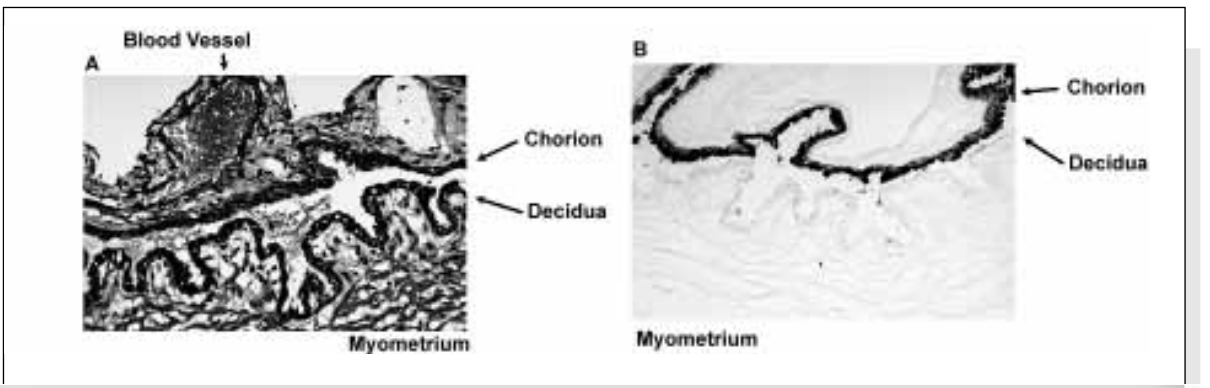

Figure 36. Immunohistochemistry for cGMP on myometrium-decidua-chorion preparations. Panel A shows a Hematoxylin-Eosin stain. Panel B shows IHC for cGMP using a specific sheep anti-cGMP primary antibody. cGMP immunoreactivity is present almost exclusively on chorion tissue. Original magnification 10X.

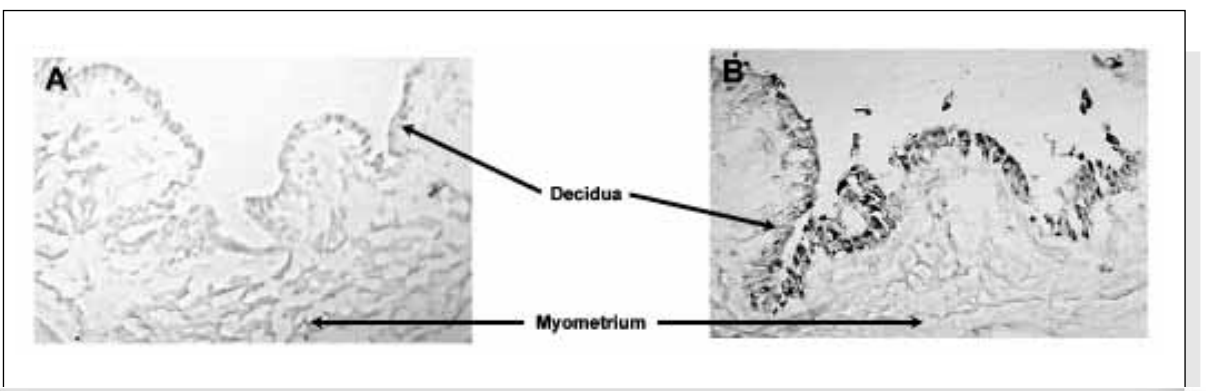

Figure 37. Immunohistochemistry for cGMP on myometrium-decidua preparations after incubation with external 1000 nM cGMP. Panel A shows a negative control (absence of primary antibody). Panel B shows IHC for cGMP using a specific sheep anti-cGMP primary antibody. cGMP immunoreactivity is present exclusively on decidua. Original magnification 10X.

DISCUSSION

This series of investigations advanced our understanding in six main areas: a.) The effect of the fetal membranes on myometrial contractility; b.) The mechanism of action underlying chorion-induced relaxation of the myometrium; c.) The effect of natriuretic peptides on myometrial contractility; d.) The mechanism of the natriuretic peptide-induced relaxation; e.) The role of cGMP in the maintenance of uterine quiescence during pregnancy; and f.) The distribution of cGMP among gestational tissues.

Chorion Releases A Substance that Inhibits Oxytocin-Stimulated Myometrial Contractility

We have demonstrated for the first time that the chorion, but not amnion, decreases oxytocin-stimulated myometrial contraction. This effect was dependent on the gestational age of the chorion (declining as pregnancy progressed), independent of the gestational age of the myometrium, and reproduced by chorion-conditioned media.

Chorion from pre-term guinea pigs produced greater inhibition of oxytocin-stimulated myometrial contraction than chorion from near-term animals. The pre-term membranes were obtained from guinea pigs at 0.7 gestation, which corresponds to 28 weeks of human pregnancy. This time period is characterized by the near absence of myometrial contraction under normal conditions. The near-term membranes studied approximated 0.9 gestation, a time period roughly equivalent to 36 weeks of human pregnancy. By this stage, myometrial activation has usually begun and the myometrium is more sensitive to contractile agonists (Norwitz et al., 1999). Since the relaxation of oxytocin-stimulated myometrium by chorion was similar regardless of the gestational age of the myometrium, the gestational age differences of chorion-induced relaxation are due to differences in the capability of the chorion to produce relaxation rather than to differences in the ability of myometrium to relax. These observations support our working hypothesis that the chorion produces a substance or substances that may have a role in the maintenance of uterine quiescence and that the generation of this substance(s) decreases near-term to allow myometrial activation.

We demonstrated that the effect of chorion-conditioned media was similar to the effect of whole chorion on oxytocin-stimulated myometrial contraction, suggesting that the inhibitory substance was released by the chorion and therefore capable of acting in a paracrine fashion. We also demonstrated that fresh chorion had the same relaxing ability as the thawed chorion, thus discarding the possibility of an artifactual effect of freezing upon the membrane.

Prior studies by other investigators have noted that the human chorion releases a substance that inhibits spontaneous and prostaglandin-stimulated, but not oxytocin-stimulated myometrial contraction of the rat uterus, suggesting a role for the fetal membranes in the maintenance of uterine quiescence (Collins et al., 1993; Collins et al., 1995; Collins et al., 1996; Emery et al., 1998). While chorion inhibited spontaneous (Collins et al., 1993) and agonist-induced contractions to prostaglandin $F_2\alpha$ (Collins et al., 1995) and the L-type calcium channel opener BAY K 8644 (Collins et al., 1996), they failed to inhibit oxytocin-induced contractions in isolated strips obtained from non-pregnant rat uterus. Emery et al proposed that a Ca^{2+}-channel blocker was released from chorion that inhibits the activation of L-type Ca^{2+} channels of myometrium (Emery et al., 1998).

In the present study, we report the new finding that guinea pig chorion releases a factor that inhibits oxytocin-induced contractility in the myometrium obtained from the pregnant guinea pig. This factor is clearly different from the one described by Collins et al since our model used myometrium from pregnant animals and the inhibited contractions were produced with oxytocin. Further, human chorion, in contrast to the Collin's report, inhibited oxytocin-stimulated contraction of myometrium obtained from pregnant guinea pigs. As the two experimental designs are similar, we presume the apparently divergent findings are due to differences in the model (pregnant vs. non-pregnant myometrium) and species (guinea pig vs. rat).

To an attempt to further differentiate our model and the rat, we investigated the effect of other contractile agonists. However, as previously reported by others, the guinea pig myometrium failed to respond to other uterotonins such as prostaglandins, endothelin and platelet activating factor with regular contractions. In our experiments, these drugs at high concentration (10^{-4} M) produced only a single, tetanic contraction lasting 3 to 5 minutes, followed by the absence of contractile activity. We were able to investigate the effect of chorion on

spontaneous activity, however. We demonstrated that spontaneous contractions, like those induced by oxytocin were also inhibited by the chorion, but not by the amnion.

The guinea pig provides several strengths compared to the rat which aids our search for an understanding of human pregnancy. First, it has hemomonochorial placentation, which is the most similar among all non-primate mammals to human placenta (Pijnenborg et al., 1981). Second, the sex hormone profile of its estrous cycle and pregnancy are similar to humans (Challis et al., 1971; Martensson, 1984). Third, the pattern of in vitro contractility of myometrium from pregnant guinea pig is more similar to human than the rat myometrium. And finally, the relatively long and stable gestational period (67 + 2 days from copulation) compared to 22 days for the rat, facilitates comparisons between different gestational ages. Additionally, our model has the advantage of using myometrium obtained from pregnant animals, which may be a central to the differences from prior publications in light of the important modifications that occur during pregnancy.

We recognize that the human and guinea pig chorion are embryologically and structurally different (Ramsey, 1975). Thus, it is reasonable to ask if the present findings in the guinea pig chorion are applicable to humans. Our preliminary data indicated that human chorion obtained from term pregnancy, prior to the onset of labor, is like the near term guinea pig chorion. Human chorion inhibited oxytocin-stimulated contraction of myometrium from pregnant guinea pigs to almost the exact same degree as the guinea pig chorion (Figure 8). As a consequence, we anticipate that the guinea pig myometrium can in the future be used as a biological model to investigate the physiology of human chorion. The clear implication of our preliminary data is that a chorion-derived substance may modulate myometrial contractility in the human.

The chemical identity of the substance(s) released by the chorion of guinea pig (and perhaps the human) that inhibits oxytocin-stimulated contractions has (have) yet to be elucidated. Several chemicals produced by the fetal membranes, decidua and myometrium itself have been studied and suggested as possible regulators of myometrial quiescence based of their known ability to relax smooth muscle (Acevedo and Ahmed, 1998; Bansal et al., 1997; Brodt-Eppley and Myatt, 1999; Downing and Hollingsworth, 1992; Ferguson et al., 1992; Itoh et al., 1993). And though a singular line of evidence

suggests that the inhibitory role of the fetal membranes, particularly the chorion, on uterine contractile activity is based on its capacity to locally degrade uterotonins and prevent their action on the myometrium (Germain et al., 1994), our findings refute this possibility since the effect of chorion was lost by washing the strips and replacing the bath with fresh buffer not containing oxytocin. Indeed, our findings suggest that oxytocin stimulates a regular pattern of activity in the myometrium that after a while becomes independent of the presence of oxytocin in the bath. After a regular pattern of contractility was stimulated by oxytocin, the replacement with fresh buffer did not abolish the contractions. The same inductory effect of oxytocin occurs in human myometrium (Germain, 1998. Personal Communication).

In summary, the chorion releases one or more factors that mediate myometrial quiescence in a paracrine fashion during pregnancy. While we document the ability of the chorion to inhibit oxytocin-stimulated myometrial contractility in vitro, the exact nature of the relaxing factor (or factors) released by chorion during gestation remains to be elucidated. The identification and characterization of the mechanism of action of this substance(s) are essential for an understanding the process of parturition and for the development of pharmacological tools useful in the management of preterm labor in humans. This was the focus of the rest of our work.

Mechanism of the Chorion-Induced Relaxation

Pregnancy changes the production and action of many agents with smooth muscle relaxing capability (Acevedo and Ahmed, 1998; Bansal et al., 1997; Dong et al., 1999; Downing and Hollingsworth, 1992; Itoh et al., 1993). Their intracellular second messengers have been studied in the myometrium and neighboring tissues (Buhimschi et al., 1995; Meera et al., 1995; Weiner et al., 1994) as related to the maintenance of uterine quiescence. No agent, however, provides a full explanation of the mechanisms underlying uterine quiescence, suggesting that several mediators may participate in the process. Considering that several relaxing pathways are proposed but not proven to be involved in the maintenance of myometrial quiescence, we investigated their possible roles in chorion-induced relaxation. Our results do not, at this time, support roles for either the cGMP-PKG pathway, nitric oxide, carbon monoxide or prostaglandins as the mediators of the paracrine action of the chorion on

myometrial contractility. Furthermore, our investigations demonstrate that chorion-induced relaxation is substantially mediated by a factor that opens large conductance Ca^{2+} activated K^{+} channels in the myometrium.

The absence of an inhibitory effect of Rp-8Br-cGMP on chorion-induced relaxation demonstrates that the chorion-induced increase in myometrial cGMP content does not mediate the chorion-induced relaxation. We also found that the chorion increases myometrial cGMP content in vitro. As a result of these findings, we now conclude that the high levels of myometrial cGMP reported during the pregnancy and its decrease prior to labor are events not causally related to myometrial quiescence.

We have demonstrated that cGMP analogs inhibit myometrial contractility (Figure 26). We studied preliminarily the effect of 10^{-5} M 8-Br-cGMP on chorion-induced relaxation, by adding this drug at the end of two chorion experiments. We observed that this cyclic nucleotide analog produced further relaxation, suggesting that its effect was additive to that of the chorion. Our experiments inhibiting PKG activity revealed that the addition of Rp-8Br-cGMP alone stimulated a mild increase in myometrial PKG activity. This paradoxical effect may be explained by a partial agonistic activity of this analog on PKG and the effect of Rp-8Br-cGMP on chorion-induced relaxation the additive result of two different relaxing pathways.

Nitric Oxide (NO) and Carbon Monoxide (CO) were proposed as putative mediators of uterine quiescence (Acevedo and Ahmed, 1998; Buhimschi et al., 1995). It is known that activation of soluble guanylate cyclase is the principal mechanism underlying the cellular actions of NO and CO (Moncada et al., 1991; Wang, 1998). Herein, we demonstrate that the inhibition of myometrial soluble guanylate cyclase, by ODQ, has no affect on chorion-induced relaxation. Thus, a role for NO and CO on chorion-induced relaxation can be almost completely discarded.

While it is accepted that the cGMP pathway is the central mechanism underlying NO induced relaxation, it has been suggested that myometrial relaxation induced by NO does not involve the cGMP pathway (Bradley et al., 1998; Hennan and Diamond, 1998). Our results are also inconsistent for a role of a non-cGMP mechanism for chorion-derived NO, since NOS inhibition in the chorion failed to alter its ability to produce relaxation of oxytocin stimulated contraction. Likewise, non-cGMP mediated CO induced smooth muscle relaxation have been reported (Wang,

1998). Our results refute this possibility given that inhibition of CO production in the chorion, by blocking hemoxygenase, failed to produce changes in chorion-induced relaxation.

Prostaglandin synthesis occurs in the fetal membranes, and its actions have been associated with an increase in myometrial contractile activity prior to parturition (Olson et al., 1995). However, it has also been shown that both excitatory and inhibitory prostaglandin receptors are expressed by the myometrium (Senior et al., 1993). In human myometrium, the prostaglandin receptor EP_2, that produces relaxation when activated increases with advancing gestation before declining prior to the onset of labor. These observations suggest a possible role for prostaglandins in generating myometrial quiescence (Brodt-Eppley and Myatt, 1999). However, a role for a chorion-derived prostaglandin was not sustained by our experiments since inhibition of chorion prostaglandin synthase by indomethacin failed to alter chorion-induced relaxation.

We previously demonstrated that the increase in myometrial cGMP evidenced during pregnancy was not related to soluble guanylate cyclase activity in the guinea pig, and suggested that an activator of particulate guanylate cyclase, such as a natriuretic peptide like substance, may be involved in the maintenance of uterine quiescence (Weiner et al., 1994). However, our present results also discard a role for cGMP.

We confirm the presence of BNP in the human chorion and hypothesize that a natriuretic peptide, perhaps BNP, may be one of the chorion-derived substances that relax the myometrium. The cleanest strategy to confirm BNP as a mediator of chorion-induced relaxation would be to test the effect of a BNP receptor blocker on chorion effect. Our experiments revealed that natriuretic peptide-induced relaxation is mediated by a novel mechanism that does not involve either activation of the known natriuretic receptors or the cGMP pathway. As we do not as yet have a drug capable of inhibiting the natriuretic peptide induced relaxation, this approach cannot be used. Thus, we cannot at this time eliminate the possibility that a natriuretic peptide contributes to the maintainence of uterine quiescence.

The action of the chorion-derived quiescent factor was blocked some 40% by the addition of K^+ channel antagonists such as iberiotoxin and tetraethyl-amonium. Considering the specificity of IbTx (Galvez et al., 1990), and the lack of an effect by the others K^+ channel

antagonists, (glibenclamide and 4-aminoperidine), we hypothesize that the chorion-derived quiescent factor acts on the myometrium by opening the large conductance Ca^{2+} activated K^+ channels (BK_{Ca}). These BK_{Ca} are present in rat and human myometrium (Khan et al., 1993). Their pharmacological properties change during labor (Khan et al., 1997), and their opening by specific drugs (eg NS-1619) inhibits oxytocin-induced myometrial contractility in vitro (Khan et al., 1998).

TEA blocks other channels in addition to the BK_{Ca}. Thus, the similar magnitude of effect for IbTx and TEA suggests that the chorion-released quiescent factor does not affect other types of K^+ channels. Further, a higher concentration of TEA produced no additional inhibitory effect on chorion-induced relaxation suggesting that the chorion releases either more than one quiescent factor, or a single substance with more than one mode of action. It is certainly possible (and teleologically likely) that there are redundant mechanisms within the chorion for this crucial activity. While whether or not the chorion releases a natriuretic factor as one of the quiescent factors remains to be determined, natriuretic peptide-induced relaxation was not inhibited by TEA. Thus, we conclude from these specific experiments that the chorion releases more than one substance with the ability to relax oxytocin-stimulated myometrial contraction. The mechanism of action of these substances involves the opening of myometrial Ca^{2+} activated K^+ channels. We have identified two candidate substances for the paracrine regulation by the chorion of myometrial quiescence: a BK_{Ca} opener and a natriuretic peptide.

To confirm the K^+ channel antagonists were acting on the myometrium and not by inhibiting the release of the chorion-derived quiescent factor, we tested preliminarily the effect of these drugs on relaxation produced by the addition of conditioned media. The average of two experiments indicated that chorion conditioned media induced relaxation was also partially inhibited by TEA, supporting our conclusion that chorion-induced relaxation was mediated by a substance that opens myometrial BK_{Ca}.

The relaxation induced by chorion increases progressively over time. We interpreted this to suggest the relaxing substance is released continuously into the media. However, the effect of chorion-conditioned media was also increased over the time. One possible explanation for this observation is that the factor released by the chorion does not directly affect K^+ channels, but rather triggers an intracellular cascade of phosphorylation

and de-phosphorylation events that culminate with an increase in the probability of an open state. Perhaps significantly, myometrial Ca^{2+} activated K^+ channel (BK_{Ca}) activity is modified by phosphorylation of the channel protein (Perez and Toro, 1994). An alternative explanation for the delayed effect of chorion-conditioned media is a necessary delay due to the specific mechanism of action of the as yet unknown quiescent factor. The activation of BK_{Ca} channels enhances membrane hyperpolarization and decreases Ca^{2+} entry through neighboring voltage-gated Ca^{2+} channels. Thus, the net result is a reduction in contractility. This mechanism would be consistent with a report that the drugs used to open BK_{Ca} channels show progressive inhibition of oxytocin-induced myometrial activity over 30 minutes of observation (Khan et al., 1998).

The nature of the substance(s) released by the chorion remains unknown and none of the substances with relaxing capabilities known to be present in the fetal membranes can explain the characteristics described herein. We suggest the chorion releases several factor(s) that modulate myometrial contractility and are responsible for the maintenance of uterine quiescence. This information provides new insights into the regulation of parturition and could ultimately lead to the development of pharmacological tools for the management of preterm labor. Specifically, drugs that modify the activity of BK_{Ca} channels are candidates for use in human diseases as the premature onset of labor.

Natriuretic Peptide-Induced Relaxation is not Mediated by Guanylate Cyclase Activation

We originally hypothesized that at least one of the chorion-derived quiescent factors produced was a natriuretic peptide. Consequently, we tested their ability to decrease oxytocin-stimulated myometrial contractility. ANP and BNP, but not CNP, inhibited oxytocin-stimulated contractions of myometrium obtained from near-term pregnant guinea pigs. And while BNP produced a greater degree of relaxation than ANP, ANP stimulated a greater increase in myometrial cGMP content. Further, anatin inhibited the increase in cGMP stimulated by both ANP and BNP, but had no effect on natriuretic peptide induced relaxation. The results of these experiments indicate that while natriuretic peptides can produce relaxation of oxytocin-stimulated myometrium, the mechanism of action does not require activation of any of the known guanylate cyclase receptors.

Six isoforms of the receptors for natriuretic peptides with guanylate cyclase activity are identified in addition to a non-guanylate cyclase coupled clearance receptor (Foster et al., 1999). Only the type A (GC-A) and type B (GC-B) receptors are reported to be present in rat (Dos Reis et al., 1995) and human myometrium (Itoh et al., 1994). The binding affinity and the order of potency for GC-A activation is ANP > BNP >>> CNP, while for GC-B is CNP >> ANP > BNP (Suga et al., 1992). While our findings regarding the stimulation by natriuretic peptides of cGMP in the myometrium are consistent with this receptor profile, they are inconsistent with natriuretic peptide-induced myometrial relaxation profile.

BNP was a more potent inhibitor of oxytocin-induced myometrial contraction than ANP, while CNP had no effect on contractility. Since CNP does not relax guinea pig myometrium, we conclude that CG-B activation is not involved the relaxation stimulated by ANP and BNP. Furthermore, anantin, a specific competitive blocker of GC-A (Weber et al., 1991) had no effect on the relaxation produced by either ANP or BNP, indicating that BNP-induced relaxation is not mediated by GC-A activation. Thus, we conclude from the available results that BNP induces relaxation of oxytocin stimulated myometrium by a mechanism independent of the activation of either GC-A or GC-B.

In contrast to relaxation, natriuretic peptides do increase myometrial cGMP via GC-A activation. The functional presence of GC-A in guinea pig myometrium from pregnant animals and its higher affinity for ANP are demonstrated by the significantly larger increase in cGMP content induced by ANP compared to BNP. Further, anantin significantly reduced the effect of ANP and BNP on cGMP content indicating that the two peptides activate GC-A to increase cGMP. CNP failed to increase cGMP content supporting our conclusion that GC-B activation was unrelated to cGMP generation in pregnant guinea pig myometrium.

Though the time course for the natriuretic peptide-induced cGMP increase paralleled the relaxing effect of the peptides on myometrial contractility, anantin had no effect on natriuretic peptide-induced relaxation despite inhibiting the cGMP increase. Further, the inhibition of cGMP-dependent protein kinase with Rp-8-Br-cGMP had no effect on the relaxation response to BNP. These results suggest that the cGMP pathway does not mediate the myometrial relaxation produced by ANP and BNP.

The observation that BNP-induced relaxation was greater than ANP induced relaxation, but the increase in cGMP stimulated by BNP was less, supports the conclusion that neither GC-A activation nor the cGMP increase mediate BNP-induced inhibition of oxytocin stimulated myometrial contraction. In summary, we conclude from the available data that the relaxation by natriuretic peptides of pregnant guinea pig myometrium stimulated to contract by oxytocin is either not receptor mediated (a mechanism that would be wholly novel), or mediated by the activation of another receptor distinct from GC-A and GC-B and free of particulate guanylate cyclase activity.

Several reports suggest that natriuretic peptides have biological actions not mediated by guanylate cyclase-coupled receptors. Rather, they function by a signaling activity via the natriuretic peptide clearance receptor (Hempel et al., 1998; Johnson et al., 1991). Activation of this signaling pathway does not result in cGMP generation (Drewett et al., 1992), but does involve a pertussis toxin sensitive G protein (Anand-Srivastava et al., 1996; Murthy and Makhlouf, 1999; Murthy et al., 1998). Activation of the clearance receptor inhibits adenylate cyclase (Drewett et al., 1992), increases phospholipase C activity (Anand-Srivastava and Trachte, 1993) and increases K^+ outward conductance (Anand-Srivastava and Trachte, 1993; Kanwal and Trachte, 1994). A relationship between clearance receptor activation and myometrial contraction / relaxation has not been shown. In the present study, we demonstrate that cANP, a specific agonist of the clearance receptor (Maack et al., 1987), did not inhibit oxytocin-induced contractility. While the presence of the clearance receptor in the myometrium of the pregnant guinea pig has yet to be demonstrated, our results do not support a role for it in the inhibition of oxytocin-induced contraction by natriuretic peptides. In addition, neither PTX (a trimeric G protein inhibitor) nor TEA (a K^+ channel blocker) interfered with BNP-induced relaxation (in contrast to their effect on chorion induced activity) further suggesting that natriuretic peptides do not activate the clearance receptor to cause myometrial relaxation. The finding that both TEA and IbTx inhibit chorion-induced relaxation of oxytocin stimulated contraction suggests that even though the chorion produces BNP, it also produces a second quiescent factor.

In summary, these experiments show that both BNP and ANP, but not CNP, inhibit oxytocin-stimulated contractions of myometrium from the pregnant guinea pig. We propose that natriuretic peptide-

induced relaxation is mediated by activation of a previously undescribed receptor that lacks guanylate cyclase activity and therefore does not involve the cGMP pathway. Our results suggest that BNP has a greater affinity for this receptor than either ANP or CNP. This pathway of relaxation is independent of GC-A or CG-B activation, cGMP generation or clearance receptor activation. Finally, we suggest that a locally produced natriuretic peptide, perhaps BNP, may be involved in generating myometrial quiescence during pregnancy via this novel mechanism.

Chorion Produced BNP may mediate Chorion Induced Relaxation

We hypothesized that at least one of the quiescent factors produced by the chorion was a natriuretic factor. We first demonstrated natriuretic peptides, especially BNP, decrease oxytocin-stimulated myometrial contraction of myometrium from the pregnant guinea pig. We next sought to confirm the presence of BNP in gestational tissues. Despite multiple alignments of the BNP gene sequence reported for a number of species to generate a large number of primers for conserved areas of the gene, we failed to identify BNP mRNA in any guinea pig tissue. We are forced to conclude at this time that either the guinea pig does not express BNP, or its gene shares little homology with the reported sequences.

We next cloned a fragment of the human BNP gene and found that among human gestational tissues, BNP was expressed, and at high levels, only in the chorion. Further, we found that the expression of BNP mRNA as quantitated by RPA, was greater in chorion from preterm (30 weeks) compared to that in term (38 and 39 weeks) pregnancies.

The presence of BNP has previously been reported in human gestational tissues (Itoh et al., 1993). This group demonstrated BNP mRNA in the amnion but not the chorion. However, they only studied term chorion. The BNP they identified in the amnion was obtained from a second trimester pregnancy (15 weeks). In agreement with our preliminary study, they were unable to amplify BNP mRNA from amnion at term (Itoh et al., 1993).

Our findings are preliminary. It remains for us to demonstrate that BNP protein is present and secreted into the conditioned media before we can make a convincing argument that BNP has a role in uterine quiescence.

Role and Tissue Distribution of cGMP During Pregnancy

The studies to date are inconclusive with respect to the role of cGMP in the maintenance of uterine quiescence. However, they suggest that the cGMP pathway is not a key mediator in the process. While the cGMP analog, 8-Br-cGMP, produced a concentration dependent reduction in oxytocin stimulated myometrial contraction, the concentration necessary to produce a 50% reduction was as high as 10^{-4} M. Furthermore, we were able in other experiments, to discard the cGMP pathway as a mediator of either natriuretic peptide or chorion-induced relaxation of oxytocin-stimulated myometrial contraction.

Prior to these studies, it was assumed the source of the myometrial cGMP was the myometrium, produced under the stimulus of a paracrine factor released by the fetal tissues. Chorion induced an increase in myometrial cGMP content, yet the effect of the chorion was not blocked by the inhibition of either soluble or particulate guanylate cyclases. Our incubation studies revealed that the amount of cGMP produced in the chorion is several times greater than that produced by the myometrium. Furthermore, we demonstrated that cGMP produced by the chorion is released into the media in high amounts. Therefore, we postulated that the cGMP measured in the myometrium during gestation might be produced in the chorion and transferred to the myometrium as a paracrine signal between these two important tissues.

The immunohistochemistry studies (Figure 36) dramatically support the postulate. Virtually all the cGMP appears to originate in the chorion. The quantity of cGMP produced by the chorion is massive strongly suggesting some important biologic function. And while we postulate that myometrial cGMP may have a role other than relaxation, a small quiescent effect cannot be excluded.

These results clarify some of our previously unexplained results. Chorion induces an increase in myometrial cGMP content measured by RIA despite the pharmacologic inhibition of myometrial guanylate cyclases. This effect of chorion was reproduced by incubation of the myometrium with exogenous cGMP. Thus, it appears from our experimental model that cGMP is released by the chorion, transferred to the media, and then to the myometrial tissue. In other models, it was reported that cGMP can be released into the extracellular space and there act as a paracrine mediator (Chevalier et al., 1996; Radziszewski et al., 1995).

In an attempt to clarify where the cGMP generated by the chorion goes, we investigated the immunolocalization of the cGMP after incubation of the tissues with exogenous cGMP. We described for the first time that cGMP in vitro is present almost exclusively in the chorion. We also found that after incubation with exogenous cGMP, this cyclic nucleotide taken up by the decidua but not myometrial cells. These studies support the likelihood of paracrine communication between the chorion and decidua but not the myometrium, an observation further rejecting a role for cGMP in the maintenance of uterine quiescence. It is possible that the cGMP content measured in biopsies of myometrium, reported by many authors reflects only the differential content of decidual cGMP.

We also cannot dismiss the possibility that this cGMP transfer demonstrated in vitro is only an experimental artifact. The experiment required the presence of IMBX to inhibit phosphodiesterase activity. Thus, the very high levels of cGMP measured in the chorion conditioned media are not reflective of the physiologic event. On the other hand, our confirmation that cGMP content changes with gestational age suggests precise control of cGMP synthesis supporting the possibility of a physiologic role.

Much work remains before our understanding of the paracrine regulation of myometrial contractility during pregnancy is complete. While the results of our work are not the final solution, they provide novel, and we believe, important new knowledge bringing us closer to the ultimate goal. We have elucidated some of the physiological mechanisms involved in the maintenance of pregnancy; identified one paracrine mechanism for myometrial relaxation, and provide studies suggesting the existence of novel substances and pathways involved in the generation of myometrial quiescence during pregnancy. These results open the door to future investigations and provide new avenues for novel therapeutic approaches to preterm labor.

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APPENDICES

Glossary of Abbreviations

4-AP	= 4-Aminoperidine
ANP	= Atrial Natriuretic Peptide
BNP	= Brain Natriuretic Peptide
BK_{Ca}	= Large Conductance Ca^{2+} activated K^{+} Channel
cANP	= Atrial Natriuretic Peptide fragment 4-23 Amide
CNP	= C-type Natriuretic Peptide
CO	= Carbon Monoxide
GC-A	= Guanylate Cyclase type A Receptor
GC-B	= Guanylate Cyclase type B Receptor
Glib	= Glibenclamide
IbTx	= Iberiotoxin
IHC	= Immunohistochemistry
NO	= Nitric Oxide
NOS	= Nitric Oxide Synthase
PKG	= cGMP-dependent Protein Kinase
PTX	= Pertussis Toxin
RT-PCR	= Reverse Transcription-Polymerase Chain Reaction
RPA	= Ribonuclease Protection Assay
TEA	= Tetraethylammonium

Pharmacological Tools Used

4-Aminoperidine	= voltage gated K^+ channel blocker
Anantin	= competitive inhibitor of GC-A receptor
cANP	= specific agonist of the natriuretic peptide clearance receptor
Glibenclamide	= ATP-sensitive K^+ channel blocker
HS 142-1	= particulate guanylate cyclase inhibitor
Iberiotoxin	= large conductance Ca^{2+} activated K^+ channel blocker
IBMX	= phosphodiesterase inhibitor
Indomethacin	= cycloxygenase inhibitor
L-NNA	= nitric oxide synthase inhibitor
ODQ	= specific blocker of soluble guanylate cyclase
Pertussis Toxin	= specific blocker of G protein α_i subunit
Rp-8Br-cGMP	= membrane permeable cGMP analog, specifically blocks PKG
8-Br-cGMP	= membrane permeable cGMP analog
TEA	= non-specific K^+ channel blocker
Tin-Protoporphyrin	= hemoxygenase inhibitor