BASIC SCIENCE: OBSTETRICS

Progesterone protects fetal chorion and maternal decidua cells from calcium-induced death

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OBJECTIVE: The purpose of this study was to determine whether progesterone exerts a protective effect in chorion and decidua cells when exposed to calcimycin.

STUDY DESIGN: Fetal membrane samples were collected from term elective repeat cesarean deliveries and chorion and decidua cells that are separated and cultured. Cells were pretreated with progesterone and exposed to calcimycin. Cell viability was determined, and percent cell viability was calculated.

RESULTS: Exposure to calcimycin resulted in a reduction of cell viability in both chorion and decidua cells in a dose-dependent fashion. In chorion and decidua cells, progesterone pretreatment followed by calcimycin increased cell viability compared with calcimycin treatment alone (chorion, 67%, vs controls, 24%; $P < .001$; decidua, 58%, vs controls, 35%; $P < .001$). The progesterone receptor antagonist, RTI 6413-49a, blocked the protective effect of progesterone in both chorion and decidua cells.

CONCLUSION: These preliminary results suggest that progesterone may provide a protective effect in fetal membrane cells and that this effect may be mediated through the progesterone receptor.

Key words: progesterone, calcimycin, cytotoxicity, fetal membrane, membrane progesterone receptor

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Rupture of fetal membranes before 37 weeks of gestation without evidence of labor, termed PPROM, occurs in approximately 2%-3% of all pregnancies but is associated with 20% of all perinatal deaths. We have demonstrated previously, in patients at term and patients with PPROM, accelerated apoptosis in the chorion cells of fetal membranes in the presence of chorioamnionitis. Using immunohistochemistry, we were able to demonstrate that subjects with histologic chorioamnionitis exhibited twice as many apoptotic nuclei in the chorion cell layer as did subjects without chorioamnionitis.[1] When we studied subjects with PPROM separately, we confirmed our previous findings and additionally found that nearly 37% of subjects with PPROM completely lacked the chorion layer of fetal membranes. The cause of the cellular mechanisms that are involved in the destruction of the membranes is not understood. It is known that exposure to lipopolysaccharide and ischemia induces cell death and that elevated intracellular calcium concentrations are an important step in the process at the cellular level.[2]

Progesterone governs a wide range of biologic processes that are related to the maintenance of pregnancy, which includes an antiapoptotic action at the cellular level in various cell types and tissues.[2,3] Functions that are attributed to progesterone in pregnancy include the stimulation of growth and differentiation of the endometrium to allow for implantation, the inhibition of myometrial contractions, and the induction of immune tolerance to the fetus.[4,5]

Progesterone also has been the focus of intense clinical research in the area of preterm birth prevention.[6,7] Although continued evidence supports the use of progesterone for the prevention of recurrent preterm birth, little is understood about the exact mechanisms by which progesterone functions in these patients. The classic physiologic effects of progesterone are mediated by the interaction of the hormone with specific intracellular progesterone receptors that are members of the nuclear receptor family of transcription factors.[8-10] The nuclear progesterone receptors consist of 2 protein isoforms that are termed A and B that are expressed from a single gene in rodents and humans.[11,12] Preliminary data from our laboratory demonstrated the expression of progesterone receptor A and B messenger RNA and protein in human fetal membranes and determined that these levels do not change in cell culture.[13]

Recently published studies indicate that progesterone may also act through a nonclassic mechanism that involves G-protein– and/or protein kinase G–coupled signaling.[14,15] The relationship of the various possible progesterone signaling mechanisms to the initiation of cell death is unclear.

As previously noted, we have demonstrated that apoptosis is common in the

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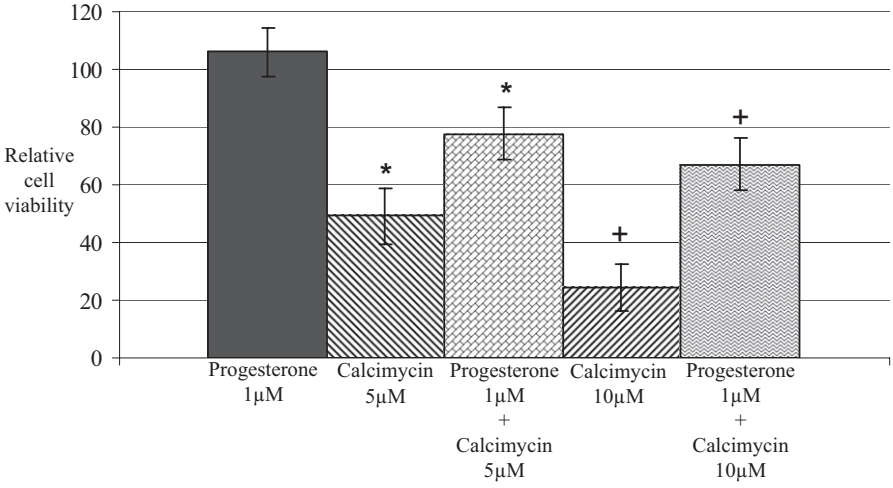
chorion layer of fetal membranes in the presence of chorioamnionitis and that this specific cell layer is destroyed in patients with PPROM.[1] Based on preliminary evidence, we believe that cell death in the fetal membranes is an important contributor to the risk for preterm delivery, specifically PPROM. We hypothesize that altered calcium homeostasis may contribute to cell death in the chorion and decidua cells of fetal membranes. Further, we hypothesize that progesterone offers a protective effect from calcium-mediated premature cell death in fetal membranes. Our objective was to determine whether progesterone protects cultured chorion and decidua cells from calcium-induced cell death.

FIGURE 1
Calcimycin vs progesterone + calcimycin in fetal chorion cells



Cells were pretreated for 24 hours with 1.0 μmol/L progesterone in DMEM and then exposed to calcimycin. Experimental conditions were repeated, and cell viability was assessed after an additional 48 hours in culture. Progesterone exposure was 1.0 μmol/L for all experimental arms that are shown. Data are shown relative to cell viability of untreated controls. Data represent mean ± SE. The *asterisk* indicates a probability value of .07 for calcimycin at 5.0 μmol/L; the *plus symbol* indicates a *P* value of <.003 for calcimycin at 10.0 μmol/L.

MATERIALS AND METHODS

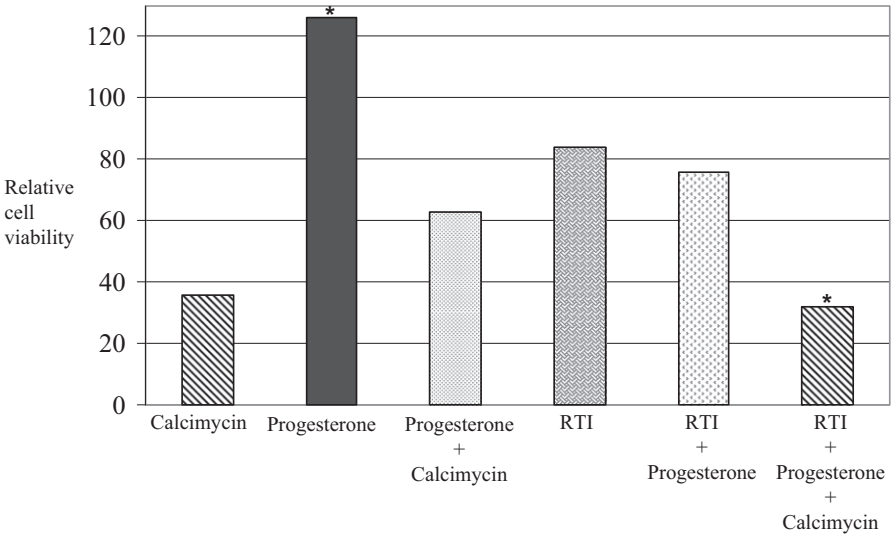
Placentas were collected from women who underwent planned cesarean delivery at term, before labor, and without rupture of membranes (n = 5). Institutional review board approval was obtained for a waiver of consent to obtain de-identified tissue that was not to be used for clinical purposes. Tissue was transported to the laboratory in Dulbecco's modified Eagle medium (DMEM)–Hams F12 media. Fetal membrane tissue was cut into 2 × 2–inch squares with forceps and scalpel. The smooth layer of amnion was pulled off manually. Separation of the decidua and chorion involved blunt dissection with forceps and scalpel. Each layer was minced by cross-cutting with scalpel blades. Tissues were processed in digestion buffer I (0.125% trypsin and 0.02% DNase I) at 37°C for 30 minutes, followed by centrifugation at 2000 rpm for 10 minutes. The cell pellet was resuspended in digestion buffer II (0.125% trypsin, 0.02% DNase, 0.2% collagenase [Sigma Aldrich, St. Louis, MO]), and incubated for 60 minutes at 37°C. Cells were filtered through 4 layers of sterile gauze and centrifuged at 2000 rpm for 10 minutes.[16] A cell-separation gradient was prepared with an Optiprep column (Sigma Aldrich), with steps that ranged from 4% to 40% of 4 mL each (4%, 6%, 8%, 10%, 20%, 30%, and 40%). Processed chorion or decidua cells were added to the top of the gradient and then centrifuged (1000*g*) at room temperature for 30 minutes. Cells between densities of 1.049 and 1.062 g/mL represented the chorion layer. Cells between densities of 1.027-1.038 g/mL represented the decidual layer. Harvested cells were washed with DMEM, centrifuged, and resuspended in DMEM. Cell viability routinely was >90%, which was verified by trypan blue staining; cells types were plated at a density of 10^6 cells/mL in DMEM/Hams F-12/10% serum plus antibiotic/antimycotics (penicillin G 100 U/mL, streptomycin sulfate 100 mg/mL, amphotericin B 1.0 mg/mL) and cultured for 24 hours. Immunohistochemistry was performed to confirm the purity of cell culture, as previously described.[13]

The cells were analyzed with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) to assess cell viability. The assay is composed of solutions of a novel tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes that are found in metabolically active cells. The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture, which is determined every 30 minutes to a total of 4 hours by a spectrophotometric plate reader (Spectra MAX 190; Molecular Devices, Sunnyvale, CA) at 37°C. All samples, including blank wells that contained media only (no cells), were run in quadruplicate The quadruplicate data points from each experimental condition were pooled to create an average after the background reading from blank wells was subtracted. The percent of viable cells relative to untreated cells was determined by dividing the average optical density for each experimental condition by the average optical density for the untreated controls.

After 24 hours, cultured cells were pretreated with progesterone for 24 hours

then exposed to calcimycin, which is a calcium ionophore (A23187) that increases intracellular calcium concentrations, for an additional 24 hours. Treatment with progesterone followed by calcimycin was repeated for an additional 48 hours (pretreatment with progesterone for 24 hours followed by calcimycin for an additional 24 hours), and cell viablility assays were performed. Dose-response experiments were performed for all conditions. Cell viability was calculated with respect to untreated chorion or decidua cells. Data from 5 separate patient membrane samples were used for analysis (n = 20 individual data points per treatment). Data were analyzed with paired *t* tests and 2-way analysis of variance between different experimental conditions, with significance defined as a probability value of <.05 (Analyse-It Software Inc, Leeds, UK). The inhibitor studies were performed in quadruplicate on a single membrane sample and were analyzed.

FIGURE 2
RTI-6413-49a abrogates the cytoprotective effect of progesterone in fetal chorion cells that are exposed to calcimycin



All experimental arms used the following doses: calcimycin 10.0 μmol/L, progesterone 1.0 μmol/L, and RTI 1.0 μmol/L. Data are shown relative to cell viability of untreated controls and are presented as the mean. The *asterisk* indicates a probability value of .001 for progesterone treatment, compared with RTI 6413-49a followed by progesterone then calcimycin.

RESULTS

Calcimycin consistently reduced relative cell viability in both chorion and decidua cells in a dose-dependent fashion. In fetal chorion cells progesterone (1.0 μmol/L) followed by calcimycin (10.0 μmol/L) significantly increased relative cell viability, compared with calcimycin treatment alone (67% vs 24%; $P < .001$). Dose-response experiments indicated that increasing calcimycin concentrations decreased relative cell viability (Figure 1). Despite dose-dependent increases in calcimyin-induced cell death, progesterone continued to provide a protective effect. When decidua cells were treated in the same fashion, there was again improved relative cell viability in the progesterone-pretreated (1.0 μmol/L) cells, compared with the calcimycin (10.0 μmol/L) alone (58% vs 35%; $P < .001$).

To determine a possible role of cross reactivity with the glucocorticoid receptor, cells were pretreated with dexamethasone and then exposed to calcimycin. When calcimycin treatment was compared with pretreatment with dexamethasone (0.1, 1.0, and 10 μmol/L) followed by calcimycin, there was a pronounced, albeit statistically nonsignificant, difference in relative cell viability (24% vs 79%; $P = .058$). Pretreatment with dexamethasone in fetal chorion cells did not improve relative cell viability when exposed to calcimycin or when compared with pretreatment with progesterone (dexamethasone [1.0 μmol/L] 79% vs progesterone [1.0 μmol/L] 90%; $P =$ not significant). In decidua cells, pretreatment with dexamethasone did not improve relative cell viability, compared with progesterone pretreatment in the same experiment (data not shown).

To further support the hypothesis that the effect of progesterone is mediated by the progesterone receptor, we attempted to block the action of progesterone using the progesterone antagonist RTI-6413-49a,[17] which specifically blocks progesterone receptor function.[18] As shown in Figure 2, 1.0 μmol/L RTI 6413-49a displayed antagonist activity and inhibited the protective effect of progesterone (1.0 μmol/L) when chorion cells were exposed to calcimycin (89.8% vs 36.7%; $P < .001$). This same effect was demonstrated in decidua cells when cells that were treated with progesterone alone were compared with cells that were treated with RTI-6413-49a plus progesterone followed by calcimycin (130% vs 30.5%; $P = .001$).

COMMENT

The results of this preliminary investigation suggest that progesterone may provide protection to calcium ion changes in human fetal chorion and maternal decidua cells and that these effects may be mediated directly through the progesterone receptor. Previously, we demonstrated through the use of real-time quantitative polymerase chain reaction and Western blot analysis that the classic nuclear progesterone receptor is present in both the chorion and decidua layers of the fetal membranes. It is possible, however, that the effects that were identified in this investigation are the results of progesterone's action on the the nuclear receptor or other nonnuclear progesterone receptors.[14,15,19]

By demonstrating a partial protective effect on both chorion and decidua cells when they were pretreated with dexa-

methasone, this investigation suggests that there may be cross reactivity between glucocorticoid and progesterone receptor action. We suspect that progesterone receptor action may be more important in this process, because we were able to block this protective effect by the administration of a specific progesterone receptor antagonist. The clinical observation that progesterone may be important in preventing recurrent preterm birth requires investigators to better understand the mechanisms involved. This investigation suggests that the presence of progesterone may protect chorion and decidua cells of the fetal membranes from calcium-induced cell death that can result from infection, ischemia, or oxidative stress. This protective effect, in part, may contribute to clinical observations and is therefore an important area of research to pursue.

It is recognized widely that many intracellular functions are mediated or regulated by calcium-binding proteins and therefore that calcium is an essential ion to cell homeostasis. The calcium ionophore calcimycin (A23187) has been used in models of calcium-dependent cytotoxicity and apoptosis.[20,21] However, the signaling pathways that control calcimycin-induced cell death are not clear.

Cell death that results from ischemia or oxidative stress is often a result of increases in cytosolic calcium levels.[22] Increases in intracellular calcium concentration because of the calcimycin (A23187) treatment may activate the permeability transition pore of the mitochondrial membrane that leads to increased intracellular calcium concentrations from both and intra- and extracellular sources. The permeability transition pore of the mitochondria constitutes the first rate-limiting event of the common pathway of apoptosis.[23] On permeability transition pore activation, apoptogenic factors from the mitochondria, which include cytochrome c and apoptosis-inducing factor, leak into the cytoplasm and begin a cascade of proteolytic activity that ultimately leads to nuclear damage (DNA fragmentation, DNA mutations) and cell death.[23] In the fetal membranes, exposure to mediators of inflammation and tissue remodeling and ischemia may also provide these same stimuli, which results in increased intracellular calcium and cell death.

The role of progesterone in the maintenance of pregnancy and the impact on the fetal membranes is not well understood, and the role of progesterone on cell viability within the fetal membranes in periods of stress has not been investigated. The initial members of the progesterone receptor family consisted of the nuclear receptors progesterone receptor–A and –B that mediate gene transcription. In addition to nuclear progesterone receptor–induced gene transcription, other receptor/signal transduction pathways could account partially or completely for the actions of progesterone that were identified in the cells of the fetal membranes in this investigation. In a recent review, Peluso[24] described the role of progesterone in the inhibition of apoptosis in granulosa cells of the ovary through protein kinase G activation and the maintenance of low intracellular calcium concentrations that are mediated through specific membrane progesterone receptors. Protein kinase G may act at several sites to regulate cytosolic calcium. One suggested mechanism involves an increase in calcium-activated potassium channel gating (BK channels).[25-27] Increased BK-channel activity hyperpolarizes the membrane and reduces intracellular calcium influx through voltage-gated calcium channels. Increased BK-channel activity appears to arise from both a direct activation by protein kinase G and increased activity of spontaneous transient outward currents that are produced by small bursts of calcium that is released from the superficial sarcoplasmic reticulum.[28] In our investigation, exposure to calcimycin that presumably led to increased intracellular calcium and subsequent cell death was blocked when cells were pretreated with progesterone. It remains for future studies to determine whether progesterone action involves a protein kinase G-dependent mechanism in cells of the fetal membranes that helps maintain normal intracellular calcium concentrations.

Exposure of the fetal membranes to stressors, which include ischemia, hypoxia, oxidative stress, and calcium overload, may occur as a result of early or chronic insults during pregnancy. Known risk factors for PPROM include smoking, bleeding during pregnancy, and infection, all of which are potential causes of these stressors. It is possible that progesterone functions to protect cells of the fetal membranes through its classic transcriptional activation mechanisms or through multiple nonexclusive signaling pathways. Much work remains to be done to evaluate these possibilities, but the results of this investigation suggest a potential protective role for progesterone on cell viability in the fetal membranes that has not been described previously. The understanding of the molecular function of progesterone at the cellular level is critical to the advancement of targeted treatment strategies and new treatment modalities in women who are at risk for preterm delivery and PPROM. ■

REFERENCES

1. Murtha AP, Auten R, Herbert WN. Apoptosis in the chorion laeve of term patients with histologic chorioamnionitis. Infect Dis Obstet Gynecol 2002;10:93-6.

2. Rotello RJ, Lieberman RC, Lepoff RB, Gerschenson LE. Characterization of uterine epithelium apoptotic cell death kinetics and regulation by progesterone and RU 486. Am J Pathol 1992;140:449-56.

3. Rueda BR, Hendry IR, Hendry Iii WJ, Stormshak F, Slayden OD, Davis JS. Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Biol Reprod 2000;62:269-76.

4. Piccinni MP, Giudizi MG, Biagiotti R, et al. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. J Immunol 1995;155:128-33.

5. Szekeres-Bartho J, Wegmann TG. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. J Reprod Immunol 1996;31:81-95.

6. Meis PJ. 17 Hydroxyprogesterone for the prevention of preterm delivery. Obstet Gynecol 2005;105:1128-35.

7. Spong CY, Meis PJ, Thom EA, et al. Progesterone for prevention of recurrent preterm birth: impact of gestational age at previous delivery. Am J Obstet Gynecol 2005;193:1127-31.

8. Conneely OM, Lydon JP. Progesterone receptors in reproduction: functional impact of the A and B isoforms. Steroids 2000;65:571-7.

9. Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol 2001;179:97-103.

10. Tsai HW, LaPolt PS, Olcott AP, Lu JK. Temporal changes occur in the neuroendocrine control of gonadotropin secretion in aging female rats: role of progesterone. Biol Reprod 2004;71:845-52.
11. Kastner P, Bocquel MT, Turcotte B, et al. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. J Biol Chem 1990;265:12163-7.
12. Kraus WL, Montano MM, Katzenellenbogen BS. Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters. Mol Endocrinol 1993;7:1603-16.
13. Mills AA, Yonish B, Feng L, Schomberg DW, Heine RP, Murtha AP. Characterization of progesterone receptor isoform expression in fetal membranes. Am J Obstet Gynecol 2006;195:998-1003.
14. Karteris E, Zervou S, Pang Y, et al. Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. Mol Endocrinol 2006;20:1519-34.
15. Peluso JJ, Pappalardo A, Losel R, Wehling M. Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. Endocrinology 2006;147:3133-40.
16. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF 3rd. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986;118:1567-82.
17. Sathya G, Jansen MS, Nagel SC, Cook CE, McDonnell DP. Identification and characterization of novel estrogen receptor-beta-sparing antiprogestins. Endocrinology 2002;143:3071-82.
18. Leonhardt SA, Edwards DP. Mechanism of action of progesterone antagonists. Exp Biol Med 2002;227:969-80.
19. Engmann L, Losel R, Wehling M, Peluso JJ. Progesterone regulation of human granulosa/luteal cell viability by an RU486-independent mechanism. J Clin Endocrinol Metab 2006;91:4962-8.
20. Matsubara K, Kubota M, Adachi S, et al. Different mode of cell death induced by calcium ionophore in human leukemia cell lines: possible role of constitutive endonuclease. Exp Cell Res 1994;210:19-25.
21. Qian T, Herman B, Lemasters JJ. The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187. Toxicol Appl Pharmacol 1999;154:117-25.
22. Ermak G, Davies KJ. Calcium and oxidative stress: from cell signaling to cell death. Mol Immunol 2002;38:713-21.
23. Crompton M, Virji S, Doyle V, Johnson N, Ward JM. The mitochondrial permeability transition pore. Biochem Soc Symp 1999;66:167-79.
24. Peluso JJ. Multiplicity of progesterone's actions and receptors in the mammalian ovary. Biol Reprod 2006;75:2-8.
25. Alioua A, Huggins JP, Rousseau E. PKG-I alpha phosphorylates the alpha-subunit and upregulates reconstituted GKCa channels from tracheal smooth muscle. Am J Physiol 1995;268:L1057-63.
26. Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, Weir EK. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc Natl Acad Sci U S A 1994;91:7583-7.
27. Fukao M, Mason HS, Britton FC, Kenyon JL, Horowitz B, Keef KD. Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. J Biol Chem 1999;274:10927-35.
28. Nelson MT, Cheng H, Rubart M, et al. Relaxation of arterial smooth muscle by calcium sparks. Science 1995;270:633-7.