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MINIREVIEW

Regulation of Uterine Function: a Biochemical Conundrum in the Regulation of Smooth Muscle Relaxation

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ABSTRACT

Premature birth accounts for the majority of fetal morbidity and mortality in the developed world and is disproportionately represented in some populations, such as African Americans in the United States. The costs associated with prematurity are staggering in both monetary and human terms. Present therapeutic approaches for the treatment of labor leading to preterm delivery are inadequate and our understanding of the regulation of myometrial smooth muscle contraction-relaxation is incomplete. The ability of nitric oxide to relax smooth muscle has led to an interest in employing nitric oxide-donors in the treatment of preterm labor. Fundamental differences exist, however, in the regulation of uterine smooth muscle relaxation and that of other smooth muscles and constitute a conundrum in our understanding. We review the evidence that nitric oxide-mediated relaxation of myometrial smooth muscle, unlike vascular or gastrointestinal smooth muscle, is independent of global elevation of cyclic guanosine 5′-monophosphate. Applying our current understanding of microdomain signaling and taking clues from genomic studies of pregnancy, we offer a framework in which to view the apparent conundrum and suggest testable hypotheses of uterine relaxation signaling that can explain the mechanistic distinctions. We propose that understanding these mechanistic distinctions in myometrium will reveal molecular targets that are unique and thus may be explored as therapeutic targets in the development of new uterine smooth muscle-specific tocolytics.

The precise physiological processes leading to birth are mysterious and the physiology of preterm labor (PTL) is unknown (Buxton et al., 2000). The majority of PTL becomes preterm delivery (PTD), accounting for 9 to 11% of births in the United States (ACOG Bulletin, 2003). If a test were available to predict PTL as well as its onset, we would fail to prevent the delivery of a preterm fetus, because there is no safe and effective means of halting labor and maintaining pregnancy until term. Although various tocolytics are in routine use, their efficacy and safety are questionable.

Thus, a basic understanding of the mechanisms regulating the contractile state of uterine smooth muscle will have immediate and important clinical utility. For several years now, we have been focused on studies of myometrial function with the goal of improving our understanding of the regulation of relaxation. Working first in guinea pig, then monkey, and now with an emphasis in human tissues, we have concluded that uterine smooth muscle is neither vascular nor gastrointestinal smooth muscle. Beyond the obvious absurdity of this statement lies a biochemical conundrum. That is, studies of receptor signal-transduction in these other muscles does not teach us what we need to know about myometrium; therefore, if the critical problem of treating PTL and PTD is to be solved, we must focus on basic studies in myometrium, preferably human myometrium. The following pages describe a conundrum in cyclic nucleotide signaling that grew out of these observations of the capacity of nitric oxide to relax myometrium.

Nitric Oxide and Uterine Function

Interest in the ability of NO donors to relax myometrium (Kuenzli et al., 1996, 1998; Bradley et al., 1998; Buxton et al.,

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ABBREVIATIONS: PTL, preterm labor; PTD, preterm delivery; sGC, soluble guanylate cyclase; BK, large conductance potassium channel; SK, small conductance potassium channel; PKG, protein kinase G; $rMLC_{20}$, 20-kDa regulatory myosin light chain; MLCK, myosin light chain kinase; MP, myosin phosphatase holoenzyme; MBS, myosin-binding subunit; PP1, myosin phosphatase; ROK, Rho kinase; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso *N*-acetyl penicillamine; PGC, particulate guanylyl cyclase; NAADP, nicotinic acid adenine dinucleotide phosphate.

2001) has led some to claim its use as a tocolytic (Lees et al., 1994). Whether NO is produced in the uterus to maintain uterine quiescence is an unanswered question. Although functional data in animals suggests a role for endogenous NO in regulating aspects of normal gestation and parturition (Tiboni et al., 2001), efforts to detect nitric-oxide synthase in human uterus using antibodies to each of the known forms of the enzyme were negative (Bartlett et al., 1999; M. E. Bradley and I. L. O. Buxton, unpublished observations), although there is data to the contrary (Bao et al., 2002). Still others have concluded that NO is not an endogenous mediator of human uterine quiescence (Jones and Poston, 1997). However, high levels of nitric-oxide synthase activity have been noted in the villous trophoblast during the first trimester, and this activity seems to decrease toward the end of gestation (Sanyal et al., 2000). The NO thus produced or theoretically available from endothelium or some other nonmuscle cell compartment would be near the uterine myometrium and might act as a paracrine agent in the maintenance of uterine quiescence during pregnancy. NO is also synthesized by macrophages associated with the decidua during pregnancy (Vince et al., 1990). Even cervical function during pregnancy seems to be at least partly under NO control (Ekerhovd et al., 1998). However, a recent review of available controlled clinical studies emphasizes that although nitroglycerin reduced the incidence of PTD, the result was not dramatic (Duckitt and Thornton, 2002). The issues of endogenous NO synthesis and the effects of NO donors in relaxing laboring myometrium are not the subject of this review. Rather, this review will explore the mechanism(s) of action of NO in modulating contractility of uterine smooth muscle.

Nitric Oxide Signaling

It is no longer accurate to attribute NO-mediated relaxation in myometrium to the actions of global elevations in cGMP. Many assume that NO acts in myometrium as a relaxing agonist in the same manner it does in other smooth muscles, through soluble guanylyl cyclase (sGC) activation and cGMP accumulation (Yallampalli et al., 1994; Buhimschi et al., 1995), even though numerous studies show that cGMP is neither necessary nor sufficient for myometrial relaxation (Diamond, 1983; Kuenzli et al., 1996, 1998; Bradley et al., 1998; Hennan and Diamond, 1998, 2001; Buxton et al., 2001; Tichenor et al., 2003). A conundrum exists: why don't global elevations of cGMP signal relaxation of myometrium as they do in vascular smooth muscle?

Data in guinea pig uterine smooth muscle demonstrated that an NO donor produced relaxation despite the inhibition of sGC by methylene blue (Kuenzli et al., 1996). Furthermore, concentrations of permeable cGMP analogs in excess of 10 μM were required to produce any demonstrable relaxation of the uterine smooth muscle. In both monkey and human myometrium, where no relaxation can be demonstrated with cGMP analogs, NO-induced relaxation is independent of a sGC-cGMP pathway (Kuenzli et al., 1998; Bradley et al., 1998; Buxton et al., 2001). These and other studies in which sGC-independent actions of NO have been noted suggest that intracellular cGMP elevation is neither necessary nor sufficient for NO-induced relaxation of the uterine smooth muscle and that other pathways, such as ion channel/pump regulation by NO, may be involved (Modzelewska et al., 1998; Buxton et al., 2001).

The notion that cGMP does not subserve all of the actions of NO is now accepted. In vascular smooth muscle, where NO and NO signaling were first worked out, it is evident that some preparations exhibit significant components of the relaxation to NO that are resistant to blockade of cGMP accumulation (Eckman et al., 1994). These exceptions are now seen in a variety of systems, such as renal arterioles (Trottier et al., 1998), cerebral microvessels and pituitary hormone secretion (Pinilla et al., 1998), regulation of neuronal cell ion channels (Ahern et al., 1999; Summers et al., 1999), and apoptosis (Brune et al., 1996). Exceptions are also seen in nonvascular smooth muscle. In canine airway, Janssen et al. (2000) have suggested that the cGMP-independent actions of NO donors can be ascribed to the chemistry of the NO species liberated. Their data, together with the work of Jones et al. (1994), suggest that the actions of NO are cGMP-dependent or -independent in airway based on the NO species delivered by a particular donor. Although such a result may be unexpected based on the chemistry of NO in warm, oxygenated physiological buffers (Stamler et al., 1992; Kishnani and Fung, 1996), their finding that there is a possible role for calcium release in the cGMP-independent actions of NO is both awkward (calcium elevation ought to signal contraction) and intriguing, and it is seen in myometrium (Tichenor et al., 2003).

The principal mechanism now established for NO signaling that is not cGMP-dependent is that of *S*-nitrosylation of proteins (Davis et al., 2001; Ahern et al., 2002). *S*-nitrosylation occurs nonenzymatically on the thiol side chains of cysteine residues. Some cysteine side chains are particularly reactive to NO as a result of accessibility at the surface of the folded protein, the specific chemical environment and a putative polar *S*-nitrosylation consensus sequence (Jia et al., 1996; Lander et al., 1997; Stamler et al., 1997). Although *S*-nitrosylation might be involved in such events as the release of calcium in microdomains in which potassium channels might be activated, no such direct evidence of this is available for myometrium.

Despite the growing evidence that there are actions of NO other than elevation of cGMP and that these other pathways are present in smooth muscle, the finding that little or no NO-mediated relaxation is caused by sGC-cGMP accumulation in myometrium is puzzling. The notion that relaxations of myometrium are entirely cGMP-independent has drawn skepticism; we too, have approached with caution the hypothesis that cGMP is neither necessary nor sufficient to relax myometrium. Our cautions notwithstanding, the hypothesis is supported by data other than our own (Diamond, 1983; Word et al., 1991; Word and Cornwell, 1998; Modzelewska et al., 1998). Efforts recently have been centered on the notion that ion channels, those carrying the major hyperpolarizing potential in myometrial smooth muscle (Fig. 1), calcium activated potassium channels (K_{Ca}), might be responsible for the actions of NO to relax myometrium (Mazzone et al., 2002). This notion is supported by the finding that scorpion toxins known to block K_{Ca}, prevent the relaxation to NO (Buxton et al., 2001) and that these channels can be activated by NO in myometrium (Shimano et al., 2000).

K_{Ca}-Channels

That K_{Ca} channels are at the root of the cGMP-independent relaxing action of NO is intriguing and is consistent with the cGMP-independent actions of NO described thus far (Ahern et al., 1999; Mazzuco et al., 2000; Yu et al., 2002). The notion that myometrial K_{Ca} are regulated directly by NO is supported by evidence in vascular (Bolotina et al., 1994) and gastrointestinal smooth muscle cells (Lang et al., 2000) that these channels can be modulated by *S*-nitrosylation. This hypothesis is challenging however, if based solely on the use of K_{Ca} channel inhibitors. Blockade of channels known to carry significant hyperpolarizing current in smooth muscle (McCarron et al., 2002) may not offer a direct assessment of the mechanism of action of NO (Kaczorowski et al., 1996). The Ca^{2+}-activated K^+ channels from human myometrium have been cloned and sequenced (GenBank accession nos. AF395661, AY040849, AY044441, AY049734, and AF39717). There are differences between those sequences and ones previously published for the human slow conductance channels (SK2 and SK3) that would be predicted to influence their voltage dependence. No evidence for a previously undescribed K_{Ca} channel has been found in myometrium, which expresses each of the known Ca^{2+}-activated K^+ channel proteins; the large conductance channel (BKα, BKβ), an intermediate conductance channel (IK), and SK2 and SK3.

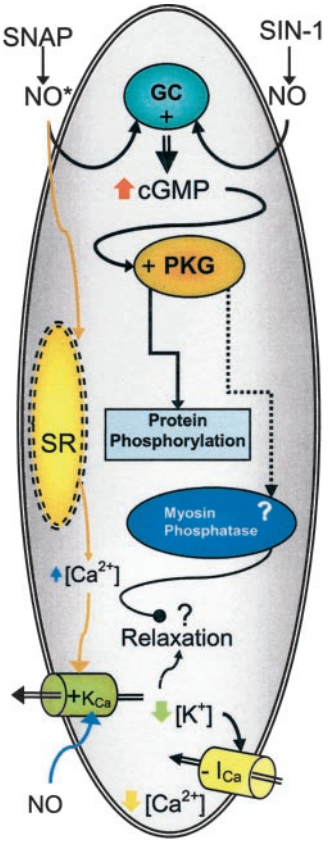

Fig. 1. Proposed model of NO signaling in a uterine smooth muscle cell. The liberation of either nitric oxide radical (NO$^{\bullet}$) or a redox form of NO* results in the expected activation of sGC and the accumulation of cGMP. Activation of PKG by cGMP results in the activation of the kinase and the subsequent phosphorylation of substrate proteins in the cell but not those associated with relaxation. The proposed protein-protein interaction that is thought to occur between PKG and the myosin phosphatase that leads to its activation and ability to dephosphorylate the myosin regulatory light chains (not shown) must be questioned in myometrium because elevation of cGMP does not relax the tissue. This interaction between PKG and myosin phosphatase is known to be critical in other smooth muscles because inhibition of the myosin phosphatase leads to increased force for a given concentration of calcium (calcium sensitization). The ability of SNAP but not SIN-1 to generate NO*-mediated release of calcium from sarcoplasmic reticulum (SR) leads to the activation of potassium channels (K_{Ca}) and the extrusion of potassium ions leading to hyperpolarization of the cell membrane. Hyperpolarization of the membrane inactivates the inward movement of calcium through the voltage-sensitive channel (I_{Ca}) lowering the intracellular calcium concentration and relaxing the muscle.

More recently, the hypothesis that K_{Ca} expression may be correlated with the timing of myometrial contraction during birth has been explored. In particular, some have proposed that expression and/or critical electrophysiological properties of the large conductance K_{Ca} (BK or Maxi-K) channel are down-regulated before birth in both animals and humans (Khan et al., 1993, 2001; Benkusky et al., 2000; Chanrachakul et al., 2003). Although down-regulation of BK channels is logical based on the fact that the BK channel carries far more hyperpolarizing current than other members of this channel family (200–300 pS versus 20–30 pS), we find no change in the expression of BKα or BKβ transcripts before labor in humans. There are, on the other hand, changes in the expression of slow conductance channels. Although the regulation of SK channel activity alone is unlikely to be at the basis of the enigma presented by the actions of NO in myometrium, it is possible that decreased SK channel expression in myometrium is part of the accommodation at term that subserves parturition (Mazzone et al., 2002).

Contributing to the conundrum, there is evidence that cGMP activation of PKG leads to modulation of K_{Ca} through both phosphorylation (White, 1999; Klyachko et al., 2001) and dephosphorylation (White et al., 1993). Because blockade of cGMP elevation does not alter the relaxation of myometrium to NO-donors, we must exclude these possibilities for myometrium unless we are to propose that cGMP elevation and kinase activation were to occur in a compartment of the cell near the membrane and that such changes occurred in the immediate proximity of the channel. Such a compartment would not be represented by global elevation of cGMP elicited by the actions of NO.

Compartmentation of Signaling

Such a notion is not all that improbable even if it is not a compartment that subserves the effects of NO, but might rather serve the actions of agonists that act by activating particulate guanylate cyclase and elevating cGMP in a discrete signaling domain such as the myocyte caveolae or lipid-rich raft region. This notion is attractive because recent studies of genes that seem to turn on between the start of pregnancy and term include the gene for uroguanylin, the endogenous activator of the C-type particulate guanylate cyclase. Exploring this notion will take time, but we think it may explain some of the confusion regarding a role for cGMP in the tissue.

If there were a compartment in the cell that signaled through cGMP (albeit not global elevations secondary to the action of NO on soluble guanylyl cyclase), it would require a compartmentation of PKG as well as the cyclase and the channel. Despite earlier assertions that PKG subserves the relaxation of myometrium to global elevation of cGMP after-treatment by NO or NO donors, little is known regarding the

isotypes of PKG in myometrium. This is particularly glaring given the role of kinase localization that explains, in part, the compartmentation of cAMP action in cardiac myocytes (Steinberg and Brunton, 2001).

Because global elevations of cGMP do not explain NO-mediated relaxation in myometrium, it is possible that there is some difference in the regulation of the smooth-muscle myosin phosphatase. Smooth muscle contraction is initiated by phosphorylation of the 20-kDa regulatory myosin light chain ($rMLC_{20}$) by a Ca^{2+}/calmodulin dependent activation of the myosin light chain kinase (MLCK) which phosphorylates $rMLC_{20}$ on Ser-18,19, leading to an acceleration of actin-myosin ATPase (Stull et al., 1991). Smooth muscle relaxation is in large measure the result of dephosphorylation of $rMLC_{20}$ by myosin phosphatase holoenzyme (MP). MP is a heterotrimer composed of a 110- to 130-kDa myosin targeting-binding subunit (MBS), a 37-kDa catalytic phosphatase subunit (PP1c), and a 20-kDa protein subunit of presently unknown function (Hofmann et al., 2000) that may be involved with subcellular localization (Takizawa et al., 2003). A large body of work has concentrated on MP, its mode of regulation, and how the phosphatase can control smooth muscle quiescence (for review, see Hartshorne et al., 1998). Inhibition of MP is thought to contribute to Ca^{2+} sensitization, a phenomenon in which greater force is produced than would result from elevation of Ca^{2+} and activation of MLCK alone (Surks et al., 1999; Hofmann et al., 2000); this is a striking aspect of contractile regulation in smooth muscle, because its corollary is that MP activity alone reduces force generation (Kitazawa et al., 1991).

The principal Ca^{2+}-independent pathway thought to increase force in smooth muscle is via inactivation of the phosphatase activity of MP. This is thought to occur through activation of Rho kinase (ROK) by membrane anchored RhoA-GTP. Active ROK has been shown to phosphorylate MBS at Thr-695, Thr-850, or both, leading to inhibition of the phosphatase and subsequent increase in MLC_{20} phosphorylation resulting in increased force without a Ca^{2+} increase (Feng et al., 1999). ROK is known to be activated by contractile agonists in smooth muscles (Somlyo and Somlyo, 2000) and to regulate expression of the smooth muscle contractile phenotype (Halayko and Solway, 2001). Although the inhibitory phosphorylation sites on MBS have been suggested to convey slight differences in the way they promote phosphatase inhibition (Velasco et al., 2002), some investigators have shown that another pathway exists to regulate the phosphatase.

Recently, a kinase thought to interact with its substrates based on the presence in their sequence of a leucine-rich domain conferring a set of repeated bends (seven) has been described in nonmuscle cells (Murata-Hori et al., 1999). In smooth muscle, the Zip-like kinase has been shown to be associated with the MP and to phosphorylate MBS at Thr-697, subsequently inhibiting the phosphatase, and thus promoting calcium sensitization (MacDonald et al., 2001a). A study appearing soon after this demonstrated that Zip kinase directly phosphorylates $rMLC_{20}$ on the same sites as MLCK but in a Ca^{2+}-independent manner and that this, rather than the phosphorylation of the MBS of MP, was the basis of the effect of Zip kinase to enhance contraction (Niiro and Ikebe, 2001). Although this controversy is not yet resolved, it is likely that both of these activities of the Zip kinase take place in phasic smooth muscle and are of interest in myometrium because the muscle must maintain tone for extended periods between relaxations during parturition. If Zip kinase exists in myometrium however, it must reside in a particulate region of the cell, because we do not find any evidence by Western blot for the presence of Zip kinase in myometrial homogenates. This could be a result of the general difficulty in finding low-abundance proteins in homogenates, in that the original description of the kinase was in samples first isolated as myofibrillar pellets. The presence and distribution of Zip kinase in myometrium will be interesting to discover because we propose that a nonzipper isoform of MBS is present in the cell soluble fraction and thus would not be expected to interact with and be phosphorylated by a Zip kinase. Indeed, with appropriate controls from gastrointestinal smooth muscle homogenates, we find no evidence for Zip kinase in the myometrial soluble fraction (S. Tichenor and I. L. O. Buxton, unpublished observations).

Whether or not the Zip kinase is present, a large (indeed bewildering) number of putative MP kinase inhibitors are thought to prevent the activity of the PP1c phosphatase activity of MP, including ROK, CPI17 (MacDonald et al., 2001b), integrin-linked kinase (Muranyi et al., 2002), PAK (Takizawa et al., 2002a), Inhibitor-4 (Shirato et al., 2000), and PPP1R14A (Li et al., 2001), to name a few. Although the matter is still controversial, phosphorylations of MP at various sites by a number of kinases result in decreased activity of the PP1c (Kimura et al., 1996) as well as decreased binding of the MBS to myosin (Velasco et al., 2002). In particular, CPI17 has recently been described in human myometrium, and its expression increased in tissue during pregnancy (Ozaki et al., 2003).

Activation of the MP must therefore involve dephosphorylation of the MBS at one or more of those sites phosphorylated (Thr-695, Thr-850, and Thr-697) by inhibitory kinases. In theory, when these sites are in a dephosphorylated state, MBS can interact with $rMLC_{20}$, removing phosphates at Ser-18 and Ser-19, thus decreasing cross-bridge cycling and force generation. What then activates MP? In particular, how are the phosphorylations on the MBS reversed? Neither of these questions has been answered in smooth muscle. Indeed, some suggest that MBS is not dephosphorylated significantly in smooth muscle (Takizawa et al., 2002b; Niiro et al., 2003), a notion that is not intellectually pleasing. Because MP is inhibited by phosphorylation of the MBS subunit by kinases such as CPI17, it stands to reason that without dephosphorylation of these sites within the time frame of contraction/relaxation, Ca^{2+} sensitization would be a permanent on-switch and thus not reversibly measurable. Are we to believe that, once phosphorylated in the first moments of function, the protein is never again to be without these inhibitory phosphorylations until replaced by new protein synthesis? These data are hard to reconcile with the bulk of data in smooth muscle showing the role of MBS phosphorylation by kinases associated with contractile agonists (e.g., RhoA). Regarding MP activation, we know that the PP1c is activated by interaction with PKG. When PKG is activated by cGMP elevation, the leucine zipper located on the N-terminal region of PKG can interact with the leucine zipper on the C-terminal region of MBS and signal $rMLC_{20}$ dephosphorylation (Hofmann et al., 2000). This interaction is not, however dependent on PKG phosphorylation of MP, although that does

occur (Nakamura et al., 1999). Indeed, the presence of a leucine zipper region in the MBS of MP is thought to be consistent with the direct interaction of these two proteins (Surks et al., 1999).

Considering that global elevation of cGMP does not relax myometrial smooth muscle, it is possible that the presence of an isoform of MBS lacking the leucine zipper (MBS_{NZ}) and residing in the non lipid-raft/caveolar region of the cell explains this lack of a cGMP-mediated response. Such an isoform is expressed in avian smooth muscle developmentally (Pfitzer et al., 1986; Khatri et al., 2001). The corollary we suggest for myometrium is that elevation of cGMP in the lipid-raft/caveolar region of the myocyte and activation of PKG in that region (proposed to be PKG II; Fig. 2) activate MP by binding to an isoform of MBS containing the leucine zipper expressed and assembled with MP holoenzyme in this region of the cell and resulting in activation of the phosphatase. Recently, Huang et al. (2004) showed that the binding of PKGI with MBS does not require the C-terminal zipper motif to be present in the MBS, whereas the dephosphorylation of $rMLC_{20}$ does. Although these data come from experiments in cultured chicken gizzard smooth muscle cells in culture, they offer an exciting context in which to consider the cGMP insensitivity of myometrium. In myometrium, perhaps the MBS_{NZ} interacts with cGMP-PKGI accumulated after NO action on the muscle.

Consistent with such a notion, some investigators have suggested that MP subunits are targeted to a region near the plasma membrane in an agonist-specific fashion (Shin et al., 2002) such that the MBS subunit remains in the periphery of the cell while the PP1c phosphatase subunit is seen in the central region of the cell. PKG is also known to be colocalized near the plasma membrane in smooth muscle cells (Koller et al., 2003). It is possible, then, that the action of NO to relax myometrium is cGMP-independent, whereas the action of other inhibitory agonists is cGMP-dependent and restricted to a particular region of the cell.

K_{ATP} Channels

Although some data implicate K_{Ca} channels in the cGMP-independent actions of NO in myometrium, there are reports that the target of NO in myometrium is the K_{ATP} channel instead (Modzelewska et al., 1998). Although mRNA has been detected for this channel in human myometrium (Chien et al., 1999; Curley et al., 2002), there is little (Hamada et al., 1994) compelling electrophysiological evidence that K_{ATP} channels are expressed there. This lack of evidence notwithstanding, it is possible that the channel exists in a closed conformation unless the metabolic state of the tissue is compromised or the channels are activated by an event such as *S*-nitrosylation. Whatever the case, the hypothesis that K_{ATP} channels are present and mediate, in whole or part, the actions of NO to relax myometrium has yet to be ruled out.

What is different about myometrium? Might it be that cGMP does not activate the cGMP-PK (PKG) known to be expressed in myometrium (Tamura et al., 1996; Word and Cornwell, 1998; Hennan and Diamond, 2001)? It has been suggested that cGMP is ineffective in pregnant myometrium because PKG is down-regulated (Word and Cornwell, 1998). Although there may be changes in PKG expression in myometrium, it is clear that cGMP elevation after NO donor

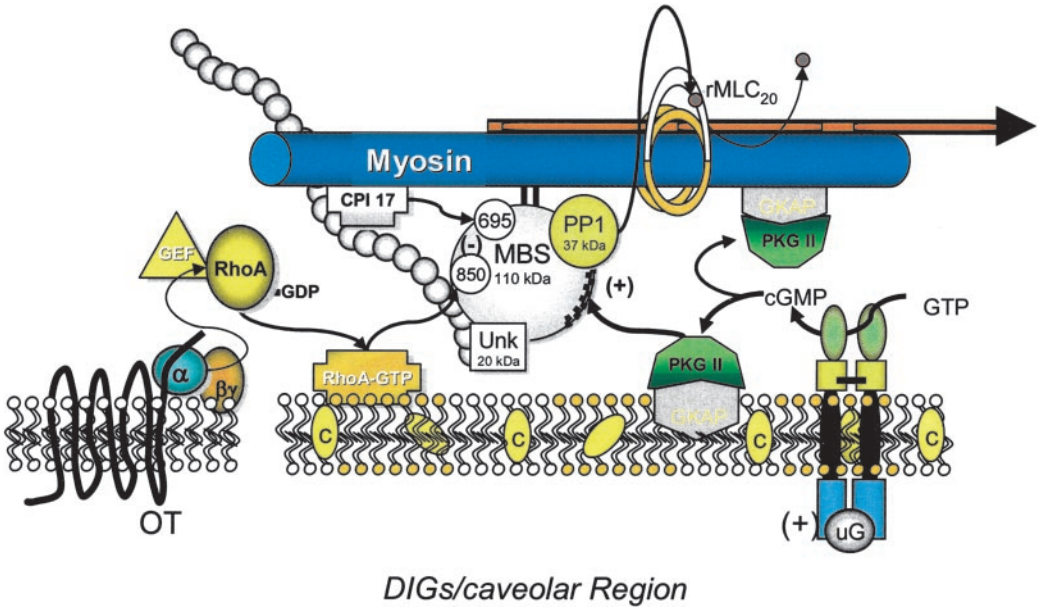

Fig. 2. Proposed compartmentation of signaling in the uterine smooth muscle myocyte. In cholesterol-rich (C), sphingolipid-rich (orange ○) regions of the myocyte membrane (detergent-insoluble glycolipid-rich domains/caveolar microdomain), signaling proteins such as the particulate guanylyl cyclase bind agonists such as uroguanylin (uG) and results in increased levels of cGMP locally. Cyclic GMP activates its cognate protein kinase (PKG) that is localized in the region of the cGMP elevation by a G-kinase anchoring protein (GKAP) and permits the phosphorylation/interaction with the MBS of myosin phosphatase (PP1) that dephosphorylates the regulatory myosin light chains (rMLC) releasing phosphate (●) and promoting relaxation (▶) of actin-myosin interaction and thus relaxation of the muscle. Contractile agonists such as oxytocin (OT) stimulate G-protein regulated activation of the kinase (RhoA) that phosphorylates MBS leading to inactivation of PP1. Other kinases, such as CPI 17, that may be activated by the oxytocin-mediated rise in intracellular calcium (data not shown) also phosphorylate and inactivate PP1. The notion that all of these events are organized in the signaling microdomain is illustrated by the presence of filamentous proteins (linked circles) that may contribute to localization of signaling proteins.

stimulation of myometrium leads to protein phosphorylation (Hennan and Diamond, 2001), just not relaxation. For example, the vasodilator associated protein VASP, a known PKG substrate in smooth muscle, is phosphorylated in response to cGMP elevation in myometrium (Tichenor et al., 2003). If cGMP activates PKG, what might explain the apparent independence of the NO-induced relaxation to global elevation of cGMP and activation of its associated kinase?

A partial answer is evident in a comparison of the actions of the *S*-nitroso thiol NO donors and non-nitroso thiol compounds such as 3-morpholinosydnonimine (SIN-1). Although both of these agents cause significant global elevations in cGMP in myometrium and other smooth muscles, in myometrium, only the *S*-nitroso compound, *S*-nitroso *N*-acetyl penicillamine (SNAP), relaxes the tissue once contracted by oxytocin (Buxton et al., 2001; Tichenor et al., 2003). These data demonstrate again that global elevations of cGMP could not be the mechanism through which NO signals relaxation of myometrium. Based on work in airway smooth muscle, Janssen et al. (2000) proposed that SNAP, unlike SIN-1, caused calcium release and that that was the basis for its action to relax airway smooth muscle, whereas SIN-1 did not and thus must work through cGMP. Although these authors did not actually measure cGMP in their study, the notion that an NO donor might cause release of intracellular calcium was, if surprising, consistent with the notion that K_{Ca} channel activation might be the basis of the action of NO in myometrium (Fig. 1). We have established in myometrium and in airway muscle that both of these donors cause global elevation of cGMP. To our surprise, SIN-1 was unable to relax myometrium, whereas SNAP was quite effective. Here then was a correlation between relaxation and NO effects on intracellular calcium release and a lack of correlation between global cGMP accumulation and relaxation (Tichenor et al., 2003). The possibility also exists that SNAP was effective only because it is an *S*-nitroso compound and able to *S*-nitrosylate critical myometrial substrates such as the K_{Ca} channel(s) or perhaps an as-yet-unknown substrate.

Leads from Genomic Studies

Genomic and proteomic studies of the physiology of parturition have begun to shed light on the mechanisms of labor. Several studies (Aguan et al., 2000; Chan et al., 2002; Bethin et al., 2003; Girotti and Zingg, 2003) seem particularly useful in the context of understanding which changes might shed light on the mechanisms of signaling regulation in the myometrium, and available work has been reviewed (Romero et al., 2002). One of the genes that is very markedly up-regulated between day 0 and term in the rat is that encoding the particulate guanylyl cyclase (pGC) activator, uroguanylin (Girotti and Zingg, 2003). Also up-regulated are transcripts for caveolin, consistent with the notion that signaling domains develop in relation to the development of quiescence during gestation.

NAADP Releasable Ca^{2+} Pool

Release of calcium from intracellular stores is established as a central component of numerous receptor-mediated signaling pathways and is obviously central to understanding the biology of smooth muscle. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a recently discovered nucleotide with intracellular Ca^{+2}-releasing properties (Lee, 1994). NAADP-induced Ca^{+2} release was first described in sea urchin egg homogenates (Chini et al., 1995) but has now been described in mammalian cells (Yusufi et al., 2001a) and tissues (for review, see Chini et al., 2002) including smooth muscle (Yusufi et al., 2002). Ca^{+2} release elicited by NAADP differs in many ways from Ca^{+2} release controlled by cyclic ADP-ribose (the endogenous ryanodine receptor agonist) and inositol 1,4,5-trisphosphate (Genazzani and Billington, 2002). Properties of NAADP-induced calcium release include: 1) the absence of regulation by Mg^{2+} and Ca^{2+}; 2) NAADP-induced Ca^{2+} release is fully desensitized by prior exposure to low concentrations of NAADP (Genazzani et al., 1996); and 3) the Ca^{2+} release induced by NAADP is insensitive to a wide range of changes in pH (Chini et al., 1998) and thus may be active in microdomain environments. These characteristics make NAADP a unique trigger of intracellular Ca^{+2} release/entry and suggest the possibility that this release pathway subserves the nitrosothiol-dependent release of Ca^{2+} in myometrium.

Synthesis of NAADP by a base-exchange reaction has been described in several mammalian tissues, including brain, heart, liver, spleen, and kidney (Chini and Dousa, 1995; Cheng et al., 2001). Furthermore, it has also been reported that ADP-ribosyl cyclase (CD38) is capable of catalyzing the synthesis of NAADP in 'smooth muscle-like' mesangial cells (Yusufi et al., 2001b). Although we are not aware of a detailed description of NAADP synthesis or its receptor in smooth muscle, CD38 is clearly expressed in myometrium (Dogan et al., 2002). It is possible that CD38 catalyzes the synthesis of NAADP in lipid-rich signaling domains of uterine smooth muscle cells. This could occur as a result of *S*-nitrosylation and activation of its synthesis via an effect on CD38 or via its receptor; this effect could subserve membrane-limited elevations in Ca^{2+} and constitute the cGMP-independent NO-mediated activation of K_{Ca} channels.

Caveolar Signaling

Lipid-enriched signaling domains such as caveolae and detergent-insoluble glycolipid-rich domains (Parton and Simons, 1995) are recognized to play roles in many cellular processes, especially in receptor signal-transduction (for review, see Anderson, 1998), explaining, at least in part, early observations of subcellular compartmentation (Buxton and Brunton, 1983) before caveolar signaling was known. Recently, studies of the regulation of receptor signaling in endothelial caveolae have suggested their role as calcium sensors (Kaiser et al., 2002). Caveolae are richly expressed in smooth muscles, including myometrium (Kwan et al., 1986), and their numbers are regulated (Turi et al., 2001). Perhaps the conundrum presented here can be resolved on the basis of the organization of receptor signaling in myometrium between detergent-insoluble glycolipid-rich domains, including rafts and caveolae (Anderson and Jacobson, 2002), and the soluble and myo-filamentous regions of the cell.

Some endogenous peptides that relax the myometrium may target the lipid-rich signaling domains of uterine smooth muscle cells. Guanylin and uroguanylin are peptides (15 and 16 amino acids respectively) homologous to the heat-stable enterotoxin of *Escherichia coli*. These peptides were

originally discovered in the gastrointestinal tract (Field et al., 1978; Hughes et al., 1978), where they regulate water and electrolyte balance through a cGMP-dependent mechanism. Enterotoxigenic strains of bacteria produce heat-stable enterotoxins that stimulate chloride secretion leading to accumulation in the gastrointestinal lumen and subsequent secretory diarrhea. In the 1980s, specific binding sites for heat stable enterotoxin peptides were discovered (membrane bound guanylyl cyclase) in intestine and other tissues such as kidney and lung (Forte et al., 1988, 1989). The discovery of receptors (pGC) in tissues not thought to be exposed to STa peptides suggested that endogenous agonists might exist. The first of these to be discovered was guanylin (Currie et al., 1992). Two other members of the family are also known in human (Nakazato, 2001), uroguanylin and lymphoguanylin, the latter of which will not be considered here. Prouroguanylin and proguanylin proteins are believed to be produced by enterochromaffin (Perkins et al., 1997) and endocrine cells (Magert et al., 1998) in the intestine and differentiated epithelial cells in the kidney (Nakazato et al., 1998). These precursor proteins are inactive and circulate in the bloodstream (Beltowski, 2001). Little is known about the actual proteolytic generation of the peptides guanylin and uroguanylin from their pro-forms, although chymotrypsin may be responsible for production of guanylin peptides in the gut (Magert et al., 1998). The fact that there is only a 20% identity in the sequences of human guanylin and uroguanylin suggests that their roles may be different.

We suggest that uroguanylin and not guanylin is produced in uterine glandular cells during pregnancy to signal to myometrium in a paracrine fashion and maintain uterine quiescence. This hypothesis is supported by the findings that prouroguanylin and proguanylin are the products of different genes (Magert et al., 1998); uroguanylin, not guanylin, gene expression is up-regulated in uterine tissue during pregnancy (Girotti and Zingg, 2003). Perhaps most interestingly, uroguanylin is thought to be 100 times more potent than guanylin in an acidic environment (Hamra et al., 1997). Our hypothesis that uroguanylin acts at the myocyte caveolae is supported both by the presence of pGC activity in myometrium (Buhimschi et al., 2000; Telfer et al., 2001) and by the notion that the receptor environment in the caveolar cleft may be acidic because of the concentration of acidic lipids (Anderson, 1998).

This hypothesis regarding the origins and actions of uroguanylin on myometrium is also supported by data from other labs (Weiner et al., 1994; Buhimschi et al., 2000; Carvajal et al., 2001; Fulep et al., 2001), although others may interpret the data differently. It is noteworthy that Weiner et al. (1994) showed that uterine cGMP is elevated over pregnancy toward term and that this accumulation of the second messenger is not caused by NO activity. Although these authors do not favor a role for pGC in this elevation (Carvajal et al., 2001), their study of natriuretic peptide-induced relaxation of oxytocin-stimulated myometrium in the guinea pig did not test the effects of uroguanylin. Studies by Buhimschi et al. (2000) and Fulep et al. (2001) demonstrate relaxant effects of natriuretic peptides on uterine contractions and a lack of effect of cGMP elevations from sGC activation. These data are useful considering previous contributions asserting that NO relaxes myometrium in a cGMP-dependent fashion (Izumi et al., 1993; Yallampalli et al., 1993, 1994; Buhimschi et al., 1995; Izumi and Garfield, 1995; Longo et al., 1999; Vedernikov et al., 2000).

Conclusions

Less is currently known about the contractile regulation of myometrial smooth muscle than other smooth muscles. Existing evidence suggests that the relaxation of uterine smooth muscle by NO and NO donors does not result from elevation of cGMP in the muscle. This disassociation of cGMP accumulation and relaxation of the muscle suggests interesting possibilities for both NO signaling and the regulation of contractile protein interactions in uterine muscle. In the case of NO signaling, myometrial smooth muscle may provide an opportunity to examine cGMP-independent signaling such as *S*-nitrosylation of proteins that may be involved in regional increases in calcium and/or direct activation of potassium channels. Because cGMP accumulation and activation of PKG is expected to relax smooth muscle in large measure via PKG activation of myosin phosphatase, the failure of cGMP accumulation to explain the actions of NO in uterine muscle suggests fundamental differences in the regulation of myosin phosphatase in myometrium.

Examining these possibilities in the context of regional signaling domains within the uterine smooth muscle cell, together with the actions of unique peptides such as uroguanylin, offers an interesting framework in which to consider existing data and plan future work.

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