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Oxytocin and parturition: a role for increased myometrial calcium and calcium sensitization?

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1. ABSTRACT

Preterm birth is associated with the majority of all death and chronic disability related to pregnancy, birth and the neonatal period. The costs to families and to the health care system are enormous. Current approaches to prevent or arrest preterm labour have been unsuccessful. This failure is largely based on our poor understanding of the regulation of the timing and maintenance of parturition. Oxytocin (OT) is the most potent known uterine stimulant. It is produced in the hypothalamus and secreted into the maternal bloodstream. However, OT also is produced within the uterine decidua in late gestation and the concentrations increase around the time of labour onset. The receptor for OT (OTR) is a G-protein coupled receptor linked through $G\alpha_{q/11}$ to phospholipase C (PLC). Activation of PLC causes increased inositol trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 activates specific receptors in the sarcoplasmic reticulum to release Ca^{2+} into the cytosol. This may induce further influx of Ca^{2+} from the extracellular space and the increased Ca^{2+}, after binding to calmodulin, activates myosin light chain kinase to phosphorylate myosin light chains (MLC) and cause contraction of the myocyte. DAG activates protein kinase C (PKC), several isoforms of which have been implicated in uterine contraction, but the substrates for this enzyme in the uterine myocyte are essentially unknown. Oxytocin may also cause "Ca^{2+}-sensitization," a process whereby there is a greater contractile force generated from a given increase in cytosolic Ca^{2+}, although the contribution of this process to myometrial contraction remains an area of debate. This phenomenon occurs mainly due to inhibition of myosin light chain phosphatase (MLCP), the enzyme that reverses the phosphorylation of MLC. There are several important potential mediators of this MLCP-inhibitory pathway in the myometrium, including the small monomeric G-protein RhoA, its downstream kinase Rho-associated kinase (ROK). and the 17-kDa PKC-potentiated inhibitor of protein phosphatase 1c (CPI-17). The roles in the myo metrium of other recently identified MLCP interacting molecules also requires further investigation. These Ca2+-sensitization pathways could be important in the mechanisms underlying pre-term or term labour. An increased understanding of the complexities of the multitude of regulatory mechanisms for uterine contractility may lead to new pharmacologic agents for the prevention or reversal of uterine contractions. This, in turn, is necessary to facilitate the development of novel and effective strategies to reduce the incidence of preterm birth.

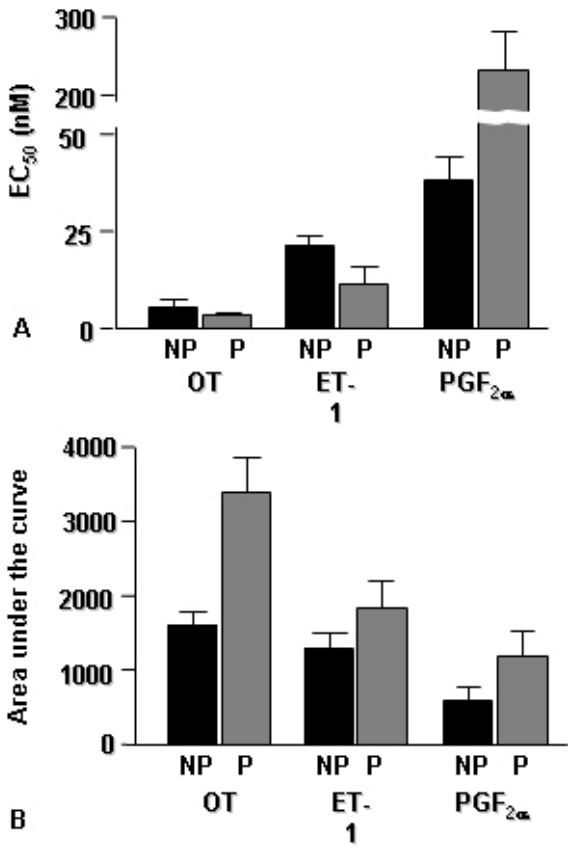

Figure 1. Sensitivity and responsiveness of mouse uterus to contractile agonists. Concentration-response curves were determined for OT, ET-1 and $PGF_{2\alpha}$ using uterine strips from non-pregnant (NP) and late pregnant (P; day18 of a 19 day gestation) mice. The sensitivity (EC_{50}: concentration giving half-maximal contraction amplitude) and responsiveness (integrated area under the contraction-response curve) were calculated. The mouse uterus is most sensitive and responsive to OT. Compared to non-pregnant animals, the sensitivity increases (lower EC_{50}) in late pregnancy for OT and ET-1 and the responsiveness increases for all agonists.

2. INTRODUCTION

The endocrine mechanisms that regulate the timing of parturition show subtle variations among species. In sheep, the fetus plays a predominant role, with increased activity in the hypothalamo-pituitary-adrenal axis leading to increased fetal cortisol, which changes the placental metabolism of steroids resulting in increase estrogen and deceased progesterone in the maternal circulation. In rodents, luteolysis results in a marked decline in maternal progesterone and a resultant increase in the ratio of estrogen to progesterone. In each species these changes cause uterine activation, a process through which the uterine muscle becomes much more sensitive and responsive to contractile agonists. In pregnant women, there is no evidence of such significant changes in maternal plasma estrogen or progesterone concentrations or in estrogen:progesterone ratios. Rather, local intrauterine paracrine mechanisms may predominate in the human. Nonetheless, as far as we presently know, the process of human myometrial cell activation occurs in a manner similar to that of the better-studied animal models. Interestingly, the contractile agonists that stimulate the uterine contractions of parturition are quite similar across species.

There are several potent contractile agonists that stimulate uterine contractions. These include oxytocin (OT), endothelin (ET-1), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and several others. The reason for the multiplicity of uterine stimulants is not clear. It is possible, however, that the multifactorial nature of myometrial stimulants enables several strategic advantages including (i) feed-forward signal amplification to enhance contractile effort at term; (ii) spatial segregation of contractile stimulation and/or (iii) the expression of compensatory contractile mechanisms in the absence of complete activation of one particular pathway. The presence of such multiplicity for a process so vital to species propagation would offer an evolutionary advantage - Each signaling pathway might be responsible for distinct proportions of the regulation of parturition to ensure successful delivery of the baby and placenta. The roles of these uterotonins may also differ between normal term labour and preterm labour. The latter may include "idiopathic" spontaneous preterm labour or may be associated with intrauterine infection, fetal compromise, maternal complications of pregnancy or other abnormalities. Here, the multifactorial nature of parturition may still be of *evolutionary* benefit – nature may deem it disadvantageous for the mother to promote a hostile intrauterine environment and therefore myriad pathways are employed to initiate preterm uterine contractions - but this also contributes to the lack of success in using single ligand/receptor-directed tocolytics to arrest early parturition. An increased understanding of the complex biochemical and physiological mechanisms involved in the stimulation of myometrial cell contraction by uterotonins is thus necessary to inform the development of improved therapies for problems of parturition.

In this review, we will focus on the role of OT and make comparisons to other stimulants of uterine contractions. We will discuss the potential role in parturition for OT synthesized within the pregnant uterus. We will describe regulation of the OT receptor (OTR) and signal transduction systems stimulated by OTR. Finally, we will discuss the proposal that mechanisms regulating the sensitivity of the uterine muscle to calcium ions (Ca^{2+}) may be as important in the mechanism of parturition as the OT-induced rise in intracytoplasmic Ca^{2+} itself.

3. OXYTOCIN AS A UTERINE CONTRACTILE STIMULANT

As noted, there are several potential uterine contractile stimulants. We have used myographic techniques to compare the sensitivity and responsiveness of the mouse myometrium to OT, ET-1 and $PGF_{2\alpha}$ (Figure 1). OT is the most potent stimulus for uterine contractions and both sensitivity and responsiveness increase at the time of

uterine activation in late gestation. In our preliminary studies with rat uterine strips, the pattern appears to be identical. This is compatible with the increasing responsiveness to OT of the human uterus during late gestation (1, 2).

3.1. Historical perspective

The uterine contractile properties of posterior pituitary extracts were well established almost a century ago. These extracts were used initially to treat post partum hemorrhage (3, 4) and were soon used for induction of labour (5). The OT molecule was purified and characterized during the 1950s and the gene was cloned 30 years later (6, 7). Oxytocin is a nonapeptide hormone that is synthesized principally in the magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus and transported through the axons to be stored in secretory granules in the posterior lobe of the pituitary, (neurohypophysis). This classical neuropeptide has several important effects within the central nervous system through neurotransmitter or paracrine mechanisms (for review see Landgraf and Neumann (8)). In this review, we will focus on the participation of OT and OTR in the mechanisms associated with the regulation of parturition. The presence of the storage granules of OT in the neurohypophysis supported the concept that OT could be released into the circulation and have endocrine effects on peripheral target organs. However, subsequent studies demonstrated the synthesis of OT in peripheral sites such as the ovary (9) and the maternal decidua during late pregnancy (10, 11). There is growing support for the hypothesis that the role of OT in parturition may arise from an intrauterine paracrine network involving OT, OTR and other mediators including estradiol and prostaglandins (12-14).

3.2. OT and parturition

The role of OT in parturition has been the subject of recent reviews (15, 16). Briefly, a large amount of evidence supports a role for the OT/OTR system in human and rat parturition. OT stimulates labour at term pregnancy that appears indistinguishable from natural labour. OT concentrations in maternal plasma have a pulsatile pattern in sub-human primates. The pulses increase in frequency and amplitude, accompanied by uterine contractions, in the days preceding overt labour onset (17-19). Further, decidual synthesis of OT in rats and humans increases at the time of parturition (10, 11, 14). An increase in OTR expression in the uterus is a hallmark of uterine activation prior to parturition in all species examined (20-23). Furthermore, a spatial regulation of the OTR occurs in human uterus and may contribute to directional contractions from the fundal to lower segment regions (14, 24). Finally, antagonists of OTR will inhibit labour-associated contractions in women (25-27), sub-human primates (17, 19, 28), and rats (29-31).

Despite the strong evidence supporting a role for OT in parturition, there have been arguments opposing the concept. For example, older data demonstrated that peak levels of maternal plasma OT occur during the second stage of labour or during fetal expulsion and only very small increases are seen prior to labour (32, 33). However, since OT is secreted from the neurohypophysis in a pulsatile manner, very frequent sampling with sensitive and precise assays is required to detect the significant increases described above. Another concern was the fact that women with known neurohypophyseal dysfunction appear to labour normally. Furthermore, the dissociation constant of OTR for OT is 1-2 nmol/L and peak concentrations of OT only reach approximately 100 pmol/L. All of these arguments were rationalized by the discovery that OT could be synthesized within the tissues adjacent to the myometrium and therefore, through paracrine mechanisms, achieve high local concentrations that are neither reflected in the maternal plasma nor dependent upon normal pituitary function.

One remaining concern regarding the role of OT in parturition is the demonstration that OT or OTR knockout mice have apparently normal gestations ending with normal parturition, though the pups die from starvation caused by failure of maternal milk ejection (34-36). However, conclusions from in vivo single gene product disruption studies in multifactorial physiological events can be misleading. Normally, redundant or 'silent' mechanisms may be up-regulated in these circumstances to promote parturition. More complete analyses of single gene knockout mice, including those targeting parturient molecular components other than OT, actually supports a permissive role of OT in the multifactorial mechanisms of labour (16, 37). In addition, OT appears to have a biphasic effect. At high concentrations, it is a potent uterine stimulant. However, given in low doses to OT null mice in late gestation, OT is luteotrophic and actually delays labour (38). The time course of this effect suggests it is a genomic mechanism. This supports the concept that, in addition to the well-characterized acute effects, OT may have longer-term genomic effects that regulate uterine responsiveness. Clearly, the effects of OT in the regulation of pregnancy and parturition are complex and currently incompletely understood. There are no reports of successful development of an OTR knockout genotype.

4. SYNTHESIS AND METABOLISM OF OT

In the hypothalamus, OT is synthesized as part of a larger precursor molecule that includes neurophysin I (Figure 2). The function of the neurophysin peptide is unknown. The larger precursor is cleaved by endopeptidase and carboxypeptidase B and then converted by an α-amidating enzyme to yield the biologically active amidated nonapeptide (39). There is a cys-cys disulfide bond, which makes a ring structure of the first 6 amino acids with a three-amino acid tail. It appears that synthesis of OT occurs through a similar pathway in peripheral sites.

4.1. Intrauterine Synthesis of OT

Expression of the OT gene in human decidua was first reported in 1991 (40). Subsequent studies confirmed this in both the human and rat (10, 11). Estrogen in both human and rat increases decidual OT mRNA and peptide and progesterone appears to augment this increase in the rat (41, 42). These effects appear to be mediated by estrogen and progesterone receptors but there is evidence that other

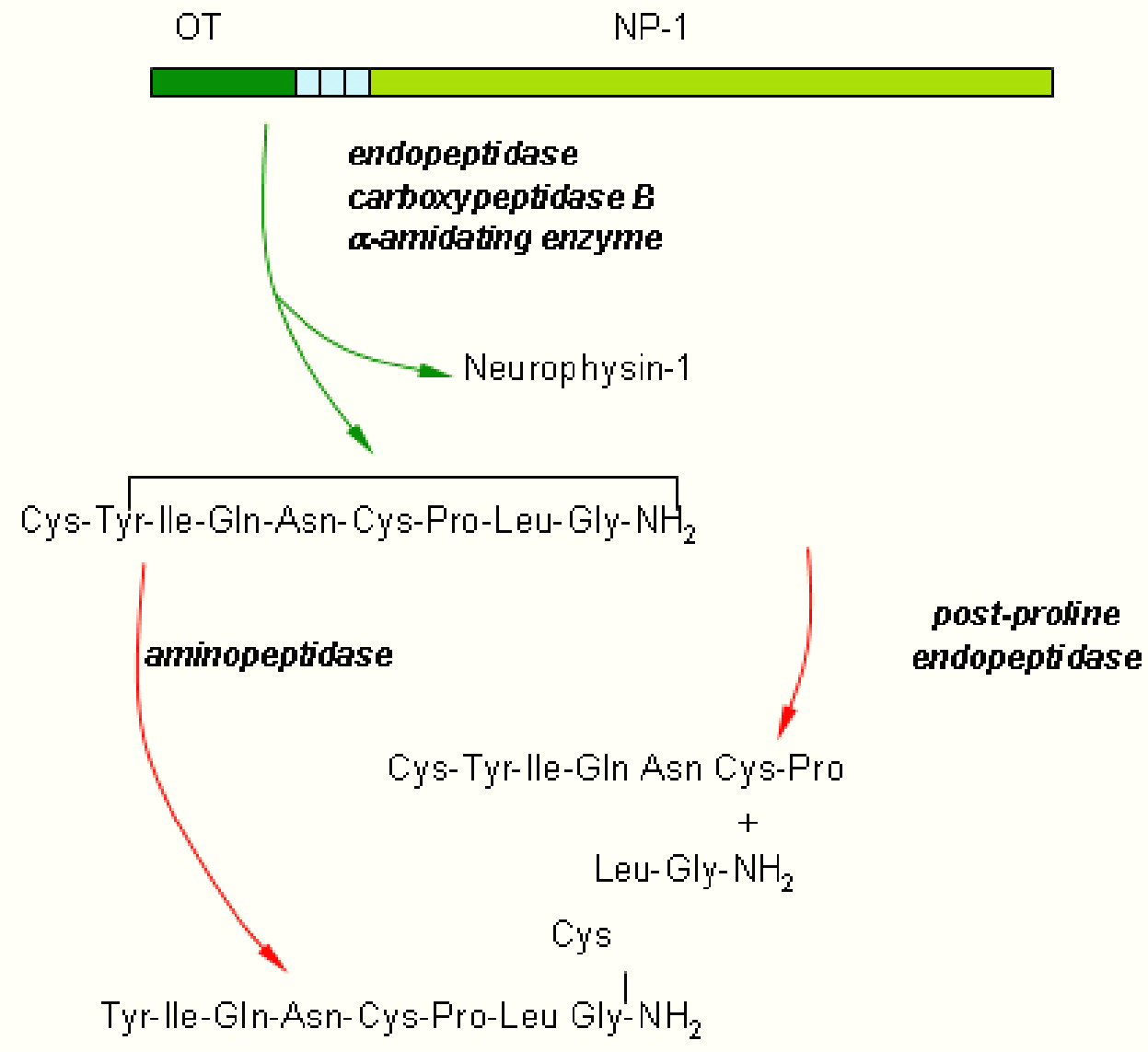

Figure 2. Synthesis and metabolism of OT. The prohormone of OT contains the neurophysin-1 (NP-1) molecule joined by the tripeptide gly-lys-arg linker. The linker is cleaved from NP-1, the lys-arg cleaved and the nonapeptide amidated to form the mature OT hormone. Catabolism of OT by aminopeptidase activity involves disruption of the ring structure created by the cys-cys disulfide bond or by cleavage of the C-terminal amino acids by post-proline endopeptidase. Both synthesis and metabolism of OT occur within human and rat decidua.

orphan nuclear receptors may also regulate OT synthesis (43, 44). Mechanical stimulation such as cervical distension or suckling induces neurohypophyseal OT release (45, 46) but it is unknown whether such stimuli influence decidual OT expression.

Following cleavage of neurophysin I from the larger precursor molecule, there remains the nine amino acids of OT with the three-amino acid linker. This gives rise to several "carboxy-extended" OT molecules, which are present in large quantities in late pregnancy rat decidua (47). We tested the hypothesis that these molecules may have OT agonistic activity or compete with OT for binding to OT and thus act as antagonists. Our results suggested that these intermediates neither possessed oxytocic effects nor competed for OTR binding.

4.2. Metabolism of OT

OT is rapidly metabolized in the maternal circulation during pregnancy resulting in its very short half-life of 2 – 4 minutes. This "oxytocinase" activity is due to an aminopeptidase enzyme that cleaves the ring structure between the cys^1 and tyr^2 amino acids, thus opening the ring and abolishing biologic activity (Figure 2). This activity increases steadily throughout pregnancy (48) and accounts for the four-fold increase in metabolic clearance rate for OT during human gestation (49). OT also is metabolized within intrauterine tissues in late human and

rat pregnancy (50, 51). There are two major pathways of metabolism: a tissue aminopeptidase activity similar to that described above as well as a post-proline endopeptidase that cleaves the last two amino acids from the tripeptide tail. We hypothesized that changes in metabolic breakdown of OT could cause accumulation of the active peptide in intrauterine tissues at the time of labour onset but could find no significant changes in metabolic activity in either human or rat around the time of parturition (50, 51). However, in the human uterus, including fundal and lower segment myometrium, OT was found almost entirely in the active α-amidated form, supporting the previous suggestions of a possible accumulation of bioactive peptide in myometrial tissue at term (14).

5. OTR – A G-PROTEIN COUPLED RECEPTOR

The classical studies of Soloff et al characterized the OTR in uterine tissues and documented key regulatory factors in gestation (20, 52-54). The human OTR gene was cloned by Kimura in 1992 (55). OTR is a member of the G-protein coupled, seven-transmembrane domain receptor superfamily. The rat OTR gene, like most species, has three exons (56) whereas the human gene has four exons resulting from division of the first exon of the rat into two in the human (57). The promoter regions of the human and rat OTR genes share many consensus response elements to transcription factors such as AP-1, NFκB, C/EBPβ and several others. Unlike the rat, the human OTR promoter has no complete palindromic response elements to the estrogen receptor but there are several scattered half response elements that could confer estrogen responsiveness as in the chicken ovalbumin gene (58). OTR stimulation appears to have at least two physiological functions: stimulation of myometrial contractions and increasing decidual production of prostaglandins (PG).

5.1. Regulation of OTR

One of the most consistent findings in the parturition literature is the marked increase in expression of the OTR gene in myometrium prior to the onset of labour. Increased information regarding the control of OTR expression could improve understanding of the whole process of uterine activation. Regulation of OTR gene expression is tissue-specific, as shown by the divergence in OTR concentrations between myometrial and mammary tissue around the time of parturition. In myometrium, OTR peaks during parturition and decreases abruptly post partum when mammary tissue OTR is peaking (20).

The sex steroids are the best-characterized regulators of OTR gene expression. Estrogen increases OTR in human rat and rabbit uterus (53, 59). The estrogen receptor antagonist tamoxifen delays rat parturition for 24 hours with an accompanying delay in the normal marked increase in OTR (22). Interestingly, the OTR levels did increase to normal parturition levels and parturition occurred despite the continued administration of the estrogen antagonist. In contrast to its effects on OT, progesterone causes a decrease in OTR expression in rat uterus and administration of the progesterone receptor antagonist RU486 causes a rapid and marked increase in rat uterine OTR (60, 61). The mechanism of action for progesterone antagonism/withdrawal on OTR expression may be indirect since there appears to be no response element for the progesterone receptor in the OTR promoter.

At the beginning of the process of parturition, there is an influx of bone marrow-derived immune cells (macrophages, large granular lymphocytes and T-cells) to uterine tissues (62, 63). Further, in cases of intrauterine infection, parturition occurs in association with high levels of TNFα, IL-1β and IL-6 (64-66). As noted above, the OTR promoter contains several response elements that may be influenced by transcription factors stimulated by the immune response. Thus, there has been considerable investigation of the influence of pro-inflammatory cytokines on OTR expression. The results have been surprisingly conflicting. Several groups have noted a decrease in OTR in human uterine myocytes treated with IL-1β *in vitro* (67-69). Further, infusion of IL-1β or TNFα into the peritoneal cavity of rats during late gestation had no effect on OTR expression (23). However, another study demonstrated a 3-fold increase in mRNA for OTR in primary uterine myocytes from late pregnancy after IL-1β treatment *in vitro* (70). Treatment of rat uterine explants with IL-6 *in vitro* increased OTR mRNA in tissue from pregnant, but not non-pregnant animals (71). There is no information on the effects of pro-inflammatory cytokines on uterine production or OT. In summary, the effect of pro-inflammatory mediators may be inhibitory and this would be in agreement with the observed increased incidence of dysfunctional labour and lack of response to intravenous OT in pregnancies complicated by intrauterine infection (72, 73). Conversely, it appears that the tremendously elevated concentrations of pro-inflammatory cytokines associated with severe intrauterine sepsis may play a role in stimulating parturition.

An additional factor of potential importance in regulation of OTR is mechanical stretch. This could be of particular importance in the high rate of preterm birth associated with twin gestation. From an elegant series of experiments in rats with pregnancies in one uterine horn only, it was concluded that mechanical stretch, whether due to the growing embryos or to an inert cylinder placed in the non-pregnant horn, caused an increase in OTR at the time of uterine activation (74). This was not evident in non-pregnant animals or in the unoccupied, unstretched horn of the pregnant animals. In pregnant sheep with natural pregnancies in one uterine horn only, there is an increase in OTR in both horns at the time of uterine activation but the increase in the gravid horn is three-fold greater (75). Application of stretch to primary cultures of human uterine myocytes causes an increase in OTR expression but only in cells obtained in late gestation prior to labour and not in cells obtained during labour or from non-pregnant women (76).

Like other G-protein coupled receptors, OTR can be down regulated in response to continuous presence of its ligand. (77) This is seen in OT-induced and augmented

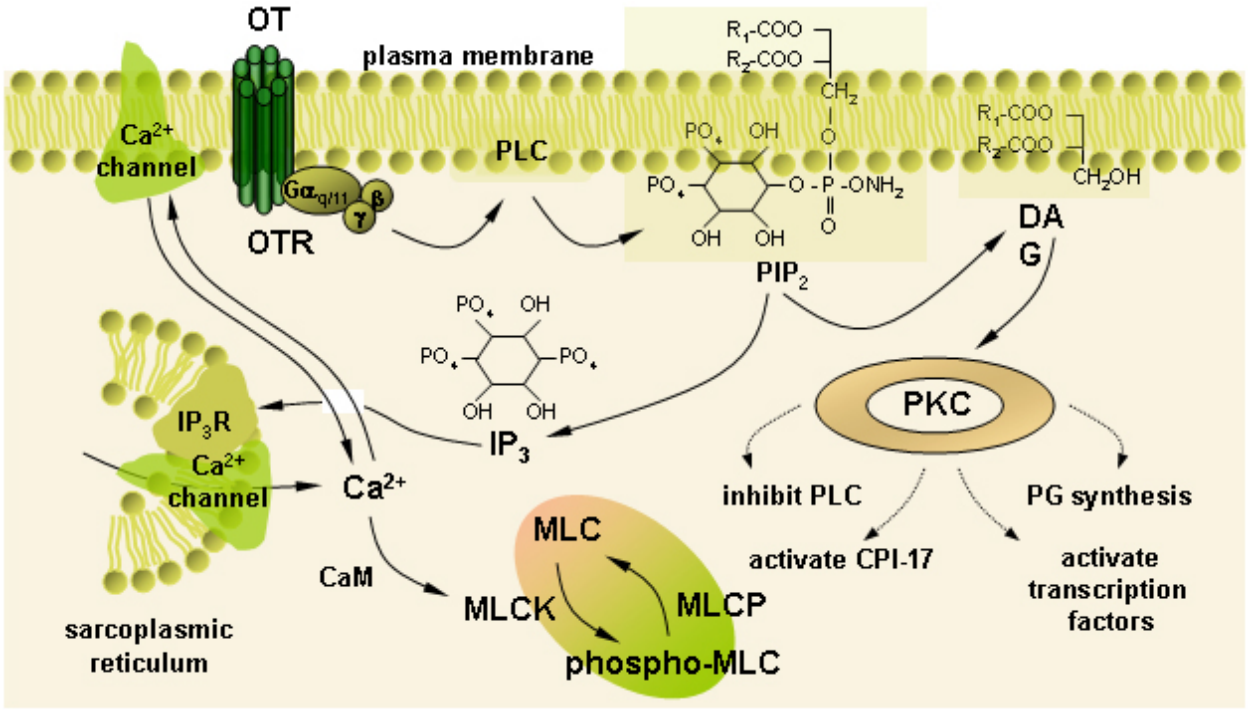

Figure 3. Signal transduction through the OT receptor (OTR). OTR is linked through $G\alpha_{q/11}$ to membrane phospholipase C (PLC) that cleaves phosphatidyl bisinosotides (PIP_2) into inositol trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 stimulates Ca^{2+} release from sarcoplasmic reticulum and this further triggers Ca^{2+} influx from extracellular stores. Increased cytosolic Ca^{2+} binds with calmodulin (CaM) to activate myosin light chain kinase (MLCK), which phosphorylates myosin light chains (MLC) to trigger the contractile machinery of the myocyte. Phospho-MLC is inactivated by MLC phosphatase (MLCP). DAG activates protein kinase C (PKC). In the uterine myocyte, the substrates for PKC are essentially unknown but could include prostaglandin-synthesizing enzymes, "feedback" inhibition of PLC, activation of transcription factors with a wide variety of genomic effects, or activation of the PKC-potentiated inhibitor of protein phosphatase (CPI-17, see text).

labours and is also observed in normal labour (78-80). It can be reproduced *in vitro* and is accompanied by a decrease in mRNA for OTR (78). This process may be mediated by an internalization procedure involving components of the clathrin-coated pits including dynamin and G-protein coupled receptor kinase 2 that are present in uterine myocytes (81, 82).

5.2. OTR – Signal Transduction

In uterine myocytes, OTR is linked through $G\alpha_{q/11}$ subunits to membrane phospholipase Cβ (PLCβ) (83, 84). The signal transduction mechanisms activated by this pathway in smooth muscle have been well reviewed (85, 86). Briefly, PLC-catalyzed hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate results in two parallel signaling pathways: inositol-1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) (Figure 3). IP_3 binds to its specific receptor on the sarcoplasmic reticulum and causes release of Ca^{2+} into the cytoplasm (85, 87). The increase in Ca^{2+} may stimulate further Ca^{2+} influx from the extracellular space into the cytoplasm through plasma membrane Ca^{2+} channels (88, 89). The relative importance of intracellular versus extracellular Ca^{2+} stores for uterine contractions is unclear but it is probable that both pools are necessary for maximal contractility. The increased Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complex activates myosin light chain kinase (MLCK). Activated MLCK catalyzes phosphorylation of myosin light chains, which activate the contractile machinery of the myocyte (90).

In contrast to the IP_3-mediated pathway, the DAG-mediated effects in the uterus are quite unclear. DAG stimulates protein kinase C (PKC). However, the substrates for activated PKC in the uterus are essentially unknown. PKC encompasses a family of serine/threonine-specific protein kinases (~ 80,000 kDa) (91). At least 11 isoforms of PKC have been identified to date. They are divided into three major groups – conventional (α, β1, β2, γ), novel (δ, ε, θ, η, μ) and atypical (λ, ζ) - according to their co-activation requirements (92). These different requirements for activation and regulation together with varying tissue expression give rise to a great variety of cellular control functions.

The DAG-PKC branch of the OTR signal transduction pathway is poorly studied in the uterus. The most commonly used pharmacological agonists are the phorbol esters. These agents are DAG mimetics that bind to PKC to increase activity. Specific inhibitors include calphostin C, which blocks the DAG-binding region (93), and the bisindolylmaleimides, which block the ATP

binding site on the catalytic subunit of PKC(94). Isoform selective inhibitors have also recently become available.

Most PKC isoforms are expressed in the pregnant uterus, with some evidence for pregnancy-induced increases in PKCβ isoforms (95-97). However, the available data regarding the role of PKC in the uterus are conflicting. Some studies suggest that PKC is necessary for the contractile effects of both OT (98) and ET-1 (99-101). Phorbol ester treatment of rat uterine strips *in vitro* had a stimulatory effect at lower concentrations and short treatment intervals (102) but at higher concentrations or for longer durations, the effects were inhibitory. Similarly, phorbol ester at high concentrations suppressed the response of rat myometrial strips to OT but at lower concentrations, the effects were less clear (103). In another study, phorbol ester treatment of human myometrial strips caused a significant stimulation of spontaneous and K^+-induced contractile activity for at least 30 minutes. This effect was greater in tissues from pregnant compared to non-pregnant women and was completely attenuated by treatment with the PKCβ isoform-specific inhibitor LY333531 (104). In contrast, pharmacologically increasing endogenous DAG concentrations caused inhibition of OT-induced contractions in rat myometrium and the effect was markedly increased during pregnancy (105). Thus, it appears the effects of PKC stimulation may depend on the isoform present, the dose and duration of stimulant and the responses may be species-specific. In addition, in several cell types, PKC can feed back to inhibit PLCβ activity through a mechanism that includes phosphorylation of PLCβ (85, 106, 107).

The PKC pathway may have another function in the process of parturition. Human decidua has high concentrations of OTR, which increases further at the time of uterine activation (21). An increase in decidual OT at this time has been described (10). Treatment of human decidual tissues with OT stimulates production of $PGF_{2\alpha}$ (21, 108, 109), which is itself a potent uterine contractile stimulant. This mechanism may involve OT-induced stimulation of the mitogen activated protein kinase (MAPK) system (110), a well-described target of PKC (111, 112).

Research on OTR signaling and uterine contractility has, to date, focused much on either the regulation of the intracellular Ca^{2+} transients that stimulate the contractile machinery of the uterine myocyte or the identity of the intracellular kinases stimulated by OT ligand binding to its receptor. Recently, there has been increasing interest in the intracellular mechanisms that may link these two events and enhance the myocyte contractile response to these Ca^{2+} transients – the phenomenon of Ca^{2+}-sensitization.

6. Ca^{2+}-SENSITIZATION

Though it is clear that myocyte contraction is stimulated by an increase in intracellular Ca^{2+}, it also is evident that, for a given increase in Ca^{2+}, there may be differing amplitudes of contractile response depending upon the specific uterotonin. The process of Ca^{2+}-sensitization was first described in the uterus by Kitizawa who noted that when permeabilized muscle is "clamped" in a constant Ca^{2+} environment, the contractile strength is greater when stimulated by an agonist compared to simple depolarization using high K^+ (93). He also noted that the agonist-induced contraction was accompanied by greater MLC phosphorylation than simple membrane depolarization. The intensity of the myocyte contraction is dependent on the balance between phosphorylated and dephosphorylated states of MLC. The phosphorylation of MLC is dependent on the activity of MLCK, which is dependent on the Ca^{2+} transient created by agonist or high K^+ treatment. The dephosphorylation of phospho-MLC is regulated by the activity of myosin light chain phosphatase (MLCP). Studies in several types of preparations have demonstrated that the mechanisms underlying Ca^{2+}-sensitization are focused on regulation of MLCP. Inhibition of MLCP activity will lead to prolongation of the effects of phospho-MLC and hence greater contractile activity. We will discuss two of the major regulatory pathways that control MLCP activity with particular attention to their possible role in OT-induced uterine contractility.

6.1. Myosin Light Chain Phosphatase

The structure and function of MLCP has been well reviewed (113). MLCP is composed of three subunits (Figure 4). The largest is the myosin binding subunit (MBS) that binds the substrate phospho-MLC. Attached to the amino terminus of MBS is the catalytic subunit that cleaves the high-energy phosphate from phospho-MLC. This subunit is a member of the protein phosphatase 1c (PP1c) family. The third subunit, called M20, is attached to the C-terminal region of MBS and its function is unknown.

In addition to the substrate-binding site, MBS has other binding motifs that may have an important influence on its function. Most importantly, there are two sites (Thr696 and Thr853 in human MBS) that, when phosphorylated, significantly inhibit MLCP activity in isolated protein studies (114). The mechanism underlying this inhibition is not proven but it is likely through interference with substrate binding to MBS and subsequent disruption of the substrate-PP1c interaction (113). The major enzyme involved with phosphorylation of Thr696 appears to be RhoA-associated kinase (ROK) and this will be discussed subsequently. The Thr853 site also may target the enzyme to myosin filaments (115), which may be the optimal intracellular location to influence contractile processes. There also is a site in the C-terminal region that binds GTP-bound RhoA (116), which is an important regulator of the enzyme activity (see below). This site, along with a myosin-binding domain in the C-terminal region of MBS may be important as docking sites to ensure proximity of the holoenzyme to its activators and substrates. Finally, interaction of acidic phospholipids with the C-terminal region of MBS inhibits PPIc activity (117).

MBS also has binding sites that may activate MLCP activity and cause Ca^{2+}-*de*sensitization. These

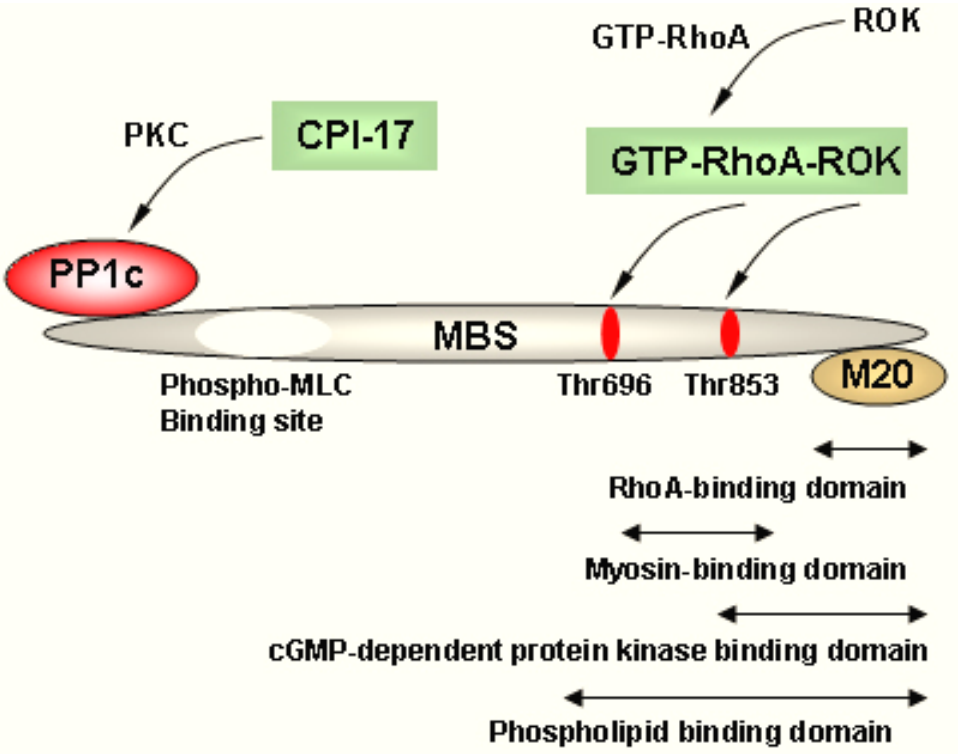

Figure 4. Mechanisms of Ca^{2+}-sensitization. Myosin light chain phosphatase is a heterotrimeric protein that consists of a myosin binding/targeting subunit (MBS) with an attached N-terminal protein phosphatase 1c (PP1c) catalytic subunit and a C-terminal subunit (M20) of unknown function. The catalytic subunit can be inhibited directly by activated CPI-17. Activated ROK can phosphorylate the MBS at Thr696 to markedly diminish enzyme activity. ROK and other kinases can phosphorylate Thr853 to inhibit the enzyme activity. The C-terminal part of MBS may also bind with other factors, as indicated, that could influence enzyme activity.

include a leucine zipper region at the C-terminus of MBS that can interact with cGMP-dependent protein kinase to increase MLCP activity (118). This may be an important mediator of cGMP-mediated relaxants such as nitric oxide. Phosphorylation of the ankyrin repeats at the N-terminal region of MBS by PKC increases binding of phospho-MLC to MBS and enhances dephosphorylation of the substrate (116).

The PP1c catalytic subunit has inherent phosphatase activity for phospho-MLC but this is significantly enhanced by complexing with the MBS and M20 subunits. One potential mechanism of inhibiting MLCP is dissociation of the PP1c subunit from MBS and this can be accomplished by arachidonic acid (119) or by PKC-mediated phosphorylation of the MBS binding site for the PP1c subunit (116). However, it appears that more important mechanisms for inhibition of MLCP involve phosphorylation reactions to impair substrate binding to MBS or direct inhibition of the PP1c catalytic subunit.

In summary, it appears that there are three potential mechanisms to cause Ca^{2+}-sensitization through inhibition of MLCP activity: disruption of the catalytic and substrate binding subunits; direct inhibition of the catalytic subunit; impairment of binding of substrate to MBS such that the interaction between catalytic site and substrate is disrupted.

6.2. RhoA – Rho Kinase Pathway

Within the last decade, it was discovered that a kinase capable of mediating phosphorylation of Thr696 in MBS, the substrate binding subunit of MLCP, is ROK (120). There are two known isoforms of ROK – ROK-1 (also called ROKβ) and ROK-2 (ROKα) (121). The phosphorylation activity of ROK is stimulated by the binding of GTP-bound RhoA.

RhoA is a member of the Rho subfamily of the Ras superfamily of small monomeric G proteins. It is involved in a variety of cellular functions including stress–fiber and focal adhesion formation (122), cell morphology, cell motility, membrane ruffling and smooth muscle contraction (123). RhoA is associated with inhibition of MLCP and hence Ca^{2+}-sensitization induced by agonist stimulation (124). RhoA exists in an inactive form in the cytosol bound to GDP in a complex including a GDP-dissociation inhibitor (GDI) (Figure 5). To be activated, these complexes are disrupted and the GDP exchanged for GTP. This is accomplished by proteins called guanine nucleotide exchange factors (GEFs), which can be activated by agonist binding to G-protein coupled receptors (125). The active GTP-RhoA associates with the plasma membrane and activates ROK (126). The hydrolysis of GTP-RhoA is catalyzed by enzymes called GTP'ase activating proteins (GAPs) (127). The resultant inactive

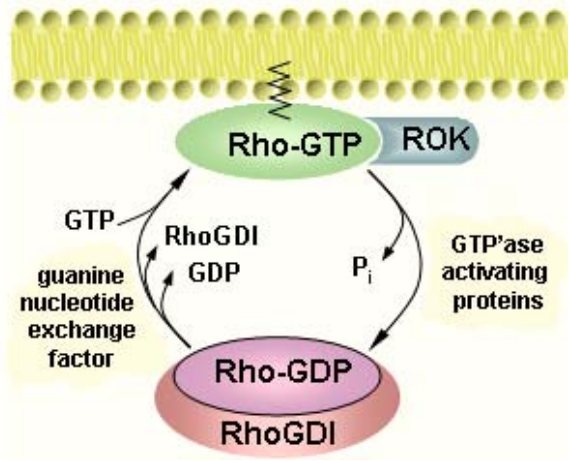

Figure 5. Activation of RhoA-associated kinase activity. RhoA exists in the non-active state in the cytoplasm bound to GDP and complexed with Rho GDP-dissociation inhibitor (GDI). Activation of G-protein coup[led receptors or other mechanisms can activate guanine nucleotide exchange factors (GEFs) that will disrupt the Rho-GDP-GDI complex and exchange GTP for GDP. The "activated RhoA-GTP is then bound to the plasma membrane where it interacts with and activates Rho-associated kinase (ROK). The hydrolysis of the GTP bound to RhoA is catalyzed by GTP'ase activating proteins (GAPs), resulting in production of inorganic phosphate and GDP-Rho, which moves to the cytoplasm and is again complexed with RhoGDI.

GDP-RhoA then leaves the membrane and forms a complex in the cytosol with GDI.

The binding of GTP-RhoA to the Rho binding domain (RBD) of ROK leads to a conformational change causing autophosphorylation and activation of ROK (128). The activated ROK then can phosphorylate one of the inhibitory sites on MBS. ROK-induced inhibition of MLCP can be prevented using ROK antagonists such as Y-27632 or H-1152, which block its activity by competing with the ATP-binding site on the enzyme (129). Interestingly, these pharmacological agents have been shown to cause relaxation of isolated human myometrium during the late third trimester of pregnancy (130).

Messenger RNA for RhoA and ROK-1/2 are present in the myometrium of pregnant women (130-132). RhoA and ROK are recruited to the plasma membrane of freshly isolated myocytes during carbachol-induced contraction (133, 134). In permeabilized cells, agonist-mediated Ca^{2+}-sensitization of force is reversed by the ROK inhibitor Y27632 (135). The Ca^{2+}-sensitisation induced by the phosphatase inhibitor microcystin-LR is unaffected by Y27632 suggesting that, like other smooth muscles, ROK-mediated Ca^{2+}-sensitization may proceed via inhibition of MLCP activity. In mouse and rat uterine myocytes, ROK expression is increased during pregnancy (136, 137) although this effect of gestation is not evident in human myometrium (135). OT treatment of rat uterine myocytes also causes ROK-mediated phosphorylation of MBS (136). In intact tissues, a marked Ca^{2+}-sensitizing action of OT has not always been observed (138, 139). However, these are contrasted by thorough examinations of the $[Ca^{2+}[_i$–force relationships in intact rat and human myometrium revealing a prominent hysteresis mediated by OT stimulation, indicative of Ca^{2+}-sensitisation of tone (140, 141). Furthermore, OT-promoted force was inhibited by Y27632 without a similar effect on $[Ca^{2+}]_i$ (141). A smaller effect of Y27632 on hypercontracted human myometrium has also been reported (139). These studies suggest that increased endogenous ROK activity may be involved in the increased contractility that occurs at the time of labour onset at term and this is in accord with the likely prevalence of multiple G-protein-coupled excitatory stimuli in addition to OT contributing to parturition. However, changes in the activity of the RhoA-ROK system through gestation, and particularly at the time of parturition, remain to be determined. Similarly unresolved, is the state of MBS phosphorylation and activation with parturition *in vivo*. As mentioned above, ROK-mediated phosphorylation on Thr696 or Thr853 was found to inhibit MLCP activity thereby elevating MLC_{20} phosphorylation and tone. However, the situation generally now appears to be more complicated. Certain vascular studies have reported no increase in Thr696 phosphorylation with ROK-dependent agonist constrictions (142-144). It is possible that this is due to high basal levels of Thr696 phosphorylation and that Thr 853 phosphorylation may be the regulatory site to certain stimuli (143, 145).

6.3. CPI-17 Pathway

Another potentially important mediator of Ca^{2+}-sensitization is the 17-kDa protein kinase C-potentiated inhibitory protein (CPI-17) (146). CPI-17 binds to PP1c and directly inhibits MLCP activity with the central domain (residues 35-120 of 147 amino acids) essential for this interaction. CPI-17 was originally identified as a substrate for PKC-dependent phosphorylation on Thr38. It was, therefore, implicated in constrictions, and Ca^{2+}-sensitisations, involving agonists coupled to phospholipase C-DAG-PKC production (146, 147). Once phosphorylated at Thr38, the inhibitory potency of CPI-17 for MLCP is increased about 1000-fold thereby enhancing net MLC_{20} phosphorylation and force (146). Interestingly, inhibitors of both PKC and ROK reduced Thr38 phosphorylation suggesting that CPI-17 could be a downstream target for ROK as well as PKC. There also may be other activators of CPI-17 (121).

The expression of CPI-17 varies for different smooth muscle. It appears to be present in higher concentrations in tonic (vascular smooth muscle) compared to phasic (vas deferens) smooth muscle (148). Nonetheless, CPI-17 mRNA and protein have been measured in the human myometrium and are higher in tissues from pregnant compared to non-pregnant women (104).

6.4. Other possible modulators of Ca^{2+}-sensitization.

Recently it has become clear that an additional intracellular signaling kinase may participate in events leading to altered MLCP activity. Integrin linked kinase

(ILK) was first identified as a β-integrin binding protein and part of a dynamic complex linking the extracellular matrix with intracellular myofilament proteins (149). Subsequently, it has been suggested to contribute to smooth muscle force production by any of three pathways: (i) direct phosphorylation of myosin light chains; (ii) MBS phosphorylation at Thr696; (iii) phosphorylation of the PP1 inhibitory proteins CPI-17 or PHI-1 (phosphatase holoenzyme inhibitor-1) (150, 151). The latter two processes would aid a Ca^{2+}-sensitization of tone. Intriguingly, PHI-1 is a homologue of CPI-17 and is also phosphorylated and activated by PKC or ROK. The upstream activators of ILK are not fully recognized but may include G-protein-coupled receptors and mechanical stretch. It has even been postulated in primary cultured smooth muscle cells that G-protein-coupled stimulation results in distinct intracellular mechanisms of CPI-17 or PHI-1 phosphorylation (152). As such, it will be interesting to elucidate the expression and role(s) of the MLCP interacting proteins ILK and PHI-1 in the myometrium.

7. PERSPECTIVE

The first half-century of research directed towards reducing the incidence of preterm birth has been a dismal failure! Not only have preterm birth rates remained stable or increased, our attempted pharmacologic interventions for preterm labour have likely caused far more maternal and fetal/neonatal morbidity and mortality than improvements in infant outcomes. Research in parturition has been predominantly focused on the regulation of concentrations of uterine contractile agonists and their receptors. We speculate that Ca^{2+}-sensitization pathways also may play important regulatory roles in uterine contractility. We have discussed two main inhibitory pathways for MLCP, the enzyme that is primarily responsible for Ca^{2+}-sensitization. Further exploration of these and additional pathways could lead to development of methods to predict, prevent or better treat preterm labour.

There is an urgent need for improved understanding of the regulation of uterine activity and the process of parturition. This will include better understanding of the roles and mechanisms of the signaling cascades stimulated by OT and other uterine agonists and relaxants. Only then will it be possible to relate pathological conditions causing preterm birth to a physiological mechanism and determine what went wrong. Only then will it be possible to design, on a scientific basis, pharmacological or other interventions that might decrease the incidence of the syndrome of preterm labour. Only then will it be possible to effectively address the high social and economic burdens of preterm birth.

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