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Commentary

Uncovering the existence of compartmentalized cAMP signals in the human myometrium

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Background

The progression of pregnancy to term (> 37 weeks') to allow optimal fetal development is underpinned by a myriad of complex interconnected maternal and fetal processes. The spontaneous initiation of labor, at any gestation, still remains incompletely understood. This area of research is highly contentious due to the multitude of factors involved and although several theories have been put forward to clarify the intricate molecular and cellular events that lead to the initiation of labor, a single, conclusive pathway has yet to be determined.

Preterm birth (PTB) and it's resulting lifelong complications remain a significant cause of neonatal morbidity and mortality globally in children below the age of 5, with a consistent rise in cases annually [1–3]. Although a definitive cause is identified in some women, specifically in instances of infection [4], it is widely recognized that preterm labor (PTL) is considered to be a complex 'syndrome', which is influenced by several pathophysiological processes involving fetal, maternal, and placental factors [5]. Tocolysis, a treatment to suppress uterine contractions, is only effective in a small subset of cases [6]. In PTL, these drugs are utilized predominately to delay delivery in order to facilitate the in-utero transfer of mothers to specialist tertiary centers or to allow for the administration of magnesium sulphate and corticosteroids for fetal neuroprotection and accelerated lung maturation [7–10]. Currently, there are no Food and Drug Administration approved tocolytic drugs. Various unlicensed medications are used globally which target specific intracellular signaling mechanisms involved in the regulation of myometrial contractility. Betamimetics, β-adrenorecept or agonists, were widely used as tocolytic drugs following their study and research in the 1960s [11] and continue to be administered worldwide, particularly in low- and middle-income countries due to their cost effectiveness [12,13].

The human myometrium expresses 3 sub-types of the β-adrenergic receptor, β1-3, which are part of the membrane-bound G protein-coupled receptor (GPCR) family [14]. Upon β-adrenoreceptor agonist stimulation, subsequent downstream intracellular signaling events initiate cyclic AMP (cAMP)-mediated protein kinase A (PKA) activation, which is a key pathway involved in myometrial relaxation [15]. The catalytic subunit of PKA has been identified to phosphorylate crucial contractile proteins, including myosin light chain kinase (MLCK), which prevents the subsequent fundamental cross-bridging interaction of actin and myosin responsible for smooth muscle shortening, thus hindering contractility [16–18]. Additionally, activated PKA inhibits the function of phospholipase C (PLC), a key regulator in the mobilization of calcium, impeding the initiation of muscle contractions [19]. The PKA-mediated phosphorylation of large-conductance Ca^{2+} activated potassium channels (BK_{Ca}), and ATP-sensitive channels (K_{ATP}) also promotes myometrial relaxation [20,21].

Subcellular compartmentalization of cAMP into receptor-specific cAMP nanodomains [22] explains how different hormones, which act via cAMP, can induce specific cellular responses [23]. These nanodomains have been characterized to be spatially distinct regions within the cell, each independently regulated, ensuring precise hormonal signaling events [24,25]. The significant advancements in the use of Fluorescence Resonance Energy Transfer (FRET) imaging and development of sophisticated biosensors have facilitated the detailed investigation of cAMP concentrations in living cells, in addition to the real-time kinetics and activity of individual molecules involved in cAMP signaling [26–29]. Several FRET biosensors have been produced to monitor cAMP signals or specifically analyze the activity of the cAMP effectors, PKA and exchange factor activated by cAMP (EPAC). The basic design of a FRET reporter encompasses a cAMP sensor, with either a single protein domain or two interacting protein domains which undergo conformational change upon cAMP binding, and two fluorescent proteins (FPs) fused to the probe, usually the cyan (donor fluorophore) and yellow (acceptor fluorophore) emitting variants of the green fluorescent protein (GFP) [30,31]. FRET efficiency relies strictly on the orientation and distance between the fluorophores, which are altered by changes in the conformation of the sensing domain upon cAMP binding [30]. This sensitive technique is unmatched in its spatial and temporal resolution, detecting discrete cAMP pools to be nanometers apart [32]. The localized pools of cAMP activate PKA embedded in macromolecular protein complexes or 'signalosomes' that are spatially confined at distinct subcellular sites, enabling selective phosphorylation of local PKA targets [32]. The targeted localization of these diverse, but highly integrated cAMP signaling units is facilitated by the multi-scaffolding functionality of A-kinase anchoring proteins (AKAPs) [33]. AKAPs mediate the direct interactions between upstream signal transduction proteins, effectors, downstream targets and also the regulatory enzymes associated with each unit [34]. These tightly coupled compartmentalized 'signalosomes' have a unique function determined by the specific combination of proteins they incorporate, such as GPCRs, adenylyl cyclases (ACs), phosphatases, and protein kinases and are regulated by a distinct subset of phosphodiesterases (PDEs), the enzyme that degrade cAMP to AMP, switching off the signal [32].

The generation of cAMP biosensors that localize to a specific location via fusion with targeting domains has facilitated enhanced mapping of the cellular organization of compartmentalized cAMP networks, including the control mechanisms governing individual cAMP signalosomes, and the physiological relevance of these localized cAMP pools [32]. Recently, evidence has suggested that disruption in the structural organization or location of these unique cAMP-dependent signalosomes, or genetic variants of key cAMP signaling components, are strongly associated with the development of several pathological conditions such as cardiac arrhythmias, cardiac failure, cancer, and certain neurological diseases [35–40].

New Insights into cAMP Compartmentalization in Human Myometrial Cells

Critical alterations in the expression levels of key cAMP signaling components and the cAMP effector system have been identified in the human myometrium, specifically in the final common pathway involved in the initiation of labor at term (as illustrated in **Figure 1** below), which may be key to the regulation of uterine function [41–43].

Similar changes have also been observed in PTL samples with different phenotypes [43]. In particular in twin pregnancies (T-PTL) and those complicated by chorioamnionitis (CA-PTL), a decrease in $PKAR_{2\alpha}$ was observed, whilst an increase in PDE4B and a reduction in AKAP79 protein levels were seen in CA-PTL [43]. Finally, an increase in EPAC1 protein occurred across all forms of PTL [43]. The potential switch in cAMP effector predominance observed in both early term and preterm twin labor with a reduction in $PKAR_{2\alpha}$ and increase in EPAC1 was considered to be driving the observed increased OTR expression promoting the onset of labor [41,43]. These changes in the cAMP system in early term labor and PTL, occurring as a result of stretch or inflammation, provide potential therapeutic targets which could be modulated by uterine selective drugs in the management of labor onset in these clinical circumstances [43]. Moreover, these findings imply that cAMP signaling may be compartmentalized in myometrial SMCs, suggesting that therapeutic interventions could potentially be targeted to individual domains, thus reducing unwanted side effects.

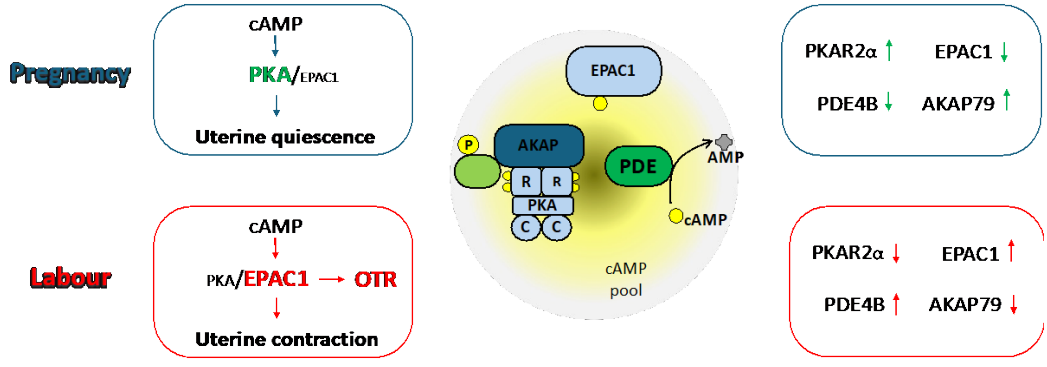

Figure 1. A schematic depicting key changes in the cAMP signaling components and proposed mechanistic theory for cAMP's action during pregnancy and at the onset of term labor. A potential switch in the cAMP effector system has been characterized whereby during pregnancy cAMP acts via a PKA-dependent pathway promoting myometrial relaxation, whilst with the onset of labor it functions via EPAC1 driving oxytocin receptor (OTR) expression and triggering a contractile response [41,43].

A recent study used FRET imaging and genetically encoded targeted cAMP reporters in human primary myometrial cells (HPMCs) to directly investigate the existence of spatially distinct cAMP domains [44]. Uncovering the physiological mechanisms that govern normal pregnancy is crucial in progressing to understand the changes that occur in labor, both at term and, more importantly, preterm. This study therefore focused specifically on myometrium obtained from women who were not in labor at term. Prior to this, there was no reliable technique to determine whether cAMP signaling is in fact compartmentalized in the human myometrium.

The use of primary cells presents certain experimental challenges, including limited cell availability and cell survival in culture [44]. In view of this, a readily available hTERT myometrial cell line (hTERT-HM) was investigated in addition to HPMCs [45]. Extensive optimization studies were conducted initially using primary cells to maximize the isolation of cells from each myometrial tissue biopsy. Additionally, FRET imaging experiments confirmed phenotypic changes in sub-cultured cells, validating the use of primary cells at passage 0 only [46].

The FRET reporters were delivered to the cells using an adenoviral-mediated infection process [47] and after confirming successful expression and localization of the sensors in both cell types, a direct comparison of the individual cell responses upon agonist stimulation was conducted at two specific cellular compartments, the bulk cytosol and the sub-plasmalemma domain [44]. Isoproterenol (ISO) and prostaglandin E2 (PGE2), which regulate myometrial relaxation during pregnancy [48,49], were used as stimuli for cAMP elevation.

This study uncovered significant differences in the cAMP signaling and PDE regulation between HPMCs and hTERT-HM cells. Detection of cAMP elevation in the presence of a non-selective PDE inhibitor, IBMX, clearly revealed distinct PDE-dependent regulation of cAMP levels at the two subcellular compartments in the hTERT-HM cells (**Figure 2A**) [44]. The cAMP pools generated in the PGE2-stimulated hTERT-HM cells were found to be spatially controlled by PDE-dependent degradation, specifically at the plasmalemma [44]. This regulatory effect was less evident for the cAMP signals triggered by β-AR stimulation [44]. In the HMPCs however, the cAMP hydrolytic activity at the two subcellular sites was found to be overall comparable for each agonist (**Figure 2B**) [44].

A limitation of the hTERT-HM cell line used in this study is that it was derived from myometrial tissue obtained from the anterior wall of the uterine fundus of a non-pregnant woman, classified as an upper segment sample [50]. The activity of PDEs during pregnancy has been shown to be reduced compared to non-pregnant myometrium, possibly due to higher levels of progesterone [51,52]. PDEs are crucial in the precise regulation of local cAMP gradients generated as they can be part of specific signalosomes, which are localized to different subcellular sites via AKAPs and incorporate a unique set of other signaling proteins [32]. Distinct PDE isoforms

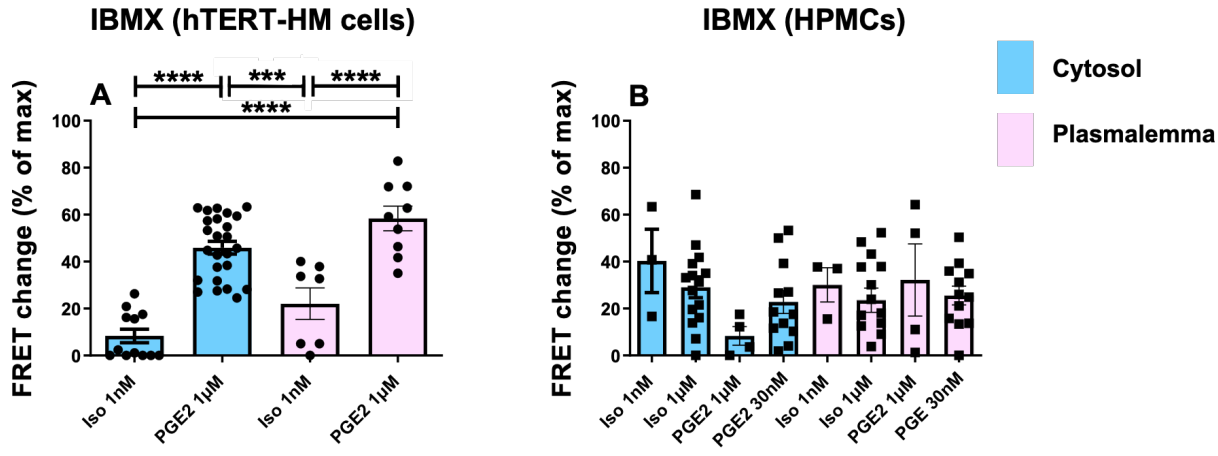

Figure 2. Comparison of the cAMP response to IBMX in the hTERT-HM cells and HPMCs in the cytoplasm and plasmalemma following agonist application. FRET-responses to IBMX (100 µM) applied following ISO or PGE2 treatment of hTERT-HM cells (**A**) or HPMCs (**B**) expressing the Epac-S^{H187} sensor (blue) or AKAP79-CUTie sensor (pink). Values were calculated as the FRET change on application of IBMX relative to maximal FRET change at saturation and are presented as mean ± SEM. For hTERT-HM, each data point (circles) represents one cell (measurements from at least three independent cultures). For HPMCs, each data point (squares) indicates average measurements from individual donors.

Experimental set up & FRET data analysis: Isolated HPMCs and hTERT-HM cells were seeded separately onto sterilized 15 mm borosilicate glass coverslips. 4.5×10^7 virus particles of EPAC-S^{H187} or 3.8×10^7 virus particles of AKAP79-CUTie sensor were added to the culture medium of each coverslip. The cells were used at 18–24 h for the EPAC-S^{H187} sensor or 48 h for the AKAP79-CUTie sensor at approximately 40% confluency to facilitate imaging of multiple cells per experiment, whilst ensuring for a region devoid of cells for background correction. Cells expressing the sensors were excited at a wavelength of 436 nm ± 10 nm and sensitized emission intensities were measured at 470 and 535 nm, respectively for YFP and CFP emission. Emission signals were monitored in real-time. Ratio and FRET-changes were calculated on background corrected emission intensities. These ratio or FRET-change values correlate to changes in intracellular cAMP concentration. Normalization to the maximal response generated by forskolin (25 µM) allowed for comparison of the two FRET reporters.

Statistical analysis: Data distribution was determined using the Shapiro-Wilk test followed by a Mann-Whitney test. Statistical significance was set at $P < 0.05$. Significance symbols: * = $<0.05<p<0.01$, ** = $0.01<p<0.001$, *** = $0.001 < p < 0.0001$, **** = $p < 0.0001$. Adapted from Varley *et al.,* 2023, cells [44,46].

have been found to preferentially modulate certain GPCRs, dependent on the cellular stimulus, thereby spatially restricting local cAMP concentrations [53]. For example, in neonatal rat ventricular myocytes, PDE3 and PDE4 are localized to different regions within the cell, with PDE4 specifically hydrolyzing cAMP pools induced by β-adrenergic receptor activation [53].

In addition to the distinct PDE-mediated regulation of the cAMP pools between the two cell types, the study also revealed striking differences in the agonist-induced responses with considerably different concentrations of agonist required to elicit a near to half-maximal FRET response [44]. Specifically, substantial cAMP responses were generated to PGE2 in the HPMCs in comparison to the hTERT-HM cells, which were found to be more sensitive to β-AR stimulation [44]. These findings suggest that the extent of cAMP synthesis may differ between the two cell types, potentially as a consequence of variations in EP receptor or β2-AR density.

Previous analyses of the gene expression profiles attained by a cDNA microarray of three immortalized cell lines derived from term pregnant human myometrium and their corresponding primary cells found that several genes associated with prostaglandin synthesis were downregulated in the cell lines [54]. Alterations in the expression of key components of the prostaglandin pathway may influence EP receptor responsiveness or certain EP receptor isoforms may have a different affinity for PGE2. This, in turn, could account for the reduced PGE2-responses observed in the hTERT-HM cell line used in this study. Of the four EP subtypes, EP1 and 3 induce the IP3/calcium pathway through activation of PLC and inhibit AC, whereas EP2 and 4 stimulate AC through coupling with GαS promoting cAMP synthesis [55]. EP2 receptor expression has been observed to be higher in pregnant myometrial tissue obtained from the lower segment of the uterus with EP2 receptor specific agonists generating a greater response compared to upper segment non-pregnant tissue where EP1 and EP3 are found to be predominantly expressed [56,57]. Conversely, β2-AR expression was increased in non-pregnant myometrial tissue with salbutamol more effectively inhibiting contractions compared to pregnant samples in functional studies [48,58]. Notable differences between the two cell types emerged from the FRET imaging experiments which likely extend beyond their physiological states (pregnant vs. non-pregnant) and anatomical origin, and as such, should be acknowledged when using myometrial cell lines as a model system.

Evidence for compartmentalization of cAMP in HPMCs was also uncovered when optimized doses of each agonist were employed [44]. Specifically, the two agonists were found to differentially affect each subcellular compartment, and the cAMP responses were shown to be independent of each other [44]. Interestingly, significant inter-patient variability in the cAMP response generated specifically to isoproterenol was also observed [44]. This finding is in line with contractility studies using different β2-AR stimulants on pregnant myometrial tissue strips which elicited a wide range of responses [59,60]. Potential explanations for this observation included differences in receptor density expression across the tissues or altered coupling with G-proteins or reduced AC activity, however this was not investigated further [60].

As illustrated in **Figure 3**, striking differences were observed in the kinetic profiles of the cAMP responses to each agonist in the cytosol and at the sub-plasmalemma compartments, which further confirmed the existence of compartmentalized cAMP handling in HPMCs [44]. In contrast to the sub-plasmalemma, where sustained responses to each agonist were observed (data not shown), in the bulk cytosol spontaneous oscillatory changes were seen, specifically for PGE2-treated cells (see **Figure 3B**). These findings may be attributed to a strong regulation of the cAMP pools generated within this compartment by PDEs [44].

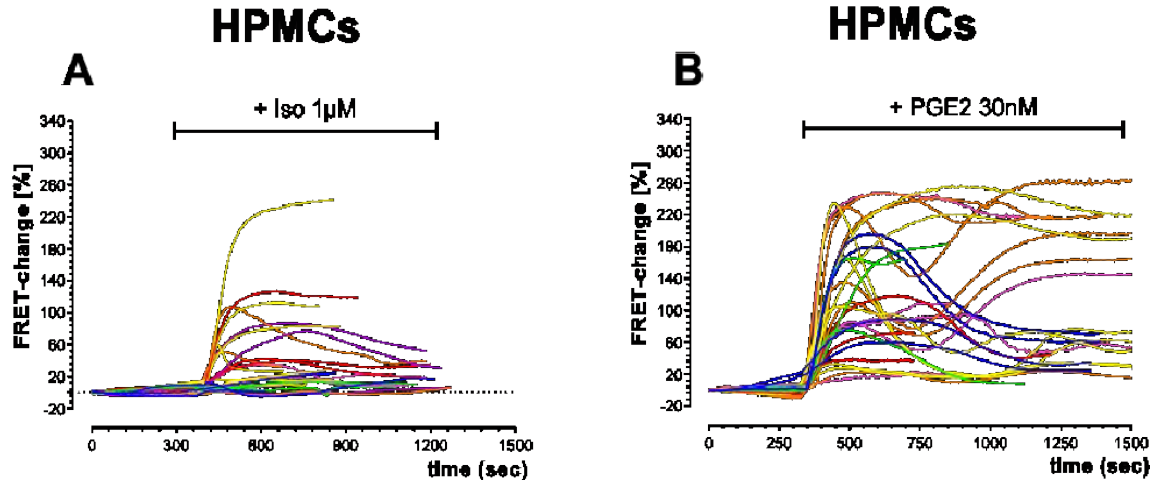

Figure 3. Kinetics of cAMP changes measured in the cytosol of HPMCs in response to ISO or PGE2 application. Kinetics of FRET- change over time, expressed as percent increase over basal values recorded in individual HPMCs expressing the cytosolic FRET sensor Epac-SH187 and stimulated with (**A**) 1 µM ISO (n=26 cells, 7 patients) or (**B**) 30 nM PGE2 (n=28 cells, 6 patients). Same color lines indicate cells from the same patient. Prior to the addition of each agonist, an initial stable baseline is established. Each pre-diluted agonist is applied evenly to the cell bathing solution. Adapted from Varley *et al*., 2023, cells [44].

Isoforms PDE4B2 and PDE4D are found to be most abundant in the human myometrium with higher levels detected in term pregnancy [61–63]. Both in non-pregnant and pregnant myometrial cells, an upregulation in PDE4 expression and activity has been observed with PGE2 treatment in a dose-dependent manner [64,65] consistent with the findings of a strong coupling of PGE2 and PDEs in the bulk cytosol of human myometrial cells, supported by these FRET imaging experiments [44].

Implications and Future Research Opportunities

The compartmentalization of cAMP signaling in the human myometrium has significant implications for our understanding of uterine physiology during pregnancy and labor. Currently, there are no effective medications capable of halting established labor once it has commenced, regardless of gestational age. Therefore, uncovering and defining the subcellular processes that regulate the onset of labor is essential for developing novel therapeutic strategies in order to facilitate the control of labor when medically required.

The pathophysiology of PTL is complex, consisting of a multi-factorial syndrome characterized by premature myometrial activation [5]. Infection is the only identifiable causal factor, but considerable evidence has been presented for an upregulation of pro-inflammatory pathways, hormonal shifts and mechanical stretch in augmenting cervical ripening and driving labor onset with uterine contractility [66,67]. The observed molecular switch in cAMP effector predominance in both term labor and PTL complicated by twin pregnancy may be a key process which determines the subsequent pro-labor cellular phenotype, via an upregulation in OTR, and thus contributes to the final common pathway of labor [41,43]. Further detailed investigation into the protein composition of the cAMP effector system within the specific, local subcellular environment, how it is regulated and the changes which occur in their binding partners with the onset of term labor is key to understanding the function of cAMP in the human myometrium. Once this is clearly defined, it is then possible to investigate the critical mechanistic changes which occur when cAMP compartmentalization is disrupted secondary to inflammation, infection, and/or hormonal dysregulation which are key contributors in premature uterine activation resulting in PTL.

The utilization of cell-region specific FRET reporters has revealed the presence of agonist-dependent compartmentalized cAMP pools in both primary cells and an hTERT-HM cell line [44], providing an opportunity for targeted therapeutic intervention. While so far only two subcellular sites have been investigated, the bulk cytosol and sub-plasmalemma regions, various alternative FRET cAMP sensors have been developed which localize to other key subcellular domains, including the nucleus, sarcoplasmic reticulum, and mitochondria [68–70]. A-kinase activity (AKAR) reporters provide a direct readout for cellular PKA activity and sophisticated constructs can localize to distinct membrane domains, such as lipid rafts, allowing interrogation of their regulation and interaction with PKA [71,72]. Exploring cAMP dynamics and PKA activity across multiple spatial locations would refine our understanding of the complex network of cAMP nanodomains underpinning myometrial physiology and their role in the transition from uterine quiescence to contractility.

Additionally, alternative labor-associated signaling pathways can be examined at distinct subcellular compartments using other GPCR agonists to assess their effects on local cAMP pools and how these change with the onset of labor. Further study is needed to understand agonist-specific cAMP production and identify the specific PDE isoforms that shape cAMP gradients in myometrial cells. Recent advancements in cell-permeable peptide disruptors have enabled the mapping of individual PDE-protein interactions within cAMP signalosomes, allowing selective modulation of specific PDE isoforms [73]. Characterizing the spatial and functional interactions between PDE isoforms, cAMP effectors, AKAPs and key signaling molecules is essential to determine how compartmentalized cAMP signaling promotes uterine quiescence. Investigating how disruptions in these interactions affect local cAMP signaling is also crucial for understanding their role in driving a contractile phenotype, particularly within the context of PTL.

Understanding the complexities of subcellular cAMP signaling in the human myometrium could pave the way for highly targeted therapeutic treatments. By achieving subcellular precision, this therapeutic strategy will limit the disruption to systemic cAMP function, minimizing side effects, improving drug efficacy and safety, and effectively managing PTL.

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