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**Investigation of the coordinated interplay among
 β_2 -adrenergic receptors, phosphodiesterase 4
and the AKAP-PKA in the cAMP dependent relaxation of
the late pregnant uterus**

Summary of Ph.D. Thesis

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Introduction

The control of uterine smooth muscle function is of vital importance during pregnancy and parturition. Worldwide, about 13 million preterm babies are born each year. There is a strong relationship between systematic or intrauterine infection and preterm delivery. The ultimate goal of treatment for preterm labour is to eliminate or reduce perinatal morbidity and mortality. High levels of preterm birth are probably due to intrauterine infection or lack of availability of drugs, such as tocolytic agents. In view of the relatively high rates of adverse maternal and fetal events and in the hope of improving the perinatal outcome, there is growing interest in experimental studies of the use of different tocolytic drug combinations.

A major signaling pathway implicated in maintaining myometrial relaxation is the cAMP/cAMP-dependent protein kinase A pathway. While β_2 -adrenergic receptor agonists stimulate the generation of cAMP in the myometrium, hence commonly used to treat preterm labor, whereas phosphodiesterase enzymes (PDEs) warrant termination of signalling by the degradation of cAMP to the inactive 5'-AMP.

Accordingly, the putative therapeutic combinations of β_2 -AR agonists and selective PDE4 inhibitors may enhance the efficacy of human tocolytic therapy.

Aims

1. The first aim of the study was to investigate the uterus-relaxing effect of the non-selective PDE inhibitor theophylline and the selective PDE4 inhibitor rolipram on isolated uterine rings from intact late-pregnant female rats (on days 20 and 22 of pregnancy) and of pregnant rats treated with LPS to evoke preterm labor (on day 20), *in vitro*.
2. By isolated organ methods our further aim was to test the uterus-relaxing effect of the selective PDE4 inhibitor rolipram in combination with the β_2 -AR agonist terbutaline, in intact and in LPS-treated rats, both *in vitro* and *in vivo*.
3. The *in vitro* uterus-relaxing effect of rolipram in combination terbutaline was tested on human uterine specimens obtained from women undergoing Caesarean section at term pregnancy or at preterm birth.

4. Our following aim was to investigate the effect of terbutaline in combination with the AKAP-PKA interaction-inhibiting molecule, FMP-API-1 on isolated uterine rings from intact pregnant rats, *in vitro*.
5. We measured the uterine cAMP levels by means of enzyme immunoassay (EIA) in the presence of rolipram and terbutaline alone, and their combination in pregnant rats and in human tissue samples at full term and at preterm birth. We also determined the changes in cAMP levels in the presence of FMP-API-1 in pregnant rat uterus.
6. The expressions of PDE4B and PDE4D proteins in the human uteri at term pregnancy and at preterm birth were also aimed to be detected, by means of Western blot.

Materials and methods

Animal studies

In vivo treatments

The animals were divided into 2 groups: (1) intact pregnant rats on day 22 of pregnancy; and (2) rats treated with LPS (i.p. 125 µg/day) to evoke preterm birth, which occurred on the early afternoon of day 20.

In vitro contractility studies

On days 20 and 22 of pregnancy the rats were killed by CO₂ inhalation, and the uteri were removed and prepared for the *in vitro* contractility assay, as it was reported previously. Briefly, the isolated uterine horns were immediately placed in an organ bath perfused with 95% oxygen and 5% carbon dioxide. Temperature was maintained at 37 °C. Rhythmic contractions were then elicited with 25 mM KCl. The effects of FMP-API-1, theophylline, rolipram and/or terbutaline on uterine rings were measured in the concentration range 10⁻¹²–10⁻⁵ M, in a cumulative manner. The tension of the myometrial rings was measured with a strain gauge transducer, and recorded and analysed with the SPEL Advanced ISOSYS Data Acquisition System. Areas under the curves (AUC) of 4-min periods were evaluated; the effects of rolipram and terbutaline were expressed as percentages of the KCl- induced contractions preceding the administration of the relaxing drugs. The maximum contraction-

inhibiting values were calculated. The results were analyzed by using one-way ANOVA with the Newman-Keuls post-test.

In vivo myographic studies

The in vivo myographic studies were done on intact rats and on LPS-treated rats. Rats were anesthetized and the jugular vein was cannulated for i.v. drug administration. After laparotomy, the left uterine horn was exposed, and an implantable force/displacement transducer was sutured onto the myometrial surface. The animals with the sensors were then placed into a Faraday cage. The amplified electric signal was detected and analyzed by the S.P.E.L. Advanced ISOSYS Data Acquisition System.

Doses of terbutaline, and rolipram, were administered i.v. and the contraction signals were recorded. Two 0.5 µg/kg doses of terbutaline were followed by 10 doses of 1 µg/kg at 5-min intervals. In combination rolipram was given in a single dose of 500 µg/kg. AUCs of 4-min periods were evaluated; the effects of terbutaline or the terbutaline + rolipram were expressed as percentages of the spontaneous activity.

Measurement of uterine cAMP accumulation

Uterine samples from intact 22-day pregnant and LPS-treated 20-day pregnant rats were incubated in de Jongh solution. The extents of cAMP accumulation were determined in the presence of terbutaline (10^{-7} M) or rolipram (10^{-6} M) alone, and also in combination, as well as in the combination with terbutaline + FMP-API-1. Uterine cAMP accumulation was measured with a commercial competitive cAMP EIA Kit, tissue cAMP levels were expressed in pmol/mg tissue. The results were analyzed by using one-way ANOVA with the Newman-Keuls post-test.

Human myometrial studies

In vitro contractility studies

30 biopsy specimens of human myometrial tissue were obtained at Caesarean section in the third trimester of pregnancy in two groups: at full term pregnancy (37-41 weeks of gestation) and at preterm birth (32-36 weeks). Tissue samples were equilibrated in an organ bath (Krebs-Henseleit solution at 37 °C), and tested in parallel. Rhythmic contractions were elicited by 10^{-6} M oxytocin. After stimulation, theophylline, rolipram, or terbutaline was added in non-cumulative manner. The effect of terbutaline was also tested in the presence of

10^{-6} M rolipram. AUCs of 4-min periods were evaluated; the effects of rolipram and terbutaline were expressed as percentages of the oxytocin-induced contractions.

Measurement of uterine cAMP accumulation

Human uterine tissue samples from full term pregnancy and preterm birth were incubated under the same conditions in organ bath as details above. In the presence rolipram (10^{-6} M; 10 min), the cAMP accumulation was stimulated by terbutaline (10^{-8} ; 10^{-6} ; 10^{-4} M; 10 min) and finally forskolin was added to the bath (10^{-5} M; 10 min).

Western blotting studies

Proteins from human uterine specimens were isolated with the Macherey-Nagel Nucleospin Kit. Protein concentrations were determined by BioSpec-nano. 60 μ g of protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units. Proteins were transferred from gels onto nitrocellulose membranes, using the iBlot Gel Transfer System. The blots were incubated on a shaker with PDE4B and PDE4D polyclonal antibodies in the blocking buffer; immunoreactivity was detected with the WesternBreeze Chromogenic Western blot immunedetection kit. The optical density (O.D.) of each immunoreactive band was determined with Kodak 1D Images analysis software. O.D. values were calculated in arbitrary units after local area background subtraction. The results were analyzed by using one-way ANOVA with the Newman-Keuls post-test.

Results

Animal studies

In vitro contractility studies

Although the non-specific PDE inhibitor theophylline had very limited effect in the uteri of intact rats, it showed significant effect in LPS-treated rats. The contraction-inhibiting effect of the specific PDE4 inhibitor rolipram exhibited a similar tendency: a declining relaxing effect towards term.

The effects of the β_2 -AR agonist terbutaline alone or in the presence of rolipram were also tested on the isolated uterine rings. (**Fig. 1/A**). In the presence of 10^{-6} M rolipram, the contractions were reduced by terbutaline to 74.02 ± 2.98 S.E.M.% ($p < 0.05$). In the LPS-treated

rats (**Fig. 1/B**), on day 20 of pregnancy, terbutaline alone decreased the uterine contractions by 64.25 ± 3.60 S.E.M.%, while in the presence of rolipram the KCl-induced contractions were reduced by 90.15 ± 5.21 S.E.M.% ($p < 0.01$).

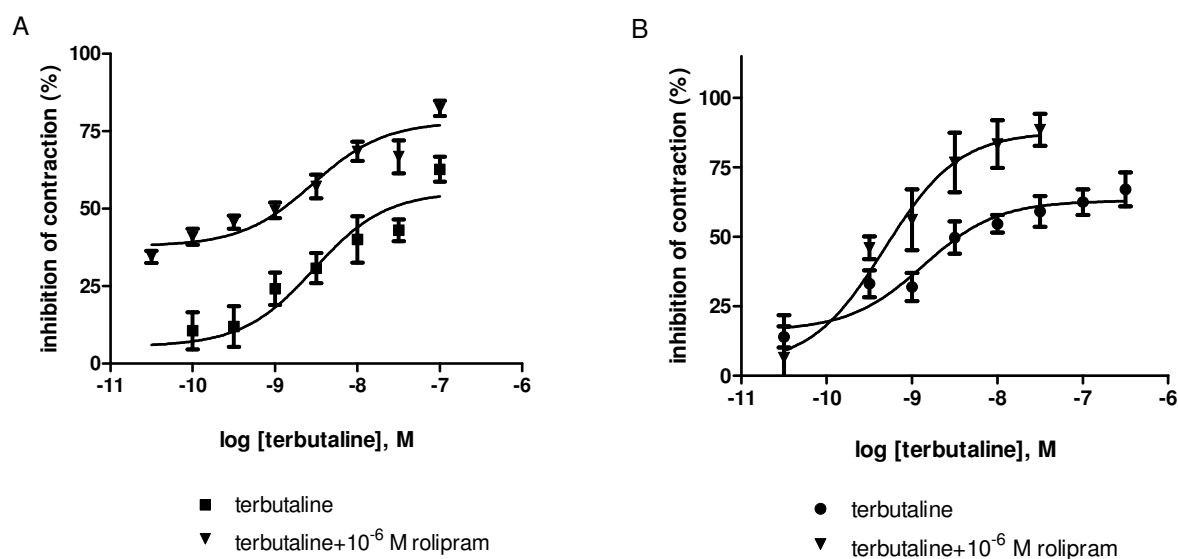


Fig. 1. Effect of rolipram on the uterine-relaxing effect of terbutaline in intact rats (■) (A) and in LPS-treated rats (▼) (B). *in vitro*. $n=8$ in each group.

The β_2 -adrenergic receptor agonist terbutaline alone elicited a 60.77% inhibition of KCl-evoked contractions, at term. In the presence of the AKAP-PKA interaction-inhibiting FMP-API-1, the maximal contraction inhibiting effect of terbutaline was increased to 75.09% ($p < 0.05$).

In vivo contractility studies

The maximal inhibitions achieved with terbutaline + rolipram were not statistically different in intact rats on day 22 of pregnancy (**Fig. 2/A**) and in LPS-treated rats on day 20 (**Fig. 2/B**). In case of the combination, rolipram potentiated the effect of terbutaline, which effect primarily prevailed at the low doses of terbutaline. In the presence of rolipram and at the lowest dose of terbutaline (0.5 $\mu\text{g}/\text{kg}$), however, the inhibition of the contractions was significantly higher ($p < 0.001$) in the LPS-treated rats (**Fig. 2/B**) than in the intact rats (**Fig. 2/A**).

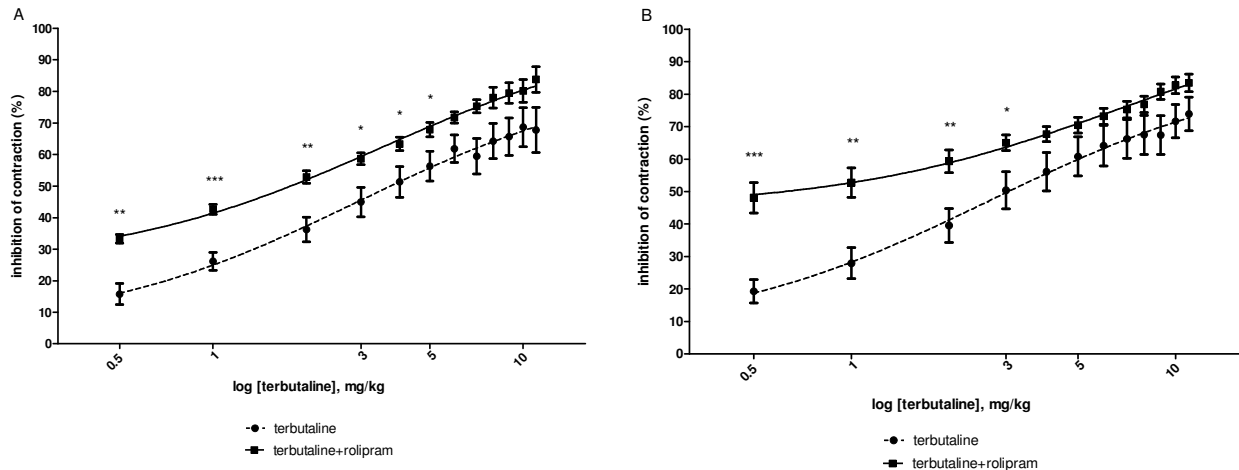


Fig. 2. Effect of rolipram on the contraction-inhibiting effect of terbutaline *in vivo*. In the presence of 500 $\mu\text{g}/\text{kg}$ rolipram (■), the contraction-inhibiting effects of 0.5-11 $\mu\text{g}/\text{kg}$ terbutaline were significantly higher in low dose both in intact rats on day 22 (A) and in LPS-treated rats on day 20 of pregnancy (B) than the effects of terbutaline alone (●). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Changes of uterine cAMP levels in rats

The cAMP generation in the pregnant rat uterus was measured in the presence of terbutaline (10^{-7} M) and rolipram (10^{-6} M) alone and of their combination (**Fig. 3**).

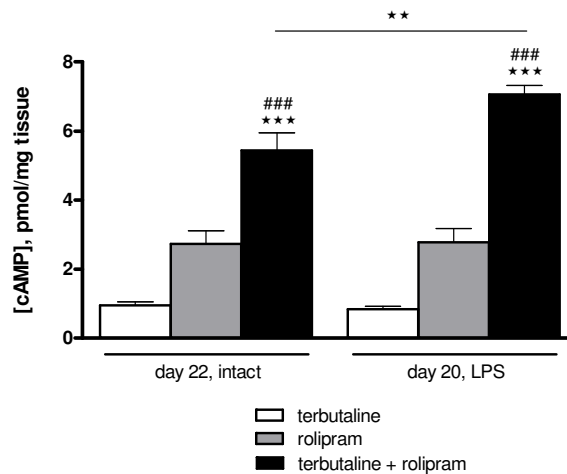


Fig. 3. Intracellular cAMP levels in the pregnant rat uterus, in the presence of terbutaline or rolipram and their combination. ***: $p < 0.001$; ###: $p < 0.001$; **: $p < 0.01$. $n = 6$ in each group.

Both in the intact and in the LPS-treated rats, the cAMP concentrations were significantly higher in the presence of terbutaline + rolipram than with only terbutaline ($p < 0.001$) or rolipram ($p < 0.001$). The combination of terbutaline + rolipram resulted in higher cAMP levels in the LPS-treated rats than in the intact rats ($p < 0.01$).

The terbutaline induced cAMP accumulations were increased significantly ($p < 0.05$) by FMP-API-1 at all the applied doses.

Human myometrial studies

In vitro contractility studies

Theophylline and rolipram reduced the oxytocin-induced contractions, respectively. The contraction-inhibiting effect of rolipram was significantly greater than that of theophylline ($p < 0.001$).

In the presence 10^{-6} M rolipram, terbutaline decreased the uterus contractions more effectively in both group. At preterm birth the effect of 10^{-10} M terbutaline was more than doubled in the presence of 10^{-6} M rolipram ($p < 0.001$) (**Fig. 4**).

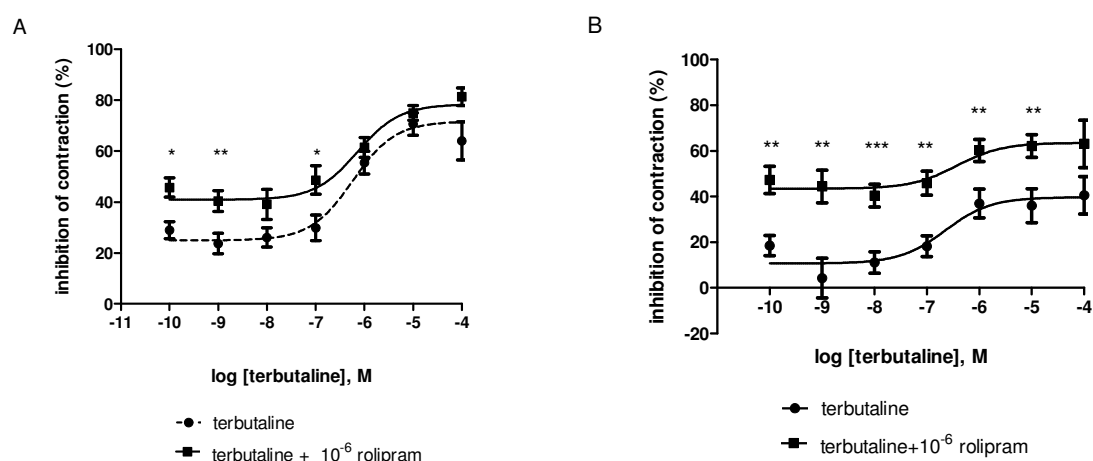


Fig.4. The contraction-inhibiting effects of 10^{-10} – 10^{-4} M terbutaline were investigated alone (●) or in combination with 10^{-6} M rolipram (■) at full term pregnancy (A) and at preterm birth (B).

cAMP levels in human uterine samples

The cAMP levels in human uterine tissue samples from preterm birth and from full term pregnancy were measured in the presence of 10^{-8} , 10^{-6} , 10^{-4} M terbutaline in combination with 10^{-6} M rolipram. There was a concentration-dependent increase of cAMP levels in both groups. Rolipram evoked a significantly higher elevation of uterine cAMP level at 10^{-6} M terbutaline in the preterm birth samples than in those from full term pregnancy ($p < 0.05$). There were no significant differences at 10^{-8} M or 10^{-4} M terbutaline.

Western blot studies

Western blot analysis revealed the expression of PDE4B and PDE4D isoenzymes in the uterine tissues from term and preterm labour. The expression of PDE4B (**Fig. 5/A**) was significantly higher at preterm labour than at full term pregnancy. The O.D. of PDE4D,

however, was significantly higher in the uterine tissues from the term pregnancies (**Fig. 5/B**) than at preterm labour.

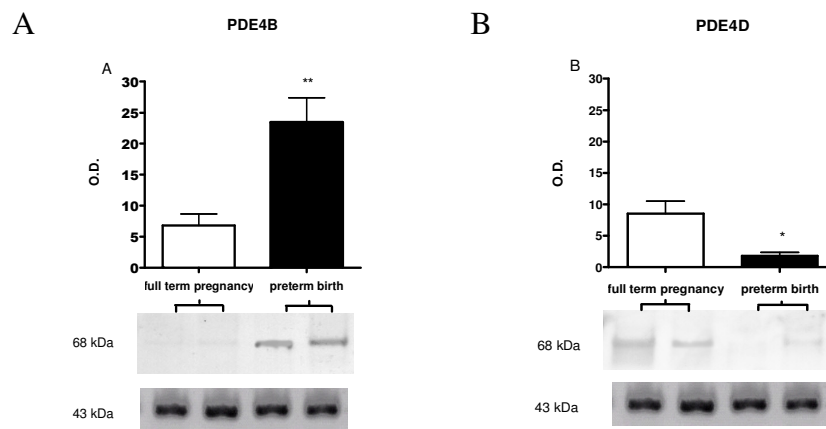


Fig. 5. The expression of PDE4B (**A**) and PDE4D (**B**) in the uterine tissues from full term pregnancies and from preterm labour. n=4 in each group

Discussion

Most of the interventions intended to reduce preterm birth rate have not achieved consistent benefit. Apart from manifest infections in late-pregnancy, when the termination of pregnancy is of utmost importance to save the mother and the infant, the causes of preterm contractions are seldom recognized before the initiation of tocolytic therapy.

In view of the role of inflammation in the provocation of preterm contractions, it appears advantageous to apply tocolytics with anti-inflammatory effects. Both β_2 -AR agonists and PDE4 inhibitors were reported to have anti-inflammatory effect. In immunocompetent cells, where PDE4 enzymes are abundant, increased cAMP levels lead to inhibition of the synthesis and release of pro-inflammatory mediators, cytokines and active oxygen species. The β_2 -AR agonists have immediate and comparable profound effects on uterine activity. Despite their unfavorable side-effects (tachycardia and the risk of pulmonary hypertension), the β_2 -AR agonists are still irreplaceable in tocolytic therapy. Recent publications have recommended the use of PDE4 inhibitors for tocolysis, but clinical experience was controversial. Monotherapy with rolipram or roflumilast was hindered by adverse effects such as unacceptable nausea and vomiting. Emesis is associated with the inhibition of PDE4D, while PDE4B has been shown to be required for LPS-evoked inflammatory responses.

Our study confirmed that the uterus-relaxing effects of low doses of terbutaline were markedly potentiated by the AKAP-PKA interaction inhibitor FMP-API-1 in intact rats, and by rolipram, both in rats and in human uterine tissue samples *in vitro* and *in vivo*. The changes in uterine cAMP levels correlated with these results. Our Western blot study demonstrated the up-regulation of PDE4B in the human samples at preterm birth. This might well be the consequence of ongoing inflammatory processes leading to preterm labor. The expression of PDE4D was decreased in samples from both term and preterm birth.

In the light of these results, we conclude that putative therapeutic combinations of β_2 -AR agonists and selective PDE4 inhibitors / AKAP-PKA interaction inhibitors may enhance the efficacy of human tocolytic therapy. Such drug combinations may have the benefit of allowing the administration of lower doses of β_2 -AR agonists, thereby delaying the desensitization of β_2 -AR receptors and preventing the early onset of adverse cardiovascular effects. We presume that the development of new PDE4B-selective inhibitors may further enhance the efficacy of tocolysis.

Articles related to the Ph.D. Thesis

1. Klukovits A, **Verli J**, Falkay G, Gáspár R. Improving the relaxing effect of terbutaline with phosphodiesterase inhibitors: studies on pregnant rat uteri *in vitro*. *Life Sci.* 87(23-26):733-7. 2010. **IF: 2,451**
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Other publications

1. Ahmed AA, Marki A, Gaspar R, Vasas A, Mudawi MM, **Verli J**, Jójárt B, Hohmann J, Falkay G. $\beta(2)$ -Adrenergic activity of 6-methoxykaempferol-3-O-glucoside on rat uterus: in vitro and in silico studies. *Eur J Pharmacol.* 2011;667(1-3):348-54.
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3. **Verli J**, Gáspár R. A kombinált β_2 -mimetikus és foszfodiészteráz 4-gátló kezelés uterus relaxáló hatásának vizsgálata terhes patkányokban. Korányi Frigyes Szakkollégium XIV. Tudományos Fórum, Budapest. 2009. március 26.
4. **Verli Judit**, Klukovits Anna, Falkay György, Kormányos Zsolt, Gáspár Róbert. A kombinált β_2 -mimetikus és foszfodiészteráz 4-gátló kezelés uterus relaxáló hatásának vizsgálata in vitro. A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése Szeged, 2010. június 16–18.
5. **Judit Verli**, Anna Klukovits, Zsolt Kormányos, Judit Hajagos-Tóth, Eszter Ducza, Attila Pál, George Falkay, Róbert Gáspár Uterus-relaxing effect of β_2 -agonists in combination with phosphodiesterase inhibitors: studies on pregnant rat in vivo and on human myometrium in vitro. *Pharmaceutical Sciences for the Future of Medicines.* Prague, Czech Republic. June 13-17, 2011.