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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

Ph.D. Thesis

by
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Szeged
2012

Contents

List of abbreviations

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: Nomenclature and symbolism for Amino Acids and Peptides (J Biol Chem 1984; 219: 345-373)

AC	adenyl cyclase
AR	adrenergic receptor
AKAP	A-kinase anchoring protein
cAMP	cyclic adenosine monophosphate
IBMX	3-isobutyl-1-methylxanthine
ic.	intracellular
IL	interleukin
G-protein	heterotrimeric guanine-nucleotide binding regulatory protein
Gi	inhibitory G-protein
Gs	stimulatory G-protein
GTPγS	guanosine-5'-O-(γ-thio)triphosphate
LPS	lipopolysaccharide
PDE	phosphodiesterase
PKA	protein-kinase A
TNF-α	tumor necrosis factor-alpha

Annex

Full papers and abstracts related to the Ph.D. thesis

I. 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.

II. Christian F, Szaszak M, Friedl S, Drewianka S, Lorenz D, Goncalves A, Furkert J, Vargas C, Schmieder P, Goetz F, Zuehlke K, Moutty M, Goettert H, Joshi M, Reif B, Haase H, Morano I, Grossmann S, Klukovits A, **Verli J**, Gaspar R, Noack C, Bergmann M, Kass R, Hampel K, Kashin D, Genieser HG, Herberg FW, Willoughby D, Cooper DM, Baillie GS, Houslay MD, von Kries JP, Zimmermann B, Rosenthal W, Klussmann E. Small molecule AKAP/PKA interaction disruptors that activate PKA interfere with compartmentalized cAMP signaling in cardiac myocytes. J Biol Chem. 286(11):9079-96. 2011.

III. **Judit Verli**, Anna Klukovits, Zsolt Kormányos, Judit Hajagos-Tóth, Eszter Ducza, Adrienn B. Seres, George Falkay and Róbert Gáspár. Uterus relaxing effect of β_2-agonists in combination with phosphodiesterase inhibitors: studies on pregnant rat *in vivo* and on pregnant human myometrium *in vitro.* Obstet Gynaecol Res. 2012.

IV. Klukovits Anna, **Verli Judit**, Falkay György. Uterus relaxáció kiváltása a foszfodoészteráz-4 enzim gátlásával terhes patkányban. Magyar Kísérletes és Klinikai Farmakológiai Társaság, A Magyar Experimentális Farmakológia III. Szimpóziuma. Budapest, 2007. június 1-2.

V. Klukovits A., **Verli J.**, Falkay G. Uterus relaxation by selective inhibition of phophodiesterase-4 in pregnant rats. 8th Congress of the European Association for Clinical Pharmacology and Therapeutics, Amsterdam, August 29-September 1, 2007

VI. **Verli J,** Gáspár R. A kombinált β2-mimetikus és foszfodiészteráz 4-gátló kezelés uterus relaxáló hatásának vizsgálata terhes patkányokban. Korányi Frigyes Szakkollégium XIV. Tudományos Fórum, Budapest. 2009. március 26.

VII. **Verli Judit**, Klukovits Anna, Falkay György, Kormányos Zsolt, Gáspár Róbert. A kombinált β2-mimetikus és foszfodiészteráz 4-gátló kezelés uterus relaxáló hatásának vizsgálata in vitro. A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése Szeged, 2010. június 16–18.

VIII. **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,

1. Introduction

1.1. Preterm birth

The control of uterine smooth muscle function is of vital importance during pregnancy and parturition. While a relative quiescence of activity is required throughout gestation, at the onset of labour powerful contractions and cervical dilatation are necessary to expel the foetus and the placenta. Any kind of imbalance in the integrated activity of the contraction and relaxation process leads to obstetrical complications such as premature labour or uterine dystochia [Wray et al., 2001].

Worldwide, about 13 million preterm babies are born each year. The preterm birth rate is defined as the percentage of babies born before 37 completed weeks of gestation. [Lawn et al., 2010]. Since the estimation of gestational age is not always correct, low birth weight is also used to define preterm birth. Defined as a birth weight of <2500 g, low birth weight is a crude indicator that includes infants who are preterm, infants who are term but small and infants who are both preterm and small for gestational age [Osrin, 2010].

Preterm birth is still one of the major reasons for neonatal mortality (70%) and morbidity (75%) [Challis et al., 2001]. Its causes may include individual-level behavioural and psychosocial factors, environmental exposures, medical conditions, biological factors, and genetics. There is a strong relationship between systematic or intrauterine infection and preterm delivery [Salminen et al., 2008]. Infants born preterm are at greater risk than infants born at term for mortality and a variety of health and developmental problems. Complications include acute respiratory, gastrointestinal, immunological, central nervous system, hearing, and vision problems, as well as longer-term motor, cognitive, visual, hearing, behavioural, social-emotional, health, and growth problems [Behrman and Butler, 2007]. The morbidity associated with preterm birth often extends to later life, resulting in enormous physical, psychological and economic costs [Beck et al., 2010].

The ultimate goal of treatment for preterm labour is to eliminate or reduce perinatal morbidity and mortality [Behrman and Butler, 2007]. High levels of preterm birth are probably due to intrauterine infection or lack of availability of drugs, such as tocolytic agents [Romero et al., 2004]. Identifying ways to address preventable causes of preterm birth should be a top priority in developing regions of the world [Beck et al. 2010].

1.2. Combinations in tocolytic therapy

The choice of first line tocolytic drugs for the treatment of preterm labour is controversial because of inconclusive information on the relative safety of the various agents. A number of agents are used clinically as tocolytics, including magnesium sulphate, indometacin, β_2-adrenergic receptor (β_2-AR) agonists, atosiban, progesterone (P4), prostaglandin (PG) synthesis inhibitors, nitric oxide donors and calcium (Ca^{2+}) channel blockers, but the efficacy of the current modes of pharmacological treatment has been questioned [Kim and Shim, 2006]. An ideal tocolytic should postpone delivery at low cost without maternal or fetal side-effects [de Heus et al., 2009]. Unfortunately, none of the currently available tocolytics fulfill these criteria. 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.

Numerous experiments have been described with combinations of different agents in tocolytic therapy. Chiossi et al. investigated the effect of PDE5 inhibitor sildenafil citrate in combination with Ca^{2+} channel blocker nifedipine [Chiossi et al., 2010], which study demonstrated that sildenafil citrate at low concentrations used for the treatment of pulmonary arterial hypotension can potentiate the tocolytic effect of nifedipine, suggesting that PDE5 inhibitors may improve the clinical effectiveness of Ca^{2+} blockers as tocolytic agents. The oxytocin antagonist atosiban with progesterone was tested by Meloni et al. Their preliminary data suggest that the vaginal administration of progesterone - after uterine activity was arrested by atosiban - could prolong pregnancy in subjects with short cervix [Meloni et al., 2009]. Hajagos-Tóth et al. investigated the tocolytic effect of β_2-AR agonists with nifedipine, in vitro and in vivo in rats. Nifedipine delayed the preterm delivery in the rats, and its effect was tripled by the addition of β_2-mimetics. However, the pretreatment with progesterone did not improve the effect of nifedipine in vivo [Hajagos-Tóth et al., 2010]. Gálik et al. reported that gestagen treatment enhances the effects of β_2-mimetics in hormone-induced preterm delivery in pregnant rats in vivo [Gálik et al., 2008].

1.3. Mechanism of cAMP mediated smooth muscle relaxation

Cyclic AMP (cAMP) is an important biological second messenger molecule involved in many biological processes [Beavo and Brunton, 2002]. Cyclic AMP elevation

instigates a wide variety of transcriptional events in many cells and tissue types, including the myometrium [Taggart et al., 2008]. Cyclic AMP is implicated in the mechanisms governing smooth muscle motility and in preventing the induction of contraction and its maintenance. The onset and progression of the inflammatory response are also sensitive to changes in the steady-state level of this cyclic nucleotide. Methods of increasing the cAMP concentration in smooth muscle and inflammatory cells have been actively pursued with a view to developing new therapeutic agents [Oger et al., 2004].

The level of cAMP in biological compartments is tightly controlled by the activity of adenyl cyclase enzyme (AC) that catalyzes the formation of cAMP. For example, the stimulation of the β_2-receptors on the myometrium leads to the activation of G_s-protein linked receptors, which stimulate the AC enzyme. The AC catalyzes the formation of cAMP from ATP, hence increases the cAMP concentration of the myometrium. On the one part, this high level of cAMP inhibits the dawn of the Ca-calmodulin complex, on the other part it inactivates the myosin light chain kinase enzyme which leads to myometrial relaxation. PDE4 inhibitors can also regulate the cAMP levels in the myometrium, thereby preventing the hydrolysis of cAMP to the inactive 5'-AMP. Any change in PDE expression or activity may alter the cAMP-mediated relaxation responses in the myometrium.

Increased levels of cAMP are translated into cellular responses through the action of cAMP-dependent protein kinase A (PKA; also known as A-kinase) [Houslay and Adams, 2003]. Stimulation of a cAMP/PKA-dependent signaling pathway, almost universally regarded as having a prorelaxant effect on the myometrium. It is now well recognized that cAMP-dependent signaling responses are compartmentalized. Compartmentalization allows spatially distinct pools of PKA to be differentially activated. It is mediated by various PKA isoforms, which are anchored at specific intracellular sites by proteins called A-kinase anchoring proteins (AKAPs). AKAP-PKA interactions play key roles in the various cellular processes.

The AKAPs directly bind to various signalling proteins such as other protein kinases, phosphatases, cAMP PDEs, GTP-binding proteins, adaptor proteins and substrate proteins of PKA [Christian et al., 2011]. In the myometrium, the agonist occupancy of the β_2-AR initially leads to coupling to G_s, which results in the activation of AC, elevated cAMP levels, and the activation of PKA, which is able to phosphorylate the β_2-AR. Concomitantly, the phosphorylation of the β_2-AR allows for the recruitment of β-arrestin together with bound PDE4. Thus, the recruited PDE4 provides a negative feedback loop,

the role of which is to attenuate local cAMP levels [Baillie et al., 2002]. In this mechanism AKAP150 and AKAP79 play a key role [Ku and Sanborn, 2002].

AKAP79 targets PKA to AC V and VI, both abundant in pregnant uterus, thereby facilitating the PKA-mediated phosphorylation and inhibition of the cyclases [Price et al., 2000; Christian et al., 2011]. This interaction of PKA and AC V and VI in the myometrium is another example of a negative feedback regulation of the β_2-AR mediated elevation of cAMP levels. In order to maximise ic. cAMP accumulation, a novel group of drugs has been developed. FMP-API-1, our novel small molecule has a dual effect: it inhibits AKAP-PKA associations and also activates PKA. The molecule binds to an allosteric site of regulatory subunits of PKA identifying a hitherto unrecognized region that controls AKAP-PKA interactions. It was demonstrated, that in cardiac myocytes of the rat, FMP-API-1 leads to an increased contractility, by controlling AKAP-PKA interactions, providing a basis for a new concept in the treatment of chronic heart failure [Christian et al., 2011].

1.4. Phosphodiesterase inhibitors

Since ubiquitously distributed in eucaryotes, the PDE superfamily represents a good target to develop new therapeutic and specific approaches, especially in diseases that remain unresolved, as much as they have multifactorial origins. Several leading pharmaceutical companies are searching for and developing new therapeutic agents on the basis of their ability to potently and selectively inhibit PDE isozymes, notably PDE4 in inflammation and PDE5 in human erectile dysfunction [Lugnier, 2006].

The PDE enzymes are encoded by 11 related gene families and are differentially expressed in human tissues [Lugnier, 2006]. Human myometrial cells [Méhats et al., 1999], amniochorionic membranes [Oger et al., 2005], monocytes and neutrophils [Wang et al., 1999] express PDE4, which promotes its role in either smooth muscle or immune functions. PDE4 (formerly known as cAMP-PDE), a cAMP-specific PDE, is the predominant isoenzyme in the majority of inflammatory cells. PDEs ensure termination of signalling by the degradation of cAMP to the inactive 5`-AMP [Klukovits et al., 2010]

There are four genetically distinct PDE4 subtypes, termed PDE4A, PDE4B, PDE4C and PDE4D [Boswell-Smith et al., 2006], with various alternative mRNA splices encoding long and short PDE4 isoforms, resulting in the expression of at least 35 different

PDE4 proteins [Lugnier 2006]. In the early 1970s, rolipram, a cAMP-PDE inhibitor, was developed as a potential drug for the treatment of depression. Although it proved to be an effective antidepressant, its side-effects (nausea and gastrointestinal disturbances) ruled out its clinical development [Boswell-Smith et al., 2006]. Other PDE4 selective ligands have been developed e.g. targeting PDE4, the enzyme responsible for metabolizing cAMP has been the focus for the development of drugs that could prove beneficial in the treatment of respiratory diseases such as asthma [Spina, 2008]. Recently, there were several rolipram analogues developed for asthma and COPD, such as piclamilast. Presently, some new PDE4 inhibitors, with lesser emetic effects, are currently under clinical investigation, such as cilomilast and roflumilast. Furthermore, PDE4 inhibition will be a new approach for schizophrenia [Lugnier, 2006], PDE4D may play an important role in regulating ic. cAMP linked to the regulation of glucagon-like peptide-1 release in therapy of diabetes mellitus. Odashima et al. reported that the specific type of PDE4 inhibitor could have a potent antiulcer effect, presumably mediated by its anti-inflammatory properties [Odashima et al., 2005].

1.5. β_2 adrenergic receptor agonists

The adrenergic system plays an important role in the control of uterine contractility. Preterm labour is commonly treated with a β_2-adrenergic receptor agonist in order to produce myometrial relaxation and prevent or delay preterm birth [Engelhardt et al., 1997]. The β_2-ARs couple via G_s protein to myometrial AC, increases the ic. levels of cAMP and activates cAMP-dependent protein kinases. This leads to myometrial relaxation through effects on the ic. Ca^{2+} concentration and myosin light kinase. Because of their role in relaxation, the β_2-ARs are still one of the main targets of tocolytic therapy.

The β_2-adrenergic receptor agonists are among the most frequently applied tocolytic agents [Andreassi and Teso, 1992]. However, their use in therapy has some disadvantages. They may have several side-effects such as tachycardia, pulmonary oedema, hypokalaemia, sodium retention and glucose intolerance, mainly as a consequence of the high doses used for uterus relaxation. Additionally, β_2-agonists can affect the life perspectives of neonates by causing respiratory distress syndrome, intracranial bleeding and neonatal jaundice [Andreassi and Teso, 1992; Smigaj et al., 1998; Gyetvai et al., 1999; Papatsonis et al., 2000]. Another disadvantage of their use is the decreased myometrial responsiveness to the β_2 AR stimulation towards the end of the pregnancy [Cruz et al 1990]. Gáspár et al. demonstrated that the uterus-relaxing effect of terbutaline on electrical field-stimulated contractions spontaneously decreases on towards the end of the pregnancy in the rat. In addition, in [^{35}S]GTPγS binding assay terbutaline decreased the amount of activated myometrial G-protein on last days of pregnancy [Gáspár et al., 2005].

Therefore, the effectiveness of these agents has been the subject of intensive debate in the literature. Some authors claim that most β_2 -mimetics can put off labour for 48–72 h [Katz and Farmer, 1999], while others conclude that their duration of action is only 24–48 h [Higby et al., 1993]. The main rationale for the use of these drugs is to delay delivery until the mother is transferred to a specialist unit. There is evidence for the increased survival of infants born preterm due to some obstetric interventions such as screening for asymptomatic bacteriuria, antenatal corticosteroid treatment, and prophylactic antibiotic treatment for group B streptococcal infections [Iams et al., 2008].

1.6. Inflammatory processes in the uterus

Preterm uterine contractions can develop as a result of several pathological processes among which intrauterine infection plays a key role. Intrauterine infection is present in approximately 25% of all cases [Romero et al., 1988], and the earlier the gestational age at delivery, the higher the frequency of intra-amniotic infection by Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, certain peptostrepococci and Bacteroides species [Yoon et al., 1999; Peltier, 2003]. It is well-known that inflammatory processes trigger a wide range of uterus contracting factors: the influx of inflammatory cells into the uterus and elevate levels of pro-inflammatory cytokines TNF-α, IL-1β, -6, -8 [Lindström and Bennett, 2005], stimulates the generation of the potent uterine-contracting prostaglandins and often results in premature rupture of membranes and preterm delivery. Despite of the intensive research on the regulation of uterine contractility, preterm birth is still the leading cause of perinatal mortality and morbidity worldwide, affecting about 8000-10.000 (roughly 8-10% of all) neonate every year in Hungary.

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 an anti-inflammatory effect [Chi et al., 2004; Hatzelmann and Schudt, 2001; Farmer and Pugin, 2000; Francischiet al., 2000].

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. LPS, an endotoxin of Gram-negative bacteria, causes preterm birth in animals and has been implicated as a factor triggering preterm labour in humans [Salminen et al., 2008]. Rolipram, the prototype of selective PDE4 inhibitors inhibits concentration-dependently the LPS-induced TNF-α release [Oger et al., 2004]. PDE4B has been shown to be essential for LPS-activated TNF-α responses. In PDE4B-deficient mice, however, LPS stimulation failed to induce TNF-α secretion and mRNA accumulation [Jin and Conti, 2002], suggesting that a selective PDE4B inhibitor would be an anti-inflammatory drug [Lugnier 2006].

β-AR agonists also modulate the production of inflammatory mediators; they have been reported to inhibit TNF-α, IL-1, and IL-6 production by human mononuclear cells [Farmer and Pugin, 2000]. Klukovits et al. demonstrated, that the rat myometrium shows

an altered responsiveness of to β_2-AR agonists as a consequence of intrauterine inflammation. Although the inflammation itself does not alter the β_2-AR density, it may lead to changes in the G_s-coupled adenylyl-cyclase activity and causes enhanced myometrial cAMP accumulation. Although the enhanced relaxing potency of β_2-agonists is a consequence of a pathological condition, it may become beneficial as regards tocolytic therapy [Klukovits et al., 2009].

The anti-inflammatory effect of a tocolytic drug is of great value because it may relieve a silent but ongoing inflammation and prolong pregnancy. The therapeutic strategy for the prevention of preterm births that might have inflammatory origin should focus on agents which can inhibit early uterine contractions and also able to control inflammation and its consequences for the mother and infant.

2. Aims

The main focus of our study was to promote the cAMP dependent relaxation in the late-pregnant uterus. In view of the role of inflammation in the provocation of preterm contractions, it appears advantageous to apply tocolytics with anti-inflammatory effects. Therefore, the following aims were set:

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.

3. Materials and methods

3.1. Animals studies

3.1.1. Housing and mating of the animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008) and under the control of ISO-9001:2008 Quality Management System.

Sexually mature female Sprague-Dawley rats (body mass: 140-160 g, 50-60 days old) were mated in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of copulation was considered to be the first day of pregnancy. The animals were housed in temperature- (20-23°C), and humidity-(40-60%) and light- (12 h of light, 12 h of dark) regulated rooms with water and food intake *ad libitum*.

3.1.2. In vivo treatments

The animals were divided into 2 groups (n=10 in each): (1) intact pregnant rats on day 22 of pregnancy; and (2) rats treated with LPS (i.p. 125 μg/day) for three consecutive days from day 18 of pregnancy, in the early morning hours, to evoke preterm birth, which occurred on the early afternoon of day 20 [Elovitz and Mrinalini, 2004].

3.1.3. In vitro contractility studies

On days 20 and 22 of pregnancy the rats were killed by CO_2 inhalation in the early afternoon hours, and the uteri were removed and prepared for the in vitro contractility assay, as it was reported previously [Klukovits et al., 2004]. Briefly, the isolated uterine horns were immediately placed in an organ bath (de Jongh solution; containing in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 MgC_{l2}, 12 $NaHCO_3$, 4 Na 2 HPO_4, 6 glucose; pH 7.4) perfused with 95% oxygen and 5% carbon dioxide; they were trimmed of fat and the foeto-placental units were removed. Temperature was maintained at 37 °C. Four rings 1 cm long were

sliced from the middle part of each horn, including implantation sites, and tested in parallel; they were mounted vertically in the above-mentioned organ bath under the same conditions. After mounting, the initial tension was set at 1.5 g and the rings were equilibrated for 60 min, with change of the buffer every 15 min. Rhythmic contractions were then elicited with 25 mM KCl. The effects of the AKAP-PKA interaction-inhibiting FMP-API-1, the non-specific PDE inhibitor theophylline, the specific PDE4 inhibitor rolipram and/or the β_2-AR agonist terbutaline on uterine rings were measured in the concentration range 10^{-12}–10^{-5} M, in a cumulative manner. The tissue samples were incubated for 4 min with each concentration. The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd, Budapest, Hungary), and recorded and analysed with the SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Budapest, Hungary). Areas under the curves (AUCs) 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.

3.1.4. In vivo myographic studies

The in vivo myographic studies were done on intact rats (on day 22 of pregnancy; at term) and on LPS-treated rats (on day 20 of pregnancy; at preterm), in the morning hours between 8 and 10 AM. Rats were anesthetized with a mixture of ketamine and xylazine (36 and 4 mg/kg, respectively) i.p.; 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 (SEN-04-FSG2; Experimetria Ltd., Budapest, Hungary) was sutured onto the myometrial surface. The animals with the sensors were then placed into a Faraday cage made from iron to filter out environmental electromagnetic noise.

The mechanical displacements elicited by the contractions of the uterus were converted to electrical impulses by the transducer, and amplified by a bridge amplifier (AMP-01-SG, Experimetria Ltd., Budapest, Hungary). The amplified electric signal was detected and analyzed by the S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

Doses of terbutaline (Sigma-Aldrich Ltd, Budapest, Hungary), and rolipram (Sigma-Aldrich Ltd, Budapest, Hungary), 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. 0.25 mg/kg of rolipram was administered similarly as terbutaline alone. In combination rolipram was given in a single dose of 500 μg/kg. The applied doses were chosen on the basis of rolipram dose-response curve (eliciting 30% relaxation). This dose shows similarity with some previous in vivo rat studies of rolipam, too [Jansson and Sandler, 1992; Lourenco et al., 2001]. Areas under the curves (AUCs) of 4-min periods were evaluated; the effects of terbutaline or the terbutaline + rolipram were expressed as percentages of the spontaneous activity.

3.1.5. 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, under the same conditions as detailed above. Forskolin (10^{-8}–10^{-6} M) -stimulated cAMP accumulation was determined in the presence of the specific PDE4 inhibitor rolipram (10^{-8} or 10^{-5} M). The extents of cAMP accumulation were also 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. After stimulation, the samples were immediately frozen in liquid nitrogen and stored until the extraction of cAMP [Gaspar et al., 2007]. Frozen tissue samples were then ground, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 1000 g for 10 min. The supernatants were extracted with 3 volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70 °C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP EIA Kit, tissue cAMP levels were expressed in pmol/mg tissue.

3.2. Human myometrial studies

3.2.1. 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; n=19) and at preterm birth (32-36 weeks; n=11). At full term pregnancy Caesarean delivery was indicated by a previous Caesarean delivery, breech presentation, suspected cephalopelvic disproportion or myopia. Parity varied from 0 to 3, mean maternal age was 30.8 years (22-41 years). None of the women received tocolytic agent, and there were no signs of labor.

Preterm delivery occurred in mothers with twin pregnancies, or labor was indicated by an ongoing infection, leukocytosis, toxaemia, fetal distress or growth restriction. In the preterm group parity varied from 0 to 3, mean maternal age was 32.7 years (26-42 years). 3 out of 11 patients received tocolytic therapy (magnesium sulfate) to arrest preterm uterine contractions, which proved to be ineffective. All the operations were performed under spinal anesthesia. The Ethical Committee of University of Szeged approved the clinical protocol (registration number: 114/2009).

Tissue samples (10x10x20 mm) from the upper edge of a lower-segment transverse incision were cut after delivering the child, but before oxytocin were given to the mothers. Tissues were stored in Krebs-Henseleit solution (containing in mM: 118 NaCl, 5 KCl, 2 $CaCl_2$, 0.5 $MgSO_4$, 1 K_2SO_4, 25 $NaHCO_3$, 10 glucose; pH 7.4) at 4 °C, and were taken into the experiment within 12 hours of collection. Four longitudinal strips (app. 3x5x10 mm) were cut from each specimen, mounted vertically in an organ bath (Krebs-Henseleit solution at 37 °C; perfused with 95% O_2 + 5% CO_2), and tested in parallel. After mounting, the strips were equilibrated for ~ 2 hours before experiments were undertaken, with a solution change every 15 min. The specimens exhibited spontaneous contractions over the incubation period. The initial tension was set to 3.00 g, which was relaxed to 1.5 g at the end of equilibration. The tension of the myometrial strips was measured with an isometric force transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with an S.P.E.L. Advanced ISOSYS Data Acquisition System.

Rhythmic contractions were elicited by 10^{-6} M oxytocin. After stimulation, theophylline, rolipram, or terbutaline was added in non-cumulative manner. After each concentrations of

the tested drug, the strips were washed 3 times, allowed to recover for 5 min, and then contracted again with oxytocin. Slight tissue fatigue was observed only before the very last dose which did not influence our measurements significantly. 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.

3.2.2. 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). After stimulation, the samples were immediately frozen in liquid nitrogen in which they were stored until the extraction of cAMP [Gáspár R et al., 2007]. Uterine cAMP accumulation was measured with a cAMP EIA Kit (Sigma-Aldrich Ltd, Budapest, Hungary) and tissue cAMP levels were expressed in pmol/mg tissue.

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3.2.3. Western blotting studies

Proteins from human uterine specimens were isolated with the Macherey-Nagel Nucleospin Kit (Izinta Ltd, Budapest, Hungary) according to the manufacturer's instructions. Protein concentrations were determined by BioSpec-nano (Shimadzu, Budapest, Hungary). 60 μg of protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Invitrogen, Hungary). Proteins were transferred from gels onto nitrocellulose membranes, using the iBlot Gel Transfer System (Invitrogen, Hungary). The blots were incubated on a shaker with PDE4B and PDE4D polyclonal antibodies (Santa Cruz Biotechnology, CA, USA; 1:200) in the blocking buffer; immunoreactivity was detected with the WesternBreeze Chromogenic Western blot immunedetection kit (Invitrogen, Hungary). 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.

3.3. Drugs

Rolipram, theophylline, terbutaline, IBMX, LPS (*E. coli* endotoxin 055:B5), forskolin, were purchased from Sigma-Aldrich Ltd, Budapest, Hungary. FMP-API-1 was kindly provided by Enno Klussmann.

3.4. Statistical analyses

In the animal and human myometrial contractility studies, AUCs were evaluated and analyzed statistically with the Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA) computer program. Group comparisons were made by one-way ANOVA tests with the Newman-Keuls post-test. The maximal inhibitory effects (E_{max}) in the curves obtained with the combinations were calculated. For statistical evaluations, data were analyzed by use of the unpaired t test. The results of the cAMP and Western blot assays were analyzed by using one-way ANOVA with the Newman-Keuls post-test.

4. Results

4.1. Animal studies

4.1.1. In vitro contractility studies

Uterine activity was characterized by the AUCs of KCl-stimulated contractions of uterine rings in vitro. Time control experiments revealed that KCl-stimulated contractions do not decrease significantly through the experiment (40–45 min). 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 **(Fig. 1).**

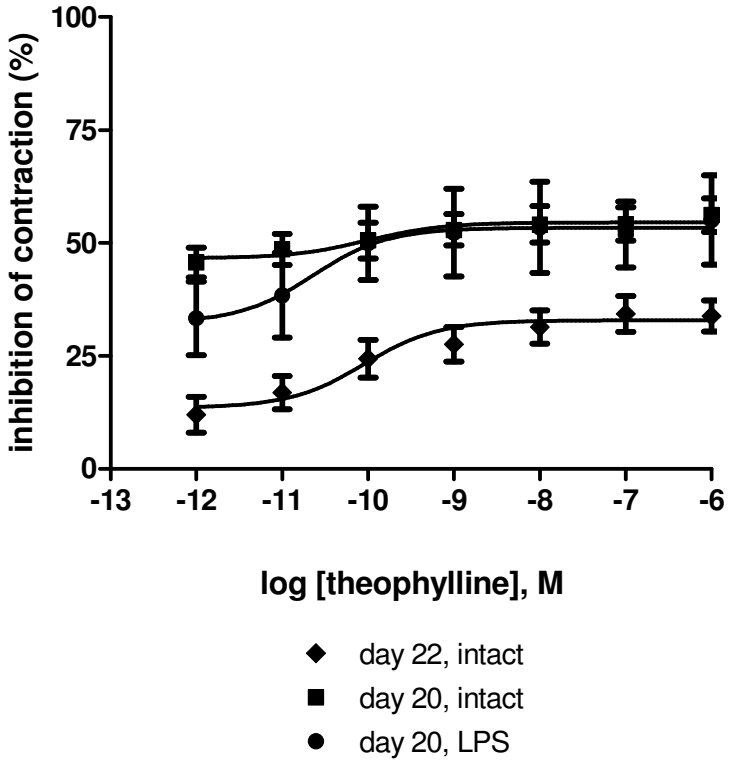


Fig. 1. Effects of theophylline on the pregnant rat uterus, *in vitro***.** The contractions were elicited with 25 mM KCl in uterine rings of intact rats on days 20 (■) and 22 (♦) of pregnancy, and in LPS-treated rats on day 20 (●) of pregnancy. n=8 in each group.

In the intact rats, the effect of theophylline varied, depending on the day of pregnancy: the KCl-induced contractions were reduced by 52.96±3.53 S.E.M.% on day 20 (2 days before physiological term) and by 25.34±2.46 S.E.M.% on day 22 (at physiological term) ($p<0.001$). In the group of rats treated with LPS, which delivered on day 20, theophylline decreased the contractions by 45.11±10.90 S.E.M.%, which was not different from that in the intact rats on day 20 ($p>0.05$) but was lower than that on day 22 ($p<0.05$). EC_{50} values of theophylline were not different ($p>0.05$) in the intact rats either on day 20 ($1.67\pm1.11\times10^{-9}$ M) or on day 22 ($4.20\pm1.81\times10^{-8}$ M), or in the LPS-treated rats ($3.52\pm2.34\times10^{-8}$ M).

The contraction-inhibiting effect of the specific PDE4 inhibitor rolipram exhibited a similar tendency: a declining relaxing effect towards term **(Fig. 2).**

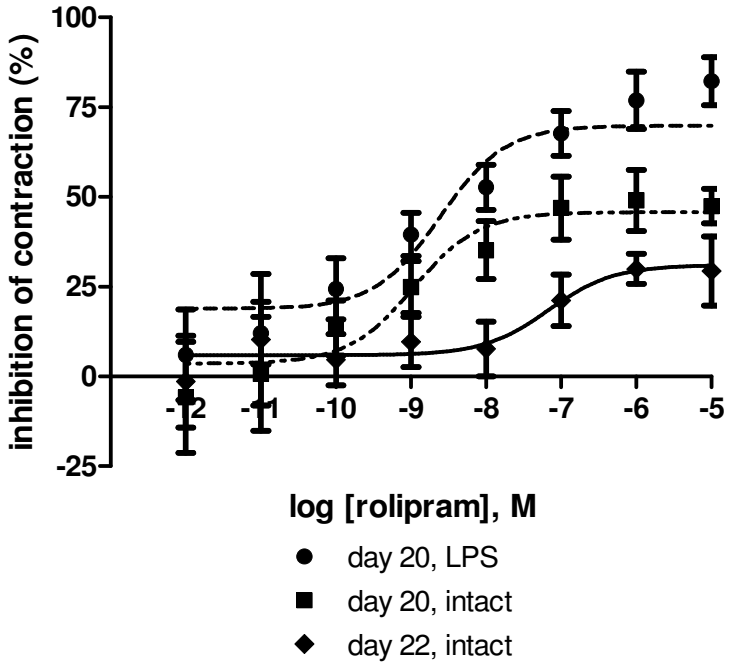


Fig. 2. Effects of rolipram on the pregnant rat uterus, *in vitro*. The contractions were elicited with 25 mM KCl in uterine rings of intact rats on days 20 (■) and 22 (♦) of pregnancy, and in LPS-treated rats on day 20 (●) of pregnancy. n=8 in each group.

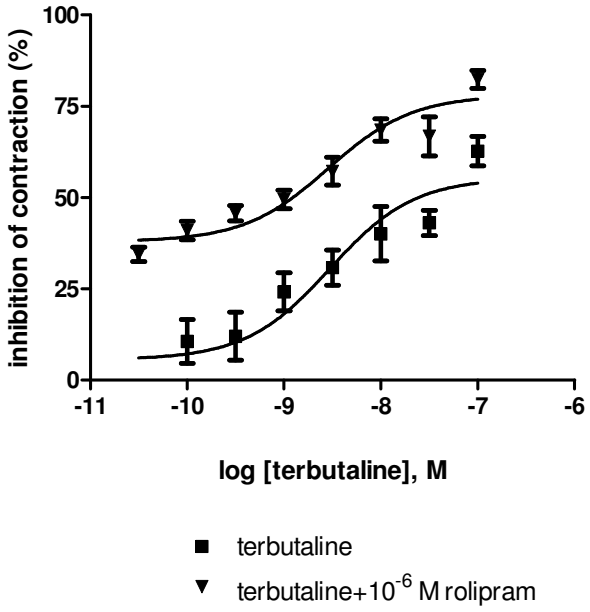
In the intact late-pregnant rats, the maximum contraction- inhibiting effect was seen on day 20, when the contractions were reduced by 45.15±4.21 S.E.M.%. The effect of rolipram was the weakest on day 22, when the contractions were reduced by 22.34±7.13 S.E.M.%, resulting in a weaker ($p<0.05$) inhibition than that on day 20. In the LPS-treated rats, however, the contractions were reduced by 80.9±4.96 S.E.M.%, resulting in a more marked inhibition than that in intact rats on day 20 ($p<0.01$). As regards the EC 50 values of rolipram, despite the increasing trend in the values towards physiological term, there were no significant differences ($p>0.05$) between the intact rats and the LPS-treated rats.

The effects of the β_2-AR agonist terbutaline alone or in the presence of rolipram were also tested on the isolated uterine rings. Terbutaline decreased the KCl-stimulated contractions by 60.77%±4.14 S.E.M.% on day 22 of pregnancy **(Fig. 3A).**

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. 3B),** 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.% ($p<0.01$).

The β_2-adrenergic receptor agonist terbutaline alone elicited a 60.77% inhibition of KCl-evoked contractions, at term **(Fig. 4).** In the presence of the AKAP-PKA interaction-inhibiting FMP-API-1, the maximal contraction inhibiting effect of terbutaline was increased to 75.09% ($p<0.05$). The EC_{50} value of terbutaline was 7.98×10^{-9} M and it was elevated to 2.98×10^{-8} M in the presence of FMP-API-1.

A



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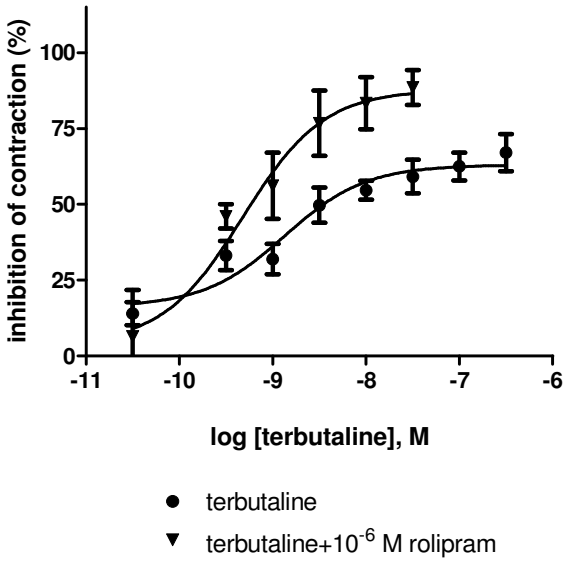


Fig. 3. Effect of rolipram on the uterine-relaxing effect of terbutaline, *in vitro***.** The contractions were elicited with 25 mM KCl in uterine rings of intact rats on day 22 (■) (A) or of LPS-treated rats on day 20 (▼) of pregnancy (B). In the presence of 10^{-6} M rolipram, the maximum contraction-inhibiting effects of terbutaline were lower both in the intact ($p<0.05$) and in the LPS-treated rats ($p<0.01$). n=8 in each group.

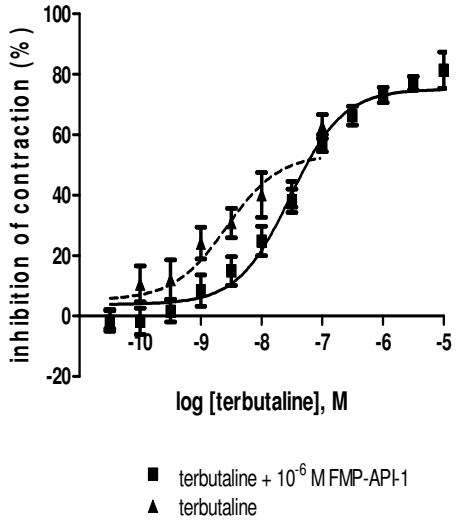


Fig. 4. Concentration-response curves of terbutaline alone and in combination with FMP-API-1 on isolated uterine rings of intact rats on day 22 of pregnancy (term). In the presence of FMP-API-1 the maximal contraction inhibiting effect of terbutaline increased significantly ($p<0.05$). $n>6$ in all cases.

4.1.2. In vivo contractility studies

The effects of the β_2-agonist terbutaline and rolipram were investigated *in vivo*. The contractions were inhibited dose-dependently by both compounds. Maximal inhibitory effect of rolipram in intact rats was 96.7 ± 3.1 S.E.M.%. **(Fig 5/A)** The maximal inhibitions achieved with terbutaline + rolipram were not statistically different in intact rats on day 22 of pregnancy (86.1 ± 8.9 S.E.M.%; **Fig. 5/B)** and in LPS-treated rats on day 20 (89.0 ± 9.5 S.E.M.%; **Fig. 5/C**). In case of the combination, rolipram (500 μg/kg i.v.) 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 μg/kg), however, the inhibition of the contractions was significantly higher ($p<0.001$) in the LPS-treated rats (48.1 ± 4.7 S.E.M.%; **Fig. 5/C**) than in the intact rats (33.3 ± 1.4 S.E.M.%**; Fig. 5/B**).

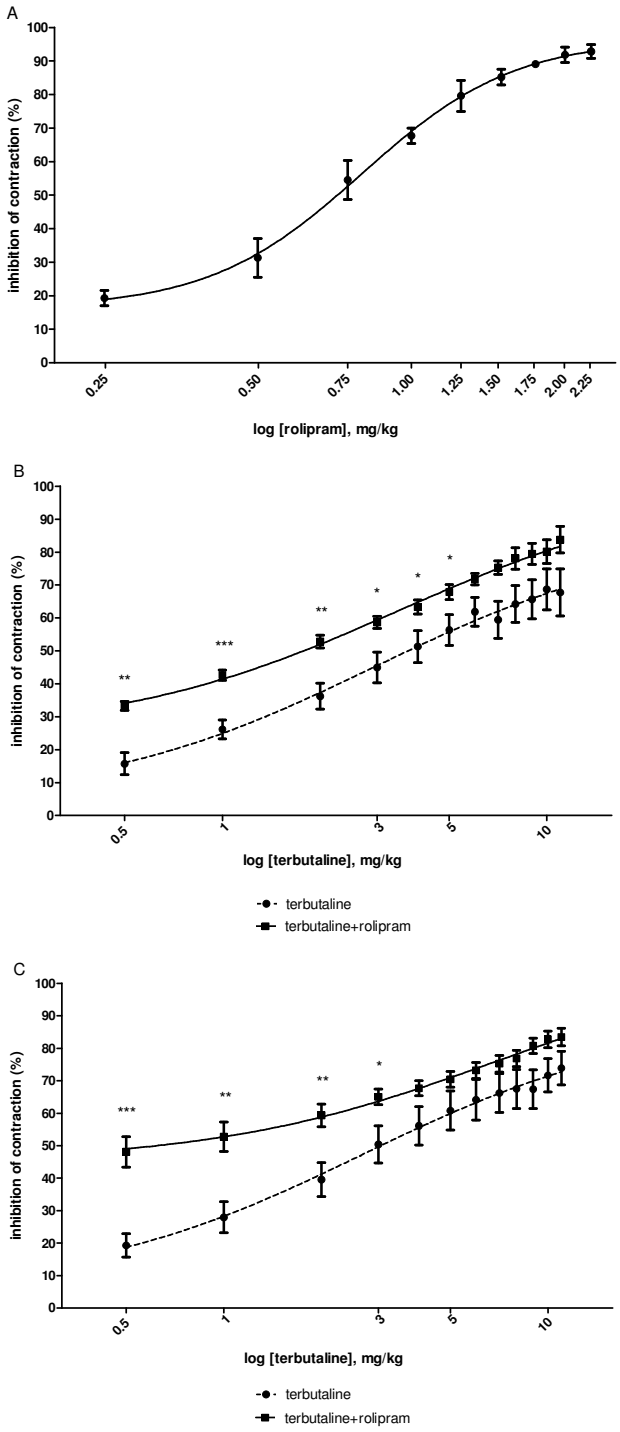


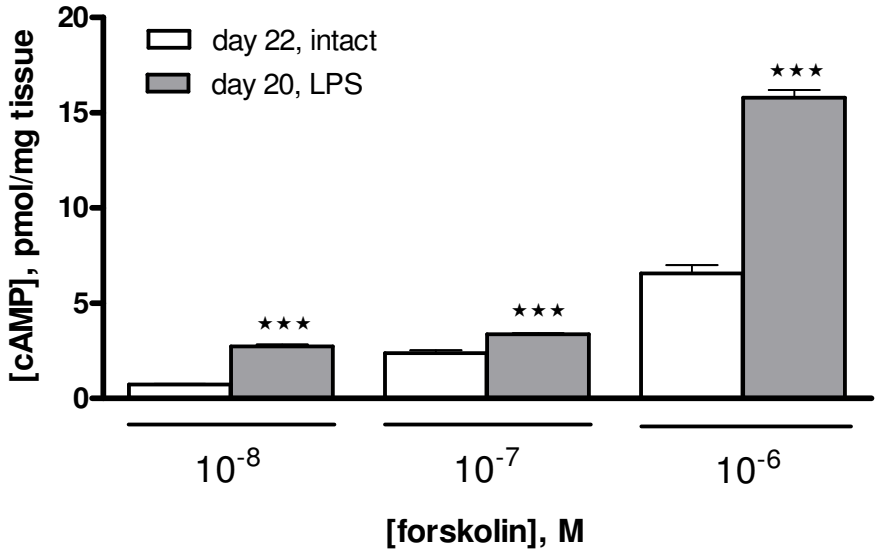
Fig. 5. Effect of rolipram on the contraction-inhibiting effect of terbutaline *in vivo*. Contraction-inhibiting effects are expressed as percentages of the spontaneous uterine activity. On the basis of rolipram dose-response curve **(A)** we determined the sufficient dose of rolipram to potentiate the contraction-inhibiting effect of terbutaline. In the presence of 500 μg/kg rolipram (■), the contraction-inhibiting effects of 0.5-11 μg/kg terbutaline were significantly higher in low dose both in intact rats on day 22 **(B)** and in LPS-treated rats on day 20 of pregnancy **(C)** than the effects of terbutaline alone (●). Group comparisons were made by unpaired t-test. n=10 in each group; *p<0.05, **p<0.01, ***p<0.001.

4.1.3. Changes of uterine cAMP levels in rats

The effects of the adenylyl cyclase activator forskolin were at first investigated in intact 22-day-pregnant and in LPS-treated 20-day-pregnant rats. In the presence of rolipram (10^{-8} M), forskolin (10^{-8}-10^{-6} M) concentration-dependently increased the uterine cAMP levels in both groups **(Fig. 6A).** In the uteri of the LPS-treated rats, 10^{-8}, 10^{-7} and 10^{-6} M forskolin evoked higher cAMP accumulation than in intact 22-day-pregnant rats ($p<0.001$). Similarly, when rolipram was present in higher concentration (10^{-5} M) **(Fig. 6B),** 10^{-8}, 10^{-7} and 10^{-6} M forskolin stimulation again resulted in significantly higher uterine cAMP concentrations in the LPS-treated than in the intact rats ($p<0.01$). At 10^{-8} and 10^{-7} (but not at 10^{-6}) M forskolin, the uterine cAMP levels were higher in the presence of 10^{-5} M rolipram than of 10^{-8} M rolipram, in both groups.

The cAMP generation in the pregnant rat uterus was also measured in the presence of terbutaline (10^{-7} M) and rolipram (10^{-6} M) alone and of their combination **(Fig. 7).** 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 ($p<0.01$).

6A



6B

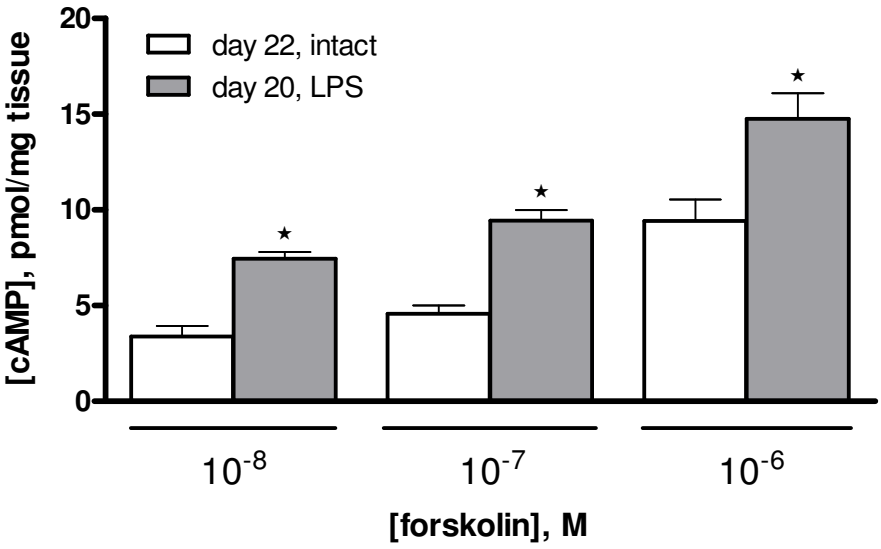


Fig. 6. Intracellular cAMP levels in the pregnant rat uterus, in the presence of forskolin and rolipram. In the presence of 10^{-8} M (A) or 10^{-5} M (B) rolipram, forskolin (10^{-8} – 10^{-6} M) concentration-dependently increased the uterine cAMP levels in intact rats on day 22 and in LPS-treated rats on day 20. In the uteri of the LPS-treated rats, the extent of cAMP accumulation was higher than in those of the intact rats. n=6 in each group; **: $p<0.01$; ***: $p<0.001$.

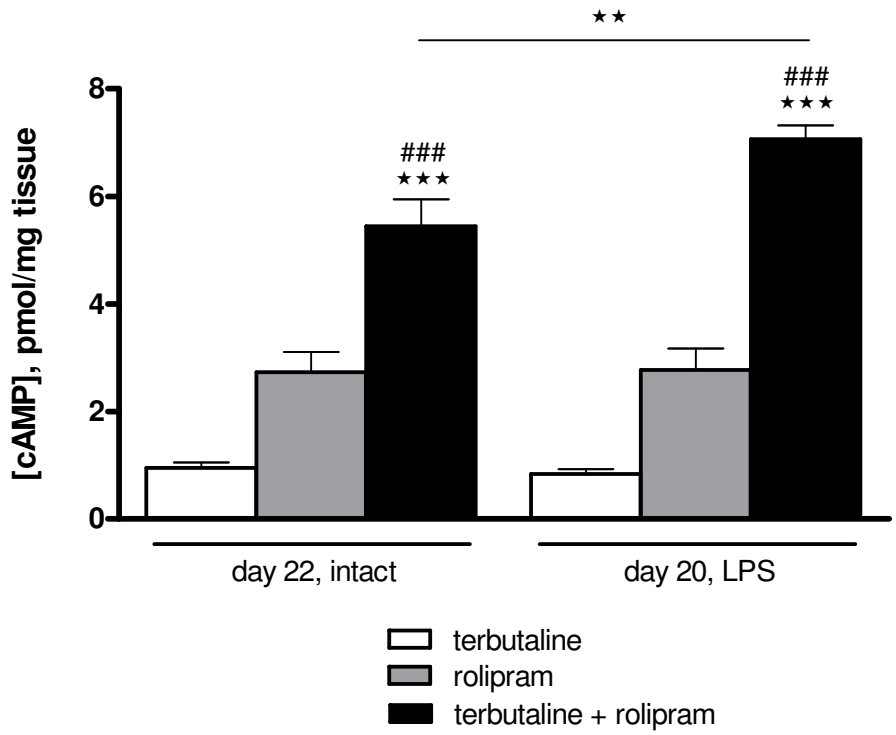


Fig. 7. Intracellular cAMP levels in the pregnant rat uterus, in the presence of terbutaline or rolipram and their combination. Both in the intact and in the LPS-treated rats, the cAMP concentrations were higher in the presence of terbutaline + rolipram than with only terbutaline (10^{-7} M; ***: $p<0.001$) or rolipram (10^{-6} M; ###: $p<0.001$). The combination of terbutaline + rolipram resulted in higher cAMP levels in the LPS-treated rats than in the intact rats (**: $p<0.01$). n=6 in each group.

The terbutaline induced cAMP accumulations were increased significantly ($p<0.05$) by FMP-API-1 at all the applied doses **(Fig 8).**

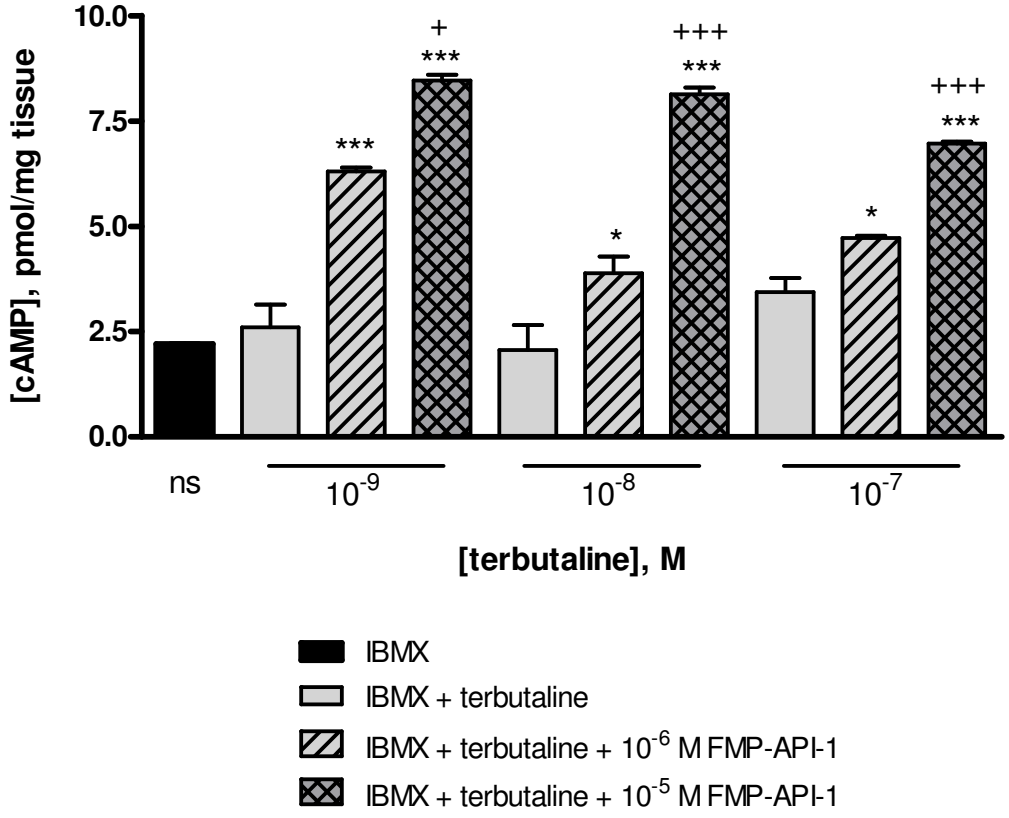


Fig. 8. Uterine cAMP accumulations in intact rats, on day 22 of pregnancy. The cAMP levels were detected in the presence of IBMX (nonstimulated controls received IBMX only; black coloumn), the cAMP accumulations in the uterine tissue samples were stimulated by terbutaline alone (light grey coloumns) or in combination with FMP-API-1 (striped and cross-hatched coloumns). At all applied terbutaline concentrations, FMP-API-1 caused a significant ($p<0.05$) and concentration-dependent elevation of tissue cAMP levels. Values are given as means ± SEM; n=6 in all groups.

* $p<0.05$; *** $p<0.001$ – as compared with terbutaline alone

+ $p<0.05$; +++ $p<0.001$ – as compared with the previous coloumn

4.2. Human myometrial studies

4.2.1. In vitro contractility studies

Uterine activity was characterized by the AUCs of the concentration-response curves of human uterine strips. Time control experiments revealed that the oxytocin-stimulated contractions did not decrease significantly through the experiment. Theophylline and rolipram reduced the oxytocin-induced contractions by 24.9 ± 3.6 S.E.M.%, and 61.9 ± 4.5 S.E.M.%, respectively. The contraction-inhibiting effect of rolipram was significantly greater than that of theophylline ($p<0.001$) **(Fig. 9).**

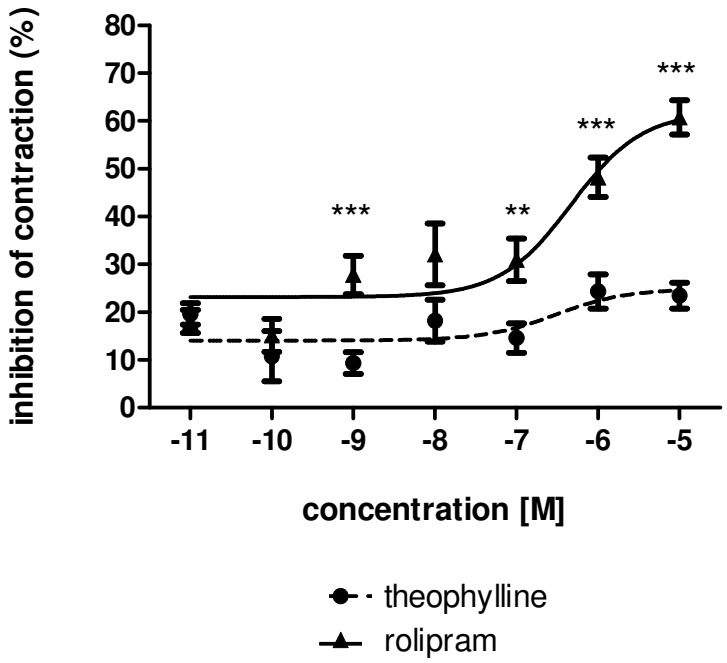
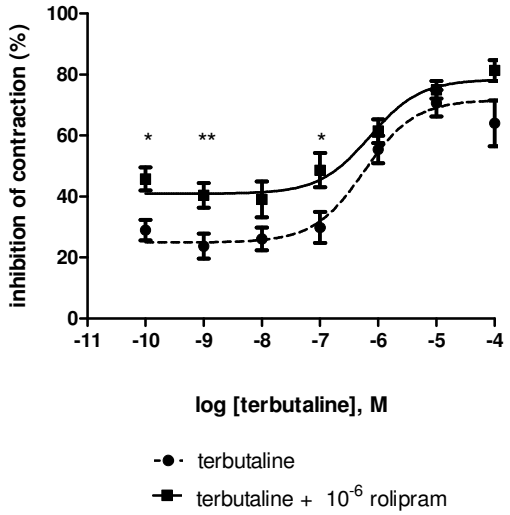


Fig. 9. Effects of selective and non-selective phosphodiesterase inhibitors on human uterine contractions at full term pregnancy, *in vitro*. The contractions were elicited with 10^{-6} M oxytocin and inhibited with 10^{-11}–10^{-5} M rolipram (▲) and theophylline (●). Group comparisons were made by unpaired t-test. n=3 in each group; **p<0.01, ***p<0.001.

The effects of the combination of terbutaline and rolipram were also tested. Whereas terbutaline alone inhibited the oxytocin-stimulated contractions by 71.6 ± 4.5 S.E.M.%, at full term pregnancy, in the additional presence of 10^{-6} M rolipram, the contractions were inhibited by 78.3 ± 4.7 S.E.M.% **(Fig. 10/A)**. At preterm birth, the

maximum contraction-inhibiting effect of terbutaline alone was 39.7 ± 4.6 S.E.M.%, but this was increased to 63.5 ± 4.7 S.E.M.% in the presence of rolipram. The effect of 10^{-10} M terbutaline was more than doubled in the presence of 10^{-6} M rolipram ($p<0.001$) **(Fig. 10/B).**

A



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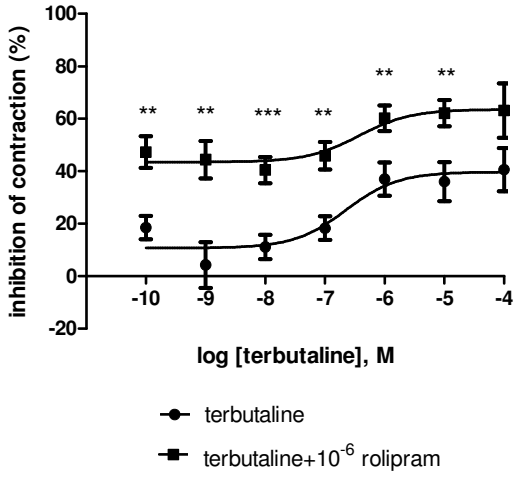

Fig. 10. Effects of rolipram on the contraction-inhibiting effects of terbutaline on human uterine samples, *in vitro*. The contractions were elicited with 10^{-6} M oxytocin. The contraction-inhibiting effects of 10^{-10}–10^{-4} M terbutaline were investigated alone (●) or in combination with 10^{-6} M rolipram (■) at full term pregnancy **(A)** and at preterm birth **(B)**. n=3 in each group

4.2.2. 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 **(Fig. 11).**

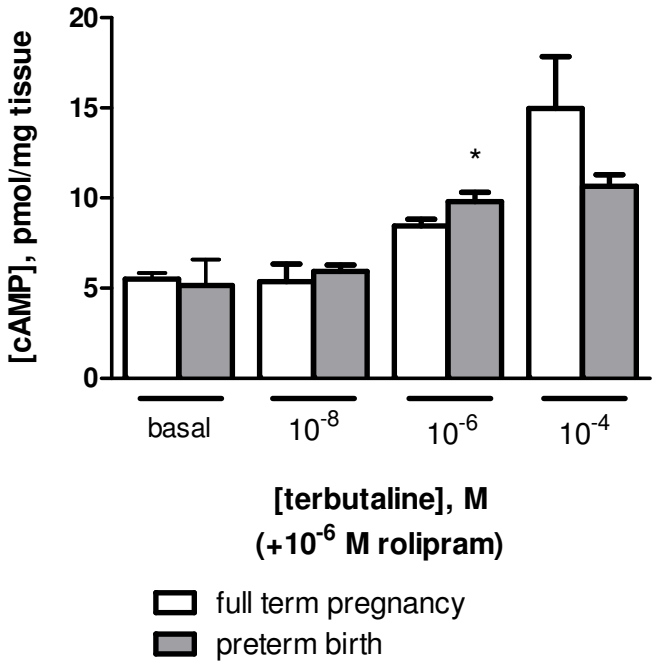

Fig. 11. Intracellular cAMP levels stimulated by 10^{-5}M forskolin, in the presence of 10^{-6} M rolipram and 10^{-8}, 10^{-6} or 10^{-4} M terbutaline in human uterine tissue samples from full term pregnancy and preterm birth. Basal cAMP levels (10^{-6} M rolipram alone) were not significantly different between the term pregnant and preterm birth groups. In the preterm birth group, at 10^{-6} M, terbutaline elevated cAMP levels ($p<0.01$) relative to the basal level. At 10^{-8} M, terbutaline elevated cAMP levels ($p<0.01$) as compared to the basal level, in both groups. At 10^{-6} M terbutaline, the cAMP levels were higher (*$p<0.05$) in the preterm birth samples than in those from full term pregnancy. n=3 in each group

4.2.3. 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. 12/A)** was significantly higher at preterm labour than at full term pregnancy (p<0.01). The O.D. of PDE4D, however, was significantly higher in the uterine tissues from the term pregnancies **(Fig. 12/B)** than at preterm labour (p<0.05).

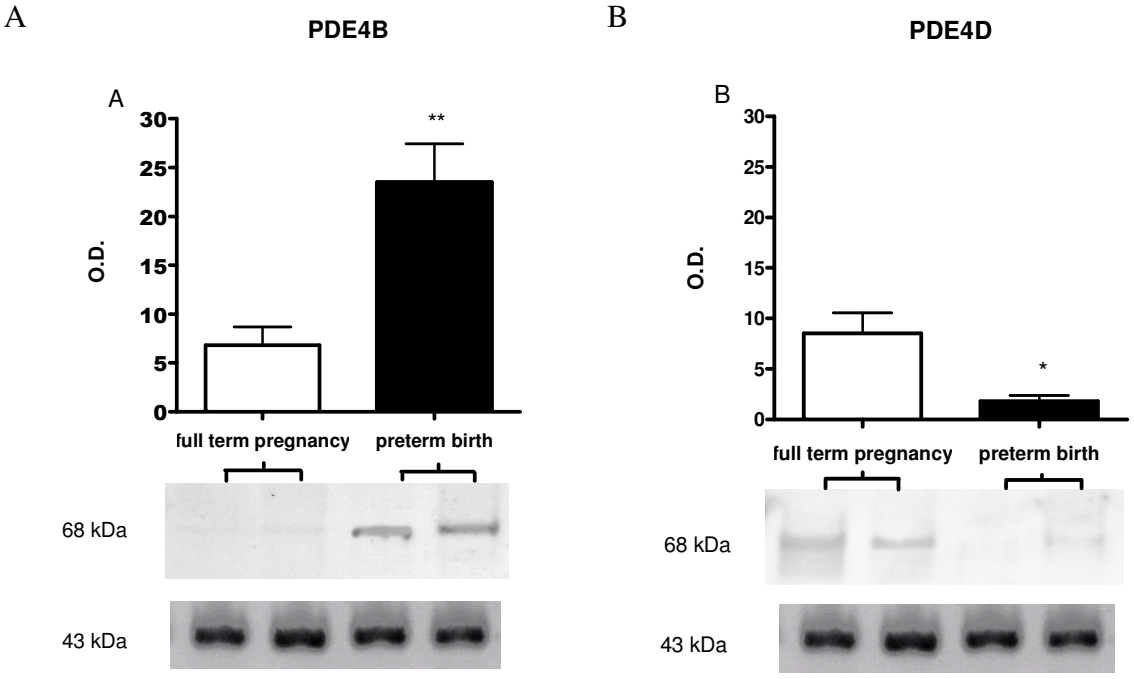

Fig. 12. Changes in PDE4B **(A)** and PDE4D **(B)** levels in human uterine samples at full term pregnancy and at preterm birth. n=4 in each group

5. Discussion

Complications of preterm birth are the leading cause of neonatal mortality, accounting for an estimated 27% of the almost four million neonatal deaths every year worldwide, and act as a risk factor for many neonatal deaths due to other causes, particularly infections [Lawn et al., 2010]. 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 [Chi et al., 2004; Hatzelmann and Schudt 2001; Farmer and Pugin 2000; Francischi et al., 2000]. 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 [de Heus et al., 2008]. Despite their unfavorable side-effects (tachycardia and the risk of pulmonary hypertension), the β_2-AR agonists are still irreplaceable in tocolytic therapy. Some publications have recommended the use of PDE4 inhibitors for tocolysis, but clinical experience was controversial [Méhats et al. 2007]. Monotherapy with rolipram or roflumilast was hindered by adverse effects such as unacceptable nausea and vomiting [Dyke and Montana, 2002].

Our study confirmed that the non-selective PDE inhibitor theophylline has only limited uterorelaxant effect in the late-pregnant rat. The selective PDE4 inhibitor rolipram, however, turned out to be much more effective to inhibit the KCl induced uterine contractions. In the late-pregnant rat uterus the administration of 25 mM KCl elicits rhythmic, sustainable uterine contractions, which do not decline over an approximately 45 min period. In this model, we could assess the effects of the PDE inhibitors and of terbutaline in a cumulative manner. In the in vitro contractility assay we observed a gestation-dependent slight decline in the effect of theophylline and rolipram, which suggests that intracellular cAMP accumulation is less appreciable towards the very end of pregnancy. In LPS-treated rats, the maximum effect of theophylline was similar to that in intact 20-day pregnant rats. Contrarily, the effect of rolipram was significantly greater in the LPS-treated rats. Since the inhibition of PDE4 does not affect cAMP production, but maintains high cAMP concentration in the myometrium cells, our results indicate that inflammation probably promotes cAMP generation in the uterus. It corresponds to our

previous findings that inflammatory processes in the late-pregnant rat uterus potentiate cAMP-related uterine relaxation via elevation of local and systemic TNF-α concentration [Klukovits et al., 2009].

To investigate the extent of cAMP production in the uterus we used forskolin to stimulate the membrane bound AC. By means of forskolin we could detect intracellular cAMP levels without direct stimulation of any membrane receptors (e.g. β-adrenergic, relaxin, prostaglandin E2, nociceptin, calcitonin gene-related peptide) which are coupled with stimulatory G-proteins, thus we rather measured the capacity of uterine smooth muscle cells to produce cAMP. We found that in the presence of rolipram the forskolin-stimulated cAMP accumulation was higher in the uteri of LPS-treated rats than in those of intact rats. The sensitization of AC in inflammation was reported formerly by our group [Klukovits et al., 2004], corresponding to earlier findings of Osawa et al [Osawa et al., 2007].

At the same time, we should note that inflammation may also decrease PDE4 activity in the myometrium, resulting in a slower turnover of cAMP and alter cAMP-mediated relaxation responses. In a recent study, however, no change has been observed in the PDE activity or PDE4B2 expression in the uteri of LPS-challenged pregnant mice [Schmitz et al., 2007]. Here we intended to focus on the uterorelaxant effects induced by the combined administration of the β_2-agonist terbutaline with the PDE4 inhibitor rolipram, and also their effect on cAMP accumulation.

We further confirmed the outstanding uterorelaxant effect of the combination of terbutaline with rolipram by measuring the tissue cAMP levels. Terbutaline alone generated a relatively small concentration of cAMP in both intact and LPS-treated rats. We note here that measuring tissue cAMP levels without a PDE inhibitor being present is against the conventional method, since cAMP is a rapidly inactivated intracellular signaling molecule. However, we considered that measuring cAMP concentration without a PDE inhibitor is of importance here, to compare the individual versus the combined effect of terbutaline and rolipram on uterine cAMP accumulation. Likewise, we detected a moderate cAMP accumulation in the presence of rolipram alone, without any receptorial stimulation. With the combination of terbutaline and rolipram, however, uterine cAMP levels were much higher, and the highest level was found in the LPS-treated rats.

We set out to investigate the effects of the above drug combination on pregnant rats *in vivo,* as well. Detection of the *in vivo* myometrial activity has the benefit of studying the

efficacy of these drugs according to the principles of integrative pharmacology [Collis, 2006]. We found that rolipram potentiated the uterus-relaxing effect of terbutaline, especially at lower doses. The extent of relaxation achieved with the first dose of terbutaline was approximately 2-times higher in intact rats and 2.5-times higher in LPS-treated rats, when it was co-administered with rolipram. Since the therapeutic action of β_2-agonists is impaired by tolerance and adverse effects, mostly associated with high-dose administration, we assume that the application of lower doses of terbutaline together with rolipram might well reduce the risk of unwanted side-effects and an inadequate response. In the course of the *in vivo* experiments, we did not observe any signs of discomfort (e.g. palpitations or dyspnea) in the tested animals. As regards the first 3 doses of terbutaline, the drug combination had a more pronounced uterus-relaxing effect at the time of LPS-induced preterm labour than at normal term, which is in accordance with our earlier *in vitro* findings.

A novel group of drugs has been developed, which inhibit AKAP-PKA associations and in this manner maximises ic. cAMP accumulation. Among them we tested the effects of FMP-API-1, a small molecule that has already been reported to increase cAMP levels in neonatal cardiac myocytes [Christian et al., 2011]. Here we investigated the effect of terbutaline + FMP-API-1 on the relaxation of pregnant rat uterus and on cAMP formation. The maximal contraction inhibiting effect of terbutaline was almost duplicated by FMP-AP-1 and the terbutaline induced cAMP accumulations were increased by FMP-API-1. The molecule enhances β-AR induced cAMP production in uterus preparations, presumably by interference with AKAP150-PKA interactions, which play a role in controlling uterine contraction [Dodge et al., 1999]. Thus attenuation of AC activity in response to β-AR agonists regularly seems to rely on AKAP-PKA interactions.

The studies on human biopsy specimens were performed in order to acquire a better picture of the applicability of this drug combination in humans. The non-selective PDE inhibitor theophylline exerted a very limited contraction-inhibiting effect on human tissue samples from full term pregnancy. The selective PDE4 inhibitor rolipram, however, proved to be much more effective in inhibiting the oxytocin-induced uterine contractions. Rolipram also potentiated the uterus-relaxing effect of terbutaline in the human uterus, its effect being most pronounced at lower terbutaline concentrations. This potentiation was more expressed in the samples from preterm birth than in those from full term pregnancy: a 2.5 versus a 1.5-times increase in the relaxing effect of 10^{-10} M terbutaline. We confirmed the contraction-inhibiting effect of the combination of terbutaline with rolipram by

measuring the tissue cAMP levels. The findings were in harmony with the results of the *in vitro* contractility assays on human uterus, suggesting that the main interaction occurs at an intracellular cAMP level.

Numerous studies have demonstrated that the four PDE4 subtypes are expressed in the human pregnant and non-pregnant myometrium, and the selective PDE4 inhibitors have been reported to exhibit greater efficacy on the pregnant than on the non-pregnant myometrium [Oger et al., 2004]. The isoenzymes of the PDE4 family play a role in inflammatory processes [Schmitz et al., 2007], but there are as yet no data as to now, whether these proteins are expressed in the human uterus at preterm birth. Unfortunately, the therapeutic promise of these compounds has been tempered by their significant side effects, particularly nausea and emesis [Oger et al., 2004].

The Western blot study demonstrated an up-regulation of the PDE4B isoform in the human uterine tissues at the time of preterm birth. This might well be a consequence of ongoing inflammatory processes that led to preterm labour. Intrauterine infection has been recognized as the primary cause of preterm delivery [Schmitz et al., 2007]. The expression of PDE4D showed a decreased signal in samples from both term and preterm birth. The low expression of PDE4D is beneficial as regards the strong emetic potential of PDE4 inhibitors that have high selectivity for PDE4D. Thus, PDE4B-selective inhibitors would be promising tocolytic drugs without adverse emetic effects.

β_2-AR stimulation and PDE4 inhibition have particular roles in inflammatory processes [Schmitz et al., 2007],. Thus, the anti-inflammatory effects of β_2-AR agonists and PDE4 inhibitors may be of great value because they can both relieve inflammation and prolong pregnancy. Besides the favorable tocolytic effect of this combination, a lower incidence of cardiac side-effects may also be expected. Although the expression of PDE4 enzymes has been reported in the cardiac ventricles, PDE4 inhibitors did not produce any positive inotropic effects [Muller et al., 1990].

Our observations are in concordance with previous finding that restraining the breakdown of cAMP with PDE4 inhibitors might act to potentiate the effects of β_2-AR agonists in asthma or COPD, which may in turn result in synergy for inflammatory outcome measures such as exacerbations [Lipworth, 2005]. It seems that, if challenged with an inflammatory reaction, the late-pregnant myometrium reacts with a higher sensitivity to cAMP-dependent relaxation mechanisms. The enhanced responsiveness of the myometrium to β_2-AR stimulation and PDE4 inhibition is probably the consequence of a pathological situation, which might become beneficial as regards tocolytic therapy.

6. Conclusions

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.

7. References

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8. Acknowledgement

I would like to express my thanks to Prof. George Falkay and to István Zupkó Ph.D. the heads of the Department of Pharmacodynamics and Biopharmacy for the possibility to take part in the Ph.D. studies.

I also would like to express my thanks to my supervisors, Róbert Gáspár and Anna Klukovits Ph.D. for their generous help and advice in the experimental work, and for critically reviewing the manuscript.

I also would like to thank my co-authors and colleagues in the Department of Pharmacodynamics and Biopharmacy for the pleasant co-operation.

I am deeply grateful for my family and friends for their patience and love.