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Ontogeny of sulfonylurea-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium

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Abstract

ATP-sensitive potassium channels (K_{ATP} channels) are composed of sulfonylurea receptors (SURs) and potassium inward rectifiers ($Kir_{6.x}$) that assemble to form a large octameric channel. This study was designed to examine the expression and role of sulfonylurea-binding regulatory subunits 1 (SUR1 (ABCC8)) and 2 (SUR2 (ABCC9)) of the K_{ATP} channels in the pregnant rat myometrium with particular regard to the contractility. RT-PCR and western blot analyses were performed to detect the presence of SUR1 and SUR2. The SUR1 levels were markedly increased in the early stages of pregnancy. The highest level was detected on day 6 of pregnancy, whereas in the late stages, the levels of SUR1 were significantly decreased. The SUR2 level remained unchanged throughout pregnancy. The SUR non-selective diazoxide and the SUR2-selective pinacidil inhibited oxytocin-induced contractions. Glibenclamide, a K_{ATP} channel blocker, antagonized both pinacidil- and diazoxide-induced relaxations. It was established that SURs are responsible for pharmacological reactivity of K_{ATP} channel openers. We conclude that both SURs are involved in the K_{ATP} channel in the pregnant rat myometrium. It may further be concluded that 'pinacidil-like' K_{ATP} channel openers may be of therapeutic relevance as tocolytic agents in the future.

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Introduction

The factors regulating myometrial function during pregnancy and labor are poorly understood. An understanding of these processes, at the cellular and molecular levels, is essential if novel therapeutic strategies are to be developed for the management of associated clinical problems such as preterm labor, the main cause of perinatal mortality and morbidity in the developed world (Byrne & Morrison 2002). The ion channels, including the potassium (K^+) channels, are central to the regulation of the cell membrane potential and contractility of the smooth muscle (Wray 1993). The opening of these channels results in an outward flow of K^+, drawing the cell membrane potential closer to the K^+ equilibrium potential and thereby reducing cellular excitability and contractility (Khan *et al.* 2001). There are several types of K^+ channels: the large-conductance calcium- and voltage-sensitive K^+ channel (BK_{Ca} channel), the ATP-sensitive K^+ channel (K_{ATP} channel), the Shaker-like voltage-gated K^+ channel (Kv channel), and small-conductance calcium-sensitive K^+ channels (SK channel; Brainard *et al.* 2007). K_{ATP} channels were first discovered in cardiac myocytes (Noma 1983) and later in many other tissues including pancreatic β-cells, skeletal muscle, smooth muscle, brain, pituitary, kidney,

and mitochondria. By linking the cell metabolic state to the membrane potential, K_{ATP} channels regulate a variety of cellular functions, including insulin secretion from pancreatic β-cells, the excitability of skeletal muscle and neurones, K^+ recycling in the renal epithelia, and cytoprotection in cardiac and brain ischemia (Inagaki & Seino 1998, Yokoshiki *et al.* 1998). K_{ATP} channels are large hetero-octameric complexes containing four subunits from the inwardly rectifying K^+ channel family ($Kir_{6.x}$: $Kir_{6.1}$ or $Kir_{6.2}$) and four regulatory sulfonylurea receptor (SUR) subunits from the ATP-binding cassette (ABC) transporter family ABCC8 (SUR1) and ABCC9 (SUR2). SUR2 has two different isoforms, SUR2A and SUR2B; these are splicing variants. Both subunits (SURs and $Kir_{6.x}$) are necessary for the channel function. $Kir_{6.x}$ comprises the K^+ channel component of the K_{ATP}, whereas the SURs are responsible for the ATP sensitivity, pharmacological properties, and trafficking of this channel (Aguilar-Bryan *et al.* 1998, Gross *et al.* 1999, Bryan *et al.* 2004, Teramoto 2006, Ko *et al.* 2008). The molecular structure of the K_{ATP} channels is different due to the heterologous expression of $Kir_{6.x}$ and the SUR subunits. This leads to different combinations and creates different types of K_{ATP} channel with distinct electrophysiological
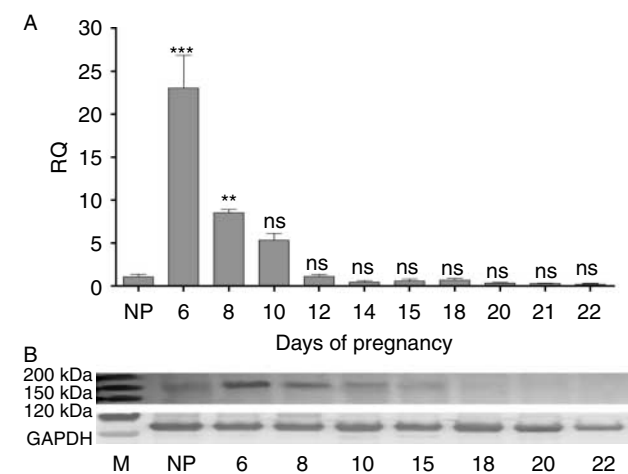
Figure 1 (A) Changes in expression of *Sur1* mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns, non-significant; **$P < 0.01$, ***$P < 0.001$. Each bar indicates the mean $\pm$ S.E.M., $n = 5$. (B) Representative western blot of SUR1 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

properties and pharmacological sensitivities that reflect the various K_{ATP} channels in native tissues. Although $Kir_{6.2}$/SUR1 constitutes the pancreatic β-cell type (Inagaki *et al*. 1995), the cardiac-type K_{ATP} channels consist of $Kir_{6.2}$/SUR2A (Inagaki *et al*. 1996) and $Kir_{6.2}$/SUR2B probably constitutes the non-vascular smooth muscle type. The vascular smooth muscle-type K_{ATP} channel comprises $Kir_{6.1}$/SUR2B (Yamada *et al*. 1997). $Kir_{6.1}$ and *Sur2b* mRNA transcripts have been identified in the rat myometrium (Chien *et al*. 1999, Sawada *et al*. 2005). There are no reports of the expression of SURs in the rat myometrium during gestation. Investigations on the human myometrium indicated that the major K_{ATP} channel is composed of $Kir_{6.1}$ and SUR2B and that downregulation of this channel may facilitate the myometrial function (Curley *et al*. 2002). Those authors were unable to delineate the exact time at which the downregulation occurs because of the ethical constraints; it was not possible to carry out serial sampling. K^+ channel-opening compounds (KCOs) are known to be potent smooth muscle relaxants and have been reported to be potent inhibitors of non-pregnant uterine contractions (Novakovic *et al*. 2007). The KCOs including diazoxide, pinacidil, cromakalim, and nicorandil are a structurally diverse group of drugs that open K_{ATP} channels in various cell types (Ashcroft & Gribble 2000*a*). It has been shown that different SUR subunits confer varying sensitivities to KCOs. For example, $Kir_{6.2}$/SUR1 channels are activated strongly by diazoxide, but not by pinacidil, $Kir_{6.2}$/SUR2A channels are activated by pinacidil and cromakalim, but only weakly by diazoxide, whereas $Kir_{6.2}$/SUR2B channels are activated by diazoxide, pinacidil, and cromakalim (Inagaki *et al*. 1995,

Isomoto *et al*. 1996, Babenko *et al*. 1998, Gribble *et al*. 1998, D'Hahan *et al*. 1999).

The objectives of this study were to investigate the expression of the SUR subunits of the K_{ATP} channels in the rat myometrium in non-pregnant animals and during pregnancy and to investigate possible correlations between SUR protein levels and the effectiveness of KCOs.

Results

### *mRNA and protein expression assays*

Relative quantitative real-time PCR and western blot analysis revealed that both SUR1 and SUR2 mRNAs and proteins are expressed in the pregnant and non-pregnant rat uteri. The mRNA and protein expression of the SUR1 subtype were found to be elevated in the early stage of pregnancy (day 6), dramatically decreased from days 8 to 12, and then remained unchanged until the end of pregnancy (Fig. 1A–B). The SUR2 mRNA and protein levels did not undergo any alterations during pregnancy (Fig. 2A–B).

Effects of SUR non-selective K_{ATP} channel opener diazoxide and K_{ATP} channel blocker glibenclamide

Diazoxide in the range 10^{-8}–10^{-4} M inhibited the oxytocin-induced contractions. The uterus-relaxant effect of diazoxide was investigated in non-pregnant and in 6-, 8-, 18-, and 22-day pregnant rat uteri. The diazoxide-relaxant effect reached its maximum
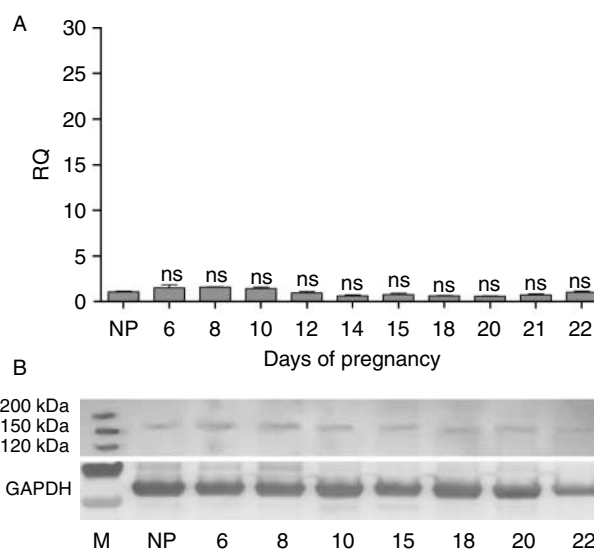
Figure 2 (A) Changes in the expression of *Sur2* mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns, non-significant. Each bar indicates the mean $\pm$ S.E.M., $n = 5$. (B) Representative western blot of SUR2 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.
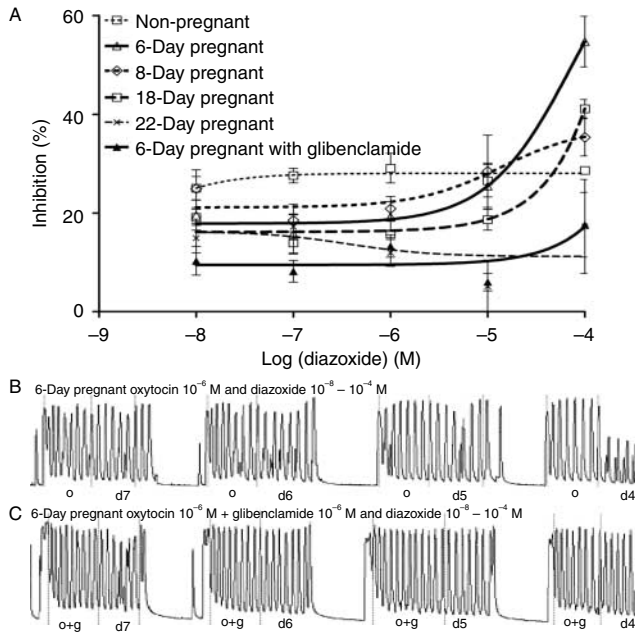
Figure 3 (A) Uterus-relaxing effect of the K$_{ATP}$ channel opener diazoxide (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 6-, 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. Values on different days of pregnancy were compared with those in non-pregnant rats. Reversal by glibenclamide (10^{-6} M) on day 6 of pregnancy. Each value denotes the mean±s.e.m., *n*=6. (B) Representative non-cumulative patterns for 6-day pregnant uterus contractions. The effect of diazoxide on oxytocin (o)-induced contractions and (C) in the presence of glibenclamide (o+g).

level on day 6 (60%) and was lower on days 8 and 18 (40%). Diazoxide had no significant effect on the uterine contractions in non-pregnant and term-pregnant animals (Fig. 3). The relaxant effect was blocked by glibenclamide 10^{-6} M on day 6 of pregnancy (Fig. 3).

Effect of SUR2-selective K$_{ATP}$ channel opener pinacidil and K$_{ATP}$ channel blocker glibenclamide and Ca-dependent K$^+$ channel blocker tetraethylammonium

The oxytocin-stimulated uterine contractions of non-pregnant and of 8-, 18-, and 22-day pregnant rats were inhibited concentration dependently by pinacidil in the range 10^{-8}–10^{-4} M (Fig. 4). The EC$_{50}$ values of pinacidil were significantly lower in the pregnant rat myometrium compared with the non-pregnant stage (Fig. 5). The E_{max} values were elevated on days 8 and 18, but on day 22, E_{max} was significantly lower, similar to that in the non-pregnant animals (Fig. 4). The uterus-relaxant effect of pinacidil was blocked by glibenclamide 10^{-6} M on days 8 and 22 (Fig. 6). The uterus-relaxant effect of pinacidil was investigated on electric field stimulation (EFS)-induced contractions in the presence of tetraethylammonium (TEA; 10^{-3} M) on non-pregnant and 22-day pregnant uterus (Fig. 7).

Discussion

The myometrial smooth muscle remains relatively quiescent throughout most of pregnancy, but at term, it undergoes a transformation that results in the development of powerful rhythmic contractions. The factors regulating these painful contractions during pregnancy and labor are poorly understood. K$_{ATP}$ channel activation has been shown to decrease the uterine tone and this is a target for the inhibition of uterine activity in the treatment of preterm labor (Piper *et al*. 1990, Brainard *et al*. 2007). K$_{ATP}$ channels are composed of two different subunits: SURs and Kir$_{6.x}$. It has been established that the SURs are responsible for the pharmacological reactivity of the KCOs on each K$_{ATP}$ channel. Previous studies reported that only the SUR2B subunit was involved in the K$_{ATP}$ channels in the rat myometrium (Chien *et al*. 1999, Sawada *et al*. 2005). Curley *et al*. (2002) found *SUR1* and *SUR2* mRNA transcripts in the human myometrium. This study was undertaken to extend our knowledge concerning the gestational changes of SUR1 and SUR2 of the K$_{ATP}$ channels in the rat uterus. In contrast to Chien *et al*. (1999) and Sawada *et al*. (2005), our results clearly demonstrated that both SUR-binding regulatory subunits were expressed in the rat myometrium during gestation. Our findings demonstrate that there is a fivefold downregulation in *Sur1* mRNA level in
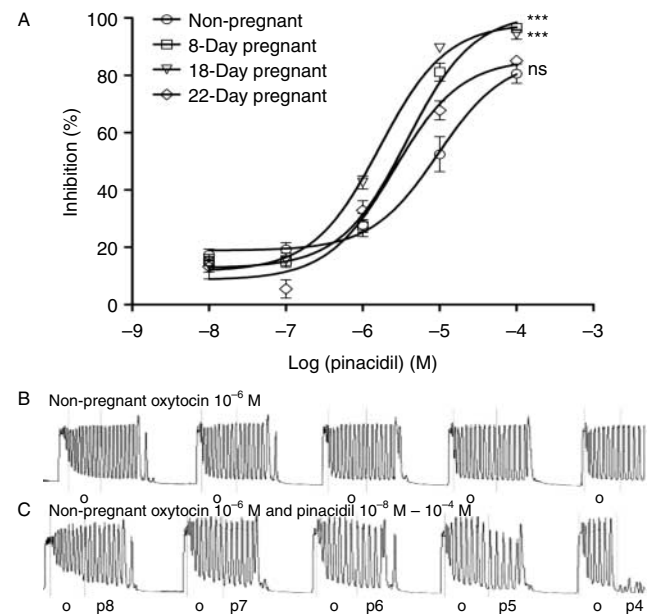
Figure 4 (A) Uterus-relaxing effect of the K$_{ATP}$ channel opener pinacidil (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. E_{max} values on different days of pregnancy were compared with those in non-pregnant rats. ***Denotes *P*<0.001, ns, non-significant. Each value denotes the mean±s.e.m., *n*=6. (B) Representative non-cumulative patterns for non-pregnant oxytocin (o)-treated uterus contractions and (C) the effect of pinacidil on oxytocin-induced contractions.
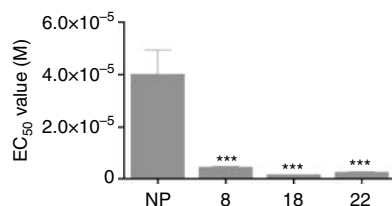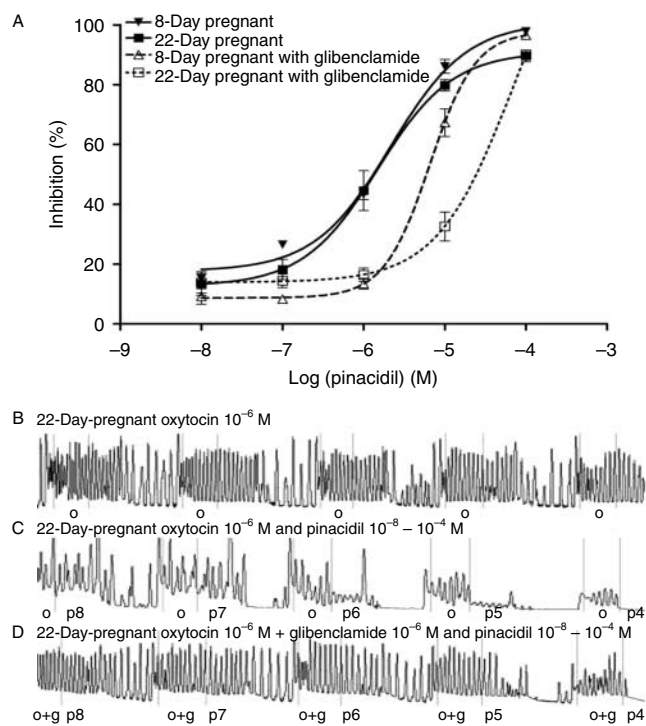
Figure 5 Changes in EC_{50} values of the K_{ATP} channel opener pinacidil on oxytocin-induced contractions in the non-pregnant (NP) and in the 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. EC_{50} values on different days of pregnancy were compared with those in non-pregnant rats. ***$P<0.001$. Each value denotes the mean $\pm$ S.E.M., $n=6$.

the rat myometrium in late pregnancy compared with the non-pregnant myometrium and ~ 80-fold decrease relative to early stages (days 6–8) of pregnancy. Similar to the results of Curley *et al.* (2002) on the human myometrium, our findings indicate that the decrease in SUR1 expression in late pregnancy may facilitate the enhanced contractility of the rat myometrium.

We have demonstrated that KCOs (diazoxide and pinacidil) are potent relaxants of the non-pregnant and pregnant rat uterus and are antagonized by glibenclamide. Diazoxide non-selectively activates K_{ATP} channels containing SUR1 or SUR2 (Inagaki *et al.* 1996, Seino & Miki 2003). Pinacidil selectively activates K_{ATP} channels containing SUR2 subunits (Yokoshiki *et al.* 1998). The uterus-relaxant effect of diazoxide was significantly stronger when the SUR1 expression was sharply increased on days 6 and 8 of pregnancy. Thus, the pharmacological reactivity of the non-selective diazoxide depends on the characteristic change in SUR1. In the case of SUR2, low mRNA expression and protein levels were found, which did not change during gestation. In spite of the low SUR2 levels, a strong uterus-relaxant effect of the SUR2 agonist pinacidil was observed on the pregnant rat uterus, whereas the relaxant effect on the non-pregnant uterus was significantly weaker. The relaxant effect of pinacidil correlates with the SUR2 level because it remained unchanged during gestation, but the difference between the pregnant and the non-pregnant stages on oxytocin-induced contractions is not clearly understood. The Ca-dependent K^+ channel (K_{Ca} channel) blocker TEA antagonized the uterus-relaxant effect of pinacidil on non-pregnant and 22-day pregnant uterus. This result confirms that the pinacidil has multiple binding sites for K^+ channels. The same results were found in the human radial artery by Gojkovic-Bukarica *et al.* (2011). Glibenclamide, a K_{ATP} channel blocker, antagonized both pinacidil- and diazoxide-induced relaxations. However, it is generally accepted that glibenclamide is a selective SUR1 blocker. Our results showed that glibenclamide selectivity in the pregnant rat myometrium is questionable. Ashfield *et al.* (1999) and Babenko *et al.* (1999) reported that both SUR subunits can bind

K_{ATP} channel blockers, but in two different ways. Whereas SUR1 has two binding sites for blockers (sulfonylurea and benzamido), SUR2 has only a benzamido-binding site. Glibenclamide contains both sulfonylurea and benzamido moieties, and it can, therefore, bind to SUR1 in two regions and to SUR2 in one region (Ashcroft & Gribble 2000*b*). Stephan *et al.* (2006) demonstrated that glibenclamide (10^{-9} M) induced complete inhibition of the pancreatic K_{ATP} channel, whereas higher concentrations (10^{-7} or 10^{-6} M) produced only partial and reversible inhibition of the cardiovascular K_{ATP} channels. These studies clearly revealed that glibenclamide is a non-selective SUR blocker. It is very likely that this mechanism exists in the pregnant rat myometrium.

In conclusion, this study provides the ontogeny of the SUR-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium. It has been established that the SUR-binding regulatory subunits play important roles in the pharmacological reactivity of KCOs. The relaxant effect of diazoxide was significantly stronger when the SUR1 expression was sharply increased on days 6 and 8 of pregnancy and did not show any appreciable effect on those gestation days when SUR1 was downregulated.

Figure 6 (A) Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions on the 8- and 22-day pregnant rat myometrium *in vitro*; reversal by glibenclamide (10^{-6} M) on days 8 and 22 of pregnancy. Each value denotes the mean $\pm$ S.E.M., $n=6$. (B) Representative non-cumulative patterns for 22-day pregnant oxytocin (o)-treated uterus contractions. (C) The effect of pinacidil on oxytocin-induced contractions and (D) in the presence of glibenclamide (o$+$g).
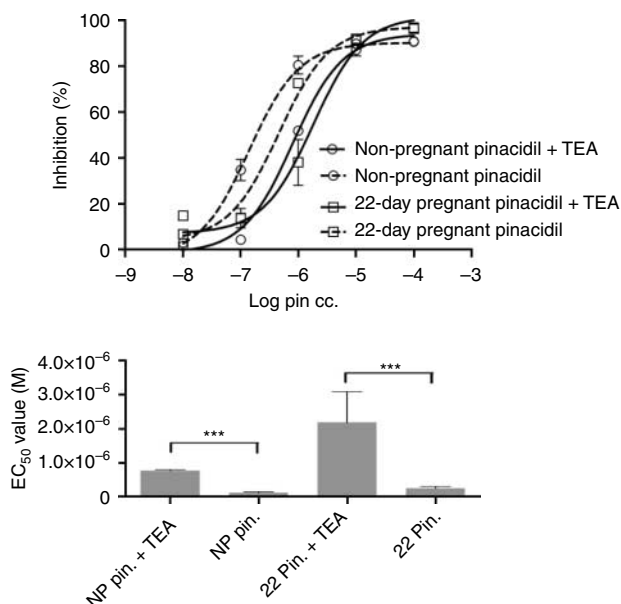
Figure 7 Uterus-relaxing effect of the K$_{ATP}$ channel opener pinacidil (10^{-8}–10^{-4} M) on EFS-evoked rhythmic contractions in the non-pregnant (NP pin.) and the 22-day pregnant (22 pin.) rat myometrium *in vitro*, reversal by TEA (10^{-3} M). ***$P < 0.001$. Each value denotes the mean±S.E.M., $n = 6$.

The downregulation of SUR1 expression in the rat uterus may contribute to the enhanced contractility associated with the onset of labor. As the uterus-relaxant effect of the SUR2-selective pinacidil on the K$_{ATP}$ channel is independent of the gestational age, it can be concluded that the development of 'pinacidil-like' uteroselective K$_{ATP}$ channel openers may be of novel therapeutic relevance in the management of preterm labor in the future. However, the main problem with KCOs is lack of specificity, resulting in undesired adverse effects. Further research in this field may shed light on the development of new drugs acting via K$^+$ channels. Whether newly developed KCOs will exhibit significant selectivity for the uterus remains to be seen.

Materials and Methods

Housing and handling 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 Ethics Committee for Animal Research (registration number: IV/01758-2/2008). Sprague–Dawley rats (Charles-River Laboratories, Budapest, Hungary) were kept at 22 ± 3 °C; the relative humidity was 30–70% and maintained on a 12 h light:12 h darkness cycle. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories) with tap water available *ad libitum*. They were killed by CO$_2$ inhalation.

Mating of the animals

Mature female (180–200 g) and male (240–260 g) rats were mated in a special mating cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Because rats are usually active at night, the separating door was opened before dawn. Within 4–5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of $1200 \times$. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day pregnant animals.

Real-time quantitative RT-PCR

Uterus tissues were separated and frozen in liquid nitrogen and the tissue was mechanically homogenized. The PARIS Kit (Protein and RNA isolation system; Life Technologies, Budapest, Hungary) was used for total RNA and protein extraction from the tissues.

The quality and the quantity of the RNA were assessed at A 260/280, and all samples displayed an absorbance ratio in the range 1.6–2.0. Two micrograms of total RNA and the High Capacity RNA-to-cDNA Kit (Life Technologies) was used for RT. PCR products were amplified with the TaqMan Gene Expression Master Mix (Life Technologies) and the ABI StepOne Real-Time cycler. The following primers were used: assay ID Rn01476318_ml for *Abcc8/Sur1* and Rn01463198_m1 for *Abcc9/Sur2* and Rn99999916_s1 for *Gapdh* as endogenous control. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle exhibiting the first significant increase in the fluorescence signal was defined as the threshold cycle (C_T).

Western blot analysis

Protein (30 µg per well) was subjected to electrophoresis on 4–12% NuPAGE Bis-Tris Gel (Life Technologies) in XCell SureLock Mini-Cell Units (Invitrogen). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Dassel, Germany) by a semi-dry blotting technique (Bio-Rad). The antibody binding was detected with the WesternBreeze Chromogenic western blot immune detection kit (Invitrogen). The blots were incubated on a shaker with SUR1, SUR2, and GAPDH polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany, 1:200) in the blocking buffer. Images were captured with the EDAS290 imaging system (KODAK, Invitrogen), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction.

Isolated organ studies

Uterus preparation

Uteri were removed from non-pregnant rats in the estrus phase (250–350 g) and from pregnant rats on day 6, 8, 18, or 22 of

pregnancy. Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml of de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH 7.4). The organ bath was maintained at 37 °C, and carbogen (95% $O_2 + 5\%$ CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before the experiments were undertaken, with a solution change every 15 min. The initial tension of the preparation was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd, Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd).

KCO studies

Oxytocin-induced contractions

Contractions were elicited with 10^{-6} M oxytocin and non-cumulative dose-response curves were constructed in each experiment in the presence of pinacidil or diazoxide (10^{-8}–10^{-4} M; Sigma–Aldrich). Following the addition of each concentration of pinacidil or diazoxide, recording was performed for 300 s. Concentration-response curves were fitted and area under curve (AUC) were evaluated and analyzed. Statistical analyses were carried out with the Prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) computer program. From the AUC values, the maximum inhibitory effects (E_{max}) of pinacidil and diazoxide were calculated on a given day of pregnancy, and the concentrations eliciting 50% of the maximum inhibitions of uterine contraction (EC_{50}) were calculated. For statistical evaluations, data were analyzed by the ANOVA Neuman–Keuls test.

Contractions induced by EFS

Uteri were removed from rats as described in the 'uterus preparation' section, except that uterus rings were vertically mounted between two platinum electrodes. Maximum rhythmic contractions were elicited with a digital, programmable stimulator (ST-02, Experimetria UK Ltd.), using different values of pulse width (PW, the duration of the electric field as a single stimulus) and period time (PP, the time interval between two stimuli). The uterus-relaxant action of pinacidil was investigated cumulatively on the non-pregnant and the 22-day pregnant uterus on EFS-induced contractions alone and in the presence of the K_{Ca} channel blocker TEA. TEA was added to the organ bath 20 min before the exposure to pinacidil. After EFS, pinacidil (10^{-8}–10^{-4} M) was added in a cumulative manner. AUC of 3 min periods were evaluated; the effect of pinacidil was expressed as a percentage of the contraction induced by EFS preceding the administration of the relaxing drug. EFS parameters were as follows: non-pregnant (PP: 30 s, PW: 50 ms) and 22-day pregnant (PP: 25 s, PW: 150 ms).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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