Pregnancy switches adrenergic signal transduction in rat and human uterine myocytes as probed by BK_{Ca} channel activity

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1. We used large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activity as a probe to characterize the inhibitory/stimulatory G protein (G_i/G_s) signalling pathways in intact cells from pregnant (PM) and non-pregnant (NPM) myometrium.

2. Isoprenaline (10 μM) enhanced the outward current (I_{out}) in PM cells and inhibited I_{out} in NPM cells. Additional application of the α_2-adrenoceptor (α_2-AR) agonist clonidine (10 μM) further enhanced the isoprenaline-modulated I_{out} in PM cells but partially antagonized I_{out} in NPM cells. Clonidine alone did not affect I_{out}. The specific cAMP kinase (PKA) inhibitor H-89 (1 μM) abolished the effects of isoprenaline and clonidine. The specific BK_{Ca} channel blocker iberiotoxin (0·1 μM) inhibited I_{out} by ~80 %; the residual current was insensitive to isoprenaline.

3. Inhibition of G_i activity by either pertussis toxin or the GTPase activating protein RGS16 abolished inhibitory as well as stimulatory effects of clonidine on I_{out}.

4. Transducin-α, a scavenger of $G_i\beta\gamma$ dimers, converted the stimulatory action of clonidine on I_{out} into an inhibitory effect. Free transducin-$\beta\gamma$ enhanced both the stimulatory and the inhibitory effects of isoprenaline on I_{out}.

5. The results demonstrate that BK_{Ca} channel activity is a sensitive probe to follow adenylyl cyclase–cAMP–PKA signalling in myometrial smooth muscle cells. Both $G_i\alpha$-mediated inhibition and $G_i\beta\gamma$-mediated stimulation can occur in the same cell, irrespective of pregnancy. It is speculated that the coupling between α_2-AR and G_i proteins is more efficient during pregnancy and that $G_i\beta\gamma$ at high levels simply override the inhibitory action of $G_i\alpha$.

It is well established that catecholamines influence the contractile state of the myometrium in response to the hormonal status (Marshall, 1981). Four adrenoceptors (α_1-, α_2-, β_1- and β_2-ARs) have been shown to be present in the pregnant and non-pregnant myometrium (Bottari *et al.* 1985). The β-ARs couple via stimulatory G proteins (G_s) to myometrial adenylyl cyclases (ACs), promote the conversion of ATP to cyclic AMP and induce, at least in the pregnant myometrium, relaxation by various mechanisms including effects on $[Ca^{2+}]_i$, myosin light chain kinase and large conductance Ca^{2+}-activated potassium channels (BK_{Ca} channels) (Anwer *et al.* 1992; Wray, 1993). Both β_1- and β_2-ARs coexist in the myometrium with a higher proportion of the β_2-subtype. During pregnancy, this subtype is selectively upregulated by progesterone at the level of gene transcription and converted to a high affinity state (Vivat *et al.* 1992). Three different ARs of the α_2-type ($\alpha_{2A\text{-}C}$) have been identified in human myometrium and an alteration of the α_{2A}/α_{2B}-subtypes expression pattern during pregnancy has been described (Mhaouty *et al.* 1995; Bouet-Alard *et al.* 1997; Adolfsson *et al.* 1998). In most cell systems investigated so far, activation of α_2-ARs inhibits AC via inhibitory G proteins (G_i), although α_2-ARs have also been reported to mediate stimulation of AC, activation of K^+ channels and of Na^+–H^+ exchange, and mobilization of intracellular Ca^{2+} (for review see Lomasney *et al.* 1991). The role of α_2-ARs in the control of pregnancy-related changes of the AC system and uterine contractility is complex. Clonidine, a potent α_2-AR agonist, potentiated the β-AR-stimulated AC activity in rat myometrium at midpregnancy by coupling to G_i proteins (Mhaouty *et al.* 1995). Conversely, a switch from the stimulatory to an inhibitory input to AC via the α_2-AR–G_i protein signalling pathway was described at late term (Mhaouty *et al.* 1995). These data suggested a differential expression of AC isoforms at midpregnancy and term. Whereas AC types II and IV are co-stimulated by $\beta\gamma$-dimers of G_i proteins in the presence of $G_s\alpha$, other cyclases are inhibited by G_i proteins (Tang & Gilman, 1991; Gao &

Gilman, 1991; Federman *et al.* 1992). Although RNA blot analysis revealed that the expression of AC transcripts in the rat myometrium remained stable over the second half of pregnancy (Mhaouty-Kodja *et al.* 1997), this cannot exclude possible changes at the protein level. At the present time this issue cannot be settled as no specific antibody that allows quantification directly of each AC type is available.

AC activity in pregnant and non-pregnant myometria is usually determined by biochemical methods on plasma membranes prepared from freshly isolated tissues. In the present study, it was our purpose to investigate the AC–cAMP cascade as influenced by β- and α_2-AR agonists in intact smooth muscle cells from pregnant and non-pregnant myometria. We show here by means of patch-clamp techniques, that the activity of BK_{Ca} channels reflects the status of the cellular AC–cAMP signalling pathway with a sensitivity superior to biochemical methods. Moreover, direct application of RGS proteins (regulators of G protein signalling), transducin-α (TDα) and transducin-$\beta\gamma$ (TD$\beta\gamma$) to the interior of cells via the patch-pipette allowed us to address the questions of differential AC expression, and of G protein subunits responsible for co-stimulatory and inhibitory effects at midpregnancy and in the non-pregnant state. In addition, the effects of adrenaline and noradrenaline, the physiological ligands on myometrial β- and α_2-ARs, were investigated.

METHODS

Materials

The catalytic subunit of cAMP kinase was prepared as described previously (Zhou *et al.* 1996). The cAMP-dependent protein kinase (PKA) inhibitor H-89 was obtained from Biomol (Hamburg, Germany), iberiotoxin (IbTX) from Latoxan (Rosans, France), (±)-adrenaline and (±)-isoprenaline from Sigma (Deisenhofen, Germany), (±)-noradrenaline and yohimbine from RBI (Natick, MA, USA), A23187 from Molecular Probes (Eugene, OR, USA), and pertussis toxin from List Biological Laboraories (Campbell, CA, USA). All drugs were dissolved in PSS (see below); solutions with IbTX contained 0·1% bovine albumin fraction V (Sigma). Collagenase type H (lot 57H6832), hyaluronidase type I-S, and papain was purchased from Sigma, and 1,4-dithio-D,L-threitol (DTT) was from Gerbu Biotechnik (Gaiberg, Germany).

Purification of proteins

His_6-RGS16 was expressed in the *Escherichia coli* strain BL21(DE3) and purified over a Ni^{2+}-NTA column (Qiagen) as described before (Chen *et al.* 1996). Bleached bovine rod outer segment (ROS) membranes were prepared from bovine retinae as described previously (Papermaster & Dreyer, 1974). The bovine retinae were obtained from a local abattoir. Transducin (TD) was eluted from the membranes by hypotonic elution in the presence of 100 μM GTP, and the subunits were separated by affinity chromatography on Blue Sepharose (Bio-Rad) (Wieland *et al.* 1991). The buffers in which the proteins were eluted from the columns were changed to the intracellular pipette solution (see below) by repeated extensive dialysis and proteins were stored in aliquots at −80 °C. The protein concentration was determined according to Bradford (1976) with IgG as standard.

Animals

All experimental procedures were carried out according to the animal welfare guidelines of the University Hospital Eppendorf. Female Wistar rats were obtained from a colony bred and maintained at the animal house of the University Hospital Eppendorf. In order to obtain myometria from pregnant animals, females were caged with males overnight, and successful mating was determined by the presence of spermatozoa in the vaginal smear (day 1 of pregnancy). Animals were killed on days 11 or 12 of gestation which corresponds to midpregnancy. Myometria were dissected from the rats following anaesthesia with halothane then cervical dislocation.

Tissue collection from pregnant and non-pregnant women

Myometrial samples were obtained from non-labouring women (37–39 weeks gestation) undergoing elective Caesarian sections. No tocolytic agents had been administered to the mother 24 h prior to Caesarian delivery. The reasons for Caesarean section included breech presentation, previous Caesarean section, and cephalo-pelvic disproportion. All biopsies were taken from the upper border of the lower isthmic uterine incision. Non-pregnant myometrium was obtained from uteri of normal cycling premenopausal patients undergoing hysterectomy for benign disease. Immediately after collection, the tissue was carried to the laboratory in ice-cold physiological saline solution (PSS (mM): 127 NaCl, 5·9 KCl, 2·4 $CaCl_2$, 1·2 $MgCl_2$, 11 glucose and 10 Hepes; adjusted to pH 7·4 with NaOH). Informed consent was obtained from the patients and the study had the approval of the Ethics Committee Hamburg, Germany.

Cell preparation

After the connective tissue and endometrium was removed, the uterine smooth muscle was cut into cubes of 1–2 mm side length and incubated at 37 °C in Ca^{2+}-free PSS including 0·7 mg ml^{-1} papain, 1 mg ml^{-1} DTT and 1 mg ml^{-1} fat-free bovine serum albumin. Thirty minutes later, the tissue pieces were transferred into PSS solution containing 50 μM Ca^{2+}, 1 mg ml^{-1} collagenase, 1 mg ml^{-1} hyaluronidase and 1 mg ml^{-1} albumin, and digested for another 10–15 min at 37 °C. Single cells were released by gentle trituration and stored in PSS at room temperature. After isolation, 30–40% of the cells were relaxed, and only these cells were used for electrophysiological studies. Experiments were conducted within 6 h of cell isolation.

Recording techniques

Standard patch-clamp recording techniques were used to measure currents in the inside-out or whole-cell patch configuration (Hamill *et al.* 1981). Patch electrodes were fabricated from borosilicate glass capillaries (World Precision Instruments) and filled with prefiltered solutions of different composition (see below). Currents were recorded at 25 °C with a List Electronics EPC-7 patch-clamp amplifier, connected via a 16 bit A/D interface to a pentium IBM clone computer. The data were filtered at 1 kHz by a 10 pole Bessel filter and sampled at 3 kHz. Data aquisition and analysis was performed with an ISO-3 multitasking patch-clamp program (MFK, Niedernhausen, Germany). The pipette resistance ranged from 2–3 MΩ in whole-cell and 8–9 MΩ for the excised-patch experiments. The amplitude of single-channel currents was derived from an amplitude distribution histogram. Average channel activity (NP_o) in patches was determined from recordings using the following expression:

$$NP_o = \left(\sum_{j=1}^{N} t_j j\right) \Big/ T,$$

where P_o is the open probability, T is the duration of the measurement, t_j is the time spent with $j = 1, 2, \ldots N$ channels open, and N is the maximum number of channels seen. NP_o was determined over a 3–5 min period immediately before and after the addition of a compound. The cell or patch under investigation was studied either after equilibration with the bath solution or during continuous superfusion by a gravity-flow microperfusion device consisting of multiple glass tubes assembling into a common tip with an 80 μm orifice and mounted on a hydraulic micromanipulator. Solution changes were achieved via remote-controlled solenoid valves (N. Graf, Planegg, Germany).

Solutions

For inside-out experiments, the bath solution (cytosolic surface of the patch) contained (mM): 134 KCl, 6 NaCl, 1·2 $MgCl_2$, 5 EGTA, 11 glucose, 3 K_2-ATP and 10 Hepes (pH 7·4); and the patch pipette (extracellular patch surface) was filled with (mM): 140 KCl and 10 Hepes (pH 7·4). Depending on the experiment, the free Ca^{2+} concentration was changed from 0 to 0·3 μM by changing the Ca^{2+} concentration in the corresponding solution. The appropriate amounts of $CaCl_2$ were added, and the pH adjusted according to a computer program (Mermi *et al.* 1991) on the basis of the binding constants of Fabiato (Fabiato, 1988) and checked by fura-2 fluorescence.

For whole-cell patch-clamp experiments, the intracellular (pipette) solution contained (mM): 126 KCl, 6 NaCl, 1·2 $MgCl_2$, 5 EGTA, 11 glucose, 3 K_2-ATP, 0·1 Na_3GTP and 10 Hepes (pH 7·4). The free Ca^{2+} concentration was 0·3 μM. The bath was superfused with PSS.

Statistics

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical significance was determined by Student's t test for paired and unpaired data. A P value less than 0·05 was considered to be significant.

RESULTS

Modulation of BK_{Ca} channel activity by PKA

In the presence of symmetrically high potassium (140 mM) and at different membrane potentials, inside-out patches of freshly isolated smooth muscle cells from non-pregnant (NPM) and pregnant (PM) rat myometrium exhibited single channel currents with distinct amplitudes. The majority of channel openings, however, conducted currents with an amplitude 4–6 times larger than those conducted by the

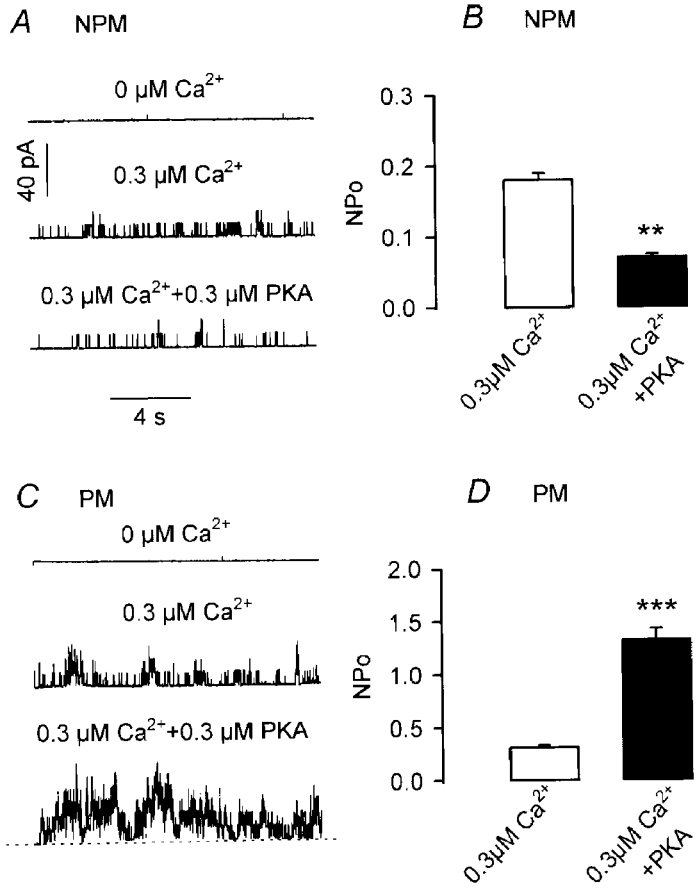

Figure 1. Differential regulation of BK_{Ca} channel activity by PKA

Repesentative single channel current recordings in inside-out patches from a non-pregnant (NPM; *A*) and a pregnant (PM; *C*) rat uterine myocyte. Channel openings are upward deflections. Dashed line in *C* indicates the closed state level. Holding potential was +40 mV and $[Ca^{2+}]_i$ is as indicated. The catalytic subunit of PKA (300 nM) was applied to the cytosolic surface of the patch. *B* and *D*, combined results from experiments with PKA in five NPM (*B*) and five PM cells (*D*), obtained from three rats in each group; $[Ca^{2+}]_i$ was 0·3 μM. ** $P < 0·01$; *** $P < 0·001$ *versus* control without PKA.

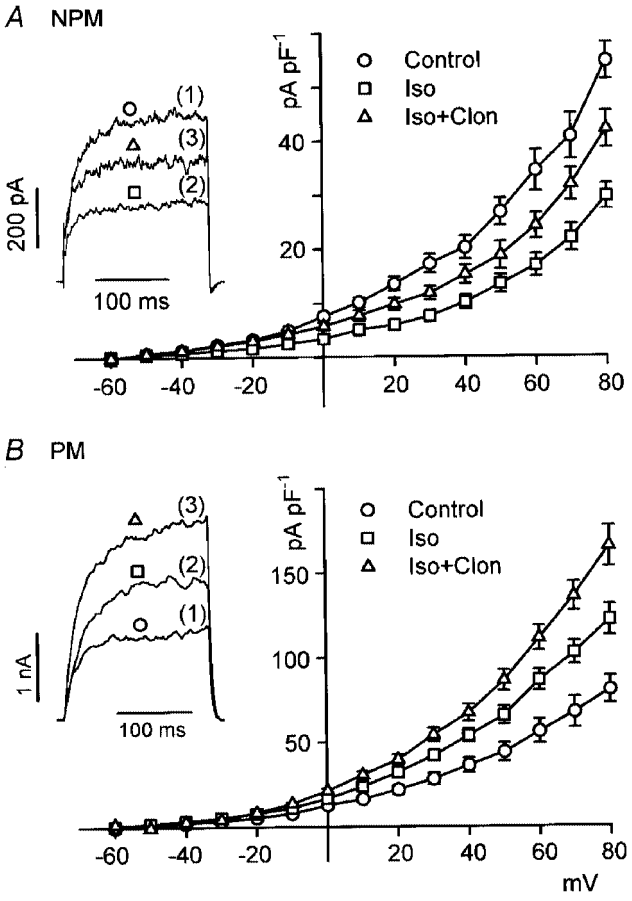

Figure 2. Opposite effects of clonidine on isoprenaline-modulated I_{out}

Current–voltage relations of I_{out} from non-pregnant (NPM; $n = 6$; *A*) and pregnant (PM; $n = 6$; *B*) rat uterine myocytes are shown. Cells were obtained from four animals in each group. Mean current densities are plotted against the respective test potential. Currents were evoked by applying a 200 ms depolarizing pulse every 10 s in 10 mV increments from a holding potential of −60 mV. Cells were superfused with 10 μM isoprenaline (Iso) first and then additionally with 10 μM clonidine (Clon). The insets show representative I_{out} recordings elicited by 200 ms pulses from a holding potential of −60 to +80 mV. Numbers in parentheses indicate the sequence of drug applications. The pipette solution contained 0·3 μM Ca^{2+}.

other channels. The current–voltage relationship of these high conductance channels was linear in the voltage range between −60 and +60 mV and showed a reversal potential close to 0 mV. The mean unitary conductances of these channels were 260 ± 10 and 267 ± 13 pS in patches excised from six NPM cells and seven PM cells, respectively. As shown in Fig. 1*A* and *C*, in the absence of Ca^{2+} in the bath solution no openings of BK_{Ca} channels could be detected. Addition of 0·3 μM Ca^{2+} resulted in the immediate appearance of channel activity with an open probability (NP_o) of 0·17 and 0·32 in the NPM and PM cells, respectively. Thus, large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} channels) were clearly identified in rat myometrial cells. Further superfusion of the cytosolic surface of the inside-out patch with 300 nM of the catalytic subunit of cyclic AMP kinase (PKA) had differential effects; NP_o decreased to 0·08 in the NPM cell (Fig. 1*A*) but increased to 1·49 in the PM cell (Fig. 1*C*). A summary of the results with PKA is presented in Fig. 1*B* and *D*; PKA decreased NP_o in NPM cells by 62% (before, 0·18 ± 0·01; after PKA, 0·07 ± 0·005; $n = 5$) and increased NP_o in PM cells by 426% (before, 0·31 ± 0·03; after PKA, 1·32 ± 0·11; $n = 5$). The results show that (1) basal NP_o is about twice as high in cells from pregnant myometrium and

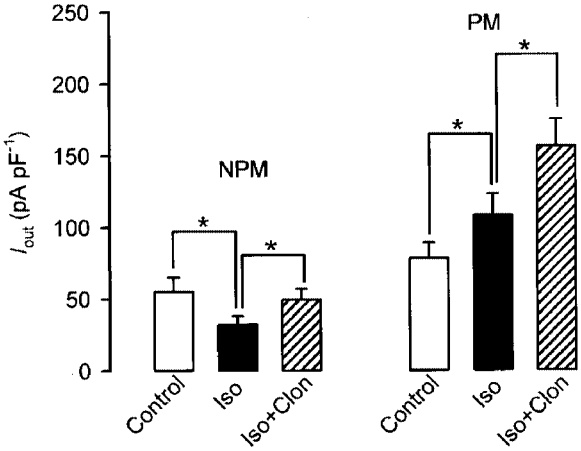

Figure 3. Effects of isoprenaline and clonidine on human uterine myocytes

I_{out} was elicited by applying a 200 ms depolarizing pulse every 10 s from a holding potential of −60 to +80 mV. The uterine myocytes were obtained from non-pregnant (NPM) and pregnant (PM) women. Cells were superfused with 10 μM isoprenaline (Iso) first and then additionally with 10 μM clonidine (Clon). Bars represent the mean current densities of four NPM and five PM cells. Each cell was from a different donor. The pipette solution contained 0·3 μM Ca^{2+}. * $P < 0·05$.

(2) PKA regulates NP_o in opposite directions with a large increase at midpregnancy.

Effects of isoprenaline and clonidine on I_{out}

Similar results to those shown in Fig. 1 were obtained when whole-cell outward currents (I_{out}) were recorded from relaxed myocytes treated with the β-AR agonist isoprenaline (Fig. 2). When cells were clamped from a holding potential of −60 to +80 mV in 10 mV increments for 200 ms, 70% of all cells responded with a non-inactivating I_{out}, whereas 30% showed a small inactivating early component superimposed on a much larger non-inactivating component as has been described before (Wang *et al.* 1998). Although I_{out} of both cell types was sensitive to IbTX (see later), cells with only a non-inactivating component were used in the present study. As shown by the current–voltage relations of Fig. 2, application of 10 μM isoprenaline induced a pronounced decrease of I_{out} in NPM cells (Fig. 2*A*), whereas an increase was observed in PM cells (Fig. 2*B*). When the effects of isoprenaline were maximal after 4 min, cells were additionally superfused with 10 μM clonidine, a selective α_2-AR agonist. As expected from its inhibitory effect on AC, clonidine antagonized the isoprenaline effect by partially restoring I_{out} in NPM cells (Fig. 2*A*). In PM cells, however, clonidine did not antagonize, but further augmented the isoprenaline-enhanced current at all potentials. Clonidine alone produced no significant effect on I_{out} when applied in the absence of isoprenaline (not shown). Representative I_{out} traces obtained from a NPM and a PM cell clamped for 200 ms to +80 mV are also presented in Fig. 2. It is shown that the control current amplitude is larger in the PM cell, which corresponds with the higher NP_o in inside-out patches, and currents did not inactivate during depolarizing steps. The enhanced current amplitude in PM cells was not due to myometrial smooth muscle hypertrophy because the difference between current amplitudes in NPM and PM cells persisted after normalizing currents to membrane capacitance as shown by the current–voltage relations in Fig. 2. Measurement of membrane capacitance of NPM and PM cells ($n = 11$ in both cases) disclosed a 2·3-fold larger cell surface of PM cells as compared with NPM cells. The stimulation and inhibition pattern of I_{out} in the presence of the α_2- and β-agonist was observed not only in rat but also in human myometria. As shown in Fig. 3, 10 μM isoprenaline decreased I_{out} of human NPM cells clamped for 200 ms from −60 to +80 mV by 42% (before, 55 ± 10 pA pF^{-1}; after isoprenaline, 32 ± 6 pA pF^{-1}; $n = 6$) and increased I_{out} in PM cells by 38% (before, 78 ± 11 pA pF^{-1}; after isoprenaline, 108 ± 15 pA pF^{-1}; $n = 5$). Additional superfusion of the cells with 10 μM clonidine resulted in an increase of I_{out} to 89% of the control (49 ± 8 pA pF^{-1}) in NPM and to 200% of the control (156 ± 19 pA pF^{-1}) in PM cells.

Selective effects of isoprenaline on the IbTX-sensitive component of I_{out}

In order to investigate to what extent BK_{Ca} channels were involved in the effects of isoprenaline, their contribution to whole-cell I_{out} was determined first. Currents were elicited in rat NPM and PM cells every 10 s by a depolarizing pulse of 200 ms from a holding potential of −60 to +80 mV. Superfusion with the Ca^{2+} ionophore A23187 (1 μM) resulted in a marked increase of I_{out} from 0·6 to 1·87 nA in the NPM cell (Fig. 4*A*) and from 1·5 to 3·1 nA in the PM cell (Fig. 4*C*).

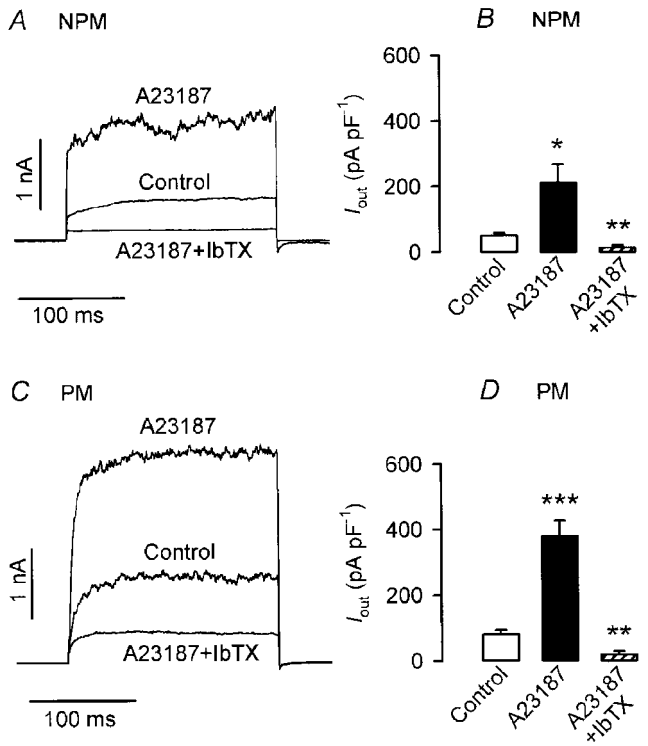

Figure 4. Calcium and iberiotoxin sensitivity of I_{out}

Currents were evoked in non-pregnant (NPM) and pregnant (PM) rat uterine myocytes by applying 200 ms depolarizing pulses from a holding potential of −60 to +80 mV. Original current recordings from a NPM (*A*) and a PM (*C*) cell exposed to 1 μM A23187 first and then additionally to 100 nM iberiotoxin (IbTX) are shown. *B* and *D*, summary of the effects of A23187 and of A23187 plus IbTX on I_{out} of five NPM and PM cells, obtained from five and four rats, respectively. Bars represent mean current densities. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ *versus* control.

Subsequent addition of 100 nM IbTX in the presence of A23187 reduced the current to 0·17 nA in the NPM and to 0·4 nA in the PM cell. A summary of effects induced by A23187 and IbTX on I_{out} is shown in Fig. 4*B* and *D*. The mean current density increased in five NPM and five PM cells from $50·6 \pm 8·9$ (control) and $82·3 \pm 14$ pA pF^{-1} (control) to $209 \pm 56·3$ and 378 ± 47 pA pF^{-1} in the presence of 10 μM A23187 and decreased to $11·6 \pm 7·4$ and $17 \pm 9·5$ pA pF^{-1} in the presence of A23187 plus 100 nM IbTX, respectively. Because 300 nM IbTX did not further decrease I_{out}, it can be concluded that the residual current of 23% in NPM cells and of 21% in PM cells was due to the opening of channels other than BK_{Ca} channels. In a second step, it was tested whether the residual current was sensitive to isoprenaline. By utilizing the same stimulation protocol as that in Fig. 4, BK_{Ca} channels were blocked by 100 nM IbTX and the effect of 10 μM isoprenaline on the amplitude of the residual I_{out} was then determined. As shown in Fig. 5*C* and *D*, 100 nM IbTX decreased the mean I_{out} density in six NPM and six PM cells from $54·3 \pm 4·6$ to $12·2 \pm 1·3$ pA pF^{-1} (i.e. by 77·5%) and from $85·8 \pm 5·2$ to $17·1 \pm 2·3$ pA pF^{-1} (i.e. by 80%), respectively. Additional superfusion of the cells with isoprenaline produced no significant effect on the amplitude of the residual current. Figure 5*A* and *B* presents original current traces from a NPM and PM cell and it is shown that in comparison with IbTX alone, neither the current amplitude nor its activation kinetics was influenced by the catecholamine. In conclusion, Fig. 5 demonstrates that about 80% of whole-cell I_{out} elicited by depolarizing steps from a holding potential of −60 to +80 mV in NPM and PM cells is conducted by BK_{Ca} channels and only this fraction of total I_{out} is modulated by isoprenaline.

Influence of H-89 on the isoprenaline- and clonidine-modulated I_{out}

Signal transduction initiated by activation of β- and α_2-ARs in myometrial smooth muscle cells is mediated via the cAMP–PKA cascade. It was therefore important to investigate whether inhibition of PKA would reverse the effects of isoprenaline and clonidine on I_{out} of NPM and PM cells. The experiments shown in Fig. 6 were carried out with 1 μM H-89, a specific inhibitor of PKA at this concentration (Hidaka & Kobayashi, 1992). As in the experiments before,

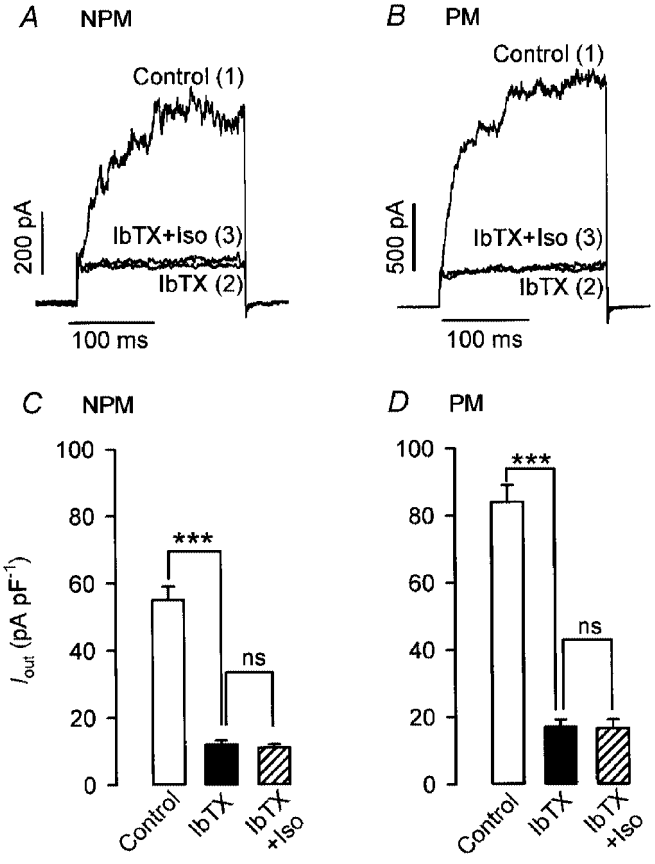

Figure 5. Isoprenaline fails to influence the iberiotoxin-insensitive I_{out}

Currents were evoked in non-pregnant (NPM) and pregnant (PM) rat uterine myocytes by applying 200 ms depolarizing pulses from a holding potential of −60 to +80 mV. Original current recordings from a NPM (*A*) and a PM (*B*) cell exposed to 100 nM iberiotoxin (IbTX) first and then additionally to 10 μM isoprenaline (Iso) are shown. Numbers in parentheses denote the sequence of drug applications. *C* and *D*, average results from six NPM and PM cells, obtained from five rats in each group. Bars represent mean current densities. *** $P < 0·001$ *versus* control; ns, not significant.

I_{out} was elicited by depolarizing the cells for 200 ms from −60 to +80 mV. It is shown that H-89 completely reversed the effect of 10 μM isoprenaline in six NPM and six PM cells (Fig. 6*A*). The same inhibitory effect of H-89 was observed in eight NPM and six PM cells which had been treated previously with isoprenaline plus clonidine (Fig. 6*B*). Thus, the findings clearly indicate that both receptor agonists produce their effects on BK_{Ca} channels exclusively via the cAMP–PKA cascade.

Influence of PTX and RGS16 on the clonidine-modulated I_{out}

In order to test whether G_i proteins are involved in both the inhibitory and stimulatory effects on I_{out} when clonidine was applied in the presence of isoprenaline, experiments with RGS16, a GTPase-activating protein for members of the $G_i\alpha$ subfamily, were conducted. Myocytes from non-pregnant and pregnant myometria were loaded via the patch-electrode with the RGS16 protein for 20 min (electrode concentration, 1 μM). Thereafter, I_{out} was elicited by depolarizing the cells from −60 to +80 mV in 10 mV increments for 200 ms to obtain current–voltage relations. As shown in Fig. 7, the inhibitory and stimulatory effects of 10 μM isoprenaline in nine NPM (Fig. 7*A*) and ten PM cells (Fig. 7*B*), respectively, were preserved in the presence of RGS16. Addition of 10 μM clonidine, however, produced no further change in current. Identical results to those shown in Fig. 7 with RGS16 were obtained in seven NPM and seven PM cells, incubated for 5 h in PSS containing 400 ng ml^{-1} pertussis toxin (PTX; data not shown). This loss of effectiveness of clonidine in the presence of RGS16 or PTX indicates that G_i proteins are involved in the inhibitory as well as in the stimulatory effect of clonidine.

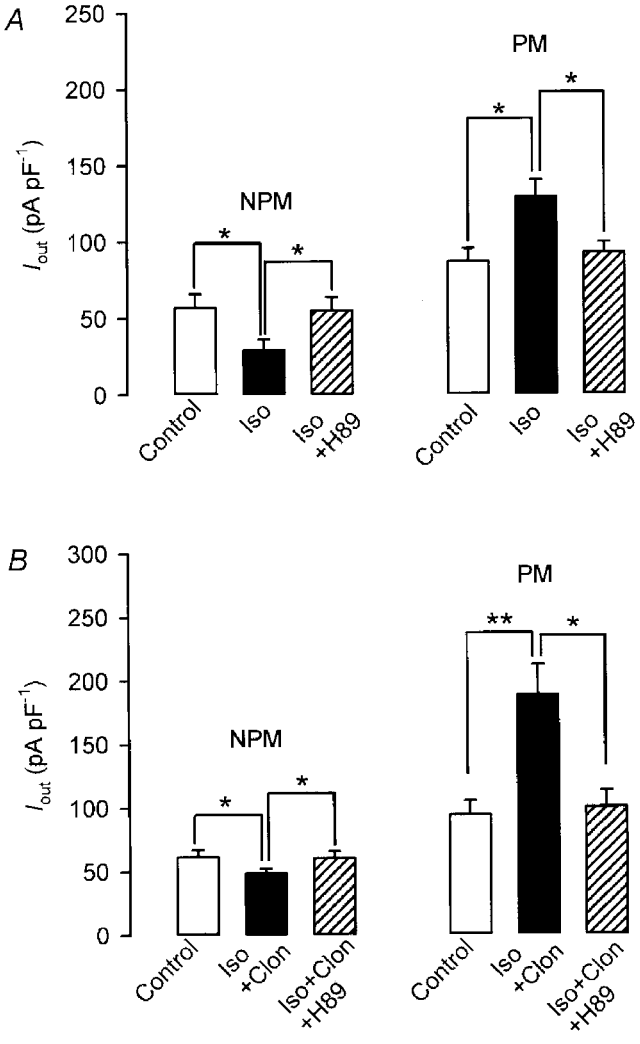

Figure 6. The PKA inhibitor H-89 inhibits the effects of isoprenaline and clonidine on I_{out}

Currents were evoked by applying a 200 ms depolarizing pulse every 10 s from a holding potential of −60 to +80 mV. After recording control currents from non-pregnant (NPM) and pregnant (PM) rat uterine myocytes, cells were superfused with 10 μM isoprenaline first and then additionally with 1 μM H-89 (*A*) or with 10 μM isoprenaline plus 10 μM clonidine first and then additionally with 1 μM H-89 (*B*). Bars represent mean current densities of six NPM and six PM cells from four rats in each group in *A* and of eight NPM and six PM cells from five and four rats, respectively, in *B*. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

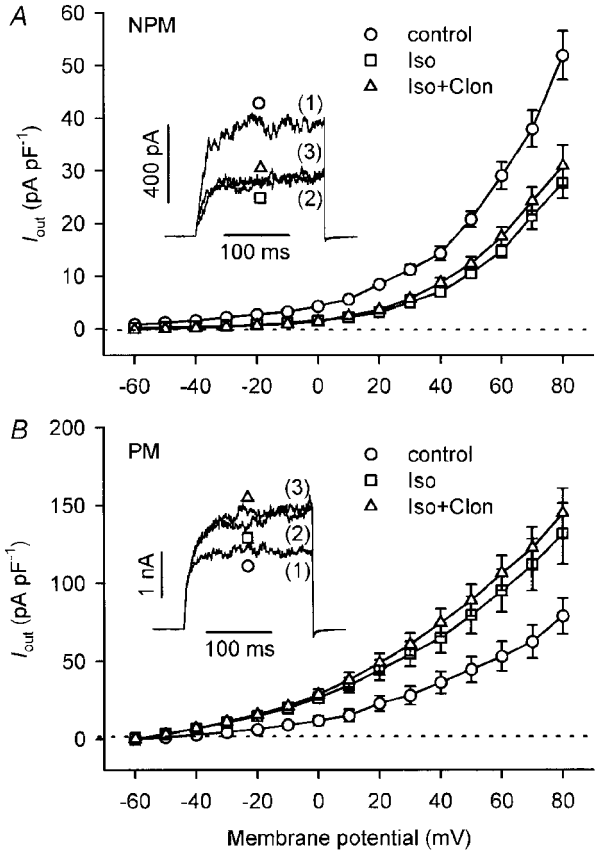

Figure 7. RGS16 abolishes the effects of clonidine on isoprenaline-modulated I_{out}

Current–voltage relations from non-pregnant (NPM; $n = 9$; *A*) and pregnant (PM; $n = 10$; *B*) rat uterine myocytes are shown. NPM and PM cells were obtained from six and seven animals, respectively. Mean current densities are plotted against the respective test potential. Currents were evoked by applying 200 ms depolarizing pulses in 10 mV steps from a holding potential of −60 mV. Cells were first dialysed via the patch-pipette for 20 min with 1 μM RGS16, thereafter superfused with 10 μM isoprenaline (Iso), followed by the additional application of 10 μM clonidine (Clon). Note that the control- and the isoprenaline-modulated I_{out} were not influenced by RGS16. The insets show representative I_{out} recordings elicited by 200 ms pulses from −60 to +80 mV. Numbers in parentheses indicate the sequence of drug applications. The pipette solution contained 0·3 μM Ca^{2+}.

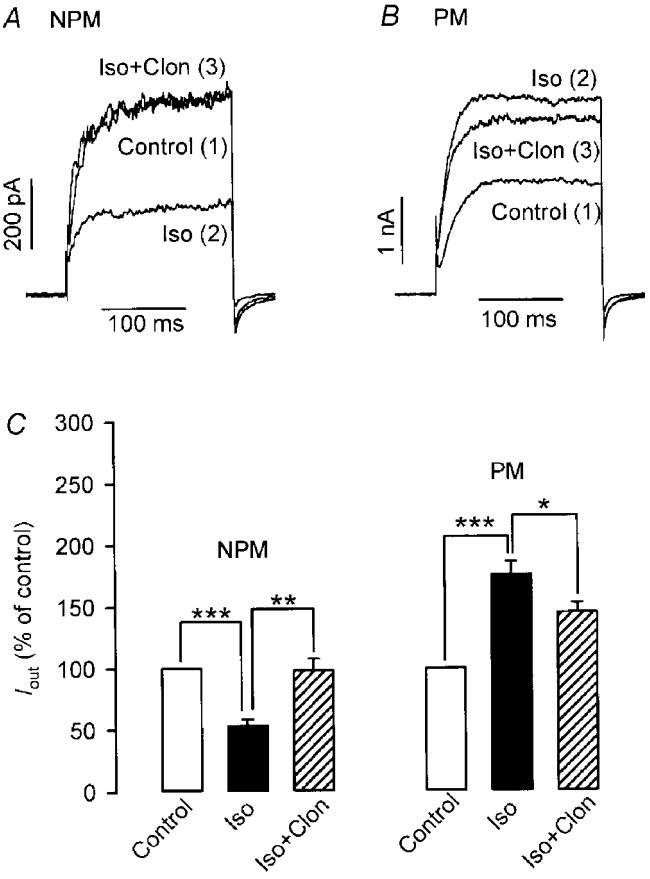

Figure 8. Transducin-α reverses the effect of clonidine on I_{out} in pregnant uterine myocytes

Currents were elicited in non-pregnant (NPM) and pregnant (PM) rat uterine myocytes every 10 s by 200 ms depolarizing pulses from −60 to +80 mV. Before drug application, cells were dialysed via the patch-pipette for 20 min with 0·3 μM transducin-α (TDα). Original current recordings showing that 10 μM clonidine (Clon) completely revoked the effect of 10 μM isoprenaline (Iso) in the TDα-loaded NPM cell (*A*) whereas the potentiating effect of clonidine was switched by TDα to an antagonistic action in the PM cell (*B*). Numbers in parentheses indicate the sequence of drug applications. *C*, average results from NPM ($n = 7$) and PM ($n = 9$) cells, obtained from five and six rats, respectively. Current densities were used to calculate the percentage values. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

Influence of TDα on the clonidine-modulated I_{out}

It is well known that both α-subunits and the βγ-dimers of G proteins regulate the activity of effectors. In order to differentiate between the two types of subunits, experiments with TDα, the α-subunit of a heterotrimeric G protein that mediates signal transduction in vertebrate photoreceptor cells, were conducted on NPM and PM cells. It was reasoned that TDα, a scavenger of βγ-dimers dissociated from heterotrimeric G_i proteins upon activation of α_2-ARs, should specifically antagonize βγ-mediated effects, whereas effects dependent on α-subunits should be preserved. The experimental design was similar to that of Fig. 7; 0·3 μM TDα was dialysed via the patch-pipette into the myocytes for 20 min before I_{out} was elicited in the absence and presence of isoprenaline and isoprenaline plus clonidine. Figure 8*A* and *B* shows the results obtained in a NPM and PM cell clamped for 200 ms from −60 to +80 mV. There were no changes in the response of both cells to isoprenaline but the clonidine effect was markedly altered in the presence of TDα. The inhibitory effect of 10 μM isoprenaline on I_{out} was completely antagonized by 10 μM clonidine in the NPM cell (before 580 pA, after isoprenaline 280 pA, plus clonidine 580 pA), whereas the stimulating effect of isoprenaline was partially reversed in the PM cell (before 1·7 nA, after isoprenaline 3·0 nA, plus clonidine 2·65 nA). Mean values obtained from seven NPM and nine PM cells are summarized in Fig. 8*C*. Note that the inhibitory effect of clonidine on I_{out} in nine PM cells exposed to isoprenaline was statistically significant ($P < 0·05$; isoprenaline 195·1 ± 23·1 % of control, plus clonidine 154·2 ± 18·3 % of control). Taken together, the results presented in Fig. 8 indicate that clonidine acts in NPM cells predominantly via $G_i\alpha$ whereas βγ-subunits of G_i proteins seem to be involved in the stimulatory effect in PM cells.

Influence of TDβγ on the isoprenaline-modulated I_{out}

Among the effector molecules regulated by G_i proteins in myometrial smooth muscle cells are different subtypes of ACs, some of which are co-stimulated by βγ-dimers of G_i proteins in the presence of stimulatory $G_s\alpha$ (induced by isoprenaline). In order to test the hypothesis that cyclases co-stimulated by βγ-dimers are specifically expressed during pregnancy, NPM and PM cells were exposed to isoprenaline and simultaneously dialysed via the patch-pipette with 1 μM transducin-βγ (TDβγ). The voltage protocol was the same as before. First, NPM and PM cells were dialysed with TDβγ and the current was recorded over 20 min (Fig. 9). Although a tendency to increase I_{out} was observed with TDβγ, it never reached statistical significance in seven cells. A second group of cells (nine NPM and PM cells, respectively) was superfused with 10 μM isoprenaline and it can be seen that the inhibitory (Fig. 9*A*) and the stimulatory effect (Fig. 9*B*) was maximal within 3 min and remained quite stable thereafter. A third group of cells (five NPM and five PM cells) was superfused with isoprenaline while the patch-pipette contained 1 μM TDβγ. Figure 9

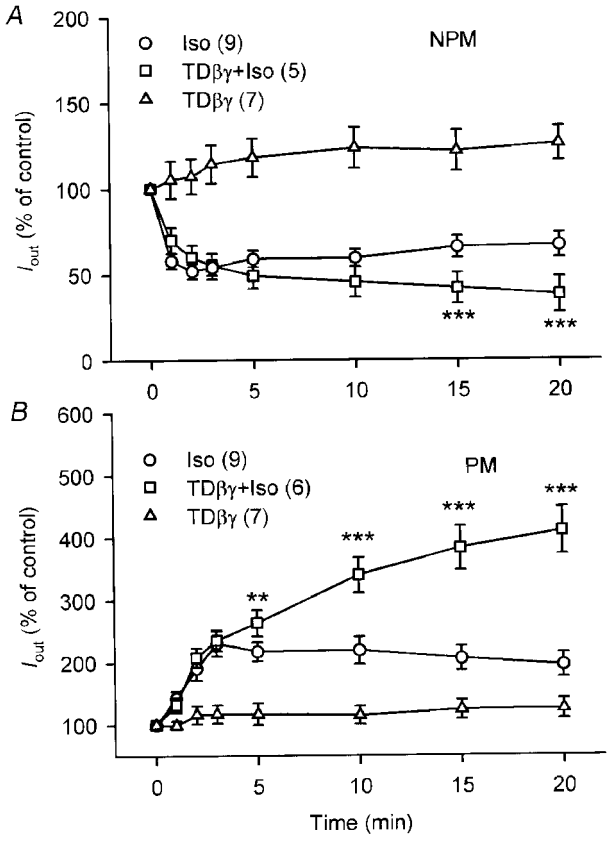

Figure 9. Transducin-βγ enhances the effects of isoprenaline on I_{out}

Currents were evoked in non-pregnant (NPM; *A*) and pregnant (PM; *B*) rat uterine myocytes by applying a 200 ms depolarizing pulse every 10 s from −60 to +80 mV. Recordings were started immediately after membrane disruption. *A* and *B*, time course of I_{out} with either 10 μM isoprenaline (Iso) in the bath, 10 μM isoprenaline in the bath and 1 μM transducin-βγ (TDβγ + Iso) in the patch-pipette, or TDβγ in the pipette but no agonist in the bath (TDβγ). Mean values from three different groups are shown in both *A* and *B*. Numbers in parentheses denote the number of cells. NPM cells were obtained from five (△), six (○) and seven rats (□), PM cells from five (△), six (○) and five rats (□). Current densities were used to calculate the percentage values. ** $P < 0·01$; *** $P < 0·001$ *versus* Iso alone.

demonstrates that the effects of 10 μM isoprenaline on I_{out} were potentiated by TD$\beta\gamma$ over a period of 20 min in both NPM and PM cells. This effect was specific, because dialysis of equimolar concentrations of TDα and TD$\beta\gamma$ (1 μM each, data not shown) did not further increase the isoprenaline-modulated I_{out}. Thus, the results suggest that both NPM and PM cells express ACs which are co-stimulated by $\beta\gamma$-dimers.

Effects of adrenaline and noradrenaline on I_{out}

The physiological ligands for α- and β-ARs are noradrenaline and adrenaline. It was therefore interesting to investigate the effects of both catecholamines on I_{out}. As shown in Fig. 10*A*, when NPM cells were depolarized for 200 ms from −60 to +80 mV, I_{out} was elicited which was unaffected by superfusing the cells with either 10 μM noradrenaline (92·0 ± 10·2% of control; $n = 7$) or adrenaline (102·5 ± 9·3% of control, $n = 6$). Subsequent addition of 10 μM yohimbine, a selective antagonist of α_2-ARs, however, disclosed the β-AR-mediated inhibitory effect of noradrenaline (52·6 ± 5·2% of control; $n = 7$) and of adrenaline (56·1 ± 5·3% of control; $n = 6$). Contrary to NPM cells, PM cells responded with a pronounced increase in I_{out} in the presence of noradrenaline (210·4 ± 20·1% of control; $n = 6$) or adrenaline (195·0 ± 19·8% of control; $n = 6$; Fig. 10*B*). Additional blockade of α_2-ARs by 10 μM yohimbine resulted in a partial decrease of the catecholamine-stimulated current to 162·4 ± 17·1% (noradrenaline) and 155·1 ± 16·3% (adrenaline) of the respective control values. The results indicate that α_2- and β-AR-mediated effects on I_{out} cancel each other in NPM cells whereas I_{out} was markedly augmented by the co-stimulatory signalling induced by the simultaneous stimulation of both adrenoceptors during pregnancy.

DISCUSSION

Myometrial smooth muscle cells are richly endowed with BK_{Ca} channels (Kihira *et al.* 1990; Anwer *et al.* 1993; Pérez *et al.* 1993; Zhou *et al.* 1998). As sensors of both voltage and intracellular Ca^{2+}, BK_{Ca} channels are responsible for membrane repolarization that follows depolarization and the accompanying increase in cytosolic free Ca^{2+} during an action potential (Parkington & Coleman, 1990; Kaczorowski *et al.* 1996). Because contraction of the myometrium is closely related to its electrical properties, which consist of characteristic spontaneous bursts of spike discharges at irregular intervals, BK_{Ca} channels are important regulators of smooth muscle contractility (Parkington & Coleman, 1990; Anwer *et al.* 1993). The membrane electrical and contractile properties of the myometrium vary markedly during the normal ovulatory cycle and pregnancy, presumably as a consequence of hormonal influences (Kao, 1989; Parkington & Coleman, 1990; Toro *et al.* 1990). For example, whereas uterine quiescence is important to maintain

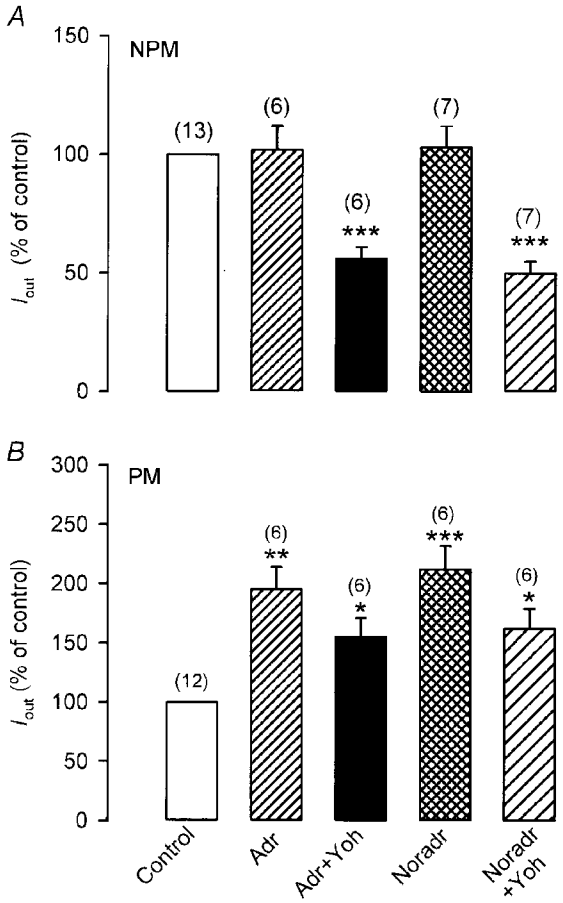

Figure 10. Adrenaline and noradrenaline enhance I_{out} only during pregnancy

Currents were evoked in non-pregnant (NPM) and pregnant (PM) rat uterine myocytes by applying a 200 ms depolarizing pulse every 10 s from −60 to +80 mV. NPM (*A*) and PM (*B*) cells were superfused with 10 μM adrenaline (Adr) or 10 μM noradrenaline (Noradr), thereafter 10 μM yohimbine (Yoh) was added. Note that yohimbine disclosed the β-AR-mediated effects of both catecholamines on I_{out}. Numbers in parentheses denote the number of cells. Cells for each group (Adr and Noradr) in *A* and *B* were obtained from five rats. Current densities were used to calculate the percentage values. The pipette solution contained 0·3 μM Ca^{2+}. * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$ *versus* control.

pregnancy, contractility is gradually activated before parturition giving rise to powerful rhythmic contractions culminating in the delivery of the fetus. Evidence has been presented that BK_{Ca} channels contribute to the myometrial tone at the different stages of pregnancy by changes in their density, voltage activation and Ca^{2+} sensitivity (Khan *et al.* 1993; Wang *et al.* 1998; Song *et al.* 1999). BK_{Ca} channels are important targets for regulation by cAMP- and cGMP-dependent protein kinases (Alioua *et al.* 1998; Nara *et al.* 1998; Zhou *et al.* 1998). Recently it has been reported that BK_{Ca} channels are differentially regulated by cAMP- and cGMP kinase in pregnant and non-pregnant myometrial cells (Pérez & Toro, 1994; Zhou *et al.* 1999). The present study confirms these observations with cAMP kinase by showing inhibition and activation of BK_{Ca} channel activity in inside-out patches from non-pregnant and pregnant rat myometrial cells, respectively. The mechanism by which BK_{Ca} channels are differentially regulated by cAMP- and cGMP kinase is presently unknown but may involve expression of isoforms of BK_{Ca} channels, different heteropolymeric assembly of channel subunits and/or expression of associated proteins with modulatory function. β-AR agonists are widely used for prevention of premature labour due to their relaxing effects on the myometrium. They are thought to act via a cascade that involves stimulatory G proteins (G_s), AC, cAMP and finally PKA activation. Myometrial relaxation is then induced by pleiotropic mechanisms including activation of BK_{Ca} channels (Anwer *et al.* 1992; Hamada *et al.* 1994). The present study shows that the β-AR agonist isoprenaline mimics the effects of PKA by augmenting I_{out} in pregnant and inhibiting the current in non-pregnant myometrial cells.

Besides β-ARs, transcripts of α_{2A}- and α_{2B}-ARs have been identified in rat myometria (Mhaouty *et al.* 1995; Bouet-Alard *et al.* 1997). It is well established that myometrial α_2-ARs are functionally linked to inhibition of AC through activation of G_i proteins (Wu *et al.* 1988; Breuiller *et al.* 1990). In accordance with this signalling pathway, clonidine, a ligand with high affinity for the rat myometrial α_2-AR (Mhaouty *et al.* 1995), partially reversed the isoprenaline effect on I_{out} in myocytes from non-pregnant myometrium. Conversely, clonidine potentiated the isoprenaline-stimulated I_{out} in cells from pregnant myometria. The latter finding corresponds with the observation that clonidine (10 μM) enhanced the effect of isoprenaline on AC activity in the rat pregnant myometrium (Mhaouty *et al.* 1995). Thus, the electrophysiological data closely resemble those of AC activity in membrane preparations. Contrary to single channel data, whole-cell I_{out} of myometrial cells is composed of various currents (Toro *et al.* 1990; Wang *et al.* 1998). Experiments with IbTX, a specific and potent blocker of BK_{Ca} channels (Galvez *et al.* 1990), disclosed that about 75–80 % of I_{out} was conducted by BK_{Ca} channels in cells from pregnant and non-pregnant myometria. This relatively high contribution of $I_{K(Ca)}$ to I_{out} differs from that reported earlier in pregnant rat myometrial cells (Wang *et al.* 1998). The difference may be due to the higher IbTX concentration used in the present study (100 nM instead of 1 nM) and to the fact that we analysed only cells displaying non-inactivating outward currents. Because isoprenaline did not significantly change the residual current, IbTX-sensitive $I_{K(Ca)}$ was the only current component sensitive to the cAMP–PKA cascade, at least under the present experimental conditions. Furthermore, the abolition of all effects on I_{out} produced by isoprenaline or isoprenaline plus clonidine by H-89, a specific inhibitor of PKA (Hidaka & Kobayashi, 1992), clearly demonstrates the causal relation between kinase activation and channel activity. As a result, $I_{K(Ca)}$ seems to be an efficient tool to monitor the intracellular cAMP–PKA cascade in myometrial cells. In contrast to data reported from rat, no stimulatory effect of isoprenaline on AC activity could be detected in membranes from human pregnant or non-pregnant myometria (Gsell *et al.* 2000). These data were explained by the low β-AR density in human as compared with rat myometrium (Cohen-Tannoudji *et al.* 1991; Engelhardt *et al.* 1997; Gsell *et al.* 2000). Contrary to AC activity, I_{out} recorded in cells from human myometrium responded to isoprenaline, although on a smaller scale, in exactly the same way as in rat cells, indicating that the electrophysiological approach is more sensitive in detecting subtle changes in cAMP–PKA signalling than the AC assay in isolated membranes. It is interesting to note that because of its high Ca^{2+} sensitivity, BK_{Ca} channel activity has been used successfully to monitor $[Ca^{2+}]_i$ in the subsarcolemmal space of arterial smooth muscle cells (Ganitkevich & Isenberg, 1996), and in several types of non-excitable cells (for review see Marty, 1989).

Positive and negative signal integration by clonidine in the presence of isoprenaline could be due to the expression of different AC isoforms in the pregnant and non-pregnant myometrium. Nine different types of ACs that differ in their regulatory mechanism and their tissue-specific distribution have been described so far (Sunahara *et al.* 1996). Among the seven ACs identified in the rat myometrium (Mhaouty-Kodja *et al.* 1997), types II and IV share the property of being stimulated by the $\beta\gamma$-subunits of G_i/G_o inhibitory proteins in the presence of activated $G_s\alpha$ (Tang & Gilman, 1991; Gao & Gilman, 1991; Federman *et al.* 1992). The demonstration that both the negative and the positive input of clonidine to I_{out} was completely abolished in cells loaded with RGS16 or treated with PTX, clearly shows that G_i/G_o proteins were involved in both signal transduction pathways (see Fig. 11). RGS16 is a GTPase-activating protein that preferentially acts on members of the $G_i\alpha$-subfamily and converts the active GTP-bound α-subunit to the inactive GDP-bound form, which then reassembles with the $\beta\gamma$-dimer (for review see Wieland & Chen, 1999), whereas PTX uncouples G_i proteins from the receptor. Apparently, when the concentration of RGS16 is sufficiently high in the cell, G_i-/G_o-mediated effects can be completely blocked, whereas the $G_s\alpha$-mediated effect of isoprenaline on I_{out} remained unchanged. In order to test which G protein subunit was responsible for the clonidine effects, we took advantage of TDα which acts as a scavenger of $\beta\gamma$-dimers of G_i/G_o

proteins (Federman *et al.* 1992). When $\beta\gamma$-dimers were released by α_2-AR activation in non-pregnant myometrial cells loaded with TDα and simultaneously superfused with isoprenaline, I_{out} was completely reversed (Fig. 8*A*) as compared with the partial recovery seen in the absence of TDα (Fig. 2*A*). This result is compatible with the view that recovery of I_{out} was due to unopposed $G_i\alpha$ because the pool of free $\beta\gamma$-dimers available for reassembling with $G_i\alpha$ protein was strongly reduced by TDα and was also not available for co-stimulation of AC isoforms. However, when the same experiment was repeated in myocytes from pregnant myometrium, clonidine produced a significant inhibition rather than a potentiation of the isoprenaline-stimulated I_{out} (Fig. 8*B*). Consequently, $G_i\beta\gamma$ must have been responsible for the synergistic effect of clonidine and isoprenaline on I_{out} when TDα was absent (Fig. 2*B*). The α_2-AR-mediated decrease of I_{out}, however, reflects very probably the unopposed inhibitory effect of free $G_i\alpha$ subunits in the presence of TDα. Thus, stimulatory and inhibitory signal transduction pathways coexist in the pregnant myometrial cell and the resulting effect depends on the prevalence of either subunit. In accordance with this view, TD$\beta\gamma$, when applied in high concentrations to the interior of myocytes, acted synergistically with isoprenaline irrespective of whether the cells were from pregnant or non-pregnant myometria. Again this finding supports the concept of opposing roles of $G_i\alpha$ and $G_i\beta\gamma$ on AC activity and is contradictory to the idea that $\beta\gamma$-stimulated AC isoforms are exclusively expressed in pregnant myometrium. We cannot exclude, however, that the amount of $\beta\gamma$-sensitive AC isoforms might be upregulated during pregnancy. Another possible explanation is a more efficient coupling between α_2-ARs and G_i/G_o, thus $\beta\gamma$-dimers at high levels may simply override the inhibitory effect of $G_i\alpha$. This interpretation is supported by an earlier report demonstrating an increase of the expression of $G_i\alpha_2$ and $G_i\beta\gamma$ in rat myometria at midpregnancy (Tanfin *et al.* 1991).

Adrenaline and noradrenaline are the physiological ligands of β- and α_2-ARs. As expected from the inhibitory coupling of the α_2-AR to AC in the non-pregnant myometrium, the β-AR-mediated reduction of I_{out} was completely prevented by either catecholamine. In the pregnant myometrium, however, where the signalling pathways of both ARs act synergistically, a strong increase in I_{out} was observed,

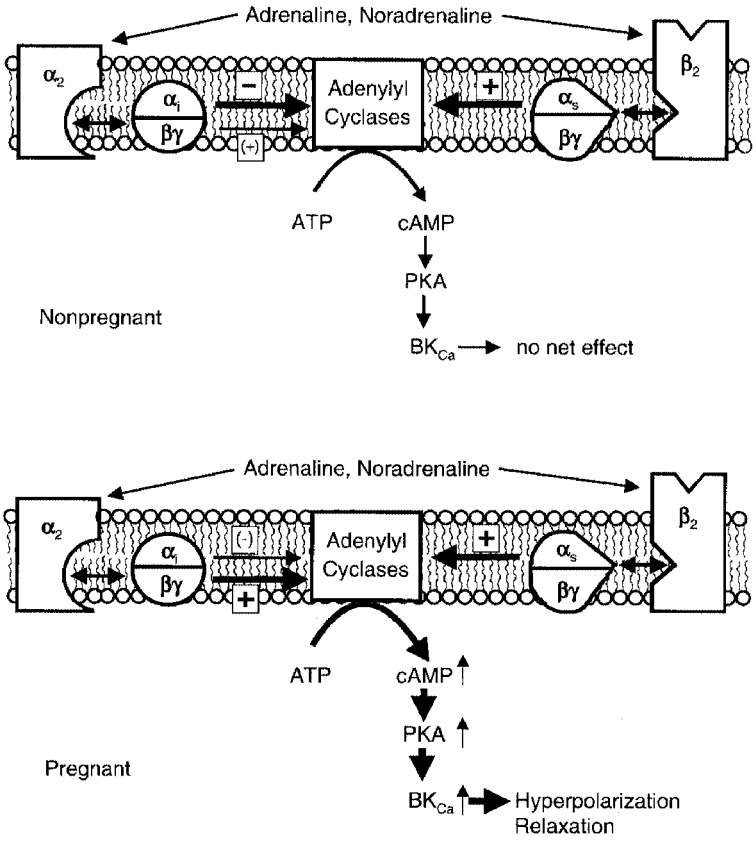

Figure 11. Scheme illustrating bivalent regulation of cAMP synthesis by G proteins

Shown is the α_2- and β-AR coupling to AC in cells of the non-pregnant (upper panel) and pregnant myometrium (lower panel). Catecholamines are the physiological AR ligands which act simultaneously through receptors coupled to stimulatory (s) and inhibitory (i) G proteins. The thickness and position of arrows between G protein subunits and AC indicate the predominant effect and the subunit involved in the respective signal transduction pathway. Note that the G proteins must bind GTP and dissociate before they become active.

exceeding that of β-AR activation alone. The female reproductive tract has a rich adrenergic innervation (Marshall, 1981) and it is therefore conceivable that the two types of regulation are physiologically meaningful, the latter by reinforcing uterine quiescence during pregnancy, the former by rendering the myometrium insensitive to the β/α_2-AR pathway in the non-pregnant state. Figure 11 summarizes the results obtained with catecholamines and in addition indicates which subunit of the G_i protein dominates in the non-pregnant and pregnant state. There are many ways by which the cAMP–PKA signalling cascade regulates myometrial tone (Wray, 1993). It should be kept in mind, however, that BK_{Ca} channels are negative feedback regulators of the membrane potential and thus play an important role in this process. The efficacy of β-AR agonists in suppressing premature labour appears to be well established. The present data suggest that their tocolytic effectiveness may be improved by the simultaneous application of α_2-AR agonists which are able to increase the level of co-stimulatory $\beta\gamma$-dimers of G_i/G_o.

In summary, the present data establish that registration of BK_{Ca} channel activity can be used as a reliable probe to monitor cAMP–PKA signalling in myometrial smooth muscle cells. The results demonstrate that the opposing effects of β- and α_2-AR agonists on BK_{Ca} channel activity in the non-pregnant myometrium switch to a synergistic action during pregnancy. This switch is probably not due to an exclusive expression of $\beta\gamma$-stimulated AC isoforms but might result from a more efficient coupling between α_2-AR and inhibitory G proteins.

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