Expression of G-protein-coupled receptor kinases in pregnant term and non-pregnant human myometrium

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Abstract

There is evidence for hormonal receptor desensitisation in human myometrium, but little is known about the mechanisms involved in the loss of myometrial response to agonists such as β_2 -adrenergic agonists, prostaglandin γ and oxytocin. It is well known that the receptors for these hormones are coupled to G-proteins. The first step of receptor desensitisation is the phosphorylation of activated receptors by a G-protein-coupled receptor kinase (GRK). GRKs are members of a multigene family and the various subtypes differ in their localisation, regulation and mode of action. We have used Western blotting and reverse transcription PCR to identify the GRKs present in human myometrium from pregnant and non-pregnant women as well as in cultured human myometrial cells. We have

found that human myometrium expresses the GRK subtypes 2, 4 γ , 5 and 6. On the other hand, GRK3 and the isoforms GRK4 α , β and δ were not found in myometrial tissue. Our data indicate that GRK2 is only expressed in pregnant term myometrium and is not found in non-pregnant tissue. Moreover, GRK6 appears to be expressed at a much higher level in pregnant term tissue than in non-pregnant myometrium. Our observations suggest that GRK2 and GRK6 may contribute to the regulation of uterine contractility at term. Further work is necessary to determine whether GRKs and receptor desensitisation play a role in disorders of uterine contractility.

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Introduction

Uterine contractility is regulated during pregnancy and labour by a variety of hormones, neurotransmitters and prostanoids which bind to specific receptors on the plasma membrane of myometrial cells. The activated receptors interact with regulatory G-proteins which stimulate (or inhibit) effector enzymes such as adenylyl cyclase or phospholipase C (PLC) that produce second messengers (e.g. cAMP, inositol trisphosphate). The cytosolic level of these messengers determines the degree of activation of the contractile machinery. Studies on a variety of G-protein-coupled receptors indicate that their repeated or prolonged stimulation results in loss of hormonal responsiveness (Liggett & Lefkowitz 1994). This phenomenon, termed desensitisation or tachyphylaxis, is a complex physiological process which prevents hyperstimulation by impairing signal transmission during prolonged receptor activation.

There is evidence for receptor desensitisation in the myometrium but little is known about the mechanisms involved in the loss of myometrial response to a variety of agonists. For example, β_2 -adrenergic agonist treatment during pregnancy provokes the loss of β_2 -adrenergic receptors, which leads to decreased cAMP levels (Berg

et al. 1985), and reduces the effectiveness of tocolytic therapy (King *et al.* 1988). Other myometrial receptors have been shown to undergo desensitisation. Treatment of rat myometrium with endothelin results in homologous desensitisation of the ET-1 receptor (Do Khac *et al.* 1994). This is characterised by a reduction of the inositol phosphate response and attenuation of the inhibitory effect on cAMP production triggered by endothelin (Do Khac *et al.* 1994). Angiotensin II AT2 receptors are also downregulated in human myometrium during pregnancy (de Gasparo *et al.* 1994). Moreover, long-term treatment of cultured human myometrial cells with oxytocin (OT) leads to homologous desensitisation characterised by a decrease in OT-stimulated activation of the PLC/calcium pathway, a substantial loss of OT-binding sites and a severe reduction of the mRNA encoding the OT receptor (OTR) (Phaneuf *et al.* 1994, 1997). Recent work in our laboratory also indicates that OTRs are desensitised *in vivo* during the progress of spontaneous and induced labour at term (S Phaneuf, B Rodríguez-Liñares, R TambyRaja, I Z MacKenzie & A López Bernal, unpublished observations).

Studies on a variety of hormonal receptors indicate that the first step of receptor desensitisation is the phosphorylation of activated receptors by a G-protein-coupled

receptor kinase (GRK) (Liggett & Lefkowitz 1994). GRKs are serine/threonine protein kinases which phosphorylate specific residues in the third intracellular loop and C-terminal cytoplasmic tail of the activated receptors. Six distinct mammalian GRKs are known and are grouped into three families based on sequence and functional similarities: (i) rhodopsin kinase (GRK1), (ii) the GRK2 family (β -adrenergic receptor kinase or β ARK) comprising GRK2 and GRK3, and (iii) the GRK4 subfamily comprising GRK4 (originally named IT-11), GRK5 and GRK6 (Inglese *et al.* 1993, Premont *et al.* 1995). To date only GRK2 and GRK3 have been described in rat myometrium (Ruzycky & DeLoia 1997).

At present there is no information on the presence of GRKs in human myometrium, and since this group of enzymes is potentially very important for receptor desensitisation in the regulation of myometrial contractility, we have undertaken this study with the aim of identifying the GRKs present in this tissue. We have detected several GRKs in human myometrial tissue from pregnant and non-pregnant women as well as in cultured human myometrial cells using Western blotting and reverse transcription PCR (RT-PCR). Important differences in the level of expression of GRK2 and GRK6 were observed between non-pregnant and pregnant myometrium.

Materials and Methods

Materials

Materials used in this study were purchased from the following sources. The enhanced chemiluminescence (ECL) detection kit, peroxidase-conjugated goat anti-rabbit antibody, 32 P-dideoxy nucleotides and thermosequenase were from Amersham International plc (Amersham, Bucks, UK). The polyvinylidene difluoride (PVDF) membranes were from BioRad (Hemel Hempstead, Herts, UK). Ampli Taq was from Perkin Elmer-Applied Biosystems (Cambridge, Cambs, UK) and the RT kit was from R & D System Europe (Abingdon, Oxon, UK). Anti-GRKs polyclonal antibodies GRK2-C-15, GRK3-C-14, GRK4-K-20, GRK4-I-20, GRK5-C-20 and GRK6-C-20 and their respective cognate peptides were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Tissue culture reagents were from Life Technologies (Inchinnan, UK). All other reagents were commercial preparations of the highest available purity.

Tissue collection and preparation

Pregnant term (38–39 weeks of gestation) human myometrium was taken from the upper border of the uterine incision during elective lower-segment cesarean sections performed for previous cesarean section or cephalopelvic

disproportion. Samples of myometrium from non-pregnant premenopausal women were obtained at hysterectomy performed for benign gynaecological disorders such as menorrhagia or dysmenorrhoea. The uteri were excised longitudinally and samples were taken from the middle of the uterine wall about 5 mm from the endometrial and serosal surfaces. This investigation had the approval of the Central Oxfordshire Research Ethics Committee and all patients gave informed consent.

For the immunodetection studies, two pools of myometrial tissues were prepared. One included specimens from 12 individual pregnant patients and another included five individual non-pregnant samples. The samples were snap frozen in liquid nitrogen and stored at -70°C until required (<1 month). The tissues in each pool were homogenised on ice in the presence of 0.25 mM sucrose, 25 mM Tris and 1 mM EDTA, pH 7.6, using a Polytron (UltraTurrax) homogeniser at full speed for three 30 s bursts. The homogenates were then centrifuged at 600 g at 4°C and the resulting supernatants were diluted with 25 mM Tris, pH 6.8 to give a protein concentration of 5 mg/ml (Bradford 1976), then mixed 1:1 with SDS sample buffer (8 M urea, 5% SDS, 8% dithiothreitol and 50 mM Tris pH 6.8). A sample of human brain was taken from a post-mortem specimen and homogenised and stored as above. For the RT-PCR assay, RNA was isolated from three separate myometrial samples obtained respectively from pregnant and non-pregnant patients and from two separate myometrial cell cultures and was assayed independently.

Cell dispersion and culture

Fresh non-pregnant myometrium was dispersed enzymatically and the myocytes were cultured in Waymouth MB 752/1 medium containing 10% (v/v) fetal calf serum, 100 IU penicillin/ml and 100 mg streptomycin/ml as described in detail previously (Phaneuf *et al.* 1993). When confluent, the myocytes were harvested with trypsin-EDTA at 37°C and washed twice with Hanks' balanced salts solution (HBSS) at 600 g. The resulting pellet was resuspended in 400 ml SDS sample buffer and passed through a 25 gauge needle to disrupt the cells (Phaneuf *et al.* 1996). The samples were snap frozen in liquid nitrogen and stored at -70°C until required (<1 month).

Immunodetection

Protein samples (50 μg) in SDS sample buffer were heated at 95°C for 15 min before PAGE, which was carried out according to Laemmli (1970), with polyacrylamide concentrations of either 7.5 or 10%. Proteins were then transferred onto PVDF membranes. Immunodetection of GRKs was carried out with polyclonal antibodies diluted 1:2000 in Tris-buffered saline containing 0.1% Tween-20, using 3% skimmed milk as blocking agent. GRKs

Table 1 GRK antisera used in this study

	Peptide sequence	Organism of origin	Epitope position
Antibody			
GRK2	LSKVPLVQRGSANGL	Human	675–689
GRK3	LSKPPLCHRNSNGL	Bovine*	675–688
GRK4-I	IPWQNEDCLTMVPSEKEVEP	Human	478–497
GRK4-K	CTILSKMPKPKPPNPSEGEQ	Human	527–546
GRK5	TSFNHHNSNHVSSNSTGSS	Human	571–590
GRK6	SRQDCCGNCSDSEELPTRL	Human	525–544

*Cross-reacts with the human antigen.

antibodies, listed in Table 1, were revealed with a peroxidase-conjugated goat anti-rabbit antibody (diluted 1:1000). The ECL detection was carried out according to the technical notes provided by the manufacturer, using Kodak X-Omat XAR film (exposure time between 30 s and 5 min).

RNA isolation and RT-PCR assay

Total RNA was prepared from individual tissue specimens or cultured cells by the guanidinium isothiocyanate/CsCl method (Chirgwin *et al.* 1979). Briefly, cells grown in 175 cm² tissue culture flasks were washed twice with HBSS, then 3.5 ml of guanidinium isothiocyanate solution (4 M guanidinium isothiocyanate, 0.5% sodium N-lauryl sarcosine (w/v), 25 mM sodium citrate, pH 7.0, 1% β -mercaptoethanol) were added to each flask. The extract was then passed through a 25 gauge needle five times, applied onto 8 ml of 5.7 M CsCl and centrifuged at 175 000 *g* for 20 h at 20 °C. Tissue specimens (300–600 mg) were homogenised separately in 3.5 ml guanidinium isothiocyanate solution with a Polytron (UltraTurrax) homogeniser at full speed for three 30 s bursts before being applied onto the CsCl gradient. RNA quality was assessed by running the samples on 1% agarose gels containing ethidium bromide. Visual examination of the ribosomal RNA bands under UV illumination did not show any differences between the samples used (data not shown).

RT-PCR was carried out as follows. Oligo dT primers (500 ng) were annealed to 2 μ g total RNA at 70 °C for 10 min, and cDNA synthesis was carried out at 37 °C with 100 U Moloney murine leukaemia virus reverse

transcriptase. The cDNA was then amplified with GRK-specific forward and reverse primers listed in Table 2. The PCR reactions were 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 64 °C for 1 min and 72 °C for 3 min, with a final elongation step at 72 °C for 5 min, except for GRK2 and GRK4AD for which the annealing temperature were 37 and 60 °C respectively. The specificity of the PCR amplification was confirmed by direct sequencing of the PCR products. Each of the GRK-derived products had 100% homology with respect to the published GRK sequences (data not shown). The primers were shown not to amplify a product from genomic DNA. Myometrial RNA from the three pregnant and non-pregnant specimens and from two myometrial cell cultures was assayed three times. Samples were loaded on 2% agarose gels containing ethidium bromide, and photographed under UV illumination. As additional controls, glucocerobrosidase and OTR cDNA fragments were amplified in the same experiments and run on the same gels (Phaneuf *et al.* 1997, Rodríguez-Liñares *et al.* 1998).

Results

Immunodetection of GRKs

Identification of the GRKs present in myometrial tissue and cultured cells was carried out by immunodetection using specific antisera raised against known GRKs sequences. Immunoblots demonstrating the presence of various GRKs are shown in Figs 1–4.

The anti-GRK2 antibody detected a major band at approximately 65 kDa in pregnant term myometrium and

Table 2 Primers used for the detection of GRKs

	Forward primer	Reverse primer	Size of amplicon (bp)
GRK			
GRK2	TCTCGCTCGTCAGCACTG	GAACCAGTCGGCACTGCT	398
GRK4AD	ATGGAGCTCGAGAACATCGT	TTTGTTACGGGTTCCTTTC	551
GRK5	CACCGGAAGCAGCTAGTTTC	TTTTTTGATAACCCCTCCC	492
GRK6	CCTCGAGCGTGAATATCACA	CGGTATTGCCTGAAGGTGTT	426

Figure 1 Immunodetection of GRK2 in myometrial tissue and cultured cells. Proteins were resolved by electrophoresis on a 7.5% polyacrylamide gel and GRK2 revealed by Western blotting and ECL detection. C, cultured myometrial cells; P, pregnant term myometrium; NP, non-pregnant myometrium. The position of the molecular mass standards is indicated on the left (kDa). The arrow indicates the GRK2 band.

in cultured myometrial cells (Fig. 1). In contrast, the antibody did not detect any immunoreactivity for GRK2 in non-pregnant myometrium (Fig. 1). Preabsorbing anti-GRK2 antibody with its cognate peptide resulted in the loss of the bands in myometrial tissue and cultured cells (data not shown).

The anti-GRK3 antibody did not detect reactivity in any of the samples analysed (data not shown). It therefore appears that GRK3 is not expressed in pregnant or non-pregnant myometrium, nor in cultured myometrial cells. GRK4 exists as four splice variants, α , β , γ and δ (Premont *et al.* 1996). They share a high level of homology and commercial antibodies available at present only allow the detection of either the α or β isoforms (antibody GRK4-K-20) and γ or δ isoforms (antibody GRK4-I-20). When used on myometrial samples, the antibody GRK4-K-20 did not detect any reactivity while the antibody GRK4-I-20 detected two bands in each of the samples tested, pregnant term and non-pregnant myometrium and myometrial cells in culture (Fig. 2). Both bands (61 and 40 kDa) disappeared when the antibody was first pre-absorbed with its cognate peptide, indicating that both polypeptides are immunologically related to GRK4 γ and/or δ isoforms. The 40 kDa band might represent a degradation product from either (or both) GRK4 γ or δ isoforms since their expected molecular sizes are 61 and 58 kDa respectively.

GRK5 is a 68 kDa protein and the GRK5-C-20 antibody detected a polypeptide of approximately

Figure 2 Immunodetection of GRK4 in myometrial tissue and cultured cells. Proteins were resolved by electrophoresis on a 10% polyacrylamide gel and GRK4 γ and δ revealed by Western blotting and ECL detection. C, cultured myometrial cells; P, pregnant term myometrium; NP, non-pregnant myometrium. The position of the molecular mass standards is indicated on the left (kDa). The arrows indicate the GRK4 bands.

75 kDa in the three sets of samples used in this study (Fig. 3). We have also found a similar molecular form in brain tissue (Fig. 3, lane B), which is known to express GRK5 (Kunapuli & Benovic 1993). Preabsorbing anti-GRK5 antibody with its cognate peptide resulted in the loss of the protein band in both myometrial tissues, brain and cultured myometrial cells (data not shown).

Using a GRK6-specific antibody, we detected the presence of GRK6 in myometrial tissues and in cultured cells (Fig. 4). GRK6 migrated as two major molecular forms, 57 and 42 kDa. Both bands were lost when the antibody was first incubated with its cognate peptide (not shown). The level of detection of GRK6 was significantly higher in pregnant term myometrium when compared with non-pregnant tissue. Again, brain tissue was used as positive control for GRK6 and only the 42 kDa species was found (Fig. 4).

Detection of GRKs by RT-PCR

RT-PCR was used to detect the presence of mRNA encoding the various GRKs. Samples from pregnant term myometrium, non-pregnant myometrium and myometrial cells in culture were tested with specific primers (Table 2). Figure 5 shows that pregnant myometrium express mRNA for GRK2, GRK4, GRK5 and GRK6. Identical results were obtained using mRNA from non-pregnant myometrial samples and myometrial cells in culture (data

Figure 3 Immunodetection of GRK5 in myometrial tissue and cultured cells and brain. Proteins were resolved by electrophoresis on a 10% polyacrylamide gel and GRK5 revealed by Western blotting and ECL detection. C, cultured myometrial cells; P, pregnant term myometrium; NP, non-pregnant myometrium; B, brain. The position of the molecular mass standards is indicated on the left (kDa). The arrow indicates the GRK5 band.

not shown). Primers were also designed to differentiate between the four GRK4 splice variants. Since the Western blotting experiment indicated that either or both the γ and δ isoforms are present in myometrial tissues, but the α and β isoforms are absent, we used primers

GRK4AD (Table 2) that would amplify a 455 bp product for the isoform δ and a 551 bp product for isoform γ . Figure 5 shows that only the 551 bp product was obtained, indicating the presence of the GRK4 γ isoform, but not of the δ isoform, in human myometrium.

Figure 4 Immunodetection of GRK6 in myometrial tissue and cultured cells and brain. Proteins were resolved by electrophoresis on a 10% polyacrylamide gel and GRK6 revealed by Western blotting and ECL detection. C, cultured myometrial cells; P, pregnant term myometrium; NP, non-pregnant myometrium; B, brain. The position of the molecular mass standards is indicated on the left (kDa). The arrows indicate the GRK6 bands.

Figure 5 Detection of GRKs by RT-PCR in pregnant term myometrium. Total myometrial RNA was reverse transcribed using oligo dT primers and the resulting cDNA was amplified by PCR with the GRK subtype-specific primers shown in Table 2. The samples were run on a 2% agarose gel and stained with ethidium bromide. The gel is representative of experiments carried out on two other pregnant and three non-pregnant myometrial specimens. The GRK subtypes are indicated by their number. B, blank (PCR of reverse-transcribed product containing no RNA); G, glucocerebrosidase control giving a band of 572 bp; OTR, control giving a band of 733 bp. The position of DNA standards is indicated on the left (bp).

For each of the primer sets used, the specificity of PCR amplification was confirmed by sequencing the purified PCR bands. Each of the GRK-derived products had 100% homology with respect to the published GRK sequences (data not shown).

Discussion

This is the first description of GRK isoforms in human myometrium. Our results demonstrate that human myometrium expresses GRK2, GRK4 γ , GRK5 and GRK6. On the other hand, GRK3 and the isoforms GRK4 α , β and δ were not found in myometrial tissue. The expression of GRK1 was not investigated because this isoform is known to be almost exclusively expressed in retina (rod and cone photoreceptor cells) and in low level in the pituitary gland (Premont *et al.* 1995).

By modulating the activity of several hormonal receptors, including β -adrenergic and prostaglandin receptors and OTRs, GRKs may play an important role in the regulation of myometrial contractility during pregnancy and during the course of labour. The formation of second messengers activated by receptors coupled to G-protein transduction systems is likely to be regulated via the phosphorylation of the receptors by GRKs, causing homologous desensitisation. Studies on β -adrenergic and muscarinic receptors indicate that phosphorylated

receptors are a target for an arrestin protein which uncouples the receptor from its G-protein, terminating signalling, and reducing the levels of functional receptors in the cell membrane. The next event is the internalisation of the receptors in intracellular vesicles. If sequestered in endosomes, the receptors may be dephosphorylated and recycled to the plasma membrane, whereas if internalised into the lysosomal compartment, they are degraded (Koenig & Edwardson 1997). It is important to note that phosphorylation of receptors by GRKs does not *per se* affect their signalling properties; however, it increases their affinity for arrestins. Only GRK-mediated phosphorylation of receptors enhances the affinity for arrestins. In contrast, phosphorylation by cAMP-dependent protein kinase A or protein kinase C has no effect on the affinity or activity of arrestins (Pitcher *et al.* 1992).

An important observation from our data is that GRK2 is only expressed in human pregnant term myometrium and is not found in non-pregnant tissue. It is interesting to note that mRNA encoding GRK2 has been found in rat myometrium and its expression increases in pregnancy to reach a maximal level 1 day post-partum (Ruzycky & DeLoia 1997). It has been shown recently that agonist occupancy of G-protein-coupled receptors promotes the association of GRK2 with tubulin and tubulin phosphorylation, which suggests a role for GRK2 in the modulation of cytoskeletal functions (Pitcher *et al.* 1998). On the other hand, our data indicate that GRK6 appears to be expressed at a much higher level in pregnant term tissue than in non-pregnant myometrium. Our observations suggest that GRK2 and GRK6 contribute to the regulation of uterine contractility at term. The specific phosphorylation sites for each GRK are not well identified but GRK2 phosphorylates three serine residues in the C-terminal cytoplasmic tail of rhodopsin (serines 334, 338 and 344) and in β_2 -adrenergic receptors (Liggett & Lefkowitz 1994). It is interesting to note that in its C-terminal tail, the OTR has three serines homologous to that of rhodopsin (Kimura *et al.* 1992, Inglese *et al.* 1993, Inoue *et al.* 1994). Moreover, OTR has two threonine and three serine residues in the third intracellular loop which are also potential targets for GRK-mediated phosphorylation. These residues may well be involved in the desensitisation process that we have described for the OTR *in vitro* (Phaneuf *et al.* 1994, 1997) and *in vivo* (S Phaneuf, B Rodriguez-Liñares, R TambyRaja, I Z MacKenzie & A López Bernal, unpublished observations).

The detection of GRK4 isoform in human myometrium, namely the γ isoform, was unexpected. Indeed, GRK4 is expressed in significant levels only in testes (Sallese *et al.* 1997). The alternatively splice forms of GRK4 differ in their domains implicated in receptor recognition (N-terminal) and membrane targeting (C-terminal) (Premont *et al.* 1995).

While GRKs are known to phosphorylate activated receptors, GRK5 exhibits a higher basal phosphorylating

activity than all the other GRKs and it autophosphorylates (Kunapuli *et al.* 1994). These properties may indicate a less stringent specificity for activated receptors and may allow GRK5 to desensitize receptors in the absence of hormonal activation. If GRK5 is expressed in myometrial tissue before term, the possibility exists that it may desensitize the β -adrenergic receptors which are important in maintaining uterine quiescence. Such events may lead to premature labour. Similarly, inactivation of stimulatory receptors such as OTR by GRK5 during the course of labour may lead to failure of labour to progress. It will therefore be important to study the expression of myometrial GRK5 at different gestational age and during the course of labour. Phosphorylation of receptors by GRKs is only part of the desensitisation process; as indicated above, it is the binding of arrestin proteins to the phosphorylated receptor that triggers receptor internalisation. We believe that study of the expression of arrestins in myometrial tissue is of paramount importance if one wants to understand how desensitisation of receptors influences the onset of labour or its slow progression. It is also imperative to determine the time course of receptor phosphorylation to explain the slow desensitisation process observed for OTR (Phaneuf *et al.* 1997).

Further work is necessary to determine whether GRKs and receptor desensitisation play a role in disorders of uterine contractility. Understanding the actions of GRKs may provoke a re-evaluation of how tocolytic and uterotonic drugs are used to prevent and promote myometrial contractions respectively. Alternative approaches to dose and administration of drugs for the management of labour, such as the use of pulsatile infusion of tocolytic or uterotonic agents, will need to take the desensitisation process into consideration.

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