Adenylyl cyclase isoforms in pregnant and non-pregnant human myometrium

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Abstract

The precise factors involved in the transition of the relaxed pregnant uterus to the contractile state at the onset of parturition remain unclear, but it is accepted that cAMP-generating pathways contribute to uterine relaxation. We have previously reported an increased expression of the adenylyl cyclase (AC)-stimulating protein G α s in human myometrium during gestation, with a corresponding increase in GTP-stimulated AC activity. However, little is known about the predominating AC isoforms expressed during pregnancy. This information is important, because although all AC isoforms are stimulated by G α s, their regulation by other signalling molecules is very different. In the present study we have identified the isoforms of AC expressed in both pregnant and non-pregnant myometrium by mRNA analysis and immunoblotting. mRNA

encoding for AC I, II, III, VIII and IX was present in non-pregnant and pregnant myometrium, and in cultured myometrial cells. Differing levels of AC protein could be detected in myometrial plasma membranes, with decreased levels of Group 1 (isoforms I, III and VIII) and Group 4 (IX) ACs allied with increased levels of Group 2 (II, IV and VII) and 3 (V and VI) ACs during pregnancy. These findings imply a role for Group 2-activating pathways, e.g. G-protein $\beta\gamma$ -subunits and protein kinase C, in the maintenance of uterine quiescence, whilst suggesting a lesser involvement of calcium-calmodulin complex, an activator of Group 1 AC isoforms, in uterine relaxation during gestation. These data may provide an alternative pharmacological approach for the attenuation of preterm labour.

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Introduction

The precise mechanisms responsible for the transition of the quiescent pregnant uterus to the contractile state, and hence the onset of parturition, remain unknown. Greater understanding of the factor, or factors, involved may ultimately assist in the anticipation and prevention of preterm labour, which whilst accounting for only 7% of total deliveries is responsible for 70% of perinatal deaths (López Bernal *et al.* 1993). Furthermore, whilst 50% of preterm babies now survive, there are a range of both short- and long-term complications, including respiratory disease and intracerebral haemorrhage, associated with deliveries occurring before 32 weeks gestation (Keirse 1995).

The second messenger cAMP is produced from ATP in response to the actions of hormones and drugs upon cell surface receptors coupled to the enzyme adenylyl cyclase (AC). cAMP influences a wide range of physiological events including metabolic processes such as gluconeogenesis, secretory activity, muscle contraction, learning, ion channel conductance and cytokine production. The ability of cAMP to promote the relaxation of myometrial cells is conferred via the activation of protein kinase A

(PKA), which phosphorylates myosin light chain kinase thus decreasing the affinity of this enzyme for the calcium-calmodulin complex (Word 1995); PKA may also promote relaxation of smooth muscle by stimulation of calcium uptake or activation of potassium channels. A wide range of agonists, including β -adrenergic agents, and some prostaglandins, such as PGE₂, are known to couple to receptors which elevate intracellular cAMP, and modifications in receptor density or G-protein-receptor coupling may assist in the maintenance of myometrial relaxation. For example, butaprost, an EP₂-receptor selective agonist, provoked potent inhibition of spontaneous activity in samples of human myometrium (Senior *et al.* 1991), and it has also been postulated that neuropeptides, such as calcitonin gene-related peptide, adrenomedullin and amylin, may act via G-protein linked receptors to contribute to the maintenance of uterine quiescence during human pregnancy (Casey *et al.* 1997). Furthermore, the density of β_2 -adrenoceptors, which positively couple to AC via G α s, has been shown to decrease in human myometrium towards term (Litime *et al.* 1989).

ACs are expressed in virtually all mammalian tissues, constituting ~0.001% of membrane proteins (Sunahara *et al.* 1996). Generally, ACs are understood to consist of a

short amino-terminal region and two cytoplasmic domains (C_1 and C_2) each ~ 40 kDa in size separated by two extremely hydrophobic domains, which have been proposed to each take the form of six transmembrane helices (Tang & Gilman 1992). The catalytic core of mammalian ACs consists of a pseudosymmetric heterodimer composed of the highly conserved portions of the cytoplasmic regions, namely C_{1a} and C_{2a} , which is able to bind one molecule of G α s, one molecule of forskolin and one molecule of ATP (Hanoune *et al.* 1997, Tesmer & Sprang 1998). Linkage of portions of these cytosolic domains yields a soluble form of AC which may be activated by both G α s and forskolin (Tang & Gilman 1995).

At least nine separate isoforms of AC have been identified, which may be allocated into one of four groups according to their modes of regulation (Hanoune *et al.* 1997). All isoforms are activated by G α s binding, primarily, to a hydrophobic negatively charged groove on the C_{2a} domain (Hurley 1998, Tesmer & Sprang 1998). Similarly, the diterpene forskolin activates all ACs, except AC IX, which lacks the required serine and leucine residues, by occupying a narrow hydrophobic crevice between C_{1a} and C_{2a} (Hurley 1998, 1999). In addition, Group 1 ACs (types I, III and VIII) are stimulated by calcium and calmodulin, whilst Group 2 isoforms (II, IV and VII) are activated by G-protein $\beta\gamma$ -subunits and by protein kinase C (PKC) phosphorylation. Group 3 ACs (types V and VI) are inhibited by low concentrations of calcium, whilst Group 4 isoforms (at present only AC IX has been characterised) are insensitive to either calcium or $\beta\gamma$ -subunits (Taussig & Gilman 1995, Hanoune *et al.* 1997), but are inhibited by the phosphatase calcineurin (Antoni *et al.* 1998a,b). Interestingly, it has been demonstrated that GTP-dependent AC activity in human myometrium increases during pregnancy and decreases at the onset of labour as a consequence of changes in the level of G α s isoform expression, but the overall activity of AC as measured by forskolin stimulation, remains unaltered (Europe-Finner *et al.* 1994). Little is known about the dominant AC isoforms present in this tissue, and it is possible that whilst overall AC membrane content remains unchanged, there may be alterations in the predominating isoforms and dominant regulatory pathways of AC, which could be implicated in the switch from uterine relaxation to contraction.

Previous studies on the AC isoforms present in myometrium have either concentrated upon alterations in AC expression in the rat uterus during gestation (Suzuki *et al.* 1997), or have utilised probes designed using a variety of non-human AC sequence data to detect AC isoforms in both human and rat myometrium (Mhaouty-Kodja *et al.* 1997). Neither study, however, demonstrated expression of the mRNA detected as membrane-associated AC protein. In this investigation we have attempted to verify the presence of RNA encoding for those isoforms of AC whose human sequences are known. In addition, we have

demonstrated the expression of this RNA as membrane-associated AC protein in both pregnant and non-pregnant human myometrium, and have identified the dominant isoforms of this enzyme.

Materials and Methods

Custom oligonucleotide primers, designed using human AC sequences recorded in the GenBank database, and tissue culture reagents were obtained from Life Technologies Ltd (Paisley, Strathclyde, UK). Specific polyclonal antibodies for AC isoforms I, II, III, IV, V, VII and VIII, and their corresponding cognate peptides, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AC IX PCR primers, anti-AC IX antiserum and cognate peptide were developed by Dr Janice Patterson and Dr Ferenc Antoni (Antoni *et al.* 1995, 1998a,b). Acrylagel and bis-acrylagel were purchased from National Diagnostics (Hull, Humberside, UK), Kaleidoscope Prestained Standards and Bio-Rad Protein reagent from Bio-Rad Laboratories (Richmond, CA, USA). Dispace (grade 2), DNA Molecular Weight Markers, 1st Strand cDNA Synthesis Kit for reverse transcriptase (RT) PCR (RT-PCR) and Complete Mini protease inhibitor cocktail tablets were obtained from Roche Diagnostics Limited (Lewes, East Sussex, UK). Hyperfilm and the enhanced chemiluminescence (ECL) assay system were obtained from Amersham International plc (Little Chalfont, Bucks, UK), $2 \times$ Protein Loading Buffer Blue from Flowgen (Lichfield, Staffs, UK), and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G from Dako (High Wycombe, Bucks, UK). Collagenase CLS (200–250 U/mg) was purchased from Lorne Laboratories (Reading, Berks, UK). All other reagents were purchased from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK).

Tissue collection

Samples of myometrium from non-pregnant premenopausal women were obtained at hysterectomies performed for benign gynaecological disorders such as menorrhagia or dysmenorrhoea. Uteri were excised longitudinally, and samples of myometrium taken from the middle of the uterine wall ~ 5 mm from either the endometrial or serosal surfaces. Pregnant term myometrium was taken from the upper border of the uterine incision during elective caesarean sections performed on women who were not in labour. All patients gave informed consent. Three pregnant hysterectomy specimens were obtained at 33, 36 and 40 weeks gestations following emergency operations for severe obstetric haemorrhage. From these specimens it was possible to obtain samples of myometrium from the fundus, corpus and lower uterine

segment. This investigation had the approval of the Central Oxfordshire Research Ethics Committee. Samples were snap-frozen in liquid nitrogen and stored at -70°C until required.

Myocyte isolation and culture

Myocytes were isolated enzymatically from samples of fresh myometrium, and maintained in culture using Waymouth's MB752/1 medium supplemented with 10% (v/v) fetal calf serum, 100 IU penicillin/ml and 100 μg streptomycin/ml as described in detail by Phaneuf *et al.* (1993).

RNA isolation and RT-PCR amplification

Isolation of RNA from myometrial tissues and cultured myocytes Total RNA was isolated from tissue samples and cultured myometrial cells by the guanidinium isothiocyanate-caesium chloride gradient method (Chomczynski & Sacchi 1987, Sambrook *et al.* 1989). Samples of myometrium from five individual patients (three non-pregnant, two pregnant) were collected and RNA extracted as described by Europe-Finner *et al.* (1996). Isolated RNA was stored in 75% ethanol at -70°C until required.

First-strand cDNA synthesis using AMV RT Total RNAs from myometrial tissues and myocytes were used as templates for first-strand cDNA synthesis using oligo-p(dT)₁₅ primer and AMV reverse transcriptase supplied in the 1st Strand cDNA Synthesis Kit for RT-PCR from Roche Diagnostics. First-strand cDNA synthesis was carried out in a 20 μl reaction volume containing 1 μg total RNA, 1.6 μg oligo-p(dT)₁₅, 1 mM deoxynucleoside triphosphates (dNTPs), 25 mM magnesium chloride, 50 units RNase inhibitor and ≥ 20 units AMV RT. Transcription was carried out (after heating at 65°C for 15 min to remove secondary structures) at 42°C for 1 h, with subsequent denaturing of AMV RT at 99°C for 5 min prior to storage at -70°C . Human brain total RNA was also prepared as described above and used as a control tissue in RT-PCR.

Amplification of first-strand cDNAs by PCR Five pairs of single-stranded oligodeoxynucleotide primers based upon the human sequences for AC I, II, III, VIII and a cardiac autoantigen demonstrating homology with G α s, published in the GenBank database, were synthesised. For AC I: one of 20 bases (forward), representing nucleotides 1441–1460 and the other, also of 20 bases (reverse) complementary to nucleotides 2247–2266 of human fetal brain AC mRNA. For AC II: one of 20 bases (forward) recognising nucleotides 136–155 and a second of 18 bases (reverse) complementary to nucleotides 743–760 of human brain AC. For AC III: one of 20 bases (forward),

representing nucleotides 1131–1150 and another of 18 bases (reverse) complementary to nucleotides 1343–1326 of human fetal brain AC mRNA. For AC VIII: one of 18 bases (forward), representing nucleotides 2031–2048 and a second of 19 bases (reverse) complementary to nucleotides 2506–2488 of human newborn brain-stem AC mRNA. For the cardiac autoantigen: one of 20 bases (forward) representing nucleotides 15–34, and a second, also of 20 bases (reverse) corresponding to nucleotides 197–178. Primers for PCR of AC IX were designed and supplied by Dr J M Paterson (MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Edinburgh, UK). Thirty PCR cycles were required to amplify mRNA for AC I and II, and G α s, 35 for AC IX and 40 for AC III and VIII.

Electrophoresis of PCR products The resultant PCR products from the amplification reaction were resolved using a 2% agarose gel, and DNA bands visualised by ethidium bromide staining and subsequent UV illumination. Five pairs of primers, namely those complementary to AC I, II, III, VIII and IX produced a single PCR product band of the expected size, which was treated with exonuclease and shrimp alkaline phosphatase before sequencing. However, primers designed to amplify cDNA coding for the cardiac autoantigen produced a double-banded product, which was purified directly from an agarose gel using the QIAquick Gel Extraction Kit (QIAGEN Ltd, Crawley, West Sussex, UK) prior to sequencing.

Sequencing of PCR products The sequences of the PCR products obtained from the amplification reaction were confirmed using a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham) and the primers designed for each individual AC isoform.

Preparation of plasma membranes

Myometrial plasma membranes were prepared using a modification of the protocol reported by Europe-Finner *et al.* (1993). Essentially, samples of myometrium from five to ten individual patients were pooled, weighed, roughly chopped and then homogenised in 3 ml/g ice-cold sucrose buffer (250 mM sucrose, 25 mM Tris, 1 mM EDTA; pH 7.6) containing one Complete Mini protease inhibitor cocktail tablet/10 ml buffer. The tissue homogenate was centrifuged for 10 min at 1000 g ; the supernatant was removed and centrifuged at 40 000 g for 45 min. The supernatant from this spin was discarded and the resulting membrane pellet gently washed with 2 ml sucrose buffer prior to resuspension in PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and one Complete Mini protease inhibitor cocktail tablet/10 ml to a membrane concentration of ~ 5 mg protein/ml. Membranes were snap-frozen and stored at -70°C until required. Protein concentration was determined using the method described

by Bradford (1976) with BSA as a standard. Human brain plasma membranes were also prepared according to this protocol, and used as a positive control in subsequent immunoblots.

Immunoblotting

Membrane proteins (100 µg) were solubilised in sample buffer (0.125 M Tris-HCl (pH 6.8), 4.4% w/v SDS, 20% v/v glycerol, 2% v/v mercaptoethanol and 0.01 mg/ml bromophenol blue) and resolved on 10% polyacrylamide gels for ~90 min at 130 V. Separated proteins were then electrotransferred onto polyvinylidene difluoride membranes at 15 V for 2 h. Blots were blocked overnight at 4 °C in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 3% skimmed milk protein prior to incubation at room temperature in a 1 µg/ml solution of primary polyclonal antibody. After 1 h, the primary antibody was removed, the blot washed thoroughly with TBS containing 0.1% Tween-20, and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, or horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G, at a dilution of 1:2000. The blot was again washed with TBS+0.1% Tween-20, and antibody complexes detected using the ECL system and Hyperfilm ECL (Amersham).

Results

mRNA expression

RT-PCR for AC I, a Group 1 isoform, demonstrated the presence of RNA coding for this isoform in non-pregnant and pregnant myometrium, and in cultured myometrial cells (Fig. 1A). Moreover, RNA encoding AC II, III, VIII and IX (Fig. 1B, C, D and E respectively) was detected in all samples, as was RNA coding for a cardiac autoantigen which has homology with *Gαs* and could be observed as a double-banded PCR product corresponding to *Gαs* large and *Gαs* small (Fig. 1F). The sequences of all PCR products were verified utilising the Thermo Sequenase radiolabelled terminator sequencing kit and the GenBank database (data not shown). Comparison of sequences obtained with the GenBank sequences used to generate primer pairs for PCR demonstrated at least 97% identity between PCR products and the published AC sequences.

Protein expression

Whilst RT-PCR demonstrated the presence in human myometrium of RNA encoding for those AC isoforms studied, Western blotting with specific polyclonal antibodies revealed significant changes in the levels of AC isoforms in pregnant compared with non-pregnant myometrium. The specificity of each anti-AC polyclonal

antibody was verified by pre-absorbing overnight with the corresponding cognate peptide. In all cases, no bands were detected when blots were subsequently treated with ECL reagents (data not shown).

AC isoforms Groups 1 and 4 Immunoblotting using a polyclonal antibody raised against AC I revealed the presence of a band with a molecular mass of ~55 kDa in both pregnant and non-pregnant tissue (Fig. 2A). This was allied with an additional faint ~61 kDa band in non-pregnant tissue, and a minor ~65 kDa band in pregnant myometrium. A greater relative expression of the 55 kDa AC I band was evident in non-pregnant myometrium compared with pregnant tissue (Fig. 2A). Similarly, the other Group 1 ACs, types III and VIII, were also more abundant in non-pregnant myometrial membranes (Fig. 2B and C respectively). A single ~65 kDa band was detected by the anti-AC III polyclonal antibody, whilst immunodetection of AC VIII revealed an ~32 kDa band. Furthermore, whilst it was not possible to detect AC IX (a Group 4 isoform) in pregnant myometrial membranes, a faint band of ~50 kDa was visible in membranes prepared from non-pregnant myometrium (Fig. 3).

AC isoforms Groups 2 and 3 Conversely, relatively greater levels of the Group 2 isoform AC II (Fig. 4A) were present in pregnant rather than non-pregnant myometrium. Seven bands of ~20, ~40, ~45, ~50, ~100, ~150 and ~200 kDa respectively, were evident in pregnant tissue, with the 20, 45 and 200 kDa bands predominating. Moreover, a single band of ~200 kDa was detected in non-pregnant tissue (Fig. 4A). A second Group 2 isoform, AC IV (Fig. 4B), was also more abundant in pregnant compared with non-pregnant myometrium. This isoform was manifested as two bands of ~50 and ~75 kDa in pregnant tissue, but as a single ~50 kDa band in non-pregnant myometrium. Similarly, a double AC VII band (~50 and ~60 kDa) could be detected in pregnant myometrium, but was absent in non-pregnant tissue (Fig. 4C). Similarly, the Group 3 isoform AC V was more abundant during pregnancy, with the density of the single ~50 kDa band detected by the polyclonal antibody being greater in pregnant than non-pregnant myometrial membranes (Fig. 5). Immunoblotting of membranes prepared from samples of myometrium taken from different regions of the uterus (fundus, corpus and lower uterine segment) from pregnant hysterectomy specimens revealed no topographical differences in the presence of the Group 2 isoforms AC II and AC IV (Fig. 6).

Discussion

This study demonstrates the presence of several different AC mRNA species and membrane proteins in human

Figure 1 Identification of myometrial AC and Gas cDNAs using RT-PCR. RT-PCR was performed as described in the text. cDNAs were resolved on a 2% agarose gel and visualised by ethidium bromide staining and subsequent UV illumination. (A) Primers recognising AC I; (B) primers recognising AC II; (C) primers recognising AC III; (D) primers recognising AC VIII; (E) primers recognising Gas; (F) primers recognising Gas. Lanes 1 and 10, DNA digest molecular markers; lane 2, control (RT performed using water rather than RNA, with subsequent PCR); lane 3, human brain, as positive control; lane 4, cultured human myometrial cells; lanes 5–7, non-pregnant human myometrium from three different patients; lanes 8 and 9, pregnant myometrium from two different patients.

Figure 2 Detection of Group 1 AC isoforms in pregnant and non-pregnant human myometrium. Plasma membranes were isolated as described in the text and membrane proteins resolved by SDS-PAGE and subsequent immunoblotting. (A) Antibody against AC I; (B) antibody against AC III; (C) antibody against AC VIII. Lane 1, pregnant; lane 2, non-pregnant; lane 3, human brain, as positive control. Pools of myometrium containing samples from five to ten different patients were used in each group. Anti-AC antibodies were used at a concentration of 1 µg/ml. An arrow indicates the theoretical molecular mass of each isoform. Molecular masses were determined using Kaleidoscope Prestained Standards.

Figure 3 Detection of the Group 4 isoform AC IX in pregnant and non-pregnant human myometrium. Plasma membranes were isolated as described in the text and membrane proteins resolved by SDS-PAGE and subsequent immunoblotting. Lane 1, pregnant; lane 2, non-pregnant; lane 3, human brain, as positive control. Pools of myometrium containing samples from five to ten different patients were used in each group. The anti-AC antibody was used at a concentration of 1 µg/ml. An arrow indicates the theoretical molecular mass of this isoform. Molecular masses were determined using Kaleidoscope Prestained Standards.

myometrium. Oligonucleotide primers specifically designed to be complementary to the cDNA sequence of human ACs, rather than rat, murine or bovine sequences, have been employed to demonstrate the presence of mRNA encoding for a variety of AC isoforms. At present, human sequence data exist for only five AC isoforms, namely AC I (Villacres *et al.* 1993), AC II (Stengel *et al.* 1992), AC III (Yang *et al.* 1999), AC VIII (Defer *et al.* 1994) and AC IX (Hacker *et al.* 1998). By utilising primers based on these published human sequences we have demonstrated the presence of mRNA encoding for Groups 1 (types I, III and VIII), 2 (AC II) and 4 (AC IX) in both pregnant and non-pregnant human myometrium. The mRNA for each AC isoform studied could also be detected in human brain (positive control) and cultured myometrial cells, indicating that myocytes themselves, rather than any vasculature associated with tissue samples, contained the relevant AC RNA.

This is the first successful demonstration of mRNA for AC I and VIII in human myometrium. Furthermore, we have demonstrated the expression of these mRNAs as membrane-associated AC proteins which were more abundant in non-pregnant than in pregnant tissue. Suzuki *et al.* (1997) concluded that whilst five isoforms of AC (namely AC II, IV, VI, VII and IX) were present in the rat uterus, the mRNA for the Group 1 isoforms AC I, III and VIII, and the Group 3 isoform AC V could not be detected. Similarly, Mhaouty-Kodja *et al.* (1997) failed to detect the presence of mRNA for AC I and VIII in either rat or human myometrium. Such disparate reports could

Figure 4 Detection of Group 2 AC isoforms in pregnant and non-pregnant human myometrium. Plasma membranes were isolated as described in the text and membrane proteins resolved by SDS-PAGE and subsequent immunoblotting. (A) Antibody against AC II; (B) antibody against AC IV; (C), antibody against AC VII. Lane 1, pregnant; lane 2, non-pregnant; lane 3, human brain, as positive control. Pools of myometrium containing samples from five to ten different patients were used in each group. Anti-AC antibodies were used at a concentration of 1 µg/ml. An arrow indicates the theoretical molecular mass of each isoform. Molecular masses were determined using Kaleidoscope Prestained Standards.

result from differences in sample collection or storage, experimental technique (Northern blotting versus RT-PCR) or stringency.

Figure 5 Detection of the Group 3 isoform AC V in pregnant and non-pregnant human myometrium. Plasma membranes were isolated as described in the text and membrane proteins resolved by SDS-PAGE and subsequent immunoblotting. Lane 1, pregnant; lane 2, non-pregnant; lane 3, human brain, as positive control. Pools of myometrium containing samples from five to ten different patients were used in each group. The anti-AC antibody was used at a concentration of 1 µg/ml. An arrow indicates the theoretical molecular mass of this isoform. Molecular masses were determined using Kaleidoscope Prestained Standards.

In addition, we have demonstrated the presence of mRNA encoding for the recently described AC IX isoform (Antoni *et al.* 1998a) in both pregnant term and non-pregnant human myometrium, and its expression as membrane-associated AC protein. We have also demonstrated the presence of mRNA for the AC-activating proteins *G α s* large and *G α s* small, using primers designed to be complementary to a cardiac autoantigen homologous to *G α s* (Eichbaum *et al.* 1994). This confirms our previous data obtained utilising a different set of PCR primers (Europe-Finner *et al.* 1996), and establishes the presence of multiple possible *G α s*-AC pathways in human myometrium. We are currently investigating the exact coupling of *G α s* to specific AC isoforms in myometrium; however, such an analysis is beyond the scope of the present manuscript.

It has previously been noted that whilst GTP-dependent and prostaglandin EP receptor-stimulated AC activity increases during pregnancy, there is no apparent alteration in forskolin-stimulated AC activity (Europe-Finner *et al.* 1994) indicating that increased coupling of EP receptors to *G α s* and of *G α s* to AC, rather than increased AC expression, may be the key factor in the maintenance of uterine quiescence. In contrast, in rat uterus an increase in overall AC activity mid-pregnancy allied with decreased AC activity near term has been demonstrated (Mhaouty-Kodja *et al.* 1997, Suzuki *et al.* 1997). However, whilst overall forskolin-stimulated AC activity may not alter during gestation, it is possible that the proportion of

Figure 6 Detection of Group 2 AC isoforms in pregnant human myometrium taken from different regions of the uterus. Plasma membranes were isolated as described in the text and membrane proteins resolved by SDS-PAGE and subsequent immunoblotting. (A) Antibody against AC II; (B) antibody against AC IV. Lanes 1, 4 and 7, fundus; lanes 2, 5 and 8, corpus; lanes 3, 6 and 9, lower uterine segment. Myometrial membranes were prepared from three different pregnant hysterectomy samples (lanes 1–3, 4–6, 7–9). Anti-AC antibodies were used at a concentration of 1 µg/ml. An arrow indicates the theoretical molecular mass of each isoform. Molecular masses were determined using Kaleidoscope Prestained Standards.

the dominant isoform, or isoforms, of AC present changes during pregnancy and parturition, implying that different signalling elements become important in the regulation of cAMP levels. In addition, the Group 4 isoform AC IX has been found to be insensitive to forskolin stimulation (Hacker *et al.* 1998, Yan *et al.* 1998) suggesting that forskolin binding studies alone are not sufficient to monitor alterations in AC expression. Using immunoblotting techniques we have now demonstrated that whilst there are no topographical differences in uterine AC isoform expression, there are changes during pregnancy in the relative expression of the various isoforms of AC as membrane-associated protein.

Thus, there are decreased levels of Group 1 and Group 4 isoforms, allied with increased membrane levels of Group 2 and Group 3 ACs in pregnant term myometrium, compared with non-pregnant tissue. The discovery of such alterations permits definition of the mechanisms responsible for the elevation of cAMP and hence the maintenance of uterine quiescence in pregnancy. Group 2 AC isoforms are known to be activated both by G-protein $\beta\gamma$ -subunit and PKC phosphorylation (Taussig & Gilman 1995, Hanoune *et al.* 1997). The elevated levels of the Group 2 ACs types II, IV and VII observed in pregnant myometrium implies a role for such factors in myometrial relaxation. This agrees with the findings of Mhaouty-Kodja *et al.* (1997) who showed that $\beta\gamma$ -subunit scavengers abolished the positive input of the Gi-protein pathway upon Gs-stimulated AC activity at mid-pregnancy in the rat. In addition, Mhaouty *et al.* (1995) speculated that at mid-pregnancy, the α_2 -adrenergic receptor potentiates cAMP production by AC II and AC IV via the release of $\beta\gamma$ -subunits from Gi₂ and Gi₃ proteins, and it is possible that such mechanisms may also

contribute to cAMP production, and hence uterine relaxation in human myometrium.

Calcium stimulates the activity of Group 1 ACs (isoforms I, III and VIII) and inhibits Group 3 isoforms such as AC V. We have observed a relative increase in Group 3 ACs in pregnant term myometrium, allied with decreased levels of Group 1 isoforms. Such findings suggest that there may be a fine balance between the expression of calcium-stimulated and calcium-inhibited ACs, which may influence the switch from uterine quiescence during gestation to uterine contraction at parturition.

The theoretical molecular masses of the various isoforms of AC, calculated according to their amino acid content, have been estimated to be ~120–150 kDa (Hanoune *et al.* 1997). In addition, it has been suggested that dimerisation, association with other proteins, such as G-proteins (Tang & Gilman 1992), or glycosylation (Hanoune *et al.* 1997) may further increase the molecular mass of ACs *in vivo*. The ~20–200 kDa immunoreactive bands detected by the anti-AC polyclonal antibodies utilised in this study may be indicative of the occurrence of proteolysis and dimerisation of AC. The Group 1 isoforms, AC I and III (Fig. 2A and B) were both manifest as ~55–65 kDa bands, suggesting that, despite the presence of a protease inhibitor cocktail, these isoforms were highly susceptible to proteolytic degradation. Similarly AC IV (Group 2), AC V (Group 3) and AC IX (Group 4) appeared to be readily degraded by naturally occurring proteases (Figs 3B, 4 and 5 respectively). However, the Group 2 isoform AC II (Fig. 3A), whilst containing low molecular mass bands indicative of proteolysis, also demonstrated a high molecular mass band of ~200 kDa, suggesting that this isoform may be more susceptible to association with other components of the signalling cascade, and to glycosylation or dimerisation.

Whilst proteolytic cleavage of AC isoforms may have occurred during the preparation of plasma membranes from samples of myometrium, it is also possible that such degradation may occur *in vivo*, implicating the cellular proteolytic system in the regulation of signalling through ACs. Indeed, Ebina *et al.* (1997) reported an isoform-dependent proteolytic activation of AC, with an ~500% increase in AC II, an ~30% increase in AC III, and an inhibition of AC V activity upon trypsin treatment. Such activation was both time- and dose-dependent and was blocked by the presence of a trypsin inhibitor. These findings imply that certain isoforms of AC may rely upon proteolytic cleavage to maximise their activation, and so be naturally more susceptible to the actions of proteases.

In conclusion, the data presented here demonstrate that relative changes in AC isoform expression in human myometrium occur during pregnancy. Such changes imply a shift from a predominance of calcium-calmodulin-stimulated isoforms, to those regulated by G-protein $\beta\gamma$ -subunits and PKC. Further studies are required to corroborate this theory, and to elucidate any changes in AC levels which may occur at the onset of spontaneous labour. Knowledge of the exact AC isoforms contributing towards the maintenance of uterine quiescence, and their modes of regulation will ultimately enable the development of more selective and efficacious pharmacological approaches to the problem of preterm labour, and hence reduce the occurrence of the complications associated with preterm deliveries.

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