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Characterization of G proteins in rat myometrium

A differential modulation of $G_i2\alpha$ and $G_i3\alpha$ during gestation

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Myometrial membranes, obtained from estrogen-dominated (day 0) rat uteri, were immunoblotted with antiserum (SG1), which recognizes the α subunits of both G_i1 and G_i2, with antiserum (LF2) specific for $G_i2\alpha$, and with 13B antiserum, specific for $G_i3\alpha$. The data revealed the absence of detectable levels of $G_i1\alpha$ and the simultaneous presence of $G_i2\alpha$ and $G_i3\alpha$ as G_i subunits in rat myometrium. The expression of G_i proteins during gestation (days 0, 12, 21) was studied with the above antibodies. No qualitative change in the nature of $G_i\alpha$ species was observed during gestation: $G_i1\alpha$ remained undetectable, $G_i2\alpha$ and $G_i3\alpha$ were both present on days 12 and 21. Of significance was the increase (100%) in the amount of $G_i2\alpha$ at midgestation (day 12) compared to days 0 and 21. A different pattern was observed with $G_i3\alpha$, which decreased with advancing gestation (day 0 > 12 > 21). Immunodetection of β subunits of G proteins indicated the presence of a 35/36 kDa doublet on days 0, 12 and 21, with an increase at midgestation. The simultaneous increase in $G_i2\alpha$ and β subunits may provide an explanation for the previously demonstrated alteration in adenylate cyclase stimulability detected at midgestation.

G_i protein; Myometrium; Gestation; Adenylate cyclase

1. INTRODUCTION

Hormonal activation of adenylate cyclase requires the specific interactions of an agonist-occupied receptor, the guanine nucleotide-binding protein (G_s or G_i) and the catalytic unit 'C'. Information from receptors, which stimulate adenylate cyclase, is mediated by G_s, whereas negative impulses are transduced to 'C' via G_i [1,2]. Multiple subtypes of $G_i\alpha$ have been identified from molecular cloning studies [3–5], viz. G_i1, G_i2 and G_i3. Each of these polypeptides is a substrate for pertussis toxin-catalysed ADP-ribosylation [6,7]. The species of G_i involved in the inhibitory arm of the adenylate cyclase cascade remains unclear, though G_i2 has been demonstrated to be a potential mediator of adenylate cyclase inhibition in some cells [8,9].

Among the components of the adenylate cyclase system, G proteins could be a locus for permissive hormone regulation [10], a phenomenon which has been observed with thyroid hormone [11,12], glucocorticoids [13] and steroids [14]. We have previously reported that at specific stages of gestation which are characterized by important fluctuations in the relative concentrations of estrogen and progesterone, the rat myometrium exhibited marked alterations in adenylate cyclase stimulability [15]. At the onset of gestation, there was a progressive attenuation of adenylate cyclase activation in response to different stimulatory agonists, with minimal responses being observed at midgestation (day 12). The advanced stages of gestation were associated with a progressive restoration of adenylate cyclase stimulability with a full responsiveness before parturition (day 21). Of interest were the findings that pertussis toxin treatment of day 12 myometrium resulted in a reversal of the attenuated cyclic AMP responses suggesting that the inhibitory protein G_i was involved. Recently, using varied α subunit-specific antisera, we reported [16] the absence of $G_i1\alpha$ and the existence of both $G_i2\alpha$ and a novel form of $G_o\alpha$ in the estrogen-dominated rat myometrium (day 0). The present study focuses on the immunological characterization and the modulation of the G_i proteins in rat uterus during gestation.

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Abbreviations: G protein, guanine nucleotide-binding protein; G_s and G_i, stimulatory and inhibitory G proteins of the adenylate cyclase system; G_o, G protein of unknown function; $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$ and $G_o\alpha$, α subunits of the corresponding G protein

2. MATERIALS AND METHODS

2.1. *Antibody characterization*

Each antiserum was produced in a New Zealand white rabbit following the procedure previously recorded [17]. SG1 is an antipeptide antiserum produced against the synthetic decapeptide

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(KNSLKDCGLF) corresponding to the C-terminal decapeptide of rod transducin [17]. This antiserum can be used to identify G_t-like proteins (G_1 and G_2). Antiserum LE2 was raised against a synthetic peptide (LERIAQSDYI) corresponding to amino acids 160–169 of $G_i2\alpha$, and is specific for $G_i2\alpha$ [17]. Antiserum 13B was raised against a synthetic peptide (KNNLKECGLY), corresponding to the C-terminal decapeptide of the α subunit of G_i3 [18]. Antiserum βN1 was raised against a peptide (MSELDQLRQE) corresponding to the N-terminal decapeptide of the β1 subunit and recognizes both β1 and β2 subunits [19].

2.2. *Tissue processing and membrane preparation*

Uteri were obtained from immature estrogen-pretreated rats or from pregnant rats at different stages of gestation. Myometrium was prepared free of the endometrium as in [15,20]. Crude membranes were obtained from different myometrial preparations at 4–6 mg of protein/ml in cold 50 mM Tris-HCl (pH 7.4) 0.5 mM EDTA buffer [16]. Crude membrane fractions were prepared from rat brain and rat glioma C6BU1 cells as described [21]. Prior to electrophoresis, samples were subjected to *N*-ethylmaleimide treatment as in [18].

2.3. *Immunological analysis*

Membrane samples were resolved by SDS-PAGE (12.5% acrylamide, 0.15% bisacrylamide (w/v)) overnight at 100 V. Proteins were transferred to nitrocellulose and blocked for 90 min at 37°C with 3% gelatin in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). Primary antiserum in 1% gelatin/TBS was then added and left overnight. After removal of the antiserum, the blot was washed with distilled water, followed by washes with TBS containing 0.1% (w/v) Tween-20 (TTBS) and TBS. Secondary antiserum (swine anti-rabbit IgG coupled to horseradish peroxidase) (1:200 dilution) in 1% gelatin/TBS was added and after 3 h incubation, the nitrocellulose was washed as detailed above. The antibody complex was detected by *O*-Dianisidine. The blots were quantitated using a scanner Desaga CD-60. Control experiments (not shown) established that for each antiserum the intensity of labeling of the corresponding band was directly proportional to the amount of myometrial membrane protein used in the assay (see Fig. 2 for example).

3. RESULTS

We have recently shown, that rat myometrial membranes contain G_i2 and are devoid of G_i1 [16]. This is further illustrated in Fig. 1(a and b), under SDS-PAGE conditions (12.5% acrylamide, 0.15% bisacrylamide) in which good resolution of distinct α subunits (α_i1, α_i2, α_i3, and α_o) can be achieved. Antiserum SG1, capable of interacting with the α subunits of G_i1 and G_i2, identified two polypeptides of 41 kDa (predominant) and 40 kDa in rat brain membranes (Fig. 1a). In contrast myometrial membranes contained only a single form of G_i, which migrated with a mobility similar to $G_i2\alpha$ (40 kDa) in rat brain (Fig. 1a). Using the selective $G_i2\alpha$ antiserum LE2 [17], we identified only low level of an immunoreactive polypeptide in rat brain and confirmed the identity of the rat myometrial SG1-detected G_i as G_i2 (Fig. 1b).

To detect the potential presence of another G_i protein in rat myometrium, we employed the antiserum 13B, which is directed against the C-terminal decapeptide of α_i3 and identified only $G_i3\alpha$ [18]. Immunoblotting of rat myometrium with 13B (Fig. 1c) led to the identification of a single polypeptide of 40.5 kDa, which migrated with the same apparent molecular mass as α_i3, present in C6BU1 cells [18] and slightly retarded compared to α_i2.

We used the specific antisera, described above to investigate the expression and an eventual quantitative change of G_i2 and G_i3 in rat myometrium during gestation. Fig. 2 displays an immunoblot of rat myometrial membranes derived from different stages of gestation

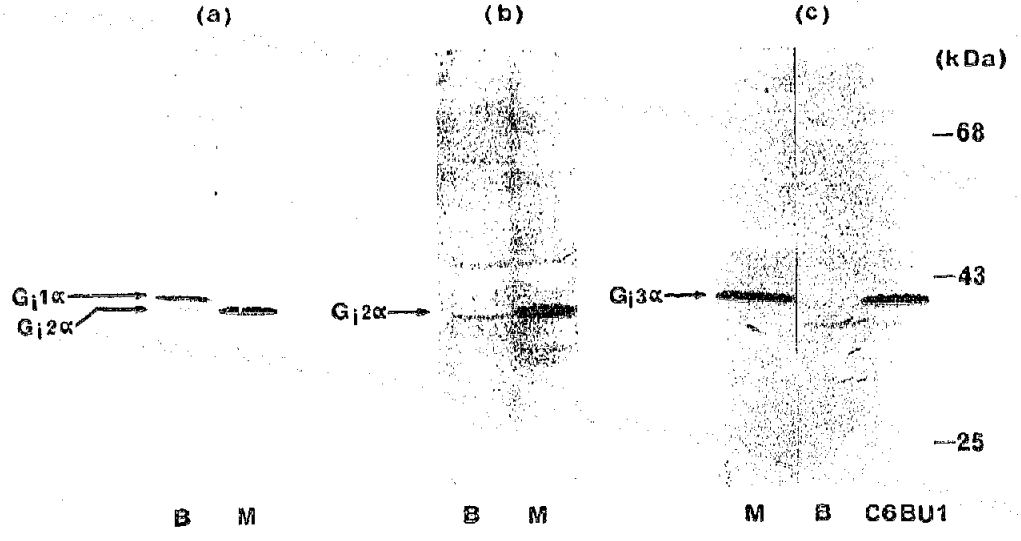

Fig. 1. Identification of G_i proteins of rat myometrium as G_i2 and G_i3. (a) Membranes from either rat brain (B) (100 μg) or estrogen-treated rat myometrium (M) (50 μg) were resolved and immunoblotted as described in section 2. The blot was developed using antiserum SG1 (1:200 dilution) as the primary reagent. (b) A similar experiment was performed except that the primary antiserum was a 1:200 dilution of LE2. (c) Membranes from either rat brain (B) (100 μg), rat glioma C6Bu1 (100 μg) or estrogen-treated rat myometrium (M) (200 μg) were resolved and immunoblotted using antiserum 13B (1:200 dilution).

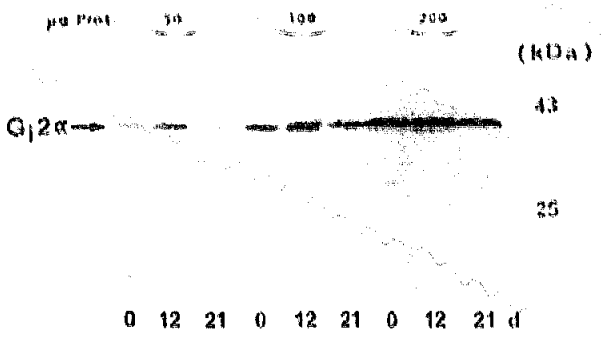

Fig. 2. Detection and modulation of the α subunit of G_i2 in rat myometrium at different stages of gestation. Different amounts of crude membranes (50 μg, 100 μg, or 200 μg) from rat myometrium (day 0, day 12 or day 21) were run on SDS-polyacrylamide gel, electroblotted and developed with SG1 antiserum (1:200 dilution) as described in section 2.

(0, 12 or 21 days), resolved by SDS-PAGE and stained with antiserum SG1. SG1 recognized a single polypeptide of 40 kDa, described as $G_i2\alpha$. It was interesting to note that for different amounts of proteins up to an amount of 200 μg, labelling of $G_i2\alpha$ was more intense in membranes of midgestation (day 12), signalling the presence of a higher quantity of $G_i2\alpha$ in these membranes compared to day 0 and day 21. Indeed, densitometric scanning of the immunoblot indicated that the level of the α subunit of G_i2 was increased in day 12 compared to day 0 by 160 ± 20% (mean ± SEM, n = 6). At the end of gestation (day 21) no significant change was observed compared to day 0. Immunoblotting experiments with the antiserum LE2, which recognizes only α_i2 (Fig. 3), revealed a single band in

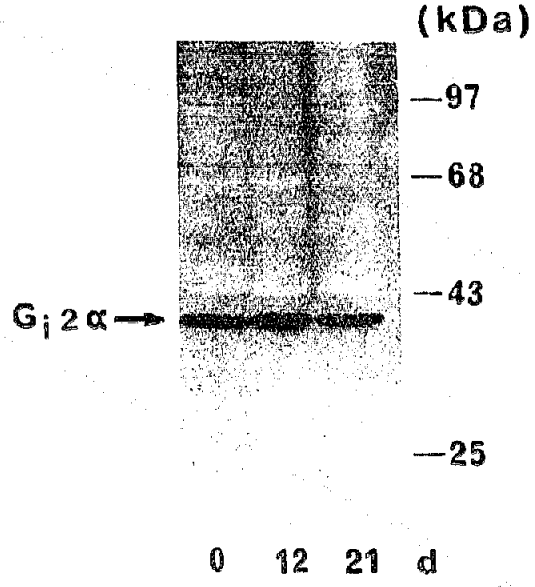

Fig. 3. Evidence for α_i2 modulation during gestation using specific antiserum LE2. Membranes from day 0, day 12 and day 21 rat myometrium (100 μg) were resolved and immunoblotted in the presence of LE2 antiserum (1:200 dilution). Experiments were carried out as described in section 2.

days 12 and 21 as well as day 0. Densitometric scanning of the immunoblot confirmed the presence of elevated levels of $G_i2\alpha$ associated with midgestation. The intensity of α_i2 labelling with LE2 was increased in day 12 compared to day 0 by 150 ± 10% (mean ± SEM, n = 3).

Data in Fig. 4 illustrate immunoblotting of rat myometrial membranes from days 0, 12 and 21 with 13B antiserum. We demonstrate that $G_i3\alpha$ was expressed throughout the gestation and furthermore that amounts of α_i3 decreased with advancing gestation (Fig. 4). Compared to day 0 membranes, some 35 ± 10% decrease at midgestation and some 75 ± 5% decrease at the end of gestation were detected by densitometric scanning (mean ± SEM, n = 4).

As G proteins are usually considered to exist as a heterotrimeric complex, $\alpha + \beta\gamma$, the status of the β subunits during gestation were investigated by immunoblotting with antiserum βN1, which recognizes both the 35- and 36-kDa-M_r forms of β subunits [19]. βN1 antiserum detected a 35/36 kDa doublet in rat myometrium, as well as in the brain membranes (Fig. 5), with a greater intensity of labeling for the higher molecular mass form. This doublet was present in membranes derived from all stages of gestation. Densitometric scanning of the immunoblot indicated that the level of both 35/36 kDa peptides was increased in day 12 compared to day 0 by 200 ± 15% (mean ± SEM, n = 3). Thus the midgestation phase was characterized by increased levels of β subunits, concomitant with the elevated levels of $G_i2\alpha$.

4. DISCUSSION

Our previous report [15] demonstrated that in rat myometrium the decline in adenylate cyclase

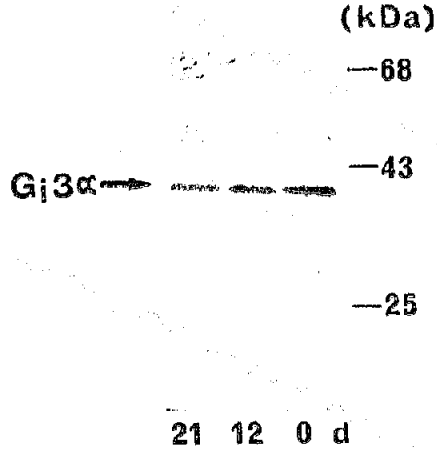

Fig. 4. Detection and modulation of $G_i3\alpha$ in rat myometrium during gestation. Different rat myometrial membranes (day 0, day 12 and day 21) (200 μg) were resolved and immunoblotted as described in section 2. The blot was developed using 13B (1:200 dilution) as primary antiserum.

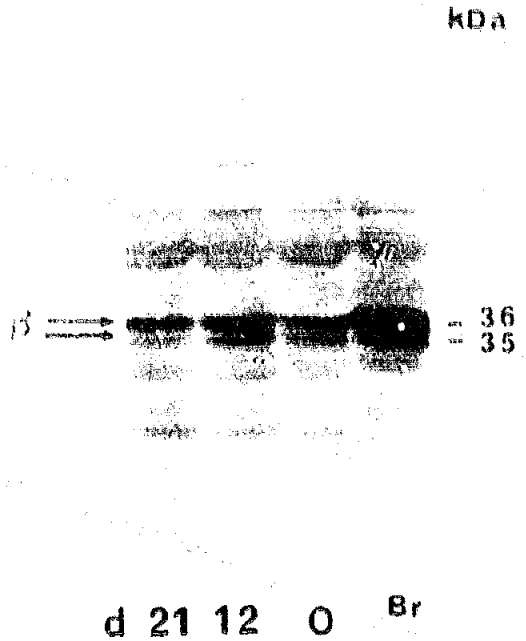

Fig. 5. Status of the β subunits of G-proteins during gestation from rat myometrium. Membranes (100 μg) from either rat brain (Br) or rat myometrium (days 0, 12 and 21) were resolved and immunoblotted using antiserum βN1 (1:200 dilution) as described in section 2.

stimulability at midgestation (day 12) could be reversed by pertussis toxin-treatment, indicating the involvement of G_i in this event. In order to characterize and tentatively evaluate the various forms of G_i, we immunoblotted myometrial membranes with different antibodies directed against distinct α_i subunits under SDS-PAGE conditions in which good resolution of these subunits can be achieved.

In the present study, we confirmed that estrogen-treated rat myometrium (day 0) did not contain detectable levels of $G_i1\alpha$ and that SG1 as well LE2 antibodies recognized the same polypeptide, which can thus be identified as $G_i2\alpha$. Additionally, the use of I3B antiserum specific for $G_i3\alpha$ led to the identification of $G_i3\alpha$ in rat myometrium. It is worth noting that I3B seems to detect an extremely weak signal in brain membranes of a polypeptide which migrated identically with rat brain $G_o\alpha$. The slight cross reactivity of the anti $G_i3\alpha$ antisera with the α subunit of G_o in rat brain has previously been recorded [22] and could be explained by the fact that α_i3 and α_o share a C-terminal tyrosine residue which is absent from α_i1 and α_i2. It remained possible that the amounts of $G_o\alpha$ present in rat myometrium [16] are sufficiently low that it is impossible to identify $G_o\alpha$ in myometrial membranes with I3B. Further evidence of the molecular identity of $G_i2\alpha$ and $G_i3\alpha$, is that the α subunits of G_i2 and G_i3 found in myometrium migrate identically with the equivalent forms of $G_i2\alpha$ in rat brain and of $G_i3\alpha$ in C6BU1 cells [18,23]. So far, these results provide evidence for the presence of $G_i2\alpha$ and $G_i3\alpha$ together with the previously described novel form of G_o in estrogen-treated rat myometrium (day 0) [16].

Experiments performed under the same conditions with membranes from days 0, 12 and 21 indicated no qualitative changes in the nature of G_i proteins, that is, undetectable level of $G_i1\alpha$ and presence of both $G_i2\alpha$ and $G_i3\alpha$ in different membrane preparations. Particularly relevant were the quantitative modifications observed during gestation in the levels of $G_i2\alpha$ and $G_i3\alpha$ which additionally appeared to be differentially regulated. Densitometric scanning of immunoblots showed that the amount of $G_i3\alpha$ progressively decreased with advancing gestation, whilst a marked increase of $G_i2\alpha$ level was critically observed at day 12, with a return to day 0 value at the end of gestation. It is important to note that the increased level of $G_i2\alpha$ at midgestation coincided with the absence of adenylate cyclase stimulability [15]. Furthermore, the decrease of $G_i2\alpha$ in day 21 myometrium to levels similar to those found in day 0 preparations, coincided with the recovery of full adenylate cyclase stimulability at term [15]. The demonstrated increase in α_i2 may thus provide a reasonable interpretation for the enhanced inhibitory pathway revealed at midgestation, particularly as we have previously demonstrated that pertussis toxin treatment of day 12 myometrium is able to restore the stimulability of adenylate cyclase [15]. It is further conceivable to propose that $G_i2\alpha$ may be the mediator of adenylate cyclase inhibition in the myometrium. Our tentative interpretation is reinforced by recent reports demonstrating that $G_i2\alpha$ was preferentially implicated in adenylate cyclase inhibition induced by α_2-adrenergic and δ-opioid receptor activation in platelets [9,24] and NG108-15 cells [8]. Interestingly, both cell types, like the myometrium, are devoid of $G_i1\alpha$ and possess both $G_i2\alpha$ and $G_i3\alpha$. The progressive decline in $G_i3\alpha$ level with advancing gestation may certainly underlie an important phenomenon, however it seems difficult to implicate it in the pattern of the altered adenylate cyclase pathway: first, the day 12 myometrium was associated with both a decrease in G_i3 level and an attenuated adenylate cyclase stimulability and second, despite the serious decline in $G_i3\alpha$, the inhibitory pathway of adenylate cyclase was normally expressed in the myometrium near term [15,25]. The functional role of $G_i3\alpha$ remains to be clarified.

Immunodetection by antiserum βN1 demonstrated the presence of 35/36 kDa M_r forms of β subunits in rat myometrium at different stages of gestation. The pattern of β subunit modulation was similar to that observed for $G_i2\alpha$: the intensity of labeling in day 12 myometrium was more pronounced compared to day 0. The simultaneous increase of β and $G_i2\alpha$ subunits at midgestation might be expected if the regulation of the expression of the different subunits of G proteins are coordinated to keep the ratio of α/β in the same range

as in control (day 0) tissues. However such an interpretation is not definitive as β subunits are thought to be part of a common pool shared by all G proteins.

In conclusion, the present observations provide a potential mechanism by which permissive regulations, linked to hormonal status during gestation, operate on G_i protein level. The data are consistent with the upregulation of G_i2 underlying the altered adenylate cyclase activity observed at midgestation; they further suggest a potential role for G_i2 in mediating adenylate cyclase inhibition in the myometrium.

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