β₂-adrenoceptor desensitization in non-pregnant estrogen-primed rat myometrium involves modulation of oxytocin receptor gene expression

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ABSTRACT

The nona-peptide oxytocin (OT) induces contraction of the myometrium by interaction with specific plasma membrane associated OT receptors (OTR), whereas stimulation of β₂-adrenoceptors (β₂AR) causes relaxation. Homologous desensitization of the myometrium to both hormones has been described. However, a possible interaction between the two systems has not been investigated.

In the present study, long-term in vivo treatment of non-pregnant estrogen-primed rats with isoproterenol decreased maximal relaxation of isolated uterine strips challenged with isoproterenol. Increased EC₅₀ values of similarly treated animals suggest that the coupling between receptor occupancy and contractile response was impaired. Since β₂AR mRNA levels were left unchanged, we conclude that the homologous desensitization to β₂ stimulation is not due to changes in β₂AR gene expression. OT infusion did not alter β₂AR mRNA levels or isoproterenol-induced relaxation of isolated uterine strips.

Treatment with OT had no effect on the amount of myometrial OTR mRNA. We have previously found that OT down-regulates OTR in the non-pregnant rat myometrium, but this therefore does not appear to take place at the level of mRNA production.

Isoproterenol treatment resulted in a three-fold increase in OTR mRNA. This was accompanied by a 91% rise in OTR binding and an augmented contractile response of isolated uterine strips to OT, suggesting that the increased production of mRNA reflects formation of active receptors. Neither OTR affinity nor EC₅₀ of in vitro strips was affected by isoproterenol treatment. We conclude that stimulation of β₂AR causes heterologous up-regulation of OTR in the non-pregnant estrogen-primed rat myometrium.

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INTRODUCTION

β-adrenomimetics are well known as potent inhibitors of myometrial contractions (Anderson et al. 1975, Berg et al. 1982, Caritis et al. 1983) and are used clinically in the treatment of pre-term labour. However, continued occupancy of β₂-adrenoceptors (β₂AR) leads to loss of myometrial responsiveness to subsequent agonist stimulation. This process, which is known as homologous desensitization, involves several steps. In frog erythrocytes (Sibley et al. 1985) and mouse lymphoma cells (Strasser et al. 1986) βAR desensitization is accompanied by receptor phosphorylation. Benovic et al. (1986) have described a cAMP-independent kinase (βAR kinase) which specifically phosphorylates β₂ARs in lymphoma cells when occupied by agonists. Lohse et al. (1989) found this kinase to be essential in phosphorylation of βARs in human epidermoid carcinoma cells. Upon phosphorylation βARs are uncoupled from stimulatory G-proteins (Benovic et al. 1988) followed by internalization and degradation (Fishman & Perkins 1988). Using receptor-binding studies, Dayes & Lye (1990) observed down-regulation of βARs in ovine myometrium challenged with isoproterenol, suggesting either receptor internalization or reduced de novo receptor synthesis.
We recently reported that pregnancy in itself induces desensitization of rat myometrium to isoproterenol and that this primarily occurs at the level of gene expression (Engstrom et al. 1997). Yeagley et al. (1987) found the degree of rat myometrial tachyphylaxis to isoproterenol to increase with advanced gestational age. These findings suggest that other factors besides β2AR stimulation are involved in the regulation of myometrial β-adrenergic desensitization in pregnancy.

Engstrom et al. (1988a) showed that in vivo infusion of the oxytocin (OT) antagonist atosiban led to up-regulation of OT receptors (OTR) in the non-pregnant estrogen-dominated myometrium. In delivering rats β2AR mRNA increased compared with day 21 of pregnancy (Engstrom T, Bratholm P, Vilhardt H & Christensen NJ, unpublished data). This increase was associated with a dramatic up-regulation of OTR, suggesting that the imposition of day 21 of pregnancy (Engstrom T, Bratholm P, Vilhardt H & Christensen NJ, unpublished data).

In the present study we have measured β2AR mRNA in estrogen-dominated rat myometrium following in vivo infusion of isoproterenol. To investigate whether β2 desensitization involves heterologous up-regulation of OTR we have concomitantly measured OTR mRNA. The physiological relevance of receptor mRNA levels was evaluated by measurements of the relaxing and contracting effects of isoproterenol and OT respectively on isolated uterine strips.

MATERIALS AND METHODS

Animals

Female Wistar rats (250–350 g) were maintained under controlled conditions in the Panum Institute Animal House. Food and water were freely available. All experiments conformed to the Guidelines on the Handling and Training of Laboratory Animals (UFAW).

Rats were anaesthetized with a mixture of Dormicur (1·25 mg, Hoffmann-La-Roche, Basel, Switzerland) and Hypnorm (0·4 ml, Janssen Chimica, Geel, Belgium). Subsequently an incision in the lower abdominal wall was made and an osmotic mini-pump (Alzet, Palo Alto, CA, USA) was introduced into the abdominal cavity. The pumps had pumping rates of 1 µl/h and were filled with OT (0·5 mg/ml, Fehrring Pharmaceuticals, Malmö, Sweden) or isoproterenol (10–20 mg/ml, Sigma Chem. Co., St Louis, MO, USA). Two days prior to decapitation the rats received an i.m. injection of 75 µg estradiol benzoate.

Primers and construction of internal mRNA standard

The oligonucleotide primers (DNA Technology, Aarhus, Denmark) used for detection of β2AR mRNA were the same as described for rat collecting duct (Mandon et al. 1995): sense primer 5′-TCT TCG AAA ACC TAT GGG AAC GGC-3′ (nucleotide 1036–1059), and antisense primer 5′-GGA TGT GCC CCT TCT GCA AAA TCT-3′ (nucleotide 1355–1378).

The primers used for OTR mRNA detection were: sense primer 5′-GGG ACG TCA ATG CGC CCA AGG AA-3′ (nucleotide 2816–2838), and antisense primer 5′-ACC AAT AGA CAC CTA ATG CA-3′ (nucleotide 3921–3940).

A basic local alignment search tool (Altschul et al. 1990) was used to search all non-redundant databases (GenBank+EMBL+DDBJ+PDB) for sequence homology. No homology with any known products other than the actual receptors was found.

The amplified DNA fragments consisted of 342 bp (β2AR) and 375 bp (OTR). The exact identities of the PCR products were confirmed by sequencing. The relevant peaks were collected from the HPLC effluent and 1/100 volume 30% acetic acid was added to adjust pH. Following addition of 50 µg glycogen the PCR products were precipitated with an equal volume of isopropanol and were finally washed with 70% ethanol and dried. Sequencing was performed by the Applied Biosystems dRhodamin sequencing kit (Perkin Elmer, Allerød, Denmark) using Applied Biosystems ABI 310 apparatus for separation and fluorescence detection.

Using a Polymerase Chain Reaction-MIMIC construction kit (Clontech, Palo Alto, CA, USA) an internal 240 bp DNA standard was constructed (Engstrom et al. 1997).

The internal standard RNAs were constructed mainly as described by Faure et al. (1995). A composite primer, comprising 37 nucleotides of bacteriophage T7 RNA-polymerase promoter followed by the sequences of our usual sense primers, was used for amplification of the 240 bp DNA sequence by PCR. The resulting 277 bp product was re-amplified using our antisense primers and a primer consisting of the initial 23 nucleotides of the T7 RNA-polymerase promoter region. Following HPLC purification the re-amplified products were used for production of RNA by in vitro transcription (Riboprobe, Promega, Madison, WI, USA). The resulting RNA standards were quantitated by
UV detection at 260 nm (Gene-quant, Pharmacia, Stockholm, Sweden). Subsequently the RNA standards underwent reverse transcription (RT) in order to verify that the resulting products were indistinguishable from the internal DNA standard.

RT-PCR
Polyadenylated mRNA from homogenized rat myometrium was isolated using the PolyATrAct system 1000 (Promega) (Engstrøm et al. 1997). RT was performed in a mixture consisting of 250 µM dNTP, 40 U MMLV-RT (Promega), 31·2 U RNA-guard, 200 pmol antisense primer and 5 µl internal RNA standard in Promega RT-buffer. Standards represented 0·024 amol (OTR) or 27 cycles consisting of 45 s at 94°C, 1·5 min at 94°C for 2 min followed by 27 cycles consisting of 45 s at 94°C, 56°C for 45 s and 70°C for 2 min (OTR). After the last cycle the incubations continued for 5 min at 72°C followed by lowering of the temperature to 4°C. PCR products were used immediately or stored at −80°C. Quantitation of PCR products was carried out by means of HPLC using a TSK DEAE–NPR column (Engstrøm et al. 1997). Following chromatography, PCR products were UV detected at 254 nm. The areas of the 240 bp PCR products of the internal standards represented 0·024 amol (β2AR) and 0·050 amol (OTR) respectively. Hence the amounts of the 342 bp and the 375 bp PCR products could be quantitated relative to these standards and they were finally related to tissue wet weight. The average value of the uterine horns of each animal was calculated to represent the amount of specific mRNA.

Binding of [³H]oxytocin to isolated myometrial plasma membranes
Preparation of rat myometrial plasma membranes was performed by subcellular fractionation (Engstrøm et al. 1988b). Subsequent binding of [³H]oxytocin ([³H]OT) was carried out as previously described (Engstrøm et al. 1988a). Plasma membranes were incubated for 60 min with concentrations of [³H]OT varying from 0·42 to 24·20 nM (Amersham International, Hillerød, Denmark; specific activity 35·0 Ci/mmol). The assay was performed at room temperature and initiated by the addition of plasma membranes. At the end of the incubation period bound ligand was separated from unbound by filtration. Specific binding of [³H]OT was calculated by subtraction of binding in the presence of a 1000-fold excess of unlabeled OT and was finally related to protein content determined according to Lowry et al. (1951).

In vitro examination of contractile force of myometrial strips
In vitro contractility was measured basically as described previously (Engstrøm et al. 1997). One uterine horn was opened longitudinally and a middle segment measuring 5 mm was mounted in an isometric myograph placed in an organ bath containing Krebs–Ringer buffer. The tissue was pre-contracted with 50 mM KCl and the relaxing effect of isoproterenol over a range of 10⁻¹⁰ to 4 × 10⁻⁶ M was examined. The addition of isoproterenol was done in a cumulative manner to obtain increasing concentrations and phenolamine (Regitin, Ciba-Geigy, Basel, Switzerland) at a concentration of 1 µg/ml was present in the incubation medium to block adrenergic α-receptors. The effect of each dose of isoproterenol was allowed to level off before addition of the next. It was ensured that the contractile response to KCl in itself did not significantly diminish during the period of the experiment. To ensure that the relaxing effect of isoproterenol was not affected by OT potentially liberated from the endometrium, experiments in which OTR were blocked with atosiban in the organ chamber were also performed.

Contractile responsiveness to OT doses in the range of 0·63 × 10⁻¹⁰ to 0·13 × 10⁻⁶ M was measured on isolated strips mounted in Munsick’s buffer, pH 7·4 (Munsick 1960). Responses following isoproterenol or OT stimulation were expressed as a percentage of the potassium-induced contraction and were plotted against the logarithm of the agonist concentration.

Data analysis
A computer program (Fig.P., Biosoft, Cambridge, UK) was used for analysis of binding assays and contractile experiments. From curve fitting of myometrial responsiveness to isoproterenol and OT maximal relaxation and contraction respectively (Fmax) and the agonist concentration giving half this effect (EC₅₀) were calculated. Kruskal–Wallis one way analysis of variance on ranks was used to
compare groups. Pair-wise comparisons were done by means of Student’s t-test or the Mann–Whitney test.

RESULTS

RT-PCR

RT-PCR with the primers used resulted in highly specific PCR-products. Products of 240 bp represented internal standards for the two primer sets whereas the β2-AR and the OTR products consisted of 342 bp and 375 bp respectively. Sequencing of both strands of each receptor product confirmed the expected identities. The sequences conformed to the published sequences, GenBank accession number L39264 for β2AR and V15280 for OTR, with the following exceptions. In L39264 nucleotides 3588 and 3589 are both As whereas we found only one A in this position. Likewise in V15280 the nucleotide 374 is a single C whereas we found a sequence of CCC corresponding to this nucleotide position. Both of these variants occurred immediately downstream of the coding sequences and therefore did not influence the structure of the gene products.

The ratio between the receptor products and the internal standards showed sigmoid dependencies of the total poly-adenylated mRNA used for RT-PCR (Fig. 1). Subsequently mRNA amounts were selected to give ratios well within the linear parts of the curves.

RT-PCR amplification of mRNA and internal standard RNA showed log-linear dependencies of the cycle number in a range from 23 to 28 cycles (Fig. 2). The curves of mRNA and internal standard RNA RT-PCR products were parallel over this range.

By comparing areas of internal standard DNA after PCR and internal standard RNA after RT-PCR and taking into account the different number of chains we were able to calculate the efficiency of the RT. This was approximately 90%.

The concentration of specific receptor mRNA was in the range of amoles when expressed per mg wet tissue weight. Coefficients of variation for isolation of mRNA and RT-PCR HPLC were 6.7% for β2-AR and 14% for OTR.

OTR mRNA

Myometrial OTR mRNA results are shown in Fig. 3. Isoproterenol treatment for the periods indicated increased mRNA values (P=0.006). High dose isoproterenol treatment (10 µg/h for 4 days or 20 µg/h for 5 days) enhanced OTR mRNA levels 1.5 times more than low dose treatment (10 µg/h for 2 days). OT treatment tended to increase mRNA levels when compared with controls. However, this difference was not statistically significant.

β2AR mRNA

Neither isoproterenol nor OT treatment affected myometrial β2AR mRNA values. Myometrial β2AR mRNA levels were of similar magnitude as OTR mRNA (0.40±0.05 vs 0.40±0.04 amol/mg tissue when vehicles were compared).

Binding of [3H]OT to isolated myometrial plasma membranes

Isoproterenol treatment for 2 days (10 µg/h) increased maximal binding of [3H]OT to isolated myometrial plasma membranes by 91% (P=0.041, Table 1). Dissociation constants (Kd) were not affected by the treatment.

Contractile activity of isolated myometrial strips

Figure 4 shows dose–response curves of isoproterenol-induced relaxation of uterine strips from rats treated with isoproterenol or OT. Maximal relaxation
decreased following isoproterenol treatment ($E_{\text{max,iso}}$) for 2, 4 or 5 days ($P=0.004$, Fig. 5). When the osmotic mini-pumps were removed 12 h prior to decapitation of the animals, $E_{\text{max,iso}}$ returned to pre-treated levels. $EC_{50}$ values increased following isoproterenol treatment in general ($P=0.016$), except when mini-pumps were removed prior to decapitation.

OT treatment did not alter maximal isoproterenol-induced relaxation and $EC_{50}$ values were left unchanged.

Since part of the isoproterenol-induced $\beta_2$-desensitization theoretically could be a result of an augmented contractile tone due to endometrial OT liberation, relaxing dose–response curves were also obtained with OTR blocked by 100 $\mu$M atosiban (a competitive OT antagonist) in the organ chamber. Neither strips from isoproterenol treated nor those from untreated rats exhibited altered dose–response relations in the presence of atosiban (data not shown).

In Fig. 6 dose–response curves from OT-stimulated uterine strips are shown. Maximal contractile effect ($E_{\text{max,OT}}$) of OT increased following isoproterenol treatment ($106.3 \pm 5.5\%$ vs...
EC$_{50}$ values were left unchanged (0.54 ± 0.08 nM vs 0.80 ± 0.15 nM, $P=0.157$).

**DISCUSSION**

Desensitization to $\beta_2$-adrenergic agonists has been described in many tissues (Lefkowitz 1979, Dayes & Lye 1990, Herman Gnjidic et al. 1994, St Onge et al. 1994). Lohse et al. (1989) reported that inhibition of the $\beta_2$AR kinase prevented homologous desensitization of $\beta_2$ARs in human epidermoid cells. Using receptor binding analysis, Dayes & Lye (1990) found a reduction in $\beta_2$AR concentrations in non-pregnant myometrium within 1 h of isoproterenol challenge. In hamsters with hereditary cardiomyopathy, isoproterenol-stimulated adenylate cyclase activity was decreased but direct stimulation of the cyclase with forskolin or fluoride was intact.
suggesting that β-desensitization reflects perturbation of the receptor-mediated stimulation (St Onge et al. 1994). In the present study β₂-desensitization of non-pregnant estrogen-primed rat myometrium in terms of \( E_{\text{max,iso}} \) approximated 50%. The process was reversed when the agonist stimulation was withdrawn prior to decapitation of the animals. Since \( E_{\text{max,iso}} \) and EC₅₀ values of isolated uterine strips challenged with isoproterenol decreased and increased respectively following long-term infusion of isoproterenol, desensitization appears to involve both a reduction in the number of active receptors and impairment of the coupling between receptor occupancy and contractile response. Further our results showed that β₂AR mRNA values remained unchanged following long-term isoproterenol treatment in vivo. This suggests that myometrial β₂AR regulation does not occur at the level of gene expression unless an altered transcription rate following isoproterenol challenge is counter-regulated by changes in the stability of mRNA. Other authors have found both up- and down-regulation of β₂AR mRNA following isoproterenol challenge. In DDT₁ MF-2 hamster vas deferens cells in culture Hadcock & Malbon (1988) and Hadcock et al. (1989) observed a time- and dose-dependent decrease in β₂-adrenergic responsiveness following isoproterenol incubation. This was accompanied by a reduction in β₂AR mRNA levels. On the other hand, in similar cell cultures Collins et al. (1989) found that short-term exposure to

**FIGURE 5.** \( E_{\text{max}} \) and EC₅₀ values obtained from dose–response curves from isolated non-pregnant uterine strips challenged with isoproterenol. Rats were pre-treated with OT (0.5 µg/h, OT), isoproterenol for 2 days (10 µg/h, iso2), 4 days (10 µg/h, iso4), 5 days (20 µg/h, iso5) or vehicle. In one experiment the pumps were removed 12 h prior to decapitation from rats treated with isoproterenol for 4 days (10 µg/h, iso4rem). Values are means ± s.e.m., \( n = 3 \) or 4.

**FIGURE 6.** Dose–response curves of isolated non-pregnant uterine strips stimulated with OT. Rats were pre-treated with isoproterenol for 2 days (10 µg/h, ○) or vehicle (●). Points are means ± s.e.m., \( n = 4 \).
epinephrine stimulated the rate of β2AR gene expression resulting in steady state levels of mRNA 3-4 fold higher than in unstimulated cultures. In rat heart cell-line H9c2, isoproterenol exposure led to a 42% down-regulation of β2AR mRNA (Dangel et al. 1996). It thus appears that desensitization to β2-stimulants involves gene expression to a varying extent depending on the tissue and species involved.

Desensitization of the myometrium to OT is far less extensively studied than β2AR desensitization. We earlier reported that prolonged exposure to OT in vivo resulted in a decreased number of OT binding sites in the estrogen-primed non-pregnant rat myometrium and that this effect was associated with a reduced maximal contractile response to OT stimulation (Engstrom et al. 1988a). Since administration of the OTR antagonist atosiban increased the number of OTRs (Engstrom et al. 1988a), it is not likely that the desensitization process is a result of receptor occupancy per se but requires activation of the OTR. Similarly Lutz et al. (1992) found vasopressin V1 receptor internalization in rat smooth muscle only in response to agonist but not antagonist binding. In the present paper treatment with OT in vivo did not change OTR mRNA indicating that the previously described homologous myometrial desensitization to OT does not include regulation at the level of gene expression.

Heterologous receptor regulation has been described in different tissues. In rat myometrium Engstrom et al. (1988b) found that long-term OT treatment down-regulated bradykinin receptors when evaluated with radioligand binding studies. Likewise the up-regulation of several receptors by estrogen is well known (Soloff 1975, Potvin & Varma 1991, Mayes et al. 1996, Parker et al. 1996). We found that isoproterenol infusion in vivo induced OTR mRNA formation in the non-pregnant estrogen-dominated rat myometrium. This effect was accompanied by a 91% rise in the number of OTR binding sites and a 23% increased maximal response of isolated uterine strips to OT. Since OTR mRNA increased more than 200%, we suggest that only a fraction of available mRNA is processed further down-stream to form plasma membrane associated receptors. The discrepancy between E_{max, OT} of uterine strips and B_{max} of the receptor binding indicates that part of the newly synthesized OTR are spare receptors. Due to the unaltered values of EC_{50} and K_d neither receptor affinity nor the coupling between OTR occupancy and contractile response appears to be affected by isoproterenol infusion.

According to the above results β2AR desensitization in the myometrium is accompanied by an up-regulation of OTR. Thus the well-known tachyphylaxis to β2-adrenomimetics following β2AR stimulation may partly be due to an augmented contractile tone imposed by newly synthesized OTR.

The mechanism behind the induction of OTR by isoproterenol treatment in estrogen-primed rats remains unclear. It has been amply demonstrated that estrogen in itself up-regulates OTR mRNA (Breton et al. 1996, Ostrowski & Lolait 1996, Breton & Zingg 1997, Quinones Jenab et al. 1997) and the effect of isoproterenol in the present study may therefore appear on the basis of an altered biological half-life of estrogens. We have, however, treated our rats with a quite high pharmacological dose of estradiol benzoate. In this context it seems unlikely that a potential change in estrogen secretion rate or turnover, induced by isoproterenol, should affect the influence of administered estrogen on the OTR gene. Jeng et al. (1995) found that activation of cAMP by forskolin increased the number of OTR in rabbit amnion cells. Although cortisol did not alter the ability of isoproterenol to increase intracellular cAMP, and cAMP did not change the number of cortisol receptors, the increase in OTR was potentiated by cortisol. The authors suggested that cAMP affects steroid receptor–DNA interaction thereby increasing OTR gene expression. A similar mechanism may underlie the effect of isoproterenol on OTR in estrogen-primed rat myometrium. cAMP regulates transcription through the cAMP response element (CRE). CRE interacts with CRE binding protein (CBP). Smith et al. (1996) reported that CBP enhanced estrogen receptor transcriptional activity 10-fold. Likewise an increase in estrogen receptors may augment OTR expression via estrogen response elements located in the OTR gene.

In summary we have shown that neither homologous β2AR nor homologous OTR desensitization in non-pregnant rat myometrium involves changes in mRNA levels and therefore they appear to be post-transcriptional events. Long-term treatment with the β2-agonist isoproterenol induced formation of OTR mRNA, increased the number of OTR and augmented maximal response of isolated uterine strips challenged with OT. We suggest that β2AR desensitization in non-pregnant estrogen-primed rat myometrium involves modulation of OTR gene expression.

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