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The Effect of Pregnancy and Contractile Activity on Bladder Muscarinic Receptor Subtypes

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Abstract

In the rabbit bladder, pregnancy and prolonged bladder contractions decrease both muscarinic receptor density and contractile response, whereas newborns show enhanced muscarinic contractile response. Although the M₂ receptor predominates in rabbit bladder, we and others have shown that the affinity of a series of subtype selective muscarinic antagonists for inhibition of muscarinic agonist-induced contractions is most consistent with the pharmacologically defined M₃ receptor directly mediating smooth muscle contraction. Bladders from fetal rabbits, gravid rabbits, and male rabbits exposed to 4 hr of induced spontaneous contractions were used to determine whether changes in receptor density and contractility are due to a selective decrease in either the M₂ or M₃ muscarinic receptor subtype. To determine organ specificity, the heart and uterus were also studied. Gravid rabbits of 3 weeks' gestation and their fetal rabbits were studied. In male rabbits, bladder contractions were induced for 4 hr by ligating the catheterized penis at its base. Muscarinic receptor density and subtype distribution were determined by radioligand binding and immunoprecipitation. Receptor density was 24% lower in gravid bladder body, unchanged in gravid bladder base, 54% lower in gravid uterus, 115% higher in fetal bladders, and 34% lower after induced bladder contractions. Immunoprecipitation showed greater M₂ receptors than M₃ in all tissues studied, whereas M₁ and M₄ receptors were undetectable. There was no difference from control in the ratio of M₂ to M₃ receptor in any tissues except that a greater proportion of M₃ receptors was found in male vs. female bladders. Changes in contractile response to cholinergic stimulation in the gravid, fetal, and experimental detrusor instability model are associated with changes in total receptor density and not solely with changes in the M₃ receptor subtype that mediates bladder smooth muscle contraction.

Keywords

bladder; uterus; muscarinic receptor subtypes; immunoprecipitation; receptor regulation; pregnancy; detrusor instability

INTRODUCTION

The primary neurotransmitter involved in the mediation of micturition is acetylcholine through its actions on bladder smooth muscle cell muscarinic receptors [Wein et al., 1987,

1988; Yalla et al., 1988; Ruggieri et al., 1990]. Muscarinic receptors have also been found to mediate contractions of myometrial and many other smooth muscle cells [Eglen et al., 1989; Leiber et al., 1989]. Advancements in molecular biology have led to the cloning of five muscarinic receptor genes (M_1 – M_5) that are expressed in multiple tissues [Bonner et al., 1987; Guizzetti et al., 1995]. Receptor subtypes M_1 – M_4 have been found to exist in numerous tissues through the use of immunoprecipitation assays using muscarinic receptor subtype specific antisera as described by Luthin et al. [1988]. Muscarinic M_5 receptor mRNA has been detected in low levels in most brain areas [Wei et al., 1994] and comprises the major subtype in microglia [Ferrari-DiLeo and Flynn, 1995]. However, a clear functional protein correlate remains elusive. Numerous investigators documented the role of the M_3 receptor subtype in mediating contraction of bladder and myometrial smooth muscle [Leiber et al., 1989; Wang et al., 1995; Eglen et al., 1996] despite the finding that the M_2 receptors far outnumber M_3 subtypes in all smooth muscle and across all species studied to date, including human. The transduction mechanism by which these receptor subtypes mediate their effect is known to involve coupling to GTP binding proteins. The M_1 , M_3 , and M_5 subtypes preferentially couple to $G_{q/11}$ leading to increased inositol phosphates and thus increased intracellular calcium. The M_2 and M_4 subtypes couple primarily to G_i and G_o , which results in inhibition of adenylyl cyclase and has been shown to contribute to bladder contractions only in the denervated bladder [Braverman et al., 1998] or under in vitro conditions of increased intracellular cAMP [Hegde et al., 1997; Braverman and Ruggieri, 1999].

In the rabbit bladder, pregnancy decreases both muscarinic receptor density and response to muscarinic stimulation [Zderic et al., 1990a; Levin et al., 1991], whereas newborn rabbit bladders show an increased contractile response but a muscarinic receptor density similar to the adult [Zderic et al., 1990b]. Furthermore, 4 hr of experimentally induced uninhibited bladder contractions decreases muscarinic receptor density and contractile response to both carbachol and field stimulation [Levin et al., 1988a; Kitada et al., 1989]. The current study reports on densities of muscarinic receptor subtypes in these models to determine whether these previously reported changes in bladder contractile response to muscarinic agonists are due to selective changes in one of the muscarinic receptor subtypes found in the bladder. The uterus was also studied since it is another smooth muscle organ that is dramatically affected by the hormonal effects of pregnancy.

MATERIALS AND METHODS

Materials

The following drugs and chemicals were obtained from the sources as indicated: tris (tris-hydroxymethyl amine), EDTA (ethylenediamine tetra-acetic acid), atropine, fine-grade Sephadex G-50, goat antimouse IgG-agarose, sodium cholate, pepstatin, leupeptin, soybean and lima bean trypsin inhibitors, aprotinin, and alpha-2 macroglobulin (Sigma Chemical Co., St. Louis, MO), pansorbin cells (Calbiochem, La Jolla, CA), digitonin (Gallard-Schlesinger Industries, Carle Place, NY), [3H]Quinuclidinyl benzilate (QNB, 43 Ci/mmol) (Dupont-New England Nuclear Research Products, Wilmington, DE), [3H]N-methyl scopolamine (NMS, 79.5 Ci/mmol) (Amer-sham Inc., Arlington Heights, IL), number 30 glass fiber filters (Schleicher & Schuell, Keene, NH), biosafe II scintillation cocktail (Fisher Scientific, Pittsburgh, PA). New Zealand White rabbits were obtained from HRP (Denver, PA): 3-week gravid, nulliparous females, and male rabbits, all 6 months old and weighing 4–5 kg.

Experimental Detrusor Instability

Male rabbits were sedated (25 mg/kg ketamine, 6 mg/kg xylazine, i.m.), and catheterized per urethra using a 8-French Foley catheter with the balloon filled with 1.5 ml of saline. The bladder was emptied and the catheter was connected in line to a Gould pressure transducer interfaced with a Grass polygraph (Grass Instruments, Quincy, MA) for continuous bladder pressure monitoring. To induce bladder contractions, the proximal portion of the catheterized penis was ligated with 2-0 silk as close as possible to the pubic symphysis. The bladder was filled with saline using a 120-ml syringe infusion pump (Stoelting, Wood Dale, IL) at 0.5 mL/min until 30 mL had been infused. At this point, >80% of the preparations with the penile ligature show significant spontaneous bladder contractile activity (detrusor instability). Preliminary studies showed that this intravesical volume induces the largest amplitude and frequency of spontaneous bladder contractions. These rhythmic contractions are stable for >4 hr during which supplemental doses of ketamine/xylazine are administered as required to maintain sedation. This procedure was repeated on a sufficient number of rabbits until bladders were obtained from a total of five rabbits that displayed these rhythmic bladder contractions. Time to bladder removal varied from 180 to 360 min after the start of contractions. The initial intent was to induce bladder contractions for the same duration (4 hr or 240 min) in all animals. Two of the animal had to be killed before 240 min (after only 180 and 220 min) because they awoke from the anesthesia. One animal underwent 6 hr of these induced bladder contractions to get an idea of whether increasing the duration of contractions would have a greater effect on receptors. The other two animals were killed after 4 hr of induced contractions as planned. Five sham control male rabbits were also sedated, catheterized, and their bladders infused with 30 mL of saline for 4 hr as described but without penile ligation. None of these animals showed any bladder contractions during this time.

Tissue Preparation

All rabbits were killed by cervical dislocation, the urinary bladder was removed and separated at the ureteral orifices into bladder base and body. All tissues were frozen on dry ice and stored at -80°C for <6 weeks prior to assay. Each tissue was weighed, thawed, and minced at 4°C in 100 mg/mL of TE buffer (10 mmol Tris, 1 mmol EDTA at pH 7.5) and 10 $\mu\text{g/mL}$ of each of the following protease inhibitors: pepstatin, leupeptin, aprotinin, trypsin inhibitors from soy and lima bean, and alpha 2-macroglobulin. The tissue then was homogenized with a Brinkman Polytron at maximum setting with three short bursts (3 sec) with 30 sec of cooling on ice between each burst.

Radioligand Binding

Homogenates were centrifuged at 20,000g for 30 min and resuspended in 100 mg/mL of TE buffer. For each binding assay, 200 μL of homogenate and 700 μL of TE buffer were each added to six varying concentrations of [^3H]QNB (43 Ci/mmol) ranging from 31 pmol to 2 nmol. For the detrusor instability tissue, binding of [^3H]NMS (79.5 Ci/mmol) at a concentration of 2 nmol was used to determine maximal binding. For each concentration of radioligand, atropine (10 μmol) was used to determine nonspecific binding. After 2–3 hr of incubation at 32°C , each sample was quenched with 5 mL of ice cold 10 mmol Tris and 150 mmol NaCl at pH 7.5. The samples were filtered under mild vacuum over glass fiber filters and washed twice with 5 mL of buffer and the filters were counted in 4 mL of scintillation cocktail. Protein concentrations were determined using the method of Lowry et al. [1951].

Immunoprecipitation

All immunoprecipitations were performed in triplicate using antisera to the M_1 , M_3 , and M_4 muscarinic receptor subtypes as characterized in detail previously [Luthin et al., 1988; Wang

et al., 1995]. The anti-M₂ receptor antibody (31-ID1) was a generous gift from Neil M. Nathanson (University of Washington, Seattle, WA). All presented data were corrected for each antibody efficiency [Wang et al., 1995].

Analysis of Data

Results are triplicate determinations performed on three to five separate animals except for the gravid bladder base where pooled tissues from four animals were necessary to perform two sets of triplicate determinations. Fetal bladder and heart required pooling of all tissues to obtain sufficient tissue for one triplicate determination. Results of triplicate determinations were treated as single datum points and presented as mean values $\pm$ standard error of the mean (SEM). Saturation isotherms were transformed using the method of Scatchard [1949]. K_d and B_{max} values were estimated by using unweighted linear regression analysis of the transformed data. Data were analyzed for statistical significance by one factor analysis of variance with post hoc Fisher's least-significant difference test for pairwise comparisons. When only two groups were compared, a two-tailed, independent Student *t*-test was used (i.e., when comparing the M₂:M₃ ratio between male and female bladder body). *P* values of 0.05 were considered to be statistically significant.

RESULTS

There was no significant difference in bladder body wet weight between female control bladders (1.57 ± 0.16 g) and gravid bladders (1.60 ± 0.40 g).

Radioligand Binding

Figure 1 shows total muscarinic receptors in gravid bladder body to be 24% less, whereas fetal bladder is 115% greater than adult female control bladder ($P < 0.05$). The affinity was unchanged (control, 0.12 ± 0.05 nmol; gravid, 0.63 ± 0.28 nmol; fetal, 0.04 nmol). Although total receptors in the gravid bladder base were 41% less, this was not statistically different from control bladder. Again, the affinities were not statistically different (gravid, 0.19 ± 0.08 nmol; control, 0.09 ± 0.004 nmol). Figure 2 shows that pregnancy induced a 54% reduction in muscarinic receptor density in another smooth muscle, the uterine fundus, however, this decrease did not reach statistical significance. As determined by Scatchard analysis, muscarinic receptor affinity for control (0.12 ± 0.08 nM) and gravid (0.14 ± 0.04 nM) uterus were not statistically different. Receptor density in the heart ventricle remains unchanged by pregnancy (gravid, 57 ± 4.6 fmol/mg; control, 50 ± 1.6 fmol/mg). Fetal heart receptor density, however, was 147% greater than control (125 fmol/mg, $P < 0.05$). The affinities, however, were not significantly different for the gravid (0.15 ± 0.01 nM), fetal (0.21 nM) or control heart tissues (0.29 ± 0.15 nM).

Induced Detrusor Instability

After ligation of the penis, bladder contractions started 58 ± 35 min later, and then continued for an average of 248 min (range, 180–360 min). Contractions occurred at a frequency of 0.72 ± 0.16 per minute, with amplitudes of 7.61 ± 0.57 cm of H₂O. Figure 1 shows that these experimentally induced in vivo bladder contractions resulted in a 34% reduction in receptor density ($P < 0.05$) with no change in affinity (0.70 vs. 0.72 nM). No statistical correlation between contraction frequency, amplitude or duration, and muscarinic receptor density was found in these five rabbits.

Immunoprecipitation

Figures 3 and 4 represent immunoprecipitation data expressed as a percentage of total solubilized receptors determined by desalting over Sephadex G50 columns. Antisera to the

M₁ and M₄ muscarinic receptors resulted in no detectable immunoprecipitation (data not shown). The relative distribution of M₂ and M₃ receptors do not statistically change for each of the gravid, fetal, or detrusor instability organ tissues, when compared to their respective controls. The M₂:M₃ ratio for the female rabbit bladder bodies, however, was found to be statistically greater than that for the male rabbit bladders (5.5 ± 1.8 vs. 2.6 ± 0.9 , $P < 0.001$).

DISCUSSION

The density of autonomic receptors including muscarinic receptors can be regulated by modifying the degree of receptor activation [Ruffolo and Kopia, 1986; Axelrod, 1983]. Muscarinic receptor agonist administration or increased neuronal stimulation decreases muscarinic receptor density and decreases responsiveness to agonists [Levin et al., 1988a; Kitada et al., 1989]. Conversely, muscarinic receptor antagonist administration increases muscarinic receptor density [Levin et al., 1988b; Lee and Wolfe, 1989; Cawley et al., 1993]. Pregnancy was chosen as a model for studying hormonal regulation of muscarinic receptors, while experimental detrusor instability was chosen as a model of neuronal regulation in the same tissue.

Fetal Bladder

One-day and 1-week-old rabbit bladders compared to mature rabbit bladders were shown to generate intravesical pressures 40% higher in response to carbachol stimulation and 266% higher in response to ATP [Keating et al., 1990]. In studying 3-week gestation fetal whole bladders, we observed a 115% greater muscarinic receptor density in the present study. This increased receptor density in near term fetal rabbits correlates with the increase in pressure responses of newborn animals. Only the M₂ and M₃ receptor subtypes can be immunoprecipitated from the urinary bladder across species [Wang et al., 1995]. In fetal bladder, immunoprecipitation assays demonstrated M₂ as the predominant receptor that did not differ with the increased age of the control bladders. As reported by us and others [Luthin et al., 1988; Lee et al., 1989; Dorje et al., 1991; Wang et al., 1995] the total percentage of precipitated receptors did not add up to 100%.

Gravid and Nulliparous Bladder

Hegde et al. [1997] demonstrated that both M₃ and M₂ receptors can mediate rat bladder contractions in vitro and possibly mediate reflex bladder contractions in vivo. They proposed that M₃ receptor activation primarily causes direct detrusor contraction whereas activation of the M₂ receptor results in indirect reversal of sympathetically mediated relaxation. During pregnancy the human urinary bladder undergoes many functional changes. These changes include an increase in residual volume, decrease in bladder tone, and increase in urinary stress incontinence. It may be possible to extrapolate our findings of decreased bladder muscarinic receptors in the gravid rabbit and the previously detailed decreased muscarinic contractile response [Zderic et al., 1990a; Levin et al., 1991] to human pregnancy. Therefore, the hormonal effects of pregnancy cause an equal decrease of both bladder muscarinic receptor subtypes, rather than one subtype, and this may be partly responsible for the clinical urologic findings seen in human pregnancy.

However, in each of the bladder tissues studied the exact contribution of each receptor subtype to bladder muscle contractility is not clear. Determining whether the M₃ or M₂ receptor is the dominant receptor mediating bladder contraction in these models is beyond the scope of this report and requires further functional pharmacologic study. Recent reports in the literature, however, suggest that the M₃ receptor is primarily responsible for normal bladder contraction [Wang et al., 1995] via metabolism of phosphoinositide as a result of receptor-G protein coupling. We showed that the M₂ receptor subtype contributes to

contractions in the denervated rat bladder [Braverman et al., 1998]. In the normal rat bladder an M₂ contribution to contraction can only be demonstrated after raising intracellular cAMP with isoproterenol and alkylating the majority of the M₃ subtypes [Eglen et al., 1996; Hegde et al., 1997; Braverman and Ruggieri, 1999].

Gravid and Nulliparous Heart

Only the M₂ receptor subtype was precipitated in fetal, gravid, and control heart. Similar to the urinary bladder, the fetal heart was found to contain significantly greater density of muscarinic receptors than either nulliparous controls or gravid adult rabbits. The physiologic significance of this increased heart muscarinic receptor density is not clear. Whether this increased fetal muscarinic receptor density is only found in tissues that contain a predominance of the M₂ subtype (bladder and heart) or is increased in all muscarinic receptor containing tissues awaits further investigation.

Gravid and Nulliparous Uterus

We confirmed that the M₂ receptor predominates (55–60%), with a small number of M₃ receptors (6–8%). However, no detectable precipitation was measured for M₁ or M₄ receptors in our series. We found a 54% decreased total muscarinic receptor density in gravid uterus. This may contribute to suppression of uterine smooth muscle contractions until the time of labor, which is essential for a successful pregnancy. As in bladder tissue, the nulliparous and pregnant uterus retained the same relative distribution of M₂ and M₃ muscarinic receptor subtypes. It appears, then, that pregnancy does not cause a selective decrease of a specific muscarinic receptor in smooth muscle, but rather a global and proportional reduction in both muscarinic receptor subtypes.

Effects of Detrusor Instability

By placing a ligature around the base of the catheterized penis, we were able to occlude the urethra without occluding the monitoring catheter within it. In this fashion we produced spontaneous bladder contractions in a similar frequency and amplitude as obtained by others [Kitada et al., 1989]. These induced bladder contractions may represent detrusor instability or reflex bladder contractions and could be the result of a selective increase in efferent discharge to the bladder. These spontaneous contractions were reported to decrease contractile response and muscarinic receptor density [Kitada et al., 1989]. We confirmed that 4 hr of spontaneous bladder contractions cause a reduction in total muscarinic density with no change in the M₂:M₃ ratio. Therefore, increased efferent nerve stimulation of the bladder causes a global rather than a selective muscarinic subtype decrease. A sex difference was found in the M₂:M₃ ratio, with a higher proportion of M₃ receptors present in the male bladders. Although the physiologic significance of this sex difference is not clear it may reflect an increased bladder contractility required to overcome a higher outlet resistance in the male imposed by the both the prostate and the greater length of the urethra. The M₂:M₃ ratio in the sham male rabbit bladder is identical to previously published data by our group [Wang et al., 1995] regarding normal male rabbit bladders. Therefore, it is unlikely that catheterization in the sham animals produced any significant amount of bladder distention or spontaneous contractions, even in the face of no overt hyperreflexia, to account for this finding.

CONCLUSION

We showed that previously noted changes in contractile response to carbachol stimulation in the urinary bladder attributed to changes in muscarinic receptor density are not due to selective regulation of a specific muscarinic subtype. Mounting reports in the literature suggest that the M₃ receptor is directly responsible for contraction of the normal bladder.

Further investigations are needed to determine the interaction between M₂ and M₃ receptors in bladder smooth muscle contractility.

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Fig. 1.

Maximal specific [^3H]QNB binding to membranes from nulliparous female control bladder body, gravid bladder body, whole bladder from pooled rabbit fetuses (mixed sexes), control female bladder base, gravid bladder base, male sham bladder body and male detrusor instability bladder body. *Significantly different from appropriate control using one factor analysis of variance with post hoc Fisher's least significant difference test for pairwise comparisons.

Fig. 2.

Receptor density for control uterus, gravid uterus, control heart, gravid heart and fetal heart.

*Significantly different from appropriate control using one factor analysis of variance with post hoc Fisher's least significant difference test for pairwise comparisons.

Fig. 3.

Distribution of muscarinic receptor subtypes in bladder tissues. Receptors were labeled with [^3H]QNB and solubilized as described previously [Luthin, 1988]. Data shown are converted to percentage of total solubilized receptors as determined by desalting over Sephadex G-50 minicolumns. The levels of M_1 , M_4 , and M_5 receptor were not determined since these were undetectable in previous studies of bladder tissue from several species including rabbit [Wang, 1995].

Fig. 4. Distribution of muscarinic receptor subtypes in uterus and heart tissues. The levels of M_1 and M_4 receptors were undetectable in these tissues.