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MUSCARINIC RECEPTOR SUBTYPES IN NORMAL, FETAL, AND GRAVID RABBIT BLADDER, HEART AND UTERUS

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Abstract

In the rabbit bladder, pregnancy has been shown to induce a significant decrease in both muscarinic receptor density and response to muscarinic stimulation. Neonatal rabbit bladders have a high muscarinic receptor density and contractile response to bethanechol stimulation. The bladders from 7 gravid rabbits, 7 age-matched virgin controls, and 32 fetal rabbits of 3 week gestation were studied. Compared to control tissue, filtration binding demonstrated receptor density to be 24.3% lower in gravid bladder dome, 41.2% lower in gravid bladder base, and 114.8% higher in fetal bladders. While total receptor density was not different from control in gravid heart, fetal hearts showed a 2.5 fold increased receptor density. There was also a 61% reduction in muscarinic receptor density in the gravid uterus.

Immunoprecipitation assays using muscarinic receptor subtype specific antisera were used to measure the relative levels of m1, m2, m3 and m4 receptors. The m2 receptor was the predominant subtype in the bladder and uterus, and the only subtype detected in rabbit heart. The m3 receptor protein was also present, but in lower levels in the bladder and uterus. The m1 and m4 receptors were not detected in any of the tissues studied. Furthermore, the relative percent of each receptor did not statistically change for the gravid or fetal rabbit bladder, uterus, or heart, when compared to its control. Differences in the contractile response to cholinergic stimulation of the gravid bladder and uterus, and of the fetal bladder then, can be attributed to changes in muscarinic receptor density and not to changes in receptor subtype.

INTRODUCTION

In the gravid rabbit, pregnancy has been shown to induce a significant (50 percent) decrease in both muscarinic receptor density and response to muscarinic stimulation (1). Neonatal rabbit bladders show a marked, yet stable muscarinic receptor density and contractile response to bethanechol and field stimulation (2). To understand the mechanism of these changes in muscarinic response, we studied the muscarinic receptor subtypes and receptor densities in the gravid, virgin, and fetal rabbit bladder. Studies were also carried out on the hearts of these animals since this organ has a similar muscarinic receptor subtype distribution as the bladder. The uterus was also studied since it is another smooth muscle organ that is dramatically affected by pregnancy.

Molecular cloning studies have identified five muscarinic receptor genes (m1–m5) that are expressed in multiple tissues (3). By using purified receptors from pig heart (m2), fusion proteins of the nonconserved segments of the third intracellular loop (m1), or c-terminal regions (m3 and m4) of these genes as antigens, subtype specific antisera have been developed, and were used here in immunoprecipitation assays (4,5). These assays provide a direct measure of the amount of the molecule directly involved in transducing the neurotransmitter signal (the receptor protein) and not merely the amount of mRNA for the receptor protein, which often does not correlate at all with amounts of receptor protein. In

addition, these immunoprecipitation assays avoid reliance on muscarinic receptor subtype selective drugs which only provide a limited degree of subtype selectivity.

MATERIALS AND METHODS

All chemicals were of analytic grade. Tris (tris-hydroxymethylamine), EDTA (ethylenediaminetetra-acetic acid), atropine, fine grade Sephadex G-50, goat antimouse IgG₁-agarose, and sodium cholate were purchased from Sigma Chemical Company (St. Louis, MO). Pansorbin cells were obtained from Calbiochem (La Jolla, CA). Protease inhibitors, pepstatin, leupeptin, soybean and lima bean trypsin inhibitors, aprotinin, and alpha-2 macroglobulin were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Digitonin was purchased from Gallard-Schlesinger Industries (Carle Place, NY). [³H] Quinuclidinyl benzilate (QNB, 43Ci/mMol) was purchased from Dupont-New England Nuclear Research Products (Wilmington, DE). Number 30 glass fiber filters were from Schleicher & Schuell (Keene, NH). Biosafe II scintillation cocktail was from Fisher Scientific (Pittsburgh, PA). Antisera to the m1-i₃ loop, m2-i₃ loop, m3-c terminal, and m4-c terminal receptor subtypes have been previously described (4,5) and were a generous gift from Dr. Gary R. Luthin (Hahnemann University, Philadelphia, PA). Tissue from six month old age-matched virgin controls and three-week gravid New Zealand White rabbits (4 to 5 kg weight), as well as from their three-week fetuses were used (HRP, Denver, PA).

Tissue Preparation

The bladder body and base, heart ventricle, and uterine fundus of 7 gravid rabbits and 7 age-matched virgin controls (6 month-old), and the whole bladders and hearts of their 32 fetuses were studied by immunoprecipitation and radioligand filtration binding assays. Each rabbit was euthanized by cervical dislocation, followed by the removal of the urinary bladder, uterus, and heart. The bladder was removed in its entirety proximal to the urethra, and separated at the level of the ureteral orifices into a bladder base and dome. From each 21 day-old fetal rabbit the whole urinary bladder and heart were removed and used for study. Each tissue was then frozen on dry ice and stored at -80 °C.

Immunoprecipitation Assay

Immunoprecipitations were performed each time in triplicate, as previously described by Luthin et. al. (5). Each tissue was weighed, thawed and minced at 4 °C in 100mg/ml of TE buffer (1mM Tris, 1mM EDTA at pH 7.5) and 10ug/ml of each protease inhibitor (PIC; pepstatin, leupeptin, aprotinin, trypsin inhibitors from soy and lima bean, and alpha 2-macroglobulin). The tissue was homogenized with a Brinkman Polytron homogenizer by two-ten second bursts at setting 10, with 2 minutes of cooling between bursts. Membranes were incubated with ³H-QNB (1.5nM) at 25 °C for 30 minutes and then centrifuged at 20,000 X g for 10 minutes at 4 °C. To solubilize the receptors, the pellet was resuspended in 1% TEDC (1mM Tris, 1mM EDTA, 1% digitonin, and 0.2% cholate) and PIC (100ug/ml) and incubated for 45 minutes at 4 °C. The homogenate was then centrifuged at 30,000 X g for 30 minutes at 4 °C and the supernate collected and transferred in 450ul volumes to microfuge tubes. 50 ul of muscarinic antisera (m1-m4) at their optimized concentration was added to the solubilized receptors. They were then incubated at 4 °C for 16-18 hours by continuous inversion on a Scientific Industries Rotator. Bound and free ligand were separated by desalting the 500ul samples over 3 ml Sephadex G-50 columns (0.7x13cm) that were equilibrated with 4ml of 0.1% TEDC buffer. Bound ligand was eluted using 1.5ml of buffer, and the receptor-antibody complexes were precipitated using pansorbin (m1, m3 and m4 receptors) or goat anti-mouse IgG₁ beads (m2 receptors). Each precipitate was then counted at 28% efficiency in 15ml of Bio-safe II scintillation cocktail, using a Beckman LS 5000TA scintillation counter. By using clonal cell lines transfected with each of the

muscarinic receptor subtypes, the relative efficiencies of the m1, m2, m3 and m4 antibodies to precipitate receptors were determined to be 100%, 75%, 85%, and 100% respectively. All data were corrected accordingly for antibody efficiency.

Radioligand binding assay

Each frozen tissue was weighed, thawed and minced at 4 °C in 100mg/ml of TE buffer (pH 7.5) then homogenized using two–ten second bursts on a Brinkman Polytron homogenizer. Homogenates were centrifuged at 20,000 X g for 30 minutes and resuspended in 100mg/ml of TE buffer. For each binding assay performed in triplicate, 200ul of homogenate and 700ul of TE buffer were each added to six varying concentrations of ³H–QNB (43 Ci/mMol) ranging from 31pM to 2nM. For each concentration of radioligand, atropine (10uM) was used to determine nonspecific binding. After 120 minutes of incubation at 32 °C, each sample was quenched with 5 ml of ice cold TEN (TE, 150mM NaCl at pH 7.5). The samples were filtered slowly under mild vacuum (1 ml/second) over glass fiber filters (#30, Schleicher & Schuell, Keene, NH) and washed twice with 5ml of TEN. Filters were counted at 28% efficiency in 4ml of Bio–safe II scintillation cocktail (Fisher Scientific). Protein concentrations of heart, bladder, and uterine membrane fractions were determined using the method of Lowry (6).

Analysis of data

The results shown in Figures 1–4 are representative triplicate determinations performed on 3 to 5 separate animals, except for the fetal tissues, where only one triplicate determination could be performed on the tissues pooled from the 32 animals. Saturation isotherms were transformed using the method of Scatchard (7). Kd and Bmax values were estimated by using unweighted linear regression analysis of the transformed data. Unless otherwise stated, all data were analyzed by analysis of variance or a two–tailed Student's t–test, where appropriate. P values < 0.05 were considered to be statistically significant.

RESULTS

No significant difference was noted in bladder dome wet weight between control bladders (1.57±0.16gm) and gravid bladders (1.60±0.40gm). Figure 1 presents filtration binding saturation curves that show gravid bladder dome maximal binding decreased by 24.3% (P<0.001) and increased in fetal bladder by 114.8% (P<0.001), with gravid Bmax at 38±1.2 fMol/mg, fetal at 109 fMol/mg, and control at 56±7.6 fMol/mg. Scatchard analysis is also presented, demonstrating essentially unchanged dissociation constants, with gravid bladder Kd at 0.09±0.01 nM, fetal at 0.04nM, and control at 0.12±0.07 nM. Figure 2 presents filtration binding for the bladder base, with 41.2% fewer muscarinic receptors in gravid bladder (33±13.2 fMol/mg; P=0.005) as compared to control bladder (56±7.6 fMol/mg). In the bladder base Kd was found to be statistically different (gravid at 0.19±0.12 nM and control at 0.09±0.01 nM; P<0.001).

Figure 3 demonstrates the effects of pregnancy on muscarinic receptor density in another smooth muscle model, the uterine fundus. A 61% reduction in density is demonstrated (15±11.3 fMol/mg vs. 39±19.6 fMol/mg; P=0.02). Pregnancy also significantly altered uterine muscarinic receptor Kd (control Kd at 0.04±0.01 nM and gravid Kd at 0.14±0.05 nM; P<0.001). Figure 4 shows that heart ventricle muscarinic receptor density remains unchanged by pregnancy, with gravid Bmax at 57±6.5 fMol/mg and control at 50±2.3 fMol/mg. Fetal heart receptor density, however, is 147% greater at 125 fMol/mg (P<0.001). The affinities, however, are not significantly different for the gravid (0.15±0.01 nM), fetal (0.21 nM) or control heart tissues (0.29±0.21 nM).

Table 1 represents the relative distribution of muscarinic receptor subtypes in the gravid and control virgin bladder dome and base, heart ventricle and uterine fundus, and in the whole fetal bladder and heart by fMol receptor precipitated per assay and by percent of total receptor determined by desalting over Sephadex G50 columns. The relative distribution of m2 and m3 receptors did not statistically change ($p>0.05$) for each of the gravid or fetal organ tissues, when compared to their respective controls. Thus, pregnancy induced changes in muscarinic receptor density in the bladder dome and base and the uterine fundus can be attributed to an overall and proportional down regulation of all muscarinic receptors detected, rather than of a specific subtype. Furthermore, differences in fetal muscarinic receptor density and responses to cholinergic agonists are then not attributable to a changes in receptor subtype.

DISCUSSION

High affinity estradiol and progesterone receptors have been clearly demonstrated in the female rabbit bladder, urethra, and uterus, with receptor density greatest in the uterus (8–10). To study the effect of these hormones on the autonomic function of the bladder, most prior studies have used exogenously administered estrogen, progesterone, or ovariectomy (11–15). The generated data has been conflicting, yet clearly shows an alteration in the physiology and pharmacology of the bladder (11–15). When given chronically, which more closely resembles a pregnancy model, when estradiol is administered by a subcutaneous osmotic pump to adult female rabbits over 3 weeks, there is a significant decrease in bladder body muscarinic receptor density, and no change in the bladder base or urethra (13). In contrast, when estradiol is given for 4 days, twice daily by injection at super physiological levels, dome and mid bladder muscarinic receptor density increases, while bladder base stays unchanged (12).

The hormonal changes of pregnancy induce changes in the physiology and pharmacology of the rabbit bladder. Despite no significant change in gravid bladder weight or in-vitro bladder capacity, pregnancy induced a 35% decrease in muscarinic receptor density, based on radioligand binding (34 fMol/mg vs 22), a 50% reduction in response to bethanechol, and a markedly increased response to ATP (16). Our data closely mirrors and reconfirms that pregnancy induces muscarinic receptor down regulation in the bladder, as well as in the uterus. These changes were again in the absence of change in bladder dome weight, as noted by others (13). This decrease in muscarinic receptor density can logically explain the functional decrease to bethanechol stimulation.

Fetal Bladder

1 day and 1 week old rabbit bladders compared to mature rabbit bladders have been shown to generate intravesical pressures 40% higher to bethanechol stimulation and 266% higher to ATP (17). With our 3 week gestation fetal whole bladders, we observed a 115% greater muscarinic receptor density. This increased density can explain the increased generated pressures. This has been confirmed by others, where muscarinic receptor density, response to cholinergic agonists, and bladder cholinergic innervation are present at high levels at birth (4 weeks gestation) and remain stable with increasing age, up to 15 weeks of life (18). In addition, neonatal bladder responses to ATP, serotonin, histamine and substance P were found to be 2.5 to 4.8 fold greater than in adult bladders (2). Nonadrenergic, noncholinergic neurotransmitters appear to play a greater role than once thought in the control of fetal and neonatal bladder function. Immunoprecipitation assays demonstrated m2 as the predominant receptor in fetal bladder, and to lesser degree m3. The distribution of fetal bladder subtypes did not differ with increased age of the control bladders. Based on prior studies in multiple species, only the m2 and m3 receptor subtypes have been isolated in the bladder, and we so limited our immunoprecipitation assays on bladder (19).

As others (4), the percent of precipitated receptors did not add up to 100 percent in all tissues studied. One possible explanation is that our data were not adjusted for nonspecific binding. Preliminary immunoprecipitation assays on rabbit bladders were performed in the presence of 1 μ M atropine, and could only account for between 1.2 to 4.5 percent nonspecific binding thus these nonspecific binding assays were subsequently not routinely performed. The antisera precipitation efficiencies for the muscarinic receptors subtypes were determined on transfected clonal cell lines which may not accurately reflect the precipitation efficiencies in rabbit tissue.

Gravid and Virgin Bladders

In general, bladder emptying is regulated pharmacologically primarily by muscarinic and purinergic mechanisms. Muscarinic cholinergic stimulation accounts for 60% of in-vitro whole bladder pressure generation in response to field stimulation and 40% by purinergic stimulation. However, 90% of in-vitro bladder emptying to field stimulation is due to muscarinic cholinergic receptors. (20).

The density of most autonomic receptors including muscarinic receptors can be regulated by modifying the degree of receptor activation (21,22). Following prolonged administration of a muscarinic receptor agonist or increased neuronal stimulation, there is a decreased muscarinic receptor density, and thus decreased response to agonists. Conversely, following prolonged muscarinic receptor antagonist administration there is an associated increase in muscarinic receptor density (23–26). As has been previously described by our laboratory and others, m2 and m3 receptors are present in the urinary bladder in multiple species (19). In the pregnant human the bladder undergoes many functional changes, among them an increase in residual volume, decrease in bladder tone and increase in urinary stress incontinence. The decreases in muscarinic receptor density in both the rabbit bladder dome and base by 24% to 41%, and the previously detailed decreases in gravid bladder contractile response to bethanechol (16), help to explain these clinical findings.

Heart

Similar to the bladder, the fetal heart was found to contain significantly greater density of muscarinic receptors than either virgin controls or gravid adult rabbits. The physiologic significance of this increased receptor density in the heart, as in the bladder is not clear. Whether this increased fetal muscarinic receptor density is only found in tissues such as the heart and bladder that contain a predominance of the m2 subtype or is increased in all muscarinic receptor containing tissues awaits further investigation.

Uterus

There is some controversy as to which muscarinic receptors are present in the uterus. Dorje et. al. (4) demonstrated by immunoprecipitation assays that the rabbit uterus predominantly has m2 (65%), m4 (15%), and to a lesser degree m3 (4.5%) receptors. We also clearly demonstrated in our rabbits that the m2 receptor predominates (55 to 60%), with a small number of m3 receptors (6.6 to 7.9%); m1 and m4 receptors were not detected in our series. With total receptors precipitated totalling only 68%, it is possible that other receptor subtypes are present (e.g. m4 or m5), but were not detected. We found a 61% decreased muscarinic receptor density in gravid uterus. This may offer an explanation of how smooth muscle uterine contractions are suppressed until the time of labor, which is essential for a successful pregnancy. As in bladder tissue, the virgin 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 down regulation of a specific muscarinic receptor in smooth muscle, but rather a global and proportional reduction in all muscarinic receptor subtypes.

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FILTRATION BINDING BLADDER DOME MEMBRANES

Figure 1.

Representative saturation isotherms of specific [^3H] QNB binding to bladder dome membranes from gravid and virgin control rabbits and to whole bladder membranes from pooled rabbit fetuses. The Scatchard replot of these data is shown in the inset.

Figure 2.
Representative saturation isotherms and Scatchard replots of specific [^3H] QNB binding to bladder base membranes from gravid and virgin control rabbits.

FILTRATION BINDING UTERINE MEMBRANES

Figure 3.
Representative saturation isotherms and Scatchard replots of specific $[^3\text{H}]$ QNB binding to uterine fundus membranes from gravid and virgin control rabbits.

Figure 4.
Representative saturation isotherms and Scatchard replots of specific [^3H] QNB binding to heart ventricle membranes from gravid and virgin control rabbits and to whole heart membranes from pooled rabbit fetuses.

TABLE 1

Distribution of Muscarinic Receptors Subtypes

Rabbit Tissue	Total Receptors (fMol/assay)	m1	m2	m3	m4
Bladder Dome					
-Control	73.69±20.43	—	52.91±19.13 (71.28±9.85)	9.35±4.77 (12.19±3.76)	—
-Gravid	66.77±18.88	—	44.44±16.31 (65.41±9.43)	9.77±3.29 (14.48±2.27)	—
Bladder Base					
-Control	66.8	—	37.4	4.7	—
-Gravid	55.8	—	32.6 (56.0)	6.1 (7.1)	—
Fetal Bladder	123.6*	—	93.5* (75.7)	20.3* (16.5)	—
Heart Ventricle					
-Control	138.2	—	122.6 (88.7)	1.2 (0.9)	—
-Gravid	79.1	—	78.7 (89.2)	0.4 (0.5)	—
Fetal Heart	199.1*	—	192.4* (96.7)	0.1 (0.1)	—
Uterus Fundus					
-Control	42.85±27.44	0.36 (0.7)	28.02±24.20 (60.82±21.22)	1.88±0.85 (5.28±2.06)	0.35 (0.7)
-Gravid	23.09±10.30	0.04 (0.2)	13.75±8.46 (58.37±14.92)	1.25±0.88 (5.28±2.06)	0.05 (0.2)

Muscarinic receptors were immunoprecipitated by subtype specific antisera. Shown are the means and standard deviations of all experiments in fMol/assay, each done in triplicate. The percentage of total receptors are shown in parentheses.

* statistically different from control, as determined by Student's t test.