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Muscarinic Receptor Transcript and Protein Density in Hypertrophied and Atrophied Rat Urinary Bladder

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Abstract

Aims—Our previous studies showed that bladder hypertrophy shifts the muscarinic receptor subtype mediating contraction from M₃ towards M₂ along with increased M₂ and decreased M₃ protein concentration. We quantified mRNA for M₁ through M₅ receptors to determine whether the changes in M₂ and M₃ protein levels was due to changes in transcription.

Methods—Bladder hypertrophy was induced by bladder outlet obstruction (BOO), major pelvic ganglion electrocautery (DEN), and major pelvic ganglion decentralization (DEC). Bladder atrophy was induced by ureteral diversion (DIV). Additional groups included denervated and diverted (DEN-DIV), sham operated (SHAM), and normal (NOR) controls. Transcripts were quantified using a multiplex ribonuclease protection assay (RPA) and receptor protein density was determined by immunoprecipitation. Receptor transcripts were expressed per unit total RNA.

Results—Although all five receptor subtype transcripts were detected in all experimental groups, the densities of M₁, M₄, and M₅ were much lower than for the M₂ and M₃ subtype. There were more M₂ receptor transcripts than all the others, consistent with M₂ protein determinations. M₂ transcripts were significantly increased in DEN and BOO bladders. Surprisingly, M₃ transcripts were also significantly increased in BOO. There was a significant correlation ($r = 0.98$, $P < 0.001$) between protein density and transcript density for the M₂ but not the M₃ receptor among the different experimental groups.

Conclusions—Changes in mRNA concentration are reflected by changes in protein density for the M₂ receptor but not for the M₃ receptor. Extrapolation of functional effects from transcript density data is invalid for M₃ mediated bladder contractions.

Keywords

atrophy; autonomic denervation; bladder; denervation; hypertrophy; receptors; muscarinic; RNA; messenger

INTRODUCTION

Classical pharmacological analysis of normal smooth muscle contraction in response to muscarinic agonists suggests that contraction is mediated primarily by the M₃ muscarinic receptor subtype [Caulfield, 1993; Eglen et al., 1996]. Our previous studies have shown that bladder pathology induces participation of the M₂ subtype in the contractile response in both the rat [Braverman et al., 1998b; Braverman and Ruggieri, 2003] and human [Pontari et al.,

2004]. Subtype selective immunoprecipitation demonstrates that the M₂ receptor protein density is about fivefold higher than the M₃ receptor protein density [Wang et al., 1995; Braverman et al., 1998b]. Similarly, the M₂ and M₃ receptor mRNA has been identified in this tissue by Northern hybridization [Maeda et al., 1988]. With the advent of the highly sensitive reverse-transcriptase polymerase chain reaction assay for the identification of mRNA, the M₁₋₄ transcripts have been shown to be present in urinary bladder smooth muscle from rats [Braverman et al., 1998a], while transcripts for all five subtypes has been demonstrated in samples of human bladder [Sigala et al., 2002].

Several groups have shown in the bladder of several different species that following selective M₃ receptor covalent inactivation, the M₂ receptor participates in contraction in an environment of increased cAMP [Hegde et al., 1997; Braverman and Ruggieri, 1999]. This has been suggested to indicate that M₂ receptor activation reverses beta adrenergic receptor mediated relaxation through opposing effects on intracellular cAMP. Our recent studies of rat bladder contraction show that various pathophysiological conditions, including denervation and outlet obstruction, or pharmacological manipulations such as exposure to thapsigargin, alter the mechanism of contraction such that the M₂ receptor directly participates in the contractile response [Braverman et al., 2002; Braverman and Ruggieri, 2003]. Similarly, in a model of acute cholecystitis in the guinea pig [Braverman et al., 2000] and in a cat model of experimentally induced esophagitis [Sohn et al., 1997], the M₂ receptor mediates smooth muscle contraction. The guinea pig gallbladder has a greater density of M₂ receptors and a lower density of M₃ receptors than the rat bladder, which may explain the finding that selective M₃ receptor alkylation results in an M₂ mediated contraction without the need to raise cAMP [Ongeri et al., 1999].

We have previously shown that the above mentioned experimentally induced pathophysiological conditions in the rat bladder result in alterations of M₂ and M₃ receptor density 3 days post-operative. The conditions which result in increased bladder stretch and hypertrophy such as DEN and BOO, have an increase in M₂ receptor density and a decrease in M₃ receptor density. The conditions which result in disuse such as bilateral ureteral diversion (DIV) and bilateral ureteral diversion with denervation (DEN-DIV) have a decrease in both M₂ and M₃ receptor density [Braverman and Ruggieri, 2003].

The goals of the current study are to determine whether alterations in muscarinic receptor subtype protein by bladder hypertrophy and denervation are accompanied by corresponding changes in mRNA as measured by ribonuclease protection assay (RPA).

MATERIALS AND METHODS

Surgery

Rats (200–250 g female Sprague–Dawley rats from Ace Animals, Inc., Boyertown, PA) were anaesthetized with 25 µg/kg buprenorphine and 2% isoflurane in oxygen and a midline incision was made in the lower abdomen. The pelvic plexus was exposed. For bilateral denervation, both the left and right major pelvic ganglion (MPG) were cauterized with a hand stitching pencil attached to a Model SSE 2 solid state electrosurgery device (Valleylab, Inc., Boulder, CO). For urinary diversions, both ureters were dissected free, cut, and sutured into the lumen of the colon. For DEN-DIV, both denervation and diversion as described above were performed. For BOO, the urethra was exposed, a 21 gauge syringe needle was placed parallel to the urethra, a 3–0 silk suture was tied around both the needle and urethra and the needle was then removed. For DEC, the nerve fibers entering the MPG from the spinal cord were severed. For sham operated animals, the abdomen was entered as described above and the urethra, ureters, and major pelvic ganglion were exposed without inducing intentional damage to these organs. The abdominal incision was closed in layers. After

surgery, urine was expressed with manual pressure on the lower abdomen twice daily. The bladders were harvested 3 days post-surgery.

Immediately prior to bladder harvesting in the DEC group, the animals were tested to ensure that spinal stimulation was ineffective in inducing a bladder contraction while MPG stimulation caused an increase in bladder pressure. For this determination, the bladder was catheterized per urethra with PE 50 tubing connected to a pressure transducer. The bladder was emptied and filled manually until an intravesical pressure of 5 cmH₂O was induced. A unipolar pith electrode grounded to the abdomen was inserted into the spinal column between the L2 and L3 vertebral bodies and stimulated with a 2–5 sec train of square wave pulses at 2 V, 30 Hz, 1 msec duration delivered by a Grass S-88 stimulator (Astro-Med, Inc., West Warwick, RI). The MPG was stimulated with bipolar electrodes separated by 4 mm with the same stimulation parameters. These stimulations consistently induced a marked bladder contraction in neurally intact animals. Any DEC animals that showed a bladder contraction to the spinal stimulation or did not show a bladder contraction to the MPG stimulation were not used.

Ribonuclease Protection Assays (RPA)

Bladders from the test groups were harvested and frozen on dry ice. RNA was isolated separately from each bladder according to protocol (Stratagene, La Jolla, CA). Tissues were chopped then homogenized using glass tissue grinders. Twenty micrograms of each sample RNA was used for the RPA. Of the 59 bladders subjected to RPA analysis, less than 20 µg but greater than 10 µg of total RNA was used in the assay for 5 of these (2 from the DIV group, 2 from the DEN-DIV group, and 1 from the DEN group). Results for these five assays were adjusted appropriately to be equivalent to 20 µg of total RNA. RPAs were done according to protocol provided by BD Pharmingen (San Diego, CA). Briefly, the ³²P labeled probes were synthesized using commercially available templates (M₁–M₅, L32, and GAPDH, BD Pharmingen) and quantified in a scintillation counter. Two microliters of probe was added to each RNA sample, then hybridized overnight followed by RNAase treatment. Probe and samples were separated by electrophoresis on 5% denaturing acrylamide gels then dried and exposed to Phosphor imaging screens (PerkinElmer Corp., Wellesley, MA) for 17 hr. Digital light units for each band were quantified using a Cyclone phospho imager and Optiquant software (PerkinElmer Corp.). The total number of digital light units for 5,000 dpm of undigested probe was used to convert digital light units to dpm.

Statistics

Statistical analysis of the data was performed using a software package (GB-STAT v8.0, Dynamic Microsystems, Inc., Silver Spring, MD). Because homogeneity of variance tests (Hartley's F-max, Cochran's C, and Bartlett's Chi-square) showed statistically significant differences in the variances between the groups, the non-parametric Kruskal–Wallis analysis of variance was used with post-hoc Wilcoxon rank sum/Mann–Whitney *U*-tests to determine statistically significant differences between experimental groups. Probability values less than 0.05 were considered statistically significant.

RESULTS

DEN, BOO, and DEC cause a significant increase ($P < 0.05$) in bladder weight, approximately twofold compared to controls (185 ± 5 , 183 ± 5 , and 179 ± 5 mg, respectively vs. 88 ± 6 for normal and 93 ± 6 mg for sham). DIV induces a significant decrease (atrophy) in bladder weight (77 ± 6 mg) while DEN-DIV has no effect on bladder weight (90 ± 6 mg).

As can be seen in Figure 1, the multiplex RPA assay used quantifies all five muscarinic receptor transcripts and two housekeeping genes (*L32* and *GAPDH*) from the same sample of RNA (20 μ g). Although these housekeeping genes were included so that their density could be used to normalize the transcripts that code for the muscarinic receptors, the density of the transcripts for the housekeeping genes per 20 μ g total RNA varies widely between experimental groups. The density of both *GAPDH* and *L32* transcripts per unit total RNA are statistically significantly increased in the hypertrophied groups (DEN and BOO) and decreased in the atrophied group (DIV) compared to both normal and sham control (Fig. 2, panel A).

Transcripts for all five receptor subtypes were detected in all groups, however, the densities of M_1 , M_4 , and M_5 transcripts are 14–56 fold lower than for the M_2 and the M_3 transcripts (data not shown). M_2 receptor transcripts are statistically significantly greater than M_3 transcript density in all groups except the DIV and DEN-DIV groups in which there is no statistically significant difference. When transcript density is normalized per 20 μ g total RNA which was constant for each sample, a statistically significant increase over sham operated controls is found for M_2 transcripts in the DEN and for M_2 and M_3 transcripts in BOO while there is a significant decrease for M_2 and M_3 transcripts in DIV (Fig. 2, panel B). M_2 transcript density increased in these groups up to 5.4-fold in the DEN bladders compared to control bladders. The increases in transcripts for the M_2 and M_3 receptors are of the same magnitude in the DEN and BOO bladders.

There are no statistically significant differences between groups in M_1 , M_4 , or M_5 transcript densities. Unlike the ratio of the M_2 to M_3 protein in these experimental groups, which ranges between 7 for control and 47 for denervated [Braverman and Ruggieri, 2003], the ratio of M_2 to M_3 receptor transcripts is lower, ranging from 0.96 for the DEN-DIV to 1.9 for the DEN group. The difference in the ratios between the protein densities and the transcript densities prompted us to determine if protein densities for the M_2 and the M_3 receptor subtypes correlates to transcript densities. As can be seen in Figure 3, a significant correlation between protein levels (as determined by subtype selective immunoprecipitation) and gene expression is found for the M_2 subtype (panel A), however, no significant correlation is found for the M_3 subtype (panel B).

Often transcript densities are reported as a the percentage of the expression of a housekeeping gene. However, the surgical models used in these studies induce significant differences in the expression of these particular housekeeping genes, making normalization of the transcript to these genes questionable. There are no differences in expression of either the M_2 or the M_3 receptor transcript compared to control when transcript density is normalized to either *L32* transcript or *GAPDH* transcript density (Fig. 4, panels A and B, respectively). Furthermore, when normalized in this fashion, there is no correlation between transcript densities and protein levels (Fig. 5).

The results of the current study demonstrate that alterations in M_2 receptor protein are accompanied by corresponding changes in M_2 receptor mRNA as measured by RPA. However, the changes in M_3 receptor protein can not be explained by changes in mRNA expression. Furthermore, the hypertrophied bladders have an increase in mRNA coding for the housekeeping genes *GAPDH* and *L32* while the atrophied bladders have a decrease in mRNA of these genes. These housekeeping genes have traditionally been used to normalize expression of genes of interest with the implicit and sometimes inaccurate assumption that there is no change in housekeeping gene expression with experimental manipulation. These results indicate that normalization of expression of the genes of interest to these housekeeping genes between tissues with differing degrees of hypertrophy is not the most appropriate way to normalize gene transcript expression.

DISCUSSION

Our previous studies [Braverman et al., 1998b, 2002; Braverman and Ruggieri, 2003] have shown a rapid response of the rat bladder to these experimental pathologies. Bladder hypertrophy was induced by DEN, BOO, and DEC and bladder atrophy was induced by DIV but not by DEN-DIV 3 days following the surgery. Since DEN by MPG electrocautery (destroying all of the neuronal cell bodies innervating the urinary bladder of the rat) induces both denervation as well as hypertrophy, additional surgical models were created in an attempt to separate hypertrophy from denervation. Hypertrophy without denervation was produced by two surgical models. DEC creates a bladder that remains innervated by the neuronal cell bodies in the MPG but these neuronal cell bodies are disconnected from the central nervous system. BOO also creates a bladder that is hypertrophied but not intentionally denervated, although a certain degree of denervation may result from the ischemia and other outlet obstruction sequella. To create a bladder that is denervated but not hypertrophied we performed MPG electrocautery together with urinary diversion (DEN-DIV) and as a control for this model we performed DIV alone which results in bladder atrophy. The hypertrophied bladders (DEN, BOO, and DEC) had an increased density of the M₂ receptor subtype protein and a shift in the mechanism of contraction such that the M₂ subtype plays a more prominent role in the contractile response whereas the M₃ receptor protein was decreased in the DEN and BOO bladders [Braverman and Ruggieri, 2003]. This leaves us with the question of whether transcript number for the M₂ and the M₃ receptor are reflective of protein density. When the protein density of these receptors is compared to the transcript densities, the M₂ receptor transcript yields a significant correlation (Fig. 3A). However, in contrast to the M₃ transcript densities, the M₃ protein density was reduced in every group except DEC compared to control, yielding no correlation between M₃ transcript density and protein density (Fig. 3B). This indicates that regulation of the protein density of the M₂ receptor subtype is different than regulation of the M₃ subtype.

The results indicate that M₂ muscarinic receptor transcript expression as a percentage of total RNA, along with GAPDH and L32 are markers for bladder hypertrophy but not denervation. Bladder hypertrophy is associated with irritative voiding symptoms and can eventually lead to a non-contractile, fibrotic bladder [Levin et al., 2000]. Bladder hypertrophy in the rat not only increases M₂ receptor density [Braverman and Ruggieri, 2003] but also changes the muscarinic receptor signal transduction pathway such that the M₂ receptor plays a more prominent role in the contractile response [Braverman et al., 2002; Doumanian et al., 2005]. If this increased dominance of bladder M₂ receptors causes the hypertrophy induced over activity and voiding symptoms, then either suppressing this induction of M₂ receptors or treatment with an M₂ selective antagonist may be therapeutically beneficial. Hypertrophy also decreases M₃ receptor density which may contribute to the development of an acontractile bladder together with the fibrosis. It is unclear whether the overactive bladder is a result of increased activity of the normal M₃ mediated bladder contraction, increased contribution of the M₂ receptor in the contractile response or to an increase in the sensory limb of micturition. It is actually not even known whether the urgency symptoms are a result of increased bladder contractile activity or increased sensitivity of the afferent limb of micturition. Thus, it is not known whether therapy for the overactive bladder should be directed towards the bladder contractile mechanism or the bladder afferent system.

It has been common practice to normalize the expression of a gene of interest to a housekeeping gene, because these housekeeping genes are thought to be constitutively expressed. The implicit assumption is that their expression remains constant with experimental manipulation, however, this must be verified. The results of our investigation demonstrate that these particular housekeeping genes increase with bladder hypertrophy and

decrease with bladder atrophy and thus cannot be used as internal standards for expression of other genes of interest such as the muscarinic receptor subtypes. GAPDH and beta-actin are often used as housekeeping genes, however, there is growing evidence that the density of these transcripts changes in response to various stimuli. GAPDH expression is substantially increased in human cancers including breast [Revillion et al., 2000], pancreatic [Schek et al., 1988], lung [Sirover, 1997], and cervical [Kim et al., 1998]. Both GAPDH and beta-actin mRNA are down-regulated up to 50% in various regions of the rat digestive tract as a consequence of fasting [Yamada et al., 1997], while GAPDH mRNA levels are increased in rat aorta about 2.5-fold as a result of oxidative stress [Ito et al., 1996]. GAPDH mRNA is up-regulated in the endometrium, but down-regulated in the liver by estradiol in ewes [Zou and Ing, 1998]. GAPDH mRNA density has also been shown to be regulated on a daily basis by the circadian clock [Shinohara et al., 1998]. Beta-actin mRNA expression is also lower in biopsy samples taken from asthmatic lung compared to biopsies from normal lung [Glare et al., 2002]. Expression of the ribosomal protein gene L32 also varies. For instance, dexamethasone treatment increases L32 transcription in rat myoblasts [Sienna et al., 2000]. L32 and GAPDH transcripts densities change as a result of mitogenic stimulation in mononuclear cells. These changes in L32 and GAPDH transcript density fluctuate differently based on the mitogen and length of mitogen exposure and thus these genes are inadequate as internal standards [Thellin et al., 1999; Bas et al., 2004].

Our results indicate that both GAPDH and L32 vary greatly between groups as a percentage of total RNA. When GAPDH transcript is increased L32 transcript density is also increased, thus the density of these transcripts correlates with each other. GAPDH is increased in the groups in which the bladders are hypertrophied and undergoing growth stimulation (DEN and BOO), and reduced in the atrophied bladders (DIV), thus making normalization of transcript density to these genes inappropriate.

The method used to isolate total RNA from the tissues was designed to obtain high quality RNA suitable for RPA. This method is not designed to quantitatively extract and thereby determine the amount of RNA per bladder. Thus, normalization of the RPA results to the total amount of RNA extracted per bladder would be highly inaccurate. Since the total RNA per bladder increases with bladder hypertrophy [Malkowicz et al., 1986] and likely decreases with atrophy, this would lead to even greater differences from control than seen in Figure 2 if gene expression were expressed per bladder.

Recently, much focus has been given to the demonstration of muscarinic receptors on bladder epithelial cells and their ability to release signaling molecules including neurotransmitters [Chess-Williams, 2002]. Many of these epithelial cells are in close proximity to sensory nerves and thus could be involved in bladder sensation. The physiological function of these receptors and their signaling is currently unclear along with any changes which may accompany pathophysiology. A better understanding of the influence of epithelial muscarinic receptors on bladder pathophysiology awaits further studies.

In conclusion, M₂ receptor protein density correlates directly with M₂ receptor transcript density expressed as a percentage of total RNA. No correlation exists between M₃ receptor protein density and M₃ receptor transcripts regardless of whether transcripts are normalized to total RNA or housekeeping gene expression. Thus there appears to be tangible differences in the regulation of protein expression between the M₂ and M₃ receptor subtypes. Direct extrapolation of transcript density to protein density and thus functional gene effects, particularly for the M₃ muscarinic receptor subtype may lead to inaccurate conclusions.

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Fig. 1.

Example of results from multiplex ribonuclease protection assay (RPA). **Lane 1** is 2,000 dpm of undigested probe while **lane 2** shows the amount of probe protected by 20 μ g of total RNA isolated from rat bladder. Images are visualized by use of a phospho imager and the bands are quantified using Optiquant software. The undigested probe lengths are 314, 285, 259, 209, 168, 141, and 124 nucleotides, while the protected probe lengths are 285, 256, 230, 180, 139, 113, and 96 nucleotides for M₁, M₄, M₅, M₂, M₃, L32, and GAPDH, respectively.

Fig. 2.

Density of transcripts for housekeeping genes *L32* and *GAPDH* (**panel A**) and *M₂* and *M₃* muscarinic receptors (**panel B**). The density of transcripts for both *L32* and *GAPDH* change significantly as a result of the experimental conditions. The density of transcripts coding for the *M₂* muscarinic receptor subtype increase significantly compared to sham in DEN and BOO while the *M₃* transcript density is increased in BOO and decreased in DIV bladders. NOR (normal, n = 9), SHAM (n = 11), DIV (n = 9), DEN-DIV (n = 8), DEN (n = 9), BOO (n = 6), DEC (n = 6). **P* < 0.05 compared to sham, ***P* < 0.01 compared to sham, +*P* < 0.05 compared to normal, ++*P* < 0.01 compared to normal.

Fig. 3.

Correlation of M₂ (**panel A**) and M₃ (**panel B**) muscarinic receptor protein density with transcript density. The density of M₂ receptor protein, as determined by subtype selective immunoprecipitation, positively correlates with the density of M₂ RNA as determined by RPA (panel A). However, there is no correlation between the density of M₃ receptor protein and M₃ receptor RNA (panel B).

Fig. 4.
Density of M₂ and M₃ muscarinic receptor transcripts when normalized to either GAPDH (panel A) or L32 (panel B).

Fig. 5. Correlation between transcript density and protein density for the M₂ (**panels A and C**) or the M₃ (**panels B and D**) receptor when normalized to either L32 (**panels A and B**) or GAPDH (**panels C and D**).