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Regulation of bladder muscarinic receptor subtypes by experimental pathologies

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Summary

1. The M₃ muscarinic receptor subtype is widely accepted as the receptor on smooth muscle cells that mediates cholinergic contraction of the normal urinary bladder and other smooth muscle tissues, however, we have found that the M₂ receptor participates in contraction under certain abnormal conditions. The aim of this study was to determine the effects of various experimental pathologies on the muscarinic receptor subtype mediating urinary bladder contraction.
2. Experimental pathologies resulting in bladder hypertrophy (denervation and outlet obstruction) result in an up-regulation of bladder M₂ receptors and a change in the receptor subtype mediating contraction from M₃ towards M₂. Preventing the denervation-induced bladder hypertrophy by urinary diversion prevents this shift in contractile phenotype indicating that hypertrophy is responsible as opposed to denervation *per se*.
3. The hypertrophy-induced increase in M₂ receptor density and contractile response is accompanied by an increase in the tissue concentrations of mRNA coding for the M₂ receptor subtype, however, M₃ receptor protein density does not correlate with changes in M₃ receptor tissue mRNA concentrations across different experimental pathologies.
4. This shift in contractile phenotype from M₃ towards M₂ subtype is also observed in aged male Sprague–Dawley rats but not females or either sex of the Fisher344 strain of rats.
5. Four repeated, sequential agonist concentration response curves also cause this shift in contractile phenotype in normal rat bladder strips *in vitro*, as evidenced by a decrease in the affinity of the M₃ selective antagonist *p*-fluoro-hexahydro-sila-diphenidol (*p*-F-HHSiD).
6. A similar decrease in the contractile affinity of M₃ selective antagonists (darifenacin and *p*-F-HHSiD) is also observed in bladder specimens from patients with neurogenic bladder as well as certain organ transplant donors.
7. It is concluded that although the M₃ receptor subtype predominately mediates contraction under normal circumstances, the M₂ receptor subtype can take over a contractile role when the M₃ subtype becomes inactivated by, for example, repeated agonist exposures or bladder hypertrophy. This finding has substantial implications for the clinical treatment of abnormal bladder contractions.

Keywords

muscarinic receptor subtypes; bladder; hypertrophy; denervation; contraction; ageing; bladder outlet obstruction

Introduction

Pharmacological mechanisms of urinary bladder contraction

It is generally accepted that acetylcholine acting on smooth muscle muscarinic receptors is the primary neurotransmitter controlling bladder emptying (Wein & Barrett, 1988). Muscarinic receptor density and the response of bladder smooth muscle to muscarinic stimulation is greatest in the dome and lowest in the base, allowing efficient emptying (Levin, Shofer & Wein, 1980). In many species, including human, neural stimulation of the bladder is only partially inhibited by the antimuscarinic agent atropine (Dean & Downie, 1978; Cowan & Daniel, 1983). In keeping with this observation in animal models, we have shown evidence for a 'purinergic' innervation that may be responsible for the atropine resistant component of parasympathetic contraction in certain human bladder specimens from patients with bladder pathologies including interstitial cystitis (IC) and neurogenic bladder (Ruggieri, Whitmore & Levin, 1990). We have shown that this atropine resistant response of the rabbit bladder is transient, and although it does not substantially contribute to the process of normal bladder emptying it may be involved in abnormal bladder contractions (Levin, Ruggieri & Wein, 1986). Other reports confirm atropine resistant, nerve evoked contractions in bladder strips from both IC (Palea, Artibani, Ostardo, Trist & Pietra, 1993) and bladder overactivity patients (Bayliss, Wu, Newgreen, Mundy & Fry, 1999). In addition, there is an age related increase in these atropine resistant, purinergic contractions in cystectomy specimens from bladder cancer patients (Yoshida et al., 2001).

Muscarinic receptor subtypes

Five intron less genes coding for muscarinic receptor subtypes have been cloned. Pharmacologically, at least three classes of muscarinic acetylcholine receptors (M_1 , M_2 and M_3) can be distinguished since subtype selective antimuscarinic agents are available that are at least 10-fold selective for each of the M_1 – M_3 subtypes and the MT3 toxin is at least 30-fold selective for the M_4 subtype (Caulfield, 1993; Caulfield & Birdsall, 1998; Jerusalinsky *et al.*, 2000). M_1 receptors have a high affinity for pirenzepine (PZP), a low affinity for methoctramine and an intermediate affinity for *p*-fluoro-hexahydro-sila-diphenidol (*p*-F-HHSiD). M_2 receptors have a high affinity for methoctramine and a low affinity for PZP and *p*-F-HHSiD. M_3 receptors have a high affinity for darifenacin, *p*-F-HHSiD and 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP), an intermediate affinity for PZP and a low affinity for methoctramine (Caulfield, 1993). The affinity of any drug represents the composite affinity at multiple receptor subtypes that may be present at uncertain levels in a tissue that expresses more than one subtype (Levine & Birdsall, 1989). As most tissue, including the urinary bladder, express more than one subtype, this concept provides an initial explanation for the often bewildering array of pharmacologic data for the action of muscarinic drugs on bladder and other tissues. There are very few studies other than our own on receptor subtype variation between different species in a given end organ such as the bladder, and this further complicates interpretation of pharmacological studies using different animal models.

One of the most fruitful avenues that has been used to clarify the pharmacology associated with muscarinic receptor subtypes has been to transfect clonal cell lines with cDNA coding for each subtype, and then to establish its properties. Based on these studies, it has been

established that M₁, M₃ and M₅ muscarinic receptor subtypes promote polyphosphoinositide (PI) breakdown, while the M₂ and M₄ subtypes inhibit adenylyl cyclase activity. The subtypes of muscarinic receptor that produce the most dramatic effects on PI metabolism, in particular the M₁ subtype, have high affinity (K_d 10 nM) for the muscarinic receptor antagonist PZP. The subtypes believed to interact with adenylyl cyclase, in particular the M₂ subtype, have much lower affinity (K_d 200–500 nM) for PZP (Maeda, Kubo, Mishina & Numa, 1988; Fukuda, Kubo, Maeda & *et al.*, 1989; Wang, Luthin & Ruggieri, 1995). The M₁ and M₂ subtypes have low and high affinities respectively for other muscarinic receptor antagonists, AFDX-116 and methoctramine.

Our early studies have resulted in the following observations regarding the bladder muscarinic receptor subtypes. No high affinity PZP binding sites are found in the urinary bladder of rat (Monferini, Firaldo & Ladinski, 1988), guinea-pig (Nilvebrant & Sparf, 1983), rabbit (Lepor & Kuhar, 1984; Ruggieri, Bode, Levin & Wein, 1987) or human (Batra, Biorklund, Hedlund, Andersson & Bjorklund, 1987; Ruggieri *et al.*, 1987). In addition, agonist stimulation of muscarinic receptors does inhibit adenylyl cyclase but fails to stimulate PI breakdown to a significant extent in rabbit bladder (Batra *et al.*, 1987; Ruggieri *et al.*, 1987). Others have shown that in the guinea-pig bladder, muscarinic agonists cause a modest but measurable accumulation of inositol phosphates (Noronha-Blob *et al.*, 1989) and this has been reported for the human bladder as well (Andersson, Holmquist, Fovaeus, Hedlund & Sundler, 1991). The M₂ and M₃ subtypes are found in bladder from human, rat, rabbit and guinea-pig, while the M₁, M₄ and M₅ subtypes are not detected using the immunoprecipitation assay. The ratio of M₂:M₃ receptors is *c.* 5:1 in rat but *c.* 3:1 in the other species (Wang *et al.*, 1995). Northern blot analysis indicates that mRNA for M₂ and M₃ but not M₁ or M₄ is expressed in porcine and rat urinary bladder (Maeda *et al.*, 1988; Fukuda *et al.*, 1989). The affinity of a series of subtype selective antimuscarinic drugs for inhibition of agonist-induced contractions in normal bladder of rat, rabbit, guinea-pig and human is most consistent with M₃ receptors mediating the contraction (Wang *et al.*, 1995). Although these biochemical effects of muscarinic receptor stimulation (increased PI turnover via M₃ stimulation and inhibition of adenylyl cyclase by M₂ stimulation) have been demonstrated, these biochemical events have not been unequivocally shown to mediate the contractile signal transduction.

There are a large number of patients who are treated with anticholinergic medications for their bladder dysfunction. These include men with lower urinary tract symptoms, women with urgency/frequency syndrome (overactive bladder), patients with uninhibited bladder contractions from a neurogenic bladder, and may even include patients with pelvic pain and voiding symptoms, i.e. IC and chronic prostatitis/chronic pelvic pain syndrome. This is a very wide range of patients and thus the potential number of patients that might be expected to benefit from this type of treatment is considered to be very large, perhaps several times the estimated 17 million patients in the US affected by overactive bladder alone (Abrams, 2001).

In the present study we examine the effects of experimental pathologies on muscarinic receptor distribution in the rat urinary bladder. Muscarinic receptors in human bladder with neurogenic dysfunction are also examined.

Methods

Materials

Drugs and chemicals were obtained from Sigma Chemical Company, St Louis, MO with the exception of darifenacin which was a generous gift from Pfizer Limited (Sandwich, Kent).

Human bladder tissue obtained from organ transplant donors was procured by the National Disease Research Interchange (Philadelphia, PA, USA).

Surgery

Female Sprague–Dawley (SD) rats (200–250 g; Ace Animals Inc., Boyertown, PA, USA) were anaesthetized with $25 \mu\text{g kg}^{-1}$ buprenorphine and 2% isoflurane in oxygen. After making a midline incision in the lower abdomen, the pelvic plexus was exposed. For bilateral denervation (DEN), 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, USA). For sham operated animals (SHM), the plexus was exposed but left intact. For urinary diversions (DIV), both ureters were dissected free, cut and sutured into the colon. For bladder outlet obstruction (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 then the needle was removed. For MPG decentralization (DEC), the nerve fibres entering the ganglion from the spinal cord were severed. The subcutaneous tissue, muscle and skin were sutured. After surgery, urine was expressed with manual pressure on the lower abdomen twice daily for 3 days.

In the DEC group, immediately prior to bladder harvesting, they 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 slowly 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- to 5-s train of square wave pulses at 2 V, 30 Hz, 1 ms duration delivered by a Grass S-88 stimulator (Astro-Med, Inc., West Warwick, RI, USA). 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.

Muscle strips

Urinary bladders were removed from rats killed by decapitation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat. The bladder was divided in the mid-sagittal plane, then cut into longitudinal smooth muscle strips (approximately 4 mm × 10 mm). The muscle strips were then suspended with 1 g of basal tension in tissue baths containing 15 ml of modified Tyrode's solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 23.8 mM NaHCO₃ and 5.6 mM glucose) and equilibrated with 95/5% O₂/CO₂ at 37 °C.

Carbachol dose–response curves

Following equilibration with the bath solution for 30 min, bladder strips were incubated for 30 min in the presence or absence of antagonist. Concentration response curves (CRC) were derived from the peak tension developed following cumulative addition of carbachol (10 nM to 300 μM final bath concentration). Only one concentration of antagonist was used for each muscle strip ($n = 6$ –8 strips per antagonist concentration). Dose-ratios were determined based on the average of the responses of antagonist free strips. An EC₅₀ value was determined for each strip using a sigmoidal curve fit of the data (Origin, Originlab Corp., Northampton, MA, USA). The EC₅₀ values determined in the presence of antagonist were used to generate Schild plots in order to calculate pA₂ values for each antagonist. If the slope of the Schild plot was not significantly different from unity (95% confidence interval),

the slope of the Schild plot was constrained to unity in order to calculate the pK_b value. To construct the Schild plot for methoctramine and *p*-F-HHSiD, doses of 0.3 and 3.0 μM were used. The Schild plot for 4-DAMP was done using 3.0, 10.0 and 30.0 nM . Because higher doses of darifenacin appeared unsurmountable and lower doses did not produce a significant shift in the CRC, a single dose of 30 nM darifenacin was used. The estimated pK_b for darifenacin was calculated using the formula $pK_b = -(\log[\text{darifenacin concentration}] - \log(\text{dose ratio} - 1))$.

Human bladder specimens

Bladder muscle specimens from seven patients with neurogenic bladder dysfunction were analysed to determine whether the muscarinic receptor subtype mediating contraction shifts from M_3 to the M_2 subtype as found in the denervated, hypertrophied rat bladder. Seven bladder specimens were analysed from six female and one male patients. Six of the patients had traumatic cervical spinal cord injuries (C4–C7) and the other patient had an L1 congenital myelomeningocele. This was compared with results from bladder specimens obtained from eight organ transplant donors. The affinities of three subtype selective muscarinic receptor antagonists for inhibition of carbachol-induced contractions were determined by Schild analysis.

Immunoprecipitations

Immunoprecipitations were performed using antibodies as previously described (Wang *et al.*, 1995; Braverman, Luthin & Ruggieri, 1998). Protein concentration in the solubilized receptor preparation was determined by a dye binding assay (Bio-Rad, Hercules, CA, USA). Muscarinic receptor density is reported as mean $\pm$ standard error of the mean (SEM) fmol mg protein^{-1} in this solubilized receptor preparation. Total muscarinic receptor levels were determined by desalting over Sephadex G-50 minicolumns. At least four determinations were performed on two different pools of bladders for all groups.

Statistical and data analysis

Contractile force is presented as absolute force generated (mN). Tension displayed is normalized to cross sectional area defined as weight divided by length (which assumes a tissue density of 1). Statistical analysis of multiple group comparison was performed by analysis of variance ($_{ANOVA}$) with a *post hoc* Newman–Keuls test or a Student's *t*-test where appropriate (GB-STAT; Dynamic Microsystems, Silver Spring, MD, USA). Statistically significant differences in the affinity values and departure from unity in the slopes derived from the Schild plots were determined using the 95% confidence intervals.

Results

Hypertrophy changes the muscarinic receptor subtype mediating bladder contraction from M_3 towards M_2

We have previously shown that denervation shifts the muscarinic receptor subtype mediating rat bladder contraction from M_3 towards M_2 (Braverman *et al.*, 1998). Rat bladder denervation is also accompanied by bladder hypertrophy. In order to determine whether the denervation (DEN) or bladder hypertrophy is responsible for this change in the muscarinic receptor subtype that mediates contraction, several additional experimental groups were studied. These included bilateral ureteral diversion (DIV) in which the ureters are diverted to the colon. These bladders have normal innervation, but are not subjected to the normal filling and emptying cycles. Denervation in conjunction with DIV (DIV-DEN) interrupts both the innervation and the filling cycles. BOO subjects the bladder to increased mechanical force and MPG decentralization (DEC) allows the postjunctional nerves to

remain intact, however, the animal is unable to void which results in the same increased stretch and hypertrophy as in DEN bladders.

Induction of hypertrophy

Figure 1a shows that neither DIV nor DIV-DEN induce hypertrophy as evidenced by an increase in bladder weight:body weight ratio compared with sham operated controls. However, DEN, BOO and DEC induce significant hypertrophy by 3 days postsurgery. Interestingly, DIV bladders are significantly smaller than sham or DIV-DEN bladders.

Electric field stimulated contraction

To determine the effect of short-term hypertrophy on electric field stimulated (EFS) contractile force, the contractile force normalized to cross sectional area was determined for each group. Fig. 1b shows that no differences in the EFS contraction to a submaximal stimulation of 8 V, 30 Hz, 1 ms is seen with DIV, BOO or MPG-DEC compared with sham operated control bladders. DIV-DEN and DEN bladders contract less to EFS than sham operated controls. Unexpectedly, DIV-DEN bladders contract greater than DEN bladders to EFS, suggesting that the nerve terminals in these bladders are not completely degenerated at 3 days.

Carbachol responses

The carbachol induced maximal contraction (Fig. 1c) is not different between any groups except that the BOO group contracts significantly greater than every other group. As the nerves degenerate or are unable to induce a maximal contraction, the ratio of the carbachol maximum contractile response to the EFS contractile response will increase. This can be used as a measure of functional denervation. DEN, DIV-DEN, DEC and BOO bladders are functionally denervated compared with sham operated bladders. DEN bladders are significantly more functionally denervated than the DIV-DEN, BOO and DEC bladders. DIV-DEN bladders are functionally denervated similar to BOO both of which are more functionally denervated than the DEC bladders. Comparing the carbachol potency between the groups, a lower carbachol EC_{50} is found in the DEN bladders compared with sham.

Muscarinic receptor subtype protein density

Total, M_2 and M_3 receptor protein density was determined by subtype selective immunoprecipitation (Fig. 2). Sham operated bladders have a significantly different total receptor density (approximately 470 fmoles mg^{-1} solubilized protein) than every group, with an M_2 to M_3 ratio of approximately 6:1. Total muscarinic receptor density in the DIV and DIV-DEN bladders are significantly less than sham operated with M_2 to M_3 ratios of approximately 9:1 and 10:1 respectively. DEN, BOO and DEC bladders have greater total receptor densities than sham with M_2 to M_3 ratios of about 34:1, 24:1 and 11:1, respectively. The hypertrophied bladders (DEN, BOO) have an increase in M_2 receptors, a decrease in M_3 receptors and an increase in total receptor density, while the hypertrophied MPG-DEC bladders have an increase in M_2 and total receptors, with no change in M_3 receptors compared with sham. The atrophied bladders (DIV) have a decrease in both M_2 and M_3 receptors which is reflected in a decrease in total receptor density. The DIV-DEN bladders, which are neither hypertrophied nor atrophied, have a decrease in M_2 , M_3 and total receptor density.

Correlation of muscarinic receptor subtype density with functional denervation

The greater the degree of functional denervation, the greater the density of the M_2 receptor subtype (Fig. 3a). No such correlation exists for the M_3 subtype (Fig. 3b). However, all groups with bladder hypertrophy have an increase in M_2 receptor density and low affinities

for M₃ selective antagonists which suggests an M₂ mediated component of contraction. The DIV and DIV-DEN bladders which are not hypertrophied have higher affinities for these M₃ selective antagonists suggesting that the M₃ subtype mediates bladder contraction.

Affinity of antagonists for inhibition of carbachol induced contractions

Based on the affinity of a series of muscarinic receptor antagonists for inhibition of carbachol-induced contractions, sham operated bladder contractions are mediated by the M₃ receptor subtype (Table 1). This is based on a high affinity for the M₃ selective antagonists *p*-F-HHSiD (7.7 ± 0.2), 4-DAMP (9.1 ± 0.2) and darifenacin (8.5 ± 0.1) and a low affinity for the M₂ selective antagonist methoctramine (6.2 ± 0.2). The affinities in DIV and DIV-DEN are also consistent with M₃ receptors mediating contraction. However, the M₃ selective antagonists have a lower affinity in the hypertrophied bladders (DEN, MPG-DEC and BOO) which is consistent with participation of the M₂ receptor in mediation of contraction. Therefore the hypertrophy and not the denervation is responsible for this change in contractile phenotype from M₃ towards M₂. Paradoxically, the M₂ selective antagonist methoctramine has a low affinity for inhibition of contraction in all groups which seems to preclude participation of M₂ receptors in contraction. This phenomenon was also seen in our earlier studies which prompted our proposal of the existence of two parallel contractile pathways, one mediated by the M₃ receptor subtype, the other mediated by the M₂ subtype.

Muscarinic receptor transcript density in hypertrophied and atrophied rat urinary bladders

As we found that the hypertrophy-induced shift in the muscarinic receptor subtype mediating contraction from M₃ towards M₂ is accompanied by an increased density of the M₂ but no change in M₃ receptor protein, we quantified mRNA for M₁ through M₅ transcripts to determine whether the changes in M₂ and M₃ protein levels was because of changes in transcription. Transcripts for M₁–M₅, and two housekeeping genes L32 and GAPDH were quantified using a multiplex ribonuclease protection assay (RPA) that we developed for these studies.

Figure 4, lane 2, shows a gel from the multiplex RPA assay in which all five muscarinic receptor transcripts and two housekeeping genes (L32 and GAPDH included as positive controls) were quantified from the same sample of bladder RNA (20 µg). These housekeeping genes were also included so that their density could be used to normalize the transcripts that code for the muscarinic receptors. The receptor transcripts are not normalized to the housekeeping genes because the density of the transcripts for the housekeeping genes varied widely between groups (Fig. 5, panel a). Lane 1 contains 2000 cpm of undigested probe used to normalize digital light units obtained using Optiquant software and a cyclone phospho imager. The density of L32 transcripts are increased in the denervated group compared with control. Similarly, the density of GAPDH transcripts are significantly increased in the denervated, bladder outlet obstructed and the decentralized groups compared to control. As a consequence, receptor transcript densities displayed in Fig. 5, panel b, are expressed per 20 lg total RNA which was constant for each sample.

Transcripts for all five receptor subtypes were detected in all groups. The densities of M₁, M₄ and M₅ transcripts are much lower than for the M₂ and the M₃ transcripts (data not shown). M₂ receptor transcripts have the highest density in all groups, consistent with the higher density of M₂ receptor protein in the rat bladder, with M₃ transcripts somewhat lower (Fig. 5, panel b). M₂ receptor transcript density is significantly increased in the denervated and the obstructed groups, while M₃ transcript density is increased in denervated, obstructed, and the decentralized groups compared with control. We correlated protein density as measured by subtype selective immunoprecipitation to transcript density to determine if alterations in transcript densities reflected changes in protein densities in these

six different surgical pathologic models. A significant correlation between protein levels and transcript amounts is found for the M₂ subtype, but, no significant correlation is found for the M₃ subtype (Fig. 6).

Aged rat studies

The affinity of the three antagonists (methoctramine, *p*-F-HHSiD and darifenacin) in the young and old male and female SD rats is shown in Fig. 7. In the old male SD rats the affinity of *p*-F-HHSiD is low, within its reported range for M₂ receptors and the affinity of darifenacin was also low and not statistically significantly different than its reported affinity range for M₂ receptors. This is not seen in young males and both young and old female SD rats in which the affinity of all three antagonists are found to be within their reported range for M₃ mediated contractions. This was also done on the Fisher strain of rats obtained from the National Institutes of Ageing (NIA) rodent colony in both 3 and 18 months old male and females. Calorie restriction of these rats after 3 months of age increases their lifespan and the Fisher strain aged rats are available from the NIA colony as both *ad libitum* fed and calorie restricted. The SD rats used in Fig. 7 were fed *ad libitum*. The antagonist affinity in the Fisher strain of rats obtained from the NIA colony is shown in Fig. 8. The affinity of the antagonists in all the F344 rats suggest that bladder contractions are mediated by the M₃ receptor subtype. There are no significant differences in the maximal contraction or the potency of carbachol in any of these groups.

Effect of repeated, cumulative, agonist exposure on subtype selective antagonist affinity

Figure 9 shows the effects of four repeated, sequential carbachol CRC on the affinity of *p*-F-HHSiD. In the absence of antagonist the first curve shows a higher maximal response and higher carbachol affinity whereas curves two to five are superimposable. *p*-F-HHSiD was added after the fourth CRC and the calculated affinity ($PK_b = 7.09$) is within the range reported for M₂ receptors mediating the contractile response.

The M₂ muscarinic receptor mediates in-vitro bladder contractions from patients with neurogenic bladder dysfunction

The affinities of the M₃ selective antagonists darifenacin or *p*-F-HHSiD were determined in six of the seven human neurogenic bladder specimens (Fig. 10, patients 2–7). The affinities were consistent with M₂ mediated contractions in four of these six specimens (patients 3, 5, 6 and 7), intermediate between M₂ and M₃ in one specimen (patient 2) and consistent with M₃ mediated contractions in one specimen (patient 4). One of these six neurogenic bladder specimens (patient 3) and the other neurogenic specimen (patient 1) were tested with the M₂ selective antagonist methoctramine and low affinities were found for both, consistent with M₃ mediated contractions. There was no striking relationship between antagonist affinity and preoperative bladder capacity, level of spinal injury, time after injury or preoperative medications.

In five bladder specimens from the organ donors (Fig. 11, donors 3–6 and 8), the affinity of both *p*-F-HHSiD and darifenacin were determined. In four of the five (donors 4–6 and 8), the affinity of *p*-F-HHSiD was within the reported range for M₂ mediated contractions while the affinity of darifenacin was not different from the range of affinities reported for the M₃ subtype. In the other specimen (donor 3), the affinity of *p*-F-HHSiD was within the reported M₂ range, and was intermediate between M₂ and M₃ range for darifenacin. In one tissue, only *p*-F-HHSiD was used (donor 1), and it had an affinity consistent with M₃ receptors mediating contraction. In one tissue (donor 7), only darifenacin was used and an affinity not different from the M₃ range was found. In one specimen (donor 2), *p*-F-HHSiD and methoctramine were used, the *p*-F-HHSiD affinity was consistent with M₂ receptors while the methoctramine affinity was consistent with M₃ receptors mediating contraction.

Strikingly, the methoctramine affinity was low (consistent with M_3 mediated contractions) in all specimens tested (patients 1 and 3, and donors 2 and 4). In every study we have previously performed, with the single exception of rat bladder following selective alkylation of M_3 receptors in the presence of isoproterenol (Braverman & Ruggieri, 1999), we have always found a low methoctramine affinity consistent with M_3 receptors mediating contraction. After confirming this in two human specimens from each group, we did not continue to determine the affinity of methoctramine using this limited patient and donor tissue.

Discussion

Our findings of bladder hypertrophy 3 days following DEN, DEC and BOO are similar to previously published reports of increased bladder weight following 1 week of denervation, DEC or BOO (Ekstrom & Malmberg, 1984; Ekstrom, Malmberg & Wallin, 1986). Because bladder atrophy was found in the DIV bladders but not the DIV-DEN bladders, development of atrophy requires an intact innervation, perhaps by neuronal release of paracrine factors that oppose bladder hypertrophy. We have previously reported that the nerve terminals in the denervated bladders undergo rapid degeneration based on the absence of EFS-induced contraction within 24 h. Although significant hypertrophy develops 24 h after denervation and there is virtually no response to EFS, there is no reduction in the maximal response to carbachol. Within 3 days following denervation there is a decrease in the affinity of M_3 selective muscarinic antagonists which is not apparent at 24 h and is maintained for at least 3 weeks (Braverman & Ruggieri, 2003). Consequently, the 3-day postsurgery time point was chosen for all of the current studies. After 3 days of outlet obstruction there is no reduction in nerve evoked contractions induced by EFS but the maximal response to carbachol is increased two-fold (Fig. 1c) indicating an adaptive compensation of the detrusor muscle to the obstruction. Both the DEN and DIV-DEN groups are functionally denervated as evidenced by a decreased response to EFS (Fig. 1b), however, because the DIV-DEN has a greater response to EFS than the DEN group this indicates that the nerve terminals do not degenerate as readily when the bladder is not exposed to increased mechanical stress.

Unlike other mammalian species, the normal rat bladder does not contain intramural ganglia so all of the neurones innervating the bladder have cell bodies in or axons passing through the MPG (Gabella & Uvelius, 1990). Because denervation by MPG electrocautery destroys all of the neuronal cell bodies and axons innervating the bladder and induces both denervation and bladder hypertrophy, it is unclear whether the hypertrophy or the denervation is responsible for the shift in muscarinic receptor subtype mediating bladder contraction. To determine this we developed surgical models that result in denervation without bladder hypertrophy as well as bladder hypertrophy without denervation. Denervation without hypertrophy was accomplished by destruction of the MPG and diverting the ureters into the colon (DIV-DEN). As a control for the latter model we also studied rats that only had their ureters diverted and were not denervated (DIV). The BOO bladder becomes hypertrophied as a result of the obstruction but is not intentionally denervated, although some degree of denervation likely ensues as a result of the ischaemia and other sequella of outlet obstruction. The DEC bladder remains innervated by the neuronal cell bodies in the MPG but these neuronal cell bodies are disconnected from the central nervous system and as a result, the bladder cannot empty voluntarily and therefore becomes hypertrophied.

The hypertrophied bladders (DEN, BOO and DEC), regardless of whether they are denervated or not, have an increased density of M_2 receptors. Because of the predominance of M_2 receptors in the bladder, this is also reflected by an increase total muscarinic receptor density. M_3 receptors are decreased by all of the experimental pathologies except DEC. The

non-hypertrophied groups (DEV and DIV-DEN) have a decrease in total, M₂ and M₃ receptor density. Thus, hypertrophy leads to an increased density of total and M₂ receptors while urinary DIV results in a decrease in density of total and M₂ receptors regardless of whether the DIV is accompanied by tissue atrophy (DIV group) or not (DIV-DEN group). As a result of this, the degree of functional denervation correlates with the density of the M₂ but not the M₃ receptor subtype (Fig. 3). All of the hypertrophied groups (DEN, BOO and DEC) have both an increased M₂ receptor density and decreased affinity of M₃ selective antagonists (Table 1) indicating an M₂ mediated component of contraction. Therefore the denervation-induced change in contractile phenotype from M₃ to M₂ mediated contractions is induced by the resultant bladder hypertrophy and not the denervation *per se* because preventing the denervation induced hypertrophy with urinary DIV prevents the change and inducing bladder hypertrophy without denervation (BOO or DEC) also causes this same change in contractile phenotype.

The hypertrophy-induced increase in M₂ receptor protein density in the DEN, BOO and DEC groups is accompanied by a commensurate increase in the tissue concentration of mRNA coding for the M₂ receptor subtype as determined by quantitative multiplex RPA (Figs 4 & 5). On the other hand the decrease in M₃ receptor protein density in all experimental pathologies except DEC is not accompanied by a parallel decrease in tissue concentration of mRNA coding for the M₃ receptor subtype. As a result there is a very close correlation between protein density and mRNA transcript density for the M₂ receptor subtype across the different experimental pathologies but not for the M₃ receptor subtype (Fig. 6). Therefore the regulation of expression from transcript to protein for the M₂ receptor subtype must be very different from regulation of the M₃ receptor subtype.

The density of M₂ muscarinic receptor protein and transcript expression as a percentage of total RNA are markers for bladder hypertrophy. As shown in Fig. 5a this is also true for transcript expression of the housekeeping genes GAPDH and L32. In the past, tissue expression of genes have been normalized to housekeeping genes such as GAPDH and L32 because these housekeeping genes are constitutively expressed and it has been assumed that housekeeping gene expression is constant with experimental manipulation. However, the fact that a housekeeping gene is constitutively expressed does not necessarily prove that the degree of cellular expression is constant under different experimental conditions. Our results with experimental bladder pathologies demonstrate that the expression of these particular housekeeping genes increases with hypertrophy and decreases with bladder atrophy and therefore they cannot be used as internal standards for expression of other genes of interest such as the muscarinic receptors. There has been a steadily increasing number of reports that the density of housekeeping gene expression does indeed change in response in various stimuli. This includes increased GAPDH expression in various cancers such as breast (Revillion, Pawlowski, Hornez & Peyrat, 2000), pancreatic (Schek, Hall & Finn, 1988), lung (Sirover, 1997), and cervical (Kim *et al.*, 1998) and decreased expression in the rat digestive tract with fasting (Yamada, Chen, Monstein & Hakanson, 1997). L32 transcription expression in rat myoblasts increases with dexamethasone treatment (Sienna, Larson & Sells, 2000). Both L32 and GAPDH transcripts densities fluctuate differently based on the mitogen and length of mitogen exposure in mononuclear cells and thus these genes are inadequate as internal standards (Thellin *et al.*, 1999; Bas, Forsberg, Hammarstrom & Hammarstrom, 2004).

Ageing is associated with a huge number of changes including effects on bladder function. We performed these studies to determine whether ageing in rats is associated with changes in the muscarinic receptor contractile phenotype similar to what we found for bladder hypertrophy. The affinity for inhibition of cholinergic contraction of our panel of subtype selective muscarinic receptor antagonist drugs was determined in male and female SD rats at

3 months of age and >12 months of age. In the old male SD rats the affinities of the M₃ selective antagonists (*p*-F-HHSiD and darifenacin) were low, within their reported range for M₂ receptors indicating that the M₂ receptor subtype participates in the contractile response. As we did not find any significant differences in antagonist affinity between 3 and 18 months old male Fisher rats fed *ad libitum* nor any differences between 18 months old female rats fed either *ad libitum* or a calorie restricted diet, we did not perform additional studies on 18 months old male Fisher rats given a calorie restricted diet. The bladder from the aged male SD rats is the first normal bladder tissue that we have found to have M₂ mediated contractions. If these results can be extrapolated to humans it would indicate that under certain conditions, ageing may be accompanied by changes in the contractile phenotype of the bladder such that the M₂ receptor subtype plays a greater role in the contractile response that is normally mediated by the M₃ receptor subtype. This would have important and substantial implications in the choice of antimuscarinic drug therapy to be used to suppress unwanted bladder contractions in this patient population.

In all of our contractility studies, each muscle strip is only exposed to a single cumulative agonist CRC so that any desensitizing effects of one CRC on subsequent agonist responses are avoided. Other investigators have used each strip as its own control, repeating agonist CRCs up to five times in succession with increasing concentrations of the antagonists or enzyme inhibitors (Fleischman, Schneider, Fetscher & Michel, 2004; Schneider, Fetscher, Kregge & Michel, 2004a; Schneider, Hein & Michel, 2004b). While this does allow each strip to serve as its own control and so the effects of inhibitors and muscarinic receptor antagonists can be expressed as a per cent of the pretreatment control response (adjusting for the physiological run down measured in time control strips), it is possible that the previous agonist CRC may have effects on the receptor or signal transduction pathway which may cloud the interpretation of the results. These investigators have found that all of the CRCs after the first curve are superimposable and thus assume that the contractile mechanisms are the same, however until now this assumption has never been tested. To test this possibility, we exposed the normal rat bladder (*n* = 16 strips) to five successive carbachol CRCs. We confirmed that the second through fifth curves are indistinguishable from each other, however, they all are somewhat functionally desensitized compared to the first curve. More importantly, we found that the affinity of the M₃ selective antagonist *p*-F-HHSiD is statistically significantly decreased from 7.6 when added after the first CRC to 7.1 when added after the fifth CRC, indicating that the repeated exposure to cumulative carbachol CRCs alters the mechanism of contraction possibly by suppression of M₃ receptor mediated signalling. Others have shown that the M₃ receptor is subject to agonist induced desensitization (Tobin, Lambert & Nahorski, 1992; Willets, Challiss, Kelly & Nahorski, 2001; Willets, Challiss & Nahorski, 2002; Griffin *et al.*, 2004). Therefore M₃ receptor desensitization may occur during the repeated agonist CRCs which could account for several discrepancies in the literature in which affinities are determined in the same muscle strips with repeated CRCs as opposed to using separate muscle strips for each antagonist concentration. *In vivo*, the bladder muscle is only exposed to neuronally released acetylcholine which is rapidly metabolized by acetylcholinesterase, thus using separate muscle strips for each agonist CRC more closely approximates the *in vivo* situation than repeated agonist CRCs.

Whereas normal detrusor contractions are thought to be mediated by the M₃ receptor subtype, in patients with neurogenic bladder dysfunction (Fig. 9) and certain organ donors (Fig. 10), contractions can be mediated by the M₂ muscarinic receptor subtype. Studies in normal bladders from all species studied to date indicate that the M₃ subtype mediates bladder contraction. These determinations are based on the assumption that only one of the receptor subtypes mediates the response. In some of our pathological human bladder specimens from patients with neurogenic bladder dysfunction or organ donors, it appears

that both M₂ and M₃ receptors may be mediating the contractile response depending on which subtype selective antagonist was used. We interpret the apparent contradictory data (i.e. a low *p*-F-HHSiD or darifenacin affinity indicating M₂ mediated contractions together with a low methoctramine affinity indicating M₃ mediated contractions, Table 1 and Figs 7, 10 & 11) to be consistent with a scenario in which either the M₂ or the M₃ receptor can mediate contraction. When the M₂ receptor is inhibited by methoctramine, the M₃ receptor mediates contraction. When the concentration of methoctramine is high enough to block both the M₂ and the M₃ receptor, contraction is inhibited. This would yield an affinity consistent with blockade of the least sensitive receptor to methoctramine, in this case the M₃. The converse is also true, i.e. *p*-F-HHSiD or darifenacin would not affect contraction until both M₂ and M₃ receptors are inhibited and thus a low affinity would be obtained. In five of the organ donor specimens, the affinity of two different M₃ selective antagonists (darifenacin and *p*-F-HHSiD) was determined. In four of these five, an affinity consistent with M₂ receptors was found using the less selective antagonist (*p*-F-HHSiD) whereas the affinity of the antagonist with a greater selectivity for M₃ receptors (darifenacin) was high, consistent with M₃ receptors mediating contractions. The relative selectivity of these two antagonists may offer some explanation of these findings.

In addition to providing confirmation of our rat studies in human bladder tissue, these findings also challenge the assumption upon which medical therapy is based for treatment of patients with voiding dysfunction. There may also be implications for drug development. If the change in function and number of receptor subtypes in denervated bladder is a common phenomenon in other pathologic states, then the therapies developed for the treatment of these conditions based on the effect in normal tissue may be clinically ineffective. Results from clinical trials with the M₃ selective receptor antagonist, darifenacin, may help to clarify the relative importance of M₂ and M₃ receptors in overactive bladder. To our knowledge, there are currently no M₂ subtype selective antagonists in development for clinical use. Thus, the present data indicate that different individuals or groups of patients might benefit from different antimuscarinic profiles, i.e. M₃ selective, M₂ selective, or non-selective drugs. Caution should be used therefore in pooling data from different patients and assuming the average results represents all patients. Currently, clinical trials require this pooling of patient data as there is currently no way of stratifying patients *a priori* into separate groups based on their potential clinical response to antimuscarinic drugs with different selectivity profiles.

Conclusion

Although the M₃ receptor subtype mediates urinary bladder contractions under normal circumstances, several pathologic conditions in human and animal models are associated with an increased participation of the M₂ receptor subtype in the contractile response. Denervation of the rat by bilateral major pelvic ganglion electrocautery induces bladder hypertrophy and a change in contractile phenotype such that the M₂ receptor mediates the contraction. Because experimental pathologies that induce bladder denervation without hypertrophy do not cause this change in phenotype and pathologies that induce bladder hypertrophy without denervation also cause this change, it is concluded that bladder hypertrophy induces this change in phenotype and not denervation *per se*.

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Figure 1.

The effect of various experimental pathologies on bladder hypertrophy (panel a), nerve-evoked bladder contractions by carbachol (panel b) and cholinergic receptor stimulated contractions (panel c). Data is presented as mean $\pm$ SEM. Top panel (a) *DEN, BOO and DEC are significantly greater than sham while DIV is significantly less than sham ($n = 9-24$ rats per group). Middle panel (b): contractile response to electric field stimulation of 8 V, 30 Hz, 1 ms, *DIV-DEN and DEN are significantly less than sham. # DIV-DEN is significantly greater than DEN ($n = 40-60$ muscle strips per group). Bottom panel (c): The maximal contractile response to carbachol, *BOO is significantly greater than every other group ($n = 40-60$ muscle strips per group).

Figure 2.

The effect of various experimental pathologies on the density of muscarinic receptor subtypes determined by immunoprecipitation. M_2 and M_3 receptors were labelled with [3H]-QNB, solubilized and immunoprecipitated as described previously (Luthin, Harkness, Artymyshyn & Wolfe, 1988). Data shown are average fmoles $\pm$ SEM of receptor mg^{-1} solubilized protein from sham operated, DIV, DIV-DEN, DEN, BOO and DEC ($n = 4-5$ triplicate determinations from six to 18 bladders). The concentration of protein in the solubilized receptor preparation was approximately 8% of the protein concentration in the crude homogenate. As compared to filtration binding, approximately 50% of the muscarinic receptors were solubilized (data not shown). Group differences were determined using ANOVA with a *post hoc* Newman-Keuls test. For total and M_2 receptor density all groups are statistically significantly different from each other. For M_3 receptor density, the sham group is statistically significantly different from DIV, DIV-DEN, DEN and BOO.

Figure 3.

Correlation between M₂ (panel a) and M₃ (panel B) muscarinic receptor subtype density with functional denervation. Functional denervation was evaluated for each individual muscle strip using the ratio of the carbachol maximum contractile response to the electric field stimulation (EFS) induced maximal contractile response. The higher this ratio is the greater degree of functional denervation. The average of the individual muscle strips for each experimental group for functional denervation is plotted vs. the immunoprecipitation data (shown in Fig. 2) for M₂ receptor density (a) and M₃ receptor density (b). The ratio of the average carbachol maximum to the average EFS contraction displayed in Fig. 2, does not equal the average of the individual ratios for the strips. The density of M₂ receptors shows a statistically significant correlation with functional denervation, however, no correlation between the M₃ receptor density and functional denervation is seen.

Figure 4.

Development of a multiplex ribonuclease protection assay for quantitative determination of tissue muscarinic receptor subtype mRNA concentration. Representative phosphor image from a polyacrylamide electrophoresis gel. Lane 1 is 2000 dpm of undigested probe while lane 2 shows the amount of probe protected by 20 µg of total RNA isolated from rat bladder. Images are visualized by use of a phospho imager and the individual bands are quantified using Optiquant software (results shown in Fig. 5). 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.

Figure 5.

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 with sham, ** $P < 0.01$ compared to sham, + $P < 0.05$ compared with normal, ++ $P < 0.01$ compared with normal.

Figure 6.

Correlation between M₂ (top panel) and M₃ (bottom panel) muscarinic receptor protein density and transcript density. The density of M₂ receptor protein, as determined by subtype selective immunoprecipitation (shown in Fig. 2), positively correlates with the density of M₂ RNA as determined by RPA (top panel). However, there is no correlation between the density of M₃ receptor protein and M₃ receptor RNA (bottom panel).

Figure 7.

Affinity of muscarinic receptor subtype selective antagonists against carbachol-induced bladder contraction in young and old Sprague-Dawley rats. Blocks with horizontal hatching indicates the M₂ affinity range for each antagonist and vertical hatching indicates the M₃ affinity range. In the old male SD rats the experimentally determined affinity of the M₃ selective antagonists and are closer to (darifenacin) or within (*p*-fluoro-hexahydro-sila-diphenidol) the M₂ affinity range of these antagonists.

Figure 8.
Subtype selective antagonist affinity in young and old rats of the Fisher strain.

Figure 9.

Effect of four sequential carbachol concentration response curves (CRCs) on the affinity of the antagonist *p*-fluoro-hexahydro-sila-diphenidol (*p*-F-HHSiD) in normal Sprague-Dawley rats. Results are shown as mean $\pm$ SEM for 16 strips (first to fourth CRC) and eight separate strips with and without 1 μ M *p*-F-HHSiD added after the fourth CRC (larger symbols). Symbols without error bars indicate that the SEM is less than the size of the symbol.

Figure 10.

Affinity of subtype selective antimuscarinics for inhibition of carbachol-induced contraction of urinary bladder muscle strips *in vitro* from spinal injury patients. The shaded areas represent the affinity ranges of antimuscarinics (darifenacin, methoctramine and *p*-fluoro-hexahydro-sila-diphenidol) for M₂ receptors (horizontal hatching) and M₃ receptors (vertical hatching) reported in the literature (Caulfield, 1993; Hegde *et al.*, 1997). The symbols refer to the affinities which were determined by Schild analysis of the bladder tissue obtained from the individual patients.

Figure 11.

Affinity of subtype selective antimuscarinics for inhibiting carbachol-induced contraction of urinary bladder muscle strips *in vitro* from organ donors. Notation is the same as indicated for Fig. 10.

Table 1

Affinity of subtype-selective muscarinic receptor antagonists for inhibition of carbachol-induced contractions of rat bladder and their reported affinity for the M₂ and M₃ receptor subtypes. The affinities for 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) and *p*-fluoro-hexahydro-sila-diphenidol (*p*-F-HHSiD) were determined by Schild analysis using three to four different antagonist concentrations at half log intervals, while an estimated pK_b for darifenacin was determined based on a single 30 nM darifenacin concentration

Group	Methoctramine	4-DAMP	p-F-HHSiD	Darifenacin
M ₂ [†]	7.8-8.3	8.0-8.4	6.0-6.9	7.0-7.4
M ₃ [†]	6.3-6.9	8.9-9.3	7.8-7.9	8.4-8.9
Sham	6.2 ± 0.2	9.1 ± 0.2	7.7 ± 0.1	8.5 ± 0.1
DIV	6.0 ± 0.2		7.4 ± 0.2	8.9 ± 0.2
DIV-DEN	6.3 ± 0.2		7.4 ± 0.2	8.9 ± 0.2
DEN	6.5 ± 0.2	8.5 ± 0.2	7.0 ± 0.2	7.9 ± 0.4
BOO	6.2 ± 0.3		6.8 ± 0.3	8.1 ± 0.2
DEC	5.0 ± 0.6		6.8 ± 0.3	7.7 ± 0.2

[†] Adapted from Caulfield (1993) and Caulfield & Birdsall (1998) which include both functional and ligand binding studies.