Muscarinic Receptor Subtypes in the Lower Urinary Tract

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Abstract
Acetylcholine acting on muscarinic $M_3$ receptors on the detrusor muscle is the principal stimulus for inducing the contractile response for urinary bladder voiding. The urinary bladder expresses, however, all cloned muscarinic receptor subtypes ($M_1$–$M_5$). In terms of quantity, the $M_2$ subtype dominates over the $M_3$ subtype in the detrusor, and its role in contraction seems to be primarily indirect, by blocking stimuli from cAMP-coupled receptors that induce relaxation. The excitatory $M_1$ and inhibitory $M_2$ and/or $M_4$ subtypes are also expressed prejunctionally. Muscarinic $M_1$ and $M_2$/$M_4$ autoreceptors facilitate and inhibit, respectively, the release of acetylcholine. The urothelium had been considered to be a passive barrier; however, during the last decade, it has been shown that the urothelium is of importance for bladder function. In a state of bladder pathology, muscarinic receptor changes occur in the detrusor, prejunctionally, and in the urothelium, but the character of the change differs between disorders. The urothelium expresses all subtypes of muscarinic receptors, and upon stimulation it releases factors affecting bladder afferents and smooth muscle. During inflammation, the expression of muscarinic $M_3$ receptors is increased, particularly in the urothelium, together with acetylcholinergic-induced production of nitric oxide in the mucosa. The present review describes signalling mechanisms, expression and functional effects of muscarinic receptors in the lower urinary tract. Their roles in physiological and pathophysiological conditions, as well as clinical implications of the occurrence of different muscarinic receptors, are discussed.

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Introduction
The functions of the urinary bladder are to store urine and to void under voluntary control. The function of the bladder is regulated by an interaction between the somatic, parasymathetic and sympathetic nervous systems. When the bladder volume is below the threshold volume for inducing voiding, the bladder gradually distends and the filling results in only minute rises in intravesical pressure [1, 2]. When the threshold volume is reached, impulses in afferents are generated through the stimulation of tension receptors in the bladder wall. The impulses are conveyed via afferent myelinated $A_b$-fibres, principally in the pelvic nerve to the spinal cord, initiating the micturition reflex [3, 4]. The activation of efferent neurons in the pelvic nerve releasing acetylcholine, which acts on muscarinic receptors on the detrusor muscle, together with an increase in intraabdominal pressure, are
the major mediators of the response that may lead to the expulsion of urine [5]. In a state when the parasympathetic input dominates, the sympathetic stimulation is quiescent, leading to relaxation of the internal urethral sphincter. Normally, the micturition is, however, under the control of the individual, since the external sphincter, innervated by the somatic pudendal nerve, relaxes voluntarily and enables bladder voiding.

The muscarinic receptor occurs in 5 subtypes (M1–M5) and the fundamental significance of muscarinic M3 receptors for micturition is well established [6, 7]. Although it has been recognized for a long period of time that other subtypes of the receptor can be found on smooth muscle cells, when examined morphologically, the functional significance of the different receptor subtypes has not been fully unravelled. It is well known that the subtypes of the receptor population interact on neuronal as well as on non-neuronal cells in the regulation of autonomic responses [8–10]. Lately, however, muscarinic receptors have also been suggested to be implicated in the control of inflammation, cell growth and proliferation [11–17].

The muscarinic receptors belong to the family of G-protein-coupled receptors [18]. The G proteins, consisting of one α-, β- and γ-subunit, are subdivided into Gα, Gβδ, Gq and G12 depending on the primary sequence homology of their α-subunits [19]. The muscarinic receptor subtypes couple differentially to the G proteins, and the subunits of G proteins activate distinct cellular pathways. Preferentially, the inhibitory muscarinic M2 and M4 receptors couple to Gα1o whereas the excitatory muscarinic M1, M3 and M5 receptors preferentially couple to Gq11 [20]. The inhibitory muscarinic M2 and M4 receptors may also affect adenylate cyclase activity, prolong the opening of potassium, as well as that of non-selective cation channels and transient receptor potential channels [21]. Muscarinic M1, M3 and M5 receptors, on the other hand, increase intracellular calcium by mobilizing phosphoinositides that generate InsP3 (inositol 1,4,5-trisphosphate) and DAG (1,2-diacylglycerol) [17, 22].

The Lower Urinary Tract

Innervation

The detrusor smooth muscle cells are supplied by an abundant quantity of autonomic nerve fibres. Animal studies show a rich innervation of cholinergic nerves in the detrusor, while noradrenergic nerves appear relatively sparse [23]. Similarly, the autonomic nerves in the human bladder contain choline acetyltransferase, and electron microscopy has demonstrated that the majority of axonal varicosities in close proximity to detrusor smooth muscle have features of cholinergic nerve terminals [24–26]. As in other species, the detrusor muscle of the human bladder is sparsely supplied with sympathetic nerve fibres [27]. Clusters of autonomic cells populate the bladder wall, most numerously in the adventitia. The choline acetyltransferase-containing neurons, and therefore presumably cholinergic neurons, receive excitatory inputs from pre-ganglionic cholinergic nerve terminals and inhibitory signals from noradrenergic nerve terminals [28]. Also, the urethral internal sphincter is innervated by cholinergic and noradrenergic nerve fibres [27, 29].

Detrusor

The contraction of the urinary bladder is primarily dependent on the activation of muscarinic receptors. In the urinary bladders of different species, including man, mRNAs for all 5 muscarinic receptor subtypes are expressed [30, 31]. In the human detrusor, Mansfield et al. [32] reported that of the total muscarinic receptor population, 70% were of the M2 subtype, 20% of the M3 subtype and 10% of the M1 subtype. The dominance of muscarinic M2 receptors is consistently reported. So, the ratio between muscarinic M2 and M3 receptors in binding studies has been estimated as 9:1 and 3:1 in rats and humans, respectively [33, 34]. Although in the minority, in several functional and knockout studies, the muscarinic M3 receptors have been linked with the entire or almost the entire cholinergic contractile response of the bladder [35–41]. Also, the muscarinic M3 receptor protein closely resembles the protein of the muscarinic M1 receptor [42], and its pharmacological effect is hard to discriminate from that of other excitatory muscarinic receptors, particularly the M3 subtype [44, 45]. Nevertheless, muscarinic M3 receptors may mobilize calcium by different pathways in the bladder; in the human bladder, by extracellular calcium through nifedipine-sensitive calcium channels and by activation of a Rho kinase [46]. Although muscarinic M3 receptors are principally responsible for the bladder contraction, in vivo knockout studies have revealed that muscarinic M2 receptors may have direct but small contractile effects [38]. However, prerequisites for direct muscarinic M2 receptor involvement in contraction are inactivated muscarinic M2 receptors and high levels of intracellular cAMP [34, 47]. Still, the predominant effect of the muscarinic M2 receptors is indirect, facilitating
contractions by opposing relaxations induced by adenylate-cyclase-coupled receptors such as the β-adrenoceptors and P1 purinoceptors [35, 48–54] (fig. 1). Cross-talks between muscarinic M₂ and M₃ receptors in the regulation of second messengers and extracellular signal-regulated kinase activity have been demonstrated in muscarinic transfected CHO cells [55]. The functional effects of the muscarinic M₂ receptors in the urinary bladder seem to be difficult to discern, since cystometric studies demonstrate that muscarinic M₁ receptor knockout mice have normal micturition parameters [39]. Also, muscarinic receptors seem to affect neuronal nitric oxide (NO) synthase activity, as shown in the rat urinary bladder [56]. Even though NO may modulate bladder responses and seems to be responsible for the main part of the inhibitory non-adrenergic non-cholinergic responses in the lower urinary tract, its functional role in the normal bladder is not established [57]. It has been suggested that NO may be an important factor for relaxation during the filling phase [58, 59]. The efferent limb in the relaxatory loop, in which NO is the last link, seems to be the hypogastric nerve [60]. The release of noradrenaline is thus suggested to induce production of NO.

In the urinary bladder, prejunctional muscarinic receptors modify the release of acetylcholine into the synaptic clefts by excitatory muscarinic M₁ and inhibitory M₂ or M₄ receptors [40, 61–65]. In view of the low specificity of the pharmacological tools used in several of the studies, the characterization of either muscarinic M₂ or M₄ receptors must be considered indecisive. Experiments on the urinary bladder in knockout mice show that the inhibitory autoreceptor is of the muscarinic M₄ receptor subtype [66]. Nevertheless, the inhibitory modulation preferentially occurs at low-intensity nerve activity, whereas, with intense activity, short-lasting facilitator modulation occurs [67]. Also, NO affects the release of acetylcholine by inhibition, as indicated by examinations of the female rabbit bladder [68]. Interactions between the parasympathetic and sympathetic nervous systems have also been demonstrated in the bladder, i.e. inhibitory muscarinic receptors located on adrenergic nerve terminals inhibit the release of noradrenaline in the rabbit bladder and urethra [69–71]. In the rabbit urinary bladder, α₂-adrenoceptors also inhibit the release of acetylcholine [67].

Urothelium

The urothelium functions as a barrier against entry of pathogens, water, ions, solutes and macromolecules into the underlying tissue [72–74]. Even if the barrier function is important, the urothelium possesses several other dynamic qualities. The urothelial cells express several types of receptors, and stimulation of these receptors may cause release of substances affecting detrusor function [75, 76]. The porcine urothelium expresses a high density of muscarinic receptors, even higher than the bladder smooth muscle [77], and, in the rat and human urothelium, the receptor proteins and mRNAs, respectively, for all muscarinic receptor subtypes (M₁–M₅) occur [31, 78]. However, the expression pattern seems to vary for the different subtypes. Principally in the human urothelium, the M₁ receptors were reported to occur on basal cells, M₂ on umbrella cells, M₃ and M₄ homogenously and M₅ with a decreasing gradient from luminal to basal cells [79]. Mansfield et al. [32] revealed by RT-PCR analysis an abundant expression of muscarinic M₂ receptors in the human bladder mucosa, but the receptors may occur at other locations in humans also, e.g. on myofibroblast-like cells [80].

Function of Muscarinic Receptor Subtypes

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Because of the proximity of the suburothelial nerve plexus to the urothelium [81–84], the urothelium is suggested to work as a mechanosensory conductor, e.g. in response to distension, it releases adenosine-5’-triphosphate (ATP) affecting underlying afferent nerve fibres via purinoceptors [85–87] (fig. 2). Furthermore, stimulation of urothelial muscarinic receptors induces release of ATP, which may consequently modify the afferent response of the bladder [88, 89]. Acetylcholine is also produced in the urothelium, but the mechanism behind its release from the urothelium does not seem to involve vesicular exocytosis, which seems to be a general feature of non-neuronal release [90–92]. The organic cation transporter 3 subtype has been suggested to be involved in the non-neuronal release and to occur in the rat urothelium [92, 93]. A muscarinic receptor negative feedback mechanism on acetylcholine release was also described in the urothelium.

Notably, it has been suggested from in vitro studies of the rat bladder, a cholinergic-induced release of a relaxant factor also occurs, but it does not seem to be released from the mucosa [95, 96]. In confirmation, during in vivo examinations at our laboratory, the removal of the mucosa did not change the cholinergic contractile bladder response of the normal rat [97]. However, it remains to be established whether this unidentified factor is just one factor or several, and, further, whether the factor(s) is released from the urothelium or from other parts of the mucosa.

The Urethra

The muscarinic receptors are also present in the urethra, but their functions have not been clarified [24, 57, 98]. The urethral sphincter tone is predominantly regulated by adrenergic nerves, but muscarinic receptors also modulate the tone [57, 98–102]. Muscarinic receptor activation mediates constriction of the urethra, but also induces urethral relaxation by the release of NO [103–105]. It seems that these two cholinergic effects are site dependent, i.e. the muscarinic receptor mediates contraction of the proximal urethra whilst mediating relaxation of the distal urethra through the release and actions of NO [101, 103]. In the pig, cholinergic urethral constriction seems to be mediated by both the muscarinic M₂ and M₃ receptor subtypes [47]. The authors reported that there seem to be differences according to the circular and the longitudinal urethral layers. Namely, muscarinic stimulation exerted considerably larger contractile responses from the longitudinal muscle than from the circular, and, further, that muscarinic M₂ receptors mostly occurred in the circular muscle layers and muscarinic M₃ receptors in the longitudinal layer. Tentatively, this could indicate an urethral stabilizing and widening function of the cholinergic response during micturition; a longitudinal muscle contraction resulting in a shorter and possibly wider urethra.

Pathophysiologica and Trophic Effects

During pathological conditions of the urinary bladder, changes in the afferent as well as efferent nervous pathways occur [106–108]. Since muscarinic receptors play a predominant role in the contraction of the bladder, several studies have assessed the cholinergic function in bladder pathology. Denervation and decentralization induce super-sensitivity to cholinergic stimuli, as been observed in the rat bladder [109], which also occurs after capsaicin treatment [110]. In the denervated rat bladder

Fig. 2. Indirect acetylcholine (ACh) effects on detrusor cells via urothelial cell function. ACh has been suggested to induce urothelial release of ATP, urothelial-derived unidentified factors (UDIF) and NO. SMC = Smooth muscle cells.
and in the bladder of spinal cord-injured rats, the expression of muscarinic M₂ receptors increases [111–113], while, in diabetic rats, a super-sensitivity to cholinergic stimuli occurs concomitantly with increments in muscarinic receptor density and in muscarinic M₃ receptor expression [114–116]. Modulations in the cholinergic bladder contraction also occur during cystitis [78, 108]. The muscarinic M₁ and M₃ receptors are upregulated, particularly in the urothelium, which seems to be coupled to the synthesis and release of NO [78, 97, 117]. Changes in the expression of muscarinic receptor subtypes can also be induced in a urothelial cell line by the pro-inflammatory substance acrolein found in the bladder [118]. Up-regulation of muscarinic receptors, as well as a parasympathetic sprouting arising in the mucosa, occur during cyclophosphamide-induced cystitis [119]. This may reflect compensatory mechanisms, as the changes in cystitis from prejunctional muscarinic receptor effects might also [120]. In chronic spinal cord injury, a prejunctional change in the muscarinic M₁, M₂ and M₃ receptor expression may be induced [121], as well as augmentation of cholinergic-induced release of ATP from the urothelium [122], and, notably, increases in muscarinic receptor expression at a non-neuronal level occur on suburothelial myofibroblast-like cells during detrusor overactivity and painful bladder syndrome [80]. The character of the expression changes thus varies according to the type of disorder. Research interest has so far mainly addressed the muscarinic M₂ and M₃ receptor subtypes in bladder disorders, while other subtypes, particularly of non-smooth muscle origin, have been overlooked or their roles are difficult to investigate.

In several species, such as the rat and the guinea pig, an atropine-resistant part of the bladder contraction exists (constituting more than 50% of the contraction [123–126]), while it is small or absent in the human bladder [127, 128]. The atropine-resistant part of the contraction is largely mediated by ATP [129, 130]. In man, ATP seems to mediate this response also [131, 132], even though other mechanisms have been suggested [133]. In the rat, the bladder contraction consists of a phasic and a tonic component in which ATP and acetylcholine play predominant roles, respectively [120, 134, 135]. In vivo studies of the rat reveal that both transmitters are important for emptying the bladder [134]. However, a postjunctional interaction between the cholinergic and the purinergic systems seems to occur; the activation of either contractile system results in a reduction in the other [48, 136]. In the bladder of diabetic rats, the cholinergic part of the parasympathetic response is decreased [137]. Conversely, other reports on partial bladder outlet obstruction and cystitis in the rat show that the atropine-sensitive part of bladder contraction is not affected or even increased [78, 138]. In man also, the non-cholinergic contractile effect increases in the functionally disturbed bladder [139]. All studies, performed in different species and during different disorders and conditions, indicate that the muscarinic receptor expression and functions may be conspicuously altered. Furthermore, in pathological conditions, NO seems to be an important molecule in the lower urinary tract [107], and seems to be of pivotal significance in interstitial cystitis [140]. In inflammation, muscarinic receptors have been suggested to couple to endothelial NO synthase activity [78], and muscarinic receptors seem to induce release of NO from the mucosa, as demonstrated in urothelium-denuded rats [97]. In view of the increased muscarinic receptor expression during cystitis, and, further, since muscarinic receptors have been shown to promote growth of tumour cells in several tissues [13, 78, 141–144], the regulation of the muscarinic receptor expression may have a role in the tumour biology of bladder cancer. To exemplify, in prostate cancer, the degree of differentiation is strongly correlated to the expression of muscarinic M₃ receptors [144].

**Clinical Aspects**

Drugs affecting muscarinic receptors have been used frequently and for a long time for treatment of diseases in the lower urinary tract. Generally, cholinergic treatments are nowadays no longer the first choice of treatment, with the exception of the urinary bladder, where anticholinergic treatments may still be used [145, 146]. Medical trials on anticholinergic treatment against overactive bladder have revealed that the placebo effect contributes markedly (by 30–50%) to the clinical improvement of patients [147]. Long-term studies on detrusor instability/overactive bladder have revealed that the 'subtype-selective' drugs of today do not have any better effect on the condition than traditional drugs [148, 149]. Even if their efficacy seems to be comparable, 'subtype-selective' drugs have fewer side effects than traditional drugs [147, 150]. Traditionally, anticholinergic treatment of overactive bladder has been considered to mediate its clinical effect by blocking muscarinic receptors on the detrusor and inhibiting contraction evoked by acetylcholine released from the parasympathetic innervation. At therapeutic doses, the anticholinergic drugs do not seem to inhibit the detrusor contractility [151, 152]. Instead, muscarinic
Antagonists are considered to act mainly during the filling phase, and to increase bladder capacity and decrease urgency. It has been hypothesized that the antagonists act by blocking urothelial muscarinic receptors, and by that inhibiting the effects of non-neuronal acetylcholine on the release of other urothelial substances, such as ATP\(^{[153]}\). Still, a muscarinic M\(_3\)-selective profile is advantageous in order to avoid cardiac, cerebral and possibly glandular side effects\(^{[154–160]}\). In spite of a M\(_3\)-selective profile, many anticholinergics are only capable of discriminating the human muscarinic M\(_3\) subtype from the M\(_1\) and M\(_5\) subtypes with an affinity factor of 1–16\(^{[161–164]}\). The common side effect of anticholinergic therapy against overactive bladder is a dry mouth\(^{[149, 165]}\), and, since muscarinic M\(_1\) receptors exert secretory effects, muscarinic M\(_3\) receptor-selective antagonists would tentatively result in fewer incidences of this adverse effect\(^{[150, 166, 167]}\).

Studies show that muscarinic receptor changes in the urothelium, as mentioned previously, may be of importance in the development of bladder disorders, i.e. in the inflamed urinary bladder and the urothelium of diabetic rats, an increased expression of muscarinic receptors occurs\(^{[78, 168]}\). In view of the expression of muscarinic receptors in the urothelium/aффerent nerves, studies have suggested that these receptors may constitute specific targets for medical treatments against an overactive bladder\(^{[31, 169]}\). A precondition for effect does not necessarily have to be muscarinic receptor subtype selectivity, but could be achieved by local administration of atropine and oxybutynin, as shown in an animal model as well as in patients\(^{[170–172]}\). Evidence for a coupling between muscarinic receptors and C fibres in the bladder has been put forward, i.e. tolterodine increases bladder capacity when it is intravesically administered, and the increase is attenuated by C fibre desensitization\(^{[173]}\). In conclusion, the efficacy of an anticholinergic drug may depend on several factors, such as muscarinic receptor-binding profile, membrane permeability and urinal active metabolites. Irrespective of the kind of disease being treated with drugs acting upon muscarinic receptors, better efficacy and less adverse effects are desirable. Muscarinic receptor subtype-selective drugs may achieve this.

**Concluding Comments**

In the urinary bladder, muscarinic M\(_3\) receptors are the principal receptor for detrusor contraction, but muscarinic M\(_2\) receptors may enhance contractions mainly by inhibition of detrusor relaxation (figure 3 indicates in a schematic illustration the expression of receptors in bladder tissues). The heterogeneous muscarinic receptor population of the urothelium/suburothelium is altered during inflammation, and in particular the expression of
muscarinic M\textsubscript{2} receptors is upregulated. Alterations in the muscarinic receptor signalling systems contribute to the pathogenesis of disorders of the functions reviewed here, including changes in acute responses and after the induction of inflammation. In view of muscarinic receptors being involved in many functions in different tissues besides the urinary bladder, knowledge about the specific receptor subtype involved in the responses provides prerequisites for effective drug treatments against bladder disorders with fewer adverse effects in the future.

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