

# Expression and Function of Muscarinic Subtype Receptors in Bladder Interstitial Cells of Cajal in Rats

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**Purpose:** To locate the muscarinic (M) M2 and M3 receptors in bladder interstitial cells of Cajal (ICCs) and to determine the effects of M2 and M3 agonists on bladder ICCs.

**Materials and Methods:** A total of 30 adult male Sprague-Dawley rats weighing 225-250 g were used in this study. Double-labeled fluorescence of muscarinic receptors and c-kit was performed for co-localization. To evaluate the effect of muscarinic agents on the excitation of bladder ICCs, we analyzed the inward current of bladder ICCs using the whole-cell patch clamp. The effect of muscarinic agents on the carbachol-induced inward currents was evaluated with the whole-cell patch clamp.

**Results:** M2 and M3 receptors were confirmed in the stroma ICCs in rats' bladders with double-labeled immunofluorescence. Spontaneous action potential was observed in freshly isolated bladder ICCs. The carbachol-induced inward Ca<sup>2+</sup> current in ICCs can be blocked by atropine. The M2 receptor antagonist methoctramine (1  $\mu$ M) showed a weak inhibitory capability on the inward Ca<sup>2+</sup> current [from  $74.8 \pm 9.6$  to  $63.3 \pm 13.8$  Pascal (pA),  $n = 12$ ,  $P = .03$ ]. While the M3 receptor antagonist 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) (1  $\mu$ M) significantly inhibited the inward Ca<sup>2+</sup> current (from  $78.4 \pm 11.2$  to  $17.3 \pm 7.9$  pA,  $n = 12$ ,  $P < .001$ ).

**Conclusion:** Bladder ICCs express M2 and M3 cholinergic receptors. Most muscarinic cholinergic receptor antagonists, especially the M3 antagonists, can effectively inhibit the carbachol-induced inward current of bladder ICCs.

**Keywords:** animals; interstitial cells of Cajal; microscopy; rats; urinary bladder; muscle; smooth; receptors.

## INTRODUCTION

The excitation and contraction of the urinary bladder are believed to be regulated neurogenically and myogenically. The bladder can autonomously contract, but it can also initiate contraction under voluntary control. Moreover, independent contraction can be observed in isolated detrusor strips.<sup>(1)</sup> It is unclear how the neurogenic and myogenic interactions coordinate. Whether other mechanisms, such as a pacemaker or other regulation center, are involved remains to be clarified.

Interstitial cells of Cajal (ICCs) are widely distributed in the visceral smooth muscle organs. They possess the ability to self-excite and produce an action potential. ICCs in the gastrointestinal tracts<sup>(2)</sup> and in the upper urinary tracts<sup>(3)</sup> are believed to behave as pacemakers. Additionally, they can receive the innervation pulse.<sup>(4)</sup> In the bladder, ICCs have been found among the detrusor smooth muscle bundles and are closely associated with bladder excitation and contraction.<sup>(5,6)</sup> Changes of bladder ICCs can impact the initiation of spontaneous electrical activity, which may result in homologous functional disturbances in bladder contraction.<sup>(7)</sup> Our previous studies have found that there were some specific changes in the quantity and physiological characteristics of bladder ICCs in bladder instability.<sup>(8)</sup> It is critical to clarify the interaction between bladder innervation and ICCs.

Cholinergic nerves are the main neurogenic factors in bladder function. The muscarinic (M) M2 and M3 receptors are the two main subtypes of muscarinic receptors in the bladder. When stimulated by cholinomimetics in vitro, ICCs generate transient calcium, which is mainly mediated by the M3 receptor agents.<sup>(9)</sup> However, the expression and function of muscarinic receptors in bladder ICC, and its potential value for treatment are still unclear. Based on our previous studies and article reports, we hypothesized that muscarinic receptors were expressed in bladder ICC, and played important role in the bladder excitation regulation. In this study, the location of M2 and M3 receptors in bladder ICCs were observed with immunofluorescence. The effects of M2 and M3 agonists on bladder ICCs were also recorded with the patch clamp technique.

## MATERIALS AND METHODS

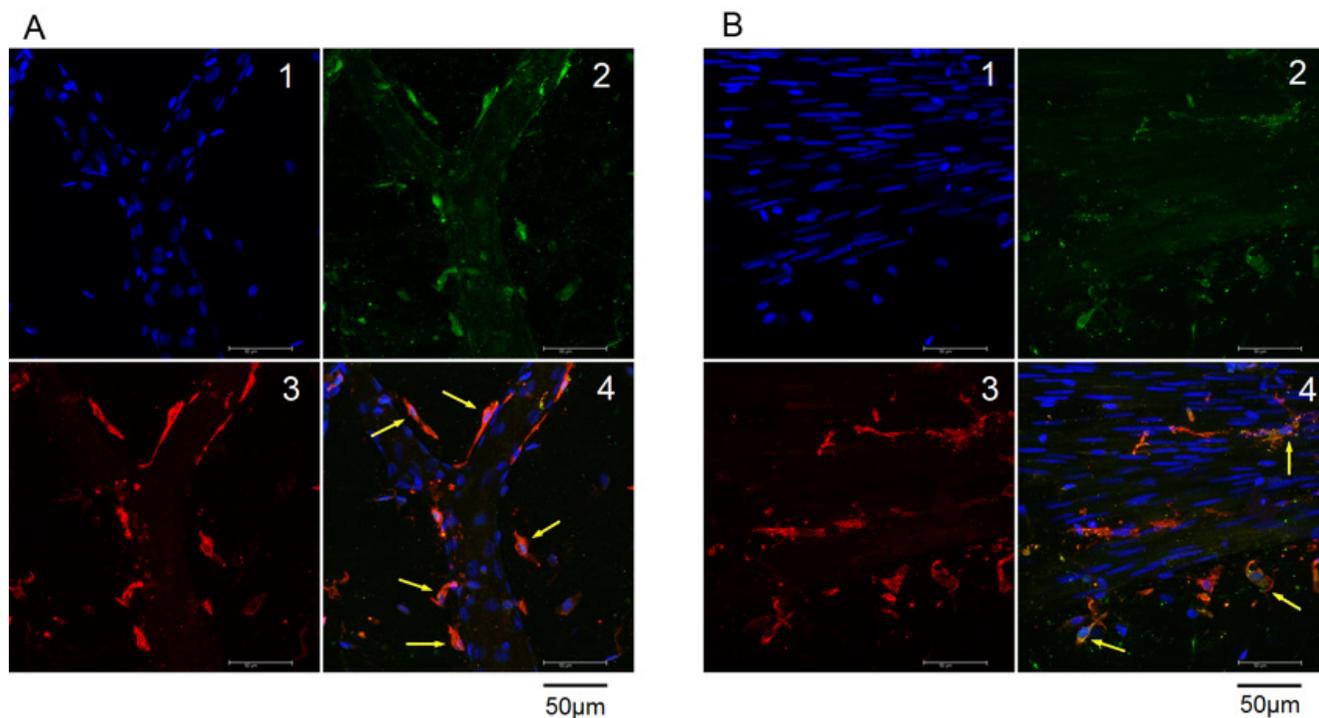
### Animals

A total of 30 adult male Sprague-Dawley rats weighing 225-

250 g were used in this study. This study was approved by the Research Council and Animal Care and Use Committee of Southwest Hospital, Third Military Medical University, China (Approval No. SYXK20070002). The experiments conformed to the guidelines for the ethical use of animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used. All animals were similarly fed in a special pathogen-free room, with free access to food and water. Their general states of health and activity were monitored closely during the experiment. The experimental unit for morphology was rat, and for functional study the unit was rat bladder ICCs. All the experimental manipulations were standardized, and accomplished by an experience experimenter.

### Double-labeled Fluorescence for Bladder ICCs and M2, M3 Receptors

To observe the location of M2 and M3 receptors in bladder ICC, 10 rats were used for this study. Double-labeled fluorescence of muscarinic receptors and c-kit was performed for co-localization. A 25% urethane (1.1 g/kg) intraperitoneal injection was used for anesthesia. Under sterile conditions, the bladder was exposed through a median abdominal incision, and the bilateral ureters were ligated. Then 4% paraformaldehyde was injected into the bladder through a transurethral epidural catheter, and the bladder neck was ligated after the bladder was filled to 20 cmH<sub>2</sub>O. The bladder was harvested and immersed into 4% paraformaldehyde solution for 6 to 8 h for continuous fixing. Under a dissecting microscope, the mucous membrane and its substratum were carefully removed. The bladder's muscular layer was longitudinally cut to 3 × 4 mm and then thoroughly rinsed with 0.01 M phosphate buffered saline (PBS) for 5 min × 3 times, 0.03% H<sub>2</sub>O<sub>2</sub>/Methanol sealing endogenous peroxidase at 37°C for 30 min, then 0.01 M PBS for 5 min × 3 times. It was then incubated in 1% bovine serum albumin (BSA) for 30 min at room temperature. The incubation solution was removed, and anti-c-Kit displaying ICC (goat anti-rat monoclonal, 1:200, Sigma) and M2 or M3 receptor antibodies (rabbit anti-rat monoclonal, 1:200°C Sigma) were added. The samples were incubated in 4°C anti-dilution for 12 h (negative control in 0.01 M PBS as collation), and secondary antibodies marked with Alexa 488 (donkey-anti-goat polyclonal, 1:200°C molecular probes) and Alexa 594 (donkey-anti-rabbit polyclonal, 1:200, molecular probes) was added for 12 h. The samples were then thoroughly rinsed with 0.01 M PBS for 5 min × 3 times and



**Figure 1.** The identification of positive muscarinic (M) receptors in ICCs of rats bladder. A: M2 receptors in rat bladder ICCs; B: M3 receptors in rat's bladder ICCs. Double-labeled immunofluorescence using anti-c-kit (green, A2) for ICC, anti-M2 (red, A3) or anti-M3 (red, B3) for M2 or M3, and DAPI (blue, A1 and B1) for nuclei. Arrows: examples of ICCs double-marked with the c-kit and M2 (A4) or c-kit and M3 (B4) receptors.

incubated with the secondary antibody at 37°C for 1 h. Then, 4',6-diamidino-2-phenylindole (DAPI) was added for 10 min for nucleus fluorescence loading. After 3 times of rinses with 0.01 M PBS (30 min each for the first two rinses and 3 h for the last time), the glass was sealed with glycerol and subjected to laser confocal microscopy. The c-kit was labeled in green under an excitation wavelength of 488 nm, and M2 or M3 receptors were labeled in red under a wavelength of 560 nm.

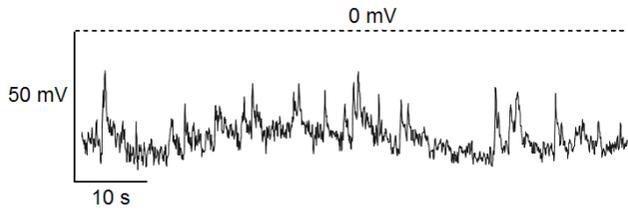
#### **Fresh Isolation and Culture of Bladder ICCs**

To evaluate the effect of muscarinic agents on the excitation of bladder ICCs, we analyzed the inward current of bladder ICCs using the whole-cell patch clamp. According to the sample size for self anterior-posterior contrast study, the number was set at 8 for atropine (effect size/estimated standard deviation = 5.12), and 12 for methoctramine and 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) (effect size/estimated standard deviation = 0.89), with power of 90%, significance level of 5%. All the variables were continuous variables. Referring to the method of McClosky (McClosky & Gurney, 2002), the urothelium layer was carefully removed, and the rat bladder was chopped into small pieces of about 1 mm<sup>3</sup>. The pieces were then incubated in a digestion solution containing (mg/mL) 1.0

Type II collagenase, 2.0 bovine serum albumin, and 1.0 trypsin inhibitor (all from Sigma) at 37°C for 30 min. The supernatant was then removed, and the isolated cells were immersed into enzyme-free Hanks' Balanced Salt Solution (HBS; R and D) and cultured at 37°C in 5% CO<sub>2</sub> and 95% O<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum and 25 ng/mL SCF until proper adherence. ICCs with typical morphology with many branches under visible light positive for c-kit staining were prepared for the subsequent patch clamp studies, as reported by McCloskey and colleagues.<sup>(10)</sup>

#### **Effects of Muscarinic Receptor Antagonists on the Carbachol-induced Inward Current**

The effect of muscarinic agents on the carbachol-induced inward currents was evaluated with the whole-cell patch clamp. Using a one-step method, soft, neutral, hollow glass tubes (Sutter Instrument, America) were drawn by a vertical puller to prepare a microelectrode with a heat value of 56. A microelectrode with a diameter of approximately 1 µm was selected for the patch clamp studies. The supernatant isolated cells was removed and the adhered recording chamber was constantly perfused with Hanks solution at room temperature and addi-



**Figure 2.** The spontaneous action potential of novel ICC samples isolated from adult rat's bladders.

tionally, the cell under study was continuously superfused by a close-delivery system. Positive pressure was loaded into the microelectrode cavity. The electrode was proceeded to the ICCs to attain negative pressure and create substantial impedance. To record the whole cell current, a pCLAMP (Axon Instruments, America) with a sampling frequency of 4 kHz and a filtering frequency of 2 kHz was used. The 3D manipulator was regulated to seal on contact, and the impedance was set to reach 1 G $\Omega$ . The cell membrane was sucked and broken to create a whole-cell recording mode. Whole-cell current recording was conducted in voltage clamp mode. All recorded data were analyzed and treated using Clampfit 9.2 at the backstage. The membrane potential was set at -50 mV with the clamp. Carbamylcholine (1  $\mu$ M and 10  $\mu$ M, Sigma) was added to induce an ICC inward Ca<sup>2+</sup> current, and various antagonists, including the M-receptor antagonist atropine (1  $\mu$ M, Sigma), the M3-receptor antagonist 4-DAMP (1  $\mu$ M, Sigma), and methoctramine, were administered to investigate their functional effect on the carbachol-induced (10  $\mu$ M, Sigma) inward Ca<sup>2+</sup> current in ICCs.

#### Statistical Analysis

All the quantitative data were confirmed to obey the normal distribution with homogeneity of variance by test of normality, and were presented as mean  $\pm$  SD. Student's *t*-test was used for comparison [statistical package for the social science (SPSS Inc, Chicago, Illinois, USA) version 13.0 software], with *P* < .05 considered significant. All the data were double-blinded, randomized and analyzed using posteriori tests.

## RESULTS

### Double-label fluorescence for M2 or M3 Receptors and Bladder ICCs

ICCs were identified by their c-kit positivity and spindle shape. M2 (Figure 1A) and M3 (Figure 1B) receptors were confirmed in the stroma ICCs in rats' bladders with double-labeled im-

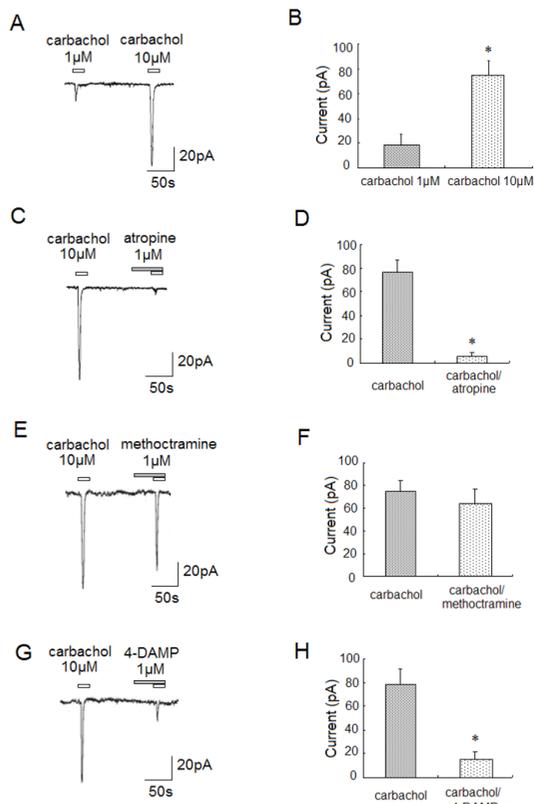
munofluorescence.

### Effects of Muscarinic Receptor Antagonists on Carbachol-induced Bladder Contraction

Spontaneous action potential was observed in freshly isolated bladder ICCs (Figure 2). ICCs can be activated with a dose-dependent inward Ca<sup>2+</sup> current induced by carbachol. The peak current values in 1  $\mu$ M and 10  $\mu$ M of carbachol are 18.3  $\pm$  8.7 Pascal (pA) and 75  $\pm$  10.9 pA, respectively, with statistical differences (*n* = 8, *P* < 0.001; Figures 3A and B). The carbachol-induced inward Ca<sup>2+</sup> current in ICCs can be blocked by atropine. When the carbachol dose was set at 10  $\mu$ M, 1  $\mu$ M atropine could significantly reduce the peak current from 77.1  $\pm$  10.0 pA to 5.8  $\pm$  2.7 pA (*n* = 8, *P* < .001; Figures 3C and D). The carbachol-induced inward Ca<sup>2+</sup> current in ICCs can also be blocked by M2 and M3 receptors antagonists. The M2 receptor antagonist methoctramine (1  $\mu$ M) showed a weak inhibitory capability on the inward Ca<sup>2+</sup> current (from 74.8  $\pm$  9.6 to 63.3  $\pm$  13.8 pA, *n* = 12, *P* = 0.03; Figures 3E and F), While the M3 receptor antagonist 4-DAMP (1  $\mu$ M) significantly inhibited the inward Ca<sup>2+</sup> current (from 78.4  $\pm$  11.2 to 17.3  $\pm$  7.9 pA, *n* = 12, *P* < .001; Figures 3G and H).

## DISCUSSION

Many studies on detrusor myocytes, receptor expression, cell-cell communication and cell phenotypic alteration have been conducted, but the mechanisms responsible for bladder excitation and contraction have not been well clarified.<sup>(11)</sup> ICCs distributing submucosally and among the smooth muscle bundles are thought to mediate signal transmission from neurons to smooth muscles.<sup>(12,13)</sup> In this study, we hypothesized and confirmed the expression and function of muscarinic cholinergic subtype receptors in ICCs with double-labeled immunofluorescence. M2 and M3 receptors were confirmed in bladder ICCs. In human and guinea pig bladder, the proportion of M2 and M3 is about 3:1, although the M3 subtype is dominant in the excitation of smooth muscle contraction via Gq/11 activating hydrolysis of phosphoinositide, IP<sub>3</sub> production, and elevation of intracellular Ca<sup>2+</sup>. Conversely, M2 receptors may indirectly enhance M3-mediated contraction, by opposing the normal effects of  $\beta$ -adrenoceptor G protein-coupled activation of adenylyl cyclase which lead to cyclic adenosine mono phosphate (cAMP) production and bladder relaxation.<sup>(14)</sup> The innervation methods of smooth muscles and skeletal muscles are different.



**Figure 3.** Effects of carbachol, atropine, methoctramine and 4-DAMP to inward Ca<sup>2+</sup> currents of bladder ICCs. A and B: Carbachol-induced inward currents in bladder ICCs. C and D: Atropin's inhibition of the inward current stimulated by carbachol. E and F: Methoctramine's inhibition of the inward current stimulated by carbachol. G and H: 4-DAMP's inhibition of the inward current stimulated by carbachol.

In skeletal muscle organs, neural impulses can directly activate the muscle cells through neuromuscular synapses. In smooth muscle organs, there are no analogous structures connecting the cells and neural terminals. When the innervation impulse reaches the nerve terminal, the secretory vesicle releases neurotransmitters, which diffuse throughout the intercellular fluid and act on the target cells. Many factors can affect excitation through this regulatory pathway. Because ICCs in the gastrointestinal tracts are considered pacemakers, ICCs in the bladder might be presumed to facilitate signal transduction from nerve terminals to detrusor myocytes. Because ICCs express the specific cell membrane surface receptor kit, the c-kit receptor identification companion and its cell shape and phenotype have been acknowledged as the standards for ICCs identification.<sup>(15)</sup> Yamanishi and colleagues found that ICC in the bladder have close structural relationships with cholinergic nerves. Freshly

dispersed detrusor ICCs and ICCs in situ respond to cholinergic stimulation by firing Ca<sup>2+</sup> transients.<sup>(8)</sup>

Among the innervation types in the nervous system, cholinergic nerves - particularly muscarinic cholinergic receptors - are the most important for bladder excitation and contraction. The parasympathetic impulse can trigger the neural transmitter and initiate bladder contraction via the muscarinic cholinergic receptor. Different subtypes of muscarinic cholinergic receptors can be classified by specific selective receptor antagonists. The expression of M2 and M3 subtype receptors has been confirmed mainly in the bladder. Muscarinic cholinergic receptors with a high affinity with AF-DX 116 are defined as Type M2, while those with a high affinity with 4-DAMP are defined as Type M3. Different blocking agents with relative selectivity for muscarinic receptors have been obtained. Among these agents, triptiramine has been found to possess the highest affinity with M2 receptors, followed by AFDX384 and imbacine. 4-DAMP possesses the highest affinity with M3 receptors, followed by darifenacin.<sup>(16)</sup> M2 receptors are more abundant than M3 receptors in the bladder, which may indirectly mediate detrusor contraction by deteriorating the detrusor dilatation mediated by the β adrenergic nerve.<sup>(17)</sup> Although M3 receptors are less prevalent, they are believed to play a central role in mediating bladder contractions.<sup>(18)</sup> In the gastrointestinal tract, ICCs in the gastric antrum and stomach can be stimulated by cholinergic neural pulses, and they can subsequently increase the slow-wave frequency. This effect can be mediated by M3 receptors, indicating that cholinergic neurotransmitters can regulate ICC excitation of the gastrointestinal tract and subsequently effect smooth muscle contraction.<sup>(19)</sup> It can be assumed that ICCs in the urinary bladder can influence bladder excitation, which can be regulated by muscarinic cholinergic nerves, especially M3 receptors.

In our functional studies, the results might indicate that bladder ICCs are mainly excited by M3 receptors, as displayed by inward currents and depolarization. It is generally believed that M3 receptors may activate phosphoinositide hydroxylation by coupling with the Gq/11 of G protein to produce IP3 and DAG as second messengers, thereby mediating the increase of the calcium ions in cells. M3 receptors also directly induce detrusor contractions,<sup>(20)</sup> but the detailed signal pathway still needs to be clarified. The functional studies on the effect of cholinergic antagonists on the carbachol-induced ICC inward current sug-

gest ICCs' important role in regulating bladder excitation from neurons to detrusor myocytes.

## CONCLUSION

Bladder ICCs may play an important role in regulating bladder excitation and contraction, presumably through the muscarinic cholinergic transmissions to the detrusor myocytes. Bladder ICCs express M2 and M3 cholinergic receptors. Most muscarinic cholinergic receptor antagonists, especially the M3 antagonists, can effectively inhibit the carbamylcholine-induced inward current of bladder ICCs.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

None declared.

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