**Full Paper**

**Are Antimuscarinic Drugs Effective Against Urinary Frequency Mediated by Atropine-Resistant Contractions?**

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Received August 2, 2010; Accepted January 11, 2011

**Abstract.** In the disease states of urinary frequency and urgency, atropine-resistant contractions are known to be involved, in addition to contractions mediated by cholinergic nerves. This study was undertaken to investigate the mechanism underlying the development of atropine-resistant contractions using the representative antimuscarinic drugs solifenacin and tolterodine and also propiverine that has Ca²⁺ channel-antagonizing activity in addition to antimuscarinic activity. Rat models of urinary frequency were established by intravesical infusion of acetylcholine (ACh) (cholinergic nerve–mediated urinary frequency model), acetic acid (AcOH) [non-adrenergic non-cholinergic nerve (NANC)-mediated urinary frequency model], or CaCl₂ (atropine-resistant contractions-mediated urinary frequency model). Cystometrograms were obtained to measure the micturition parameters following oral administration of the aforementioned drugs. Propiverine increased the micturition weight in all the urinary frequency models. Solifenacin and tolterodine increased the micturition weight in the ACh-induced urinary frequency model but neither had any effect in the AcOH- or CaCl₂-induced urinary frequency models. While antimuscarinic drugs are, in general, effective for the control of urinary frequency and incontinence, use of drugs possessing inhibitory effects on contractions mediated by cholinergic as well as NANC nerve transmission or Ca²⁺ influx into smooth muscles is recommended for management of the symptoms in disease states in which atropine-resistant contractions, such as Ca²⁺- and capsaicin-sensitive sensory nerves, are involved.

**Keywords:** urinary frequency, overactive bladder, propiverine, antimuscarinic, atropine-resistant contraction

**Introduction**

In recent years, drugs with high selectivity for muscarinic receptors have been developed and demonstrated to yield gratifying clinical results in the management of urinary frequency and incontinence. Over time, antimuscarinic agents have come to be used for the treatment of urinary frequency and urgency in patients with overactive bladder (OAB) or neurogenic bladder all over the world. The underlying mechanism of action of these drugs consists of inhibition of detrusor overactivity by blocking the binding of acetylcholine to muscarinic receptors (1).
nerves, Ca\(^{2+}\) influx is involved in smooth muscle contractions. Therefore, it is important for the control of such symptoms as urinary frequency and urgency to also inhibit atropine-resistant contractions; however, the commonly used muscarinic receptor–specific drugs do not inhibit these contractions.

Under these circumstances, we conducted this study using an atropine-resistant contractions-mediated urinary frequency model to verify whether antimuscarinic agents currently in use in the clinical setting might be useful for the management of urinary frequency and urgency in which atropine-resistant contractions are also involved. The drugs assessed included solifenacin and tolterodine, both of which exhibit high specificity for the muscarinic receptor, and propiverine that is endowed with an anti-

The measurements were carried out on Day 7 after the operation. A swivel (22 ga single channel stainless steel swivel; Instech Laboratories, Inc., Plymouth Meeting, PA, USA) was coupled to the catheter connected to the bladder of the rat and the other end of the catheter lumen was divided into two branches with a three-way stopcock. One branch was connected via a pressure transducer (Disposal Blood Pressure Monitoring Life Kit DX-360; Nihon Kohden Corporation, Tokyo) to a pressure amplifier (Blood Pressure Amplifier AP-641G combined with the main unit case RMP-6008M, Nihon Kohden Corporation) to measure the intravesical pressure. The intravesical pressure was recorded with a PowderLab 16/30 System (Chart v5.2.2 for Windows; ADInstruments Pty Ltd., Sydney, Australia). The urine weight was recorded with the PowerLab 16/30 System simultaneously in terms of changes in the urine weight measured using an electronic balance (HF200; A and D Company Co., Ltd., Tokyo) placed directly underneath the cage. The other branch was connected to a syringe fitted to a continuous injector (Micro syringe pump KDS200; KD Scientific, Colorado Springs, CO, USA).

Intravesical infusion of physiological saline was performed at the rate of 3.0 mL/h. The test drugs were administered orally after achieving stabilization of the intravesical pressure, and at the same time, the physiological saline infused intravesically was switched to physiologically saline containing 30 mM ACh (Wako Pure Chemical Industries, Ltd., Osaka), 0.3% AcOH (Wako Pure Chemical Industries, Ltd.), or CaCl\(_2\) (30, 50, 80 mM; Wako Pure Chemical Industries, Ltd.) (3, 6). The cystometric measurements were initiated 30 min after the dosing and continued for over 2.5 h with the animals in a conscious state without restraint.

**Materials and Methods**

**Animals**

Female 11-week-old Sprague-Dawley rats were used. The animals, purchased from Charles River Japan, Inc. (Yokohama), were accommodated in cages in an environmentally controlled room at an ambient temperature and relative humidity of 20°C – 26°C and 30% – 70%, respectively, under a 12-h light/dark cycle (lights from 6:00 to 18:00 h). Water and food were provided ad libitum. All experiments were conducted in accordance with the Laboratory Animal Care Code of Ethics of Taiho Pharmaceutical Co., Ltd.

**Cystometry**

After induction of anesthesia, the body temperature of the animals was maintained at 37°C using a heating board (PS-53; Sakura Finetek Japan, Tokyo) during the operation. The abdomen was opened and the bladder was exposed from the abdominal cavity, followed by a small incision of the bladder vertex. A polyethylene tube (PE-50; Becton Dickinson, Franklin Lakes, NJ, USA) was inserted into the urinary bladder and fixed therein, followed by suture of the incised area. The catheter was passed subcutaneously and led out of the occipital region to be passed into a spring.

Drugs and administration

Atropine sulfate (1 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), verapamil hydrochloride (3 mg/kg; Wako Pure Chemical Industries, Ltd.), propiverine hydrochloride (1 – 30 mg/kg, Taiho Pharmaceutical Co., Ltd., Tokyo), solifenacin succinate (0.3 – 10 mg/kg; Taiho Pharmaceutical Co., Ltd.), and tolterodine tartrate (0.1 – 3 mg/kg; Taiho Pharmaceutical Co., Ltd.) were dissolved in purified water to prepare the dosing solutions at the time of switching of the intravesical infusion fluid. Capsaicin (125 mg/kg, Sigma-Aldrich) was dissolved with ethanol at a concentration of 200 mg/mL and mixed with an equal volume of Tween 80 (Sigma-Aldrich). This mixture was mixed with 8 times its volume of physiological saline to prepare the dosing solution. For desensitization with capsaicin, the prepared injection fluid was ad-
ministered subcutaneously at 50 mg/kg in the morning 5 days before the measurements, at 25 mg/kg about 12 h afterwards, and at 50 mg/kg in the morning 4 days before the measurements. All injections were performed while the animals were under halothane anesthesia according to the method of Chuang et al., and the anesthesia was maintained for approximately an hour after the capsaicin injection attenuated the pain (3).

Phenylalkylamine (PAA) sensitive L type Ca\(^{2+}\) channel affinity
The rat cerebral cortex was used for PAA-sensitive L-type Ca\(^{2+}\) channels. After the tissue was excised, it was homogenized and suspended in 19 volumes of 50 mM Tris-HCl, pH 7.4 (Wako Pure Chemical Industries, Ltd.) and used as the receptor preparation. \(^{3}\)H]-Desmethoxyverapamil (Amersham Biosciences, Little Chalfont, UK) was used as the tracer substance, and methoxyverapamil (from 1 × 10\(^{-8}\) M to 3 × 10\(^{-6}\) M, Sigma-Aldrich) was used as the positive control. The Ki values were computed using the formula: Scatchard analysis of the results of the binding tests, and the Ki values were computed using the formula: 

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Ki = IC_{50} / (1 + L / Kd).
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Statistical analyses
The waveforms of the micturition weight and intravesical pressure measurement data were analyzed by using PowerLab Chart v5.2.2. For all the parameters assessed, the mean and standard error (S.E.M.) of the measurements from the first micturition to the last micturition were calculated. The paired \(t\)-test was used for comparison of the pre and post model responses. Comparison between the results in the normal group infused with physiological saline and those in each of the ACh-, AcOH-, and CaCl\(_2\)-induced urinary frequency models was performed by Student’s \(t\)-test. Comparison between the results in each model group and each of the antimuscarinic agent–treated groups was carried out using Williams’ test. Increase of the micturition weight as compared with that in the control group was observed in the antimuscarinic agent–related group, and the percent effective ratio was calculated. The ED\(_{50}\) was determined by linear regression analysis. For the statistical analyses, EXSAS (EXSAS 7.10; Arm Systex, Osaka) was used, with the \(P\)-value for significant difference set at <0.05 in the Dunnett’s test and Student’s \(t\)-test and at <0.025 in the Williams’ test.

Results

Effects of the agents examined in the normal rat
The experiment was conducted on rats that had been operated on 7 days previously. Cystometric measurements in the animals were performed in the usual manner to record the voiding behavior. The effects of the drugs were assessed during the course of continuous intravesical infusion of saline following administration of distilled water or one of the drugs. Thus, the effects of purified water, atropine (1.0 mg/kg), verapamil (10 mg/kg), desensitization (capsaicin, 125 mg/kg), propiverine (30 mg/kg), solifenacin (10 mg/kg), or tolterodine (3 mg/kg) were evaluated. The micturition weights in the groups given purified water, atropine, verapamil, capsaincin, propiverine, solifenacin, and tolterodine were 1.09 ± 0.13, 1.15 ± 0.06, 1.17 ± 0.06, 1.37 ± 0.10, 1.34 ± 0.12, 1.37 ± 0.14, and 1.40 ± 0.11 g, respectively; and the maximal voiding pressures were 14.5 ± 2.2, 11.6 ± 1.6, 17.2 ± 1.5, 19.3 ± 2.6, 15.6 ± 1.0, 14.9 ± 1.2, and 15.7 ± 0.4 mmHg, respectively.

Effects of antimuscarinic agents in the ACh-induced urinary frequency model
The effects of the drugs were assessed during the course of continuous intravesical infusion of ACh following administration of distilled water or each drug. Typical charts are presented in Fig. 1. In regard to the control of the urinary frequency in the ACh-induced urinary frequency model, atropine caused a 76.0% increase of the micturition weight (\(P = 0.002\)) and verapamil produced a 46.0% increase, but the difference was not statistically significant (\(P = 0.061\)). Desensitization with capsaicin was ineffective (\(P = 0.193\)). None of these treatments had any effects on the intravesical pressure (Fig. 1). Then, the effects of propiverine (1 – 10 mg/kg), solifenacin (0.3 – 3 mg/kg), and tolterodine (0.1 – 1 mg/kg) were explored. In the ACh-induced urinary frequency model, propiverine given at the dose of 10 mg/kg, solifenacin at the dose of 3 mg/kg, or tolterodine at the dose of 1 mg/kg caused a significant increase in the micturition weight without affecting the intravesical pressure (Fig. 2). The ED\(_{50}\) of propiverine, solifenacin, and tolterodine were 3.05, 0.56, and 0.35 mg/kg, respectively.
Antimuscarinics in Pollakiuria Models

The effects of antimuscarinic agents on AcOH-induced urinary frequency were explored in this animal model of urinary frequency evoked by stimulation of capsaicin-sensitive nerves. Desensitization with capsaicin abolished AcOH-induced urinary frequency without affecting the intravesical pressure. Atropine and verapamil were ineffective ($P = 0.981$, $P = 0.948$) (Fig. 3).

Then, the effects of propiverine (3 – 30 mg/kg), solifenacin (1 – 10 mg/kg), and tolterodine (0.3 – 3 mg/kg) were assessed. Propiverine given at the dose of 30 mg/kg caused an increase in the micturition weight without affecting the intravesical pressure. Solifenacin and tolterodine had no appreciable effect on the micturition parameters (Fig. 4).

Assessments in the CaCl$_2$-induced urinary frequency model

Rats receiving continuous intravesical infusion of CaCl$_2$ at various concentrations were observed for changes in their voiding behavior. Continuous infusion of 30 mM CaCl$_2$ did not produce any change in the voiding behavior as compared with the observations in the rats administered intravesical infusion of physiological saline. Infusion of 50 mM CaCl$_2$ caused a reduction in the micturition interval, that is, induced urinary frequency, without affecting the intravesical pressure. Infusion of 80 mM of CaCl$_2$ further shortened the micturition
interval, so that it was difficult to identify micturition at all in occasional animals (Fig. 5). Based on these results, a CaCl₂ concentration of 50 mM was adopted for this urinary frequency model. Verapamil produced a 72.3% increase \( (P = 0.004) \) of the micturition weight without exerting any influence on the intravesical pressure in the rats with urinary frequency induced by 50 mM CaCl₂. Neither atropine nor desensitization with capsaicin had produce any effect (Fig. 6).

**Effects of antimuscarinic agents in the CaCl₂-induced urinary frequency model**

The effects of propiverine (1 – 10 mg/kg), solifenacin (0.3 – 3 mg/kg), and tolterodine (0.1 – 1 mg/kg) on CaCl₂-induced urinary frequency were explored. As compared with the observations in the control group, propiverine at 10 mg/kg significantly increased the micturition weight without exerting any effects on the intravesical pressure. The micturition weight tended to increase in response to administration of solifenacin or tolterodine at the doses tested (Fig. 7). In view of this finding, we further assessed the effects of these two drugs at higher dose levels in a preliminary study, but neither drug produced any significant improvements (data not shown). The ED₅₀ of propiverine was 3.29 mg/kg and the values for the other antimuscarinic agents could not be calculated.
Fig. 5. Effects of each concentration of intravesical CaCl₂ on the intravesical pressure and micturition weight in conscious freely-moving rats. Upper panel shows the intravesical pressures, and the lower panel shows the micturition weight. The dots represent the micturition points. A: 0 mM, B: 30 mM, C: 50 mM, D: 80 mM.

Fig. 6. Effects of intravesical instillation of 50 mM CaCl₂ stimulation on intravesical pressure and micturition weight in conscious freely-moving normal rats and desensitized rat. Upper panel shows the intravesical pressure, and the lower panel shows the micturition weight. The dots represent the micturition points. A: atropine (1 mg/kg), B: verapamil (10 mg/kg), C: desensitized rat.
controls, atropine at the dose of 0.32 mg/kg p.o. was reported to produce a significant decrease of the blood pressure in normal rats (14); therefore, these doses of atropine and verapamil were selected for this study. Desensitization with capsaicin was performed in the usual manner (3). The administration of each drug and the desensitization had no significant effect on the micturition weight or intravesical pressure in normal animals.

In the evaluation of antimuscarinic agents, atropine exerted a conspicuous ameliorative effect in the ACh-induced urinary frequency model, whereas the Ca\textsuperscript{2+} antagonist verapamil produced some improvement, but not statistically significant, of urinary frequency in this model. Verapamil was demonstrated to reduce the maximal contractile response to carbachol (pD\textsubscript{2} = 6.98) in a study on isolated urinary bladder (15), and this effect may underlie the improvement of the urinary frequency in the ACh-induced urinary frequency mode. It was thus inferred that in addition to the antimuscarinic activity, the Ca\textsuperscript{2+} channel–antagonizing activity might also contribute to the control of ACh-induced urinary frequency. In the ACh-induced urinary frequency model, the antimuscarinic agents used in this study were demonstrated to cause a dose-dependent increase in the micturition weight; this finding was consistent with previously reported data obtained in a rat model of cerebral infarction (16).

We also conducted assessments in the AcOH-induced urinary frequency model established via stimulation of capsaicin-sensitive nerves (3). None of the drugs, including atropine, the other two specific antimuscarinic drugs examined (solifenacin and tolterodine) and verapamil, improved urinary frequency, whereas significant improvement of the urinary frequency was observed in the propiverine-treated group and the group that was subjected to capsaicin desensitization. It has been demonstrated that propiverine directly binds to transient receptor potential vanilloid subtype 1 (TRPV1) and thereby inhibits capsaicin-stimulated Ca\textsuperscript{2+} influx (17). The inhibition of AcOH-stimulated urinary frequency observed in the present study was considered to be consequent to inhibition of C-fiber hyperfunction via inhibition of Ca\textsuperscript{2+} influx caused by propiverine binding to TRPV1. It was inferred from these findings that antimuscarinic and Ca\textsuperscript{2+} channel–antagonizing activities are ineffective for the control of urinary frequency caused by C-fiber stimulation, whereas propiverine alleviates the urinary frequency in this model via TRPV1.

A CaCl\textsubscript{2}-induced urinary frequency model in which concentration-dependent urinary frequency can be

Affinity of antimuscarinic agents for PAA-sensitive L-type Ca\textsuperscript{2+} channel

In order to corroborate their efficacy in the CaCl\textsubscript{2}-induced urinary frequency model, we investigated the binding affinity of the anticholinergic drugs for PAA-sensitive L-type Ca\textsuperscript{2+} channels. The positive control methoxyverapamil inhibited [\textsuperscript{3}H]-desmethoxyverapamil binding in a concentration-dependent manner, and the Ki value for the PAA-sensitive Ca\textsuperscript{2+} channels was 4.97 × 10\textsuperscript{-8} M. It was confirmed that all of the anticholinergic agents also inhibited [\textsuperscript{3}H]-desmethoxyverapamil binding in a concentration-dependent manner under these conditions. The Ki values of propiverine, solifenacin, tolterodine, and atropine for PAA-sensitive Ca\textsuperscript{2+} channels were 1.61 × 10\textsuperscript{-6}, 2.91 × 10\textsuperscript{-6}, 6.38 × 10\textsuperscript{-7}, and 4.18 × 10\textsuperscript{-5} M, respectively. Based on these results it was concluded that propiverine, solifenacin, and tolterodine possess affinity for the PAA-sensitive Ca\textsuperscript{2+} channels and exhibit antagonist activity.

Discussion

The present study represents an attempt to examine whether antimuscarinic agents currently in use in the clinical setting might be useful in the management of urinary frequency mediated by atropine resistant contractions, by comparing the drugs in three different urinary frequency animal models. The doses of the antimuscarinic agents employed in this study were determined by reference to human and rat plasma concentrations of these drugs (7 – 12). Of the drugs used as positive controls, atropine at the dose of 0.32 mg/kg p.o. was reported to improve the symptom of urinary frequency in rats with ibotenic acid–induced destruction of the nucleus basalis magnocellularis (13), and verapamil at the dose of 10 mg/kg, p.o. was reported to produce a significant decrease of the blood pressure in normal rats (14); therefore, these doses of atropine and verapamil were selected for this study. Desensitization with capsaicin was performed in the usual manner (3). The administration of each drug and the desensitization had no significant effect on the micturition weight or intravesical pressure in normal animals.

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A CaCl\textsubscript{2}-induced urinary frequency model in which concentration-dependent urinary frequency can be
evoked by continuous intravesical infusion of CaCl₂ has been newly established. The CaCl₂-induced urinary frequency model was considered as a non-cholinergic urinary frequency model in as much as improvement of the urinary frequency in this model was obtained by administration of atropine. The Ca²⁺-channel antagonist verapamil, on the other hand, also improved the urinary frequency, indicating that the model represents a Ca²⁺-induced urinary frequency model as well. Desensitization with capsaicin had no effect on the urinary frequency in this model, suggesting the absence of significant involvement of capsaicin-sensitive sensory nerves. Zhang et al. (18) obtained evidence showing that ATP could also be an NANC mediator in the bladder because ATP-induced urinary frequency was not inhibited by resiniferatoxin desensitization. The results would be strengthened by investigating the involvement of purinergic nerves in the CaCl₂-induced urinary frequency. However, it was difficult to prove that the CaCl₂-induced urinary frequency model was equivalent to the only purinergic nerve-mediated urinary frequency model based on only this study.

This model was considered to reproduce a disease state in which Ca²⁺ homeostasis plays a significant role, in that there have been reports demonstrating the effectiveness of Ca²⁺ antagonists in the management of urinary urgency, frequency, and incontinence (19). Meanwhile, the Ca²⁺ channel antagonist verapamil has been documented to increase the bladder capacity and at the same time, induce arrhythmia when administered intravenously in rats with bladder outlet obstruction (20). On account of the noticeable influence of the clinically used Ca²⁺ antagonists on the cardiovascular system, we considered that Ca²⁺ antagonists with high selectivity for the urinary bladder relative to that for the cardiovascular system would be preferable for the management of urinary frequency and urgency associated with abnormalities of Ca²⁺ homeostasis. Of the antimuscarinic drugs used in this study, propiverine produced attenuation of the urinary frequency in the CaCl₂-induced urinary frequency, while the other agents proved less effective. Propiverine is known to have an inhibitory effect on ACh-, KCl-, or CaCl₂-induced contractile responses of human and rat detrusor muscles (2, 21). Furthermore, propiverine has been demonstrated to inhibit Ca²⁺ uptake in experiments conducted with isolated guinea-pig urinary bladder and murine urinary bladder myocytes and is known to be endowed with both antimuscarinic activity and Ca²⁺ antagonistic activity (5, 22).

When we calculated the Ki values of atropine and the other antimuscarinic agents for the PAA sensitive L-type Ca²⁺ channels in order to corroborate efficacy of these agents in the CaCl₂-induced frequency model, a more than 100,000-fold difference in the binding affinity of atropine for Ca²⁺ channels and muscarinic receptors was found (23). Among the anticholinergic drugs, the Ki value of propiverine for the PAA sensitive L-type Ca²⁺ channels was 4.6-fold higher than for the M₃ muscarinic receptor (24), which is thought to be the reason for the close ED₅₀ values obtained between the ACh-induced frequency model and CaCl₂-induced frequency model. Thus, propiverine was confirmed as a drug capable of improving urinary frequency at clinical dose levels both via its antimuscarinic effect and its effect of inhibiting Ca²⁺ influx into the urinary bladder myocytes. A more than 100-fold difference was observed in the binding affinity of tolterodine and solifenacin between the M₃ muscarinic receptors and the PAA sensitive L-type Ca²⁺ channels (24), which was inferred to be the reason why effect of these drugs in ameliorating urinary frequency was inadequate at the doses used in this study. The efficacy of administering solifenacin at 100 mg/kg and tolterodine at 30 mg/kg was investigated in a preliminary study; however, the increase in amount of the micturition weight decreased. Moreover, the effect of solifenacin and tolterodine, although weak, in ameliorating urinary frequency is likely to be attributable to their anticholinergic actions.

In the present experiments, the urinary frequency symptom due to cholinergic nerve stimulation was found to be responsive to solifenacin, tolterodine, and propiverine. Urinary frequency due to stimulation with AcOH or Ca²⁺, and hence not mediated by the cholinergic nervous system, on the other hand, was not responsive to the highly selective muscarinergic receptor antagonists, whereas propiverine, endowed with both antimuscarinic and Ca²⁺ channel-antagonizing activities, produced marked improvement.

It has been documented in contraction experiments on detrusor strips that ATP- and CaCl₂-induced contractions were conspicuously enhanced in the bladder of patients with neurogenic bladder, whereas ACh-stimulated detrusor contractions were essentially comparable between patients with neurogenic bladder and healthy subjects (25). According to a report by Yoshida et al., ATP-induced detrusor contractions in humans increased progressively with advancing age in humans (26). It has also been demonstrated in contraction inhibition tests with atropine and αβ-methylene ATP on detrusor contractions evoked by electrical field stimulation in humans and rats that the cholinergic nerve system–mediated contractile elements diminished and the purinergic nerve system–mediated contractile elements increased progressively with advancing age in humans (26). Therefore, it is generally thought that the effects of antimuscarinic drugs are weak in patients with neurogenic bladder and elderly
subjects and that inhibition of atropine-resistant contractions including the NANC nervous system-mediated contractions is important for improving the urinary frequency and urgency associated with an OAB neurogenic bladder in elderly patients. Attention is being focused increasingly on the role of atropine-resistant contractions in the treatment of urinary frequency and urgency due to OAB or neurogenic bladder and numerous studies have been conducted (27, 28). In the clinical setting, intravesical resiniferatoxin or botulinum toxin A— injection therapy has been shown to be successful for the control of abnormal excitation of atropine-resistant contractions in patients with symptoms refractory to antimuscarinic drugs (29).

Although the cholinergic nervous system is considered to be the principal nervous system involved in the development of urinary frequency and urgency, increase in atropine-resistant contractile elements are evident in certain disease states manifesting with the symptoms of urinary frequency and urgency, in which the efficacy of antimuscarinic agents is diminished. Thus, the use of drugs having inhibitory effects on both atropine-sensitive and atropine-resistant contractions, for example, propiverine, is recommended for the management of urinary frequency and urgency in such patients.

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