



Excitatory effect of acotiamide on rat and human bladder: Implications for underactive bladder treatment

Nishant Singh^a, Shinsuke Mizoguchi^a, Takahisa Suzuki^a, Irina Zabbarova^b, Youko Ikeda^b, Anthony Kanai^b, Christopher Chermansky^a, Naoki Yoshimura^a, Pradeep Tyagi^{a,*}

^a Department of Urology, University of Pittsburgh, Pittsburgh, PA, United States of America

^b Department of Medicine, University of Pittsburgh, Pittsburgh, PA, United States of America

ARTICLE INFO

Keywords:

Underactive bladder
Acotiamide
Antimuscarinic
Nerve evoked
Bethanechol

ABSTRACT

Objective: To evaluate whether approved gastroprokinetic agent, acotiamide exerts a direct excitatory effect on bladder to help explain the reported meaningful reduction of post-void residual urine volume (PVR) in detrusor underactivity (DU) patients after thrice daily oral intake of acotiamide 100 mg for 2 weeks.

Methods: Effect of acotiamide [1–16 μ M] was assessed on nerve-mediated contractions evoked by electrical field stimulation (EFS) for 5 s with 5 ms pulse trains of 10 V in longitudinal, mucosa intact rat and human bladder strips to construct frequency response curve (1–32 Hz) and repeat 10 Hz stimulation at 60s interval. Effect of acotiamide 2 μ M on spontaneous and carbachol evoked contractions was also assessed.

Results: Acotiamide 2 μ M significantly enhanced the Atropine and Tetrodotoxin (TTX)-sensitive EFS evoked contractions of rat and human bladder at 8–32 Hz (Two-way ANOVA followed Sidak's multiple comparison; $*p < 0.01$) and on repeat 10 Hz stimulation (Paired Student's *t*-test; $*p < 0.05$), while producing a modest effect on the spontaneous contractions and a negligible effect on the carbachol evoked contractions.

Conclusions: Enhancement of TTX-sensitive evoked contractions of rat and human bladder by acotiamide is consistent with the enhancement of excitatory neuro-effector transmission mainly through prejunctional mechanisms. Findings highlight immense therapeutic potential of antimuscarinics with low M3 receptor affinity like acotiamide in Underactive bladder (UAB)/DU treatment.

1. Introduction

Underactive bladder (UAB) is characterized by hesitancy, prolonged urination with or without a sensation [1] of filling or incomplete bladder emptying, a slow stream and large post-void residual (PVR). Often, UAB is associated detrusor underactivity (DU) - a urodynamic analog of detrusor overactivity (DO) - defined by the International continence society as a detrusor contraction of inadequate strength and/or duration resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying, in the absence of urethral obstruction. Although, currently available drugs (α 1-blockers) are effective in managing obstructive urinary retention (urinary hesitancy and prolonged urination) [2], their efficacy in the treatment of non-obstructive urinary retention is poor. Poor efficacy prompts the search for newer drugs and motivates the efforts to repurpose already approved drugs.

Appearance of an analogous relationship between DU and DO

prompted some experts to argue that UAB is an antithesis of overactive bladder (OAB) symptoms. A strong association of DO with lower acetylcholinesterase (AChE) activity in bladder of rodents [3] and patients [4] supports the argument that the rate of AChE mediated ACh degradation [5] determines the functionally active acetylcholine (ACh) levels available at the neuromuscular junction [6–9], which ultimately determines the strength and duration of detrusor contraction [10]. Furthermore, successful management of responsive and treatment refractory OAB symptoms through either competitive blockade of ACh action (antimuscarinics) or via inhibition of ACh release by onabotulinumtoxin A [11] supports the association of enhanced ACh activity with the etiology of OAB symptoms.

In contrast to OAB, deficiency in cholinergic neurotransmission [12] is a plausible factor in the diminished sensation of bladder filling [1] and a weaker detrusor contraction of UAB patients. Indeed, impaired voiding function of aged rodents is demonstrably linked to the cholinergic deficiency [13]. An apparent analogous etiologic relationship

* Corresponding author at: Department of Urology, University of Pittsburgh, E313 Montefiore Hospital, 3459 Fifth Ave., Pittsburgh, PA 15213, United States of America.

E-mail address: tyagip@upmc.edu (P. Tyagi).

<https://doi.org/10.1016/j.lfs.2020.118179>

Received 28 February 2020; Received in revised form 27 July 2020; Accepted 28 July 2020

Available online 03 August 2020

0024-3205/ © 2020 Elsevier Inc. All rights reserved.

between OAB and UAB may have motivated the inversion of a successful OAB pharmacological approach to accomplish an excitatory effect in patients afflicted with non-obstructive urinary retention. Thus, the development of a synthetic cholinomimetic (bethanechol) or AChE inhibitor (distigmine) [10] for an excitatory effect in bladder appears analogous to the development of antimuscarinics for OAB. However, the search for an UAB treatment approach analogous to the mechanism of action of onabotulinumtoxin A in OAB [11] is still ongoing. An indirectly acting cholinergic agonist that seeks to augment the activity of endogenously released ACh instead of directly stimulating the post-junctional muscarinic receptors would fit that bill.

An indirect cholinergic approach tried so far in UAB is the competitive inhibition of AChE by distigmine, which was able to reduce PVR significantly enough to obviate the need for intermittent self-catheterization [10]. Most importantly, increase in the PVR of UAB patients after discontinuation of distigmine treatment suggests a mechanistic link between increased PVR and the increased AChE activity [10]. Another approach that is mechanistically akin to distigmine is to augment the ACh activity by targeting the autoregulation of nerve evoked ACh release for stronger voiding contraction [14]. It is known that pre-junctional M_1 auto-receptors exert a positive auto-feedback on evoked ACh release [15] but pre-junctional M_2 , M_3 and M_4 auto-receptors exert a negative feedback on the evoked ACh release [16] at the neuromuscular junction.

Detrusor contraction for voiding can be driven by the sole stimulation of post-junctional M_3 receptors is clearly demonstrated by the carbachol evoked contraction of human bladder [17]. Since pre-junctional muscarinic autoreceptors exert an inhibitory action on cholinergic neuro-effector transmission, we surmised that muscarinic antagonist lacking functional antagonism of M_3 receptors can potentially enhance voiding function. Indeed, Acotiamide, a non-selective muscarinic antagonist having an amide bond (Fig. 1) in its pharmacophore instead of an ester bond of acetylcholine or the carbamate bond of Bethanechol and cholinesterase inhibitors fails to functionally antagonize recombinant M_3 receptors expressed on oocytes in concentrations up to 100 μM [18]. Furthermore, daily oral treatment of acotiamide (approved gastroprokinetic agent in Asian countries) led to a significant reduction of PVR in DU patients [19–21] without any effect on post-junctional M_3 receptor signaling. Following study sought to answer the question, whether acotiamide (Z-338) [22] has a direct excitatory effect on nerve-mediated bladder contractions via pharmacological antagonism of prejunctional muscarinic receptors.

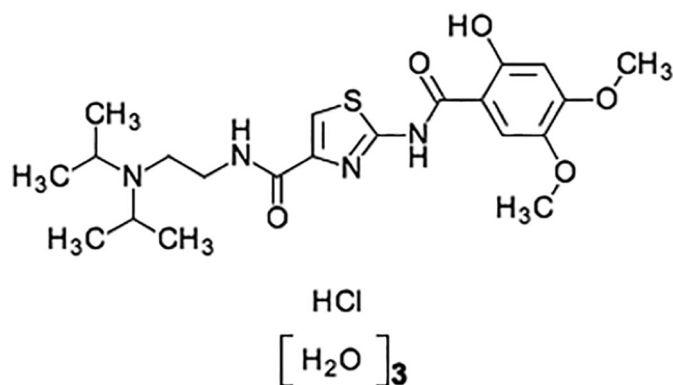


Fig. 1. Chemical structure – Acotiamide has an amide bond in pharmacophore instead of the ester bond of acetylcholine and the carbamate bond of Bethanechol and cholinesterase inhibitors.

2. Material and methods

2.1. Drugs

Acotiamide dihydrochloride (Cat no. SML 1569) was procured from Sigma-Aldrich (St. Louis, MO, USA) and aqueous, stock solution was kept at -20°C until the day of experiment. Carbachol (Cat no. C4382) and Bethanechol (Cat no. C5259) for control experiments were also procured from Sigma-Aldrich.

2.2. Isolated rat bladder

Adult male disease-free Sprague-Dawley rats (10–12 weeks old) were obtained from Envigo. Whole bladder removed from ten rats was euthanized by carbon dioxide overdose as per American Veterinary Medical Association. Removed bladders were dissected in pre-oxygenated cold Krebs's physiological solution into four to five longitudinal, mucosa intact strips ($\sim 4 \times 10$ mm) [3,23], mounted vertically between platinum ring electrodes in organ bath chambers containing 20 mL Krebs's physiological solution composed of 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 1.2 mM, CaCl_2 , 2.5 mM, 1.2 mM KH_2PO_4 and 11.5 mM glucose, warmed to 37°C and constantly bubbled with 95% oxygen-5% carbon dioxide. Strips were stretched slowly to achieve a final isometric tension of 10 mN and then washed three times with fresh solution every 20 min during an equilibration period of 60 min. Isometric tension generated by the strips was measured using force transducers connected to a bridge amplifier (World Precision Instruments) and digitized by PowerLab Software. Electrical field stimulation (EFS) for 5 s was delivered via pulse trains of 10 volts (V) in amplitude with pulse duration of 5 ms at ascending frequency of 1, 2, 4, 8, 16 and 32 Hz (one stimulation at each frequency) at 60 s intervals. Pulses were delivered using bipolar platinum electrodes placed approximately 1 cm apart of tissue strip from constant-current Grass S88 stimulator. Frequency-response curves with the above stated EFS parameters were constructed on equilibrated strips at baseline and 30 min after the addition of acotiamide 1–16 μM in the bath. Separate strips were used for evaluating the effect of acotiamide at different concentrations on EFS. EFS evoked contractions sensitive to 1 μM tetrodotoxin (TTX), a neuronal Na^+ channel blocker and atropine 5 μM were considered nerve evoked (neurogenic) [23]. Considering that atropine sensitive (ACh mediated) EFS contractile response are dominant at EFS frequencies ≥ 10 Hz [6,23], we also assessed the contractile responses to repeat 10 Hz stimulation every 60 s until a stable plateau of 4–5 contractile responses. Repeat 10 Hz stimulation for 5 s each was performed with trains of 5 ms rectangular pulses at 10 V before and after addition of Acotiamide 1–16 μM and evoked contraction amplitude was normalized to the peak response obtained after application of isoosmolar solution of 120 mM potassium chloride. In separate strips, cumulative concentration-response (0.05–10 μM) of Carbachol (CCH) was evoked in absence and presence of acotiamide 2 μM . Concentration dependent effect of acotiamide and bethanechol on the spontaneous contractions evoked by 10 mN of tension was compared. Additional strips were left untreated to monitor any time dependent changes in contractility.

2.3. Human bladder

Human bladder from deceased organ donors ($n = 3$) were procured from the local tissue bank in compliance with the tissue bank IRB#0506140 and the Committee for Oversight of Research and Clinical Training Involving Decedents (CORID # 400) within 4 h of brain death. Honest broker system of tissue bank does not permit them to share disease information of the organ donors and the bladder specimens were therefore presumed normal. Removed whole bladders were kept in ice-cold Krebs's solution till its dissection for isometric tension studies which were performed within 24 h of organ removal. Bladder was cleaned of fat and connective tissues and mucosa intact

strips were immersed in 20 mL organ baths for frequency response curve and repeat 10 Hz stimulation as described for rat bladder.

2.4. Statistical analysis

Values are expressed as mean \pm standard deviation and differences were considered statistically significant with $p < 0.05$. Statistical significance for frequency response curve in EFS was analyzed by two-way ANOVA followed by Sidak's multiple comparison test, preferred over Tukey's test for its higher power. Paired Student's *t*-test was used for analyzing the contraction amplitude differences evoked by repeat 10 Hz stimulation. Carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data analyzed by GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Rat bladder

Marked inhibition of frequency-response curves of mucosa intact rat bladder strips in presence of 1 μ M TTX demonstrate that evoked contractile responses were of neurogenic origin in our findings. Prior incubation of the strips with acotiamide 2 μ M significantly enhanced the EFS contractions evoked at 8–32 Hz by $> 50\%$ with respect to the EFS evoked contraction amplitude measured before the addition of acotiamide (Two-way ANOVA followed by Sidak's multiple comparison with respect to only control group; $p < 0.01$). Differences in the number of separate strips used for evaluating acotiamide effect at different concentrations together with the absence of any excitatory effect of acotiamide $> 2 \mu$ M on EFS evoked contraction precluded the comparison between different drug concentrations (Fig. 2A–B). TTX sensitivity of the acotiamide 2 μ M mediated enhancement on EFS evoked contraction and the absence of any enhancement $> 2 \mu$ M together imply that antagonism of pre-junctional receptors by Acotiamide up to 2 μ M produces a maximal excitatory effect, whereas dominance of antagonist action on post-junctional receptors at higher concentrations may counter the excitatory effect of acotiamide on evoked contractions

noticeable at lower concentrations. A similar lack of concentration dependent effect with respect to acotiamide was also noted on spontaneous contractions as described later.

Considering that atropine sensitive (ACh mediated) nerve evoked contractile response is dominant at EFS frequencies ≥ 10 Hz [6,23], we performed paired analysis of responses evoked by repeat 10 Hz EFS at different concentrations of Acotiamide (Fig. 3A). Paired analysis revealed $\sim 50\%$ enhancement with Acotiamide 2 μ M (Two tailed Student's *t*-test; $p < 0.05$) (Fig. 2B). Considering that only a slight $\sim 5\%$ enhancement is observed at higher concentrations, observed findings argue for a prejunctional action of acotiamide, which is supported by the absence of any excitatory effect in presence of TTX (Fig. 3A) or atropine (data not shown).

3.1.1. Effects of acotiamide on carbachol response

Complete overlap of cumulative carbachol concentration-response curve constructed in the absence and in presence of acotiamide 2 μ M indicates that acotiamide 2 μ M does not oppose post-junctional muscarinic receptor stimulation [17]. Carbachol concentration necessary for generating a 50% of maximal response (EC50) only showed an insignificant increase from 171 nM to 197 nM in presence of acotiamide without any change in the maximal response (Fig. 4A–B). These findings are consistent with earlier findings where acotiamide in concentration up to 100 μ M failed to functionally antagonize recombinant M3 receptors expressed on oocytes [18].

3.1.2. Effects of acotiamide on spontaneous contractions

Cumulative concentration dependent addition of acotiamide only moderately affected the spontaneous contractions (Fig. 3C) compared to bethanechol, which mimics the Carbachol effect (Fig. 4A). Tracings and the summarized integral force calculations (Fig. 4D) together indicates that acotiamide fails to generate a concentration dependent increase in the tonic activity as observed with Carbachol (Fig. 4A–B) or Bethanechol. Since a minimal effect of acotiamide on the spontaneous phasic activity becomes more pronounced in presence of TTX, it can be argued that a pre-junctional action of acotiamide may counter a direct excitatory effect of Acotiamide on post-junctional muscarinic receptors

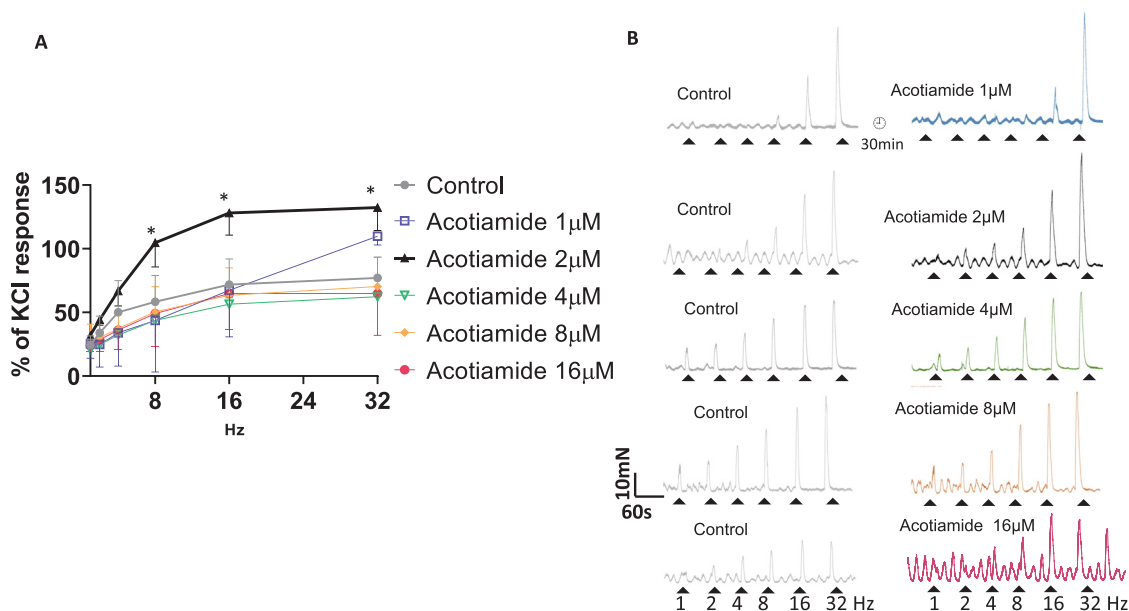


Fig. 2. Effect of acotiamide on EFS frequency response curve of rat bladder at different concentrations. Panel A - Acotiamide 2 μ M (black curve) shifted the frequency response curve to the left of control (grey curve). A $> 50\%$ increase in the magnitude of EFS contractions evoked at 8–32 Hz (Two-way ANOVA followed by Sidak's multiple comparison with respect to only the control group; $p < 0.01$; $n = 7$ strips) was dramatically reduced for contractions evoked at frequencies < 8 Hz. Panel B - Raw traces of TTX-sensitive EFS evoked contractile responses in separate strips evoked before (control grey traces) and in presence of acotiamide at different concentrations (colored traces) illustrates that the prejunctional action of acotiamide at concentration $> 2 \mu$ M is countered by action at other receptors.

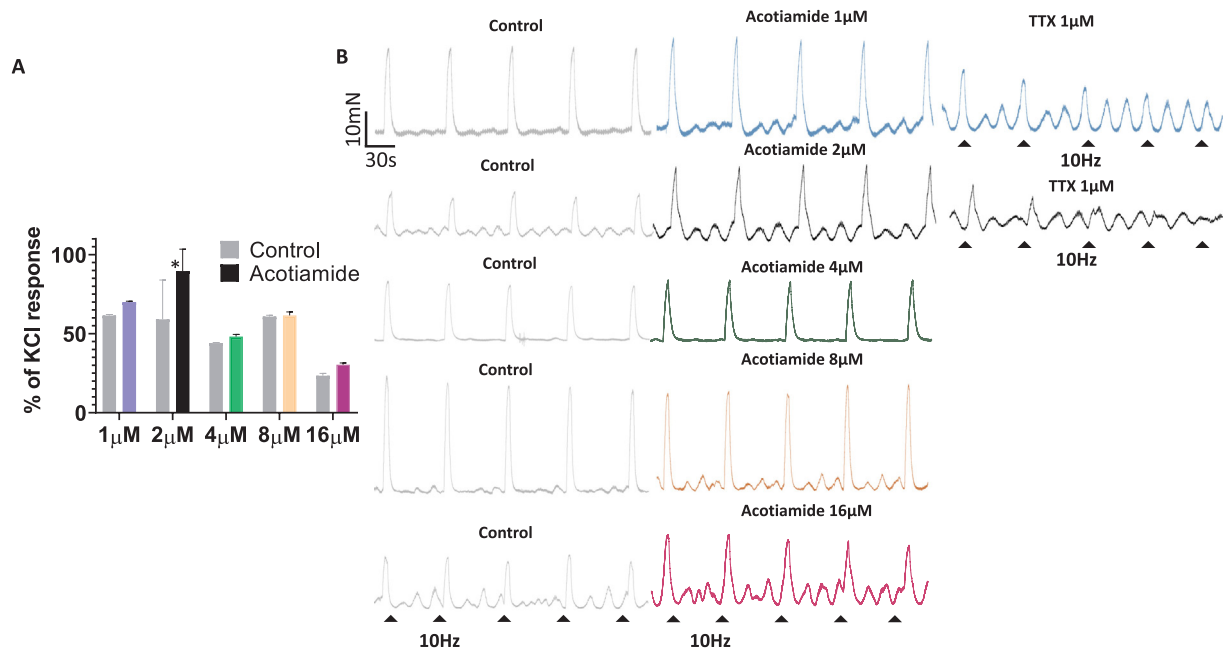


Fig. 3. Effect of acotiamide on nerve evoked contraction elicited by repeated 10 Hz stimulation. Panel A – Paired analysis of contractile responses evoked by repeat 10 Hz EFS at different acotiamide concentrations revealed a significant enhancement after 30 min of incubation with acotiamide 2 μM (Two tailed Student's *t*-test. **p* < 0.05; *n* = 4 strips) but not at higher concentrations. Panel B - Raw traces of EFS evoked contractile responses in separate strips evoked by repeat 10 Hz EFS before (control grey traces) and in the presence of acotiamide at different concentrations and after addition of TTX 1 μM together support a pre-junctional action site for acotiamide and the neurogenic origin of elicited responses.

for eliciting spontaneous phasic contractions. Importantly, TTX does not influence the modest increase in baseline tone generated by acotiamide which suggests that rise in basal tension and phasic contractions are likely mediated by the action of acotiamide on pre-junctional

and post-junctional receptors, respectively (Fig. 4C–D).

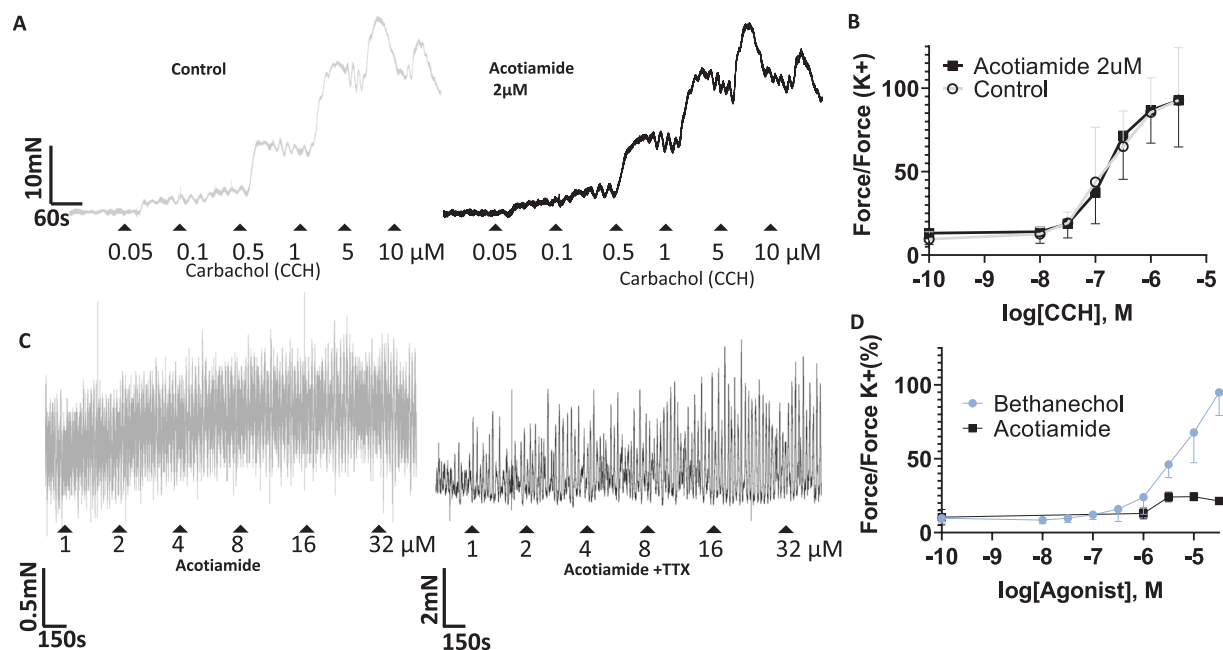


Fig. 4. Effect of acotiamide on carbachol evoked and spontaneous contractions. Panel A - Raw traces for cumulative carbachol concentration-response curve constructed in absence (grey tracing) and in presence of acotiamide 2 μM (black tracing). Panel B - Logarithmic concentration response curve of Carbachol evoked contraction in absence (grey curve) and in presence of acotiamide 2 μM (black curve) did not change appreciably. EC₅₀ and amplitude of maximum contraction remained unchanged in *n* = 4 strips. Panel C - Concentration dependent effects of acotiamide on spontaneous contractions of isolated rat bladder strips. Raw traces of spontaneous contractility following cumulative addition of acotiamide 1–32 μM in absence (grey trace) and in presence of TTX 1 μM (black trace). Acotiamide raised the basal tension two-fold relative to pre-drug levels but failed to produce a concentration dependent rise in tension associated with Carbachol/Bethanechol. Panel D - Summarized integral force calculations indicate that compared to bethanechol, acotiamide does not produce a concentration dependent increase in tonic activity. Notice the difference in scale bar for the contraction force in panel C relative to panel A and B.

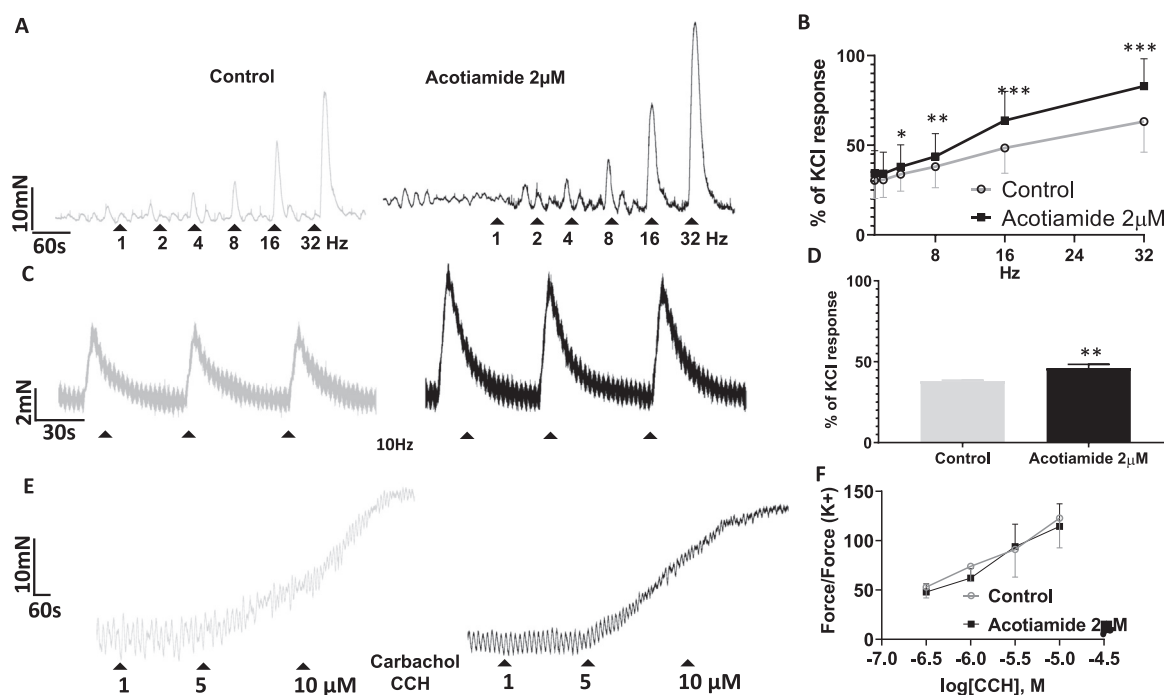


Fig. 5. Excitatory effect of acotiamide 2 μ M on nerve evoked contraction of human bladder. Panel A - Raw traces of EFS frequency-response curves 1–32 Hz constructed before (grey tracing) and in presence of acotiamide 2 μ M (black tracing). Panel B - Acotiamide 2 μ M (black curve) shifted the frequency response curve to the left of control (grey curve) with a significant $\sim 20\%$ enhancement of atropine sensitive (ACh mediated) nerve evoked contractile responses elicited by 16–32 Hz stimulation ($***p < 0.0001$; Two-way ANOVA followed by Sidak's multiple comparison; 4 strips). Panel C - Raw traces for repeat 10 Hz stimulation before (grey tracing) and after addition of acotiamide 2 μ M (black tracing). Panel D - Paired analysis of contractile responses demonstrate a significant enhancement by acotiamide 2 μ M (Two tailed paired Student's *t*-test. $*p < 0.01$). Panel E - Raw traces for cumulative carbachol concentration-response curve constructed in absence (grey tracing) and in presence of acotiamide 2 μ M (black tracing). Panel F - Concentration response curve of Carbachol induced contraction in absence (grey curve) and in presence of acotiamide 2 μ M (black curve) did not change appreciably. Higher magnitude of carbachol evoked response relative to EFS evoked response is evident from the differences in scale for y-axis in panel F with respect to panel B and D.

3.2. Human bladder

Frequency-response curves of human bladder constructed before and after incubation of acotiamide 2 μ M showed a significant enhancement of the evoked contractile response > 4 Hz (Two-way ANOVA followed by Sidak's multiple comparison; $p < 0.05$; Fig. 5B). Importantly, the magnitude of enhancement was $\sim 20\%$ in the atropine sensitive (ACh mediated) range for nerve evoked contractile responses elicited with 16–32 Hz stimulation ($p < 0.0001$) as well as on repeat 10 Hz stimulation (Two tailed paired Student's *t*-test. $p < 0.01$) (Fig. 5D). Limited availability of human bladder precluded the evaluation of acotiamide effect at higher concentrations. Acotiamide 2 μ M did not oppose the human bladder contractility evoked by carbachol with only a minor change in EC₅₀ (Fig. 5E), which was in the range reported by other groups [17]. Effect of acotiamide 2 μ M on carbachol evoked response and the absence of any effect in absence of EFS is consistent with a prejunctional site of action for acotiamide in human bladder.

4. Discussion

This is a first report demonstrating a direct excitatory effect of Acotiamide on the neural modulation of rat and human bladder contractility mainly through prejunctional mechanisms [24]. Acotiamide is known to decrease the PVR of DU patients and resolve urinary retention of female patients with thrice daily oral treatment of 100 mg for 2 weeks [19–21]. Clinically used dose of acotiamide [20,21] is said to generate plasma concentration in the range of 2 μ M [25]. Since we tested acotiamide within a concentration range around 2 μ M in an ex vivo experimental setup, the findings described here are easily translatable to the bedside.

Several studies have demonstrated that voiding contraction in mammals can be experimentally evoked by EFS of parasympathetic nerve with 1 ms pulses of 50 V at 32 Hz [26], which is considered to elicit the release of ACh [5,11,27] and therefore support the use of EFS for testing the excitatory effect of acotiamide in bladder. Given that EFS evokes a frequency dependent release of neurotransmitters from post-ganglionic nerves of bladder with the dominant release of ATP and of ACh at frequencies ≤ 10 Hz [6] and at ≥ 10 Hz, respectively [23], we infer that the significant excitatory effect of acotiamide 2 μ M on EFS evoked contractions of rat and human bladder elicited by EFS frequencies > 8 Hz represent an enhancement of cholinergic neurotransmission in bladder.

Since prejunctional receptors on cholinergic nerve terminals exerts either a positive [6,15] or a negative [16] auto-feedback on evoked ACh release, an excitatory effect of acotiamide 2 μ M on TTX-sensitive evoked contractions of rat and human bladder implicates a suppression of autoinhibitory mechanisms involved in ACh release. The implication is supported by the absence of any excitatory effect on rat and human bladder contractility in absence of EFS. An inhibitory role for the prejunctional muscarinic receptors in bladder was first noted with the inhibition of EFS evoked ACh outflow by the non-selective muscarinic agonist, carbachol [15]. Simultaneous measurement of EFS evoked ACh outflow and contraction amplitude uncovered that non-selective agonism of the prejunctional M₂ and M₄ auto-receptors by carbachol or by its congener (bethanechol) exerts a negative feedback on the EFS evoked ACh release [15], which eventually reduces the detrusor contraction amplitude [16,27] to produce lower voided volumes and bladder filling compliance during cystometry [28]. A separate study suggested that a decrease in PVR of bethanechol treated obstructed rats was caused by an increase in voiding frequency [29].

Acotiamide is known to bind to most muscarinic receptors with the

IC50 in the range of 1.8–10 μM [18] except for M_3 receptors, which rules out any direct stimulation of post-junctional receptors in the excitatory effect of Acotiamide 2 μM , reported here. Earlier studies on *Xenopus* showed that Acotiamide competitively inhibits the inwards currents mediated by cloned M_1 and M_2 receptors and uncompetitively inhibits the nicotinic ACh receptor-mediated inward currents at concentrations $> 10^{-6}\text{M}$ [30]. The lack of antagonism for M_3 receptors by acotiamide may contribute to the observed acotiamide mediated enhancement of excitatory neurotransmission in bladder as well as in gastrointestinal tract [18]. Lower affinity for M_3 receptors justifies the use of acotiamide as a useful tool to distinguish M_1 and M_2 receptor-mediated responses from muscarinic M_3 receptor-mediated responses. Since concentrations of acotiamide $> 2 \mu\text{M}$ is likely to antagonize both pre- and post-junctional muscarinic receptors [6], classical receptor antagonism studies are not possible with Acotiamide through measurement of isometric tension only.

Excitatory effect of acotiamide on evoked contractions of rat and human bladder is also supported by a similar enhancement in the amplitude of twitch-like contractions and excitatory junction potentials (EJPs) evoked by single or repetitive EFS in the circular muscle strips of the guinea-pig stomach [24]. The excitatory effect of acotiamide is linked to the acceleration of TTX-sensitive and extracellular Ca^{2+} dependent EFS evoked ACh release in the [^3H]-choline-preincubated gastric antrum and body of rat and dog at doses $> 10^{-6}\text{M}$ [24]. Moreover, acotiamide mediated enhancement of EFS evoked ACh release was higher in the presence of physostigmine, which suggests that direct AChE inhibition by acotiamide [18] may have modest contribution to the efficacy of acotiamide in DU patients [19–21] and in the findings described here.

Cholinergic transmission is not only critical for voiding but is also critical in maintenance of the bladder tone during storage phase. It is reported that nerve evoked ACh release [7] for accomplishing voiding is 300 times higher than the ACh released in basal mode for volume sensation and maintenance of bladder tone during the storage phase [9]. Infact, several studies have now suggested that age dependent changes in the release of ATP and ACh [12] are linked to the defective volume sensation and impaired voiding in aged male rat [13] and in aged UAB/DU patients [1]. Quantitative differences in the ACh release during storage and voiding phase may explain the inferiority of a direct cholinergic agonist like bethanechol [28] over an indirect agonist, because an indirect agonist can efficiently modulate the intensity of cholinergic stimulation from low to high level for storage and voiding phases, respectively whereas, a similar intensity of exogenous cholinergic stimulation during storage and voiding phase may contribute to the ineffectiveness of a direct cholinergic agonist like bethanechol in PVR reduction of UAB patients [10].

Moderate excitatory effect of acotiamide on spontaneous contractions compared to bethanechol argues against the non-selective agonism of pre-junctional and post-junctional muscarinic receptors in UAB treatment. Modest effect of acotiamide on basal tone in contrast to bethanechol suggests that the excitatory action of acotiamide on spontaneous ACh release via antagonism of pre-junctional M_2 receptors [9] is countered by the simultaneous antagonism of post-junctional M_2 receptors. Therefore, a direct excitatory effect of acotiamide on bladder is in agreement with the expression of M_2 muscarinic receptors in rat and human bladder [31] and the purported functional role of pre-junctional M_2 receptors in homeostatic regulation of voiding contraction [32]. However, these results of acotiamide do not exclude the possible involvement of muscarinic M_4 receptors [33].

Taking our experimental findings and the clinical results [19–21] together, it is likely that the excitatory effect of acotiamide in bladder may involve action on nicotinic autoreceptors on cholinergic nerve terminals [34], which warrants investigation in future studies. Several clinical studies have now compared the direct and indirect cholinergic agonists in UAB patients with intact voiding reflex and reported that indirect cholinergic agonist like Distigmine works better than

Bethanechol [10]. Although cholinomimetic agonism of the post-junctional muscarinic receptors by carbachol or bethanechol is desirable during voiding phase, same cholinomimetic action during storage phase is suggested to increase the basal tone [16], which decreases the bladder compliance and contributes to the ineffectiveness of Bethanechol in UAB patients [10]. Nonetheless, bethanechol effectively decreases the duration of urethral catheterization following transient loss of voiding reflex during postoperative and postpartum nonobstructive urinary retention and in patients with acontractile detrusor [35]. Acotiamide is an indirect cholinergic agonist like distigmine [10] and clinical experience with acotiamide in DU patients is predictive of its efficacy in UAB patients. In fact, acotiamide was effective in reducing the PVR of DU patients non-responsive to distigmine [21], suggesting that differences in mechanism of action of acotiamide and distigmine is of therapeutic relevance in aged UAB patients.

5. Conclusions

This is the first report demonstrating the direct excitatory effect of Acotiamide on neural modulation of rat and human bladder contractility in an *ex vivo* setup. These findings are consistent with PVR reduction in Acotiamide treated DU patients and highlight that anti-muscarinics selective for M_3 receptors and those lacking any action on M_3 receptors are promising for OAB and UAB treatment, respectively.

Declaration of competing interest

None of the authors have any disclosures.

Acknowledgements

This project was supported by grant awarded by National institute of Aging - AG062971.

References

- [1] P.P. Smith, D.J. Chalmers, R.S. Feinn, Does defective volume sensation contribute to detrusor underactivity? *Neurourol. Urodyn.* 34 (2015) 752–756.
- [2] M. Oelke, A. Bachmann, A. Descasez, M. Emberton, S. Gravas, M.C. Michel, et al., EAU guidelines on the treatment and follow-up of non-neurogenic male lower urinary tract symptoms including benign prostatic obstruction, *Eur. Urol.* 64 (2013) 118–140.
- [3] M. Kashyap, S. Pore, M. Chancellor, N. Yoshimura, P. Tyagi, Bladder overactivity involves overexpression of MicroRNA 132 and nerve growth factor, *Life Sci.* 167 (2016) 98–104.
- [4] I.W. Mills, J.E. Greenland, G. McMurray, R. McCoy, K.M. Ho, J.G. Noble, et al., Studies of the pathophysiology of idiopathic detrusor instability: the physiological properties of the detrusor smooth muscle and its pattern of innervation, *J. Urol.* 163 (2000) 646–651.
- [5] O. Yossepowitch, G. Gillon, J. Baniel, D. Engelstein, P.M. Livne, The effect of cholinergic enhancement during filling cystometry: can edrophonium chloride be used as a provocative test for overactive bladder? *J. Urol.* 165 (2001) 1441–1445.
- [6] A.S. Braverman, I.J. Kohn, G.R. Luthin, M.R. Ruggieri, Prejunctional M_1 facilitatory and M_2 inhibitory muscarinic receptors mediate rat bladder contractility, *Am. J. Phys.* 274 (1998) R517–R523.
- [7] G. D'Agostino, M.C. Chiari, E. Grana, Prejunctional effects of muscarinic agonists on 3H-acetylcholine release in the rat urinary bladder strip, *Naunyn Schmiedeberg's Arch. Pharmacol.* 340 (1989) 76–81.
- [8] C.P. Smith, T.B. Boone, W.C. de Groat, M.B. Chancellor, G.T. Somogyi, Effect of stimulation intensity and botulinum toxin isoform on rat bladder strip contractions, *Brain Res. Bull.* 61 (2003) 165–171.
- [9] V.P. Zagorodnyuk, S. Gregory, M. Costa, S.J. Brookes, M. Tramontana, S. Giuliani, et al., Spontaneous release of acetylcholine from autonomic nerves in the bladder, *Br. J. Pharmacol.* 157 (2009) 607–619.
- [10] K. Izumi, A. Maolake, Y. Maeda, K. Shigehara, M. Namiki, Effects of bethanechol chloride and distigmine bromide on postvoiding residual volume in patients with underactive bladder, *Minerva Urol. Nefrol.* 66 (2014) 241–247.
- [11] P. Tyagi, M. Kashyap, N. Yoshimura, M. Chancellor, C.J. Chermansky, Past, present and future of chemodenervation with botulinum toxin in the treatment of overactive bladder, *J. Urol.* 197 (2017) 982–990.
- [12] M. Yoshida, K. Miyamae, H. Iwashita, M. Otani, A. Inadome, Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging, *Urology* 63 (2004) 17–23.
- [13] W. Zhao, T. Aboushwareb, C. Turner, C. Mathis, C. Bennett, W.E. Sonntag, et al.,

- Impaired bladder function in aging male rats, *J. Urol.* 184 (2010) 378–385.
- [14] D.K. Kim, Current pharmacological and surgical treatment of underactive bladder, *Investig Clin Urol* 58 (2017) S90–S98.
- [15] G.T. Somogyi, M. Tanowitz, W.C. de Groat, M1 muscarinic receptor-mediated facilitation of acetylcholine release in the rat urinary bladder, *J. Physiol.* 480 (Pt 1) (1994) 81–89.
- [16] G. D'Agostino, M.L. Bolognesi, A. Lucchelli, D. Vicini, B. Balestra, V. Spelta, et al., Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M4 receptor subtype, *Br. J. Pharmacol.* 129 (2000) 493–500.
- [17] C. Fetscher, M. Fleischman, M. Schmidt, S. Kregge, M.C. Michel, M(3) muscarinic receptors mediate contraction of human urinary bladder, *Br. J. Pharmacol.* 136 (2002) 641–643.
- [18] Y. Doi, O. Murasaki, M. Kaibara, Y. Uezono, H. Hayashi, K. Yano, et al., Characterization of functional effects of Z-338, a novel gastroprokinetic agent, on the muscarinic M1, M2, and M3 receptors expressed in *Xenopus* oocytes, *Eur. J. Pharmacol.* 505 (2004) 31–35.
- [19] K. Sugimoto, T. Akiyama, N. Matsumura, T. Minami, S. Uejima, H. Uemura, Efficacy of acotiamide hydrochloride hydrate added to alpha-blocker plus cholinergic drug combination therapy, *Int. J. Urol.* 26 (2019) 848–849.
- [20] K. Sugimoto, T. Akiyama, N. Shimizu, N. Matsumura, M. Hashimoto, T. Minami, et al., Acotiamide hydrochloride hydrate added to combination treatment with an alpha-blocker and a cholinergic drug improved the QOL of women with acute urinary retention: case series, *Res Rep Urol* 9 (2017) 141–143.
- [21] K. Sugimoto, T. Akiyama, N. Shimizu, N. Matsumura, T. Hayashi, T. Nishioka, et al., A pilot study of acotiamide hydrochloride hydrate in patients with detrusor underactivity, *Res Rep Urol* 7 (2015) 81–83.
- [22] P. Tyagi, S. Mizoguchi, C. Chermansky, N. Yoshimura, Excitatory effect of acotiamide on nerve evoked contractions of rat and human bladder, *Neurourol. Urodyn.* (2019) 192.
- [23] M.P. Kashyap, S.K. Pore, W.C. de Groat, C.J. Chermansky, N. Yoshimura, P. Tyagi, BDNF overexpression in the bladder induces neuronal changes to mediate bladder overactivity, *Am. J. Physiol. Renal Physiol.* 315 (2018) F45–F56.
- [24] M. Ogishima, M. Kaibara, S. Ueki, T. Kurimoto, K. Taniyama, Z-338 facilitates acetylcholine release from enteric neurons due to blockade of muscarinic autoreceptors in guinea pig stomach, *J. Pharmacol. Exp. Ther.* 294 (2000) 33–37.
- [25] K. Yoshii, M. Iikura, M. Hirayama, R. Toda, Y. Kawabata, Physiologically-based pharmacokinetic and pharmacodynamic modeling for the inhibition of acetylcholinesterase by acotiamide, a novel gastroprokinetic agent for the treatment of functional dyspepsia, in rat stomach, *Pharm. Res.* 33 (2016) 292–300.
- [26] J. Zeng, C. Pan, C. Jiang, S. Lindstrom, Cause of residual urine in bladder outlet obstruction: an experimental study in the rat, *J. Urol.* 188 (2012) 1027–1032.
- [27] G. D'Agostino, A. Maria Condino, P. Calvi, Involvement of beta3-adrenoceptors in the inhibitory control of cholinergic activity in human bladder: direct evidence by [(3)H]-acetylcholine release experiments in the isolated detrusor, *Eur. J. Pharmacol.* 758 (2015) 115–122.
- [28] H. Nagabukuro, S. Okanishi, T. Doi, Effects of TAK-802, a novel acetylcholinesterase inhibitor, and various cholinomimetics on the urodynamic characteristics in anesthetized guinea pigs, *Eur. J. Pharmacol.* 494 (2004) 225–232.
- [29] T. Hashimoto, H. Nagabukuro, T. Doi, Effects of the selective acetylcholinesterase inhibitor TAK-802 on the voiding behavior and bladder mass increase in rats with partial bladder outlet obstruction, *J. Urol.* 174 (2005) 1137–1141.
- [30] Y. Kanemoto, H. Ishibashi, A. Doi, N. Akaike, Y. Ito, An electrophysiological study of muscarinic and nicotinic receptors of rat paratracheal ganglion neurons and their inhibition by Z-338, *Br. J. Pharmacol.* 135 (2002) 1403–1414.
- [31] S. Tyagi, P. Tyagi, S. Van-le, N. Yoshimura, M.B. Chancellor, F. de Miguel, Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor, *J. Urol.* 176 (2006) 1673–1678.
- [32] A.S. Braverman, L.R. Doumanian, M.R. Ruggieri Sr., M2 and M3 muscarinic receptor activation of urinary bladder contractile signal transduction. II. Denervated rat bladder, *J. Pharmacol. Exp. Ther.* 316 (2006) 875–880.
- [33] T. Takeuchi, N. Yamashiro, T. Kawasaki, H. Nakajima, Y.T. Azuma, M. Matsui, The role of muscarinic receptor subtypes in acetylcholine release from urinary bladder obtained from muscarinic receptor knockout mouse, *Neuroscience* 156 (2008) 381–389.
- [34] C. Prior, S. Singh, Factors influencing the low-frequency associated nicotinic ACh autoreceptor-mediated depression of ACh release from rat motor nerve terminals, *Br. J. Pharmacol.* 129 (2000) 1067–1074.
- [35] I. Hirotsu, C. Hayano, T. Tani, Effect of muscarinic agonist on overflow incontinence induced by bilateral pelvic nerve transection in rats, *Jpn. J. Pharmacol.* 76 (1998) 109–111.