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Constitutively active PKA regulates neuronal acetylcholine release and contractility of guinea pig urinary bladder smooth muscle

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Submitted 19 January 2016; accepted in final form 27 March 2016

Xin W, Li N, Fernandes VS, Petkov GV. Constitutively active PKA regulates neuronal acetylcholine release and contractility of guinea pig urinary bladder smooth muscle. Am J Physiol Renal Physiol 310: F1377–F1384, 2016. First published March 30, 2016; doi:10.1152/ajprenal.00026.2016.—Autonomic and somatic motor neurons that innervate the urinary bladder and urethra control the highly coordinated functions of the lower urinary tract, the storage, and the emptying of urine. ACh is the primary excitatory neurotransmitter in the bladder. Here, we aimed to determine whether PKA regulates neuronal ACh release and related nerve-evoked detrusor smooth muscle (DSM) contractions in the guinea pig urinary bladder. Isometric DSM tension recordings were used to measure spontaneous phasic and electrical field stimulation (EFS)- and carbachol-induced DSM contractions with a combination of pharmacological tools. The colorimetric method was used to measure ACh released by the parasymptathetic nerves in DSM isolated strips. The pharmacological inhibition of PKA with H-89 (10 μM) increased the spontaneous phasic contractions, whereas it attenuated the EFS-induced DSM contractions. Intriguingly, H-89 (10 μM) attenuated the (primary) cholinergic component, whereas it simultaneously increased the (secondary) purinergic component of the nerve-evoked contractions in DSM isolated strips. The acetylcholinesterase inhibitor, eserine (10 μM), increased EFS-induced DSM contractions, and the subsequent addition of H-89 attenuated the contractions. H-89 (10 μM) significantly increased DSM phasic contractions induced by the cholinergic agonist carbachol. The inhibition of PKA decreased the neuronal release of ACh in DSM tissues. This study revealed that PKA-mediated signaling pathways differentially regulate nerve-evoked and spontaneous phasic contractions of guinea pig DSM. Constitutively active PKA in the bladder nerves controls synaptic ACh release, thus regulating the nerve-evoked DSM contractions, whereas PKA in DSM cells controls the spontaneous phasic contractility.

protein kinase A; acetylcholine; muscarinic receptor; detrusor smooth muscle; H-89

THE STORAGE AND RELEASE of urine are subjected to a complex control by autonomic and somatic motor neurons that innervate the bladder and urethra (3, 4). To facilitate detrusor smooth muscle (DSM) phasic contractions and bladder voiding, the postganglionic parasympathetic nerves release ACh and ATP, which activate DSM muscarinic ACh receptors (mACHRs) and purinergic receptors, respectively (2, 7). ACh is the primary excitatory neurotransmitter for nerve-evoked voiding contractions of human DSM (10, 20).

The neuronal release of ACh is regulated by both PKA and PKC (26). Particularly, the phosphorylation of certain synaptic vesicle proteins by endogenous neuronal PKA is key for exocytosis in the basal state, and such phosphorylation is required for neurotransmitter release (15). More specifically, the phosphorylation of the vesicle protein, synapsin, by PKA is required for the ACh release from presynaptic neurons (9). In various tissues, the activation or inhibition of PKA can increase or decrease neuronal ACh release, respectively (11, 18, 23, 24).

Whereas it is well established that ACh released from parasympathetic nerves in the bladder initiates voiding contractions (2), the function of PKA in bladder nerves has not been well studied. A previous study suggests that PKA activity might be involved in idiopathic detrusor instability associated with a reduction in the neuronal density (29). Activation of PKA can increase the release of nerve growth factors in the human urinary bladder (29). Furthermore, a mouse model of overactive bladder caused by a genetic deletion of the large conductance voltage- and Ca2+-activated K+ (BK) channels develops compensatory changes by overexpression of PKA in DSM cells (5). The aim of the present study was to investigate the physiological roles for PKA in the regulation of ACh release from parasympathetic neurons and the subsequent modulatory effects on the nerve-evoked contractions of guinea pig DSM.

MATERIALS AND METHODS

Ethical approval. Experimental procedures were carried out in accordance with the Animal Use Protocol #2186, reviewed and approved by the University of South Carolina Institutional Animal Care and Use Committee.

DSM tissue collection. A total of 25 male Hartley Albino guinea pigs (Charles River Laboratories, Raleigh, NC), age 3–5 mo, was used in this study. The guinea pigs had free access to food and water while exposed to 12/12 h light/dark cycles. The animals were euthanized by CO2 inhalation using an automated CO2 delivery system (Smarbox; Euthanex, Palmer, PA). Upon a follow-up thoracotomy, the bladders were cut open above the bladder neck and transferred to a Petri dish containing Ca2+-free dissection solution. DSM strips (~2–3 mm wide and ~5–8 mm long) were prepared by removing the mucosa and were used for isometric DSM tension recordings.

Isometric DSM tension recordings. Isometric contractions of DSM-isolated strips were measured, as previously described (30). In brief, DSM strips were secured to isometric force-displacement transducers.
and were suspended in temperature-controlled (37°C), water-jacketed tissue baths containing 10 ml physiological saline solution, aerated with 95% O2-5% CO2, pH = 7.4. The DSM strips were initially subjected to basal tension of 10 mN and washed with fresh physiological saline solution every 15 min during an equilibration period of 45–60 min. Electrical field stimulation (EFS)-induced, spontaneous, phasic, and carbachol-induced contractions were recorded using a MyoMED myograph system (MED Associates, St. Albans, VT). To eliminate the effects of neurotransmitters released from neurons, the experiments on carbachol-induced contractions were performed in the presence of 1 μM tetrodotoxin, a selective blocker of the neuronal voltage-gated Na⁺ channels.

Nerve-evoked contractions were induced by EFS using a pair of platinum electrodes mounted in the tissue baths parallel to the DSM strips. The EFS pulses were generated using a PHM-152I stimulator (MED Associates). The EFS pulse parameters were as follows: 0.75 ms pulse width, 20 V pulse amplitude, and 3 s stimulus duration, and polarity was reversed for alternating pulses. After the equilibration period, the DSM strips were subjected to continuous, repetitive EFS with a frequency of 20 Hz at 1 min intervals or to increasing EFS frequencies from 0.5 to 50 Hz at 3 min intervals. The cholinergic and purinergic components of EFS-induced contractions were recorded in the presence of suramin and α,β-methylene-ATP, or atropine, respectively. The acetylcholinesterase effect on EFS-induced contractions was blocked using eserine.

ACh measurements. Total ACh in the buffer, collected from the isometric DSM tension recording experiments and stored at −20°C, was measured with a choline/ACh assay kit (ab65345; Abcam, Cambridge, MA) using the colorimetric method following the manufacturer’s instructions. For each DSM strip, three samples (3 × 100 μl) were collected from the tissue bath containing 10 ml physiological saline solution: sample 1 at time t₁, 20 min before the addition of H-89 (10 μM); sample 2 at time t₂, right before the addition of H-89; and sample 3 at time t₃, 20 min after the addition of H-89. Fresh physiological saline solution (100 μl) was added into the tissue bath after each sample collection. The sample of 50 μl was then mixed with 50 μl of the reaction mixture in a 96-well plate and allowed to incubate at room temperature and was protected from the light for 30 min. The absorbance of 570 nm light by the solution was measured using the ELx808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT). The total choline concentration was calculated as a product of the amount of choline ([AChₜ₃]) in the sample, determined from the standard curve and the volume of sample (Sv) used in the reaction ([ACh] = [AChₜ₃]/Sv), where [ACh] is ACh concentration, and was expressed as nanomole/milliliter. The ACh release rate under conditions: ([ACh]ₜ₃ - [ACh]ₜ₂)/20, expressed as nanomole/milliliter/minutes, was compared with the rate in the presence of H-89 ([ACh]ₜ₃ - [ACh]ₜ₂)/20.

Data analysis and statistics. The parameters of DSM spontaneous phasic and EFS-induced contractions were analyzed using Mini-Analysis software (Synaptosoft, Decatur, GA). The following DSM contraction parameters were studied: amplitude (the difference between the force-time baseline curve and the peak of the contraction), muscle force (calculated by integrating the area under the phasic contraction force-time baseline curve), duration (the width of contraction at 50% of the height of the amplitude), frequency (contractions/min), and muscle tone (the changes in force-time baseline curve). Contraction parameters were normalized to the control (100%) and expressed as percentages. The effect of H-89 on 20 Hz EFS-induced contractions was measured after the contractions reached a steady state for at least 10 min. Data were analyzed with GraphPad Prism 4.03 software (GraphPad Software, La Jolla, CA) and expressed as means ± SE; n = the number of DSM strips, and N = the number of guinea pigs. Statistical significance was performed using Student’s t-test, and P < 0.05 was considered significant.

Solutions and drugs. The Ca²⁺-free dissection solution contained (in mM) the following: 80 monosodium glutamate, 55 NaCl, 6 KCl, 2 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, and 10 d-glucose, pH was adjusted to 7.3 with NaOH. The Ca²⁺-containing physiological saline solution, a modified Krebs solution, was prepared daily and contained (in mM) the following: 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄, and 11 d-glucose and was aerated with 95% O₂ and 5% CO₂.

Fig. 1. Pharmacological inhibition of PKA with H-89 attenuates EFS-induced contractions of DSM isolated strips. A and B: original recordings illustrating that H-89 (10 μM) decreased the EFS-induced contractions of DSM isolated strips. C: summary data showing that H-89 (10 μM) significantly decreased the 20-Hz EFS-induced contraction amplitude and muscle force, respectively (n = 15, N = 12, **P < 0.05, ***P < 0.001). D and E: summary data showing that H-89 (10 μM) significantly decreased extended 0.5–50 Hz EFS-induced contraction amplitude and muscle force at the frequencies higher than 20 Hz (n = 14, N = 8, *P < 0.05).
to obtain pH 7.4. Tetrodotoxin citrate, H-89, carbachol, suramin, α,β-methylene-ATP, and eserine were purchased from Sigma-Aldrich (St. Louis, MO). H-89 was dissolved in DMSO as stock solutions. The maximal DMSO concentration in the baths was <0.1%. All other chemicals were dissolved in double-distilled water.

RESULTS

The pharmacological inhibition of PKA with H-89 attenuated nerve-evoked contractions of DSM isolated strips. EFS induces ACh release from parasympathetic nerve terminals, and the subsequent activation of mAChRs increases DSM contractions. The inhibition of PKA with H-89 (10 μM) decreased the EFS-induced contraction amplitude and muscle force. H-89 (10 μM) inhibited the 20-Hz EFS-induced contraction amplitude and muscle force to 47.4 ± 5.7% and 43.2 ± 5.6% of the control values, respectively (n = 15, N = 12, P < 0.05; Fig. 1, A and C).

H-89 (10 μM) significantly inhibited the EFS-induced contraction amplitude and muscle force at frequencies higher than 20 Hz when the DSM strips were stimulated with increasing frequencies in a range of 0.5–50 Hz (n = 14, N = 8, P < 0.05; Fig. 1, B, D, and E). The EFS-induced contraction amplitude and muscle force at 50 Hz were reduced to 78.9 ± 4.4% and 80.4 ± 3.5% of the control values, respectively (n = 14, N = 8, P < 0.05; Fig. 1, B, D, and E). These data suggest that neuronal PKA plays a key regulatory role in the nerve-evoked DSM contractions.

Pharmacological inhibition of PKA with H-89 attenuated nerve-evoked contractions, while it simultaneously increased the spontaneous phasic contractions in DSM isolated strips. Our previous study showed that the constitutive PKA in DSM cells is essential for maintaining the BK channel activity and the spontaneous phasic contractions of DSM isolated strips and that the inhibition of PKA increases the spontaneous phasic contractions of DSM isolated strips (30). The current study shows that inhibition of PKA with H-89 (10 μM) significantly decreased the EFS-induced DSM contraction amplitude and muscle force (n = 6, N = 3, P < 0.05; Fig. 2), while it

Fig. 2. Pharmacological inhibition of PKA with H-89 attenuates EFS-induced contractions, while it simultaneously increases the myogenic spontaneous phasic contractions of guinea pig DSM strips. A: an original recording illustrating that H-89 (10 μM) inhibited the EFS-induced contractions, while it stimulated the spontaneous phasic contractions of guinea pig DSM strips. B: summary data showing that H-89 significantly increased spontaneous phasic contraction amplitude, duration, and muscle force and decreased the contraction frequency (n = 6, N = 3, *P < 0.05 vs. control). C and D: summary data showing that H-89 significantly inhibited the EFS-induced contraction amplitude and muscle force (n = 6, N = 3, #P < 0.05 vs. control).
simultaneously increased the spontaneous phasic contraction amplitude, muscle force, and duration (n = 6, N = 3, P < 0.05; Fig. 2). These data support the concept that the reduction of the nerve-evoked contractions upon PKA inhibition is mediated by signaling pathways located in bladder neurons rather than in DSM cells.

**Pharmacological inhibition of PKA with H-89 attenuated the cholinergic component of the nerve-evoked contractions of DSM isolated strips when purinergic receptors were blocked.** EFS-induced DSM contractions have cholinergic (main) and purinergic (secondary) components. Suramin (10 μM), a purinergic receptor antagonist, and α,β-methylene-ATP (10 μM), which desensitizes purinergic receptors, reduced 20 Hz EFS-induced DSM contraction amplitude to 87.6 ± 5.2% of the control value, whereas they increased the contraction duration to 114.5 ± 4.0% of the control value (n = 13, N = 4, P < 0.05; Fig. 3). In the presence of purinergic receptor blockers (suramin and α,β-methylene-ATP), the PKA inhibitor H-89 reduced the EFS-induced contraction amplitude at 50 Hz to 59.0 ± 4.6% of the control (n = 14, N = 5, P < 0.05 vs. suramin + α,β-methylene-ATP; Fig. 3). These data suggest the critically important functional role of PKA for the regulation of nerve-evoked cholinergic contractions of DSM.

**Pharmacological inhibition of PKA with H-89 increased the purinergic receptor-mediated component of nerve-evoked contractions.** Atropine (1 μM), a competitive antagonist of mAChRs, attenuates EFS-induced contractions (Fig. 4). When

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**Fig. 3.** The inhibition of PKA is highly effective in the attenuation of EFS-induced contractions of DSM isolated strips when purinergic receptors are blocked with suramin and α,β-methylene-ATP. A and B: original recordings illustrating that the inhibition of PKA with H-89 (10 μM) decreased the EFS-induced contractions in DSM isolated strips when the purinergic receptors are blocked. C: summary data showing that the inhibition of PKA significantly decreased the 20-Hz EFS-induced DSM contraction amplitude, duration, and muscle force in the presence of suramin and α,β-methylene-ATP (α,β-M-ATP; n = 13, N = 4, *P < 0.05 vs. control, #P < 0.05 vs. suramin + α,β-methylene-ATP). D: summary data showing that the inhibition of PKA significantly decreased the extended EFS-induced DSM contraction amplitude when purinergic receptors are blocked (n = 14, N = 5, #P < 0.05 vs. suramin + α,β-methylene-ATP).
mAChRs were blocked, the inhibition of PKA with H-89 increased the purinergic receptor-mediated DSM contractions induced by EFS (Fig. 4). Inhibition of PKA with H-89 (10 μM) increased the 20-Hz EFS-induced contraction amplitude, muscle force, and duration to 117.8 ± 5.9%, 149.9 ± 8.8%, and 126.1 ± 3.0%, respectively, of the control measured in the presence of 1 μM atropine (n = 10, N = 5, P < 0.05; Fig. 4). In the presence of atropine (1 μM), H-89 (10 μM) increased the extended EFS-induced DSM contraction amplitude at frequencies higher than 3.5 Hz (n = 10, N = 5, P < 0.05; Fig. 4). These data reveal that PKA differentially regulates mAChR- and purinergic receptor-mediated contractions in DSM isolated strips (Figs. 3 and 4).

Pharmacological inhibition of PKA with H-89 attenuated the nerve-evoked contractions in DSM isolated strips in the presence of eserine. Eserine, an acetylcholinesterase inhibitor, blocks the degradation of ACh. Eserine (10 μM) significantly increased the 20-Hz EFS-induced contractions, and the subsequent addition of H-89 (10 μM) inhibited the contraction amplitude, muscle force, duration, and tone to 53.8 ± 3.8%, 35.3 ± 4.1%, 72.0 ± 5.4%, and 53.1 ± 5.0%, respectively (n = 10, N = 3, P < 0.05; Fig. 5). These data suggest that H-89 inhibits the ACh release from nerve terminals in DSM, independently of acetylcholinesterase activity, thereby inhibiting EFS-induced contractions in DSM isolated strips.

Pharmacological inhibition of PKA with H-89 potentiated carbachol-induced DSM phasic contractions. Carbachol (0.1 μM), a chemically stable mAChR agonist, induces phasic contractions in DSM isolated strips. H-89 (10 μM) increased carbachol-induced contraction amplitude and muscle force to 143.0 ± 9.9% and 143.6 ± 10.6%, whereas it inhibited the contraction frequency to 69.0 ± 2.7% of the control values (before the addition of H-89; n = 12, N = 5, P < 0.05; Fig. 6). These results indicate that the inhibition of PKA potentiates the mAChR-induced phasic contractions in DSM isolated strips and that the inhibition of PKA does not directly affect DSM mAChR activity.

Pharmacological inhibition of PKA with H-89 attenuated the EFS-induced release of ACh in DSM isolated strips. To investigate further the role of PKA on ACh release in DSM tissue, we measured the amount of ACh released following DSM nerve stimulation by EFS using the colorimetric method. The inhibition of PKA with H-89 decreased the ACh released from DSM strips. H-89 (10 μM) decreased the ACh release from 0.013 ± 0.003 nmol·ml⁻¹·min⁻¹, under control conditions, to 0.0047 ± 0.001 nmol·ml⁻¹·min⁻¹ (n = 5, N = 4, P < 0.05; Fig. 7). These results suggest that neuronal PKA activity is critically important for the synaptic ACh release in guinea pig DSM tissue.

DISCUSSION

The current study revealed a novel regulatory mechanism by which constitutive neuronal PKA controls nerve evoked DSM contractions induced by the excitatory neurotransmitter, ACh. The nerve-evoked DSM contractions during the bladder-voiding phase are primarily induced by ACh, released by parasympathetic efferent nerves, but also by ATP as a secondary excitatory neurotransmitter (12, 14). The activation of mAChRs and purinergic receptors by ACh and ATP, respec-

![Fig. 4](http://ajprenal.physiology.org/)
tively, leads to the depolarization of the DSM cell membrane potential and an increase of intracellular Ca\textsuperscript{2+} levels, which consequently stimulates DSM contractions (20). The current study revealed that constitutively active PKA in the bladder nerves regulates the synaptic ACh release, thus controlling the nerve-evoked DSM contractions. The inhibition of neuronal PKA decreases the nerve-evoked DSM contractions by reducing ACh release, whereas the inhibition of PKA in DSM cells increases the myogenic DSM contractions by attenuating BK channel activity (30). The current data, along with our previous report (30), provide novel evidence that the constitutively active PKA in bladder nerves and DSM cells differentially controls nerve-evoked and spontaneous phasic contractility, respectively.

Some forms of bladder dysfunction are attributed to abnormal mAChR activity (21). The underlying causes may include excessive neuronal ACh release, which is regulated by various cellular signaling pathways in bladder nerve terminals. Regulation of neurotransmitter release is thought to involve protein phosphorylation, and the activation of the cAMP–PKA pathway has been shown to facilitate neurotransmission in mammalian neuromuscular synapses (18). Furthermore, it has been suggested that nitric oxide acts presynaptically to facilitate vagal neurotransmission involving mechanisms that enhance cAMP–PKA-dependent phosphorylation of presynaptic N-type Ca\textsuperscript{2+} channels. This pathway may augment the presynaptic Ca\textsuperscript{2+} influx and thereby, vesicular release of ACh (13).

The localized synaptic PKA signaling pathways control presynaptic release of neurotransmitter vesicles (22). All isoforms of the vesicle synapsin have a short N-terminal region containing the phosphorylation site for PKA, and the PKA-mediated phosphorylation plays a central modulatory role in the synaptic exocytosis of presynaptic neurons (6, 19). Genetic or pharmacological modulation of PKA has an important impact on the neurotransmission (27). In peripheral nerves, activation of presynaptic \(\beta\)-adrenoceptors stimulates adenylate cyclase and increases neuronal cAMP levels, which leads to PKA activation and enhanced neurotransmitter release (17). It has been shown that the PKA inhibition by H-89 can attenuate the evoked ACh release in levator auris longus muscle in rats (24) and rat motor nerve terminals (25).

The current study demonstrates that PKA has a key regulatory function in the synaptic transmission of ACh in the guinea pig urinary bladder. Regardless of whether acetylcholinesterase is blocked or not, the inhibition of PKA still attenuates the EFS-induced contractions. In fact, ACh release increases in the presence of the acetylcholinesterase inhibitor, eserine (28). This finding suggests a possible decrease of synaptic ACh

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**Fig. 5.** In the presence of the acetylcholinesterase inhibitor eserine, the inhibition of PKA with H-89 attenuates the EFS-induced contractions in DSM isolated strips. *A*: an original recording illustrating that H-89 (10 \(\mu\)M) reduced the 20-Hz EFS-induced contractions of DSM isolated strips in the presence of 10 \(\mu\)M eserine. *B*: the original recording in *A* shown in an enlarged time scale. *C*: summary data, illustrating that eserine (10 \(\mu\)M) significantly increased, and the subsequent inhibition of PKA with H-89 significantly reduced the EFS-induced contraction amplitude, muscle force, duration, and tone of DSM isolated strips \((n = 10, N = 3, *P < 0.05)\).
release rather than an increase of ACh degradation. In addition, it has been reported that EFS-induced ACh release is also enhanced by muscarinic antagonists, such as atropine, as well as the cAMP-elevating agents, such as forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1). Moreover, inhibition of constitutive PKA activity in DSM cells and the consequent increase in the spontaneous phasic contractions of DSM (30) further enhanced DSM contractions following stimulation with carbachol. Collectively, all of these findings indicate that the inhibition of PKA does not directly block mAChRs. Therefore, the reduction of EFS-induced contractions is most likely due to the blockade of ACh release by the inhibition of PKA. This concept was further confirmed by direct measurements of ACh showing that the rate of ACh release was reduced upon PKA inhibition.

Intriguingly, our data showed that PKA inhibition by H-89 increased the purinergic component of EFS-induced DSM contractions. Purinergic signaling predominates at lower frequencies (2), which is consistent with our observations that H-89 did not reduce the contractions induced by EFS below 10 Hz; the two effects (increase vs. decrease) compensate for each other, and the net effect is zero. This suggests that PKA might also be involved in regulating the purinergic signaling in the bladder; however, the underlying mechanism needs further investigation in future studies.

One limitation of the present study is that H-89 might also have some effects on other non-DSM, non-neuronal cell types located within the detrusor layer, such as bladder interstitial cells. Although H-89 might have some inhibitory effects on other protein kinases, such as protein kinase G, a previous study has demonstrated that the constitutive protein kinase G plays a negligible role in the regulation of DSM contractions (30). Therefore, the observed H-89 effects in DSM are primarily due to an impact on PKA.

Neurotherapeutics aim to normalize the excessive release of neurotransmitters associated with various types of neurological disorders, including neurogenic detrusor overactivity (8). One clinical implication is the local injection of botulinum toxin A (BoNT/A), which has been used to treat some human disorders associated with hyperactivity of motor or autonomic nerves (8). BoNT/A works by locally inhibiting ACh release from bladder nerve endings (16). The high-affinity binding of BoNT/A with the synaptic vesicle protein blocks exocytosis, thereby attenuating the release of ACh from bladder cholinergic nerve terminals (8). BoNT/A can significantly reduce overactive bladder symptoms caused by spinal cord injury or multiple sclerosis and has been recently approved by the U.S. Food and Drug Administration for overactive bladder treatment in patients with neurogenic detrusor overactivity (16). Inhibition of PKA diminishes the phosphorylation of synaptic vesicle proteins, thus blocking the exocytosis of synaptic vesicles, and attenuates the ACh release (9).

In conclusion, the current study revealed a novel mechanism regulating synaptic release of ACh by neuronal PKA in the urinary bladder. Specifically, the study revealed that the inhibition of localized presynaptic PKA effectively attenuates ACh release in the bladder. Therefore, targeted pharmacological modulation of neuronal pathways involving PKA may represent an important research area for future investigations in the context of improved treatments for neurogenic detrusor overactivity.

ACKNOWLEDGMENTS

The authors thank Mr. Biao Chen for his help with some of the experiments, as well as Dr. Kiril L. Hristov and Mr. Aaron Provence for the critical evaluation of the manuscript.

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GRANTS

Support for this study was provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Grants R01 DK084284 and R01 DK106964 to G. V. Peikov).
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W.X. and G.V.P. conception and design of research; W.X., N.L., V.S.F., and G.V.P. performed experiments; W.X., N.L., V.S.F., and G.V.P. analyzed data; W.X., N.L., V.S.F., and G.V.P. interpreted results of experiments; W.X. and G.V.P. prepared figures; W.X. and G.V.P. drafted manuscript; W.X., N.L., V.S.F., and G.V.P. edited and revised manuscript; W.X., N.L., V.S.F., and G.V.P. approved final version of manuscript.

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