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# Diabetic plasticity of non-adrenergic non-cholinergic and P2X-mediated rat bladder contractions

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## Abstract

We investigated the plasticity effects of diabetes mellitus and diuresis on the non-adrenergic non-cholinergic (NANC) and purinergic (P2X-type) contractile responses in longitudinal rat bladder strips. Female Sprague-Dawley rats received streptozotocin to induce diabetes, or sucrose in water to induce diuresis as a control condition for polyuria. Experiments were carried out at four weeks after treatments, using bladders from not-treated rats as control. Urinary bladder strips were electrically stimulated throughout the experiments to generate neurally evoked contractions (NEC). In all cases, P2X-mediated purinergic contractions were evaluated at the beginning and end of the stimulations with α,β-Methylene Adenosine Triphosphate (α,βMeATP). The NANC responses were assessed by using two independent protocols. First, cholinergic receptors were activated with carbachol (CCh), followed by inhibition of the muscarinic component with atropine. In the second protocol, the application order for CCh and atropine was reversed. The NANC response, unmasked with the application of atropine, and the P2X purinergic contractions were analyzed. NANC contractions in diabetic bladder strips are more resistant to the desensitizing effects caused by activation of cholinergic receptors. In early stages of experimental diabetes, NANC responses in diabetic strips are less sensitive to functional inhibition mediated by the cholinergic activation. However, P2X-mediated purinergic contractions are more sensitive to desensitization in diabetic or diuretic bladders. For instance preventing muscarinic receptor activation with atropine does not counteract the desensitization of purinergic contractions in either diabetic or diuretic strips. We suggest that diabetes may induce a plasticity of the NANC and P2X-mediated bladder contractile responses. The first one may be associated with diabetic neuropathic damage to bladder nerves, while impaired P2X purinergic contractions might be associated with detrusor hypertrophy observed in diabetic and diuretic strips.



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**Conflict of interest**

Authors do not have any Conflict of Interest to declare.

**Keywords**

Diabetic bladder dysfunction; purinergic receptor; cholinergic receptor; non-adrenergic non-cholinergic response; neurally evoked contraction; detrusor muscle

## 1. Introduction

Neurogenic detrusor contractions are driven by the parasympathetic release of several transmitters, among them acetylcholine acting on cholinergic receptors to initiate the efferent side of the micturition reflex [8, 31]. Furthermore, activation of M3 muscarinic receptors by acetylcholine released from efferent nerve terminals is fundamental for bladder contractions [1]. Experimentally, the application of an electrical field stimulation to urinary bladder strips generates a neurally evoked contraction (NEC) characterized by high levels of acetylcholine acting on detrusor muscarinic receptors [23]. Although nor-epinephrine is also released by electrical stimulation [22], in the rat bladder it activates β3-adrenergic receptors to facilitate detrusor relaxation rather than adrenergic contractions [27, 31].

The pharmacological inhibition of cholinergic receptors using atropine [13] or specific M-type muscarinic antagonists such as 4-Diphenylacetoxy-*N*-methylpiperidine (4-DAMP), unmasks a remaining NEC known as the non-adrenergic non-cholinergic (NANC) contractile response [2, 12]. In rat detrusor the NANC response is mostly mediated by the activation of P2X-type purinergic receptors by co-released ATP, and can be revealed by pharmacological inhibition of cholinergic transmission [9, 13, 26].

We previously reported that activation of cholinergic receptors with carbachol (CCh) decreases NANC contractions via a desensitization mechanism most probably involving P2X-type purinergic receptors in intact rat bladders [13]. Moreover, the specific inhibition of bladder M-type muscarinic receptors with 4-DAMP prevents the attenuation of the NANC responses, thus suggesting that activation of muscarinic receptors may desensitize a purinergic contractile component mediated by P2X-type receptors [12, 13, 26].

In this report we used detrusor strips from diabetic, diuretic and normal rats to expand our understanding between cholinergic and purinergic signaling in regulating the plasticity of NANC and P2X-mediated bladder contractions in pathological conditions. By following a similar approach as in previous studies [12, 13], we calculated the changes in NEC and NANC by measuring strip contractile changes while preventing muscarinic receptor activation with a high dose of atropine. We also determined the degree of desensitization for the purinergic component by comparing P2X purinergic receptor contractions at the beginning and end of the stimulation protocols.

## 2. Materials and Methods

Animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, and followed the research standards suggested by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### 2.1. Experimental animal groups

Female Sprague-Dawley rats weighing 250 to 300 g were use during the course of the experiments. Diabetes was induced with a single intravenous tail injection of 50 mg/Kg streptozotocin (STZ; VWR, Radnor, PA), prepared in 100 mM citrate buffer at pH 4.0 followed by a subcutaneous glucose injection (1.0 g/Kg) to prevent severe hypoglycemia. In a second group of rats serving as diuretic control for bladder hypertrophy, diuresis was

induced by complementing *ad-libitum* drinking water with 5% sucrose [15]. Control animals received regular water and i.v. injected with vehicle alone. Blood glucose was weekly monitored with a One Touch Ultra glucometer (LifeScan; Milpitas, CA). STZ-injected rats with persistent blood glucose levels above 250 mg/dl were included in the study.

### 2.2. Preparation of rat bladder strips for contractile evaluations

Contractile experiments were performed 4 weeks after the induction of diabetes or diuresis. At this time point rats were euthanized and bladders removed into an oxygenated Krebs solution containing (in mM): NaCl (113); KCl (4.7); $CaCl_2$ (1.25); $MgSO_4$ (1.2); $NaHCO_3$ (25); $KH_2PO_4$ (1.2); D-glucose (11.5). Solution temperature was set to 37°C and the pH was kept at 7.4 by a continuous bubbling of a 95% O2/5% CO2 gas mixture. After dissecting the whole bladder above the trigone region, two longitudinal strips were obtained from each bladder and mounted in tissue baths filled with oxygenated and warmed Krebs solution. For comparison purposes with our previous studies, special care was taken to preserve urothelial integrity in the bladder strips. Contractile experiments were run in parallel for diabetic/diuretic, diabetic/control or diuretic/control group combinations. A pretension of 10 mN was applied to each strip. After a recovery period of 30 minutes, isometric contractions were determined with a force transducer connected to a 4-channel trans-bridge amplifier (both from WPI; Sarasota, FL). NEC contractions were induced via platinum wire electrodes that generated an electrical field stimulation with square wave impulses (0.25 ms; 20Hz; 200 shocks every 100 s) at 100 V using an S-88 dual channel stimulator (Grass Technologies; West Warwick, RI). This voltage was enough to generate a supra-maximal contractile response from the detrusor strips. Data were collected in real time using the Windaq acquisition software (DataQ; Akron, OH) at a sampling rate of 20 Hz. Chemically induced P2X-type purinergic contractions were determined without electrical stimulation as described below.

### 2.3. Evaluation of NANC and purinergic contractions

As performed in our previous studies, two stimulation protocols were achieved on each half bladder [12, 13]. In all cases the intact P2X purinergic contractile response was evaluated with the application of the specific P2X agonist α, β-Methylene-Adenosine Triphosphate (αβMeATP) using a single dose of 50 μM at the beginning of each contractile experiment. The stimulation protocols for NEC, NANC and the application of 50 μM carbachol (a cholinergic agonist) or 1 μM atropine (a muscarinic receptor antagonist) are illustrated in figure 1. Briefly, after an initial P2X stimulation (i.e. $\alpha\beta MeATP_1$) and subsequent wash-out, NECs were induced until the amplitude of these contractions was stable (as indicated with the boxed-B). Then, the NANC bladder contractions were unmasked by adding atropine after either the application of CCh (figure 1A; CCh to atropine group) or by atropine followed by CCh application (figure 1B; atropine to CCh group).

The percentage of the residual contraction that remained after atropine administration (boxed R in figure 1) was considered as the NANC contractile response. To determine the degree of purinergic contraction desensitization induced by activation of cholinergic receptors, a second application of the same P2X purinergic receptor agonist (i.e. $\alpha\beta MeATP_2$) was performed without washing-out the CCh/atropine mixture of the organ bath. Purinergic contractions were evaluated approximately 60 minutes apart. Bladder strip viability was verified by raising the extracellular potassium concentration to 140 mM $KCl^+$. For comparison, we calculated the percent of the NANC contraction change as the % of the R/B proportion. The degree of purinergic contractile desensitization evoked by αβMeATP was determined as the percentage of the $\alpha\beta MeATP_2/\alpha\beta MeATP_1$ quotient.

### 2.4. Reagents

Krebs salts, αβ-MeATP, sucrose, carbachol and atropine were from Sigma-Aldrich (St. Louis, MO).

### 2.5. Statistical analysis and figure preparation

Bladder strips from 6 control, 6 diabetic or 6 diuretic rats received were stimulated with the protocols shown in figure 1 and included for analysis (i.e. N=6 per experimental condition). Data are expressed as mean +/− S.E.M. Statistical analysis was performed by one-way ANOVA followed by a Tukey's post-test to compare NANC and purinergic contractions in control, diabetic and diuretic groups for each of the two stimulation protocols. Contractile traces were imported from the Windaq acquisition software (DataQ; Akron, OH). Figure preparation and statistical analysis were performed with Prism 6.0 (GraphPad Prism; San Diego, CA). Statistical significance was considered at $p<0.05$.

## 3. Results

### 3.1. Diabetes and diuresis effects on body weight, blood glucose and neurally evoked contractions

Four weeks after destruction of β-cells with STZ, the body weight of diabetic rats was 243+/−4 g (*$p<0.05$ vs control) while no statistical differences were observed between control (302+/−12 g) and sucrose fed (289+/−10 g) animals (figure 2A). Blood glucose levels were significantly elevated in STZ-treated rats (424+/−21 mg/dl; ***$p<0.001$) in comparison with control (108+/−8 mg/dl) or diuretic (133+/−9 mg/dl) rats (figure 2B). Figure 2C shows the distribution for the NEC amplitude for all the strips receiving the stimulation protocols shown in figure 1, that is, contractions at the time indicated by the boxed-B. The mean value +/−S.E.M. for the magnitude of the initial NEC response in control (1.71+/−0.4 mN/mg), diabetic (0.95+/−0.2 mN/mg) or diuretic strips (2.31+/−0.3 mN/mg) is also illustrated. A statistical difference in the NEC amplitude was observed between diabetic vs diuretic bladder strips (**$p<0.01$).

### 3.2. Cholinergic receptor activation is more effective in decreasing the NANC response in control and diuretic, but not in diabetic bladder strips

The amplitude of the NANC response in control bladder strips was attenuated 56% when cholinergic receptors were activated with CCh and later inhibited with atropine (figure 3A; **$p<0.01$ vs atropine to CCh). On the contrary, the NANC responses in diabetic bladder strips were less sensitive to the inhibitory effects caused by cholinergic receptor activation (figure 3B; 34%; *$p<0.05$ vs atropine to CCh). The degree of NANC inhibition in diuretic strips was the same as in control strips (figure 3C; 55%; **$p<0.01$). A statistical analysis for the NANC responses when stimulating with CCh before blocking muscarinic receptors with atropine, showed that the NANC contractions in diabetic bladder strips were more resistant to the desensitizing effect produced by CCh (figure 3D; $^{\#\#}p<0.01$ vs both control or diuretic CCh to atropine condition). The application of atropine before cholinergic stimulation with CCh significantly prevented the reduction of the NANC contractile amplitude in control (2% decrease), diabetic (8% decrease) or diuretic (9% decrease) rat bladder strips (figure 3A–C, empty bars; $p>0.05$ vs NEC responses).

### 3.3. Effects of cholinergic receptor activation on P2X-mediated purinergic bladder contractions

During control contractile experiments we found that a second P2X stimulation (i.e. $\alpha\beta MeATP_2$) approximately 60 minutes after a first αβMeATP application produced a contraction of identical magnitude only if the first P2X agonist was washed-out and no other

electrical or chemical stimulation induced (not shown). The evaluation of the P2X purinergic contractions before (i.e. $\alpha\beta$-MeATP$_1$) and after (i.e. $\alpha\beta$-MeATP$_2$) the stimulation protocol described in figure 1A (CCh to atropine) showed that both diabetic (57%; *$p<0.05$ vs control CCh to atropine) and diuretic (53%; *$p<0.05$ vs control CCh to atropine) bladder strips have a higher degree of desensitization than control strips (31%; figure 4A). Following the stimulation protocol described in figure 1B (atropine to CCh), we found that both purinergic contractions had the same contractile magnitude in control strips. However, they were significantly desensitized in diabetic (39%; $^{###}p<0.001$ vs control atropine to CCh) and diuretic (21%; $^{\#}p<0.05$ vs control atropine to CCh) bladder strips independently of the cholinergic inhibition (figure 4B).

## 4. Discussion

Our results show that NANC contractions in diabetic bladder strips are more resistant to the desensitizing effects caused by activation of cholinergic receptors with CCh. In contrast to normal bladders, the P2X-mediated purinergic contractions are more susceptible to desensitization induced by cholinergic receptor activation in both diabetic and diuretic bladder strips. These observations suggest that preventing cholinergic receptor activation with atropine does not effectively counteract, as observed in normal rats, the desensitization of purinergic and NANC contractions in either diabetic or diuretic bladder strips. Changes in bladder weight and cystometric properties of diabetic rats have demonstrated that these animals develop a specific bladder cystopathy that may involve detrusor and nerve dysfunction [15, 18]. In fact, neuropathy is one of the most common complications of diabetes, affecting the quality of life of patients by decreasing nerve functionality and therefore peripheral sensation [29]. Under hyperglycemic conditions nerve fibers progressively degenerate in diabetic neuropathy affecting autonomic nerve functions [20], for instance the release of different transmitters regulating bladder contractions.

The main origin of NEC and NANC responses is pre-synaptic, as demonstrated by applying tetrodotoxin during electrical field stimulation [7]. We have perform continuous electrical stimulations for periods of 60–90 minutes using the parameters indicated and did not see a significant decrease in NEC responses. Thus, we are confident that the stimulation protocol by itself does not generate a contractile desensitization. Studies in diabetic animal models have found that diabetes can induce either an increase [25] or a decrease [10] in the amplitude of the NEC. We found that NECs have a significant trend towards reduced contractile magnitudes in diabetic strips. This may be explained by reduced efferent nerve activation during electrical stimulation, verified by the application of tetrodotoxin, and associated with a chronic hyperglycemia during diabetes [14]. The specificity of this diabetes-induced damage is supported by the observation that either sucrose-induced diuresis or STZ-provoked diabetes generate a hypertrophy of both detrusor and urothelium, however, only the diabetic bladders show significant increased levels for markers of oxidative stress that may affect nerve function [30]. We hypothesize that impaired NANC and P2X-mediated contractions in diabetic cystopathy are a consequence of a significant reduction in the nerve density at the detrusor layer. This is supported by the observation that diabetic bladders present an important reduction in the expression of neurofilament 200-immunoreactive nerves when normalizing by the detrusor area. This neuropathic diabetic effect was statistically significant at 9 or 20 weeks, with a reduction trend already evident by one week after diabetes induction [16]. The similar magnitudes for the NECs in control and diuretic strips in our study suggests that after 4 weeks of STZ-treatment, contractile mechanisms in diabetic bladders have been already affected, mainly by oxidative stress [30].

Other regulatory players may be affected by diabetes thus contributing to impaired NANC and P2X purinergic contractions. For example, urothelial cells are important contributors of

ATP release for the mediation of bladder sensation during filling [3, 11, 17]. One of the main actions for the ATP released from the urothelium is the activation of ionotropic purinergic receptors in nerve terminals to convey sensory transmission, especially through P2X3-type ionotropic purinergic receptors, to the central nervous system [6, 19]. The tissue bath application of αβMeATP can activate either nerve terminal/urothelial P2X3- receptors as well as detrusor P2X1 purinergic receptors. Consequently, with our *in vitro* preparation an afferent effect of P2X3 activation on the strip contractile purinergic response is not expected, thus it is possible to suggest that the observed desensitizing effect mainly involves desensitization of P2X1-type purinergic receptors expressed in detrusor.

The desensitization of P2X1 purinergic contractions on the diuretic and diabetic detrusor seems to be enhanced by the application of CCh, confirming a specific muscarinic receptor inhibitory interaction taking place in smooth muscle cells [12, 13]. In fact, the $IC_{50}$ doses of atropine for M2/M3 muscarinic receptor inhibition are in the nM range [4], thus we predict that 1 μM was enough for blocking all muscarinic receptor isoforms, supporting the idea that muscarinic receptor-activation is a key event in mediating the impairment of NANC and P2X-mediated bladder contractions. Nevertheless, in contrast to NANC responses from rats with spinal cord injury [12], the plasticity of the NANC contractions in bladder strips from diabetic rats appear to be less sensitive to muscarinic receptor activation. Because we used the same CCh dose in control or diuretic strips, it is possible to suggest that perhaps a higher expression of muscarinic receptors in diabetic bladders [5, 24] may be supporting the lower desensitizing effect observed in diabetic strips. Additionally, the reduced degree of desensitization for the NANC response in diabetes may not entirely depend on muscarinic receptor activation at the detrusor, but also on reduced neural components affected by diabetes [14, 16]. We believe that the small increase in the contractile tone when applying CCh after atropine is produced by a non-specific activation of other cholinergic receptors that present atropine resistance.

Additional studies have shown that in rats with neurogenic or diabetic bladder overactivity the release of ATP from the urothelial bladder layer is enhanced [18, 21], and might be an important contributor to altering the micturition reflex by generating a high frequency of non-voiding contractions via the activation of P2X1 receptors on the detrusor [6]. Similarly, the inhibition of P2X3-formed purinergic receptors in bladder afferent nerve fibers of rats with spinal cord injury can decrease neural sensory activity and the occurrence of non-voiding contractions [19]. In any case, the inhibition of bladder purinergic receptors, and consequently modulation of the NANC purinergic component, may be an important pharmacological target to ameliorate urinary bladder dysfunction in different human pathological states [28, 31].

## 5. Conclusions

Our results suggest that diabetes produces a plasticity effect on detrusor contractile components in opposite directions to create a more resistant NANC response but a P2X-mediated contraction with higher degree of desensitization. Targeting cholinergic pathways in bladder dysfunction may alter the responsiveness of non-cholinergic components. However, it is possible that an early intervention targeting NANC and/or P2X-mediated bladder contractile responses in diabetic cystopathy may be helpful to ameliorate bladder dysfunction.

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## Abbreviations

| | |
|---|---|
| **NEC** | neurally-evoked contraction |
| **NANC** | non-adrenergic non-cholinergic |
| **4-DAMP** | 4-Diphenylacetoxy-*N*-methylpiperidine |
| **ATP** | Adenosine Triphosphate |
| **α, βMeATP** | α,β-Methylene- Adenosine Triphosphate |
| **P2X** | ionotropic purinergic receptor |
| **CCh** | carbachol |

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## Highlights

- Activation of cholinergic receptors inhibits P2X and NANC bladder contractions
- Neurally evoked contractions are smaller in diabetic when compared with diuretic bladder strips
- Diabetic NANC responses are less sensitive to inhibition by cholinergic receptor activation
- P2X-mediated contractions are more desensitized in diabetic and diuretic bladder strips

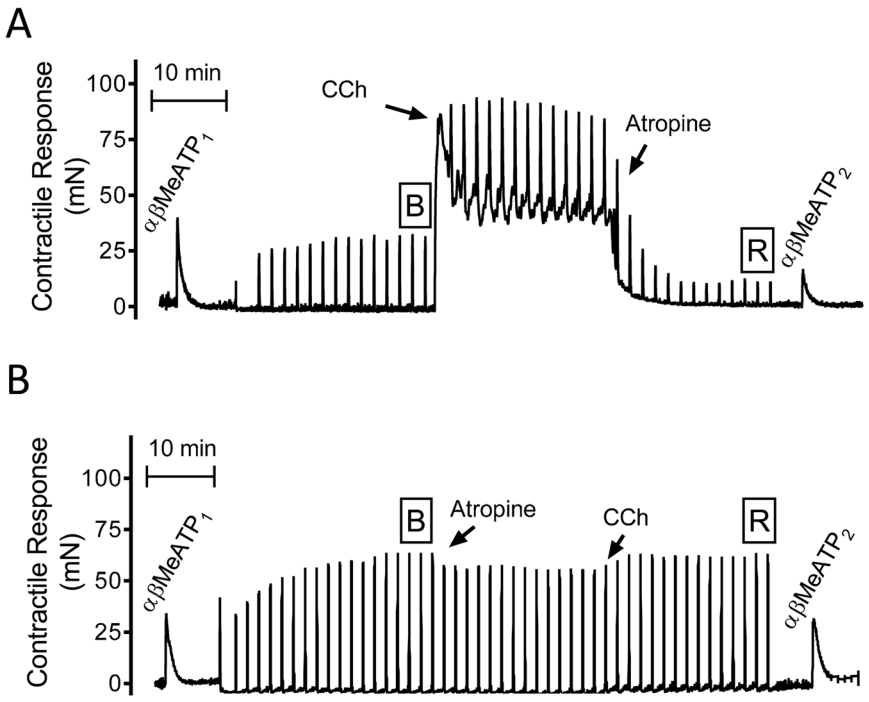


**Figure 1.**

Stimulation protocols for generation and evaluation of purinergic, neurally evoked (NEC) and non-adrenergic non-cholinergic (NANC) contractions. (A) Representative contractile responses for the protocol where carbachol (CCh) was applied before atropine. (B) Representative contractile responses for the protocol where atropine was applied before CCh. See the corresponding material and methods section for a detailed description of the evaluation protocols.

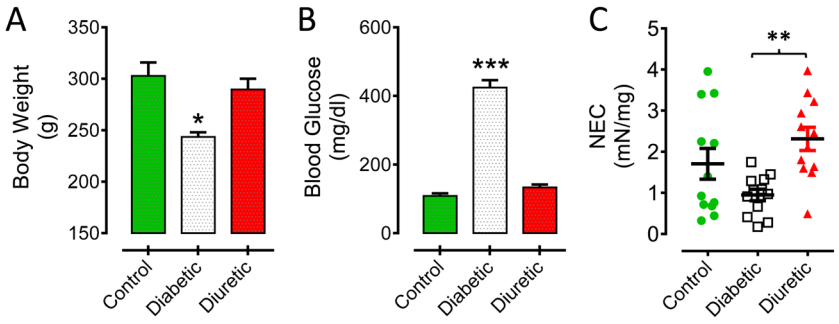


**Figure 2.**

Physiological effects of diabetes and diuresis on: (A) body weight (*p<0.05 vs control and diuretic); (B) blood glucose (***p<0.001 vs control and diuretic) and (C) NEC amplitude (**p<0.01 for diabetic vs diuretic bladder strips).

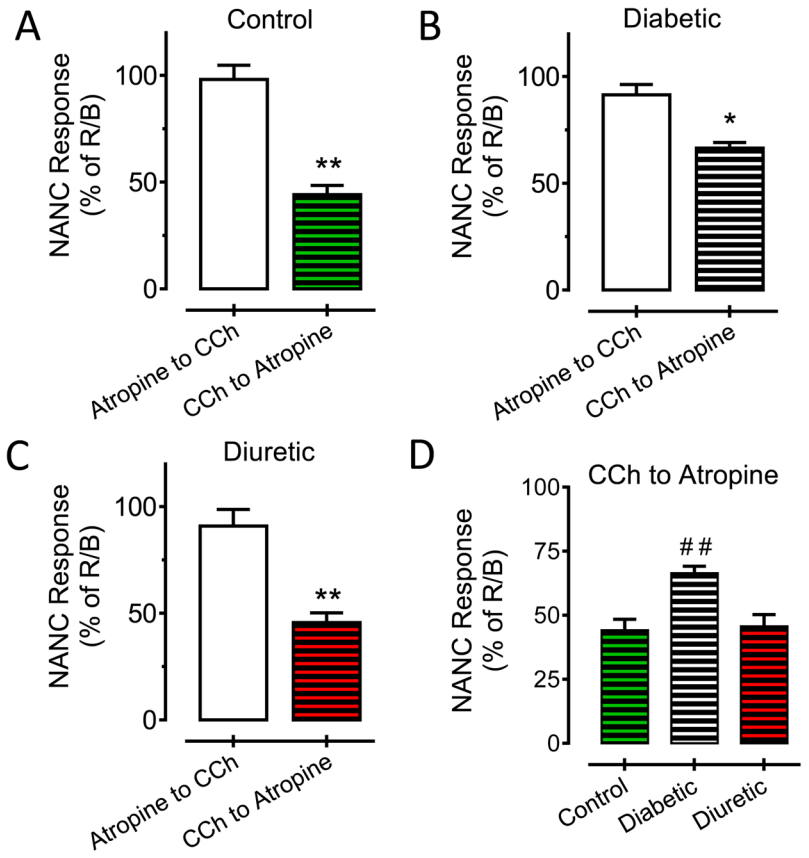


**Figure 3.**

Diabetic effects on non-adrenergic non-cholinergic bladder contractions. (A) NANC responses in bladder strips from control rats (**$p<0.01$ vs control atropine to CCh condition). (B) NANC responses in bladder strips from diabetic rats (*$p<0.05$ vs diabetic atropine to CCh condition). (C) NANC responses in bladder strips from diuretic rats (**$p<0.01$ vs diuretic atropine to CCh condition). (D) Comparisons for the NANC responses during the CCh to atropine protocol in bladder strips from control, diabetic and diuretic rats ($^{\#\#}p<0.01$ vs control or diuretic strips).

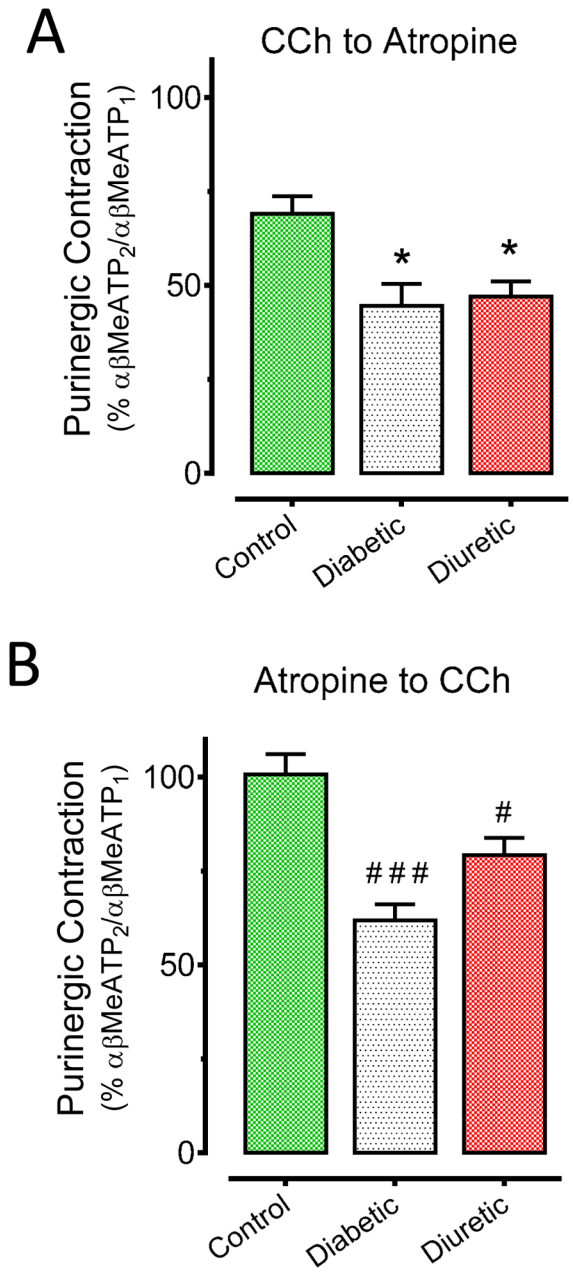


**Figure 4.**

Effects of carbachol and atropine on P2X-mediated purinergic contractions. (A) Activation of cholinergic receptors produces a higher purinergic desensitization in diabetic and diuretic bladder strips than in controls (*$p<0.05$ vs control CCh to atropine condition). (B) Cholinergic inhibition with atropine does not entirely prevent purinergic desensitization in diabetic or diuretic bladder strips after application of CCh ($^{###}p<0.001$ and $^{\#}p<0.05$ vs control atropine to CCh condition).