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The Inhibitory Mechanism on Acetylcholine-Induced Contraction of Bladder Smooth Muscle in the Streptozotocin-Induced Diabetic Rat

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Abstract

Most diabetic patients experience diabetic mellitus (DM) urinary bladder dysfunction. A number of studies evaluate bladder smooth muscle contraction in DM. In this study, we evaluated the change of bladder smooth muscle contraction between normal rats and DM rats. Furthermore, we used pharmacological inhibitors to determine the differences in the signaling pathways between normal and DM rats. Rats in the DM group received an intraperitoneal injection of 65 mg/kg streptozotocin and measured blood glucose level after 14 days to confirm DM. Bladder smooth muscle contraction was induced using acetylcholine (ACh, 10^{-4} M). The materials such as, atropine (a muscarinic receptor antagonist), U73122 (a phospholipase C inhibitor), DPCPX (an adenosine A_1 receptor antagonist), udenafil (a PDE5 inhibitor), prazosin (an α_1-receptor antagonist), papaverine (a smooth muscle relaxant), verapamil (a calcium channel blocker), and chelerythrine (a protein kinase C inhibitor) were pre-treated in bladder smooth muscle. We found that the DM rats had lower bladder smooth muscle contractility than normal rats. When prazosin, udenafil, verapamil, and U73122 were pre-treated, there were significant differences between normal and DM rats. Taken together, it was concluded that the change of intracellular Ca^{2+} release mediated by PLC/IP3 and PDE5 activity were responsible for decreased bladder smooth muscle contractility in DM rats.

Key Words: Bladder, Contractility, Diabetes, Smooth muscle, PLC

INTRODUCTION

Patients with diabetes mellitus (DM), a hyperglycemia and increase in insulin resistance, often have additional metabolic disorders, such as atherogenic lipidemic symptoms, hypertension, and inflammation (Weissman, 2006; Tuttle *et al*., 2014; Amaral and Okonko, 2015; Pecoits-Filho *et al*., 2016; Rizos *et al*., 2016). In addition, one of the main complications of diabetes mellitus that affects quality of life is impaired bladder function. In fact, over 50% of diabetic patients have bladder dysfunction (Van Den Eeden *et al*., 2009). The symptoms of diabetic bladder dysfunction include increased residual urine volume after voiding and impaired detrusor contractility, including clinical evidence (Bradley, 1980; Ioanid *et al*., 1981; Kaplan *et al*., 1995).

The walls of the bladder are mainly composed of the detrusor muscle that allows the bladder to contract to excrete urine or relax to hold urine. The detrusor muscle is under the control of the autonomic system and is formed of smooth muscle (Sam and LaGrange, 2018). Muscarinic receptors are principally responsible for mediating bladder contraction, with M2 muscarinic receptors representing approximately 90% of the total muscarinic receptors in the bladder smooth muscle in rats (Wang *et al*., 1995). There are several receptors in the rat bladder including adrenergic and purinergic receptors (Braverman *et al*., 2006; Kullmann *et al*., 2011; Vesela *et al*., 2011). In the lower urinary tract, nitric oxide has the potential to function as a transmitter for various organs because nitric oxide synthase (NOS) is expressed in afferent and efferent nerves, as well as in the smooth muscle, urothelium, and striated muscle. It activates guanylate cyclase, which results in increased levels of cyclic guanosine monophosphate (cGMP), leading to smooth muscle relaxation (Johansson *et al*., 2002; Artim *et al*., 2009; Matsumoto *et al*., 2010). Hydrogen sulfide (H_2S) can

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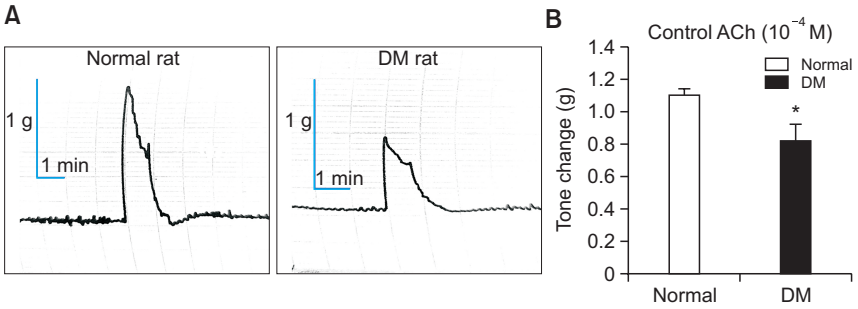

Fig. 1. Representative traces and tension comparison for ACh-induced contraction of bladder smooth muscle in normal and DM rats. (A) ACh-induced contraction. (B) Average tension for ACh-induced contraction in normal and DM rats. The left- and right-hand sides of 1A represent normal and DM rats, respectively. ACh was added at a concentration of 10^{-4} M. Each point represents the mean ± SEM (n=13). **p*<0.05 by Student's *t*-test.

also induce contraction of the bladder smooth muscle, and furthermore, H_2S synthetic enzymes are known to be expressed in the bladder (Patacchini *et al*., 2004, 2005; Dombkowski *et al*., 2006). It has been reported that PDE5 inhibitors increase the contractile force of normal rat bladder. PDE5 inhibitors are known to stimulate bladder smooth muscle relaxation by inhibiting the degradation of cGMP by PDE5 (Matsumoto *et al*., 2010).

In a DM model, a hypocontractile state has been reported, especially in rats (Daneshgari *et al*., 2006). This decrease in bladder contractility occurs because of a variety of reasons. A decrease in sensitivity to ACh has been reported in DM rats (Longhurst and Belis, 1986), and the change in calcium sensitivity has been suggested to be related to changes in bladder contractility (Belis *et al*., 1991; Waring and Wendt, 2000). It is known that NO production increases in hyperglycemia and diabetic cystopathy (Poladia and Bauer, 2003; Adela *et al*., 2015). Therefore, it was suggest that G protein–coupled receptors (GPCRs), intracellular signaling pathways, and NO production play major roles in bladder contractility in DM rats.

The purpose of this study was to examine the role of GPCRs, intracellular signaling pathways, and NO production in bladder smooth muscle contractility in DM rats. The goal was to understand the mechanism underlying the changes in bladder smooth muscle contraction in rats with DM. The pharmacological inhibitors used in this study were: atropine (a muscarinic receptor antagonist), U73122 (PLC inhibitor), DPCPX (an adenosine A1 receptor antagonist), udenafil (a PDE5 inhibitor), prazosin (an α_1-receptor antagonist), papaverine, NaHS (a smooth muscle relaxant), verapamil (a calcium channel blocker), and chelerythrine (a protein kinase C [PKC] inhibitor).

MATERIALS AND METHODS

Animals

Male Sprague–Dawley (SD) rats weighing 250-280 g were supplied by Samtako Bio (Osan, Korea). The animals were group-housed in cages with wire-net floors in a temperature controlled room (24-25°C), with controlled humidity (70-75%), and a 12 h light-dark cycle. Rats were fed a normal laboratory diet from Samtako Bio, and were fasted for 24 h prior to the experiment. The experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care Use Committee of Chung-Ang University (IACUC 2017-00072).

Drugs & chemicals

ACh, prazosin, udenafil, NaHS, atropine, papaverine, U73122, DPCPX, chelerythrine, verapamil, and STZ were all purchased from Sigma (St. Louis, MO, USA). The composition of the Krebs buffer was as follows: 133 NaCl mM, 4.7 KCl mM, 2.5 $CaCl_2$, 1.35 mM NaH_2PO_4, 0.6 mM $MgSO_4$, 16.3 mM $NaHCO_3$, and 7.8 mM dextrose.

Induction of diabetes

STZ is often used in medical research to produce an animal model of hyperglycemia, as well as diabetes. Experimental diabetes was induced in overnight-fasted rats using a single dose (65 mg/kg) of STZ (Sigma). STZ was dissolved in normal saline and was administered intraperitoneally to the rats. The blood glucose levels of rats were checked two days after STZ administration using a CareSens II glucose meter from i-SENS (Seoul, Korea). Rats with blood glucose levels higher than 300 mg/dL were considered to have DM.

Organ bath experiment using bladder strips

Two weeks after STZ administration, rats were sacrificed using CO_2. The bladder was removed and placed in a Krebs solution. The bladder was then cut longitudinally into equal strips (2 mm×7 mm). A mixture of oxygen and CO_2 gas was supplied continuously to the strips. Transversely oriented muscle strips were taken from the rat bladder. The strips were then cut into 2-3 minor strips, and silk ligatures were tied at both ends. The muscle strips were then mounted in separate 1 mL muscle chambers. One wire was fixed to the bottom of the muscle chamber, whereas the other was attached to a force transducer (FT03, Grass Instruments Co., Quincy, MA, USA). Changes in isometric force were recorded on a polygraph (Model 79, Grass Instruments Co.). They were initially stretched using 1 g to bring them near to the conditions for optimal force development, and were equilibrated for over 60 mins while continuously being perfused with oxygenated Krebs solution.

Data analysis

Contraction response to ACh is expressed as percentage (%) versus control. Data are expressed as mean ± standard error of the mean (SEM). Statistical differences among the

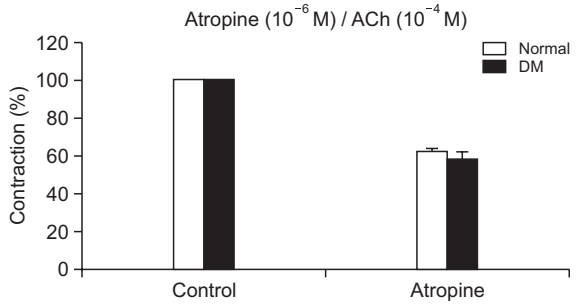

Fig. 2. Changes in contraction following atropine treatment of bladder smooth muscle from normal or DM rats. ACh-induced contraction. Atropine was added at 10^{-6} M. ACh was added at 10^{-4} M. Each point represents the mean ± SEM (n=6).

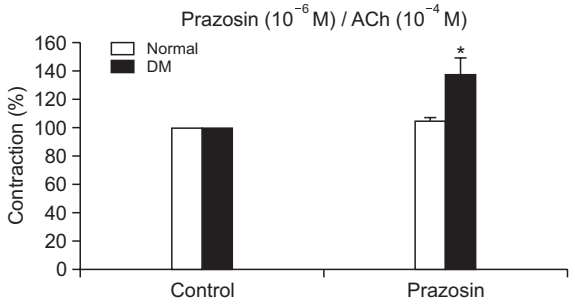

Fig. 3. Changes in contraction following prazosin treatment of bladder smooth muscle from normal or DM rats. ACh-induced contraction. Prazosin was added at 10^{-6} M. ACh was added at 10^{-4} M. Each point represents the mean ± SEM (n=6). *p<0.05 by Student's t-test.

groups were analyzed using a two-tailed Student's t-test and a two-way repeated measures ANOVA test. A p-value less than 0.05 was considered statistically significant.

RESULTS

Comparisons of tension in normal and diabetic rats

Representative tracings of tension in normal and diabetic rats are shown in Fig. 1A. The average contractility is shown in Fig. 1B where the contractility of the bladder is expressed in terms of change in tension (g). The contraction response was induced using an ACh (10^{-4} M). Student's t-test was used to compare the average contractility upon ACh stimulation. In DM rats, there was a significant decrease in ACh-induced contraction.

GPCR-mediated signaling on ACh-induced contraction in bladder smooth muscle

As shown in Fig. 2, atropine inhibited ACh-induced contraction of bladder smooth muscle in both normal and DM rats. Rat bladder smooth muscle was pretreated with atropine (10^{-6} M, a muscarinic receptor antagonist) for 15 min before the experiment. The percentage of contraction compared to the control was 62% in the normal group and 58% in the DM group. However, there was no significant difference between the two groups.

The effect of prazosin (10^{-6} M, an α_1 adrenoceptor antagonist) on the contraction of bladder smooth muscle is shown in Fig. 3. Rat bladder smooth muscle was pre-treated with prazosin for 15 min before the experiment and contractility was measured. The contraction response to ACh was higher in DM rats than the contraction in normal rats, and these differences were statistically significant.

As shown in Fig. 4, there were no differences in ACh groups when the muscle strips were treated with DPCPX (10^{-6} M, an adenosine A_1 receptor antagonist). Rat bladder smooth muscle was pre-treated with DPCPX for 30 min before the experiment. In addition, there were no differences between the two groups. Experiments examining the role of GPCR-mediated signaling pathways showed that the effect of α_1 adrenoceptor on contraction responses was significantly different between normal and DM rats.

The signals of PLC on ACh-induced contraction in bladder smooth muscle

The effect of verapamil (10^{-6} M, a calcium channel blocker) on bladder smooth muscle contraction is shown in Fig. 5A. Rat bladder smooth muscle was pre-treated with verapamil for 5 min before the experiment. The contraction response was significantly decreased in response to Ach in normal rats more than that in DM rats. The effect of U73122 (10^{-6} M, a PLC inhibitor) on bladder smooth muscle contraction is shown in Fig. 5B. Rat bladder smooth muscle was pre-treated with U73122 for 20 min before the experiment. For ACh-induced contraction, there was only a small change in contraction in both groups compared to the control and these differences were not statistically significant. As shown in Fig. 5C, there were no differences in contraction when the controls were compared with ACh groups when chelerythrine (10^{-6} M, a PKC inhibitor) was used to treat the muscle strips. Rat bladder smooth muscle was pre-treated with chelerythrine for 20 min before the experiment.

In these experiments examining intracellular signaling pathways, modulators of calcium channels, PLC had significantly different effects on bladder smooth muscle contraction responses between normal and DM rats.

Effect of Udenafil, NaHS, and papaverine on ACh-induced contraction in bladder smooth muscle

The effect of udenafil (10^{-6} M, a PDE5 inhibitor) on bladder smooth muscle contraction is shown in Fig. 6A. Rat bladder smooth muscle was pre-treated with udenafil for 15 min before the experiment. For the ACh-induced contraction, the DM rats also had a higher percent contraction than normal rats. As shown in Fig. 6B, there were no differences in contraction between normal and DM rats for ACh-induced contraction when NaHS (10^{-6} M, a smooth muscle relaxant) was used to treat the muscle strips. Rat bladder smooth muscle was pre-treated with NaHS for 15 min before the experiment. In addition, there were no significant differences between the controls in each group.

The effect of papaverine (10^{-6} M, a smooth muscle relaxant) on bladder smooth muscle contraction is shown in Fig 6C. Rat bladder smooth muscle was pre-treated with papaverine for 15 min before the experiment. Papaverine had a smooth muscle relaxing effect on ACh-induced contraction in both normal and DM rats. For ACh-induced contraction, the inhibition

of contraction was greater in normal rats than in DM rats.

DISCUSSION

Bladder dysfunction is one of the main complications in DM (Golbidi and Laher, 2010). A decrease in detrusor contractility is a common symptom in bladder dysfunction (Bradley, 1980). Many causes can influence bladder smooth muscle contraction. The present study was conducted to investigate which receptor or enzymes affect the contraction of bladder smooth muscle in a rat DM model.

To examine the role of GPCRs in bladder contraction in DM, atropine, prazosin, and DPCPX were used. It is well-known that α_1-adrenergic receptors, especially the α_{1A} subtype are present in rat bladder. Agonists that act on the α_1-adrenoceptor are known to enhance the release of ACh in the isolated rat bladder. It has also been reported that α_1-adrenoceptor agonists produced contractions that are approximately 10-43% of those achieved using muscarinic stimulation (Michel and Vrydag, 2006). In addition, an enhanced adrenoceptor receptor response has been observed in the DM rat bladder (Kudlacz *et al*., 1989). These results suggested that α_1-adrenoceptors may have a role in bladder contraction in DM.

Adenosine A_1 receptors are known to be expressed in the uroepithelium of the bladder (Yu *et al*., 2006). DPCPX (8-cyclopentyl-1, 3-dipropylxanthine) is a drug that acts as a selective antagonist of the adenosine A_1 receptor (Lohse *et al*., 1987; Martinson *et al*., 1987). It has been reported that adenosine A_1 receptors can activate PLC, resulting in an increase in cytoplasmic Ca^{2+} concentrations, and that they can also inhibit adenylyl cyclase (Schulte and Fredholm, 2003; Bucheimer and Linden, 2004). ATP can also induce contraction through activation of P2X purinergic receptors (Inoue and Brading, 1990). Adenosine-induced relaxation is primarily thought to occur through P1A1 receptors because an antagonist of the P1A1 receptor (DPCPX) has been shown to decrease adenosine-induced relaxation in a normal rat bladder (Vesela *et al*., 2011). In this study, prazosin had a significantly different effect on contraction between normal and DM rats.

Udenafil (PDE5 inhibitor), which is used for the erectile dysfunction treatment. Interestingly, the mRNA encoding PDE5 has been shown to be expressed in the rat bladder (Zhu *et al*., 2017). It has also been suggested that treatment with vardenafil, another PDE5 inhibitor, increases the bladder smooth muscle contraction. This effect is thought to be mediated through changes in cGMP that lead to increases in intracellular calcium levels, thus increasing bladder contraction (Rybalkin *et al*., 2003). This study also suggests that the cGMP pathway might be involved in the control of relaxation of bladder smooth muscle in DM.

It has been reported that the density of muscarinic receptors in the rat bladder increases during the early stages of DM (Tong *et al*., 1999). It has also been reported that M_3 muscarinic receptors cause bladder contraction in a PLC-independent mechanism (Sand and Michel, 2014). The main pathway for muscarinic stimulation of contraction of bladder smooth muscle involves the activation of PLC, which leads to the generation of inositol-1, 4, 5-trisphosphate (IP_3) (Andersson and Arner, 2004). IP_3 induces Ca^{2+} release and leads to bladder contraction (Somlyo and Somlyo, 2003). Many studies have observed alterations in calcium channel sensitivity and calcium concentration in DM rats. In particular it has been shown that intracellular calcium levels are elevated in animal models of DM (Belis *et al*., 1991; Levy *et al*., 1994; Waring and Wendt, 2000).

Papaverine is an anti-spasmolytic drug that has long been known to have a smooth muscle relaxing effect (Ferrari, 1974). It has been reported that papaverine relaxes smooth muscle by reducing the activity of cyclic nucleotide PDEs, as well as reducing calcium transport (Huddart *et al*., 1984; Diederichs, 1991). Our data demonstrated that the percentage decrease in bladder contraction was larger in normal rats treated with

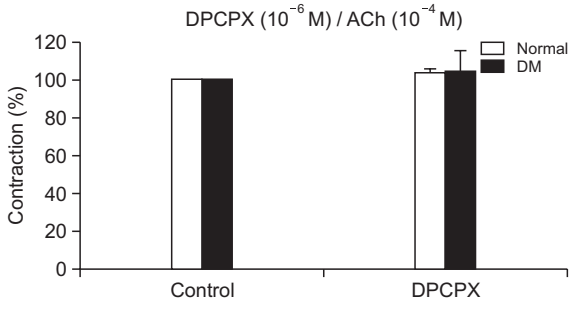

Fig. 4. Changes in contraction following DPCPX treatment of bladder smooth muscle from normal or DM rats. ACh-induced contraction. DPCPX was added at 10^{-6} M. ACh was added at 10^{-4} M. Each point represents the mean ± SEM (n=6).

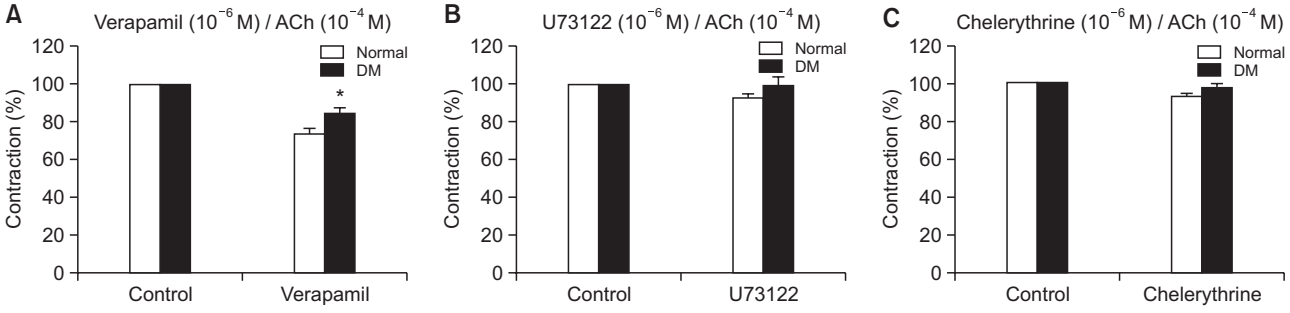

Fig. 5. Changes in contraction following verapamil treatment of bladder smooth muscle from normal or DM rats. ACh-induced contraction. (A) Verapamil, (B) U73122, (C) Chelerythrine was added at 10^{-6} M. ACh was added at 10^{-4} M. Each point represents the mean ± SEM (n=6). **p*<0.05 by Student's *t*-test.

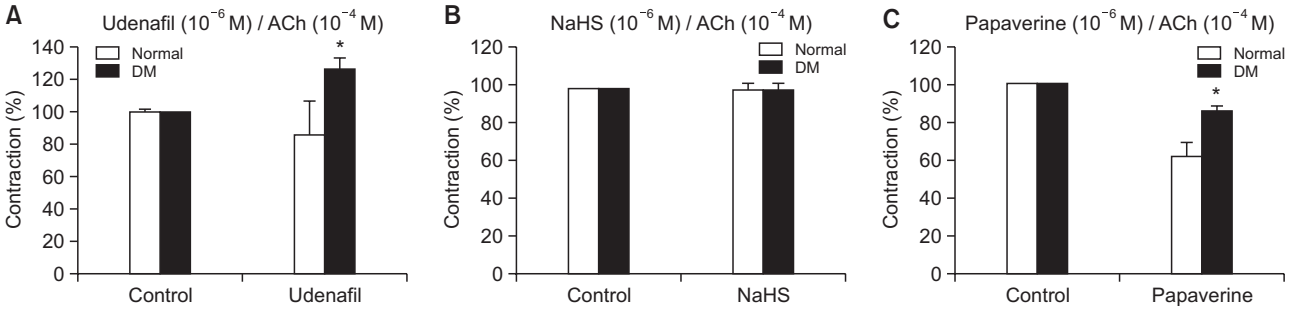

Fig. 6. Changes in contraction following udenafil treatment of bladder smooth muscle from normal or DM rats. ACh-induced contraction. (A) Udenafil, (B) NaHS, (C) Papaverine was added at 10^{-6} M. ACh was added at 10^{-4} M. Each point represents the mean ± SEM (n=6). **p*<0.05 by Student's *t*-test.

papaverine than in DM rats. NaHS also induces the relaxation of bladder smooth muscle, and it is known that H_2S synthetic enzymes are expressed in the bladder. However, we found no significant differences in smooth muscle bladder contraction between normal and DM rats following NaHS treatment.

Several studies have investigated the effect of DM on contraction in cardiac muscle and colonic smooth muscle. It has been reported that the cholinergic response and the contractility of colonic smooth muscle were reduced in DM rats. The contractile response of the proximal colon to carbachol, an acetylcholine receptor agonist, was also found to be significantly weaker in diabetic rats. (Kim *et al*., 2011). Another study in DM using female GK rats demonstrated that these rats had a reduced contraction of the left ventricular muscle (Iltis *et al*., 2005).

Our study has several limitations. First, only male rats were used. Male rats have organs that do not exist in female rats, such as the penis and the prostate. These male-specific organs may have effects on bladder smooth muscle contraction that we did not take into account. Second, the effect of age was not studied because all of the rats used here were of a similar age (10 weeks). In this regard, it has been reported that the contractility of rats changes over time (Daneshgari *et al*., 2006). Third, a hypercontractile state was also observed in DM rats. There might be various other factors that affect the contraction of bladder smooth muscle. Accordingly, additional experiments will be required to address these limitations.

In conclusion, the results of the present study show that the bladder contractility is decreased in DM rats. While atropine has no inhibitory effect in DM rats, the responses to a PLC inhibitor, a calcium channel blocker, and a PDE5 inhibitor were significantly different between normal and DM rats. Therefore, the change of intracellular Ca^{2+} release mediated by PLC/IP3 and PDE5 activity are responsible for decreased bladder smooth muscle contractility.

CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

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