

Effects of Diminazene Aceturate on Isolated Rat Bladder Tissue: Role of Nitric Oxide and Cyclooxygenase Pathways

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İzole Sıçan Mesane Dokusunda Diminazen Aseturatın Etkileri: Nitrik Oksit ve Siklooksijenaz Yolaklarının Rolü

SUMMARY

Diminazene aceturate (DIZE) is a compound reported to activate ACE2. A local renin-angiotensin system, including ACE2 and Mas receptors, has been demonstrated in bladder tissue. This study aimed to investigate the effects of DIZE on isolated rat bladder tissue and to evaluate the potential involvement of nitric oxide (NO) and cyclooxygenase (COX) pathways. Isolated rat bladder strips were mounted in organ baths. The effects of cumulative DIZE administration were examined on basal tone and on carbachol-precontracted tissues. Neurogenic contractions were assessed using electrical field stimulation (EFS) after incubation with vehicle or DIZE, and under DIZE conditions in the presence of L-NAME (NOS inhibitor) or flurbiprofen (COX inhibitor). ACE2 and Ang (1-7) levels were quantified in bladder homogenates by ELISA. In carbachol-precontracted strips, DIZE induced a concentration-dependent relaxation. This relaxant response was not modified by L-NAME or flurbiprofen. In EFS experiments, DIZE attenuated frequency-dependent neurogenic contractions, with more pronounced inhibition at higher frequencies. Under DIZE incubation, L-NAME partially reversed the suppression of high-frequency EFS responses, whereas flurbiprofen did not produce a comparable reversal. In ELISA analyses, ACE2 and Ang (1-7) tissue levels were not significantly altered by DIZE under the conditions tested. DIZE modulates rat bladder contractility by relaxing muscarinic tone and dampening neurogenic contractions. The direct relaxant effect appears largely independent of NOS and COX pathways, whereas NO may contribute to DIZE's modulation of high-frequency neurogenic responses. The lack of major ELISA changes suggests stable, steady-state ACE2/Ang (1-7) levels in the healthy bladder, warranting further studies.

Keywords: Diminazene aceturate, ACE2, bladder, nitric oxide, prostaglandin.

ÖZ

Diminazen aseturat (DIZE), ADE2'yi aktive ettiği bildirilen bir bileşiktir. Mesane dokusunda ACE2 ve Mas reseptörlerini içeren lokal bir renin-angiyotensin sisteminin varlığı gösterilmiştir. Bu çalışmanın amacı, DIZE'nin izole sıçan mesane dokusu üzerindeki etkilerini araştırmak ve nitrik oksit (NO) ile siklooksijenaz (COX) yolaklarının olası katkısını değerlendirmektir. İzole sıçan mesane şeritleri organ banyolarına asılmıştır. Kümülatif DIZE uygulamasının bazal tonus ve karbakol ile önceden kastırılmış dokular üzerindeki etkileri incelenmiştir. Nörojenik kasılmalar, vehikül veya DIZE inkübasyonu sonrasında ve ayrıca DIZE varlığında L-NAME (NOS inhibitörü) ya da flurbiprofen (COX inhibitörü) eşliğinde elektriksel alan uyarımı (EFS) kullanılarak değerlendirilmiştir. Mesane homojenatlarında ADE2 ve Ang (1-7) düzeyleri ELISA yöntemiyle ölçülmüştür. Karbakol ile önceden kastırılmış şeritlerde DIZE, konsantrasyona bağlı gevşeme oluşturmıştır. Bu gevşetici yanıt, L-NAME veya flurbiprofen ile anlamlı olarak değişmemiştir. EFS deneylerinde DIZE, frekansa bağlı nörojenik kasılmaları azaltmış; bu inhibisyon özellikle yüksek frekanslarda daha belirgin olmuştur. DIZE inkübasyonunda L-NAME, yüksek frekanslı EFS yanıtlarındaki baskılanmayı kısmen geri çevirmiştir; bu durum özellikle alan ölçümünde belirgin bulunmuştur. Buna karşılık flurbiprofen benzer bir geri çevirici etki göstermemiştir. ELISA analizlerinde, test edilen koşullar altında DIZE'nin doku ADE2 ve Ang (1-7) düzeylerini anlamlı olarak değiştirmediği saptanmıştır. DIZE, sıçan mesanesinde muskarinik tonusu gevşeterek ve nörojenik kasılmaları baskılayarak kontraktiletiyi modüle etmektedir. Doğrudan gevşetici etkinin büyük ölçüde NOS ve COX yolaklarından bağımsız olduğu, buna karşın NO'nun DIZE'nin yüksek frekanslı nörojenik yanıtlar üzerindeki etkisine katkıda bulunabileceği düşünülmektedir. ELISA'da belirgin değişiklik saptanmaması, sağlıklı mesanede ADE2/Ang (1-7) düzeylerinin kararlı durumda kaldığını düşündürmektedir. Mekanizmaların daha ayrıntılı olarak ortaya konabilmesi için ileri in vivo modellerde yapılacak çalışmalara gereksinim vardır.

Anahtar Kelimeler: Diminazen aseturat, ADE2, mesane, nitrik oksit, prostaglandin.

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INTRODUCTION

The renin-angiotensin system (RAS) is a widely distributed homeostatic system that regulates various physiological processes, including sodium balance and blood pressure. It comprises angiotensin peptides (e.g., angiotensin II [Ang II], angiotensin 1–7 [Ang (1–7)]), enzymes (e.g., angiotensin-converting enzyme [ACE], angiotensin-converting enzyme-2 [ACE2]), and receptors (e.g., AT1, AT2, Mas). ACE2 converts Ang II to Ang (1–7), which activates Mas receptors and counteracts AT1-mediated actions (Cantero-Navarro et al., 2021). A local RAS has been demonstrated in human and rat bladder tissue, including ACE2 and Mas receptor expression, particularly in bladder epithelial cells (Lamy et al., 2022). Notably, ACE2 gene expression in the human bladder is reported to be nearly as high as in lung tissue (Al-Benna, 2021), supporting the bladder as a potential target for pharmacological modulation of the ACE2/Ang (1–7)/Mas axis in genitourinary diseases. Ang (1–7) and ACE2 have been linked to nitric oxide (NO) signaling by influencing nitric oxide synthase (NOS) expression and NO release (Rabelo et al., 2016; Vassiliou et al., 2021). In addition, prostaglandins have been discussed in relation to ACE2 regulation during COVID-19 (Robb et al., 2020), and COX-derived prostaglandins have been reported to mediate antihypertensive actions of Ang (1–7) (Iyer et al., 2000). The role of the ACE2/Ang (1–7) axis and the interaction of NO and prostanoids in bladder function remains insufficiently understood. Diminazene aceturate (DIZE) is a veterinary antiparasitic drug approved for trypanosomiasis and babesiosis (Peregrine & Mamman, 1993). Beyond antiparasitic activity, DIZE modulates proinflammatory cytokines, suggesting potential utility in inflammatory conditions (Kuriakose & Uzonna, 2014; Passaglia et al., 2024). Following its identification as an ACE2 activator, DIZE has been extensively studied in experimental disease models, with many reports describing beneficial cardiovascular effects attributed to ACE2 activation

(Coutinho et al., 2022; Velkoska et al., 2016). However, some studies suggest ACE2-independent actions, indicating alternative mechanisms may contribute (Esfahani et al., 2022). In isolated organ bath studies, DIZE produces concentration-dependent relaxation in rat aorta and rabbit arteries, with reported involvement of Mas receptors and NO signaling (De Maria et al., 2015; Matsoukas et al., 2022). However, the precise mechanism of DIZE's relaxant effects remains unclear, and its relationship with ACE2 and Ang (1–7) in these effects has yet to be fully understood. Given the presence of a local bladder RAS and the established roles of NO and prostanoids in urinary physiology, investigating how DIZE affects bladder contractility and neurogenic responses may provide mechanistic insights relevant to genitourinary disorders. Therefore, this study aimed to evaluate the effects of the ACE2 activator DIZE in rat bladder tissue and to determine the contribution of NO synthase and cyclooxygenase pathways. In addition, ACE2 and Ang (1–7) levels were assessed to support mechanistic interpretation and to inform future disease-model and *in vivo* studies.

MATERIALS AND METHODS

Animals

In this study, 14 adult male Wistar albino rats (250–300 g) were used. The animals were maintained in controlled, well-ventilated rooms with a 12-hour light/dark cycle and were provided *ad libitum* access to standard laboratory chow and tap water. All animal procedures were carried out in accordance with the European Community Directive (86/609 ECC) regarding the care and use of laboratory animals. This study was approved by Nesa Experimental Animals Laboratory Local Ethics Committee (05.12.2024/058).

Chemicals

Carbachol was obtained from Macklin (Shanghai, China). Diminazene aceturate, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), and flurbiprofen were obtained from Cayman Chemical

(Michigan, USA). Physiological saline was used as the solvent for DIZE (Macedo et al., 2015). The composition of the Krebs-Henseleit solution is (mM) NaCl 118; KCl 4.7; NaHCO_3 25; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.9; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.26; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5; and glucose monohydrate 11. The Krebs-Henseleit solution was prepared fresh daily based on our previous studies (Okcay et al., 2024).

Tissue preparation and isolated organ bath protocol

The animals were anesthetized using 90 mg/kg ketamine and 10 mg/kg xylazine, followed by euthanasia through cervical dislocation. The bladders were rapidly excised and immersed in Krebs-Henseleit solution. The adipose and connective tissues around the bladder were carefully dissected, and the bladder was cut into 6 equal strips, which were then suspended in 10 ml organ baths containing Krebs-Henseleit solution, continuously aerated with a 95% O_2 and 5% CO_2 mixture at 37 °C. Tissue responses were obtained and recorded isometrically using an isometric force transducer (FDT10-A, May IOBS 99; COMMAT Communication Co.) connected to a computer via a data acquisition system (MP36; BIOPAC Systems Inc., Santa Barbara, CA, USA) with software (BSL PRO v 3.6.7; BIOPAC Systems Inc.).

Experimental protocols

At the onset of the experiment, the bladder strips were subjected to an initial tension of 1 g, followed by a 1-hour equilibration period during which the tissues were rinsed with fresh Krebs-Henseleit solution every 15 minutes. Tissue viability was assessed by the administration of 80-mM KCl-containing Krebs solution.

The effects of diminazene aceturate on bladder strips

Assessment of constriction effect: Following the assessment of tissue viability, the tissues were rinsed with Krebs-Henseleit solution every 15 minutes for further equilibration, and cumulative concentrations of DIZE (10^{-9} M – 3×10^{-5} M) were administered to investigate the constricting effects of DIZE (n = 3).

Assessment of relaxation effect: After testing tissue viability, the tissues were rinsed with Krebs-Henseleit solution every 15 minutes for further equilibration and then contracted with carbachol (10^{-6} M). Once contraction responses reached a plateau, cumulative concentrations of DIZE (10^{-9} M – 3×10^{-4} M) were applied, and concentration-response curves were obtained. The tissues were then rested for 1 hour, rinsed every 15 minutes, and contracted again with carbachol. Then, the cumulative DIZE administration was repeated in the presence and absence (n=5) of antagonists (nitric oxide synthase inhibitor (NOS) L-NAME (10^{-4} M) (n = 5) and cyclooxygenase (COX) inhibitor flurbiprofen (10^{-6} M) (n = 5). The effect of antagonists was investigated by comparing DIZE relaxation alone and in the presence of an antagonist in the same tissue. Additionally, control experiments were conducted using vehicle (n = 5), and spontaneous basal tonus reduction (n = 4) was also tested with carbachol precontraction. The relaxation responses were expressed as a percentage of the carbachol-induced contraction.

The effects of diminazene aceturate on electrical field stimulation-induced contractions

After testing tissue viability, the tissues were rinsed with Krebs-Henseleit solution every 15 minutes for further equilibration. Subsequently, the tissues were incubated for 20 minutes with either DIZE alone (10^{-5} M) (n=6) or in combination with L-NAME (10^{-4} M) (n = 6) or flurbiprofen (10^{-6} M) (n = 6). Electrical Field Stimulation (EFS) was then administered to the tissues via platinum electrodes, with 10-second pulses of 60 V, 1 ms at frequencies of 1, 2, 4, 8, 16, and 32 Hz, administered every 2 minutes, to obtain frequency-response curves (İlhan et al., 2022). The same protocol was repeated for control tissues treated with the vehicle. The concentrations of the substances used were determined based on similar studies in the literature. The contractile responses were given as the percentage of 80 mM KCl contraction.

Investigation of ACE2 and Ang (1-7) levels on rat bladder tissue homogenates

After euthanasia, bladders were rapidly excised, rinsed in physiological saline, snap-frozen on dry ice, and stored at -80°C . On the day of analysis, ~ 0.1 g tissue was homogenized in 0.9 mL 140 mM KCl buffer (Otto Scientific, OttoC1983) with zirconium beads (Allsheng; 3200 rpm, 30 s x 2 cycles with a 45-s interval) and centrifuged at 4°C for 5 min at 7000 rpm. The clarified supernatant was collected; all steps were performed on ice, and freeze-thaw cycles were avoided. Homogenates were incubated with vehicle (saline) or DIZE (10^{-5} M), with or without L-NAME (10^{-4} M) and flurbiprofen (10^{-6} M), prior to ELISA. ACE2 (Elabsience, E-EL-R2453) and Ang-(1-7) (Elabsience, E-EL-R1138) levels were measured according to the manufacturer's instructions and read on an ELISA plate reader (BioTek ELX800). Kit sensitivities were 0.19 ng/mL (ACE2) and 9.38 pg/mL (Ang-(1-7)).

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism 8.02. Normality was assessed by the Shapiro-Wilk test. Concentration-response curves were fitted by nonlinear regression (log(agonist) vs. normalized response, variable slope). Group differences across concentrations and frequencies were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ELISA data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons.

Data are presented as mean $\pm$ S.E.M., and $p < 0.05$ was considered significant.

RESULTS

Effects of diminazene aceturate on isolated bladder tissue

The contraction experiments revealed that DIZE did not elicit any detectable contractile response in the bladder tissue (data not shown). The relaxation experiments demonstrated that DIZE induced relaxation in bladder tissues precontracted with carbachol by 56.37 ± 7.29 maximally. The relaxation effect of DIZE significantly differed from the vehicle group at the concentrations of 10^{-4} ($**p < 0.01$) and 3×10^{-4} M ($***p < 0.001$) ($n=5$) (Figure 1.). The second administration of DIZE and vehicle to the same tissue revealed similar relaxation responses. The semi-logarithmic concentration-response relationship of DIZE was analyzed in rat bladder strips ($n=16$) that received DIZE at the first administration. Nonlinear regression analysis determined that the logarithm of the half-maximal effective concentration (logEC50) was -3.742 , corresponding to an EC50 of 0.0001811 M. The Hill slope, which describes the steepness of the curve, was calculated as 0.4280. The maximal relaxation response achieved by DIZE was $58.32 \pm 4.37\%$. Original traces of cumulative DIZE administration on isolated rat bladder precontracted with carbachol are given in Figure 6A.

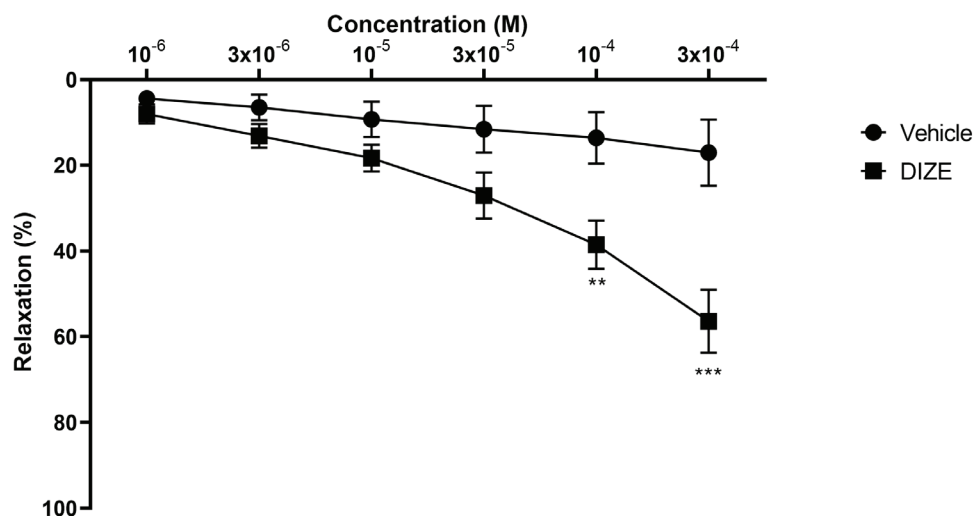


Figure 1. Relaxation responses to diminazene aceturate and its vehicle (physiological saline) in isolated rat bladder tissues precontracted with carbachol (10^{-6} M)

Responses were given as the percentage of carbachol contraction. Two-way ANOVA followed by Bonferroni's multiple comparison test was performed. ** $p < 0.01$, *** $p < 0.001$; significant difference between groups. DIZE: diminazene aceturate (n = 5).

Contribution of L-NAME and flurbiprofen on the relaxation effect of DIZE on isolated rat bladder tissue

The effects of each concentration of DIZE were compared in the presence of antagonists, and the mean responses of all DIZE concentrations were also evaluated. Figure 2 shows the effects of L-NAME (A) and flurbiprofen (B) on DIZE-induced concentration-dependent relaxation in isolated rat bladder tissues. Incubation with L-NAME or flurbiprofen

did not significantly alter the relaxation response. The pD₂ of cumulative DIZE administration before and after L-NAME and flurbiprofen incubation were presented in Table 1. There was a slight decrease in potency after L-NAME incubation, as indicated by a lower pD₂ value and a higher EC₅₀. In contrast, after flurbiprofen incubation, DIZE's potency tended to increase, with a higher pD₂ value and a lower EC₅₀ compared to the pre-incubation values. However, none of the differences were statistically significant as compared in the nonlinear regression analysis.

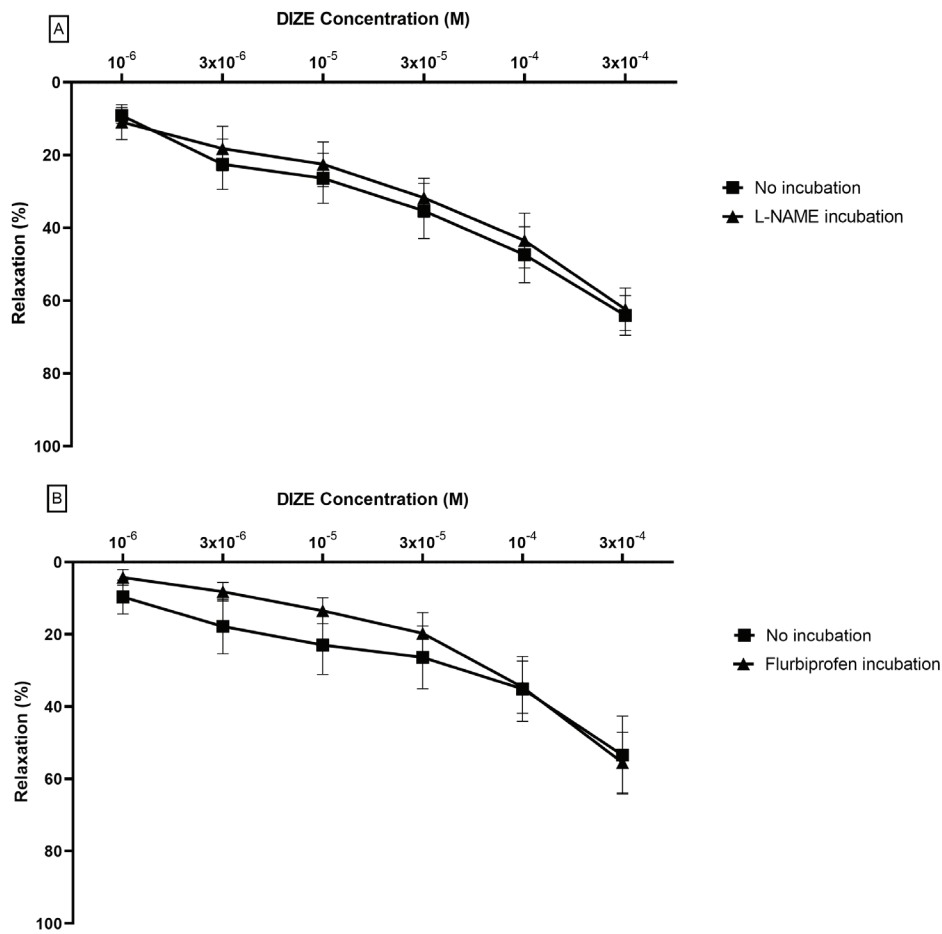


Figure 2. Relaxation responses to diminazene aceturate in the presence of L-NAME (A), and flurbiprofen (B) in isolated rat bladder tissues precontracted with carbachol (10^{-6} M)

Responses were given as the percentage of carbachol contraction. Two-way ANOVA followed by Bonferoni's multiple comparison test was performed. * $P < 0.01$; significant difference compared to the group without L-NAME incubation. DIZE: diminazene aceturate, FLUR: flurbiprofen. Values expressed as $\pm$ S.E.M. ($n = 5$).

Table 1. The pD2 (-logEC50) values of cumulative DIZE administration in isolated rat bladder strips precontracted with carbachol (10^{-6} M) in the presence and absence of antagonists

Cumulative DIZE relaxation response		pD2 (-logEC50)
L-NAME	-	3.993 ± 0.1402
	+	3.865 ± 0.1367
Flurbiprofen	-	3.498 ± 0.2904
	+	3.606 ± 0.1168

Nonlinear regression analysis with log(agonist) vs. normalized response model - variable slope was performed ($n = 5$).

Effects of DIZE on the EFS-induced contractile responses in isolated rat bladder tissue

Incubation with DIZE reduced frequency-dependent EFS-evoked contractions compared with vehicle (Figure 3.). In terms of peak-to-peak amplitude (Figure 3A.), contractile responses increased with stimulation frequency in both groups; however, DIZE produced a modest suppression of contractions that became most evident at the highest frequency. At 32 Hz, peak-to-peak amplitude was significantly lower in the DIZE group than in the vehicle group ($p < 0.05$). Analysis of the contraction area (Figure 3B.) showed a similar frequency-dependent increase in both groups, but DIZE caused a clearer inhibition at higher frequencies. The contraction area was significantly reduced by DIZE at 16 Hz ($p < 0.05$) and was further decreased at 32 Hz ($p < 0.01$) compared with the vehicle.

To evaluate the contribution of NO and COX pathways under DIZE conditions, contractile responses obtained with DIZE alone were compared with those obtained in the presence of L-NAME or flurbiprofen. For peak-to-peak amplitude (Figure 4A.), co-incubation with L-NAME or flurbiprofen did not cause significant differences compared to DIZE incubation alone. For contraction area (Figure 4B.), L-NAME co-incubation significantly increased the response at 32 Hz compared with DIZE alone ($p < 0.05$), suggesting that NOS inhibition partially reverses the DIZE-associated suppression of neurogenic contractile activity at high-frequency stimulation. In contrast, flurbiprofen co-incubation did not significantly alter contraction area relative to DIZE alone at the tested frequencies. Original traces of EFS-induced contractions on isolated rat bladder are given in Figure 6B.

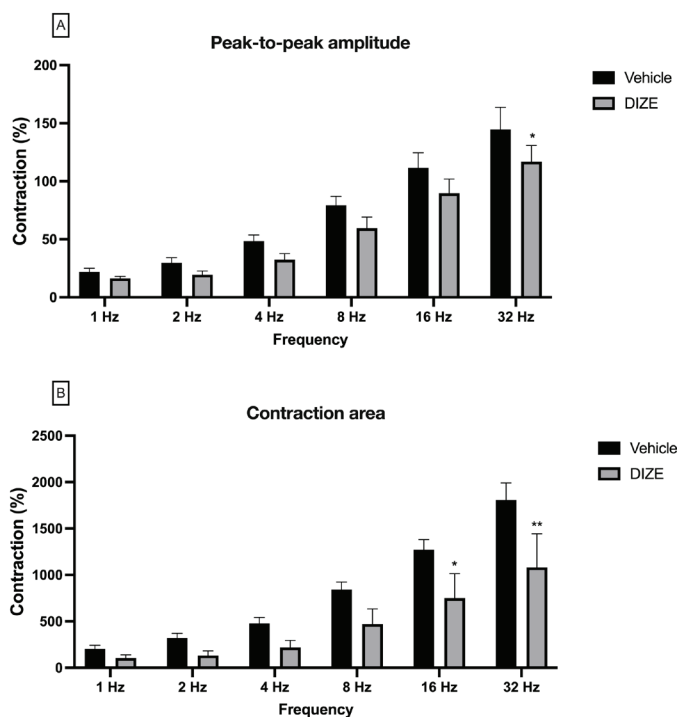


Figure 3. Effects of vehicle and DIZE incubation on peak-to-peak amplitude (A) and contraction area values (B) of frequency-induced contractions in isolated rat bladder

Responses were given as the percentage of KCl contraction. Two-way ANOVA followed by Bonferroni's multiple comparison test was performed. $*p < 0.05$, $**p < 0.01$; significant difference compared to vehicle incubated tissues. DIZE: diminazene aceturate. Values expressed as $\pm$ S.E.M. (n = 6).

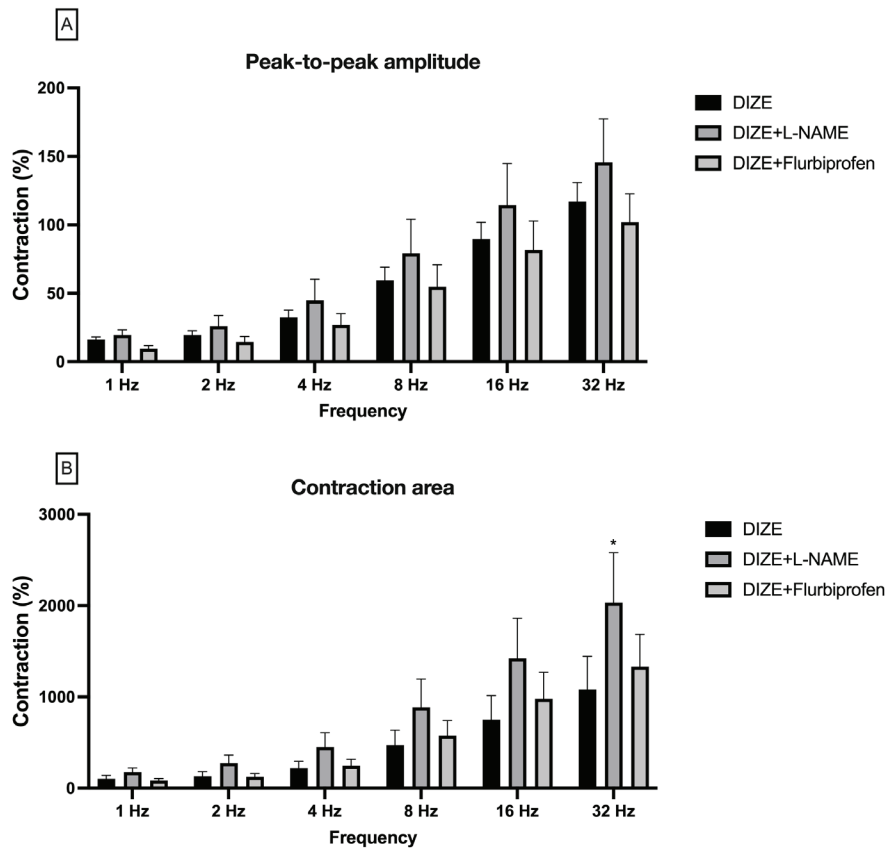


Figure 4. Effects of DIZE, DIZE+L-NAME, and DIZE+Flurbiprofen incubation on peak-to-peak amplitude (A) and contraction area values (B) of frequency-induced contractions in isolated rat bladder

Responses were given as the percentage of KCl contraction. Two-way ANOVA followed by Bonferroni's multiple comparison test was performed. * $p < 0.05$; significant difference compared to DIZE incubated tissues. DIZE: diminazene acetate. Values expressed as $\pm$ S.E.M. ($n = 6$).

Effects of DIZE on ACE2 and Ang (1-7) levels in rat bladder tissue homogenates with and without L-NAME and flurbiprofen incubation

In rat bladder homogenates, ACE2 concentrations were comparable between the vehicle and DIZE groups (Figure 5A.), indicating no marked change in ACE2 levels following DIZE incubation under the conditions tested. When DIZE was co-incubated with inhibitors, ACE2 levels showed a numerical decrease in the L-NAME + DIZE group and a further down-

ward trend in the flurbiprofen + DIZE group relative to DIZE alone, none of which reached statistical significance. Similarly, Ang (1-7) concentrations were broadly similar between vehicle and DIZE (Figure 5B.). Co-incubation with L-NAME + DIZE produced a modest reduction in Ang (1-7) compared with DIZE alone, whereas the effect of flurbiprofen + DIZE was comparable to DIZE alone. No-incubation and antagonist-only (without DIZE) conditions were also tested for ACE2 and Ang (1-7); no significant differences were detected (data not shown).

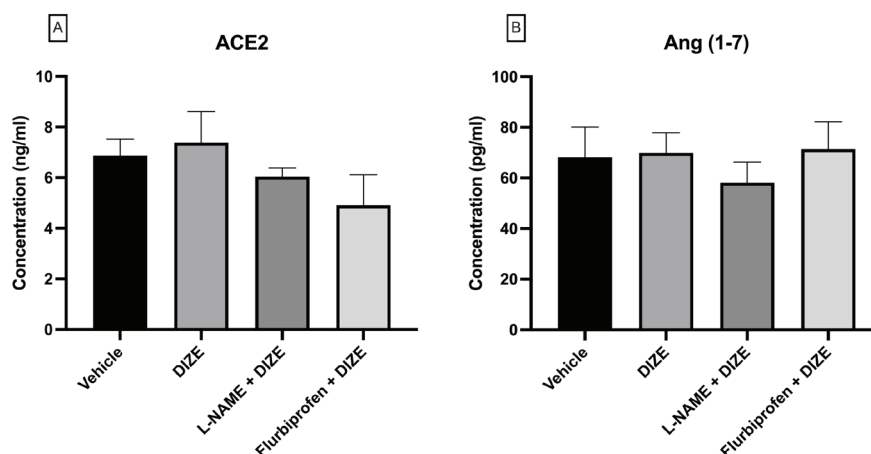


Figure 5. Effects of DIZE alone or combined with L-NAME or flurbiprofen on ACE2 and Ang-(1-7) levels in rat bladder homogenates

Kruskal-Wallis, followed by Dunn's multiple comparison test, was performed. DIZE: diminazene aceturate. Values expressed as $\pm$ S.E.M. (n = 5-6).

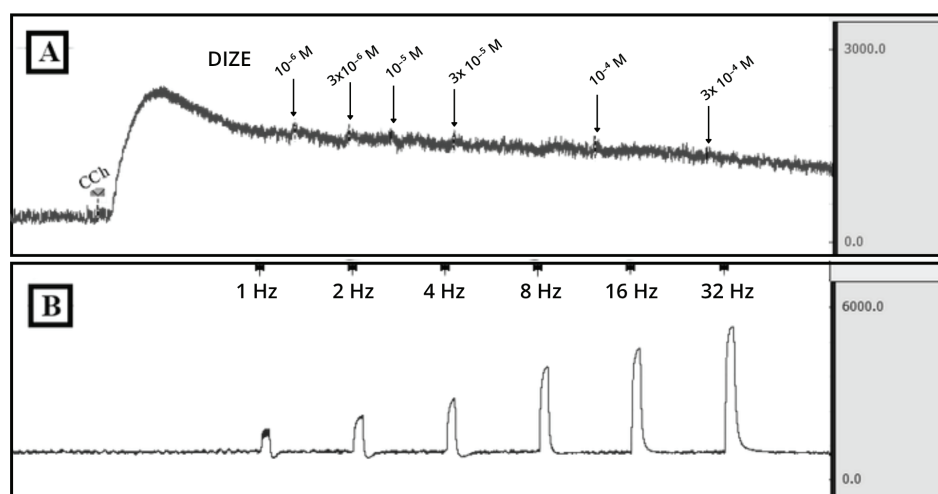


Figure 6. Representative original traces from isolated rat bladder strips. (A) Relaxation responses to cumulative concentrations of DIZE in precontracted tissues. (B) Typical contractile responses induced by electrical field stimulation (EFS)

Concentrations were given as M (A) and EFS was given in 1-32 Hz. CCh: carbachol.

DISCUSSION

This study is the first to investigate the effects of DIZE on isolated rat bladder tissue. In this study, cumulative DIZE administration had no observable constricting effect on the basal tone of the bladder, suggesting that it does not interact with receptors involved in bladder contraction. In tissues precon-

tracted with muscarinic agonist carbachol, DIZE induced a concentration-dependent relaxation, which was significantly different than vehicle, with a more pronounced effect observed at higher concentrations. The Hill slope indicated a gradual loss of basal tension, suggesting a partial DIZE-induced relaxation, possibly due to incomplete activation of relaxant pathways.

Incubations with L-NAME and flurbiprofen did not result in significant differences in the concentration-response of DIZE. The ACE2/Ang-(1-7)/Mas axis interacts closely with NO signaling in vascular tissues, but this relationship is bidirectional and context dependent: ACE2 activation can increase eNOS phosphorylation/NO production, whereas ACE2 overexpression or deficiency has also been reported to alter NOS pathways in opposite directions (Jin et al., 2012; J. Kuriakose et al., 2021; Rabelo et al., 2016). Sustained NOS inhibition with L-NAME may trigger compensatory upregulation of the ACE2/Ang (1-7) axis (Inaba et al., 2011), potentially limiting oxidative stress and inflammation when NO is reduced (Tsai et al., 2021). Despite this complex crosstalk, in our experiments L-NAME did not modify DIZE-induced relaxation, suggesting that NO is not a major mediator of the direct smooth-muscle relaxant response in rat bladder, consistent with reports that NO may play a limited role in bladder relaxation (Fujiwara et al., 2000).

EFS experiments further extended these findings by showing that DIZE also modulates neurogenic bladder activity. While frequency-dependent contractions increased progressively in both groups, incubation with DIZE attenuated the EFS-evoked responses compared to vehicle, with the inhibitory effect becoming most evident at higher stimulation frequencies. This pattern suggests that DIZE does not merely reduce muscarinic tone but can also dampen stimulus-driven contractility under conditions that more closely reflect nerve-mediated bladder activation. Importantly, the reduction was clearer when responses were assessed as integrated contractile activity rather than peak-to-peak amplitude, indicating that DIZE may influence not only the maximal force of each twitch but also the overall contractile response during high-frequency stimulation. While L-NAME did not modify DIZE-induced relaxation in carbachol-precontracted strips, it did partly reverse the DIZE-associated suppression of contractions during EFS at higher frequencies, suggesting that NO contributes

mainly to DIZE's modulation of nerve-evoked activity rather than to its muscarinic relaxant effect. COX inhibition with flurbiprofen did not produce a comparable reversal in EFS responses, implying a limited role for prostanoid signaling in the DIZE effect under the stimulation parameters used. NO has been suggested to regulate bladder afferent firing, and its inhibition may trigger unstable bladder contractions and increased overactivity (Andersson & Persson, 1994). Consistent with this, in a bladder outlet obstruction model, L-NAME increased contractile responses to EFS, suggesting that NO inhibition heightens bladder excitability and contractility (Pereira et al., 2017). However, another study found that L-NAME did not affect detrusor strip contractions at 20 Hz (Persson et al., 1992), indicating that NO's role in contraction may be frequency-dependent. Our results align with this, as L-NAME increased contraction only at the highest frequency (32 Hz). This finding suggests that NO plays a more prominent role in modulating nerve-induced contractions under conditions of strong stimulation, while lower frequencies may not be as dependent on NO signaling in the presence of DIZE. Since this effect occurs at high frequency, it is likely due to changes in smooth muscle contractility rather than the neurogenic response. We propose that NO inhibition primarily influences smooth muscle activity at high frequencies, whereas at lower frequencies, the neurogenic response plays a larger role. The absence of effects across all parameters, with significant findings only observed at higher frequencies, suggests that DIZE may not exert a dominant role in neurogenic responses but could instead have a subtle modulatory effect under conditions of high-frequency neural stimulation. Although studies have shown that single incubation with L-NAME (Cao et al., 2020) and flurbiprofen (Vural et al., 2025) does not significantly alter EFS responses in bladder tissue, a limitation of the present study is the inability to assess the individual effects of these antagonists. Future studies should aim to further explore the detailed interactions.

In the present study, ELISA-based quantification of ACE2 and Ang (1–7) in rat bladder homogenates complemented the functional organ bath data. Under our experimental conditions, DIZE did not significantly alter ACE2 or Ang (1–7) tissue levels compared with vehicle. Although DIZE is commonly considered an ACE2 activator, the lack of a detectable ELISA change may indicate that ACE2/Ang (1–7) signaling is not strongly modulated in healthy bladder tissue under these conditions, or that regulatory factors in the tissue/homogenate environment mask subtle effects. This interpretation is consistent with reports showing that ACE2 responses to RAS-modulating interventions can vary markedly across tissues and models, and with the concept that ACE2 protein levels do not necessarily reflect enzymatic activity (Tang et al., 2021; Wysocki et al., 2020). In addition, Ang (1–7) measurements may be challenging because locally produced peptides can redistribute into extracellular compartments, and ELISA-based detection has been questioned in terms of sensitivity/accuracy in some settings (Chappell et al., 2021). Given the well-established role of prostanoids in bladder physiology and the conflicting literature on NSAIDs and ACE2 regulation (including reports of no effect with flurbiprofen *in vitro*) (de Bruin et al., 2022; Valenzuela et al., 2021), our finding that flurbiprofen did not reverse DIZE's functional effects further supports the idea that any COX–ACE2 interaction may be context dependent and potentially more evident in inflammatory/pathological bladder states. Therefore, future studies using disease-relevant models and complementary readouts (e.g., ACE2 activity assays and additional RAS peptides) are warranted to clarify whether DIZE exerts stronger ACE2-linked effects when the system is activated.

CONCLUSION

In conclusion, this study is the first to characterize DIZE in isolated rat bladder tissue. DIZE did not induce contraction but produced concentration-dependent relaxation in carbachol-precontracted strips,

which was not significantly affected by NOS or COX inhibition, indicating pathway-independent relaxation under these conditions. DIZE reduced high-frequency EFS-evoked contractions, and this inhibition was partly reversed by L-NAME, implicating NO in strong neurogenic activation, whereas flurbiprofen had no comparable effect. In ELISA analyses, ACE2 and Ang (1–7) levels were not significantly affected by DIZE, suggesting that tissue levels of these mediators remain stable in the healthy bladder under the present conditions. These findings highlight the potential modulatory effects of DIZE on bladder smooth muscle and suggest that further *in vivo* research is necessary to better understand its therapeutic implications for bladder dysfunctions.

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AUTHOR CONTRIBUTION STATEMENT

Investigation, Data Curation, Formal Analysis, Visualization, Writing – Review & Editing (YO), Investigation, Data Curation, Writing – Review & Editing (ZR), Conceptualization, Methodology, Supervision, Writing – Review & Editing (EA), Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review & Editing (IMV).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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