Full paper

Mineralocorticoid receptor stimulation induces urinary storage dysfunction via upregulation of epithelial sodium channel expression in the rat urinary bladder epithelium

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ABSTRACT

We aimed to evaluate mineralocorticoid receptor (MR) expression in rat bladder and the physiological role of the MR-epithelial sodium channel (ENaC) pathway in controlling bladder function in 10−12-week-old, male Sprague−Dawley rats. First, we examined the mRNA expression of MR and localization of MR and ENaC-α proteins in the urinary bladder. MR mRNA expression was observed in untreated-rat urinary bladders, and MR and ENaC-α proteins were localized in the epithelium. Next, rats were treated with vehicle (controls) or fludrocortisone (an MR agonist) for 3 days, and ENaC-α protein expression levels and bladder function were evaluated on day 4. ENaC-α protein expression was significantly higher in fludrocortisone-treated rats than in controls. In addition, cystometry was performed during intravesical infusion of saline and amiloride (an ENaC inhibitor). While intercontraction intervals (ICIs) during saline infusion were significantly shorter in the fludrocortisone group than in the controls, infusion of amiloride normalized the ICIs in the fludrocortisone group. However, no intra- or inter-group differences in maximum intravesical pressure were observed. Taken together, MR protein is localized in the rat urinary bladder epithelium, and may regulate ENaC expression and bladder afferent input. The MR-ENaC pathway may be a therapeutic target for ameliorating storage symptoms.

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1. Introduction

Overactive bladder syndrome (OAB) exhibits urinary urgency and is typically accompanied by urinary frequency and nocturia (1,2). Anticholinergic drugs are currently the most common treatment for urinary incontinence and frequent urination in patients with OAB (1). However, adverse events are often associated with anticholinergic drug use, including dry mouth, constipation, and urination disorders, which can affect patient adherence (3,4). A selective β₃-adrenoceptor agonist, mirabegron, has recently been approved for the treatment of OAB in several countries (5). However, the treatment of some clinical cases has been difficult even by using this new drug. Therefore, a new therapeutic strategy for storage dysfunction (including OAB) is needed.

The pathology of bladder dysfunction involves altered bladder epithelial function (6), and a number of ion channels that are expressed at the bladder epithelium are reportedly involved in mechanosensation during bladder filling (e.g., TRPA1, TRPV4, or the epithelial sodium channel [ENaC]) (7,8). In the rat urinary bladder

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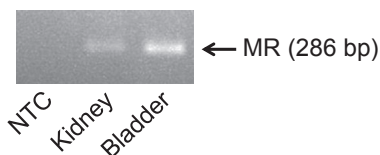Peer review under responsibility of Japanese Pharmacological Society.

Fig. 1. Reverse transcriptase-polymerase chain reaction and agarose gel electrophoresis to analyze mineralocorticoid receptor expression in the rat urinary bladder. Mineralocorticoid receptor (MR) mRNA expression was observed in the rat urinary bladder. The kidney was used as the positive control, and the non-template control (NTC) was used as the negative control.

epithelium, ENaC is known to play a key role in releasing ATP, which is an important neurotransmitter for evoking detrusor contractions in response to mechanical stretch in the storage phase (9). Furthermore, among patients with bladder outlet obstruction and benign prostatic hyperplasia, bladder mRNA levels of the ENaC α, β, and γ subunits is significantly correlated with storage symptoms using the International Prostate Symptom Score (IPSS) (10). In addition, ENaC expression is regulated by mineralocorticoid receptors (MRs) in renal, colonic, and fetal lung alveolar type 2 epithelial cells (11,12); MRs are also expressed in the toad urinary bladder (13). Aldosterone-bound to MRs regulated the unidirectional transport of sodium from the mucosal phase to the serosal phase via the activation of amiloride-blockade sodium channels (13). However, to the best of our knowledge, no studies have shown that MRs are expressed in the bladder of rats or other mammals and the MR-ENaC pathway plays a physiological role in regulating bladder function. Therefore, the present study investigated whether MRs are expressed in the rat urinary bladder, and whether MR-ENaC signaling regulates bladder afferent nerve activity and bladder function.

2. Materials and methods

2.1. Animal and tissue preparation

We used 10−12-week-old male Sprague−Dawley rats (SLC Inc., Shizuoka, Japan) for all experiments. The rats were kept in a temperature- and humidity-controlled room, with a 12-h/12-h light/dark cycle and free access to laboratory chow and normal water. To prepare bladder tissue samples for the in vitro analyses, the rats were euthanized via excessive anesthesia, and their bladders were collected for reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescent immunohistochemistry analysis. All animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of the Science and International Affairs Bureau of the Japanese Ministry of Education, Culture, Sports, Science and Technology. The study design was reviewed and approved by the Ethics Committee of Nagoya City University.

2.2. RT-PCR

Total RNA was extracted from the whole kidney without the urothelium and the whole bladder by using the ISOGEN reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's recommended protocol. The RNA concentration and quality were measured via spectrophotometry at 260 nm and 280 nm. Then, 1 μg of total RNA was reverse transcribed using the ReverTra Ace-α-kit (Toyobo, Osaka, Japan) according to the manufacturer's recommended reaction protocol, with oligo $(dT)_{20}$ as the primer. The cDNA was mixed with AmpliTaq Gold DNA Polymerase in Buffer II and $MgCl_2$ (Applied Biosystems, Foster City, CA, USA), and the solution was combined with two primers that are specific for the MR gene: MR forward 5′-GTGGACAGTCCTTTCACTACCG-3′ and MR reverse 5′ -TGACACCCAGAAGCCTCATCTC-3′. The specificity of these primers was confirmed by a BLAST search of the rat genome database. The reaction mixtures were incubated at 95 °C for 10 min, which was followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and finally held at 72 °C for 7 min. Non-template control DNA was used as the negative control. The PCR products were subjected to electrophoresis on a 2% agarose gel, then stained with ethidium bromide and imaged under ultraviolet light. An automated DNA sequencer was used to confirm the DNA sequence of the purified PCR products, using the sequence of the Nr3c2 gene available in the online database.

2.3. Fluorescent immunohistochemistry

Extirpated urinary bladders and kidneys of untreated rats were fixed in 4% paraformaldehyde, which was replaced with 10%, 20%, and 30% sucrose solutions for >12 h, and then frozen in an optimal cutting temperature compound (Sakura Finetechnical Co., Ltd.,
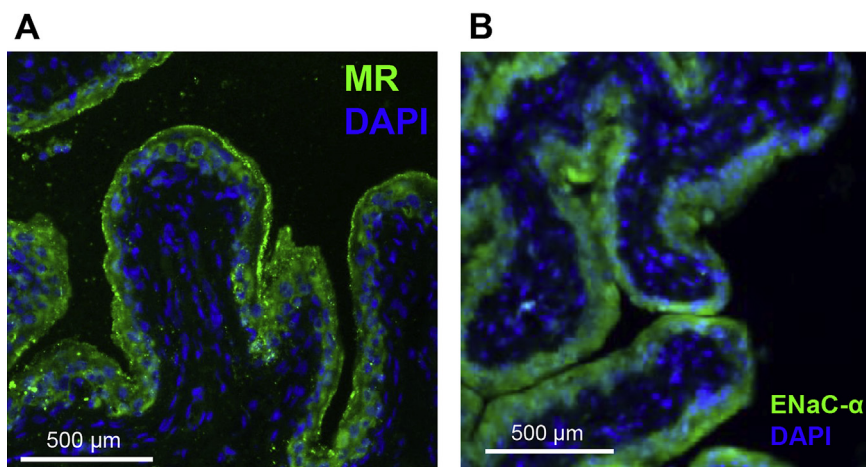

Fig. 2. Expression and localization of mineralocorticoid receptor protein and epithelial sodium channel-α protein in the rat urinary bladder. Blue staining indicates 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei. (A) Green staining indicates localization of the mineralocorticoid receptor (MR) protein at the rat urinary bladder epithelium. (B) Green staining indicates localization of the epithelial sodium channel-α (ENaC-α) protein at the rat urinary bladder epithelium. Scale bar = 500 μm.

Table 1
Baseline data of the control and FC groups.

	Control group	FC group
Bladder-to-body weight ratio (mg/g)	0.28 ± 0.01	0.28 ± 0.02
Heart rate (beats per min)	358.0 ± 10.2	336.1 ± 9.2
Systolic blood pressure (mmHg)	127.2 ± 5.0	125.1 ± 3.5
Mean blood pressure (mmHg)	102.7 ± 3.8	104.4 ± 2.1
Diastolic blood pressure (mmHg)	90.6 ± 4.1	94.3 ± 1.7

Data are expressed as mean ± standard error. Three days following administration, no statistically significant differences were observed between the control and fludrocortisone groups on day 4.
FC: Fludrocortisone.

Tokyo, Japan). Serial 10-μm sections were cut and mounted on glass slides, and the slides were washed with phosphate buffered saline (PBS) and then blocked with 5% skim milk in PBS for 1–2 h. After washing with PBS, the sections were incubated for 30 min with a goat anti-MR polyclonal antibody (1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or overnight with a rabbit anti-ENaC-α polyclonal antibody (1:200, Bioss Inc., Woburn, MA, USA). Visualization of MR protein was performed for 30 min using the Alexa Fluor 488 Donkey Anti-Goat IgG (H + L) antibody (1:250, Molecular Probes, Eugene, OR, USA), and ENaC-α protein was visualized using the Alexa Fluor 488 Goat Anti-Rabbit IgG (H + L) antibody for 30 min (1:250, Molecular Probes). The nuclei were then stained for 3–5 min with Cellstain DAPI solution (Dojindo Laboratories, Kumamoto, Japan).

Extirpated urinary bladders and kidneys of rats from Control, FC and FC + EPL groups were fixed in 10% formaldehyde, which was replaced with 70% ethanol, and the fixed samples were then embedded in paraffin. Serial sections (4-μm thickness) were deparaffinized and rehydrated in an ethanol series, and antigens were activated by the microwave method. The sections were blocked with 5% skim milk in PBS for 1 h at room temperature and incubated with a goat anti-MR polyclonal antibody (1:50) and rabbit anti-ENaC-α polyclonal antibody (1:200), which were mixed and diluted in Reagent 1 of IMMUNO SHOT (Cosmo Bio Co., Ltd., Tokyo, Japan) at 4 °C overnight. Costaining of MR and ENaC-α was performed for 30 min by using Alexa Fluor 488 Donkey Anti-Goat IgG (H + L) antibody (1:250) and Alexa Fluor 594 Donkey Anti-Rabbit IgG (H + L) antibody (1:1000, Molecular Probes), which were mixed and diluted in Reagent 2 of IMMUNO SHOT. The nuclei were then stained with Cellstain DAPI solution (Dojindo Laboratories, Kumamoto, Japan).

Kidney tissues were used as a positive control, and the same protocol without the primary antibodies was used as a negative control.

2.4. The activated MR model

The rats were randomized into the control or fludrocortisone (FC, a potent MR agonist) groups. The FC group was treated orally with FC (6 mg/kg/day) in 1% hydroxypropyl methylcellulose (Sigma–Aldrich Japan, Tokyo, Japan), once daily for 3 days, based on previous reports (14,15). The control group was treated with the vehicle solution once daily for 3 days. After the 3-day treatment period, the systemic arterial blood pressure and heart rate of the rats in each group were measured using a noninvasive automatic device (BP-98A-L, Softron, Tokyo, Japan), and the rats were maintained at 37 °C during the measurements using a warmer (THC-31, Softron). Each measurement was performed three times per rat (while awake), and the mean value was used for analysis.

2.5. Protein extraction and western blotting

After the 3-day treatment period, protein from the urinary bladder of the rats was extracted using the PRO-PREP Protein Extraction Solution (iNtRON Biotechnology Inc., Gyeonggi-do, Korea), and the total protein concentration was quantified using the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA). Samples containing 25 μg of total protein were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were then transferred onto polyvinylidene difluoride membranes (ImmobilinTM; Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% skim milk, incubated with the primary rabbit anti-ENaC-α polyclonal antibody (1:500; Bioss Inc., Woburn, MA, USA) in Tris-buffered saline and Tween 20 (TBST), and then incubated in TBST with the secondary anti-rabbit immunoglobulin G (IgG), which was conjugated to horseradish peroxidase (1:5000; GE Healthcare, Little Chalfont, UK). The protein bands were visualized using ECL (GE Healthcare, Little Chalfont, UK) and an ImageQuant LAS 4000 mini (GE Healthcare), the membranes were washed with TBST, and the bound primary and secondary antibodies were removed using Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA). Next, the membranes were re-blocked with 5% skim milk, incubated with the primary mouse anti-β-actin monoclonal antibody (1:2500; Sigma, Saint Louis, MO, USA) in 5% skim milk, and then incubated with the secondary anti-mouse IgG (1:5000; GE Healthcare). The protein bands were visualized using ECL and ImageQuant LAS 4000 mini (GE Healthcare). The band densities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and expressed as the ratio of the ENaC-α protein to the β-actin protein.
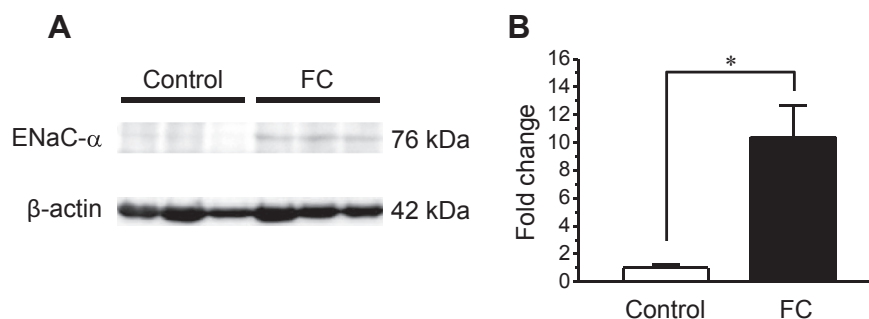

Fig. 3. Western blot analysis of the expression of epithelial sodium channel-α protein in the rat urinary bladder after three days of fludrocortisone administration. (A) Representative immunoblots of epithelial sodium channel-α (ENaC-α) and β-actin proteins. (B) Densitometric quantification of the corresponding bands revealed that ENaC-α protein in the fludrocortisone (FC) group was significantly upregulated, compared with the control group. The mean expression level of ENaC-α protein in the control group was set to 1.0-fold for the comparative analysis. Each bar indicates mean ± standard error values; n = 3 in the control group (vehicle administration), and n = 7 in the FC group. *P < 0.05.
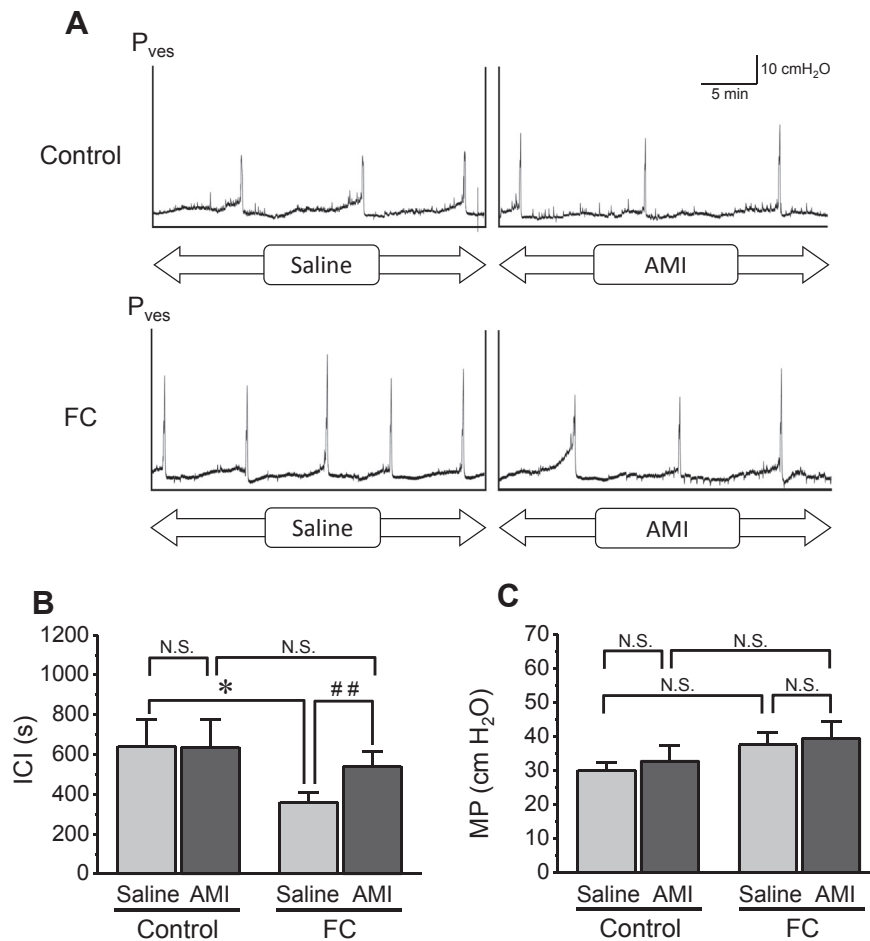
Fig. 4. Cystometry after three days of fludrocortisone administration. (A) Representative cystometrogram during intravesical infusion of saline and the subsequent amiloride (AMI) treatment in the control and fludrocortisone (FC) groups. (B) Results of the intercontraction intervals (ICI) analysis. The FC group exhibited significantly shorter ICIs during the saline infusion, compared with the control group. The subsequent infusion of amiloride significantly prolonged the ICIs in the FC group, although it did not change the ICIs of the control group. (C) The maximum intravesical pressure (MP) during saline and amiloride infusion in each group. No intra-group or inter-group changes in MPs were observed during infusion of saline and amiloride. Each bar indicates mean ± standard error values (panels B, C); n = 4 in the control group (vehicle administration), and n = 7 in the FC group. *P < 0.05 vs. control. ##P < 0.01 vs. saline. N.S., not significant. Pves, intravesical pressure.

2.6. Bladder-filling cystometry

Under inhalational anesthesia with 3% isoflurane for induction and 2% isoflurane for maintenance, the bladders of the rats were exposed, and polyethylene-50 tubing (with holes in the sides) was inserted into the bladder dome and sealed with 5−0 silk sutures. The tubing was connected to a syringe pump that was connected to a pressure transducer (UD5500, Dantec, Denmark), which allowed for saline infusion, and cystometry was performed while the rats were awake in a restraining cage. Each group received saline

(0.08 mL/min at room temperature), followed by 1 mM amiloride hydrochloride hydrate (an ENaC inhibitor, 0.08 mL/min at room temperature), for analysis of the intercontraction intervals (ICIs, the time between two voiding contractions) and the maximum intravesical pressure (MP, the maximum bladder pressure during a micturition cycle). We then compared the intra- and inter-group changes in ICI and MP during the amiloride and saline infusions. In addition, to confirm the involvement of MR in bladder function, we used the MR-selective blocker eplerenone (EPL) (Tokyo Chemical Industry, Tokyo, Japan). FC (6 mg/kg/day) with EPL (75 mg/kg/day) in 1% hydroxypropyl methylcellulose (Sigma−Aldrich Japan, Tokyo, Japan) was orally administered to the rats once daily for 3 days, based on previous reports (16). Then, cystometry was performed as described above. Rats did not move during cystometry; hence, intravesical pressure was assumed equal to detrusor pressure and residual urine volume was not be observed.

Table 2
Intercontraction intervals of control, FC, and FC + EPL groups.

Group	Intercontraction intervals (s)
Control	746.3 ± 73.8
FC	394.0 ± 60.5**
FC + EPL	509.2 ± 110.1

Data are expressed as mean ± standard error.
** P < 0.01 vs. control group.
FC: fludrocortisone, EPL: eplerenone.
n = 5−11.

2.7. Statistical analysis

The results are expressed as mean ± standard error of the mean. The two-sided Student's t-test was used to compare the two groups, while the paired t-test was used to compare pre- and post-
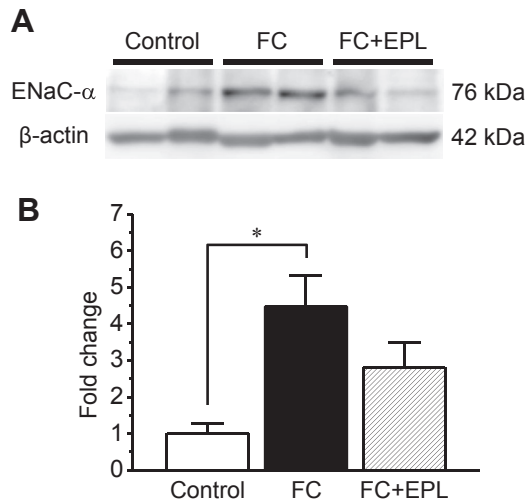
Fig. 5. Western blot analysis of the expression of epithelial sodium channel-α protein in the rat urinary bladder after three days of vehicle, fludrocortisone or fludrocortisone plus eplerenone administration. (A) Representative immunoblots of epithelial sodium channel-α (ENaC-α) and β-actin proteins. (B) Densitometric quantification of corresponding bands revealed that ENaC-α was significantly upregulated in the fludrocortisone (FC) group compared with the control group. Co-administration of EPL with FC attenuated this effect of FC, although the difference was not statistically significant. The expression levels in the FC + EPL group was not significantly different from those in the control group. The mean ENaC-α expression in the control group was set to 1.0 for the comparative analysis. Each bar indicates mean ± standard error values; n = 4 in the control group (vehicle administration), n = 7 in the FC group and n = 5 in the FC + EPL group. *P < 0.05.

intervention differences. Bonferroni multiple comparison tests were used to determine significantly differences between means. Differences with a P-value of <0.05 were considered statistically significant.

3. Results

3.1. MR mRNA expression in the urinary bladder

Positive MR mRNA expression was observed in the kidney (positive control) and rat urinary bladder, and no mRNA expression was observed in the negative controls (Fig. 1). The base sequence of the PCR product was identical to the previously reported sequence (17).

3.2. MR and ENaC-α protein localization

As the ENaC-α subunit is critical for formation of functional channels (18), we selected the ENaC-α protein as an indicator of ENaC protein expression. Immunofluorescence revealed that the MR and ENaC-α proteins were clearly localized in the rat urinary bladder epithelium (Fig. 2A, B). The positive (kidney tissue) and negative (no primary antibodies) controls revealed that both primary antibodies were sensitive and specific (data not shown).

3.3. Baseline data for the control and FC groups

The control and FC groups had similar bladder-to-body weight ratios, heart rates, systolic blood pressures, mean blood pressures, and diastolic blood pressures on day 4 (Table 1).

3.4. The effect of FC on ENaC-α protein expression and bladder function

After treatment with the vehicle or FC, we evaluated ENaC-α expression in the rat urinary bladder via western blotting on day 4; ENaC-α expression was higher in the FC group than in the control group (P < 0.05) (Fig. 3). Cystometry revealed that the FC group had a significantly shorter mean ICI during saline infusion, compared
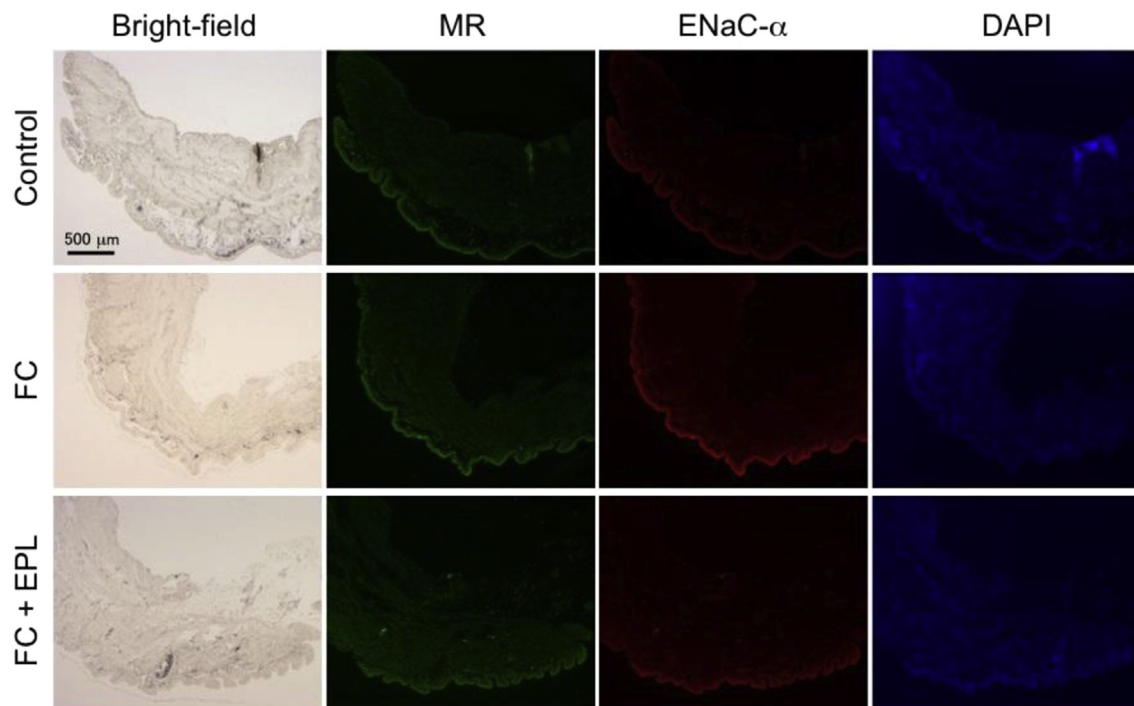

Fig. 6. Immunofluorescent analysis of mineralocorticoid receptor protein and epithelial sodium channel-α protein in the rat urinary bladder after three days of vehicle, fludrocortisone or fludrocortisone plus eplerenone administration. Blue, green, and red staining indicates 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei, mineralocorticoid receptor (MR) protein, and epithelial sodium channel-α (ENaC-α) protein, respectively, in the rat urinary bladder. MR and ENaC-α were localized at the rat urinary bladder epithelium. The staining patterns were not altered between control, FC, and FC + EPL groups. Scale bar = 500 μm.

with the control group (360.0 ± 50.8 s vs. 639.5 ± 138.0 s, respectively; P < 0.05). The amiloride infusion did not cause an intra-group change in the ICI of the control group. However, the amiloride infusion significantly prolonged the ICI in the FC group compared with that in the FC group during the saline infusion (538.4 ± 79.1 s vs. 360.0 ± 50.8 s, respectively; P < 0.01). No significant difference in the ICI was observed between the control and FC groups during the amiloride infusion (635.0 ± 140.5 s vs. 538.4 ± 79.1 s, respectively) (Fig. 4A, B). The amiloride infusion did not create any intra-group or inter-group differences in the MP between the control and FC groups (Fig. 4C).

3.5. The effect of MR blockade

After treatment with the vehicle, FC, or FC plus EPL, we evaluated bladder function via cystometry. Cystometry revealed that the FC group had a significantly shorter mean ICI during the saline infusion, compared with the control group. Co-administration of EPL with FC extended the ICI compared with that of the FC group, although the difference was not statistically significant. Hence, the ICI of the FC + EPL group was not significantly different from the ICI of the control group (Table 2).

In addition, we examined MR and ENaC-α protein localization and ENaC-α protein expression levels by western blotting. ENaC-α protein expression in the FC group was significantly higher than that in the control group. Co-administration of EPL with FC slightly prevented the up-regulation of ENaC-α, although not statistically significant (Fig. 5).

According to immunohistochemistry results, in all groups, both MR and ENaC-α were localized only at the urothelium and not in the smooth muscle or vessels (Fig. 6).

4. Discussion

A previous study reported that MRs are expressed in the urinary bladder of toads. MRs bind aldosterone and regulate unidirectional transport of sodium from the mucosal phase to the serosal phase via activated amiloride-blockade sodium channels (13). However, MR expression in the mammalian urinary bladder is not well studied. This is the first study to reveal that functional MRs are expressed in the rat urinary bladder epithelium. In this context, the oral administration of FC (a potent synthetic MR agonist), elicited frequent urination without affecting the bladder-to-body weight ratio, blood pressure, or detrusor muscle contractile activity of rats. Furthermore, this frequent urination was attenuated by intravesical infusion of amiloride (an ENaC inhibitor). Co-administration of EPL (a selective MR blocker) with FC tended to improve this frequent urination.

In renal, colonic, and fetal lung alveolar type II epithelial cells, aldosterone-bound MR is translocated into the nucleus, where it regulates the expression and activity of ENaC (11,12). Furthermore, ENaC is reported to have a mechanosensitive property in *Caenorhabditis elegans* (19). ENaC is reportedly expressed in the rat and rabbit urinary bladder epithelium and plays a role in the mechanosensory transduction of the bladder by responding to mechanical stretch during the urine storage phase (7,9,20). In addition, a previous study reported that seven of nine patients with bladder outlet obstruction and benign prostatic hyperplasia exhibited involuntary detrusor contraction and increased ENaC expression in the urinary bladder epithelium (10). Furthermore, the IPSS storage symptom score is significantly correlated with ENaC expression levels (10). In our study, as expected, systemic FC administration increased ENaC-α protein expression and induced frequent urination. This frequent urination was attenuated by intravesical

infusion of amiloride (an ENaC inhibitor). Thus, upregulated ENaC may play a role in FC-induced storage dysfunction.

To confirm the involvement of MR, we co-administered FC and a selective MR blocker, EPL. Blockade of MR signaling using EPL, tended to ameliorate FC-induced storage dysfunction and prevented the up-regulation of ENaC-α, although these differences were not statistically significant. These results further suggested that the MR-ENaC pathway is involved in bladder function.

Furthermore, we demonstrated that MP did not change after FC administration and/or amiloride infusion. Therefore, these findings indicate that MR activation in the bladder epithelium affects ENaC expression without affecting the contractile activity of the detrusor muscles; thus, this pathway may be an effective target for managing storage symptoms.

Based on these findings, it appears logical that patients with diseases that result in elevated aldosterone levels (e.g., primary aldosteronism) would experience storage symptoms. Indeed, patients with primary aldosteronism experience nocturia, which is a manifestation of storage symptoms. However, nocturia is caused by a decrease in the kidneys' ability to concentrate urine, which is induced by low serum potassium levels and which causes the subsequent polyuria (21). Thus, although these patients may exhibit bladder overactivity that is derived from pathological changes in the epithelium, it is impossible to distinguish between bladder overactivity and polyuria in these cases. Metabolic syndrome or obesity is also related to elevated serum aldosterone levels or activation of the renin—angiotensin—aldosterone system via obesity-associated insulin resistance or hypertension (22—24). Associations between metabolic syndrome and lower urinary tract symptoms have recently been reported. In particular, both basic (25) and clinical research (26—28) have revealed a relationship between metabolic syndrome and storage dysfunction. Therefore, the MR-ENaC pathway might be a novel target for storage symptoms in these diseases.

5. Conclusions

In conclusion, the MR protein is localized in the rat urinary bladder epithelium, and the MR-ENaC pathway in the bladder epithelium might contribute to storage dysfunction (e.g., overactive bladder) via these pathological mechanisms. This pathway may be a novel therapeutic target for ameliorating storage symptoms.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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