Store-operated Ca\(^{2+}\) entry suppresses distention-induced ATP release from the urothelium

Kazumasa Matsumoto-Miyai,1 Ai Kagase,1 Erika Yamada,1 Masaru Yoshizumi,1 Manabu Murakami,2 Takayoshi Ohba,2 and Masahito Kawatani1

Departments of ¹Neurophysiology and ²Cell Physiology, Akita University Graduate School of Medicine, Akita, Japan

Submitted 31 August 2010; accepted in final form 13 December 2010

Matsumoto-Miyai K, Kagase A, Yamada E, Yoshizumi M, Murakami M, Ohba T, Kawatani M. Store-operated Ca\(^{2+}\) entry suppresses distention-induced ATP release from the urothelium. Am J Physiol Renal Physiol 300: F716–F720, 2011. First published December 15, 2010; doi:10.1152/ajprenal.00512.2010.—Epithelial cells in the urinary bladder (urothelium) trigger sensory signals in micturition by releasing ATP in response to distention of the bladder wall. Our previous study revealed the distinct roles of extracellular Ca\(^{2+}\) and the Ca\(^{2+}\) stores in the endoplasmic reticulum (ER) in urothelial ATP release. In the present study, we investigated the regulation of urothelial ATP release by Ca\(^{2+}\) influx from the extracellular space and Ca\(^{2+}\) release from the ER using a distention assay of the mouse bladder wall in a small Ussing chamber. Stimulation of Ca\(^{2+}\) release from the ER in the mucosal side of the bladder induced significant ATP release without distention. Blockade of the inositol 1,4,5-trisphosphate receptor reduced distention-induced ATP release, suggesting that Ca\(^{2+}\) release from the ER is essential for the induction of urothelial ATP release. On the other hand, blockade of store-operated Ca\(^{2+}\) entry (SOCE) from the extracellular space significantly enhanced distention-induced ATP release. Thus Ca\(^{2+}\) release from the ER causes urothelial ATP release and depletion of Ca\(^{2+}\) stores in the ER, which in turn causes the depletion-inducing SOCE to suppress the amount of urothelial ATP release.

THE UROTHELIUM RELEASES ATP in response to distention of the bladder wall during the phase of storage (3, 5). ATP receptor P2X3 is expressed at theafferent nerve terminals in close proximity to the urothelium and is crucial for control of urinary bladder volume reflex. P2X3-deficient mice show urinary bladder hyporeflexia, such as attenuated response of bladder afferents to distention (26), and a decrease in voiding frequency with an increase in bladder capacity (9). The pain response behavior was also reduced in P2X3-deficient mice (9). Thus urothelial ATP release could play a key role in both volume- and noxious stimulus-evoked reflex. Hyperreflexia by excessive urothelial ATP release might cause frequent urination or bladder pain in the case of interstitial cystitis or an overactive bladder. In fact, an excess of urothelial ATP release was observed in interstitial cystitis (4, 23, 24). In a group of female overactive bladder patients, those with a high ratio of urinary ATP/creatinine showed a higher symptom score of urinary frequency than those with a low ratio (22). Therefore, elucidation of the molecular mechanisms underlying urothelial ATP release would contribute to drug development for the therapeutic alleviation of storage symptoms or bladder pain.

Previous studies show that activation of either TRPV1 or TRPV4 channels, which induce Ca\(^{2+}\) influx, or the stimulation of chemical mediator receptors, which induce Ca\(^{2+}\) release from the endoplasmic reticulum (ER), triggers urothelial ATP release (4, 6, 12–14, 18, 27). This suggests that an increase in cytosolic Ca\(^{2+}\) is essential for the induction of ATP release. On the other hand, our previous study suggested a different regulatory role of Ca\(^{2+}\) influx in urothelial ATP release, with an elevation of extracellular Ca\(^{2+}\) resulting in decreased amounts of ATP release (17), implying that some types of Ca\(^{2+}\) influx from the extracellular space might suppress the ATP release. To date, the distinct functions of Ca\(^{2+}\) on urothelial ATP release are still unknown. In the present study, we investigated the dual roles of Ca\(^{2+}\) in distention-induced ATP release from the urothelium using a dissected, opened urinary bladder mounted in an Ussing chamber. We adopted the change in ATP amount in the mucosal side of the chamber as an index of urothelial ATP release, because previous studies showed that l) stimuli evoke ATP release from muscle-removed urinary bladder strips but not from mucosa-removed strips (11); and 2) stretching the urothelium induces ATP release into both the mucosal and serosal sides in a similar way (15). Among various types of Ca\(^{2+}\) influx, store-operated Ca\(^{2+}\) entry (SOCE), a Ca\(^{2+}\) influx induced by depletion of Ca\(^{2+}\) stores in the ER (19, 20, 25), is known to play important, unique roles in nonexcitable cells (19, 20). However, the function of SOCE in the urothelium remains to be understood. Here, we focused on SOCE as a candidate of suppressive function on urothelial ATP release and investigated the opposite role of SOCE on distention-induced urothelial ATP release compared with that of Ca\(^{2+}\) release from the ER.

MATERIALS AND METHODS

Animals. Eight- to 12-wk-old C57BL/6 male mice were used in this study. The Animal Research Committee of Akita University approved the protocols for the experiments, and the guidelines of the American Physiological Society for animal research were followed.

ATP release assay using the Ussing chamber. A total of 97 male mice were used for the Ussing chamber assay. The urothelial ATP release assay was performed as described previously (17). In brief, isolated urinary bladders were opened vertically from the urethra to the apex. The opened bladder was mounted to act as a diaphragm of 7 mm\(^2\) between the two halves of a customized small Ussing chamber. The mucosal (urinary) side of the chamber had a volume of 700 μl. Chambers were filled with Krebs solution (117 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl\(_2\), 24.8 mM NaHCO\(_3\), 1.2 mM NaH\(_2\)PO\(_4\), and 11.1 mM glucose) including 0 mM or 2.5 mM CaCl\(_2\) with 95% O\(_2\)/5% CO\(_2\). Bubbling. In the chemical stimulation assay, Ca\(^{2+}\) release from the ER was assessed by

Address for reprint requests and other correspondence: K. Matsumoto-Miyai, Dept. of Neurophysiology, Akita Univ. Graduate School of Medicine, 1-1-1 Hondo, Akita, Akita 010-8543, Japan (e-mail: kmiyai@med.akita-u.ac.jp).
subtracting the ATP content before administration from that after administration.

We performed two types of distention stimulation assays. In the first assay, we applied hydrostatic pressure at 45 cmH2O to the serosal (smooth muscle) side for 20 min twice. Fifty microliters of Krebs solution was sampled from the mucosal side before and after application of pressure. Heparin sodium salt (Sigma), 2-aminoethyl diphenylborinate (2-APB; Sigma), or 1-(5-chlorophthalene-1-sulfonfonyl)-1H-hexahydro-1,4-diazepine (ML-9; Sigma) was administered to the mucosal side 30 min before the second pressure application. The effects of drugs were assessed by the ratio of the second pressure-induced change in ATP content to the first. The experimental design of the distention stimulation assay is also illustrated in Figs. 2A and 3A. In the second assay, 5 cmH2O of pressure was applied to the serosal side for 20 min once, which is thought to reflect the physiological range of pressure during urine storage. We sampled 50 μl of Krebs solution in the mucosal side of chamber before and after application of pressure. Heparin or ML-9 was administered 30 min before the pressure was applied. The ATP release was assessed by the subtraction of ATP content before administration from that after administration. The experimental design of the distention stimulation assay was determined according to previous studies about Ca2⁺ functions (4, 7, 21).

Measurement of ATP concentration. The ATP content in 50 μl of Krebs solution in the mucosal side of the chamber was assayed by means of the luciferin-luciferase method (Kikkoman, Tokyo, Japan) according to the manufacturer’s protocol. Standard lines were constructed in each experiment using 3 × 10⁻⁷, 3 × 10⁻⁸, 3 × 10⁻⁹, and 3 × 10⁻¹⁰ M ATP.

Data analysis. Stimuli-elicited changes in ATP content were calculated by subtracting the value before the administration of chemicals or pressures. In the first distention stimulation assay, measuring the ratio of second pressure-induced ATP release to the first pressure-induced ATP release was performed to access the effects of the inhibitors and to eliminate individual differences in bladder samples. Statistically significant differences were detected using the unpaired t-test. All data were expressed as means ± SE.

RESULTS

Ca2⁺ release from the ER drives ATP release from the urothelium. We stimulated Ca2⁺ release from the ER by administering the Ca2⁺ ionophore A23187 (10 μM) or the ryphaneside receptor agonist caffeine (10 mM) to the mucosal side of the Ussing chamber for 30 min. To exclude the effects of Ca2⁺ influx, the bladder walls were incubated in Ca2⁺-free Krebs solution. Although the vehicle for A23187, DMSO (final concentration 0.1%) had a weak effect on increasing ATP (0.34 ± 0.54 nM), the administration of A23187 significantly elevated ATP content (1.95 ± 0.39 nM), much more than DMSO without hydrostatic pressure for distention (Fig. 1A). Administration of Krebs solution slightly decreased ATP content (−0.42 ± 0.41 nM) by the constitutive ATP degradation activity of ecto-ATPase, whereas caffeine, dissolved in Krebs solution, also induced a significant ATP release (1.00 ± 0.27 nM) from the mucosal side of the bladder wall without distention (Fig. 1B).

Blockade of Ca2⁺ release from the ER inhibits distention-induced urothelial ATP release. The involvement of Ca2⁺ release from the ER in distention (45 cmH2O of pressure)-induced ATP release from the urothelium was investigated next. To exclude variance among bladder samples, we assessed the ratio of the second pressure-evoked ATP increase to the first pressure-evoked ATP increase in the same tissue. The

![Fig. 1. Endoplasmic reticulum (ER) Ca2⁺-releasing factors evoke urothelial ATP release. The effect of A23187 (10 μM; A), caffeine (10 mM; B), or vehicle (DMSO in A and Krebs in B) on ATP release to the mucosal side of the chamber is shown.](http://ajprenal.physiology.org/)

isotol 1,4,5-triphosphate (IP₃) receptor blocker heparin (100 μg/ml) or vehicle (H₂O) was administered to the mucosal side for 30 min before the second pressure application (Fig. 2A). The ratio of the second ATP to the first ATP by vehicle only was 0.58 ± 0.14 in the presence of 2.5 mM CaCl₂ in Krebs solution (Fig. 2B). Administration of heparin significantly reduced the second pressure-evoked ATP release by ~30% (0.18 ± 0.05; Fig. 2B). The reducing effect of heparin was also detected in the Ca2⁺-free Krebs solution. Although the ratio by addition of vehicle was lower in the absence of CaCl₂ (0.20 ± 0.07), heparin significantly decreased the ratio (~0.19 ± 0.16; Fig. 2B). The minus value of the ratio means that the second pressure application decreased the ATP content in the mucosal side of chamber, which is due to smaller ATP release than constitutive ATP degradation.

Blockade of SOCE enhances distention-induced urothelial ATP release. We focused on SOCE in various types of Ca2⁺ influx from the extracellular space, because SOCE plays important, unique roles in nonexcitable cells (19, 20). To investigate the effect of SOCE on urothelial ATP release induced by 45 cmH2O of pressure, we administered 2-APB (100 μM), an inhibitor of SOCE (25), into the mucosal side of the chamber between the first and second pressure applications (Fig. 3A). The effect of 2-APB was evaluated by the ratio of the second pressure-evoked ATP increase to the first pressure-evoked ATP increase. Administration of heparin (final 0.1%), the vehicle for 2-APB, showed a similar ratio of the second ATP release (0.66 ± 0.04 in 2.5 mM CaCl₂, 0.28 ± 0.07 in 0 mM CaCl₂) to 0.1% H₂O, which was used as a vehicle for heparin (see Fig. 3B). 2-APB significantly enhanced ATP release by 2.47-fold when Krebs solution containing 2.5 mM CaCl₂ was used (1.62 ± 0.37; Fig. 3B). The enhancing effect was diminished in the absence of CaCl₂ in Krebs solution (0.24 ± 0.03; Fig. 3B), indicating that the effect of 2-APB was dependent on the presence of extracellular Ca2⁺.
To confirm the involvement of SOCE in ATP release, we assessed the effect of a potent myosin light chain kinase inhibitor, ML-9, which is also reported to inhibit SOCE (25). As shown in Fig. 4C, administration of ML-9 (100 μM) facilitated ATP release in a similar way to 2-APB. Although DMSO (0.1%; the vehicle for ML-9) reduced the ratio of the second ATP (0.17 ± 0.05 in 2.5 mM CaCl₂, 0.19 ± 0.05 in 0 mM CaCl₂) compared with 0.1% ethanol, a significant enhancing effect by ML-9 was detected (up to 2.74-fold) when it was in the presence of 2.5 mM CaCl₂ (0.47 ± 0.12; Fig. 3C). In Ca²⁺-free Krebs solution, ML-9 did not affect the ratio of the second ATP release (0.22 ± 0.03; Fig. 3C), which was the same for 2-APB.

Ca²⁺ release from the ER and SOCE also regulate urothelial ATP release induced by physiological range of pressure during urine storage. Previously, we found that ATP was released in proportion to 15–45 cmH₂O of hydrostatic pressure (17), implying that the physiological range of pressure-induced ATP release could be also regulated by Ca²⁺ in a similar way. To confirm this possibility, we examined the effect of heparin and ML-9 on the ATP release induced by 5 cmH₂O of pressure.

Distention for 20 min by 5 cmH₂O increased ATP content in the mucosal side of chamber under conditions of vehicle administration (0.13 ± 0.06 nM in 0.1% H₂O, Fig. 4A; 0.11 ± 0.09 nM in 0.1% DMSO, Fig. 4B). However, administration of heparin (100 μg/ml) oppositely decreased ATP content in the presence of extracellular Ca²⁺ (~0.09 ± 0.06 nM, Fig. 4A), meaning that ATP release did not exceed the constitutive degradation of ATP by ecto-ATPase in mucosa. On the other hand, ML-9 (100 μM) significantly enhanced the ATP increase when extracellular Ca²⁺ was present (0.41 ± 0.08 nM, Fig. 4B). These data showed that the physiological range of pressure-induced ATP release was driven by Ca²⁺ release from the ER, but was suppressed by SOCE.

DISCUSSION

In this study, we demonstrate the distinct role of Ca²⁺ release from the ER and SOCE in urothelial ATP release using dissected urinary bladders of adult mice. Different types of ER Ca²⁺-releasing factors, a Ca²⁺-ionophore (A23187) or a ryanodine receptor agonist (caffeine), increased ATP content to ~1.4–1.6 nM in Ca²⁺-free Krebs solution without any other stimulation (Fig. 1). This suggests that Ca²⁺ release from the ER is capable of inducing urothelial ATP release. The inducing effect of A23187 or caffeine was slightly less than that of 15 cmH₂O of hydrostatic pressure (~2 nM) in our previous study (17). Conversely, blockade of Ca²⁺ release from the ER by heparin significantly suppressed distention-induced ATP release in this study (Figs. 2B and 4B). A similar effect of heparin has also been reported using primary cultures of feline urothelial cells (4), supporting the notion that the effect of heparin is due to its action on urothelial cells. Heparin reduced the hydrostatic pressure (45 cmH₂O)-induced ATP release to ~30% (under conditions of 2.5 mM CaCl₂ in Fig. 2B), meaning that most of the distention-induced ATP release is triggered by Ca²⁺ release from the ER. The reminder of ATP release could be dependent on Ca²⁺ influx through mechanosensory channels such as TRPV1 or TRPV4, because both TRPV1- and TRPV4-deficient mice show impairment of stretch-evoked increases in intracellular Ca²⁺ and ATP release from the urothelium (6, 12). In agreement, the second pressure for distention evoked almost no increase in ATP under conditions of 0 mM extracellular CaCl₂ with heparin (Fig. 2B).

Previously, we found an additional role for extracellular Ca²⁺ in the regulation of urothelial ATP release (17). Physiological levels of extracellular Ca²⁺ have an inhibitory effect on ATP release, which is distinct from the driving role of Ca²⁺ release from the ER or from Ca²⁺ influx through mechanosensory channels. Using dissected bladder tissues, the removal of extracellular Ca²⁺ enhanced the distention-evoked ATP release. In primary culture of urothelial cells, an increase in extracellular Ca²⁺ reduced the amount of chemically induced ATP release, indicating that extracellular Ca²⁺ directly acts on urothelial cells, not through suburothelial or smooth muscle cells. As a distinct suppressive effect of extracellular Ca²⁺ on urothelial ATP release, we focused on the SOCE, which is induced by the depletion of Ca²⁺ stores in the ER (19, 20, 25). The molecular components of store-operated Ca²⁺ channels are supposed to be the TRPC and Orai family members. Of these members, the most evidence comes from studies showing that TRPC1, Orai1, or Orai2 can form store-operated Ca²⁺...
channels (2, 25). In the present study, we found that the mucosal administration of different types of SOCE blockers, 2-APB or ML-9 (25), significantly enhanced distention-evoked urothelial ATP release (Fig. 3). The involvement of SOCE in ATP release is also supported by the dependency of the effects of 2-APB or ML-9 on extracellular Ca\(^{2+}\) (Fig. 3, B and C). These data indicate that SOCE suppresses urothelial ATP release in contrast to Ca\(^{2+}\) release from the ER, although they both result in an increase in intracellular Ca\(^{2+}\). Previous studies show that the SOCE has a discrete role from Ca\(^{2+}\) influx either through mechanosensory or voltage/ligand-gated channels or from Ca\(^{2+}\) release from the ER (19). For example, the SOCE specifically, directly regulates the activity of enzymes such as adenylate cyclase and nitric oxide synthase (NOS), which are held at the plasma membrane (19, 20). The activity of adenylate cyclase type VI is inhibited by SOCE, but not by other types of Ca\(^{2+}\) influx or Ca\(^{2+}\) release from the ER (8, 10). SOCE evokes the generation of 10-fold more nitric

Fig. 3. Blockade of store-operated Ca\(^{2+}\) entry (SOCE) enhances distention-induced urothelial ATP release. A: schematic representation of the experimental procedure for the distention-induced ATP release assay. Hydrostatic pressure (45 cm H\(_2\)O for 20 min) was applied twice to the same bladder wall. ATP content was measured at the time point indicated by the arrows. 2-Aminoethyl diphenylborine (2-APB), 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), or vehicle (ethanol for 2-APB or DMSO for ML-9) was administrated 30 min before the second pressure application. B and C: changes in ATP content in the mucosal side of the chamber 20 min after administration. B: heparin (100 µg/ml) diminished 5 cmH\(_2\)O of pressure-induced increase in ATP content when Krebs solution containing 2.5 mM CaCl\(_2\) (P < 0.05 vs. vehicle by unpaired t-test) was used. Error bars indicate SE; n = 4 in all conditions. C: ML-9 (100 µM) significantly enhanced 5 cmH\(_2\)O of pressure-induced increase in ATP content in the presence of 2.5 mM CaCl\(_2\) in Krebs (P < 0.05 vs. vehicle by unpaired t-test). Error bars indicate SE; n = 4 in vehicle and 5 in ML-9.

Fig. 4. Ca\(^{2+}\) release from ER and SOCE also regulate physiological pressure-induced urothelial ATP release. A: schematic representation of the experimental procedure for the distention-induced ATP release assay. Hydrostatic pressure (5 cm H\(_2\)O for 20 min) was applied once. ATP content was measured at the time point indicated by the arrows. Heparin, ML-9, or vehicle (H\(_2\)O for heparin or DMSO for ML-9) was administrated 30 min before the pressure application. B and C: changes in ATP content in the mucosal side of the chamber 20 min after administration. B: heparin (100 µg/ml) diminished 5 cmH\(_2\)O of pressure-induced increase in ATP content when Krebs solution containing 2.5 mM CaCl\(_2\) (P < 0.05 vs. vehicle by unpaired t-test) was used. Error bars indicate SE; n = 4 in all conditions. C: ML-9 (100 µM) significantly enhanced 5 cmH\(_2\)O of pressure-induced increase in ATP content in the presence of 2.5 mM CaCl\(_2\) in Krebs (P < 0.05 vs. vehicle by unpaired t-test). Error bars indicate SE; n = 4 in vehicle and 5 in ML-9.

AJP-Renal Physiol • VOL 300 • MARCH 2011 • www.ajprenal.org

Downloaded from http://ajprenal.physiology.org/ by 10.220.33.6 on April 13, 2017
oxide than ionomycin-induced Ca\(^{2+}\) influx (16). Such specific regulation might be achieved by proximity in the same microdomain; TRPC1, adenylyl cyclase type VI, and endothelial NOS are localized to lipid rafts or caveolae (1, 28). The distinct suppressive function of SOCE in urothelial ATP release might therefore be mediated by its regulation of these enzyme activities.

Although the level of pressure at 45 cmH\(_2\)O is higher than the normal pressure range during urine storage, we have previously shown that urothelial ATP release was proportional to the pressure range of 15–45 cmH\(_2\)O (17). In addition, the inhibitory effect of heparin and the enhancing effect of ML-9 were also demonstrated in ATP release induced by 5 cmH\(_2\)O of pressure in this study (Fig. 4). These data indicate that the same regulatory system by Ca\(^{2+}\) works in urothelial ATP release by the physiological pressure during the filling stage. The common functions of Ca\(^{2+}\) release from the ER and SOCE at a broad range of pressure imply that this mechanism might be involved in both micturition and nociception pathways.

In summary, we have shown the dual, opposite roles of Ca\(^{2+}\) in the regulation of distention-induced urothelial ATP release. Ca\(^{2+}\) release from the ER drives ATP release, whereas SOCE inhibits it. Since SOCE is preceded by Ca\(^{2+}\) release from the ER, SOCE might suppress the amount of ATP release triggered by Ca\(^{2+}\) release from the ER and adjust it to a physiologically adequate level.

GRANTS
This work was supported by Grants-in-Aid for Scientific Research (C) (KAKENHI 21600001) from the Japan Society for the Promotion of Science and the discretionary budget earmarked for the President of Akita University.

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

REFERENCES