The regulation of distention-induced ATP release from urothelium by the adenylyl cyclase-cyclic AMP pathway

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ABSTRACT
Distention of the bladder during urine storage induces ATP release from urothelium, thereby facilitating transmission of visceral sensory signals to afferent nerve fibers. An excess of urothelial ATP release was found in interstitial cystitis, a condition accompanied by hyperesthesia of the urinary bladder; it remains unclear which signals are involved in this upregulation. The present study demonstrated that the adenylyl cyclase pathway enhances distention-induced ATP release in mouse bladder. In the absence of distention, adenylyl cyclase activation by forskolin or cyclic AMP increased by rolipram did not induce significant ATP release. However, forskolin or rolipram significantly enhanced ATP release from urothelium by a physiologically normal urine storage pressure (5 cmH₂O). Blockade of adenylyl cyclases did not alter pressure-induced ATP release in normal condition. Thus, the adenylyl cyclase-cyclic AMP pathway might be activated in pathological conditions and cause an excess of ATP release.

Recent studies revealed that urinary bladder epithelium (urothelium) functions as a visceral sensory organ as well as a barrier against urine (1, 3, 6). Distention of the bladder wall during urine storage causes a release of adenosine triphosphate (ATP) from the urothelium. Released ATP could act through the purinergic receptor P2X₃, which is expressed on afferent nerve terminals in close proximity to urothelial cells. Studies using P2X₃-deficient mice revealed that P2X₃ is essential for control of the urinary bladder volume reflex (11, 32); the pain response behavior was also reduced in these mice (11). These studies indicate that urothelial ATP release could play a key role in both volume- and noxious stimulus-evoked reflexes. In fact, an excess of urothelial ATP release was detected in interstitial cystitis or overactive bladder, conditions accompanied by visceral hyperesthesia leading to frequent urination or bladder pain (2, 28–30). However, the precise signaling cascade involved in the pathological increase of urothelial ATP release remains unknown. Identification of this signaling cascade may lead to improved strategies for clinical management of storage symptoms or bladder pain.

Urothelial ATP release is mediated by various signal transduction pathways (24). In these, Ca²⁺ plays an important role in the induction or regulation of ATP release. Urothelial ATP release was shown to be triggered by activation of transient receptor potential (TRP) V1 or TRPV4 (4, 22, 25), which are ion channels permeable to cations such as Ca²⁺. Knockout mice lacking TRPV1 or TRPV4 showed impairment of stretch-evoked increases in intracellular Ca²⁺ and in ATP release from the urothelium (4, 15). Furthermore, Ca²⁺ release from the endoplasmic reticulum (ER) is involved in induction of urothelial ATP release (23). On the other hand, store-operated Ca²⁺ entry, which is driven by depletion of Ca²⁺ stores in the ER, exerted an opposite, suppressive effect (23). Involvement of Ca²⁺ implies that ATP...
release is mediated by regulatory exocytosis from storage vesicles, a hypothesis that was partially supported by a previous study (2).

In addition to Ca²⁺, cyclic adenosine monophosphate (cAMP) might regulate urothelial ATP release. Vesicular exocytosis is enhanced by cAMP in various cell types (7, 13, 19). Metabotropic receptors that regulate cAMP are found in urothelium: G-protein Gα or Gβγ-coupled receptors, such as α2 and β-adrenergic (26, 27, 31) and muscarinic M2 and M4 (8, 21, 34) receptors. Furthermore, store-operated Ca²⁺ entry specifically inhibited adenylyl cyclase type 6 (10, 14). These findings raise the possibility that adenylyl cyclase-cAMP could be involved in upregulation of urothelial ATP release. In the present study, the effects of activation/inhibition of adenylyl cyclases or inhibition of phosphodiesterase 4 (PDE4) on distention-induced ATP release was investigated.

MATERIALS AND METHODS

Animals. Six- to 10-week-old C57BL/6 male mice were used in this study. All protocols were approved by the Animal Research Committee of Akita University, and followed the American Physiological Society guidelines for animal research.

ATP release assay using the Ussing chamber. The urothelial ATP release assay was performed as described previously (22, 23). In brief, isolated urinary bladders were opened vertically from the urethra to the apex. The opened bladder was mounted to act as a 7-mm² diaphragm 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 MgCl₂, 24.8 mM NaHCO₃, 1.2 mM NaH₂PO₄, and 11.1 mM glucose) with 2.5 mM CaCl₂; a continuous stream of 95% O₂/5% CO₂ was infused through the chamber solution. In the chemical stimulation assay, forskolin (Sigma) or rolipram (Sigma) was added to the mucosal side for 30 min; 50 μL of the mucosal-side chamber solution was sampled before and after stimulation, and ATP content was assayed by the luciferin-luciferase method (Kikkoman Co. Ltd., Tokyo, Japan) according to the manufacturer’s protocol. Release was determined by subtracting ATP content before from that after stimulation.

In the distention stimulation assay, 5 cmH₂O pressure was applied to the serosal (smooth muscle) side of the chamber for 20 min, which simulates a physiological range of pressure during urine storage; 50 μL of Krebs solution in the mucosal side of chamber was sampled before and after pressure application. Forskolin, rolipram, 2′,5′-dideoxyadenosine (DDA) (Sigma), or 9-(Tetrahydro-2-furyl) adenine (SQ22536) (Sigma) was added 30 min before applying pressure, and resulting ATP release to the mucosal side was determined. The experimental design of the distention stimulation assay is illustrated in Fig. 2A.

The amount of ATP release was determined using standard curves, which were constructed for each experiment using 3 × 10⁻⁷, 3 × 10⁻⁸, 3 × 10⁻⁹, and 3 × 10⁻¹⁰ M ATP. Data were analyzed using unpaired t tests. Statistical significance was assigned to differences having P values less than 0.05. All data are expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Adenylyl cyclase-cAMP pathway did not cause urothelial ATP release in the absence of distention

Investigation was made into whether activation of adenylyl cyclase-cAMP causes ATP release. Administration of vehicle (dimethylsulfoxide; DMSO) at a final concentration of 0.1% slightly increased ATP content in the mucosal chamber without applied pressure (0.22 ± 0.04 nM; Fig. 1). An activator of adenylyl cyclase, forskolin (50 μM), did not induce any significant change in ATP release (0.13 ± 0.07 nM) (Fig. 1). A similar result was obtained by administration of rolipram (10 μM; 0.12 ± 0.08 nM), a blocker...
of PDE4, which catalyzes the hydrolysis of cAMP (Fig. 1). Thus, neither activation of adenylyl cyclase nor an increase in cAMP evoked ATP release in the absence of distention of the bladder wall tissue.

**Adenylyl cyclase-cAMP pathway facilitated physiological distention-induced urothelial ATP release**

The effects of added forskolin or rolipram on distention-induced ATP release by pressure simulating normal physiological conditions during urine storage (5 cmH$_2$O for 20 min) was assessed (Fig. 2A); this induced 0.19 ± 0.03 nM of ATP release (Fig. 2B). Preincubation with forskolin significantly enhanced distention-induced ATP release (0.33 ± 0.05 nM, $p < 0.05$ vs. vehicle; Fig. 2B). Administration of 0.1 μM rolipram resulted in a 36% increase in distention-induced ATP release (0.27 ± 0.06 nM; Fig. 2B), and 10 μM of rolipram significantly facilitated the release (0.44 ± 0.14 nM, $p < 0.05$ vs. vehicle; Fig. 2B).

**Blockade of adenylyl cyclases did not affect distention-induced urothelial ATP release**

To determine whether the enhancement by adenylyl cyclases was in fact driven by the physiological distention of the bladder wall, inhibitors of adenylyl cyclases, SQ22536 (100 μM) or DDA (100 μM), were added to assess their effects on urothelial ATP release. Although SQ22536 decreased distention-induced ATP release to 77.5% (0.18 ± 0.03 nM) in comparison to vehicle (H$_2$O; 0.23 ± 0.03 nM), the difference was not significant ($p = 0.267$; Fig. 3A). Similarly, use of another inhibitor, DDA, decreased distention-induced ATP release to 59.4% (0.12 ± 0.02 nM) compared to vehicle, which was also not significant ($p = 0.094$; Fig. 3B).

**DISCUSSION**

The present study demonstrated that the adenylyl cyclase-cAMP pathway facilitated distention-induced ATP release from urothelium (Fig. 2). Since activation of adenylyl cyclases by forskolin or blockade of hydrolysis of cAMP by rolipram did not cause ATP release in the absence of distention (Fig. 1), this pathway contributes only to the upregulation of distention-induced ATP release, and not to its induction.

The adenylyl cyclase-cAMP pathway enhances vesicular exocytosis in a wide variety of secretory cell types. For example, cAMP-dependent protein kinase (PKA) enhances regulatory exocytosis of secretion vesicles in pancreatic beta-cells or neurons (13, 19). In urothelium, fusion of fusiform vesicles to the apical membrane is induced during the accumulation of urine (20). This fusion contributes to an enlargement of the bladder surface area during urine storage.
storage, and to a reduction of intracellular uropathogenic *E. coli* in fusiform vesicles, and was enhanced by forskolin (7). Because previous studies suggested the possibility that urothelial ATP release to basal membrane also occurs through vesicular exocytosis (2), this process might be also facilitated by the adenylyl cyclase-cAMP pathway. Consistent with this speculation, ATP release from dorsal root ganglion neurons was stimulated by \(\beta_3\) adrenergic receptors inducing a cAMP increase and PKA activation (18). On the other hand, it was reported that ATP release was mediated by ABC transporters (9) or pannexin hemichannels (12); therefore the regulation of these transporters/channels by adenylyl cyclase-cAMP pathway should also be considered for the release of ATP from urothelium.

The adenylyl cyclase-cAMP pathway is upregulated by \(G_i\) protein. In urinary bladder, \(\beta_1\), \(\beta_2\), and \(\beta_3\)-adrenoceptors, which are \(G_i\)-coupled-receptors, are expressed in urothelium (27, 31). Among adrenoceptor subtypes in urothelium, \(\alpha_1\) is involved in the induction of ATP release (17), and \(\beta\) activation triggered a production and release of nitric oxide (5). In the detrusor, \(\beta_3\)-adrenoceptor stimulation induced relaxation of the smooth muscle fibers (16, 33). In addition to these functions, urothelial \(\beta\)-adrenoceptors might facilitate ATP release. On the other hand, \(G_{i/o}\)-coupled-receptors, such as the \(\alpha_2\)-adrenoceptor (26) or the muscarinic receptors M2/M4 (8, 21, 34), are also expressed in urothelium; their functions remain controversial, but might involve suppressing the system that leads to enhanced ATP release.

Inhibition of adenylyl cyclases did not affect distention-induced ATP release in this study. Whereas a trend toward a decrease in ATP release by adenylyl cyclase blockade was detected in the 5 cmH\(_2\)O-pressure, the decrease was not statistically significant (Fig. 3). These results suggest that mechanical stimuli do not directly activate the adenylyl cyclase-cAMP pathway in the isolated, normal urinary bladder. One possibility is that the adenylyl cyclase-cAMP pathway might contribute to neurogenic or hormonal modulation of mechanically-evoked ATP release. Another is that it might be activated in pathological conditions, causing an excess of ATP release.

Excessive distention-induced ATP release from urothelium was detected in interstitial cystitis, a condition that is accompanied by frequent urination or bladder pain (2, 29). Possible involvement of the adenylyl cyclase-cAMP pathway in pathological visceral hyperesthesia of the urinary bladder should be ascertained in future studies.

In summary, we have demonstrated involvement of adenylyl cyclase-cAMP in the upregulation of urothelial ATP release using dissected urinary bladders of adult mice. Although the adenylyl cyclase system was not directly activated by distention, it enhanced urothelial ATP release evoked by physiologically normal distention. The adenylyl cyclase-cAMP pathway might be involved in facilitating the micturition reflex, under neurogenic or hormonal control, or it might cause an excess of ATP release in pathological conditions causing frequent urination.

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