MaxiK channel-triggered negative feedback system is preserved in the urinary bladder smooth muscle from streptozotocin-induced diabetic rats

Tsutomu NAKAHARA¹, Akiko MITANI¹, Yuko KUBOTA¹, Takeshi MARUKO¹, Kenji SAKAMOTO¹, Yoshio TANAKA², Katsuo KOiKE², Koki SHIGENOBU³ and Kunio ISHI¹

¹Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
²Department of Chemical Pharmacology
³Department of Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1 Miyama, Funabashi City, Chiba 274-8510, Japan

Abstract

MaxiK channel, the large-conductance Ca²⁺-sensitive K⁺ channel, facilitates a negative feedback mechanism to oppose excitation and contraction in various types of smooth muscles including urinary bladder smooth muscle (UBSM). In this study, we investigated how the contribution of MaxiK channel to the regulation of basal UBSM mechanical activity is altered in streptozotocin-induced diabetic rats. Although the urinary bladder preparations from both control and diabetic rats were almost quiescent in their basal mechanical activities, they generated spontaneous rhythmic contractions in response to a MaxiK channel blocker, iberiotoxin (IbTx). The effect of IbTx on the mechanical activity was significantly greater in diabetic rat than in control animal. Similarly, the basal mechanical activity was increased with apamin, an inhibitor for some types of small conductance Ca²⁺-sensitive K⁺ channels, and this effect was more pronounced for diabetic rat. However, in both control and diabetic animals, IbTx action was stronger than that of apamin. Diabetes also enhanced the responses to BayK 8644, an L-type Ca²⁺ channel agonist. The extent of this enhancement in diabetic bladder vs. control was, however, almost the same as that attained with IbTx. Expression levels for MaxiK channel as well as apamin-sensitive K⁺ channels and L-type Ca²⁺ channel were not altered by diabetes, when determined as their corresponding mRNA levels. These results indicate that diabetes can potentially increase the basal UBSM mechanical activity. However, in diabetic UBSM, the main negative-feedback system triggered by MaxiK channel is still preserved enough to counteract the possible enhancement of this smooth muscle mechanical activity.

Key words: diabetes, large-conductance Ca²⁺-sensitive K⁺ channel (MaxiK channel), rhythmic contraction, streptozotocin, urinary bladder
Introduction

Diabetes mellitus, a metabolic disease caused by a deficiency of pancreatic insulin secretion and/or impaired tissue responsiveness to this hormone, causes various short- and long-term complications in different systems in the body (Öztürk et al., 1996). Owing to significant developments in antidiabetic therapy, mortality rates due to diabetes mellitus itself and its acute complications have been greatly reduced. By contrast, its long-term complications resulting from such as chronic elevation of blood glucose and neuropathy are still serious problems for diabetic patients. In many cases, these long-term complications due to diabetes are ultimately associated with morphological and functional changes produced in various tissues consisted of smooth muscles (Öztürk et al., 1996). Urinary bladder is one of the tissues, structures and functions of which are impaired due to diabetes (Buck et al., 1974; Turner and Brading, 1997).

In diabetes, urinary bladder is structurally altered, and its weight and urinary capacity are increased with chronically residual urine (Buck et al., 1976; Ellenberg, 1980). The hypertrophy of diabetic urinary bladder may be an organ adaptation to hyperdiuresis due to nephropathy and increased water intake (Longhurst et al., 1991). In contrast, diabetes-induced increase in the total volume of urinary retention is associated with the decreased urinary bladder function to micturate. The dysfunctions of urinary bladder in diabetes mellitus may be largely related to, or as a result of both afferent and efferent (autonomic) neuropathy (Ellenberg and Weber, 1966; Faerman et al., 1973; Buck et al., 1976). However, contrary to the decrease in integral tissue functions, smooth muscle of diabetic urinary bladder itself seems to acquire in general more excitable mechanical activity in responses to chemical stimuli such as acetylcholine and substance P (Kolta et al., 1985; Latifpour et al., 1989, Latifpour et al., 1991; Dahlstrand et al., 1992; Kamata et al., 1993). The diabetic hyperreactivity of urinary bladder smooth muscle (UBSM) to biologically active substances may be ascribed to the denervation due to this disease. On the other hand, independently of autonomic nerves, UBSM itself generates spontaneous rhythmic contraction, which is myogenic in nature (Brading, 1992; Herrera et al., 2000; Imai et al., 2001). It has not been elucidated, however, whether this rhythmic contraction is enhanced in the diabetic UBSM.

The mechanisms by which UBSM generates spontaneous rhythmic contraction also still remain to be established. However, the activation and/or deactivation of some ion channels responsible for the formation of spontaneous action potential in this tissue seem to be significant in the induction of the mechanical activity (Brading, 1992). These ion channels include L-type Ca\(^{2+}\) channel and several types of K\(^{-}\) channels; the Ca\(^{2+}\) channel inhibitors diminish both spontaneous action potential and smooth muscle contraction whereas K\(^{-}\) channel inhibitors enhance both the electrical and the mechanical events (Brading, 1992; Heppner et al., 1997; Herrera et al., 2000; Imai et al., 2001). Among the K\(^{-}\) channels, role of large-conductance type of Ca\(^{2+}\)-sensitive K\(^{-}\) (MaxiK) channel is distinctively evident since the scorpion toxin blocker specific for this channel (iberiotoxin, IbTx) dramatically enhances the generation of action potential and contraction (Heppner et al., 1997; Herrera et al., 2000; Imai et al., 2001). This type of K\(^{-}\) channel is activated by both membrane depolarization and by intracellular Ca\(^{2+}\) (Toro et al., 1998; Orio et al., 2002; Tanaka et al., 2004), and thus should function as the key negative
feedback element to limit Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channel and to inhibit mechanical activity of this smooth muscle (Herrera et al., 2000; Imai et al., 2001; Tanaka et al., 2004). If MaxiK channel in UBSM is functionally impaired, it would enhance the mechanical activity of this muscle. However, it still remains unknown how the contribution of MaxiK channel to the regulation of urinary bladder mechanical activity is altered in diabetes mellitus.

Therefore, in the present study, we examined 1) whether diabetes changed the basal mechanical activity of UBSM and 2) how diabetes affected the negative feedback regulation of UBSM mechanical activity by MaxiK channel using streptozocin (STZ)-induced diabetic rats.

**Methods**

**Animal model of diabetes**

Male Wistar adult rats weighting 210–280 g were maintained on standard rat chow and tap water at libitum with 12:12-h light: dark cycles in a quite environment. Diabetes was induced by a single intraperitoneal injection of STZ (80 mg/kg) dissolved in sodium citrate buffer (pH 4.5). Age-matched control rats were treated with an injection of an equal volume of vehicle. Induction of diabetes was ascertained by determination of serum glucose concentrations 1 week after STZ and confirmed by a serum glucose concentration >300 mg/dl at death.

**Preparation of rat urinary bladder strips**

At 8–10 weeks after treatment with STZ or vehicle (sodium citrate buffer), rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The urinary bladder was removed and placed in ice-cold physiological saline solution (PSS, composition in mM: NaCl, 119; KCl, 4.7; MgSO\(_4\), 1.2; KH\(_2\)PO\(_4\), 1.2; CaCl\(_2\), 2.5; NaHCO\(_3\), 24; glucose, 11.0 and ethylenediaminetetraacetic acid, 0.023) (pH = 7.4). Four equally sized longitudinal strips (approximately 1–2 mm in width \(\times\) 10 mm in length) were isolated from the bladder body. These were used for measurement of mechanical responses.

**Measurement of mechanical activity**

One end of each strip was attached to an isometric force displacement transducer (model TB-611T, Nihon Kohden, Tokyo, Japan) by a cotton thread, and the other end was tied to a stainless-steel holder. Tension recorded was digitized at a sampling rate of 2 Hz with the use of a 12-bit analog-to-digital converter (model AD12-8(PM), Contec, Osaka, Japan) interfaced with a dedicated laboratory computer system (PC9821 Nr150, NEC, Tokyo, Japan). Strips were mounted in 20-ml of jacketed organ baths filled with PSS gassed with 95\% O\(_2\)–5\% CO\(_2\) at 37°C. The preparations were placed under an initial load (1 g) and the resting tension was adjusted every 15 min. The tissues were allowed to equilibrate for 60 min and the bath solution was exchanged every 15 min with fresh PSS.

After this period, contractile responses of the strips to iberiotoxin (IbTx), apamin or BayK 8644 were measured by applying these ion channel modulators in a cumulative fashion. Atropine, phentolamine, propranolol, tetrodotoxin and \(\alpha\), \(\beta\)-methylene ATP (1 \(\mu\)M each) were present throughout the experiments to measure the changes in basal mechanical activities.
without nerve-mediated contractile components, as described previously (Herrera et al., 2000).

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

The rat urinary bladders were homogenized in TRIZOL reagent (GibcoBRL Life Technologies) and total RNA was extracted. Single-stranded cDNAs were synthesized from total RNA using oligo dT priming and Superscript II Reverse Transcriptase (GibcoBRL Life Technologies).

Primers for the amplification of the rat KCa channel γ subunit gene (sense 5'-GGACTTAGGGATGTTG-3'; antisense 5'-GGGATGGAGTGGAGAGA-3'), those for the rat KCa channel δ1-subunit gene (sense 5'-ACCAGTGTCGCCCTGTT-3'; antisense 5'-AGGTGGCCAGAAAGGAGA-3'), those for the rat SK2 channel gene (sense 5'-TACCACGCGAGGAAATACA-3'; antisense 5'-CACCAGCCAGCAAGGAG-3'), those for the rat SK3 channel gene (sense 5'-ACAACGGGAAAAACCAAC-3'; antisense 5'-AGAGGGGTTAGAGAAGGAG-3') and those for the rat L-type Ca2+ channel α1C subunit gene (sense 5'-AGGCTGTGAATGTCAGTG-3'; antisense 5'-CTCATGTTGCAAGGATGC-3') were used. KCa channel γ subunit and δ1-subunit primer sets, SK2 and SK3 channel primer sets, and rat L-type Ca2+ channel α1C subunit primer sets were designed using previously published sequences (Genbank Nos. U55995, U55498, U69882, U69884 and M67515, respectively). As a control for cDNA synthesis, α-actin-specific primers (sense 5'-GTGGGCCGCCCTAGGCACCA-3'; antisense 5'-TTAATGTCACGCACGATTTC-3') were used.

Polymerase chain reaction (PCR) was performed in a final volume of 50 µl containing 5 µl PCR buffer (10×), 4 µl dNTPs (2 mM), 1 µl of each sense and antisense primer (50 pmol/µl), 1 µl cDNA, and 0.25 µl AmpliTaq Gold DNA polymerase (5 U/µl; Applied Biosystems), using a programmable thermal controller (PTC-100, MJ Research).

The PCR conditions were initial denaturation for 10 min at 96°C, followed by 25–35 cycles of 1 min at 96°C, 1 min at 60°C, and 1 min at 72°C and a final elongation for 10 min at 72°C. Ten-microliter aliquots of the PCR products were separated on a 1.2% agarose gel and stained with 0.1% ethidium bromide. The density of the bands was analyzed by NIH image software.

**Data analysis and statistics**

Data were expressed as mean ± s.e.mean with N = number of rats and n = number of preparations. Because bladders of diabetic rats weighed more than normal rat bladders, we normalized contractility data as milligrams of tension generated per milligram of tissue weight. Data were analyzed using Student’s t-test. A P value less than 0.05 was considered significant.

**Drugs**

The following drugs were used: atropine sulfate, α, β-methylene ATP, S(-)-Bay8644, phentolamine hydrochloride, DL-propranolol hydrochloride, streptozotocin (STZ), tetrodotoxin, (Sigma Chemical, St. Louis, MO, USA), apamin, and iberiotoxin (IbTx) (Peptide Institute, Osaka, Japan). Stock solutions were diluted appropriately using KRB.
Results

Effects of STZ-induced diabetes in rats

Some of the characteristics of the rats used in this study are shown in Table 1. All control rats showed an increase in body weight, whereas the diabetic rats failed to gain weight over 8–10 week period following STZ injection. The plasma glucose level in the diabetic rats was significantly higher than that in normal rats. Bladder weight in absolute terms was increased in the diabetic rats by approximately 2.0 times that of the controls.

<table>
<thead>
<tr>
<th>Plasma glucose (mg/dl)</th>
<th>Control (N=11) 189 ± 10</th>
<th>Streptozotocin (N=11) 573 ± 22 a</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>419 ± 4</td>
<td>279 ± 5 a</td>
</tr>
<tr>
<td>Bladder weight (mg)</td>
<td>137 ± 7</td>
<td>238 ± 8 a</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.mean. *P<0.01 vs. corresponding controls values.

Contractile responses to IbTx, apamin and BayK 8644

The spontaneous mechanical activities under basal conditions were not dramatically different between the control and diabetic bladder preparations (3.16 ± 0.81 mg tension/mg wet weight vs. 3.59 ± 0.50 mg tension/mg wet weight, n = 17–19). In both preparations, addition of IbTx enhanced the amplitude of rhythmic contractions in a concentration-dependent manner (10–100 nM) (Fig. 1). The increasing effect of IbTx was significantly greater in diabetic rats.
than in controls (Fig. 2A). On the other hand, contraction frequency was not affected by diabetes (for example, at 100 nM, control 7.7 ± 1.1 contractions/min vs. diabetes 7.3 ± 0.5 contractions/min, n = 7–8). Apamin (3–100 nM) also enhanced the mechanical activities in both normal and diabetic urinary bladders. However, the effects of apamin were more pronounced for diabetic urinary bladder and markedly smaller than those of IbTx (Fig. 2B).

Figure 3 shows the effects of BayK 8644 on the contractile responses of bladder strips. As shown clearly in Fig. 3, increasing effect of BayK 8644 on the mechanical activity was conspicuously enhanced in diabetic urinary bladder, which was also evident from the summaries in Fig. 4.

Figure 5 shows the relationships for the developed tensions with tested three different ion channel modulators (IbTx, apamin and BayK 8644) between control and diabetic urinary bladders. As shown in the figure, diabetes potentiated urinary bladder mechanical activities almost to the similar degree for these three types of modulators.

**RT-PCR analysis of mRNA levels in rat urinary bladder**

We examined the expression of mRNAs for γ and δ1-subunit of MaxiK channel, two subtypes (SK2 and SK3) of SKCa channel and α1C subunit of L-type Ca\(^{2+}\) channel in isolated rat urinary bladders using RT-PCR. As shown in Fig. 6A, these mRNAs were detected in both normal and diabetic bladders. The expression levels of the mRNAs were not different between normal and diabetic bladders (Fig. 6B).

**Discussion**

In the present study, we showed that rhythmic contraction of UBSM is enhanced with IbTx.
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much more strongly in diabetic rat whereas this mechanical activity is almost quiescent apparently in both normal and diabetes rats. Diabetic urinary bladder was also shown to generate spontaneous contraction more vigorously in response to the L-type Ca\(^{2+}\) channel agonist, BayK 8644. These findings indicate that UBSM is potentially prone to generate spontaneous contraction in diabetes. However, possible increment of the mechanical activity in diabetic UBSM can be suppressed since MaxiK channel-triggered inhibitory regulation mechanism is still preserved enough to counteract diabetes-induced hyperactivity of this tissue.

**Fig. 3.** Typical recordings of effect of BayK 8644 on the urinary bladder preparation from a control (A) and a diabetic rat (B). BayK 8644 was added cumulatively. Each arrow shows point of application of BayK 8644, whose concentrations are given above arrows.

**Fig. 4.** Effect of BayK 8644 on the contractility of rat urinary bladder preparations from control and diabetic rats. Each column with a vertical bar represents mean ± s.e.mean from five separate preparations. *P<0.05, **P<0.01 vs. corresponding control values.
Fig. 5. Relationship between the developed tensions with iberiotoxin, apamin and BayK 8644 in control bladders and those in diabetic bladders from the data shown in Fig. 2 and Fig. 4. The relationships obtained from these agents were practically identical.

Fig. 6. Detection of mRNAs for α- and β1-subunit of MaxiK channel (BKα and BKβ), two subtypes (SK2 and SK3) of SKCa channel, α1C subunit of L-type Ca2+ channel (VDCC) and β-actin in isolated rat urinary bladders using RT-PCR. Representative examples (A) and quantitation of signals for MaxiKα, MaxiK β, SK2, SK3 and VDCC (B). The intensity of each band was quantified by densitometry: the data are normalized by β-actin mRNA. Data expressed as mean ± s.e.mean obtained from four to five bladders.
Our results from rat urinary bladder showed that the mechanical activity of this smooth muscle was increased more strongly with IbTx than apamin though their stimulatory effects were both substantial. This tendency was the same in both control and diabetic urinary bladders. Similarly, spontaneous mechanical activity is enhanced with both IbTx and with apamin in the urinary bladder smooth muscles from guinea-pigs (Suarez-Kurtz et al., 1991; Herrera et al., 2000; Imai et al., 2001) and pigs (Buckner et al., 2002). However, also in the bladders of these species, IbTx increases contraction amplitude more strongly than apamin (Herrera et al., 2000; Imai et al., 2001; Buckner et al., 2002). These results indicate that as compared to apamin-sensitive SKCa channels (SK2 and SK3) (Dale et al., 2002), MaxiK channel contributes more profoundly to the inhibitory mechanism for the mechanical activity of urinary bladder smooth muscle, and suggests that this is not a species-specific phenomenon, but is applicable over the species differences.

Alterations in the mechanical function of urinary bladder due to diabetes have been thought to be associated with autonomic neuropathy. Therefore, enhanced sensitivity to IbTx of the diabetic urinary bladder may be attained through the dysfunctions of autonomic nerves contained in this tissue. However, in our present study, muscle mechanical activities were obtained in the presence of atropine (muscarinic receptor antagonist), phentolamine (α-adrenoceptor antagonist), propranolol (β-adrenoceptor antagonist), α, β-methylene ATP (purinergic receptor antagonist) and tetrodotoxin (Na+ channel blocker). Therefore, possible contribution of neuronal components can be ruled out to account for the different sensitivities to IbTx between control and diabetic urinary bladders at least in our present experimental condition.

One possibility to account for the more prominent effects of IbTx on the diabetic UBSM would be the enhanced expression of the protein complexes for MaxiK channel due to this disease. Therefore, we first compared the expression levels for the pore-forming γ-subunit of this channel as mRNA level. Using RT-PCR analysis, we detected mRNA transcripts encoding for MaxiK channel pore-forming δ-subunit protein in the urinary bladder smooth muscles from normal and STZ-induced diabetic rats. However, mRNA levels for the channel α-subunit were substantially the same in both rats. Thus, elevated expression of MaxiK channel α-subunit cannot explain the diabetes-induced enhancement of urinary bladder contraction to IbTx. Secondly, we examined the diabetic urinary bladder for possible alterations in the expression of mRNA for MaxiK channel regulatory γ (δ1)-subunit. This is because MaxiK channel is a protein complex of the pore-forming γ subunit and the regulatory α subunit (Knaus et al., 1994; Tanaka et al., 1997; Toro et al., 1998). Among MaxiK channel γ subunit subfamily (δ1–δ4), δ1-type is dominant in smooth muscle cells (Orio et al., 2002), and its presence increases Ca²⁺ sensitivity and voltage-dependence of this channel (McManus et al., 1995; Meera et al., 1996). Especially, the expression level for δ1 subunit is high in UBSM (Jiang et al., 1999). Therefore, it seems possible that δ1 subunit expression is elevated in diabetic urinary bladder smooth muscle, which may explain the augmented sensitivity to IbTx. However, mRNA levels for MaxiK channel δ1 subunit were not significantly affected by diabetes, and thus, this possibility can also be ruled out. Taken together, apparent increment of MaxiK channel contribution to the regulation of diabetic UBSM contractility cannot be ascribed to the enhanced expression of this type of ion
channel protein complexes. However, at present, we cannot completely rule out this possibility since we have not yet directly confirmed this at the channel protein level; diabetes might increase the expression of MaxiK channel proteins through a post-transcriptional mechanism.

Other possibilities to explain our present findings would include the alterations in the membrane potential and intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]) since MaxiK channel is a voltage-dependent ion channel to possess Ca\(^{2+}\) sensitivities (Toro et al., 1998; Orio et al., 2002). Concerning to these possibilities, we obtained an interesting finding that muscle sensitivity to BayK 8644 was strongly increased in diabetic urinary bladder tissue. This implies that 1) diabetes elevates the expression levels for L-type Ca\(^{2+}\) channel and/or 2) resting membrane potential of UBSM cells is more depolarized in diabetes. However, diabetes did not significantly alter the expression level for L-type Ca\(^{2+}\) channel, when determined as the mRNA for its major pore-forming \(\alpha (\alpha_{1c})\) subunit in smooth muscle cell (Kuriyama et al., 1998). Therefore, enhanced sensitivity of diabetic urinary bladder to BayK 8644 cannot be accounted for the increased expression of L-type Ca\(^{2+}\) channel. The second possibility (resting membrane potential changes) might be also ruled out or its contribution seems to be marginal since STZ-induced diabetes does not affect resting membrane potential of UBSM cells (Hashitani and Suzuki, 1996). Therefore, possible changes in resting membrane potential cannot fully account for the enhancement of MaxiK channel roles in the regulation of diabetic urinary bladder smooth muscle.

On the other hand, the possibility of alternation in the Ca\(^{2+}\) concentration in UBSM cells cannot be completely excluded, because diabetes may affect the local Ca\(^{2+}\) concentration in the subplasmalemma area by impairing function of Ca\(^{2+}\) buffering system, although the [Ca\(^{2+}\)] level of UBSM cells is not elevated significantly due to diabetes (Waring and Wendt, 2000). Furthermore, in diabetic urinary bladder smooth muscle, changes in the Ca\(^{2+}\) sensitivity of contractile apparatus should be taken into account; in rat urinary bladder, diabetes increases the sensitivity of the contractile apparatus to Ca\(^{2+}\) (Belis et al., 1991; Kamata et al., 1992; Longhurst et al., 1992; Waring and Wendt, 2000). Some investigators have reported, however, that contractile responsiveness of rat urinary bladder is diminished rather than potentiated in diabetes (Longhurst and Belis, 1986; Gupta et al., 1996). If the former is the case in our experiment, a larger contraction may be generated with IbTx as well as with apamin and BayK 8644 in diabetic urinary bladder smooth muscle, and these changes should be caused to similar extents with all stimulations. Actually, when the extents of the diabetes-induced enhancement of urinary bladder mechanical activity were compared for these ion channel modulators, they were found to be practically identical (Fig. 5). These findings seem to strongly indicate that Ca\(^{2+}\) sensitivity of the contractile apparatus is increased in diabetic urinary bladder smooth muscle, and thus this may account for the apparent enhancement of this tissue contractility to IbTx. However, since MaxiK channel can also be activated by other factors such as protein kinases and membrane stretch, changes of the channel biophysical properties by these factors should be also considered.

Finally, physiological and pathological significances of the present findings will be mentioned. Generally, the role of MaxiK channel in control of smooth muscle mechanical activity is considered to provide a negative-feedback mechanism to these tissues. This can also
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be applied to urinary bladder mechanical function since detrusor muscle shows overactivity when this channel is inhibited with IbTx. If this is also true in in vivo system, possible urinary frequency is suppressed owing to the presence of UBSM cell MaxiK channels, and thus, this channel role is significant to preserve normal urinary bladder function. This channel role in the regulation of urinary bladder mechanical activity seems to be more important in diabetes; in this disease, diuresis can be enhanced due to an increased hydropisia, and thus a large volume of urine is stored in the urinary bladder. If the urinary bladder shows a hyperreactivity, this will result in an increased urinary frequency. However, this can be inhibited since the possible overactivity of UBSM is suppressed due to the preserved negative-feedback system triggered by MaxiK channel.

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References


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