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The structure, function and distribution of the mouse TWIK-1 K^+ channel

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Abstract The two P domain K^+ channel mTWIK-1 has been cloned from mouse brain. In *Xenopus* oocytes, mTWIK-1 currents are K^+-selective, instantaneous, and weakly inward rectifying. These currents are blocked by Ba^{2+} and quinine, decreased by protein kinase C and increased by internal acidification. The apparent molecular weight of mTWIK-1 in brain is 81 kDa. A 40 kDa form is revealed after treatment with a reducing agent, strongly suggesting that native mTWIK-1 subunits dimerize via a disulfide bridge. TWIK-1 mRNA is expressed abundantly in brain and at lower levels in lung, kidney, and skeletal muscle. In situ hybridization shows that mTWIK-1 expression is restricted to a few brain regions, with the highest levels in cerebellar granule cells, brainstem, hippocampus and cerebral cortex.

Key words: Heterologous expression; *Xenopus* oocyte; In situ hybridization; Two P domains

1. Introduction

Many functionally different K^+ channels are involved in regulating electrical activity in the nervous system [1–3]. Most of these channels fall into two main classes: the depolarization-activated (or outward rectifier) K^+ channels and the channels which are not activated by depolarization and are collectively referred to as inwardly rectifying K^+ channels. Inward rectifier channels conduct large inward currents at membrane potentials negative to the potassium equilibrium potential (E_K) and small outward currents at more positive potentials. They are mainly involved in the maintenance of the resting conductance but also in more diverse cellular processes such as K^+ homeostasis, synaptic inhibition, and neuronal firing [2]. Different members of this class have been cloned and expressed (for review see [4]). Each channel subunit has two transmembrane domains and one conserved domain, designated as the P domain, which is involved in the formation of the potassium-selective aqueous pore. These channels are either 'strong' inward rectifiers (i.e. they pass essentially no current at potentials more positive than E_K), such as the classical IRKs and the G-coupled K^+ GIRKs, or 'weak' inward rectifiers such as ROMK1. The rectification has been shown to be caused by internal Mg^{2+} and polyamines (for reviews see [5,6]).

Recently, a new type of weak inward rectifier K^+ channel called TWIK-1 has been cloned from human [7]. It shows considerable differences in structure from other classical inward rectifiers (K_{IR}) but also from outward rectifier K^+ channels (Kv). It has four putative transmembrane segments, instead of two for K_{IR} and six for Kv channels, and it comprises two pore-forming domains instead of only one as is the rule for all previously cloned mammalian K_{IR} and Kv channels. The equivalent mouse TWIK-1 channel has now been cloned and expressed. The tissue distribution, the developmental properties, and the brain localization of the channel have now been characterized.

2. Materials and methods

2.1. cDNA cloning and sequencing

A mouse brain cDNA library (Stratagene) was screened under mild stringency conditions using a random primed ^{32}P-labelled DNA probe corresponding to the human TWIK-1 coding sequence (Genbank accession number U33632). Filters were hybridized and washed as previously described [8]. The positive λZAPII phages were converted to plasmid cDNAs by rescue excision (Stratagene). The cDNA inserts were characterized by restriction enzyme analysis and by partial or complete sequencing on both strands using the dye terminator kit and an automatic sequencer (Applied Biosystems model 373A).

2.2. Expression in Xenopus oocytes

The coding sequence of mTWIK-1 was amplified with a low error rate DNA polymerase (Pwo DNA polymerase, Boehringer) and subcloned into pEXO, a plasmid designated for expression in *Xenopus* oocytes [9]. The sequence integrity of pEXO-mTWIK-1 was checked by sequence determination of the insert. Plasmid was linearized by *Eco*RI and capped TWIK-1 cRNAs synthesized in vitro using the T7 RNA polymerase (Stratagene). *Xenopus laevis* preparation, cRNA injection, and electrophysiology were carried out as previously described [10].

2.3. Northern blot analysis

Poly(A)$^+$ RNAs were isolated from adult mouse tissues and blotted onto nylon membranes as previously described [11]. The blots were probed with the ^{32}P-labelled insert of pBS-mTWIK-1 in 50% formamide, 5×SSPE (0.9 M sodium NaCl, 50 mM sodium phosphate pH 7.4, 5 mM EDTA), 0.1% SDS, 5×Denhardt's solution, 20 mM potassium phosphate (pH 6.5) and 200 μg/ml denatured salmon sperm DNA at 55°C for 18 h and washed stepwise to a final stringency of 0.1×SSC (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0), 0.3% SDS at 55°C.

2.4. In situ hybridization

Riboprobes were generated by in vitro transcription by using [α-^{33}P]UTP (3000 Ci/mmol, ICN Radiochemicals) with T7 (antisense probe) and T3 (sense probe) RNA polymerases (Boehringer) from linearized plasmid containing a 0.7 kb DNA fragment of mTWIK-1 cDNA. Adult BALB/c mice were killed after a transcardial perfusion with a phosphate-buffered 4% paraformaldehyde solution. Dissected tissues were further fixed in the same solution for 2 h and immersed in 20% sucrose overnight. Frozen tissue sections (12 μm) were cut on a cryostat (Microm). Prehybridization included treatment with glycine 0.1 M, Triton X-100 0.3%, proteinase K (10 μg/ml, 15 min), and triethanolamine 0.1 M/acetic anhydride 0.25%. Hybridization was carried out at 60°C overnight in a buffer containing 50% formamide, 5×Denhardt's solution, 20 mM $NaPO_4$, 10% dextran sulfate, 5% sarcosyl, and 2×SSC. Following hybridization, sections were washed

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twice (45 min) in 1×SSC, treated during 10 min with RNase A (10 μg/ml), and washed in 0.1×SSC at room temperature (30 min). Specimens were then dehydrated, air dried, and exposed to Hyperfilm β-max (Amersham) for 10 days.

2.5. Western blot analysis

Brains from adult mice were isolated and homogenized with a Potter homogenizer (20 strokes at 800 rpm) in 10 volumes of an ice-cold buffer containing 50 mM Tris pH 8.0, 10% sucrose, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 800×*g* for 20 min, the nuclear pellet was discarded, and the supernatant was centrifuged at 19000×*g* for 20 min. The pellet (microsomal fraction) was resuspended in 5 mM Tris pH 8.0, 0.1 mM PMSF, left on ice for 1 h, then manually homogenized in a Potter. Synaptic membranes were isolated by combined flotation sedimentation gradient centrifugation as described [12]. The synaptic membrane interphase was recovered, pelleted at 100000×*g* for 40 min, and the membranes were resuspended in 5 mM Tris pH 8.0. After determination of the protein concentration, the synaptic membranes were stored at −70°C until use.

For Western blot analysis, aliquots of membrane preparations were mixed with Laemmli's sample buffer in the presence or absence of β-mercaptoethanol and incubated at 37°C for 10 min. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond C-extra, Amersham). The blots were probed with affinity-purified rabbit anti-TWIK-1 antibodies (1:1000) [13] and a secondary goat anti-rabbit antibody (1:10000) conjugated to horseradish peroxidase (Jackson ImmunoResearch). The immune complexes were visualized with the SuperSignal substrate following the manufacturer's instructions (Pierce).

3. Results

3.1. Primary structure and functional expression of TWIK-1

The screening of a mouse cDNA library with a human TWIK-1 probe under low stringency conditions resulted in the isolation of 8 cDNAs of approximately 2–2.1 kb. All cDNAs shared an identical open reading frame (ORF) of 1011 nucleotides encoding a 336 amino acid polypeptide (Fig. 1). The nucleotide sequence of the ORF and the deduced amino acid sequence were 88.6% and 94% identical to that of the human TWIK-1 channel, respectively. This mouse channel was designated mTWIK-1. It contains 4 putative transmembrane segments (M1–M4) and two putative pore-forming domains (P1 and P2). mTWIK-1 has one potential *N*-linked glycosylation site in the loop M1P1 (residue 95), one potential phosphorylation site for protein kinase C (PKC) in the linker M2M3 (residue 161), one for calcium calmodulin kinase II in the cytoplasmic N-terminus (residue 19), and one for casein kinase II in the C-terminus (residue 303).

Fig. 2A illustrates TWIK-1 currents obtained from one oocyte that was injected with mTWIK-1 cRNA 48 h before the recording. In standard ND96 solution, mTWIK-1 currents are almost instantaneous and non-inactivating. mTWIK-1 currents present a weak inward rectification. The current-voltage relationship for the steady-state current is linear up to 0 mV then saturates for stronger membrane depolarization (Fig. 2B). mTWIK-1 is highly selective for K^+. The relationship between the reversal potential and the external K^+ concentration is close to the predicted Nernst value (49 mV/decade, $n=4$), as illustrated in Fig. 2C,D. As expected for a K^+-selective channel active at every membrane potential, mTWIK-1 is capable of driving the membrane resting potential (E_m) close to the K^+ equilibrium potential (E_K) ($E_m = -76 \pm 1$ mV in injected oocytes ($n=20$) versus $E_m = -41 \pm 2$ mV in non-injected oocytes ($n=10$)).

The effects of various drugs on the current elicited by voltage pulses to +30 mV (or +0 mV with the same results) were studied with the double microelectrode method. As for hTWIK-1, the mTWIK-1 currents were inhibited by quinine (35% of the current inhibited by 100 μM quinine, not shown) and by Ba^{2+} ($IC_{50} = 35.2$ μM). Less than 5% inhibition was obtained with the following K^+ channel blockers: Cs^+ (1 mM), TEA (1 mM), glibenclamide (10 μM), 4-AP (100 μM), apamin (10 nM), dendrotoxin (100 nM) and charybdotoxin (10 nM). Moreover, known K^+ channel openers like P1060 (100 μM), lemakalin (100 μM) and pinacidil (100 μM) were without effect. The class III antiarrhythmic amiodarone (100 μM) and tedisamil (100 μM) were also without effect. Fig. 2E shows that the mTWIK-1 currents are decreased upon internal acidification caused by CO_2 bubbling, with 61 ± 12% ($n=3$) of inhibition after 7 min of perfusion. mTWIK-1 currents are positively modulated by activation of PKC with the phorbol ester PMA (40 nM) which produced a channel activation of 46 ± 2% ($n=4$) (Fig. 2F) while its inactive analogue, PDA (40 nM), was ineffective on mTWIK-1 currents (not shown). Exposure to 40 nM PMA during 10 min is not accompanied by changes in membrane capacitance (124 ± 10.7 μF ($n=5$) before PMA application, and 123 ± 10 μF after PMA application). The variation of internal Ca^{2+} obtained with the ionophore A23387 (10 μM) was without effect on mTWIK-1 currents. Finally, the channel is probably not sensitive to cAMP since perfusions of an activator of adenylate cyclase, forskolin (10 μM), or of an indirect inhib-

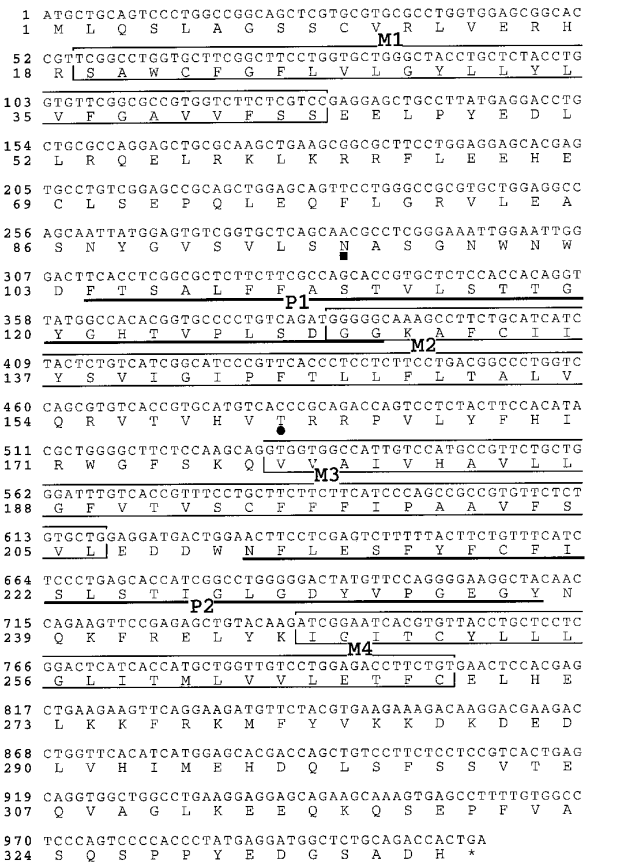

Fig. 1. Nucleotide and deduced amino acid sequences of mTWIK-1. The hydrophobic segments (M1–M4) are boxed and the P domains are underlined. ●: threonine residue in a consensus protein kinase C recognition site. ■: potential *N*-linked glycosylation site.

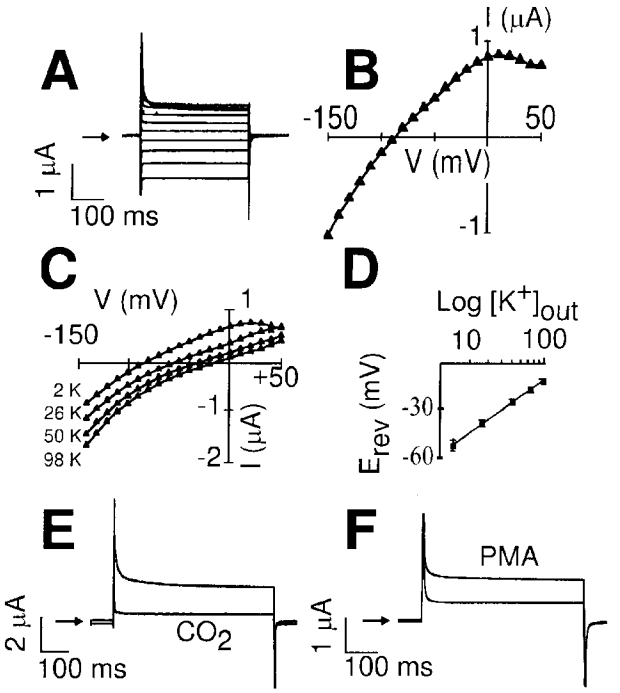

Fig. 2. Functional expression of mTWIK-1 channels in *Xenopus* oocytes. A: mTWIK-1 currents elicited by 350 ms voltage pulses from −130 to +50 mV in 20 mV steps from a holding potential of −80 mV in standard ND96 solution. B: Current-voltage relation measured at the end of voltage pulses as in A, from −150 to +50 mV, in 10 mV steps. C: mTWIK-1 current-voltage relationships as in B, in various external K^+ concentrations (external K^+ was substituted by *N*-methyl D-glucamine). D: Reversal potentials of mTWIK-1 currents as a function of external K^+ concentration ($n=4$). E: mTWIK-1 current, elicited by 500 ms voltage pulses to +30 mV, before and after 7 min perfusion of a solution bubbled with CO_2, which leads to internal acidification of the oocyte. F: mTWIK-1 current, elicited by 500 ms voltage pulses to +30 mV, before and after 7 min perfusion with 40 nM PMA. In A, E, and F the zero current levels are indicated by arrows.

itor of adenylate cyclase in the *Xenopus* oocyte, progesterone (10 µM), were also without effect.

3.2. Distribution of mouse TWIK-1 mRNA

The expression of TWIK-1 in various adult mouse tissues was examined by RNA blot analysis. As shown in Fig. 3, the TWIK-1 probe detected a single band of mRNA with an approximate estimated size of 2.1 kb. TWIK-1 mRNA is expressed at the highest level in the brain, at moderate levels in kidney and lung, and at a low level in skeletal muscle (Fig. 3A). No positive signal was obtained from heart and liver. In the brain, the expression increased from 2 to 8 postnatal days and became stable from 8 to 120 postnatal days (Fig. 3B).

In situ hybridization experiments revealed that TWIK-1 mRNA is localized to a few brain regions (Fig. 4). The strongest hybridization signals are observed in the hippocampal formation, cerebral cortex and cerebellum. In the hippocampal formation, strong signals are observed in all the layers including the dentate gyrus. The signal in the granule cells of the dentate gyrus seems stronger than in the CA1, CA2, and CA3 subfields (Fig. 4A,D). A strong signal is obtained in all regions and throughout the entire thickness of the cerebral cortex. In the cerebellum a high signal is seen in the granular layer. The deep cerebellar nuclei (Fig. 4C), and several nuclei

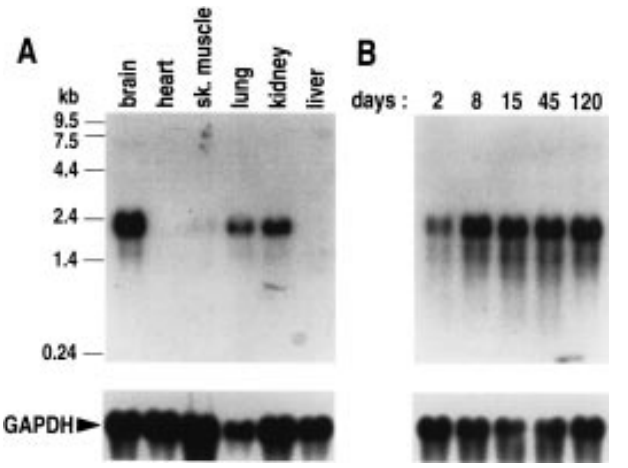

Fig. 3. Northern blot analysis of mTWIK-1 expression in tissues and developing brain. RNA was prepared from adult mouse tissues (A) and from brain at postnatal days 2, 8, 15, 45, and 120 (B). 5 µg of poly(A)$^+$ RNA was loaded per lane. Autoradiograms were exposed for 48 h. For control, blots were reprobed with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene.

of the brainstem including the pontine nucleus (Fig. 4A) and nuclei within the reticular formation (Fig. 4C) also display moderate signals. A very weak signal is obtained in the thalamus (Fig. 4D), and faint to background signals are seen in all other regions including the olfactory bulb (Fig. 4E), caudate-putamen and substantia nigra.

3.3. Western blot analysis of the mTWIK-1 protein in brain. Implication for TWIK-1 structure

We have previously shown that the recombinant TWIK-1 protein expressed in insect cells dimerizes via a disulfide bond to give a glycosylated complex with an apparent molecular weight (MW) of approximately 80 kDa [13]. However, it had not been shown that the native TWIK-1 channel is also a covalent dimer. To verify that the 81 kDa band detected by anti-TWIK-1 antibodies in the brain membranes corresponds to a disulfide-bridged dimer, brain synaptic membrane proteins were analyzed in the absence or presence of the reducing agent β-mercaptoethanol (β-ME). As expected, the 81 kDa band gave rise to a new band of approximately 40 kDa under

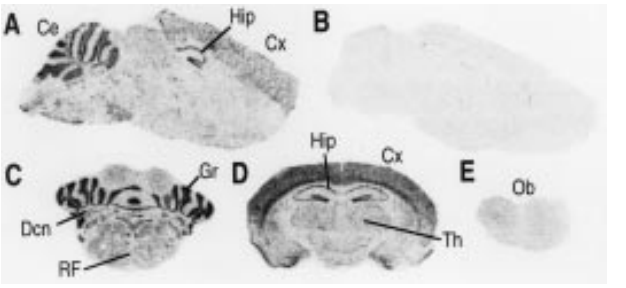

Fig. 4. Localization of mTWIK-1 in adult mouse brain by in situ hybridization. Photomicrographs of autoradiograms of brain sections after in situ hybridization histochemistry. A, B: Sagittal sections hybridized with mTWIK-1 antisense (A) and sense (B) riboprobes. C–E: Coronal sections at the level of cerebellum (C), hippocampus and thalamus (D), and olfactory bulb (E). Abbreviations: Ce, cerebellar cortex; Cx, neocortex; Dcn, deep cerebellar nuclei; Gr, cerebellar granular layer; Hip, hippocampal area; Ob, olfactory bulb; RF, reticular formation; Th, thalamus.

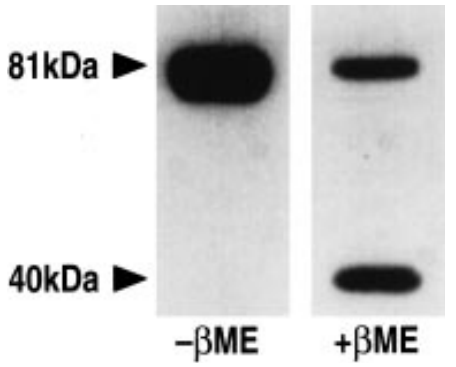

Fig. 5. Western blot analysis of the mTWIK-1 protein. Synaptic membrane preparations (25 μg of proteins) from mouse whole brain were probed with affinity-purified anti-TWIK-1 antibodies. Before analysis, proteins were incubated either in the presence (lane: +βME) or in the absence (lane: −βME) of the reducing agent β-mercaptoethanol. This treatment resulted in the detection of an additional band at approximately 40 kDa. The labelled bands were abolished by preincubating the antibodies with the antigenic TWIK-1 fusion protein.

reducing conditions (Fig. 5). A longer treatment of the membrane proteins with β-ME resulted in a complete conversion of the 81 kDa band into the 40 kDa (data not shown). The MW of 40 kDa is in good agreement with the calculated MWs of the mouse TWIK-1 protein (37 kDa). This result strongly suggests that the TWIK-1 K^+ channel subunit dimerizes via a disulfide bond in the brain as previously observed in heterologous expression systems [13].

4. Discussion

Recently, we have characterized a new structural class of K^+ channels with four transmembrane segments and two P domains. TWIK-1 was originally cloned from human [7] and TREK-1, the second member of this family, was isolated from the mouse [14]. Despite a similar overall structure, these two channels have totally different functional properties. The most obvious difference resides in the rectification properties. K^+ currents generated by TWIK-1 are inwardly rectifying while K^+ currents generated by TREK-1 are outwardly rectifying. In order to compare the properties of both channels in the mouse, TWIK-1 was cloned from mouse brain by screening a cDNA library under low stringency. Expressed in *Xenopus* oocytes, this mouse channel has the same biophysical, pharmacological and regulation properties as the human TWIK-1. Briefly, currents are time-independent, non-inactivating and weakly inwardly rectifying. They are inhibited by quinine and Ba^{2+}, down-regulated by internal acidosis, and up-regulated by PKC. These results clearly establish that the cloned channel is the product of the orthologous gene of TWIK-1 in the mouse and it was called mTWIK-1. Northern blot analysis showed a wide distribution of mTWIK-1 mRNA among adult mouse tissues. mTWIK-1 is particularly well expressed in the brain but is also expressed in kidney, lung, and skeletal muscle. The expression of mTWIK-1 in the brain reaches a maximal level from 8 days after birth. Such an increase in expression levels in the mouse developing brain has been previously observed for various other K^+ channels between day 2 and adulthood [11]. Using anti-TWIK-1 antibodies and brain synaptic membranes, we have shown that the native mTWIK-1 channel is an oligomer of 81 kDa which is probably made up of two subunits covalently assembled via a disulfide bond.

In situ hybridization experiments were carried out to determine the localization of TWIK-1 channels in the mouse central nervous system. TWIK-1 mRNA distribution was shown to be restricted to a few brain regions. The strongest hybridization signals were seen in the hippocampus in the CA1, CA2 and CA3 pyramidal cells, in granule cells of the dentate gyrus, and in the cerebellar granule and Purkinje cells. A moderate signal was obtained within all regions and layers of the cerebral cortex, in the cerebellar nuclei and in various nuclei in the brainstem and mesencephalon. The expression of TWIK-1 mRNAs is concentrated to relatively few areas. This is unlike most inward rectifier K^+ channels of the IRK or GIRK family [15–26]. The most remarkable differences from the distribution of IRKs and GIRKs are the high concentration of TWIK-1 in the cerebellum, in both Purkinje and granule cells, and the low abundance in most areas of the forebrain. Like TWIK-1, TREK-1 was shown to be expressed in all pyramidal cells in the CA1, CA2, and CA3 fields of Ammon's horn, and in cerebellar granule cells [14]. The presence of TWIK-1 and TREK-1 in these cells as well as their biophysical properties (time independence of activation and setting of the resting membrane potential near E_K) suggest that these channels generate the leak currents that underlie the background K^+ conductance which is involved in maintenance of the membrane resting potential in these neuronal cells.

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