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Postsynaptic zinc potentiation elicited by KCl depolarization at hippocampal mossy fiber synapses

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Abstract. The hippocampal mossy fibers contain a substantial quantity of loosely-bound zinc in their glutamatergic presynaptic vesicles, which is released in synaptic transmission processes. Despite the large number of studies about this issue, the zinc changes related to short and long-term forms of potentiation are not totally understood. This work focus on zinc signals associated with chemically-induced mossy fiber synaptic plasticity, in particular on postsynaptic zinc signals evoked by KCl depolarization. The signals were detected using the medium affinity fluorescent zinc indicator Newport Green. The application of large concentrations of KCl, 20 mM and 60 mM, in the extracellular medium evoked zinc potentiations that decreased and remained stable after washout of the first and the second media, respectively. These short and long-lasting enhancements are considered to be due to zinc entry into postsynaptic neurons. We have also observed that following established zinc potentiation, another application of 60 mM KCl only elicited further enhancement when combined with external zinc. These facts support the idea that the KCl-evoked presynaptic depolarization causes higher zinc release leading to zinc influx into the postsynaptic region.

Key words: Postsynaptic zinc — KCl depolarization — Newport Green (NG) — Mossy fiber synapses — Hippocampal CA3 area

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DMSO, dimethyl sulfoxide; KA, kainate; NMDA, N-methyl-D-aspartate; NG, Newport Green.

Introduction

Zinc is one of the most important divalent cations that are present in the mammalian forebrain (Frederickson 2000; Sensi et al. 2011). Only a small amount of zinc is concentrated in the presynaptic boutons of zinc-containing neurons (Frederickson 1989), being the larger fraction of zinc found in metalloproteins, which form complexes with zinc with very high-affinity (Jacob et al. 1998). One of the most important zinc releasable pools is found in hippocampal mossy fibers (Choi et al. 1998), which have large boutons and are located very close to the apical dendrites of CA3 neurons, suggesting that they are part of a uniquely strong synapse (Bischofberger et al. 2006). Mossy fiber synapses se-

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quester, accumulate and release zinc from their glutamatergic presynaptic vesicles that contain the zinc transporter ZnT-3, which pumps zinc into the vesicles and is expressed exclusively in the brain (Palmiter et al. 1996; Frederickson et al. 2005). The depolarization of zinc-containing neurons leads to calcium-dependent glutamate and zinc co-release *via* the exocytosis of their vesicles (Howell et al. 1984; Perez-Clausell and Danscher 1986). Large depolarizations, evoked by electrical or chemical stimulation, can result in the formation of long-term potentiation (LTP) (Bliss and Collingridge 1993; Bortolotto and Collingridge 1993). This form of synaptic plasticity consists of a long lasting enhancement of synaptic transmission and is considered to be involved in learning and memory processes in the brain (Malenka and Bear 2004). LTP can be induced by high-frequency stimulation (tetanus) and also by the application of large amounts of extracellular potassium in hippocampal slices (Fleck et al. 1992; Bernard et al. 1994; Roisin et al. 1997) and in dissociated neuronal cultures (Appleby et al. 2011). Potassium-induced LTP shares some properties with tetanus-induced LTP in hippocampal CA1 area (Fleck et al. 1992; Bernard et al. 1994). For example, the population EPSP amplitudes had similar enhancements in both cases (Fleck et al. 1992). Other forms of chemically-evoked LTP include the TEA-LTP (Suzuki and Okada 2009) and also LTP induced by the application of 4-amino pyridine, mediated by the inhibition of voltage-dependent potassium channels, which causes significant cell depolarization (Bancila et al. 2004). The depolarization associated with chemically-induced LTP may activate simultaneously all potentiable mossy fiber synapses (Zhao et al. 2012). It was observed that the induction of tetanically-evoked mossy fiber LTP in CA3 hippocampal area, is accompanied by significant zinc release from mossy fibers (Quinta-Ferreira et al. 2004; Qian and Noebels 2005; Quinta-Ferreira and Matias 2005; Matias et al. 2010). Thus, intense high-frequency stimulation causes an increase of zinc in the synaptic cleft, which may reach 10–100 μM, and also an enhancement of postsynaptic intracellular zinc (Vogt et al. 2000; Li et al. 2001a, 2001b; Ueno et al. 2002; Paoletti et al. 2009). Potassium-induced depolarization evokes, as well, a postsynaptic zinc increase (Li et al. 2001a, 2001b; Ketterman and Li 2008), which may, at least in part, be explained by zinc entry through voltage-gated calcium channels and calcium-permeable glutamate receptors, as observed applying exogenous zinc in cell cultures (Sensi et al. 1997; Marin et al. 2000). Cytoplasmic zinc enhancements were also observed in non-neuronal cells, following membrane potassium depolarization (Slepchenko and Li 2012). In both cortical and non-neuronal cells, there is also evidence that zinc is taken up in intracellular stores upon stimulation, being considered that it could be stored in the endoplasmic reticulum, the Golgi apparatus and mitochondria (Saris and Niva 1994; Sensi et al. 2000; Stork and Li 2010; Qin et al. 2011; Sensi et al. 2011). Because of its complexity and the large number of mechanisms involved, the characterization of zinc dynamics associated with chemically-induced synaptic potentiation remains to be clarified.

The aim of this work was to address intracellular zinc changes associated with potassium-evoked mossy fiber synaptic plasticity in CA3 hippocampal area. For this purpose, hippocampal slices were loaded with the permeant form of the zinc selective fluorescent probe Newport Green (NG) (Haugland 1996) being the cells depolarized with different concentrations of extracellularly applied KCl.

Most of the present findings have been reported in abstract form.

Materials and Methods

Data were collected in the synaptic system mossy fibers – CA3 pyramidal cells of hippocampal slices obtained from pregnant Wistar rats (10–13 weeks old). The animals were sacrificed by cervical dislocation and the isolated brain was rapidly cooled (5–8°C) in artificial cerebrospinal fluid (ACSF). The slices (400 mm thick) were cut transversely and transferred to a container with ACSF at room temperature, saturated with a gas mixture (95% O_2, 5% CO_2). They remained there at least 1 hour before being used in an experiment. The ACSF medium had the following composition (in mM): NaCl 124; KCl 3.5; $NaHCO_3$ 24; NaH_2PO_4 1.25; $MgCl_2$ 2; $CaCl_2$ 2 and D-glucose 10; pH 7.4. The slices were subsequently transferred to the experimental chamber where they were perfused with ACSF, at a rate of 1.5 to 2 ml/min, at temperatures in the range 30–32°C. The KCl solutions consisted of ACSF with higher concentrations of KCl, 20 mM and 60 mM. In some experiments $ZnCl_2$ (1 mM) was added to the 60 mM KCl medium. All media were perfused for periods of 10–30 min.

Experimental setup and optical measurements

The measurement of optical signals was performed using a fluorescence microscope (Zeiss Axioskop) with a transfluorescence arrangement, including a halogen light source (12 V, 100 W), a narrow band (10 nm) excitation filter (480 nm) and a high-pass emission filter (>500 nm). The light was collected by a water immersion lens (40×, N.A. 0.75) and then focused on a photodiode (Hammamatsu, 1 mm^2), passing its signal through a current/voltage converter (I/V) with a 1 GΩ feedback resistance. The signals were digitally processed by means of a 16 bit analog/digital converter, at a frequency of 0.017 Hz and analyzed using the Signal Express™ software from National Instruments. For measuring zinc changes the hippocampal slices were incubated for 1 h in a medium containing the permeant form of the zinc indicator Newport

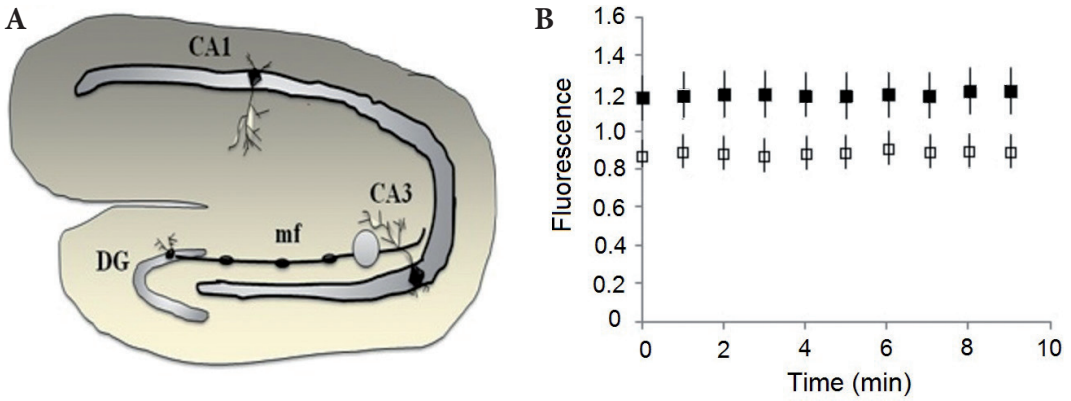

Figure 1. Diagram of the hippocampal slice, autofluorescence and basal fluorescence. **A.** Schematic representation of the hippocampal slice. The circle indicates the region from where the optical signals were recorded. **B.** Fluorescence from non-incubated and from Newport Green containing slices. Autofluorescence (open symbols) and fluorescence signals from slices incubated with 5 mM of the zinc indicator Newport Green (closed symbols) (n = 16). The points represent the mean ± SEM. mf, mossy fibers; DG, dentate gyrus.

Green (NG) (5 µM). This solution was obtained dissolving 1 mg NG in 250 µl of DMSO and then diluting 5 µl of this mixture (DMSO+NG) in 5 ml of ACSF containing 5 µl of pluronic acid F-127. This indicator has a moderate affinity for zinc (K_d ~ 1 µM) and a relatively low affinity for calcium (K_d > 100 mM (Haughland 1996). The optical data consist of fluorescence values represented at 1 minute intervals, in ACSF or in a KCl medium. The signals were corrected for the autofluorescence component, evaluated as the average of ten data points obtained from an equivalent region of dye-free slices, perfused with the normal solution. All measurements are presented as mean ± SEM. Statistical significance was evaluated using the Mann-Whitney U test ($p < 0.05$).

Drugs used were NG, Pluronic acid F-127 (Life technologies, Carlsbad, CA); DMSO (Sigma-Aldrich, Sintra, PT).

All experiments were carried out in accordance with the European Communities Council Directive. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Results

The fluorescence signals were collected from the *stratum lucidum* of CA3 hippocampal area, as shown in Fig. 1A. It was observed that dye-free slices have a significant autofluorescence, triggered by 480 nm incident light and detected for wavelengths above 500 nm. In order to evaluate the contribution of autofluorescence to the signals detected from NG-containing slices, both types of data are indicated in Fig. 1B. It can be noticed that autofluorescence is a major part of the total fluorescence, representing about 75% of it. Thus, all signals were corrected subtracting the autofluorescence component, which was obtained from non-incubated slices. The remaining fluorescence is due to the formation of the NG-zinc complex (Fig. 2A). Since the permeant form of NG is hydrolyzed in the intracellular medium, becoming

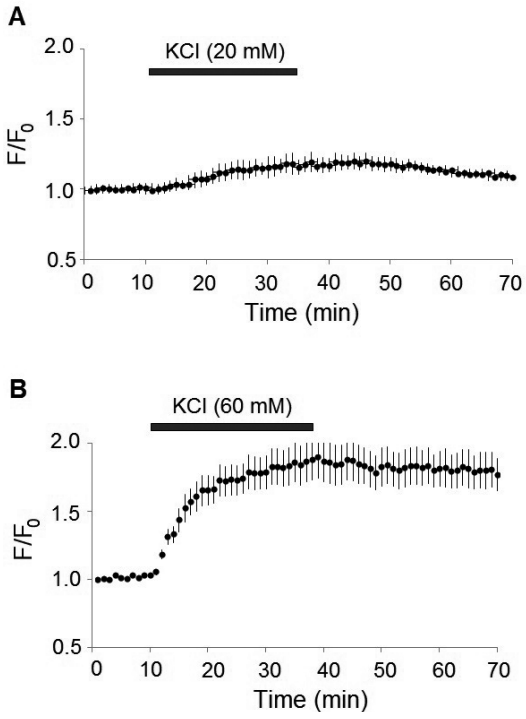

Figure 2. Pooled data of KCl-induced zinc changes obtained with Newport Green. Application of 20 mM KCl (**A**, n = 3) and 60 mM KCl (**B**, n = 7) evoked a rise in the NG fluorescence that was reverted upon washout ($p < 0.05$). All values were normalized by the average of the first 10 responses and represent the mean ± SEM. F, fluorescence; F_0, autofluorescence.

charged, it cannot permeate the vesicular membranes and is thus unable to detect presynaptic zinc in the vesicles (Li et al. 2001b). For this reason, it is considered that the corrected optical signals have a postsynaptic origin.

The perfusion of the medium containing 20 mM KCl caused a rise in the zinc signals to 119 ± 5%, at 35–40 min ($n = 3$, $p < 0.05$), that is partially reverted after a 30 min period, upon returning to the initial ACSF solution, as shown in Fig. 2A. However, the medium with a higher concentration of KCl, 60 mM, evoked a zinc potentiation that is maintained following washout. In Fig. 2B it can be observed that the amplitude of the zinc signals obtained in the presence of 60 mM KCl increased to 184 ± 14%, at 35–40 min ($n = 7$, $p < 0.05$). These signals remained stable following the withdrawal of KCl, revealing the establishment of a KCl-induced persistent zinc potentiation measuring 181 ± 13%, at 65–70 min ($n = 7$), with respect to baseline.

The following experiments were designed to study the effect of repeated applications of the KCl media considered before. A second addition of 20 mM KCl caused similar zinc changes to those induced by the first one, i.e. an enhancement in the presence of that medium followed by a decrease in its absence (Fig. 3A). In the case of the 60 mM KCl solution the repeated perfusion did not induce further potentiation (Fig. 3B). The results in Fig. 3C rule out the possibility of saturation of the indicator (NG) by zinc, since the application of extracellular zinc (1 mM) accompanying KCl (60 mM) resulted in further zinc potentiation that was maintained upon returning to ACSF.

Discussion

In this study we observed zinc signals associated with potassium-induced depolarization of hippocampal mossy

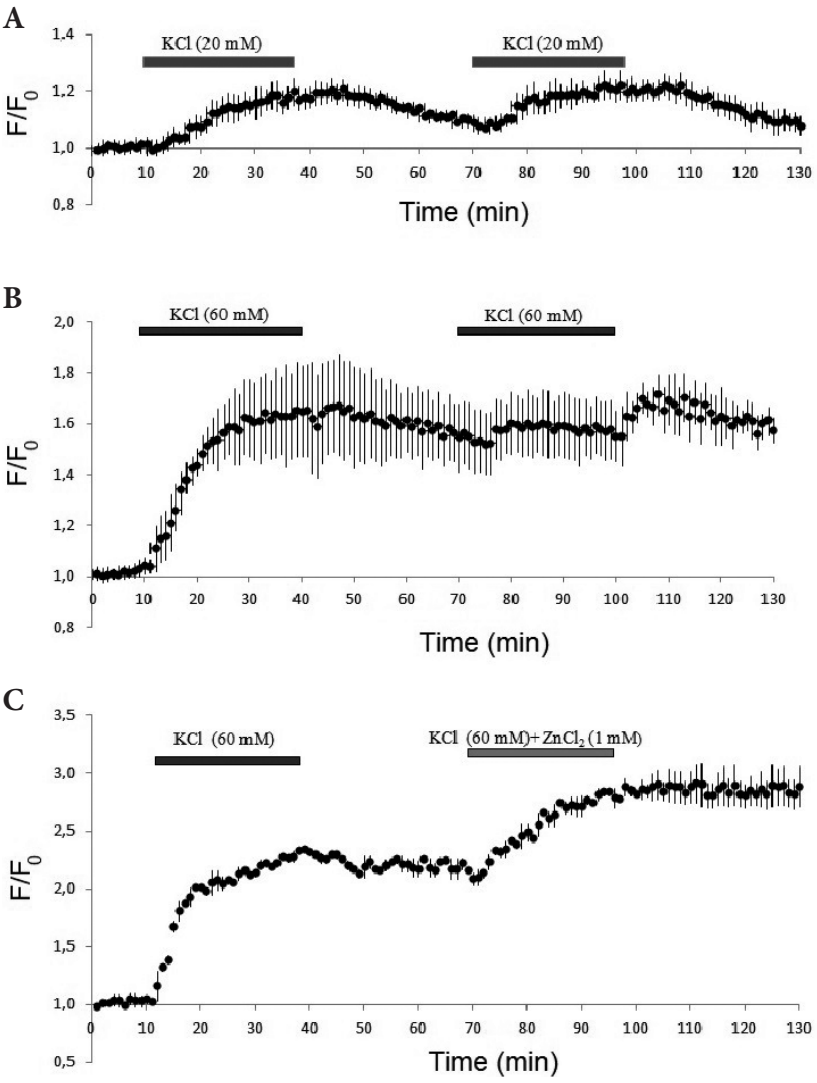

Figure 3. Zinc signals during consecutive applications of KCl media. **A.** Repeated perfusion of 20 mM KCl induced similar transient potentiations ($n = 3$, $p < 0.05$). **B.** Subjecting the slices a second time to 60 mM KCl caused no further zinc enhancement ($n = 3$, $p > 0.1$). **C.** Subsequent zinc potentiations in slices exposed first to KCl (60 mM) and then to a mixture of KCl (60 mM) and $ZnCl_2$ (1 mM) ($n = 2$, $p < 0.05$). All values were normalized by the mean of the first 10 responses and represent the mean ± SEM. F, fluorescence; F_0, autofluorescence.

fibers. It has been shown that zinc is released from these fibers into the extracellular medium when electrical stimuli are delivered (Li et al. 2001a; Quinta-Ferreira et al. 2004; Khan et al. 2014; Vergnano et al. 2014) and that it enters to postsynaptic neurons following intense electrical or chemical stimulation (Vogt et al. 2000; Li et al. 2001a, 2001b; Ueno et al. 2002; Ketterman and Li 2008). The exposition of the slices to a high concentration of exogenous potassium causes an enhancement of the measured fluorescence signals, considered to be associated with postsynaptic zinc changes (Li et al. 2001b; Ketterman and Li 2008). The potassium-induced increase in the postsynaptic zinc concentration may be explained by a rise in synaptic activity, caused by the potassium-evoked shift of the presynaptic membrane potential. In the presence of the 20 mM and 60 mM KCl solutions, the resting values increase to about –54 mV and –33 mV, respectively, thus leading to cell depolarization (Bancila et al. 2004). This causes intense co-release of glutamate and zinc, followed by zinc entry into the postsynaptic area, through several types of receptors and channels. The subsequent depolarization of the spine region evoked by glutamate binding to postsynaptic AMPA, NMDA and calcium permeable AMPA/kainate receptors causes the opening of their channels and also of voltage dependent T- and L-type calcium channels which are located in the same membrane. Except for the AMPA channels, all the others are permeable to zinc. For the calcium permeable AMPA/kainate channels, the permeability ratio P_{Ca}/P_{Zn} was about 1.8 (Weiss and Sensi 2000; Jia et al. 2002). This allows zinc entry to the postsynaptic region through the mentioned zinc permeant channels, namely L- and T-type VDCCs, NMDA and calcium permeable AMPA/kainate receptors (Sensi et al. 1997, 1999; Takeda et al. 2009). There is also experimental evidence that zinc can be released from intracellular stores following the blockade of postsynaptic endoplasmic reticulum calcium pumps (Stork and Li 2010). In the present work, after removal of the KCl solution, the zinc signals decreased in the 20 mM medium and remained unchanged in the 60 mM one. It was also observed that, after the induction of the long-lasting zinc potentiation, another application of KCl (60 mM) did not induce further zinc enhancement. However, when KCl (60 mM) was added in combination with extracellular zinc (1 mM), a second zinc potentiation was elicited, with similar magnitude. The mossy fiber boutons contain a huge amount of synaptic vesicles (~16,000), with about 20 active zones, being up to 1400 vesicles ready to undergo exocytosis (Hallermann et al. 2003; Rollenhagen and Lubke 2010). However, the inexistence of the second potentiation in the absence of exogenous zinc might be due to the lack of additional ready releasable vesicles, caused by the previous intense release. Overall the results suggest that the evoked zinc potentiations are due to zinc entry in the postsynaptic area.

It was previously shown that KCl depolarization induces LTP in CA1 hippocampal area (Fleck et al. 1992; Bernard et al. 1994; Roisin et al. 1997). That potentiation may be evoked by an enhancement of the glutamate release process or may be due to persistent modifications of postsynaptic channels permeabilities eventually by an increase in the number of AMPA receptors in the hippocampal neurons (Malenka and Bear 2004). Thus, the potassium-induced long-lasting potentiation, that is a form of LTP, may be expressed pre- or postsynaptically. There are a large number of studies that characterize mossy fiber LTP as presynaptically expressed, being mediated by enhanced glutamate release (Johnston et al. 1992; Malenka and Bear 2004). However, some studies are in favor of the hypothesis of a postsynaptic *locus* for mossy fiber LTP expression (Yamamoto et al. 1992; Yeckel et al. 1999; Quinta-Ferreira et al. 2004; Suzuki and Okada 2009). The main argument in favor of the presynaptic nature for mossy fiber LTP is the reduction of the paired-pulse ratio (the ratio of the amplitude of the second excitatory postsynaptic response to that of the first in two consecutive pulses), i.e. of paired-pulse facilitation, which is inversely correlated with the transmitter release probability (Zalutsky and Nicoll 1990; Zucker and Regehr 2002). However, changes in paired-pulse ratio are not exclusively mediated by modifications of the presynaptic release probability. For example, they can be influenced by postsynaptic receptor desensitization and lateral diffusion (Frischknecht et al. 2009). Further support for the presynaptic *locus* of mossy fiber LTP comes from quantal analysis, since the failure rate is negatively correlated with the average release probability. Thus, a lower failure rate after LTP induction means a higher probability of glutamate release (Malinow and Tsien 1990). However, that conclusion can only be achieved assuming a constant number of synapses. The discovery of postsynaptically silent synapses provided an explanation for the mentioned lower failure rate after LTP (Isaac et al. 1995). More experimental evidence in favor of the presynaptic hypothesis for the expression of mossy fiber LTP is the effect of cAMP which mediates presynaptic mossy fiber LTP processes (Tong et al. 1996). Assuming a purely presynaptic *locus* for mossy fiber LTP, the zinc released from mossy fibers should rise after electrically- or chemically-induced depolarization, since it is generally accepted that zinc is co-released with glutamate. However, there are experimental results showing that zinc release is not enhanced after high-frequency mossy fiber stimulation (Budde et al. 1997; Quinta-Ferreira et al. 2004) and also following exposure to high-potassium concentrations (Ketterman and Li 2008). Thus, the lack of enhancement of zinc release after LTP induction argues in favor of the contribution of postsynaptic mechanisms for the expression of mossy fiber LTP. Furthermore, the fact that the blockade of postsynaptic T-type VDCCs prevents the expression of

this form of LTP is another strong argument in line with the postsynaptic hypothesis (Suzuki and Okada 2009). As expected, in CA1 hippocampal area, it was already shown that the potassium-induced LTP is mainly mediated by postsynaptic mechanisms (Roisin et al. 1997). The possible postsynaptic expression of mossy fiber LTP might be mediated by zinc influxes into postsynaptic neurons. However, there is still controversy about the role of zinc in mossy fiber LTP, existing studies in favor (Lu et al. 2000; Li et al. 2001a) and against it (Vogt et al. 2000; Matias et al. 2006). The reason for these different results may be the variety of experimental approaches used that may lead to different intracellular zinc availability and metal/chelator complexes, some of which are potentially toxic (Armstrong et al. 2001). Another possible explanation is that the chelators used may be neuroprotective or neurotoxic, in pathological or normal situations (Cuajungco and Lees 1997; Armstrong et al. 2001). Further support for the role of zinc in mossy fiber LTP comes from the existence of signal transduction pathways that are modulated by zinc (Frederickson and Bush 2001). Our results support the idea that the zinc signals are due to the formation of postsynaptic zinc-NG complexes, since they increase with extracellular zinc that may permeate the postsynaptic membrane. They also suggest that the zinc potentiation associated with a long-term enhancement of synaptic activity is expressed postsynaptically.

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