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Changes of Ca^{2+}/calmodulin - dependent protein kinase-II after transient ischemia in gerbil hippocampus

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Abstract. Transient cerebral ischemia induces, besides delayed neurodegeneration in selected brain structures, a number of early responses which may mediate ischemic injury/repair processes. Here we report that 5 min exposure to cerebral ischemia in gerbils induces a rapid inhibition and subsequent translocation of Ca^{2+}/calmodulin--dependent protein kinase II (CaMKII). These changes were partially reversible during a 24 h post-ischemic recovery. Concomitantly the total amount of the enzyme protein, as revealed by Western blotting (α-subunit specific), remained stable. This is consistent with our previous hypothesis, that the mechanism of ischemic CaMKII down-regulation involves a reversible posttranslational modification-(auto)phosphorylation, rather than the degradation of enzyme protein. The effectiveness of known modulators of postischemic outcome in counteracting CaMKII inhibition was tested. Three of these drugs, namely dizocilpine (MK-801), N-nitro-L-arginine methyl ester (L-NAME) and gingkolide (BN52021), all significantly attenuated the enzyme response to ischemia, whereas an obvious diversity in the time-course of their actions implicates different mechanisms involved.

Key words: CaMKII, brain ischemia, neuroprotection, Ca^{2+} -signal transduction

INTRODUCTION

Two temporal phases of neuronal damage are evident after ischemia: an acute phase, where damage is visible almost immediately after the insult, and a late phase, which occurs around 24 h and then progresses. The first phase is not specifically linked to any brain regions or to cell population. The general metabolic response to a rapid blood flow interruption is typical. Ischemic steady-state is reached after two minutes regardless of the investigated area (Domańska-Janik et al. 1985) and when prolonged would lead to ischemic pannecrosis (infarct). In contrast, selective ischemic death of neurones, which occurs some hours later, cannot be explained merely by energy deprivation and membrane dysfunction mechanisms. In order to explain this delayed reaction additional processes have been recently suggested, such as initiation of the intrinsic apoptotic death program (Goto et al. 1990, Volpe et al. 1995) or microglial- or macrophage-connected cytotoxicity (Giulian et al. 1989).

The hippocampus is one of the brain structures preferentially sensitive to ischemic damage. This sensitivity mainly involves the large pyramidal neurones of the CA1 region, which suffer a delayed death after a relatively short ischemic insult. This slow process undoubtedly creates a great challenge to clinical and pharmacological researchers to find new and satisfactory therapeutic strategies.

Most reports published so far indicate that an increase in intracellular calcium, evoked mainly by activation of glutamatergic receptors, mediates development of acute as well as delayed phases of postischemic brain injury (Siesjö and Bengtsson 1989, Salińska et al. 1991, Łazarewicz 1996). The further intracellular propagation of calcium-initiated signals involves phosphorylation/dephosphorylation of many strategic proteins by calcium-activated protein kinases and phosphatases. Recent studies performed in our laboratory (Domańska-Janik and Pylowa 1992, Domańska-Janik and Zalewska 1992, Domańska-Janik and Zabłocka 1993, Zabłocka and Domańska-Janik 1993, Gajkowska et al. 1994, Zabłocka et al. 1994, Zalewska et al. 1995) suggest that this particular step may play an important role in the brain injury/repair processes associated with ischemia.

In these experiments we have focused on the role of calcium/calmodulin-dependent kinase (CaMKII) in transient forebrain ischemia in gerbils. This enzyme, associated with a wide variety of neuronal processes, will be compared to previously studied protein kinase C (PKC) behaviour in the same experimental model.

The role of both kinases in pre- and postsynaptic actions has been described and is consistent with their function in neural signalling (Hara et al. 1993, Soderling et al. 1994). They phosphorylate proteins intimately involved in vesicular release at nerve terminals (Nichols et al. 1990, Zabłocka and Domańska-Janik 1994) and have been implicated directly in the induction of gene transcription by phosphorylation of transcription factors and their components (Sheng et al. 1991, Hara et al. 1993, Zabłocka et al. 1994). Physiologically, PKC and CaMKII are both required for the induction of long-term potentiation (LTP) in the hippocampus (Linden and Routtenberg 1989, Malinow et al. 1989).

It has been reported that brain ischemia down-regulates CaMKII activity and concomitantly recruits the enzyme protein to the plasma membrane (Aronowski et al. 1992, Yamamoto et al. 1992, Hu and Wieloch 1995, Zalewska et al. 1995), to which it probably must move in order to be activated. There are reports that these alterations may be either a persistent or transient phenomenon (Westgate et al. 1994, Hu et al. 1995), depending on the type of ischemia and the sensitivity of the investigated structure.

In this work we have compared CaMKII alterations during the first minutes of ischemia and recovery in two brain structures (hippocampus and cerebral cortex), which differ in their sensitivity to ischemic insult. In order to evaluate the importance of CaMKII in the development of delayed neuronal damage we have investigated the effect of some known neuroprotective drugs on the enzyme activity after 3 h postischemic recovery. It was previously reported that certain pharmacological treatments could, besides reducing neuronal dam-

age (Diemer et al. 1990, Prehn and Krieglstein 1993, Huang et al. 1994), inhibit several early cellular responses connected with ischemia, in particular the attenuation of ischemia-stimulated ability of specific oligonucleotide consensus sequence binding transfactor - AP1, to react with nuclear protein as revealed by electrophoretic mobility shift analysis (Zabłocka et al. 1994). The same drugs in parallel blocked postischemic translocation of PKC toward membranes. Therefore, the involvement of PKC-dependent protein phosphorylation in upregulation of the assortment of nuclear AP1-like transcription factors after ischemia was postulated.

In this work we have tested whether CaMKII postischemic changes would respond also to the same mode of neuroprotective treatments with:
MK-801 (5-methyl-10,22-dihydro-5H-dibenzo(a,d) cyclohepten-5,10-iminemaleate), a noncompetitive antagonist of the NMDA (N-methyl-D-aspartate) receptor/channel complex.

L-NAME (N-nitro-L-arginine methyl ester), a competitive inhibitor of nitric oxide synthase in the brain (Knowles et al. 1989).

BN52021, an inhibitor of platelet-activating-factor (PAF) receptor belonging to group of gingkolides (Spinnewyn et al. 1987).

METHODS

The ischemic model and sample preparation

Male Mongolian gerbils (Merinos unquiculatus), 50-70 g, were used in the experiments. The ischemic insult was performed by 5 min bilateral ligation of the common carotid arteries under light anaesthesia (2.5% halothane inhalation in a mixture of O_2/NO 30:70 by vol., which was reduced to 1% during the operation). Where indicated the ischemia period was shortened to 0.5 and 2 min. Sham operated animals, not exposed to ischemia but either treated or untreated with the drugs, served as controls. Animals subjected to ischemia were allowed to recover up to 72 h. During ischemia and immediately after the insult (up to 3 h postischemia) the body temperature was monitored and kept constant. At the pointed recirculation time animals were decapitated (under light halothane anaesthesia) and the brains were cooled, dissected and frozen at -80°C until use. The tissue was homogenised using a Teflon - glass homogenizer in ice-cold 50 mM HEPES (pH 7.5) containing 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycero-phosphate, 25 mM NaF, 0.1 mM leupeptin, 75 μM pepstatin A, 0.05 mg/ml aprotinin, 1 mM dithiothreitol and 0.1% Triton X-100. The homogenates were centrifuged at 12,000 g for 10 min at 4°C. The supernatants of these Triton-treated homogenates were collected and used for kinase activity measurements. For the studies of CaM-KII distribution the homogenization buffer did not contain Triton X-100. In this case both fractions - the supernatant (soluble fraction) and the pellet (later called the membrane fraction) resuspended in the starting volume of the homogenization buffer, were used for electrophoresis.

Administration of drugs

The PAF antagonist BN52021, in a dose of 10 mg/kg daily, was given as a suspension (1.5 mg./ml of 5% ethanol in water) by oral route for three days. The last treatment took place 1 h before ischemia.

MK-801 (0.8 mg/ml physiological saline) in a dose of 0.8 mg/kg body weight was administered i.p. 30 min before ischemia and repeated 5 min after the insult.

L-NAME (5 mg/ml physiological saline) was administered in a dose of 10 mg/kg body weight i.p. 30 min before ischemia and additionally 5 mg/kg body weight 5 min after the insult.

CaMKII assay

CaMKII activity was determined in a standard assay by incorporation of [^{32}P]phosphate into syntide-2 as described previously (Zalewska et al. 1995). Briefly, the phosphorylation reaction mixture (25 μl) contained 50 mM HEPES (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 0.05 mM [γ-^{32}P] ATP (3,000 cpm/pmol), 20 μM syntide-2, 1 mg/ml bovine

serum albumin (BSA), 5 μM protein kinase inhibitor (Sigma, P 0300). To measure the total CaMKII activity, the assay medium contained 1 mM $CaCl_2$ and 3 μM calmodulin, while 1 mM EGTA was added to determine the Ca^{2+}-independent activity, as described by Fukunaga et al. (1989). The reaction was started by the addition of 3 μl of the supernatant fraction corresponded to 1 μg of protein. Incubation was performed at 30°C for 1 min and was stopped by spotting 15 μl of the reaction mixture on P-81 cellulose paper and processing as described previously (Zalewska et al. 1995).

Immunoblotting

The amount of enzyme was evaluated using a Western blot analysis. Samples of protein (15 μg per lane) were separated using SDS-PAGE on 10 % polyacrylamide gels according to Laemmli (1970). After electroblotting onto nitrocellulose paper (Hybond C extra) blots were probed with monoclonal CaMKII antibodies specific to the 50 kDa subunit, which was visualised with peroxidase - linked anti-mouse IgG. The results of densitometric analysis of ECL (enhanced chemiluminescence) developed Western blots were expressed as a related percentage of the subcellular enzyme distribution. Each experimental group consisted of three animals. The same control samples were processed together with every set of experimental samples to serve as an internal standard.

Protein concentration was determined by the method of Bradford (1976) with BSA as a standard.

Statistics

Results are presented as mean ±SD with differences between means analysed by ANOVA followed by the Student's (t) test according to the Pharmacological Calculation System Program.

RESULTS

Our studies confirmed that CaMKII phosphorylating activity is extremely sensitive to ischemia. Immediately after 5 min blood flow interruption the total enzyme activity decreased more than 80% as compared with control values (Fig.1). This rapid loss of activity during ischemia and early reperfusion was followed by its limited restoration, more pronounced in brain cortex than in the hippocampus.

The calcium-independent kinase activity, which is connected with sequential autophosphorylation and occurrence of the so-called "autonomous" form of the enzyme (Miller and Kennedy 1986), changed less dramatically. However, the relative contribution of this form to total CaMKII activity increased considerably and rapidly after 5 min ischemia and

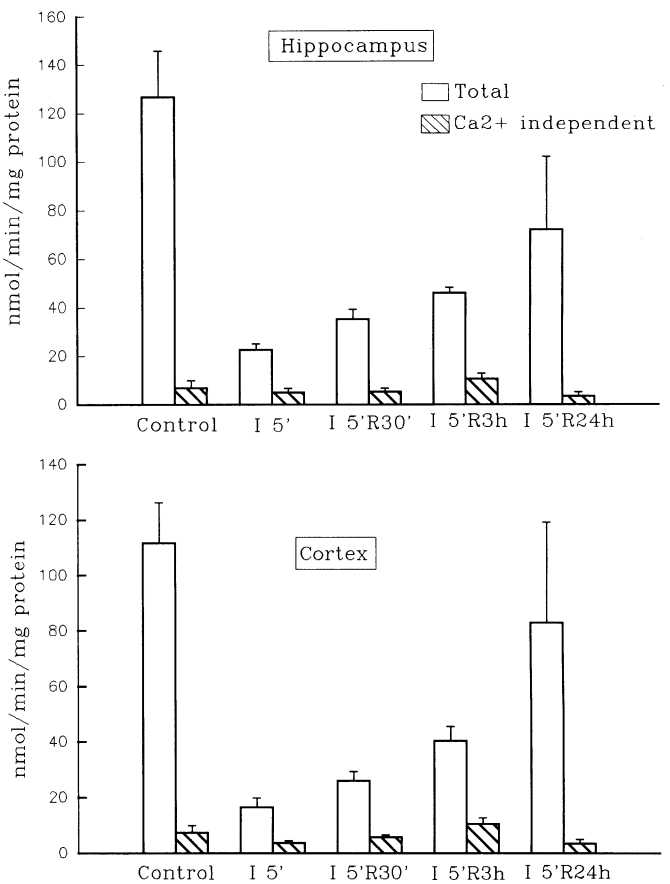

Fig. 1. Effect of brain ischemia on CaMKII activity in gerbil brain. CaMKII activities were assayed in the presence of calcium and calmodulin (total activity) or in the presence of EGTA (Ca^{2+}-independent activity) under standard conditions (as described in Methods section). Data for control, 5 min ischemia (I 5') and different recovery periods (R) are shown as means ±SD for six animals for each experimental and 16 animals for control groups.

was elevated during the whole recovery period studied.

In spite of the above-mentioned inhibition of CaMKII activity after ischemia, we did not find any decrease in the total amount of this enzyme protein. On the other hand, there was an extensive translocation of CaMKII 50 kDa subunit from the cytosol toward membranes (Fig. 2). This postischemic subcellular CaMKII redistribution, believed to be driven by autophosphorylation of the enzyme molecules (Zalewska et al. 1995), was unrelated to the brain structure (hippocampus versus cerebral cortex) and had an obvious tendency to normalization during 72 h recovery.

The next question was to determine if the postischemic changes in CaMKII activity described above would respond to neuroprotective drug treatments. It has been shown that all neuroprotective compounds tested are able partially to protect CaMKII activity against its postischemic inhibition. However, the effect of MK-801 could be noticed only after relatively short, 30 s ischemic insult (Fig. 3). As it is demonstrated here, this antagonist of NMDA receptor/channel was able at this time point to protect completely CaMKII activity and, in addition,

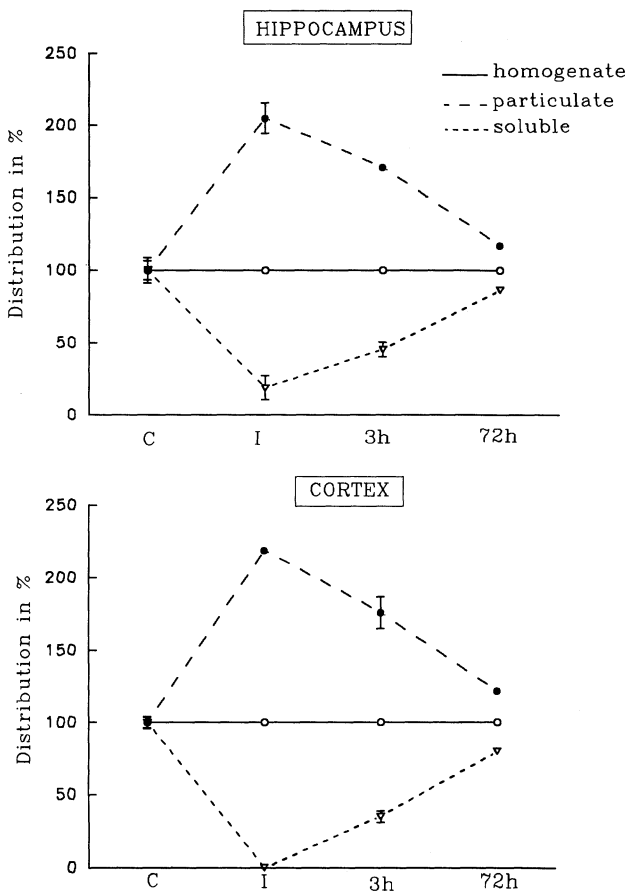

Fig. 2. Quantification of CaMKII protein in homogenate, soluble and particulate fractions after 5 min ischemia and subsequent recovery period. The amount of CaMKII α subunit was determined densitometrically on Western blots (as described in Methods) and expressed as a percentage of appropriate homogenate. Each point represents mean value ±SD from 4-6 blots. The variations are visualised when they exceeded 5%. C, control, I, ischemia.

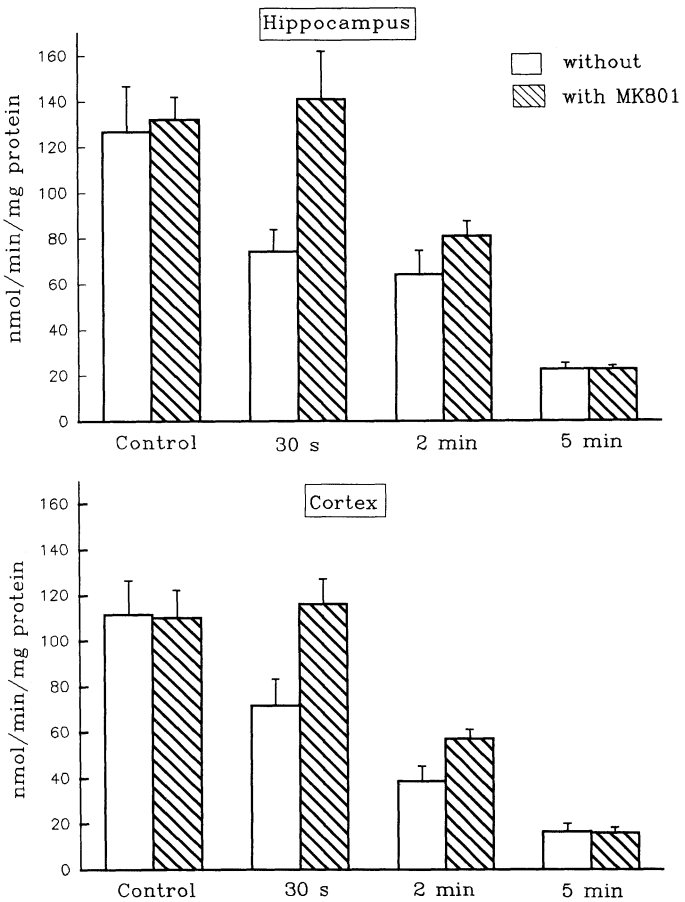

Fig. 3. Effect of MK-801 on short term brain ischemia-induced changes in CaMKII activity. Total CaMKII activity was measured as described in Methods. The animals were treated with MK-801 in a dose of 0.8 mg/kg body weight i.p. 30 min before ischemia. All experiments with control and MK-801 treated animals were performed simultaneously. Mean ±SD from at least 4 animals in each group.

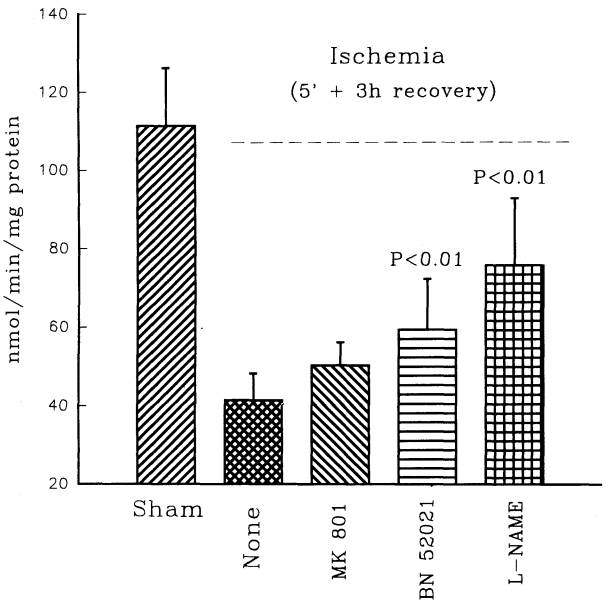

Fig.4. Effect of different drugs on ischemia-induced changes in CaMKII activity. Animals were treated with appropriate drugs indicated on the bottom of figure and total CaMKII activity was measured at 3 h after 5 min ischemia, as described in Methods. Mean values from 4 to 8 animals in each experimental group SD compared with the mean value from 16 controls.

counteract its translocation toward membranes (results not shown). When blood flow was interrupted for 2 min, MK-801 also significantly attenuated the enzyme down-regulation. In contrast, MK-801 was without effect on CaMKII behaviour after 5 min ischemia as well as after 3 h recovery (Figs. 3 and 4). On the other hand, significant reduction of the extent of CaMKII down-regulation was achieved at 3 h recovery after 5 min ischemia with the other neuroprotective treatments. In this action L-NAME was found to be slightly more effective than BN52021 treatment (Fig. 4).

DISCUSSION

The CaMKII inhibition induced by transient, 5 min forebrain ischemia in gerbils resembles, in the acute postischemic phase, the reactions reported by other authors dealing with different experimental models (Taft et al. 1988, Churn et al. 1990, Morioka et al. 1992, Hu et al. 1995, Zalewska et al. 1995). The decrease of total enzymatic activity measured in the presence of calcium and calmodulin was rapid and significant as early as 30 s after blood flow interruption, then progressed down to 80% during 5 min of ischemia. This inhibition was followed by the reversible translocation of CaMKII toward plasma membrane as revealed by Western blotting. The absence of changes in the quantity of the enzyme protein (Fig. 2), its transient and reversible translocation as well as the substantial restoration of the enzyme catalytic activity during 24 h recovery, could indicate reversible modification of the enzyme molecule rather than its proteolytic degradation. This corresponds with the results of Westgate et al. (1994), where, besides the apparent stimulation of calpains under *in vitro* ischemia, no changes in the amount of CaMKII protein were noticed.

As was previously suggested, one possible post-traslational CaMKII modification leading to CaMKII inhibition may involve extensive (inhibitory) enzyme phosphorylation during ischemia (Aronowski et al. 1992, Zalewska et al. 1995). It can be mentioned here that the initial, Ca^{2+}/CaM-dependent autophosphorylation of the kinase at Thr286 residue is known to be necessary for its activation and translocation. The physiological roles of the other, sequential CaMKII phosphorylation are still unclear at present. A number of cytosolic enzyme proteins, among them kinases, proteinases and adapter proteins, are switched on by recruiting to specific regions of plasma membrane. Some of them, e.g. CaMKII, are concomitantly and obligatorily phosphorylated. The others, like PKC, are translocated in a calcium- and ligand-dependent manner. Then, after phosphorylation of the target proteins, these kinases are deactivated by means of different routes. In the case of CaMKII, this is probably achieved by its further autophosphorylation and/or interactions with autoinhibitory domain (Colbran 1992), while PKC seems to be proteolytically degraded (Zabłocka and Domańska-Janik 1993). The whole process may be governed by intracellular calcium, which activates a number of phosphokinases as well as phosphatases and calcium-dependent proteases.

The apparent reversibility of CaMKII translocation and inhibition supports our previous suggestion that phosphorylation/dephosphorylation of the enzyme molecule may determine its alterations during and after ischemia (Zalewska et al. 1995). However, this suggestion must be contrasted with a recently reported persistent decrease of CaMKII message and protein noticed only in certain, discrete, vulnerable regions of postischemic hippocampus (Hu et al. 1995). This, together with the observed translocation of the enzyme to postsynaptic densities (PSD), would indicate the possible contribution of an additional, protein degradation--involving mechanism, restricted to defined cells or subcellular structures. As we have not observed any correlation between vulnerability of the selected structures to ischemia and responsiveness concerning CaMKII in our experiments, it is possible that only the above more subtle changes would be pathognomonic for irreversible ischemic injury.

In our experiments substantial protection against postischemic CaMKII inhibition was achieved by administration of the NMDA receptor antagonist MK-801, the presynaptic PAF receptor antagonist, BN52021, or the nitric oxide synthase inhibitor L-NAME. As reported previously all these treatments were effective in the counteracting of postischemic changes in PKC and ornitine decarboxylase (ODC) activity as well as in blocking AP-1 binding protein activation (Zabłocka et al. 1995). Whether altered function of all these proteins, together with CaMKII, is an actual part of the ischemic cell death program or whether they trigger the necessary steps leading to it is for the present moment unknown. It is known, however, that the treatments reducing postischemic neuronal damage are able to block some up-stream cellular responses, like AP1 or ODC activation, and concomitantly reduce postischemic changes of calcium-dependent kinases: PKC and CaMKII (Zabłocka et al. 1995 and this report). Immediately the question arises as to the exact point at which activation of the investigated kinase would be involved in the above chain of events. The high concentration of CaMKII in PSD structure together with the recently observed excessive enzyme translocation to the synaptic junctions during ischemia (Hu and Wieloch 1995) suggests that CaMKII would change the sensitivity of postsynaptic signalling system.

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