A cell-shrinkage-induced non-selective cation conductance with a novel pharmacology in Ehrlich–Lettre-ascites tumour cells

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Abstract In whole-cell recordings on Ehrlich–Lettre-ascites tumour (ELA) cells, the shrinkage-induced activation of a cation conductance with a selectivity ratio $P_{Na}:P_{Li}:P_K:P_{choline}:P_{NMDG}$ of $1.00:0.97:0.88:0.03:0.01$ was observed. In order of potency, this conductance was blocked by Gd^{3+} = benzamil > amiloride > ethyl-isopropyl-amiloride (EIPA). In patch-clamp studies using the cell-attached configuration, a 14 pS channel became detectable that was reversibly activated upon hypertonic cell shrinkage. It is concluded that ELA cells express a shrinkage-induced cation channel that may reflect a molecular link between amiloride-sensitive and -insensitive channels. In addition, because of its pharmacological profile, it may possibly be related to epithelial Na^+ channels (ENaCs).
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1. Introduction

In most systems, hypertonic cell shrinkage elicits a regulatory volume increase (RVI) that is achieved by the uptake of NaCl and osmotically obliged water. Ion pathways employed in this process are Na^+–K^+–$2Cl^-$ symport, Na^+/H^+ antiport, as well as cation channels (the latter two working in parallel to Cl^-/HCO_3^- antiport and anion channels, respectively) [1–3]. Activation of cation channels upon cell shrinkage has been proven to be one of the most efficient mechanisms of RVI [4]. The first group of these channels is non-selective for monovalent cations and it is clearly insensitive to the diuretic amiloride in concentrations up to 10^{-4} M. Quite typically, however, these cation channels are inhibited by the anti-inflammatory drug flufenamate (10^{-4} M) as well as by Gd^{3+} (10^{-5}–10^{-3} M). Channels of this type have been described for a variety of preparations and, most typically, their conductance is in the range of 15–25 pS [5–10]. The molecular organisation of these channels is completely unclear.

The second group of shrinkage-activated cation channels is inhibited by amiloride [11–16] but (with one exception [17]) insensitive to Gd^{3+} and flufenamate. These amiloride-sensitive channels are, in many instances, also non-selective for monovalent cations. Interestingly, evidence was reported that they may be related to epithelial Na^+ channels (ENaCs); this is of note, because ENaCs are typically highly Na^+ selective [18–20]. In rat hepatocytes, for instance, injection of specific anti-α-ENaC antisense DNA oligonucleotides [21] reduced the hypertonic activation of a cation channel with a P_{Na}/P_K of 1.4 by some 70%; moreover, all three ENaC subunits could be detected in this system on the RNA and protein level [15]. On the other hand, the pharmacological profile of the channel was EIPA (ethyl-isopropyl-amiloride) > amiloride > benzamil [15,22] which is precisely opposite to the one found for ENaCs [18,19]. In the ciliary epithelium of the eye, α-ENaC could be detected on the RNA level and benzamil was an effective blocker of RVI [16].

In the present study, we characterise a shrinkage-activated cation channel in Ehrlich–Lettre-ascites tumour (ELA) cells that is non-selective for monovalent cations. Interestingly, the channel is inhibited by blockers of both groups that are typically used to classify the shrinkage-induced cation channels reported so far, i.e. Gd^{3+} on the one hand, and amiloride and its derivatives on the other. The channel may thus reflect a possible molecular link between these two groups that may be of use for the understanding of channel architectures. Furthermore, the sensitivity profile of the channel of benzamil > amiloride > EIPA is suggestive of a molecular correlation to ENaCs.

Parts of this study have been presented in abstract form [23].

2. Materials and methods

2.1. Cell culture

ELA cells were grown on collagen-coated gas-permeable supports (Petriperm®; Bachofer, Reutlingen, Germany) in RPMI-1640 medium (Gibco, Invitrogen, Karlsruhe, Germany) fortified with 10% foetal calf serum and 100 U/ml penicillin–100 µg/ml streptomycin at 37°C and 5% CO_2. Cells were passaged every week and used from passage 12 to 32.

2.2. Patch-clamp techniques

Membrane currents were recorded from single ELA cells in culture in the fast whole-cell or the cell-attached mode of the patch-clamp technique. Patch pipettes were pulled from thin- or thick-walled borosilicate glass capillaries of 1.5 mm outside diameter (OD) and 1.17 or 0.86 mm inside diameter (ID) (Clark Electromedical, Reading, UK), respectively, on a programmable multi-stage pipette puller (DMZ-Universal Puller; Zeitz-Instrumente, München, Germany). After final heat polishing, pipettes had resistances in the range of 3.5–5.5 MΩ when filled with intracellular solution (whole-cell measurements; see below) and from 4.5 to 6.5 MΩ when filled with isotonic bath solution (cell-attached recordings). A motorised micromanipulator (mini 25; Luigs and Neumann, Ratingen, Germany) was used for the positioning of pipettes and an Ag–AgCl wire served as the reference electrode.

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Pipette offsets, series resistances (not exceeding 10 MΩ) and capacitive transients were compensated on the patch-clamp amplifier (Axopatch 200, Axon Instruments, Foster City, CA, USA). Only recordings with less than 10% changes in access resistance (as judged from the compensatory circuit settings) were accepted. Currents were digitised with an AD-converter (DigiData 1200A, Axon) at either 5 or 10 kHz and filtered with the built-in four-pole Bessel filter at 0.5 or 1 kHz. Data acquisition and analysis were done with the pClamp 6.0 software package (Axon). In the whole-cell recordings, the holding voltage was −40 mV and voltage ramps from −80 to +80 mV and of 900 ms duration were applied every 6 s.

For the experiments, culture dishes were rinsed with isotonic control solution (see below) and pieces of approximately 1 cm^2 were cut from the bottom of the culture dishes. They were then transferred to a recording chamber that was mounted on the stage of an inverted microscope (Aviovert 10; Zeiss, Oberkochen, Germany). Cells were continuously superfused at a rate of 5 ml/min with a complete exchange of the bath solution every 4 s.

2.3. Solutions and chemicals

The isotonic control solution (300 mOsm, pH 7.4) contained (in mM): NaCl, 135; KCl, 10; MgCl$_2$, 1; CaCl$_2$, 1; HEPES, 10. In the hypertonic test solution, osmolarity was increased to 350 mOsm by addition of sucrose. The intracellular pipette solution (300 mOsm, pH 7.2) contained: NaCl, 20; K-gluconate, 120; MgCl$_2$, 1; ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1; HEPES, 10; Na$_2$-adenosine triphosphate (ATP), 1; Na$_2$-guanosine triphosphate (GTP), 1. In the cell-attached measurements, pipettes were filled with isotonic control solution. The pH of solutions was adjusted by addition of 1 M Tris and the osmolarity was routinely checked with an osmometer (Knauer, Berlin, Germany). All experiments were performed at room temperature (18–21°C).

2.4. Statistical analysis

Data are presented as means ± S.E.M. with n denoting the number of cells tested. For comparison of data sets, Student's t-tests for paired and unpaired data were employed as appropriate.

3. Results and discussion

3.1. Shrinkage-induced whole-cell currents in ELA cells

In the first series of whole-cell recordings, the responsiveness of single ELA cells to hypertonic stress was monitored. It was found that in some 62% of the cells tested (i.e. in 100 out of a total of 162), increasing extracellular osmolarity from 300 to 350 mOsm led to a sizable increase of membrane current from −38 ± 24 pA to −175 ± 22 pA (at $V_{hold} = -40$ mV), i.e. by 360 ± 45% of control (Fig. 1A). Within this group of cells, the pattern of current activation was highly reproducible and a constant maximal level was achieved after approximately 160 s of hypertonicity. This is in line with an early report on the closely related Ehrlich-ascites tumour cells, where Na$^+$ conductance was found to be augmented under hypertonic conditions [24]. The increase in current observed in the present investigation coincided with a shift in zero-current voltage from −39.6 ± 5.8 mV to 0.5 ± 4.2 mV (Fig. 1B). In conjunction with the observed low selectivity of the shrinkage-induced cation conductance for small monovalent cations observed in the ion substitution experiments (see Section 3.2), this reversal potential close to 0 mV strongly suggests that other ion conductances are not changing. Upon return to isotonic conditions, membrane currents declined to 146.8 ± 2.2% of the pre-stimulus level. In parallel, zero-current voltage was shifted to −20.4 ± 2.9 mV. There was a certain variability concerning rectification with an almost linear current–voltage relation found in some cells (not shown) and a slight outward rectification in others (Fig. 1B).

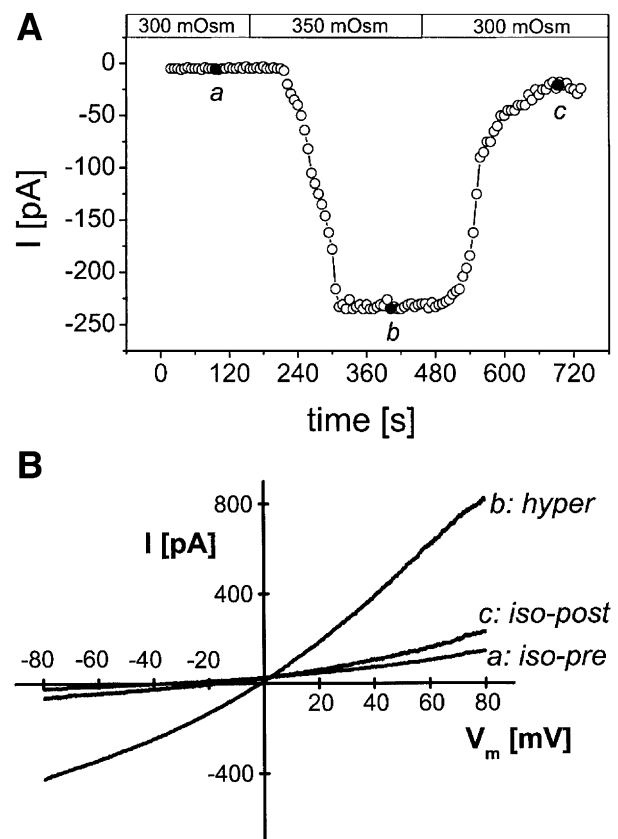In some 38% of the cells used in this study, hypertonic

Fig. 1. Shrinkage-induced membrane currents in single ELA cells. Currents were measured in the whole-cell mode of the patch-clamp technique. A: Membrane currents determined at $V_{hold} = -40$ mV. For the times indicated, the extracellular osmolarity was increased from 300 to 350 mOsm by addition of sucrose. Filled circles at times a, b, and c refer to the corresponding current–voltage relations shown in B. B: Current–voltage relations before (a: iso-pre), during (b: hyper), and after (c: iso-post) hypertonic exposure of cells.

conditions neither changed currents nor zero-current voltages. These cells were not considered in our analysis.

3.2. Ion selectivity of shrinkage-induced currents

In this series of measurements, extracellular Na$^+$ was completely replaced by various substitutes and the resultant changes of shrinkage-induced current and zero-current voltages were determined. Fig. 2A shows a typical ion replacement experiment. It is seen that Na$^+$ substitution by Li$^+$ or K$^+$ only slightly decreased inward currents to 96.2 ± 2.0% ($n = 6$, not significant) and 86.4 ± 4.1% ($n = 6$, $P < 0.05$), respectively, already indicative of a comparable permeability of the shrinkage-induced conductance in ELA cells to monovalent alkali cations. In contrast, in the presence of choline$^+$ and N-methyl-D-glucamine (NMDG)$^+$, currents were reduced to 4.1 ± 2.4 and 2.8 ± 3.3%, respectively ($n = 6$, $P < 0.001$ each; Fig. 2A). In line with these observations, the reversal potentials in the presence of Na$^+$, Li$^+$ and K$^+$ amounted to −1.5 ± 1.7 mV, −2.0 ± 2.0 mV and −5.3 ± 1.7 mV, respectively, whereas those obtained with choline$^+$ and NMDG$^+$ were −38.5 ± 4.8 mV and −41.8 ± 6.0 mV (Fig. 2B). From these data and by use of the Goldman–Hodgkin–Katz equation, a permeability sequence $P_{Na}:P_{Li}:P_K:P_{choline}:P_{NMDG}$ of

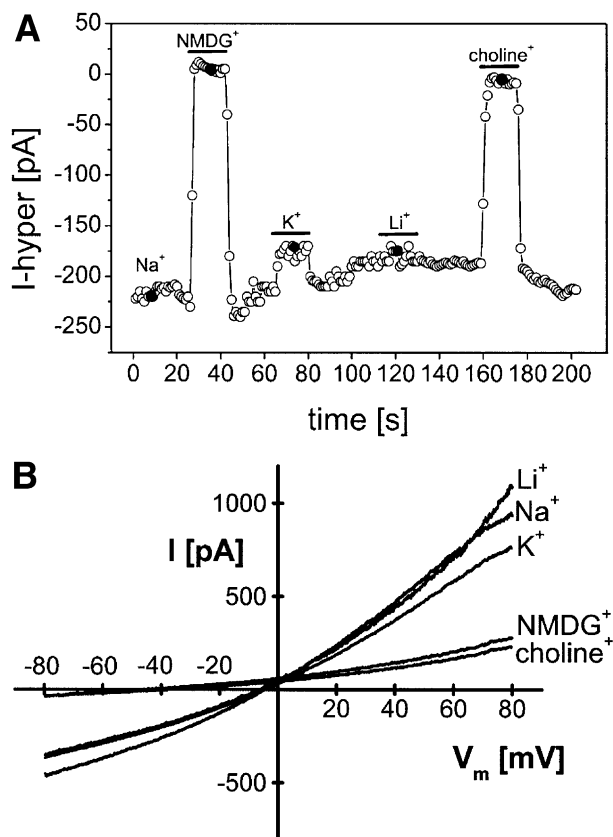Fig. 2. A: Effects of extracellular Na^+ replacements on hypertonicity-induced membrane current (hyper minus iso-pre). Typical experiment, $V_{hold} = -40$ mV. Filled circles indicate the times at which the current–voltage relations shown in B were taken. B: Current–voltage relations in the presence of Na^+ and after Na^+ substitution by various cations as indicated.

1.00:0.97 (not significant):0.88 ($P < 0.05$):0.03 ($P < 0.001$): 0.01 ($P < 0.001$) can be computed.

3.3. Pharmacology

To further characterise the shrinkage-induced cation conductance in ELA cells, its pharmacological profile was determined. All compounds used were tested in the same concentration range, namely at 0.1, 1 and 10 μM (with $n = 5$–7 for every condition), and in all instances their effects were clearly dose-dependent. Gd^{3+} which is one of the classical inhibitors of the shrinkage-induced (but amiloride-insensitive) conductance in various systems (see Section 1) inhibited currents by $78.1 \pm 9.5\%$ at 10 μM ($P < 0.001$; Fig. 3). Much to our surprise, however, amiloride exhibited significant effects as well with an inhibition that (at 10 μM) amounted to $52.4 \pm 7.7\%$ ($P < 0.001$). Moreover, its derivative benzamil which is the most efficient blocker of the epithelial type of Na^+ channels (ENaCs) [18,19] was even more effective with a maximal inhibition by $68.8 \pm 8.5\%$ ($P < 0.001$; Fig. 3). EIPA, the second amiloride derivative tested (and the most effective blocker of the shrinkage-induced cation conductance in rat hepatocytes [15,22]) was found to be a less potent inhibitor than its congeners with a maximal reduction of currents by $27.2 \pm 11.6\%$ ($P < 0.05$). The amiloride sensitivity of the shrinkage-induced cation channel in ELA cells and, furthermore, the observed sensitivity profile benzamil > amilori-

de > EIPA, which is quite typical for ENaCs, suggests a possible molecular correlation to these channels.

Flufenamate, a second blocker commonly used in the analysis of shrinkage-activated non-selective (and amiloride-insensitive) cation channels (see Section 1) could not be tested in ELA cells. This is due to significant side effects of the compound, namely a swelling of cells and a concomitant increase in K^+ conductance (data not shown).

Interestingly, 0.1 μM Gd^{3+}, 1 μM benzamil, and 10 μM amiloride which were found to reduce hypertonicity-induced currents with almost equal efficiencies (i.e. by 45–50%; see Fig. 3) shifted reversal potentials by virtually identical amounts, i.e. by -18.2 ± 5.0, -21.6 ± 4.9, and -19.0 ± 3.8 mV, respectively. These results already suggest that, in ELA cells, it is a single population of cation channels that is activated by hypertonic stress.

3.4. Single-channel events

To obtain information about the channels underlying the shrinkage-induced cation conductance in ELA cells, patch-clamp experiments in the cell-attached configuration were performed. Under *isotonic* conditions, various types of channels were observed that appeared to be similar to those described for the closely related Ehrlich-ascites tumour cells. Among these were the stretch-activated non-selective cation channel of intermediate conductance (that was supposed to be Ca^{2+}-permeable), intermediate and small conductance K^+ channels, as well as small conductance Cl^- channels as was judged from conductivities and gating patterns [25]. Under *hypertonic* conditions, in seven out of 21 recordings, the activation of a 14 pS channel could be observed (see Fig. 4A). The channel had a linear current–voltage relation and its open probability did not depend on membrane voltage (Fig. 4B). Single-channel currents reversed at a pipette potential of 42.0 ± 2.8 mV. This is in accordance with the reversal potential close to zero that was determined for the whole-cell current (Fig. 4B). Given the high asymmetry of Cl^- under these conditions, the channel is also very unlikely to be Cl^- selective. Channel
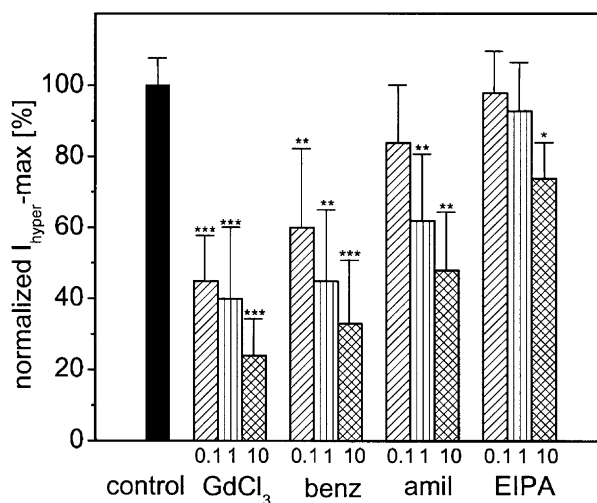

Fig. 3. Pharmacological profile of the shrinkage-induced current in ELA cells. Test solutions were applied for periods of 30 s during maximal current activation. $n = 5$–7 per data point except for control conditions ($n = 74$). Inhibitor concentrations are given in μM. *, **, and ***: significantly different from control at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
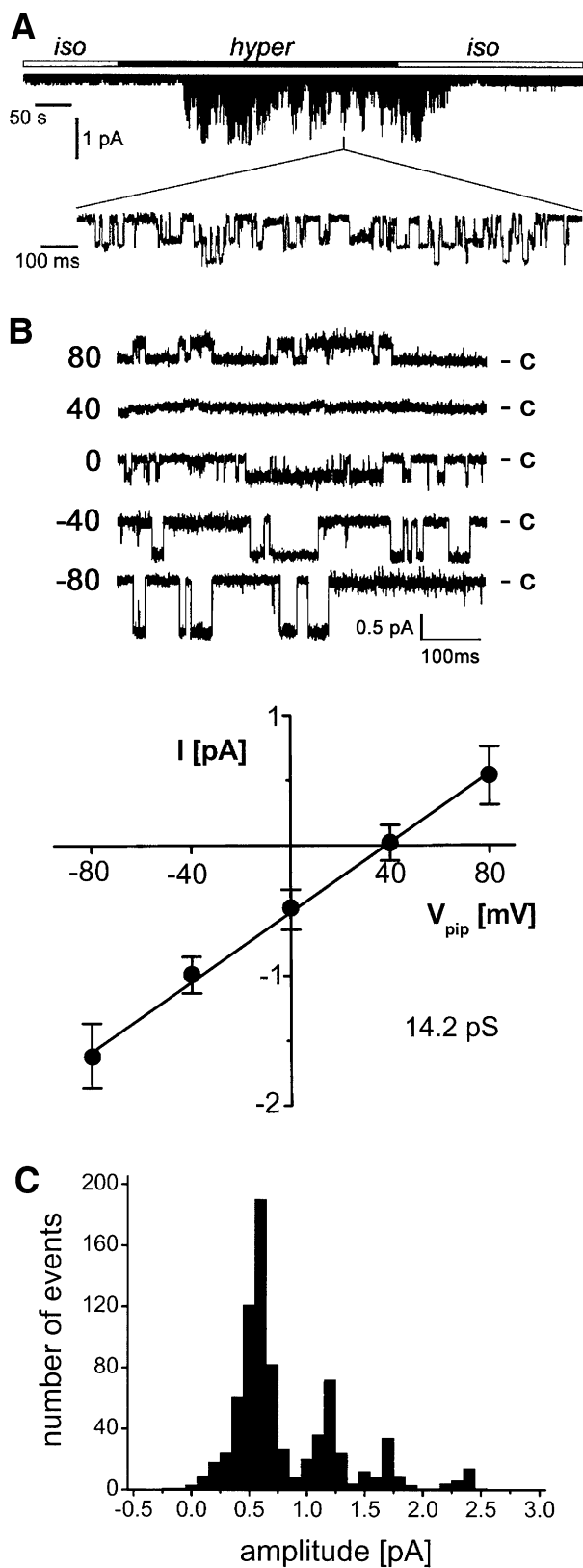
Fig. 4. A: Cell-attached recording (at $V_{hold} = V_m$) exemplifying the induction of ion channel activity under hypertonic conditions. B: Current–voltage relation of single channels in ELA cells. Top, representative recording; bottom, average data. C: Amplitude histogram of single-channel currents derived from a patch containing four channels (at $V_{hold} = V_m$). Note that the distance between maxima is virtually identical yielding individual conductance levels very close to 14 pS.

activation was reversible (Fig. 4A) and the channel was never observed under isotonic conditions ($n = 42$). As is exemplified in Fig. 4C, channel conductances, in every instance, were close to 14 pS. Moreover, from a kinetic analysis, mean open and closed times of 25 ± 7 and 318 ± 40 ms were obtained, respectively. The high reproducibility of these data provides strong further evidence that, in fact, a single population of cation channels is activated.

After excision of patches, resolvable channel openings disappeared within seconds.

4. Conclusions

In the present study, we observe the hypertonic activation of an ion conductance in ELA cells that is non-selective for monovalent alkali cations. This cation conductance exhibits a novel pharmacological profile in that it is inhibited by Gd^{3+} as well as by amiloride. Moreover, among amiloride and its derivatives it shows a sensitivity sequence that very much resembles that of ENaCs, namely benzamil > amiloride > EIPA. Also of note in this respect is the 40% inhibition by benzamil at a concentration as low as 100 nM. Thus, the pharmacology of the conductance is suggestive of a possible correlation to the ENaC (or its subunits) whereas its non-selectivity to alkali ions clearly is not. It is this combination of features that renders the ELA channel a novel and interesting member of the group of shrinkage-activated cation channels that may be specifically useful for the molecular understanding of the actual channel architectures. It might be interesting in this context that recent mutagenesis studies on ENaC show that the selectivity filter of this channel involves a stretch of three conserved amino acids and that mutation of a S589 in the α-subunit is sufficient to allow larger monovalent as well as divalent cations to pass [20].

With respect to single-channel characteristics, the cation conductance in ELA cells is likely to reflect the shrinkage-activated 14 pS channel that was observed in the cell-attached measurements. Based on this parameter, it is clearly different from the shrinkage-activated channels found in most systems that exhibit a conductance close to 25 pS [4,6,7,9]. It may be related, however, to the channel observed in BSC-1 cells (derived from the kidney of African green monkey) or HT-29 (human colon carcinoma) cells, where single-channel conductances amounted to 15 and 17 pS, respectively [9]. Unfortunately, in no study reported hitherto it was possible to determine the pharmacology of shrinkage-induced cation conductances on the single-channel level.

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