Hypocalcin from Stannius corpuscles inhibits gill calcium uptake in trout

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Lafeber, F. P. J. G., G. Flik, S. E. Wendelaar Bonga, and S. F. Perry. Hypocalcin from Stannius corpuscles inhibits gill calcium uptake in trout. Am. J. Physiol. 254 (Regulatory Integrative Comp. Physiol. 23): R891-R896, 1988.—Bidirectional whole body flux and branchial Ca\(^{2+}\) influx were measured in freshwater rainbow trout. Intra-arterial injections of homogenates of Stannius corpuscles (CS) as well as of a 54-kDa isolated product (hypocalcin) exerted an inhibitory effect on whole body Ca\(^{2+}\) influx, but did not affect Ca\(^{2+}\) efflux. Hypocalcin was more effective in reducing Ca\(^{2+}\) influx in trout acclimated to low-calcium freshwater than in fish from normal-calcium water. We conclude that the isolated product (hypocalcin) represents the hypocalcemic principle of the CS. Similar doses of hypocalcin caused quantitatively similar decreases in Ca\(^{2+}\) influx in vivo and in the isolated perfused head preparation. This indicates that the gills form the principle target for hypocalcin in trout. The branchial transepithelial potential did not change during hormone treatments. Possible mechanisms of hypocalcin action are suggested.

calmodulin; rainbow trout; hypocalcin action

TELHOST FISH such as trout are able to regulate their blood calcium levels efficiently (2, 11). It is well known that fish absorb calcium directly from the water and that this uptake route can account for their entire calcium requirement (5). The gills are a major site of calcium exchange between water and fish, and, therefore, the gills play a predominant role in the maintenance of the fish's calcium balance (3, 5, 11). One may reason, then, that the gills will be the primary target for calcitropic hormones in fish (3).

An important calcitropic factor in fish is the hypocalcemic principle from the corpuscles of Stannius (CS) (see Ref. 22 for a recent review). The mechanisms of action of the CS principle are less well known, although the gills have been anticipated as one of its target organs (11). In isolated perfused eel gill preparations from stanniomized fish an increase in Ca\(^{2+}\) uptake was demonstrated (4, 16). Perfusion of CS tissue homogenate caused a reduction of Ca\(^{2+}\) uptake (10, 17).

Recently, we have reported on the isolation and identity of a 54-kDa product of trout CS (9). Injection of this principle in hypocalcemic stanniomized eels results in a decrease of plasma calcium levels (unpublished results). We have called this principle hypocalcin, the name proposed by Pang et al. (12) for the hypocalcemic principle of the CS. Here we demonstrate that hypocalcin exerts an inhibitory control on the branchial Ca\(^{2+}\) influx in trout that may account for the hypocalcemic action of this hormone. Because it was shown that the hypocalcemic action of the CS principle is noticeable only when it is injected into fish adapted to low-calcium water (21), we have examined its effect on whole body Ca\(^{2+}\) in- and efflux in fish acclimated to both normal- and low-calcium freshwater. To establish the relative importance of the gills and their control by hypocalcin for whole body Ca\(^{2+}\) flux, the effects of hypocalcin on Ca\(^{2+}\) influx were also determined in an isolated head preparation.

MATERIALS AND METHODS

Animals

Trout (Salmo gairdneri) used for collection of CS and isolation of hypocalcin were purchased from a commercial trout farm (The Netherlands). The fish used for (in vitro) branchial flux experiments (173 ± 6 g; n = 19) were acclimated to Nijmegen tap water as described in detail by Verbost et al. (18).

Trout used for (in vivo) whole body Ca\(^{2+}\) flux experiments (183 ± 4 g (SE) body wt; n = 158) were obtained from a commercial dealer (Ontario, Canada). The fish were acclimated for at least 3 wk to dechlorinated Ottawa tap water containing (in mM) Ca\(^{2+}\) 0.5, Cl\(^{-}\) 0.1, Na\(^{+}\) 0.1, K\(^{+}\) 0.02; this water is referred to as normal-calcium water. A group of fish were acclimated to low-calcium water for at least 3 wk. Low-calcium water was prepared by titrating demineralized water with CaCl\(_2\)·2H\(_2\)O to achieve a final Ca\(^{2+}\) concentration of 0.04 mM. The Cl\(^{-}\), Na\(^{+}\), and K\(^{+}\) concentrations were adjusted to the levels of normal-calcium water. Our setup allowed a constant flow of low- or normal-calcium water through 1,000-liter fiber glass tanks. Water temperature was 15 ± 3°C, and the water pH varied between 6.3 and 7.4. The photoperiod was a 12-h light-dark cycle. Fish were fed daily to satiation with commercial trout pellets.

Analytical Methods

Water and plasma total calcium contents were determined with a commercial calcium kit (Sigma Chemical). Combined calcium-phosphate standards (Sigma Chemical) were used as reference.

Protein content was measured with a commercial reagent kit (BioRad) using bovine serum albumin (BioRad)
as a reference.

$^{40}$Ca contents of water samples and of plasma samples were determined after addition of ScintiVerse II (Fisher) by liquid scintillation analysis. $^{42}$Ca contents of water and plasma samples were determined by gamma-ray spectrometry.

Isolation of Hypocalcin

Hypocalcin was isolated as described recently (9), using concanavalin A (ConA) affinity chromatography. Material bound to ConA consisted of at least 98% of a 54-kDa glycoprotein, which we named hypocalcin. The material without affinity to ConA (crude CS homogenate minus hypocalcin) is referred to as residue protein and was devoid of hypocalcin judged by sodium dodecyl sulfate-gel electrophoresis.

Determination of Whole Body $Ca^{2+}$ Flux

Catheterization. Fish were anesthetized in sodium bicarbonate-buffered (pH 7.4) ethyl aminobenzoate (MS222; 0.1 g/l). During the operation, which lasted up to 10 min, fish were kept wet and the gills were irrigated with MS222-containing water. Dorsal aortic catheterization was subsequently performed using standard techniques. Catheters were filled with slightly modified Cortland saline containing 10 U/ml ammonium heparin and were closed with a stainless steel pin. Fish were allowed to recover on the operating table by irrigation of the gills with plain water. Next, they were left for at least 24 h before experimentation in individual flux boxes supplied with running normal- or low-calcium water.

Experimental procedures. Whole body flux rates were determined using catherized fish kept in 3-liter opaque Perspex boxes. At the start of an influx experiment, water flow through the boxes was discontinued, and $^{40}$Ca was added to the water ($5 \times 10^7$ Bq/l; ICN, Canada). Efficient mixing of the $^{40}$Ca with the water was assumed by vigorous aeration of the flux boxes. After a 4-h exposure period, the fish were killed with an overdose of MS222 (0.8 g/l). Next, the fish were rinsed in tap water (1 min) and transferred for 3 min to water containing 10 mM CaCl$_2$ to exchange $^{40}$Ca adsorbed to the integumental surfaces. To determine total body $^{40}$Ca activity, fish were microwave cooked (1.5 min) and homogenized in a commercial blender with 65% body wt of distilled water. Quintuple tissue samples (~0.5 g weighed to the nearest 3 decimals) were dissolved at 60°C overnight in 2 ml of tissue solubilizer (NCS; Amersham). Subsequently, the samples were neutralized with glacial acetic acid, 5 ml of distilled water were added, and the $^{40}$Ca activity was determined.

For efflux experiments, fish were injected (via their catheter) with $2.22 \times 10^6$ Bq of $^{42}$Ca in a volume of 1 ml 6% NaCl 24 h before the start of the flux period. Ten minutes before the start of an experiment, water flow through the boxes was discontinued and the $^{42}$Ca appearance rate in the water monitored (see below). After a flux period of 4 h, the $^{42}$Ca-containing water was replaced by tracer-free water until $^{42}$Ca became undetectable. Subsequently, a second 4-h flux period was performed. The first flux measurement served to establish individual control values; the second flux period was used to determine the effects of vehicle or hormone treatment.

Flux determinations were performed according to Flik et al. (5) and Perry and Wood (15). For influx measurements we assumed a constant $^{40}$Ca accumulation rate by the fish for at least 4 h. Flik et al. (5) showed that in tilapia, the $^{40}$Ca accumulation rate under comparable conditions was constant for that time period. Water $^{40}$Ca sp act did not decrease significantly over the 4-h flux period. For efflux determinations a constant $^{40}$Ca tracer appearance rate in the water for at least 4 h (measurements were performed every 15 min) for control as well as hormone-treated fish was observed ($r = 0.999$; significance of the slope $P = 0.01$). Renal calcium excretion was either constant or, when intermittent, did not influence water $^{42}$Ca sp act because no fluctuations in $^{40}$Ca appearance rate in the water were observed. A 24-h period of internal $^{42}$Ca distribution was sufficient to ensure a constant plasma $^{45}$Ca sp act ($<5\%$ change) during the flux period.

Product administration. All injections (200 µl) were given intra-arterially via the catheter at time ($t = 0$) of the flux period. Three types of treatments were given: 1) crude CS tissue homogenates; 2) hypocalcin, and 3) residue proteins. The amounts of protein injected per 100 g fish were 235 µg CS tissue homogenate, 50 µg hypocalcin, and 100 µg residue; these doses equal the amount of material obtained from 3.75 (crude tissue homogenate) and 6.5 fish of similar body weight, respectively. Cortland saline injections served as controls.

Blood and water sampling. For influx as well as efflux measurements 500-µl blood samples were withdrawn via the catheter immediately before hormone injection ($t = 0$) and on completion of the flux experiment ($t = 4$ h). During influx experiments 5-ml water samples were taken at $t = 15$ min and at the end of the flux period ($t = 4$ h). For efflux determinations, water samples were taken at the start, middle, and end of the flux period ($t = 0, 2, \text{and} 4$ h, respectively). Blood samples were centrifuged in heparinized 1.5-ml tubes. Plasma (100 µl) and water (5 ml) samples were analyzed in triplicate for $^{42}$Ca sp act.

Calculations. Influx of $Ca^{2+}$ was calculated from the total $^{40}$Ca activity of the fish after 4 h of exposure to $^{40}$Ca-containing water and the mean sp act of the water, and was normalized to fish weight. $Ca^{2+}$ efflux was calculated from the slope of the time curves of the $^{40}$Ca appearance in the water and the mean $^{42}$Ca sp act of the plasma and was normalized to fish weight. Flux is expressed in micromoles per hour per kilogram fish.

Determination of Branchial $Ca^{2+}$ Influx

Experimental procedures. To determine branchial $Ca^{2+}$ influx the isolated head preparation was used as described by Payan and Matty (13), with some modifications according to Perry and Wood (15). Procedures for $Ca^{2+}$ influx calculation and determination of transepithelial potential (TEP) have been described in detail recently by Verbost et al. (18). At the start of the exper-
iment ⁴⁷Ca (2.9 × 10⁶ Bq/l) was added to the water. The Ringer solution was well aerated throughout the experiments.

**Product administration.** CS crude tissue homogenates, hypocalcin, and residue proteins were added to the Ringer solution used to perfuse the gills. A 30-min perfusion with plain Ringer solution to determine control influx was followed by a 15-min perfusion with hormone containing Ringer solution, using separate containers connected by a three-way valve. Hormone was added in concentrations of 50 μg/100 ml Ringer solution for the isolated product, 100 μg/100 ml Ringer solution for residue proteins, and 150 μg/100 ml Ringer solution for crude tissue homogenate.

**Perfusate and water sampling.** For Ca²⁺ influx the ⁴⁷Ca appearance rate in the perfusate collected from the dorsal aorta was determined (the dorsal aortic output represents 90-95% of the total (arterial and venous) ⁴⁷Ca influx; 15). Perfusate samples (100 μl) were taken every 1 min for the first 10 min and every 5 min thereafter until t = 30 min, followed by 10 1-min interval samples and a final sample at 45 min. Water samples (1 ml) were taken at the same time. These samples were used to determine the water ⁴⁷Ca sp act.

**Calculations.** Ca²⁺ influx was calculated on the basis of tracer appearance in the perfusate and the water ⁴⁷Ca sp act. Control influx was calculated at t = 25-30 min; influx after hormone treatment was calculated at t = 40-45 min.

**Statistical Analysis**

Data are presented as means ± SE. For statistical evaluation the Mann-Whitney U test, one tailed, was used. Significance was accepted at P < 0.05.

**RESULTS**

**Whole Body Ca²⁺ Flux**

**Controls.** Ca²⁺ influx values in untreated fish show only small variations for normal-calcium acclimated fish as well as for low-calcium acclimated fish. No significant variation of Ca²⁺ influx is observed over the 2-mo period in which the experiments were performed.

**Low-calcium acclimation.** In Fig. 1, influx, efflux, and net flux of Ca²⁺ are shown for low-calcium acclimated fish, and these are compared with flux values for normal-calcium acclimated fish. Under normal- and low-calcium conditions, net flux of Ca²⁺ does not differ significantly, although both influx and efflux are lower in low-calcium acclimated trout.

**Hormone effects.** Figures 2 and 3 show the effects of the various treatments on the Ca²⁺ in- and efflux values in normal- and low-calcium acclimated fish compared with the controls (saline injected). Crude CS tissue homogenates and the isolated hypocalcin inhibit Ca²⁺ influx in both groups. Similar doses of hypocalcin yield significantly greater inhibition in low-calcium acclimated trout than in normal-calcium acclimated trout. Injection of the residue proteins do not affect Ca²⁺ influx.

CS crude tissue homogenates, hypocalcin, and residue proteins do not alter Ca²⁺ efflux compared with the saline-treated or untreated fish (Figs. 2 and 3). Saline infusion (control c) does not alter Ca²⁺ efflux when compared with Ca²⁺ efflux in untreated fish (control c; Figs. 2 and 3).

Net Ca²⁺ flux from the water in low- and normal-calcium acclimated trout are indicated in Figs. 2 and 3 (m). Injection of crude CS tissue homogenates and of hypocalcin, but not of the residue, results in a decrease of net Ca²⁺ flux. Actually, fish show a net loss of calcium after injection of crude tissue homogenate as a result of the decreased Ca²⁺ influx (2.0 and 1.55 μmol-h⁻¹·kg⁻¹ for normal- and low-calcium acclimated fish, respectively). The same doses of hypocalcin resulted in significantly greater inhibition of the Ca²⁺ net flux in the low-calcium acclimated fish (97%) than in the normal-calcium acclimated fish (52%).
FIG. 3. Effects of Stannius corpuscles crude homogenate, hypocalcin, and residue proteins on Ca\(^{2+}\) influx (in) and Ca\(^{2+}\) efflux (out) for low-calcium acclimated fish. For efflux, control flux (c; fish without treatment) is shown in green, and experimental flux (e; fish after treatment) is indicated on right side of bars. \(\square\), Ca\(^{2+}\) net flux. Mean values \(\pm SE\) are given; within bars indicate \(n\). * Significant difference from control.

TABLE 1. Effects of Stannius corpuscle crude homogenate, hypocalcin, and residue proteins on branchial Ca\(^{2+}\) influx

<table>
<thead>
<tr>
<th></th>
<th>(n)</th>
<th>Branchial Ca(^{2+}) Influx, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>92.7±0.4</td>
</tr>
<tr>
<td>Crude</td>
<td>4</td>
<td>80.6±3.1*</td>
</tr>
<tr>
<td>Hypocalcin</td>
<td>7</td>
<td>79.3±2.7†</td>
</tr>
<tr>
<td>Residue</td>
<td>4</td>
<td>92.7±4.1</td>
</tr>
</tbody>
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Values are means \(\pm SE\); \(n\), no. of fish. One hundred percent of prehormone Ca\(^{2+}\) flux = 7.28 ± 1.13 \(\mu\)mol \(\cdot\) h\(^{-1}\) \(\cdot\) kg\(^{-1}\). * \(P < 0.014\). † \(P < 0.003\).

Branchial Ca\(^{2+}\) Influx

Hormone effects. As shown in Table 1, crude tissue homogenates as well as isolated hormone, but not the residue proteins, result in a statistically significant decrease of branchial Ca\(^{2+}\) influx in the isolated head preparation. A considerable variability in the values for Ca\(^{2+}\) flux between individual fish was observed. Therefore, in each fish the posthormone treatment flux is expressed as a percentage of the prehormone treatment flux (designated to 100%). The average pretreatment value was 7.28 ± 1.13 \(\mu\)mol \(\cdot\) h\(^{-1}\) \(\cdot\) kg\(^{-1}\) \((n = 19\); range 3.24–8.40 \(\mu\)mol \(\cdot\) h\(^{-1}\) \(\cdot\) kg\(^{-1}\)).

Hormone and control injections did not affect the TEP. For the control, crude tissue homogenate-, hypocalcin-, and residue-treated fish the TEP was \(-0.05 \pm 1.19\), \(-1.62 \pm 0.76\), \(-0.65 \pm 0.77\), and \(-0.09 \pm 0.39\) mV, respectively. The average value for all fish was \(-0.58 \pm 0.70\) mV \((n = 19)\).

Treatment with crude tissue extracts resulted in an increase of systemic blood pressure of 20.3 ± 6.3%. No blood pressure effects were observed in control-, hypocalcin-, or residue-treated isolated head preparations (nondetectable, 4.6 ± 2.8 and \(-1.0 \pm 3.3\), respectively).

DISCUSSION

The following conclusions are drawn from the data presented in this paper. 1) In freshwater rainbow trout, hypocalcin, the 54-kDa glycoprotein isolated from the CS, inhibits Ca\(^{2+}\) uptake from the water by inhibition of Ca\(^{2+}\) influx; Ca\(^{2+}\) efflux is not affected. 2) Hypocalcin injections appear more effective in inhibiting Ca\(^{2+}\) influx in fish acclimated to low-calcium water than in fish from normal-calcium water. 3) Hypocalcin inhibits Ca\(^{2+}\) influx in the isolated head preparation, thereby establishing the gill as an important target for hypocalcin. 4) Hypocalcin does not alter the branchial TEP; we suggest, therefore, that the inhibition of Ca\(^{2+}\) influx is due to a specific modulation of the Ca\(^{2+}\) uptake mechanism in the gill.

Our values for whole body Ca\(^{2+}\) flux and branchial Ca\(^{2+}\) flux determined with the isolated head technique are in agreement with Ca\(^{2+}\) flux values reported in the literature (8, 13–15).

We showed here that trout hypocalcin inhibits whole body Ca\(^{2+}\) uptake in intact trout. Thus the reduction of plasma calcium levels caused by injections of crude CS tissue homogenates observed in freshwater fish (21) could be explained, at least in part, to result from the hypocalcin-induced reduction of whole body Ca\(^{2+}\) uptake.

The inhibition of Ca\(^{2+}\) influx by hypocalcin in adult trout was a consistent phenomenon in our study. Wagner et al. (20) reported that the hypocalcemic principle of salmon CS exerts inhibitory effects on \(^{44}\)Ca influx in juvenile rainbow trout, but only when Ca\(^{2+}\) influx in these fish is high (19). We compared whole body Ca\(^{2+}\) flux in fish from normal- and low-calcium water (with high and low Ca\(^{2+}\) influx rates, respectively) and found that an inhibitory effect of hypocalcin on Ca\(^{2+}\) influx was always apparent.

We calculated that on protein basis four times more crude tissue extract than purified hormone was needed to obtain the same degree of inhibition of whole body Ca\(^{2+}\) influx. This indicates that hypocalcin was purified from crude tissue extracts by a factor of about four. This seems a rather low purification factor. It is, however, in good agreement with the relatively large amount of hypocalcin present in crude tissue homogenates of CS of trout and eel when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (at least 12% on protein basis, 9) and with the abundance of secretory granules observed in electron microscope slides of freshwater trout CS (9, 22).

In trout injections of hypocalcin did inhibit Ca\(^{2+}\) influx but did not induce significant hypocalcemia within the 4-h period of the flux determination. This may be expected, since the net whole body Ca\(^{2+}\) flux from water to fish is still positive. After injection of crude tissue extracts, a net efflux of Ca\(^{2+}\) is observed. Assuming an extracellular fluid volume of 300 ml/kg body wt (8), a loss of 8 \(\mu\)mol calcium as observed in a 4-h period is only 1.3% of the total calcium present in the extracellular fluid compartment of the fish (assuming a calcium concentration of 2 mM in this compartment) and, therefore, may not be detectable at the level of a change in plasma calcium. The same dose of trout hypocalcin (6.5 fish equivalents), however, does evoke a hypocalcemic re-
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sponse in 15 days stannicnomized eels (Lafeber et al., unpublished data).

In trout adapted to low-calcium water, the effect of hypocalcin was more effective to inhibit Ca²⁺ influx than in fish from normal-calcium water. This is consistent with observations of Wendelaar Bonga et al. (21) on killifish and tilapia. These authors showed that CS homogenates hardly influence plasma calcium levels in fish from normal-calcium freshwater. Conversely, significant hypocalcemia could be induced in fish from low-calcium freshwater. It was suggested that in low-calcium water the circulating levels of the active hypocalcemic principle of the CS will be low, which enables exogenous hormone to be more effective (21). This reasoning may also be applicable to our results.

We showed that both Ca²⁺ influx and efflux were lower in fish from low-calcium water than in fish from normal-calcium water, whereas total Ca²⁺ uptake was similar in both groups. In this respect trout differ from tilapia, in which increased Ca²⁺ influx and efflux were reported, resulting in an increased Ca²⁺ uptake in low-calcium water (6). Apparently the control of calcium homeostasis is different in the two species.

The reduction of whole body Ca²⁺ uptake by hypocalcin appears to be caused by a specific inhibition of branchial Ca²⁺ influx; whole body Ca²⁺ efflux is not affected. These results imply that an effect of hypocalcin on calcium excretion via the kidney (1) must quantitatively be of less importance. It has been reported that Ca²⁺ uptake by the gut is also inhibited by hypocalcin (23). The relative contribution of the gut to Ca²⁺ uptake is small (3, 5). Therefore, the effect of hypocalcin on the intestinal Ca²⁺ uptake will be of less importance for the whole body Ca²⁺ uptake.

The inhibition of hypocalcin on gill Ca²⁺ influx, as we observed in the isolated head, can account for the effect observed in vivo. In the isolated head, 21% inhibition was obtained with 50 μg/100 ml Ringer solution. In the intact fish, 52% inhibition was obtained with 50 μg/100 g body wt. Assuming that the hypocalcin distribution space in the fish is equivalent to the extracellular fluid volume (300 ml/kg body wt; 8), the in vivo dose of 50 μg/100 g body wt corresponds to a dose of 150 μg/100 ml extracellular fluid. These calculations indicate that the in vivo-to-in vitro dosage ratio (0.42) is comparable to the in vivo-to-in vitro percentage inhibition ratio (0.35). Thus the inhibition of gill Ca²⁺ uptake can be explained by the inhibition of branchial Ca²⁺ uptake. This further substantiates that the gills are an important effector for the hypocalcemic action of hypocalcin.

Because no pressor effects were observed with hypocalcin or residue proteins, we conclude that the increase in branchial vascular resistance is an effect specific for CS crude tissue extracts. The substance involved in pressure control must be smaller than 5 kDa (such small proteins are lost during our isolation procedure; 9) or inactivated during the isolation procedure, since no effect is observed after treatment with residue proteins. Already in 1969, Chester Jones and co-workers (1) suggested an involvement of CS in vasopressor control. Apparently, hypocalcemic as well as vasoactive subststances are present in the CS. Our data confirm the suggestion by Chester Jones et al. (1) but show that vasopressor activity is not related to hypocalcin.

Hypocalcin is involved in the inhibition of branchial Ca²⁺ uptake. But how does hypocalcin affect branchial Ca²⁺ flux? A model for branchial Ca²⁺ uptake in fresh water trout is postulated by Perry and Flik (14). This model essentially corroborates the model originally postulated for branchial Ca²⁺ uptake in tilapia (5, 7). In this model Ca²⁺ uptake is thought to include a passive apical Ca²⁺ influx via calcium channels and an active basolateral Ca²⁺ extrusion from the cytosol to the blood via a high-affinity Ca²⁺-ATPase. In our studies on the hormonal control of the branchial Ca²⁺ uptake mechanism in fresh water fish, evidence is accruing, although no decisive answers can be given yet, that hypocalcin does not act on the basolateral Ca²⁺-ATPase. We, therefore, hypothesize that the hypocalcin-induced inhibition of branchial Ca²⁺ influx involves modulation of calcium channels in the apical membranes of the calcium-transporting cells.

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