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Effect of 2-deoxy-D-glucose on acetylcholine and histamine levels in gastric juice of pylorus-ligated rats anesthetized with urethane

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Abstract

Acetylcholine (ACh) in gastric juice was detected and measured by pretreatment of acetylcholinesterase inhibitor, 1 m*M* eserine (1 ml/rat, p.o.), in pylorus-ligated rats, by liquid chromatography with electrochemical detection. In order to elucidate whether or not the ACh level in gastric juice reflects the activity of cholinergic neurons, the effect of 2-deoxy-D-glucose (2-DG), a vagus stimulant, on the levels of ACh, histamine and gastric acid in gastric juice was investigated in pylorus-ligated rats anesthetized with urethane (1.25 g/kg, i.p.). Under the non-anesthetic condition, ACh, histamine and gastric acid levels were 100±25 pmol/h, 120±10 ng/h, and 240±32 μequiv./h, respectively. These levels were completely inhibited by urethane anesthesia. Under the anesthetized condition, 2-DG (50–200 mg/kg, i.v.) significantly increased ACh and histamine levels in gastric juice, as well as acid secretion. The 2-DG (200 mg/kg, i.v.)-induced increases in these levels were completely inhibited by vagotomy. These results suggest that ACh level measured in gastric juice reflects the activity of cholinergic transmission. Furthermore, these results also support the conclusion that vagus stimulation facilitates not only cholinergic transmission but also histaminergic transmission related to gastric acid secretion.

Keywords: 2-Deoxy-D-glucose; Acetylcholine; Histamine; Urethane

1. Introduction

Until now, experiments investigating gastric acetylcholine (ACh) and histamine releases were carried out under in vitro conditions using isolated stomach preparations [1,2] and gastric strips [3] or by measuring these contents in gastric mucosa [4,5]. However, there are few reports indicating detection of ACh in gastric juice under in vivo conditions although there are some reports regarding histamine [6]. In the present study, we tried to detect ACh and histamine in gastric juice of pylorus-ligated rats, which are widely used as an animal model for in vivo experiments of gastric secretion.

2-Deoxy-D-glucose (2-DG), a stimulant of vagus nerve via central nervous system, induces hypersecretion of gastric acid in the perfused stomach of anesthetized cats [7] and rats [8]. Furthermore, cholinergic agonists are known to induce histamine

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release from gastric mucosa [9]. Therefore, vagus stimulation by 2-DG is expected to increase both ACh and histamine outputs or levels in the gastric juice, as well as gastric acid secretion, if ACh and histamine levels detected in gastric juice reflect the activity of cholinergic and histaminergic neurotransmissions.

In the present study, the effect of 2-DG on levels of ACh, histamine and gastric acid was investigated in urethane-anesthetized pylorus-ligated rats, to elucidate whether ACh and histamine levels in gastric juice reflect the activity of gastric acid secretion.

2. Experimental

2.1. Animals

Male Wistar rats purchased from SLC Japan (Hamamatsu, Japan) were housed in a facility at a temperature of 24±1°C, relative humidity of 55±5%, and controlled lighting with lights on from 07:00 to 19:00 daily. The animals were allowed free access to water and standard laboratory food (MF, Oriental Yeast, Tokyo, Japan).

Experimental protocols were approved by the Committee for Animal Experimentation at Gunma University School of Medicine and met the guidelines of the Japanese Association for Laboratory Animal Science.

2.2. Experimental schedule

After a 24-h fast, rats weighing 240–260 g were pylorus-ligated with or without gastric vagotomy, under 2.0% halothane anesthesia induced by a small animal anesthetizer (Model TK-4, Bio Machinery, Funabashi, Japan). Vagotomy was carried out by cutting bilateral (anterior and posterior) gastric branches of the celiac vagus [10]. The rats awakened within 5 min after the surgical anesthesia was stopped. In the experiment for the determination of ACh level in gastric juice, 1 ml of 1 m*M* eserine sulfate in distilled water (Wako, Osaka, Japan) was orally administered to the awakened rats. The pylorus-ligated rats pre-treated and non-treated with eserine were anesthetized by intraperitoneal (i.p.) injection of urethane (1.25 g/2 ml distilled water/kg, Sigma, St. Louis, MO, USA). Various concentrations of 2-DG (Sigma) dissolved in saline were injected into the jugular vein of the urethane-anesthetized rats. Four hours following the injection of 2-DG, the stomachs of the rats were removed after the cardia was ligated (in non-anesthetized rats, the stomachs were removed under halothane anesthesia). Gastric juice was collected and centrifuged at 12 000 rpm and 4°C for 10 min. After measuring the volume of gastric juice, an aliquot of gastric juice was used for the determination of ACh, histamine or gastric acid levels.

2.3. Detection and determination of ACh level in gastric juice

A 1-ml volume of gastric juice was neutralized to pH 7.4 by 1 *M* NaOH. Ethylhomocholine iodide (EHC, 1 μmol/20 μl), an internal standard of ACh, was added. The mixture was centrifuged at 12 000 rpm and 4°C for 15 min. The supernatant was purified by passage through a 0.45-μm Millipore filter. An aliquot, typically 50 μl, of the filtrate was injected into a high-performance liquid chromatography (HPLC) system (Nanospace, Shiseido, Tokyo, Japan) equipped with an electrochemical detection device using post-column enzyme reaction for the determination of ACh level [11].

In this system, an ACh Separation analytical column (3 μm, 60×4 mm, polymeric styrene-based packing materials, Bioanalytical Systems; BAS, Tokyo, Japan) was used for separation of EHC and ACh. An immobilized column (5×4 mm, BAS) containing acetylcholinesterase (AChE) and choline oxidase was used for post-column reaction. A glassy carbon pre-column (10×4 mm, Irica, Kyoto, Japan) was set up between the injector and the analytical column to trap the interfering compounds. The mobile phase was 0.05 *M* phosphate buffer, pH 8.4, containing 1.0 m*M* disodium EDTA and 0.4 m*M* sodium 1-octanesulfonate. The flow-rate was set at 0.70 ml/min. The potential of the platinum working electrode was set at +0.50 V vs. an Ag/AgCl reference electrode. Temperatures of the pre-column, separation column and post-column were all maintained at 35°C. A Shiseido Nanospace workstation was employed for data collection and processing.

The retention times were 4.82 min for EHC and 9.32 min for ACh. The detection limit of ACh in the injected sample was 0.5 pmol. ACh output was expressed as pmol/h and was calculated as follows: ACh output (pmol/h)=[volume (ml/4 h)×ACh level (pmol/ml)]/sampling time (4 h).

To determine whether or not the peak detected in the gastric juice in this system was indeed ACh, 10 units/20 μl of AChE (EC 3.1.1.7, Sigma: one unit hydrolyzes 1.0 μmol of ACh to choline and acetate per minute at pH 8.0 at 37°C) was added to 1.0 ml of some neutralized gastric juices. The mixture was incubated for 5 min at 37°C. After centrifugation, the supernatant was filtrated using a Millipore filter and then an aliquot of the filtrate was injected into the ACh assay system.

2.4. Determination of histamine level in gastric juice

Spermidine-3HCl (Sigma) (100 μl of a 1.0 mg/ml solution) as an internal standard for histamine, 120 μl of 5 *M* NaOH, 400 mg of NaCl and 3.5 ml of *n*-butanol were added to 1.0 ml of gastric juice. The mixture was centrifuged at 3000 rpm and 4°C for 5 min. After removing the lower layer, the upper layer was washed twice with 2 ml of 0.1 *M* NaOH in NaCl-saturated solution by repeating mixing and centrifugation. The butanol layer (upper layer), 3 ml, was transferred to the other test tube, and 4 ml of *n*-heptane as well as 2.5 ml of 0.1 *M* HCl were added to it and mixed. After 5 min, the upper layer was removed by aspiration. An aliquot (100 μl) of the lower layer was injected into the HPLC system (LC-10AD, Shimazu, Kyoto, Japan) equipped with fluorescent detection using post-column derivatization with *o*-phthalaldehyde (OPA), for the determination of the histamine level [5].

In this system, a STR ODS-II reversed-phase column (150×4.6 mm I.D., Shimazu) was used for separation of histamine and spermidine. The mobile phase consisted of a mixture (A–B, 2:1) of A solution (100 m*M* sodium tartaric acid buffer, pH 4.4, containing 10 m*M* sodium 1-octanesulfonate) and B solution (99.7% methanol for HPLC). The flow-rate was maintained at 1.0 ml/min. The effluent from the column was mixed with a post-column solution (400 m*M* sodium borate buffer, pH 9.2–10 m*M* OPA in methanol, 2:1) at a flow-rate of 0.5 ml/min. The mixture of the effluent and the post-column solution flowed to a reaction coil (4.0 m×0.5 mm I.D., stainless steel tubing) at a final flow-rate of 1.5 ml/min, and passed through a fluorescent detector where the OPA-derivatives of histamine and spermidine were detected at excitation level of 360 nm and emission at 440 nm. Temperatures of the separation column and the reaction coil were maintained at 50°C. A Shimazu LC-10A workstation was employed for data collection and processing. The retention times were 4.40 min for histamine and 7.89 min for spermidine. The detection limit of histamine in the injected sample was 10 ng.

Histamine output was expressed as ng/h and was calculated as follows: histamine output (ng/h)=[volume (ml/4 h)×histamine level (ng/ml)]/sampling time (4 h).

2.5. Determination of acidity in gastric juice

Phenolphthalein (10 μl of a 1% solution) in ethanol solution as an indicator was added to 1.0 ml of gastric juice diluted in water (ratio gastric juice:water=1: 9). Acidity was measured by titration with 0.01 *M* NaOH and expressed as mequiv./l. Acid output was expressed as μequiv./h and was calculated as follows: acid output (μequiv./h)=[volume (ml/4 h)×acidity (mequiv./l)]/sampling time (4 h).

3. Results

3.1. Detection of ACh observed in gastric juice

Fig. 1 shows typical chromatograms obtained in the ACh analysis system from standard solution (A), eserine-treated gastric juice of non-anesthetized rat (B) and AChE-treated gastric juice (C). The retention time (t_R: 10 min) of the ACh peak observed in Fig. 1B corresponded to that of authentic ACh in Fig. 1A. The detected ACh peak disappeared following the addition of AChE into the gastric juice as shown in Fig. 1C.

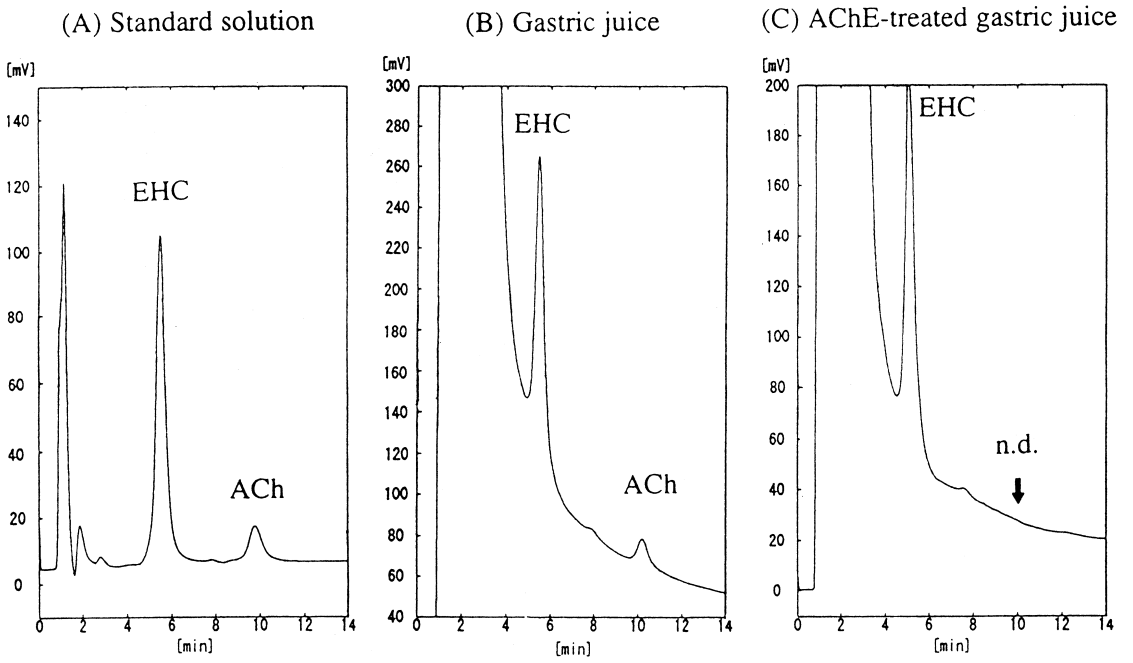

Fig. 1. Typical chromatograms obtained in the ACh analysis system from standard solution (A), eserine-treated gastric juice of non-anesthetized rat (B) and AChE-treated gastric juice (C). The n.d. shown in (C) stands for ''not detected''.

3.2. Effects of 2-DG on ACh level in gastric juice in anesthetized pylorus-ligated rats

The results are shown in Fig. 2. Under the non-anesthetic condition, ACh (100±25 pmol/h) was detected in gastric juice by eserine treatment following pylorus ligation, although ACh was not detected under the eserine-free condition. Urethane anesthesia inhibited eserine-detected gastric ACh level. Under the anesthetized condition, 2-DG (50–200 mg/kg, i.v.) significantly increased the ACh level. 2-DG (200 mg/kg)-induced increase in the ACh level was completely inhibited by vagotomy.

3.3. Effects of 2-DG on histamine level in gastric juice in anesthetized pylorus-ligated rats

Fig. 3 shows typical chromatograms obtained in the histamine analysis system from standard solution (A) and gastric juice of non-anesthetized rat (B). The retention time of the histamine peak detected in the gastric juice corresponded to that of authentic histamine. As shown in Fig. 4, histamine levels in

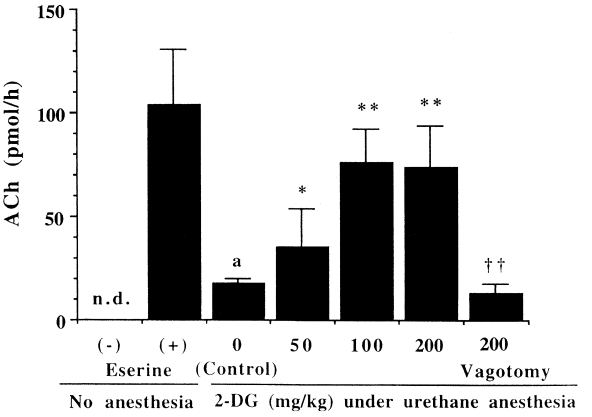

Fig. 2. Effect of 2-DG on ACh level in gastric juice of anesthetized pylorus-ligated rats. Effect of anesthesia on gastric ACh level was assessed by Student's *t*-test: $^{a}P<0.01$ compared to normal (non-anesthetized) rats. Effect of 2-DG on ACh level in anesthetized rats was assessed using a one-way ANOVA, followed by Bonferroni multiple-comparison test: $^{*}P<0.05$ and $^{**}P<0.01$ compared to the control group. Effect of vagotomy on 2-DG (200 mg/kg)-induced increase in ACh output was assessed using Student's *t*-test: $^{\dagger\dagger}P<0.01$ compared to 200 mg/kg 2-DG-treated group.

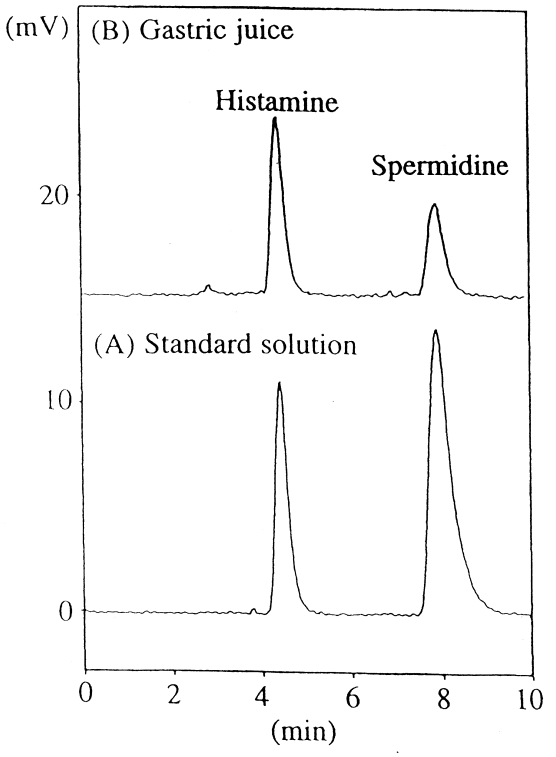

Fig. 3. Typical chromatograms obtained in the histamine analysis system from standard solution (A) and gastric juice of non-anesthetized rat (B).

gastric juice of non-anesthetized pylorus-ligated rats were 120±10 ng/h. The levels were completely inhibited by urethane anesthesia. Under the anesthetized condition, 2-DG (50–200 mg/kg, i.v.) significantly increased gastric histamine level. 2-DG (200 mg/kg, i.v.)-induced increase in the histamine level was completely inhibited by vagotomy.

3.4. Effects of 2-DG on gastric acid secretion in anesthetized pylorus-ligated rats

The results are shown in Fig. 5. Gastric acid output in non-anesthetized pylorus-ligated rats was 240±32 μequiv./h. The output was completely inhibited by urethane anesthesia. Under the anesthetized condition, 2-DG (50–200 mg/kg, i.v.) significantly increased the gastric acid output. 2-DG (200 mg/kg, i.v.)-induced increase in the output was completely inhibited by vagotomy.

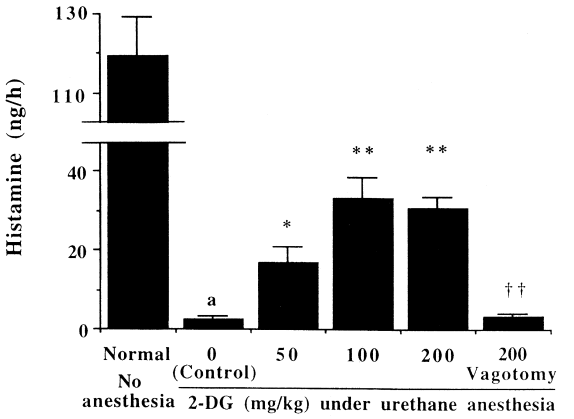

Fig. 4. Effect of 2-DG on histamine level in gastric juice of anesthetized pylorus-ligated rats. Effect of anesthesia on gastric histamine level was assessed by Student's *t*-test: $^{a}P<0.01$ compared to normal (non-anesthetized) rats. Effect of 2-DG on the histamine level in anesthetized rats was assessed using a one-way ANOVA, followed by Bonferroni multiple-comparison test: $*P<0.05$ and $**P<0.01$ compared to the control group. Effect of vagotomy on 2-DG (200 mg/kg)-induced increase in histamine level was assessed using Student's *t*-test: $^{††}P<0.01$ compared to 200 mg/kg 2-DG-treated group.

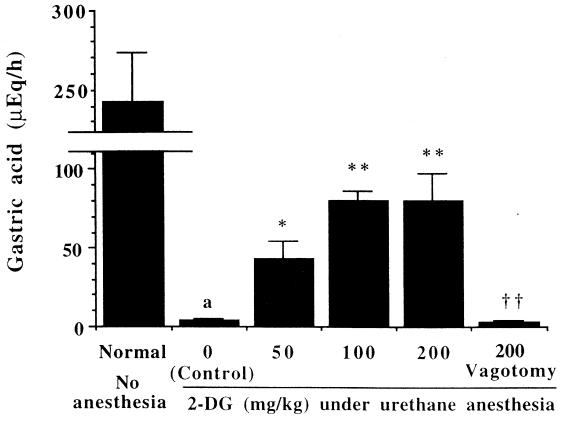

Fig. 5. Effect of 2-DG on gastric acid secretion in anesthetized pylorus-ligated rats. Effect of anesthesia on gastric acid output was assessed by Student's *t*-test: $^{a}P<0.01$ compared to normal (non-anesthetized) rats. Effect of 2-DG on the acid output in anesthetized rats was assessed using a one-way ANOVA, followed by Bonferroni multiple-comparison test: $*P<0.05$ and $**P<0.01$ compared to the control group. Effect of vagotomy on 2-DG (200 mg/kg)-induced increase in the acid output was assessed using Student's *t*-test: $^{††}P<0.01$ compared to 200 mg/kg 2-DG-treated group.

4. Discussion

In the present study, we could detect ACh in 1 m*M* eserine-treated gastric juice of pylorus-ligated rats under in vivo conditions, as the peak of ACh was not observed without using AChE inhibitor. The retention time of the gastric ACh peak in the chromatogram corresponded to that of authentic ACh. The peak disappeared after addition of a hydrolytic enzyme, AChE, to the collected gastric juice. From these results, the peak was identified as ACh. As there had been no prior report demonstrating ACh level in gastric juice of pylorus-ligated rats under in vivo conditions, the present levels (100 ± 25 pmol/h) could not be compared to others. However, the amount of spontaneous overflow of ACh in the perfusate from the isolated rat stomach in the presence of 100 μM physostigmine is reported to be 21.7 ± 0.6 pmol/2 min [2]. Tonini et al. [12] have reported that resting release of ACh from antral strips of rat stomach is 23.52 ± 3.70 pmol/g/min in Krebs solution containing 3 μM eserine. Thus, although ACh release levels differ with experimental conditions, these findings clearly suggest that ACh is detected in gastric juice in the presence of an esterase inhibitor.

To clarify the significance of the ACh level measured in gastric juice, we examined the effect of 2-DG on the levels of ACh, histamine and gastric acid in gastric juice. 2-DG stimulates secretion of gastric acid by increasing efferent activity in gastric vagus nerve via glucopenia in hypothalamic neurons [13,14]. These responses are thought to be mediated vagally, because 2-DG-induced effects are entirely neutralized by vagotomy or by prior administration of atropine [15,16]. It was also reported that restraint and water-immersion stress or administration of 2-DG increased the activity of both gastric choline acetyltransferase and AChE, and gastric ACh metabolism in rats [17,18]. Yokotani et al. [2] have demonstrated using an isolated rat stomach that the vagal nerve stimulation-evoked ACh overflow in the perfusate is completely neutralized by tetrodotoxin or calcium removal from the perfusion medium. Therefore, if the ACh level measured in gastric juice reflects the activity of gastric cholinergic neurons, the level of ACh must be increased by 2-DG. In fact, ACh levels increased in parallel with an increase in gastric acid secretion by stimulating vagus nerve with 2-DG, and these increased levels were completely inhibited by vagotomy. These results suggest that the ACh level in gastric juice clearly reflects the activity of gastric cholinergic transmission. As ACh (35.7 ± 10.1 nmol/g tissue) has been detected in the gastric mucosa of rats [4], ACh detected in gastric juice may be the result of the leakage or diffusion of mucosal ACh.

There are few reports examining histamine output in gastric juice of pylorus-ligated rats. The output level (120 ± 10 ng/h) in the present study was in agreement with the results obtained by radioimmunoassay [6]. Main and Pearce [19] have demonstrated that histamine is released from isolated gastric mucosa in rats. Typical and atypical populations of mast cells have been observed and purified in isolated cells obtained from the gastric mucosa of dogs [20,21]. Recently, Ishizuka et al. [22] have demonstrated that histamine is released from cultured mucosal mast cells. Thus, it is believed that histamine derives from histamine storing cells such as enterochromaffin-like (ECL) cells or mast cells which are present in gastric mucosa [23]. Therefore, histamine level measured in gastric juice may also be due to the leakage or diffusion of mucosal histamine, as well as gastric ACh.

It has been widely accepted that distinct receptors for ACh, gastrin and histamine are present on acid-secreting parietal cells [24]. In addition, recently, ACh and gastrin were proven to be capable of releasing histamine from mucosal stores [6,9,25]. ACh and gastrin increase the level of cytosolic calcium, whereas histamine increases the level of cAMP. The two transduction pathways converge on the H^+–K^+ ATPase, hydrogen pump of the parietal cells. Therefore, in the present study we suggest that 2-DG-evoked vagus stimulation induced not only cholinergic – but also histaminergic neurotransmission as well as increased gastric acid secretion. This conclusion is supported by the fact that these increased levels were completely inhibited by vagotomy.

The present report is the first study to detect and measure ACh levels in gastric juice of pyrolus-ligated rats, under in vivo conditions. We conclude that ACh and histamine outputs in gastric juice of pyrolus-ligated rats are useful indicators of gastric

activity related to mechanisms responsible for gastric acid secretion.

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