THE ROLE OF THE POLYMORPHIC EFFLUX TRANSPORTER P-GLYCOPROTEIN ON THE BRAIN ACCUMULATION OF d-METHYLPHENIDATE AND d-AMPHETAMINE

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ABSTRACT:
The psychostimulant medications methylphenidate (MPH) and amphetamine (AMP), available in various ratios or enantiopure formulations of their respective active dextrorotary isomers, constitute the majority of agents used in the treatment of attention-deficit/hyperactivity disorder (ADHD). Substantial interindividual variability occurs in their pharmacokinetics and tolerability. Little is known regarding the potential role of drug transporters such as P-glycoprotein (P-gp) in psychostimulant pharmacokinetics and response. Therefore, experiments were carried out in P-gp knockout (KO) mice versus wild-type (WT) mice after intraperitoneal dosing (2.5 mg/kg) of d-MPH or (3.0 mg/kg) of d-AMP. After the administration of each psychostimulant, locomotor activity was assessed at 30-min intervals for 2 h. Total brain-to-plasma drug concentration ratios were determined at 10-, 30-, and 80-min postdosing timepoints. The results showed no statistically supported genotypic difference in d-AMP-induced locomotor activity stimulation or in brain-to-plasma ratio of d-AMP. As for d-MPH, the P-gp KO mice had 33% higher brain concentrations (p < 0.05) and 67.5% higher brain-to-plasma ratios (p < 0.01) than WT controls at the 10-min postdosing timepoint. However, in spite of elevated brain concentrations, d-MPH-induced locomotor activity increase was attenuated in P-gp compared with that for WT mice. These data indicate that P-gp has no apparent effect on the pharmacokinetics and pharmacodynamics of d-AMP. In addition, d-MPH is a relatively weak P-gp substrate, and its entry into the brain may be limited by P-gp. Furthermore, the mechanism by which d-MPH-induced locomotor activity was attenuated in P-gp KO mice remains to be elucidated.

Attention-deficit/hyperactivity disorder (ADHD) is the most common neurobehavioral disorder affecting school-aged children. ADHD is generally characterized by varying degrees of inattention, hyperactivity, and impulsivity (Biederman and Faraone, 2005). Although recently established practice guidelines and algorithms have not identified a specific medication of first choice (Pliszka et al., 2000b; American Academy of Pediatrics, 2001; Greenhill et al., 2002), essentially all of these published guidelines identify one of the primary psychostimulants methylphenidate (MPH) or amphetamine (AMP) as initial pharmacotherapy. Of these two compounds, various formulations exist in differing biopharmaceutical delivery systems and isomeric content (Greenhill et al., 1999; Pliszka et al., 2000a; Faraone et al., 2002; Patrick et al., 2005) and aggregately constitute approximately 80% of all medications presently used in the treatment of ADHD. Large interindividual differences in drug response and dose have been noted (Wilens and Biederman, 1992; Greenhill et al., 2002), and predicting a therapeutic response in individual patients remains problematic, with most research finding no neurological, psychological, or physical characteristics as reliable predictors of response (Greenhill et al., 2002).

The ability of drug transporters to significantly influence drug absorption and disposition and potential role in explaining interindividual differences in therapeutic response or toxicity is increasingly recognized (Ho and Kim, 2005). The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of efflux transport proteins contains the subfamily B, of which the most thoroughly studied is P-glycoprotein (P-gp).

The multidrug resistance (MDR) gene ABCB1 encodes for P-gp, which is present in numerous normal tissues, including the apical membrane of the gastrointestinal tract, the biliary canalicular membranes of hepatocytes, and luminal membranes of endothelial cells in cerebral capillaries forming the blood-brain barrier (BBB) (Lin and Yamazaki, 2003). The biological function of P-gp is thought to be serving a protective role for major organs by limiting cellular uptake of xenobiotics by extruding these compounds into bile, urine, and intestinal lumen, thus limiting brain accumulation (Lösch and Potschka, 2005). P-gp has broad substrate specificity and is able to

ABBREVIATIONS: ADHD, attention-deficit/hyperactive disorder; MPH, methylphenidate; AMP, amphetamine; ABC, ATP-binding cassette; P-gp, p-glycoprotein; MDR/mdr, multidrug resistance; BBB, blood-brain barrier; CNS, central nervous system; KO, knockout; WT, wild type; ANOVA, analysis of variance; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; WTV, wild type injected with vehicle; WTD, wild type injected with drug; P-gp KOV, P-gp KO mice injected with vehicle; P-gp KOD, P-gp KO mice injected with drug.
actively transport a wide variety of structurally unrelated compounds out of cells. P-gp is also highly polymorphic (Schwab et al., 2003), and its expression in the BBB limits substrate access to the central nervous system (CNS), thereby influencing activity of some psychoactive drugs (Lee and Bendayan, 2004; Löscher and Potschka, 2005). Therefore, individual differences in P-gp expression could have therapeutic implications for patients receiving pharmacotherapy for ADHD. Furthermore, because a variety of medications and other substances may inhibit or induce the expression of P-gp, substrates of this transporter may become subject to potential drug interactions that may compromise outcomes (Lin, 2003; Fricker and Miller, 2004; Balayssac et al., 2005).

Although drug transporters can have profound effects on the disposition of and response to various CNS therapeutic agents (Wang et al., 2004a,b; Löscher and Potschka, 2005), to date there are little data available with regard to psychostimulants as substrates for P-gp or other drug transporters. In the mouse, P-gp is encoded by both Abcb1a (localized in brain capillaries) and Abcb1b (localized in brain parenchyma), which have 90% sequence homology to each other and 80% to human MDR1 (Ambudkar et al., 1999; Löscher and Potschka, 2005). Thus, double-knockout mice (i.e., lacking both Abcb1a and Abcb1b) are completely devoid of P-gp and allow for the assessment of the influence of P-gp upon candidate substrates (Schinkel, 1999).

Therefore, the present BBB transport studies used Abcb1a−/−, Abcb1b−/− double knockout (KO) mice lacking P-gp, and genetically matched wild-type (WT) Abcb1a+/−, Abcb1b+/− FVB mice as an in vivo model for studying the role of P-gp in brain penetration or accumulation of the major active isomers of MPH and AMP, d-MPH and d-AMP, respectively (Fig. 1). In addition, these pharmacokinetic studies were complemented by standard pharmacodynamic measures that assessed differences in animal response to psychostimulant dosing potentially mediated by P-gp status.

Materials and Methods

Compounds and Reagents. (+)-Amphetamine or d-AMP and (-)-amphetamine or l-AMP, as their respective sulfate salts, were gifts from the National Institute on Drug Abuse Research Resource Drug Supply System; (+)-methylphenidate (d-MPH) and (−)-methylphenidate (l-MPH), d-dl-MPH (methyl-labeled), 1-methyl-3-phenylpropylamine, and pentafluoropropionic anhydride were obtained from St. Louis, MO, 4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl chloride was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and pentobarbital (Nembutal) was from Abbott Laboratories (Abbott Park, IL). All other agents were of high analytical grade and obtained through Fisher Scientific Co. (Fairlawn, NJ).

Research Animals. Eight- to 9-week-old male Abcb1a−/−, Abcb1b−/− KO mice functionally devoid of P-gp and genetically matched WT Abcb1a+/−, Abcb1b+/− FVB mice aged 8 to 10 weeks weighing 20 to 30 g were obtained from Taconic (Germantown, NY). Animals were housed in groups in translucent polypropylene cages with wood-chip bedding. A separate rodent colony room was maintained at 70–74°F on a 12-h light cycle (lights on: 7:00 AM; lights off: 7:00 PM), and all animals had ad libitum access to tap water and a standardized rodent chow (Harlan Teklad Rodent Diets, Indianapolis, IN). Animals were allowed to acclimate to their cages and colony room for a minimum of 7 days after their arrival before any study procedure. All animal studies and procedures were performed in accordance with the US Public Health Service Policy for the Care and Use of Laboratory Animals. In addition, the Medical University of South Carolina Animal Care Committee approved all experimental studies.

Dose-Finding Studies. Before performing the primary pharmacokinetic and pharmacodynamic experiments, preliminary dose-finding studies were conducted to determine doses of the respective psychostimulants that would best capture any pharmacodynamic consequences of P-gp deficiency and might allow correlation of behavioral effects with pharmacokinetic measurements in blood and brain. The goal of these preliminary dose finding experiments was to identify a dose of each compound that would allow detection of either enhancement or attenuation of drug-induced locomotor stimulation for P-gp deficient relative to control mice. Because dose-response studies indicate that higher doses of each drug produce stereotypic behavior that results in a reduction of locomotor activity, stereotypy was assessed to insure that reductions in locomotor activity reflected attenuated rather than enhanced pharmacodynamics. Thus, in separate dose finding experiments, after a 30-min habituation period, three groups of WT control mice (n = 6 each) were dosed i.p. with saline vehicle (0.01 ml/g b.wt.), 2.5 mg/kg, or 5.0 mg/kg doses of d-MPH (Exp-1) and d-AMP (Exp-2). Doses ultimately selected were 2.5 mg/kg for d-MPH and 3.0 mg/kg for d-AMP.

Locomotor Activity Determinations. After the determination of optimal doses of each psychostimulant (2.5 mg/kg for d-MPH and 3.0 mg/kg d-AMP), naive WT control mice [Abcb1a+/−, Abcb1b+/−] (n = 18) and P-gp KO mice [Abcb1a−/−, Abcb1b−/−] (n = 18) were used to assess the influence of P-gp reduction on the stimulatory effects of d-MPH and d-AMP. The mice in each experiment were naive to experimental manipulations. The experimental design was a 2(WT versus KO) × 2(psycho-stimulant versus vehicle) factorial. Mice were habituated to the activity chambers for 30 min, removed, and injected with the respective psychostimulant and placed back into the activity chamber for an additional 120 min. Activity data were collected in 5-min increments. Data were analyzed with an analysis of variance (ANOVA) appropriate for the 2 × 2 factorial design.

Dosing and Blood and Brain Sample Collection. Pharmacokinetic studies to compare blood and brain tissue drug concentrations in Abcb1a−/−, Abcb1b−/− mice versus the control animals (n = 6 mice per time point/per group) were completed on mice used for the motor activity experiments at 10, 30, or 60 min after i.p. dosing with 2.5 mg/kg for d-MPH and 3.0 mg/kg for d-AMP. At the selected time points after psycho-stimulant dosing, mice were injected i.p. with pentobarbital 100 mg/kg to produce deep anesthesia before exposure of the heart. The right atrium was then punctured and approximately 0.3 ml of blood was withdrawn via syringe equipped with a 22-gauge needle (BD Biosciences, San Jose, CA). Direct heart blood was obtained to avoid possible contamination with gastric contents associated with trunk blood sampling that could influence drug concentration measurements. Collected whole blood samples were immediately transferred to light gray-stoppered glass collection tubes (Vacutainer; BD Biosciences) stored on ice that contained sodium oxalate to prevent clotting and sodium fluoride to prevent hydrolysis of MPH. Blood samples were then centrifuged at 3000g for 20 min to separate plasma from other blood components. The plasma portion was then removed and stored at −70°C until analysis. Whole brains were likewise immediately collected by dissection, weighed, and placed in plastic conical tubes on ice and subsequently transferred to a −70°C freezer until analysis. Stored whole brains were later thawed and homogenized in a 5-fold volume of an Hanks’ balanced salt solution buffer containing 0.02 M MOPS, pH 7.2, with a Polytron PT 1200 handheld homogenizer (Brinkmann Instruments, Westbury, NY) before extraction procedures.

Gas Chromatographic-Mass Spectrometry Analysis. MPH determinations were made for both d- and l-MPH as pentafluoropropionyl derivatives

![Chemical structures of d-MPH and d-AMP.](Image)
using a gas chromatographic-mass spectrometry (GC-MS)-negative ion chemical ionization method. d_{3}-dL-MPH was incorporated as the internal standard. Instrumentation was an Agilent model 6890 GC-5973 MS fitted with a 5% phenylmethylpolysiloxane column (HP-5MS, 30 m × 0.25 mm, 0.25 μm film; J and W Scientific). In brief, 100 μl of plasma per sample timepoint and 100 μl of brain sample homogenate were analyzed. d_{3}-MPH was added as the internal standard at 0.25 μg per plasma sample and 1 μg per brain sample. Sodium chloride (0.5 g) was added to brain samples to prevent emulsion. Ammonium hydroxide (28%, 100 μl) was added, followed by extraction with butyl chloride/acetonitrile (4:1, 2 ml). The organic phase was transferred to sample residues were derivatized by adding 10 μl of propionic anhydride (50 μl) and 2-MPH in plasma and brain was 10 ng/ml and 40 ng/g, respectively.

High-Performance Liquid Chromatography Analysis. Both d- and L-AMP determinations were made using an high-performance liquid chromatography (HPLC) method based on that of Al-Dirbash et al. (1998). In brief, 100 μl of the sample and 50 μl of internal standard 1-methyl-3-phenylpropylamine (2 μM) were added and mixed, followed by the addition of 500 μl of 0.05 M borate buffer (pH 10.0) and 1.5 ml of ethyl acetate. Samples were mixed for 5 min and then centrifuged for 10 min at 2000 g. The organic layer (1 ml) was transferred to fresh tubes and evaporated to dryness under nitrogen. The residue was reconstituted with heptane (50 μl) and 2-MPH injections were made by the pulsed-splitless mode. The helium carrier gas linear velocity was 50 cm/s. The oven was held at 90°C for 1.5 min and then ramped to 280°C at 20°C/min and held for 5 min. Methane was used as the ionization buffer. Detection was by selected ion monitoring of m/z 339 for MPH-pentafluoropropionyl [M-(2HF)] and m/z 342 for the internal standard [M-(2HF)]. These compounds eluted at 8.97 and 8.96 min, respectively. The lower limit of quantitation for d-MPH in plasma and brain was 10 ng/ml and 40 ng/g, respectively.

Data Analysis. Quantitation of d-MPH and d-AMP was achieved by comparing peak areas of each psychostimulant to their respective internal standards with ratios derived from calibration curves of standards containing known amounts of drug extracted from spiked mouse plasma and brain as described above. Three timepoints were examined after dosing of each drug expressed as nanograms per milliliter for blood concentrations and nanograms per gram for whole brain. An unpaired t test was used to compare the differences between concentrations of the respective psychostimulants in the Abcb1ab(/−/−, /−/−) mice versus the control animals (i.e., Abcb1ab(+/+,-/+,-/+)) and their brain-to-plasma ratios. A two-tailed t test was used with a level of statistical significance was set at P < 0.05. With regard to behavioral activity measures of Abcb1ab(/−/−, /−/−) mice versus control animals, motor activity was expressed as total distance traveled (centimeters) during a 30-min habituation before drug or vehicle injections to determine potential genotypic differences in basal motor activity and then at 30-min intervals for 2 h after injections of drug or vehicle. Basal motor activity was analyzed with a one-way ANOVA for the four groups: wild-type controls injected with vehicle (WT-V) or with drug (WTD) and P-gp KO mice injected with vehicle (P-gp KOV) or drug (P-gp KOD). For the first experiment, the drug was d-MPH, and for the second, d-AMP. Data after drug or vehicle injections were analyzed with 2(genotype) × 2(drug) × 4(30-min time interval) ANOVAs for each experiment. Significant interaction terms were resolved with additional one-way ANOVAs and Tukey tests of differences between means. Sample size for the different groups in the d-MPH experiment were eight for WT-V, seven for P-gp KOD, six for WTD, and six for P-gp KOD. For the d-AMP experiment, sample sizes were eight for WTV, eight for P-gp KOV, seven for WTD, and eight for P-gp KOD.

Results

Locomotor Activity. Locomotor activity during the 30-min habituation period and the 2-h test period after psychostimulant injections are summarized in Fig. 2 for d-MPH and Fig. 3 for d-AMP. To determine whether P-gp deletion influenced basal motor activity levels, data generated during the 30-min habituation period before injection of psychostimulant were analyzed with one-way ANOVAs for each experiment. To assess the affect of P-gp deletion on the stimulatory effect of d-MPH and d-AMP, data generated after drug injection were analyzed with 2(genotype) × 2(drug) × 4(30-min time interval) ANOVAs for each experiment. Basal motor activity during the 30-min habituation period did not differ for Abcb1ab(/−/−, /−/−) P-gp KO mice and Abcb1ab(+/+,-/+,-/+,-/+)+) WT controls. The ANOVA on the habituation data indicated no significant difference among the four groups for either the d-MPH injection of psychostimulant were analyzed with one-way ANOVAs for each experiment. Significant interaction terms were resolved with additional one-way ANOVAs and Tukey tests of differences between means. Sample size for the different groups in the d-MPH experiment were eight for WT-V, seven for P-gp KOD, six for WTD, and six for P-gp KOD.
achieve the desired response. P-gp expressed in BBB provides a protective physiological barrier capable of limiting a variety of CNS drugs from entering brain and prevents or minimizes their pharmacological effects. The abca1a/b knockout mouse model has been widely used to study the role of P-gp in the brain. In the current study, we aimed to investigate the effect of P-gp on the pharmacokinetics and pharmacodynamics of psychostimulants, specifically d-MPH and d-AMP, in mice with and without P-gp.KO mice were injected with d-MPH and d-AMP, and their pharmacokinetic parameters were compared with those of wild-type (WT) mice. The results showed that the plasma clearance and brain to plasma ratios of d-MPH were significantly higher in P-gp KO mice compared to WT mice, indicating that P-gp plays a role in the uptake of d-MPH into the brain. However, the pharmacokinetics of d-AMP were not significantly different between the two groups. These findings suggest that P-gp may influence the pharmacokinetics of psychostimulants, potentially affecting their efficacy in the treatment of ADHD.

**Discussion**

For any therapeutic agent intended to act as a psychotherapeutic agent, adequate drug concentrations must be attained in the brain to achieve the desired response. P-gp expressed in BBB provides a protective physiological barrier capable of limiting a variety of CNS drugs from entering brain and prevents or minimizes their pharmacological effects. The abca1a/b knockout mouse model has been widely used to study the role of P-gp in the brain. In the current study, we aimed to investigate the effect of P-gp on the pharmacokinetics and pharmacodynamics of psychostimulants, specifically d-MPH and d-AMP, in mice with and without P-gp. K0 mice were injected with d-MPH and d-AMP, and their pharmacokinetic parameters were compared with those of wild-type (WT) mice. The results showed that the plasma clearance and brain to plasma ratios of d-MPH were significantly higher in P-gp KO mice compared to WT mice, indicating that P-gp plays a role in the uptake of d-MPH into the brain. However, the pharmacokinetics of d-AMP were not significantly different between the two groups. These findings suggest that P-gp may influence the pharmacokinetics of psychostimulants, potentially affecting their efficacy in the treatment of ADHD.
versus WT animals has become a standard experimental approach to determine whether the tested drugs are P-gp substrates and limited from entering into brain. At least one previous study assessed the AMP derivative methamphetamine, an infrequently prescribed Food and Drug Administration-approved agent for treating ADHD in the United States (Desoxyn), in a mouse P-gp KO model (i.e., single knockout, mdr1a−/−) relative to control animals (Mann et al., 1997). In this investigation, differential effects on the dopamine transporter were observed in the P-gp-deficient mice relative to control animals, suggesting that AMPs and structurally related compounds could serve as P-gp substrates. However, unlike the present study, no drug concentrations were measured. Doran et al. (2005) recently reported the results of a comprehensive screening of over 30 CNS active agents in the Abcb1a/b−/− mouse model, including racemic MPH (i.e., dl-MPH) at subcutaneous doses of 3 mg/kg, which effectively delivers only 1.5 mg/kg of the active d-isomer per dose. These investigators collected blood (plasma), brain, and cerebral spinal fluid samples at four timepoints after dosing, 0.5, 1.0, 2.5, and 5 h and calculated the area under the time versus concentration curves for Abcb1a/b−/− versus control animals. No statistically significant differences in drug concentration in any sampled tissues were reported when comparing the two groups. However, significant differences were found when they compared the ratio of brain-to-plasma and cerebral spinal fluid-to-plasma concentrations of dl-MPH.

In the present study, the concentrations of dl-MPH and d-AMP in brain and plasma were measured at 10-, 30-, and 80-min postdosing timepoint in P-gp KO and WT mice. The results showed that only at the 10-min postdosing timepoint, P-gp KO mice had significant higher values of the dl-MPH brain concentrations and brain-to-plasma ratios than WT control. However, these increments in P-gp KO mice were minor relative to other CNS drugs known to be P-gp substrates. With regard to d-AMP, no significant differences in the drug concentrations of brain and plasma and the ratios of brain to plasma were observed at any timepoints between P-gp KO mice and WT mice. These data indicate that dl-MPH is not likely to be a P-gp substrate, whereas dl-MPH may be a relatively weak P-gp substrate. The present data are consistent with and support previous findings with racemic MPH.

Locomotor activity assessment is a well known and widely accepted research paradigm to evaluate behavioral effects of stimulants in rodents. Coupled measures of motor activity with brain and blood concentrations of the respective psychostimulants were investigated to document the potential behavioral consequences of any P-gp-mediated differences in the brain penetration of the studied compounds in Abcb1a/b−/− mice versus matched control animals. As expected, in a comparison of the increase of locomotor activity stimulated by d-AMP in a 120-min period, there was no statistical difference between P-gp KO and WT mice. However, in spite of elevated dl-MPH concentrations in the brain of P-gp KO mice compared with WT mice, drug-induced stimulation was attenuated rather than enhanced as was hypothesized. Possible reasons for this unanticipated result are speculative but include the possibility that regioselective differences in dl-MPH concentrations could have differed among the genotypes that would have gone undetected in our whole-brain analyses. An alteration in the volume of distribution and/or residence time could also potentially contribute to the increase of the brain/plasma ratio of dl-MPH in KO animals versus WT animals. In addition, as has been recently reported, the possibility of compensatory increases in genetic expression and synthesis of other transporters in P-gp-deficient mice cannot be excluded (see Cisternino et al., 2004). Other possible explanations include the down-regulation of other systems affecting dl-MPH disposition and pharmacodynamic response, or attenuated activity in Abcb1a/b−/− mice could be a physiological role for P-gp in removing neuroactive metabolites.

**Fig. 5.** Blood (A) and brain (B) concentrations and brain-to-plasma ratios (C) of d-AMP in P-gp KO mice (solid bars) and WT mice (open bars) at 10, 30, and 80 min after an i.p. injection of 3.0 mg/kg d-AMP. The concentrations of d-AMP were determined using HPLC analysis. Values are shown as mean ± S.D. (n = 6).
Although l-isomers of MPH and AMP are viewed as being pharmacologically inactive to mildly active relative to their respective d-isomer counterparts (Patrick and Markowitz, 1997), the P-gp substrate properties of l-MPH and l-AMP, as well as d-isomers of MPH and AMP, were initially studied in vitro using the human MDR1 cDNA-transfected cell line LLC-PK1/MDR1 and its parental cell line LLC-PK1 (the experimental details and results for these and other ADHD therapeutic agents are being presented in full elsewhere). No differences in the accumulation of tested compounds were observed between LLC-PK1/ MDR1 and LLC-PK1 cells except that a 97% increase of the accumulation of d-MPH in LLC-PK1 cells occurred compared with that in LLC-PK1/MDR1 cells. These results corroborate our in vivo findings and suggest that neither d- or l-AMP nor l-MPH are P-gp substrates and that d-MPH may be, at best, a relatively weak P-gp substrate (unpublished observation). Considering the minor differences between d- and l-isomers of MPH and AMP with regard to being P-gp substrates coupled with the weak pharmacological activity of the respective l-isomers, no further in vivo experiments of l-MPH and l-AMP in P-gp KO mice were carried out. Both MPH and AMP are known to be biotransformed into CNS-active compounds. However, the amounts of these metabolites produced relative to circulating parent drugs in humans is so small as to be generally viewed as insignificant (see Patrick and Markowitz, 1997). Thus, further evaluation of their respective metabolites was not deemed worthwhile at present and not undertaken. In conclusion, these data indicate that P-gp has no effect on the pharmacokinetics and pharmacodynamics of d-AMP. In addition, d-MPH seems to be a relatively weak P-gp substrate, and its entry into the brain may be limited to a minor extent by P-gp. Finally, the mechanism by which d-MPH-induced locomotor activity was attenuated in P-gp KO mice remain unknown. It seems that if active transport of d-AMP and d-MPH does occur within the BBB, one or more drug transporters other than P-gp governs brain penetration.

References


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