Immunopharmacological Studies on TBX, a New Antiallergic Drug

(1) Inhibitory Effects on Passive Cutaneous Anaphylaxis in Rats and Guinea Pigs

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Abstract—The effects of 9-methyl-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one potassium salt (TBX), a new antiallergic drug, on passive cutaneous anaphylaxis (PCA) mediated by homologous IgE or IgG antibody were investigated in rats and guinea pigs. TBX (i.v. and p.o.) clearly inhibited IgE- and IgGα-mediated homologous PCAs in rats, without showing any inhibition of the skin reactions caused by histamine, serotonin and bradykinin in contrast to the inhibition of prostaglandin E1-induced skin reaction. Neither adrenalectomy nor propranolol treatment modified TBX’s inhibition of the former PCA. With regard to tachyphylaxis to TBX, it was demonstrable in IgE-mediated homologous PCA in rats when they were pretreated with TBX (0.5 mg/kg, i.v.), followed 60 min later by a second dose of the drug (0.05 mg/kg, i.v.). There was no cross-tachyphylaxis between disodium cromoglycate (DSCG) and TBX. Homologous PCA caused by guinea pig IgE was also inhibited in a dose-dependent fashion by i.v. and p.o. administrations of TBX, although higher doses of TBX were needed to inhibit guinea pig PCA than the rat one. Interestingly, TBX showed more potent inhibition of both rat and guinea pig homologous PCAs than DSCG or tranilast. The results obtained indicate that TBX is an orally effective antiallergic agent displaying no antagonistic actions on the chemical mediators released.

IgE antibodies are well-known to play a major role in the pathogenesis of atopic diseases such as bronchial asthma; cross-linking of surface IgE bound to Fc receptors for IgE expressed on tissue mast cells and blood basophils results in the massive release of chemical mediators responsible for asthmatic attacks. Recently, the pharmacotherapy of bronchial asthma has been quite successful because of considerable progress in the development of antiallergic drugs showing the inhibition of chemical mediator release. One of the typical drugs effective for controlling allergic asthma through the inhibition of IgE-mediated chemical mediator release from mast cells is disodium cromoglycate (DSCG), known to be effective by inhalation but not oral administration (1). Attempts have thus been made to develop orally active agents; for this purpose, tranilast, which is chemically unrelated to DSCG, was first shown to be useful for experimental and clinical trials (2–4). Subsequently, drugs such as ketotifen (5) and oxatomide (6, 7), displaying antagonistic actions on chemical mediators, particularly histamine, have been also reported to inhibit mediator release, possibly by preventing both Ca uptake and Ca2+ release from the intracellular Ca2+ store. In addition to these drugs, azelastine (8), amlexanox (9) and repinast (10) are now available for clinical use in Japan as antiallergic drugs. Note that only azelastine has ketotifen- or oxatomide-like activity.
In the present study, the effects of a newly synthesized compound, 9-methyl-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one potassium salt (TBX, see structural formula shown in Fig. 1) on passive cutaneous anaphylaxis (PCA) mediated by homologous IgE or IgG antibody were investigated in rats and guinea pigs. The results obtained here indicate that TBX, an orally effective agent, markedly inhibits both rat and guinea pig homologous PCAs, as compared with DSCG and tranilast. The data further suggest that the PCA inhibitory action of this agent is due to the inhibition of chemical mediator release from skin mast cells, presumably in a manner different from that of DSCG.

Materials and Methods

Animals: Male Wistar rats weighing 200 to 300 g and male Hartley guinea pigs weighing 350 to 450 g purchased from Shizuoka Laboratory Animal Center were used.

Drugs: TBX (Tokyo Tanabe) is a yellowish white crystalline powder, highly soluble in water. Other drugs used were DSCG (Fuji-sawa), tranilast (Kissei), isoproterenol bitartrate (Sigma) and propranolol hydrochloride (Sumitomo). Agents except tranilast were dissolved in physiological saline for i.v. or p.o. administration. Tranilast was suspended in 0.5% tragacanth for p.o. administration.

Antigens: Ascaris extract coupled with the 2,4-dinitrophenyl group (DNP-As) was prepared by the method of Tada and Okumura (11). Bovine serum albumin (BSA, Armour) was coupled with DNP group according to the method of Lee and Sehon (12), and DNP20-BSA was obtained.

Rat anti-DNP IgE serum: Rats were immunized s.c. with 1 mg protein of DNP-As mixed with killed organisms (2×10^{10}) of Bordetella pertussis (Chiba Serum Institute) into the four foot pads and were boosted i.m. by 0.5 mg protein of DNP-As alone 5 days later, according to the method of Tada and Okumura (11). They were then bled 3 days after the booster injection. The anti-DNP IgE antibody titer of the antiserum was 1:256 as determined by 48-hr homologous PCA in rats challenged with DNP-BSA.

Rat anti-DNP IgGα serum: Rats were immunized by s.c. injection of 4 mg of DNP-As emulsified with complete Freund’s adjuvant (CFA, Difco). Two weeks later, they were boosted s.c. by an emulsion containing DNP-As and CFA, and then bled 2 weeks after the booster. The antiserum obtained was heated at 56°C for 1 hr. The anti-DNP IgGα antibody titer of the antiserum was 1:128 as estimated by 3-hr homologous PCA in rats.

Guinea pig anti-DNP IgE serum: Guinea pigs were immunized by i.p. injection of 10 μg of DNP-As absorbed on Al(OH)₃ gel (alum), 6 times at intervals of a month, according to the method of Levine et al (13). The anti-DNP IgE antibody titer of the antiserum was 1:1024 as assessed by 8-day homologous PCA in guinea pigs.

IgE- or IgGα-mediated homologous PCA in rats: Rats were injected intradermally at 3 sites with 0.1 ml of homologous anti-DNP IgE serum at 1/64 dilution or anti-DNP IgGα serum at 1/50 dilution on the shaved back. The animals were passively sensitized with the former antiserum 48 hr or the latter one 3 hr before antigen challenge. PCA was elicited by i.v. injection of 1 ml of physiological saline containing 2.5 mg DNP-BSA and 5 mg of Evans blue (Merck) under conditions of both light anesthesia with ether and non-restriction. The animals were sacrificed 30 min after antigen challenge, and the skin samples were removed for the colorimetric measurement of the bluing spot. The amount of the dye leaked into the PCA sites was determined as described by Katayama et al. (14).

Skin reactions induced by chemical mediators in rats: The skin reactions were induced intradermal (i.d.) injection of 5 μg/site of histamine dihydrochloride (Wako), 0.1 μg/site of serotonin creatinine sulfate
(Wako), 1 μg/site of bradykinin (Wako), and 1 μg/site of prostaglandin E₁ (PGE₁, Sigma), immediately after i.v. injection of 2.5 ml/kg of 1% Evans blue solution. The animals were killed 30 min later, and the skins were removed for the colorimetric measurement of the bluing spot.

**IgE-mediated homologous PCA in guinea pigs:** Guinea pigs received i.v. injection of 0.1 ml of homologous anti-DNP IgE serum at 1/64 dilution at 3 sites on the shaved back. After 8 days, PCA was provoked by i.v. injection of 1 ml of physiological saline containing 2.5 mg of DNP-SBA and 5 mg of Evans blue under identical conditions described in the rat PCA, and the skins were removed for the colorimetric measurement of the bluing spot.

**Statistical analysis:** Results are expressed as the mean±S.E. Statistical significance was determined by Student's t-test. The ID₅₀ was obtained by the logit method.

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**Fig. 2.** Time course of inhibitory effect of TBX on IgE-mediated 48-hr homologous PCA in rats. TBX was administered i.v. (A) and p.o. (B) at various times before the antigen challenge. Each point represents the mean±S.E. of 5 to 10 animals. The amounts of Evans blue leaked in the control group were 35.9±5.57 μg/site and 31.3±3.61 μg/site for (A) and (B), respectively.

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**Results**

IgE-mediated 48-hr homologous PCA in rats: The study pertaining to the optimal time of TBX administration was first performed using two doses (0.05 mg/kg, i.v. and 0.2 mg/kg, p.o.) of the drug. As shown in Fig. 2, the highest inhibition of PCA was observed when TBX was administered i.v. 0 min before and p.o. 5 min before antigen challenge, respectively. Second, the efficacy of TBX was compared with that of DSCG or tranilast. As illustrated in Fig. 3, the PCA was inhibited in a dose-dependent fashion by TBX (0.0025 to 0.025 mg/kg, i.v.) and by DSCG (0.5 to 5 mg/kg, i.v.) when the drugs were administered simultaneously with antigen. In addition, TBX (0.01 to 0.1 mg/kg, p.o.) showed dose-dependent inhibition of the PCA when given 5 min prior to antigen challenge. Tranilast (50 to 250 mg/kg, p.o.), administered 60 min before challenge, was also found to dose-dependently inhibit the PCA. Note that the
PCA inhibitory activity of TBX was much more potent than that of DSCG or tranilast. The third experiment was carried out to study whether TBX exerted its inhibitory influence on the PCA in adrenalectomized rats. TBX was administered i.v. simultaneously with antigen to adrenalectomized or sham-operated animals. As indicated in Fig. 4, TBX
(0.01 mg/kg, i.v.) strongly inhibited the PCA in both adrenalectomized and sham-operated rats, and no significant difference in inhibition by TBX was observed between the two groups. Fourthly, the effect of TBX on the PCA was investigated in rats pretreated with propranolol (0.25 mg/kg, i.v.). As shown in Fig. 5, the inhibition of PCA by TBX (0.025 mg/kg, i.v.) was not affected by pretreatment with propranolol, whereas isoproterenol (0.05 mg/kg, i.v.)-induced inhibition of PCA was almost completely abrogated by such pretreatment. Finally, tachyphylaxis to TBX and cross-tachyphylaxis between DSCG and TBX were investigated in passively sensitized rats. They received i.v. injection of TBX (0.01 to 0.5 mg/kg, i.v.) or DSCG (50 mg/kg, i.v.), followed 60 min later by a second administration of TBX (0.05 mg/kg, i.v.). The results in Fig. 6 indicate that pretreatment with TBX (0.5 mg/kg, i.v.) but not with DSCG (50 mg/kg, i.v.) significantly reduced the inhibitory influence on the PCA of a second dose of TBX. Similarly, DSCG pretreatment significantly reduced the ability of the drug (5 mg/kg, i.v.) to inhibit the PCA.

IgG,,-mediated 3-hr homologous PCA in rats: The effect of TBX on 3-hr homologous PCA in rats was examined and compared with that of DSCG or tranilast. As shown in Fig. 7, 3-hr homologous PCA was inhibited in a dose-dependent fashion by TBX (0.0025 to 0.025 mg/kg, i.v.) as well as by DSCG (0.5 to 5 mg/kg, i.v.) when the drugs were administered simultaneously with antigen. In addition, TBX (0.025 to 0.5 mg/kg, p.o.) showed the dose-dependent inhibition of the PCA when given 5 min prior to challenge, whereas tranilast (100 and 250 mg/kg, p.o.), administered 60 min before challenge, displayed the slight inhibition of this PCA. Again, it was demonstrated that TBX was much more potent than DSCG or tranilast.

Skin reactions induced by chemical mediators in rats: The results are shown in Table 1. TBX (0.01 to 0.25 mg/kg, i.v.) displayed no inhibition of the skin reactions caused by histamine, serotonin and bradykinin. In contrast, PGE,,-induced skin reaction was significantly inhibited by TBX (0.05 and 0.25 mg/kg, i.v.).

IgE-mediated 8-day homologous PCA in guinea pigs: The optimal administration time to inhibit 8-day homologous PCA in guinea pigs was investigated employing TBX (50 mg/kg, p.o.). As illustrated in Fig. 8, TBX, administered orally 2 hr before antigen challenge, showed the highest inhibition of PCA.

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![Fig. 5. Effects of TBX and isoproterenol on IgE-mediated 48-hr homologous PCA in rats pretreated with propranolol. Each column represents the mean±S.E. of 5 animals. **: Statistically significant difference from the control at P<0.01.](image-url)
Fig. 6. Development of tachyphylaxis to TBX but not of cross-tachyphylaxis between DSCG and TBX in the inhibition of IgE-mediated 48-hr homologous PCA in rats. Each column indicates the mean±S.E. of 5 to 8 animals. The amount of Evans blue leaked in the control group was 30.2±3.51 μg/site. *, **: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.

Fig. 7. Inhibitory effects of TBX, DSCG and tranilast on IgGα-mediated 3-hr homologous PCA in rats. In the case of i.v. administration, TBX and DSCG were administered simultaneously with the antigen, while in the case of p.o. administration, TBX and tranilast were given 5 and 60 min before the antigen challenge, respectively. Each point represents the mean±S.E. of 5 or 6 animals. The amount of Evans blue leaked in the control group was 34.2±2.88 μg/site.
Next, the efficacy of TBX on the PCA was compared with that of DSCG or tranilast. The results are shown in Table 2. The PCA was inhibited in a dose-dependent fashion by TBX (0.25 to 5 mg/kg, i.v. and 5 to 50 mg/kg, p.o.) when administered i.v. simultaneously with antigen or p.o. 2 hr prior to the challenge. In contrast, high doses of DSCG and tranilast were needed to inhibit this PCA; for example, DSCG in a dose of 10 mg/kg (i.v.), showing the complete inhibition of rat homologous PCA mediated by IgE, exhibited only 28.5% inhibition of this guinea pig homologous PCA at the same dose, although its inhibition was not statistically significant.

**Table 2. Effects of TBX on skin reactions induced by chemical mediators in rats**

| Group | Dose (mg/kg, i.v.) | No. of animals | Histamine (5 μg/site) | Serotonin (0.1 μg/site) | Bradykinin (1 μg/site) | PGE₁ (1 μg/site) |
|-------|-------------------|----------------|----------------------|------------------------|-----------------------|----------------|
| Control | —                 | 6              | 28.8±3.1             | 16.1±2.1               | 14.1±2.7              | 22.3±3.0       |
| TBX    | 0.01              | 6              | 27.1±1.8             | 19.9±0.7               | 14.1±1.4              | 17.3±2.0       |
|        | 0.05              | 6              | 24.2±2.4             | 20.9±1.4               | 10.8±0.7              | 11.4±1.9*      |
|        | 0.25              | 6              | 23.3±2.2             | 13.2±1.9               | 11.8±1.5              | 9.4±1.5**      |

* ***: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.

Next, the efficacy of TBX on the PCA was compared with that of DSCG or tranilast. The results are shown in Table 2. The PCA was inhibited in a dose-dependent fashion by TBX (0.25 to 5 mg/kg, i.v. and 5 to 50 mg/kg, p.o.) when administered i.v. simultaneously with antigen or p.o. 2 hr prior to the challenge. In contrast, high doses of DSCG and tranilast were needed to inhibit this PCA; for example, DSCG in a dose of 10 mg/kg (i.v.), showing the complete inhibition of rat homologous PCA mediated by IgE, exhibited only 28.5% inhibition of this guinea pig homologous PCA at the same dose, although its inhibition was not statistically significant.

**Fig. 8.** Time course of inhibitory effect of TBX (50 mg/kg, p.o.) on 8-day homologous PCA in guinea pigs. Each point represents the mean±S.E. of 7 or 8 animals. The amount of Evans blue leaked in the control group was 12.7±2.52 μg/site.

Next, the efficacy of TBX on the PCA was compared with that of DSCG or tranilast. The results are shown in Table 2. The PCA was inhibited in a dose-dependent fashion by TBX (0.25 to 5 mg/kg, i.v. and 5 to 50 mg/kg, p.o.) when administered i.v. simultaneously with antigen or p.o. 2 hr prior to the challenge. In contrast, high doses of DSCG and tranilast were needed to inhibit this PCA; for example, DSCG in a dose of 10 mg/kg (i.v.), showing the complete inhibition of rat homologous PCA mediated by IgE, exhibited only 28.5% inhibition of this guinea pig homologous PCA at the same dose, although its inhibition was not statistically significant.

**Table 3. ID50 values in rat and guinea pig PCAs:**

ID50 values were determined in 48-hr homologous PCA mediated by rat anti-DNP IgE serum, 3-hr homologous PCA by rat anti-DNP IgGₐ serum and 8-day homologous PCA by guinea pig anti-DNP IgE serum. In the case of i.v. administration, TBX and DSCG were administered simultaneously with antigen to rats and guinea pigs; In the case of p.o. administration, TBX and tranilast were given at 5 and 60 min before antigen challenge to rats and were administered 2 and 1 hr prior to the challenge to guinea pigs, respectively. The results are summarized in Table 3. ID50 values of TBX (i.v.) were 7 μg/kg, 6 μg/kg and 0.9 mg/kg in 48-hr homologous PCA (rat), 3-hr homologous PCA (rat) and 8-day homologous PCA (guinea pig), respectively. In the case of p.o. administration, they were 27 μg/kg, 107 μg/kg and 6.8 mg/kg in 48-hr homologous PCA, 3-hr homologous PCA and 8-day homologous PCA, respectively. Note that TBX strongly inhibited both rat and guinea pig PCAs and was much more potent than DSCG or tranilast.

**Discussion**

The present results clearly indicate that TBX, a newly synthesized compound, is an orally effective drug capable of strongly inhibiting both rat and guinea pig PCAs mediated by homologous IgE or IgG antibody. Interestingly, these PCAs were inhibited by smaller doses of TBX, regardless of the administration route, as compared with those of DSCG and tranilast. For instance, the potency of TBX to inhibit IgE-mediated 48-hr homologous PCA in rats was approximately
Table 2. Effects of TBX, DSCG and tranilast on 8-day homologous PCA in guinea pigs

| Group | Route | Dose (mg/kg) | No. of animals | Amount of dye (µg/site) | % Inhibition |
|-------|-------|--------------|----------------|------------------------|--------------|
| Control | Vehicle | 5 | 17.2±1.7 | — |
| TBX | i.v. | 0.25 | 5 | 10.4±1.2** | 39.5 |
| | | 1 | 5 | 9.4±1.2** | 45.3 |
| | | 5 | 5 | 5.2±0.6** | 69.8 |
| DSCG | i.v. | 10 | 5 | 12.3±2.5 | 28.5 |
| Control | Vehicle | 50 | 11 | 16.9±1.5 | — |
| TBX | p.o. | 5 | 8 | 9.7±1.0** | 42.6 |
| | | 10 | 7 | 6.6±1.1** | 60.9 |
| | | 25 | 7 | 6.2±0.8** | 63.3 |
| | | 50 | 6 | 4.0±0.6** | 76.3 |
| Tranilast | 250 | 7 | 9.9±1.5** | 41.4 |

In the case of i.v. administration, TBX and DSCG were administered simultaneously with the antigen, while in the case of p.o. administration, TBX and tranilast were given 2 and 1 hr before the challenge, respectively. **: Statistically significant difference from the control at P<0.01.

Table 3. ID50 or % inhibition of TBX, DSCG and tranilast in rat and guinea pig homologous PCA

| Recipient | Antibody | Route | ID50 or % inhibition |
|-----------|----------|-------|----------------------|
| Rat | Rat IgE | i.v. | 7 µg/kg | 1.3 mg/kg | NT |
| | (48-hr PCA) | p.o. | 27 µg/kg | NT | 101.7 mg/kg |
| Rat | Rat IgGa | i.v. | 6 µg/kg | 1.1 mg/kg | NT |
| | (3-hr PCA) | p.o. | 107 µg/kg | NT | 24.2% at 250 mg/kg |
| Guinea pig | Guinea pig IgE | i.v. | 920 µg/kg | 28.5% at 10 mg/kg | NT |
| | (8-day PCA) | p.o. | 6.8 mg/kg | NT | 41.4% at 250 mg/kg |

Each experiment included 5 to 8 animals. NT: not tested.

200 times as high as DSCG when the drugs were administered i.v. simultaneously with antigen, and TBX was at least 3700 times more potent than tranilast when TBX and tranilast were given p.o. at 5 and 60 min prior to antigen challenge, respectively (Table 3). Similar results were obtained with TBX’s inhibition of IgGα-mediated 3-hr homologous PCA in rats. In addition, 8-day homologous PCA induced by guinea pig IgE antibody was also inhibited by i.v. and p.o. administration of TBX, although higher doses of TBX were needed to inhibit guinea pig PCA than the rat one. It should be emphasized that TBX showed more potent inhibition of guinea pig PCA than DSCG or tranilast did, since DSCG is known to exert weak influences on guinea pig models (15, 16). From the results showing that TBX in doses capable of completely inhibiting rat PCAs displayed no inhibition of rat skin reactions caused by histamine, serotonin and bradykinin, it is obvious that the inhibition of rat and guinea pig PCAs by TBX was not due to antagonistic actions on chemical mediators immunologically released by skin mast cells. In contrast, TBX dose-dependently inhibited only the skin reaction mediated by PGE1, known to non-immunologically induce histamine release from mast cells (17). However, larger doses of TBX were required to significantly inhibit the PGE1-induced skin reaction than rat homologous PCAs. These
findings strongly suggest that TBX inhibits chemical mediator release induced by both immunological and non-immunological stimuli. In addition, it is interesting to note that neither adrenalectomy nor propranolol treatment modified TBX's inhibition of IgE-mediated homologous PCA in rats, indicating that this drug has no ability to release intrinsic adrenal hormones and to stimulate β-adrenergic receptors. Thus it is suggested that the inhibition of chemical mediator release from mast cells is involved in the PCA inhibitory action of TBX. This suggestion is further supported by the observation described in the companion paper (18) demonstrating that TBX inhibited IgE-mediated histamine release from rat peritoneal mast cells.

The occurrence of tachyphylaxis to DSCG has been documented mainly in the rat system (19–22). In addition, other antiallergic drugs chemically unrelated to DSCG have been also reported to show tachyphylaxis and to occasionally exhibit cross-tachyphylaxis with DSCG in regards to the inhibition of IgE-mediated allergic reactions in vivo and in vitro (23–26). Indeed, pretreatment with TBX (0.5 mg/kg, i.v.), followed 60 min later by a second administration of the drug (0.05 mg/kg, i.v.), resulted in a significant decay in the inhibition of IgE-mediated homologous PCA in rats (Fig. 6). Similar results were obtained with DSCG. However, it ought to be mentioned that there was no cross-tachyphylaxis between DSCG and TBX, suggesting the possibility that TBX does not share a common mechanism of antiallergic action with DSCG. Although less is known about the mechanism of tachyphylaxis to the inhibitory actions of drugs on rat PCA, a few hypotheses have been proposed based on in vitro findings demonstrated in DSCG-induced inhibition of histamine release from rat peritoneal mast cells and lung fragments. For example, Kusner et al. (27) proposed that a smaller or negligible amount of factor(s), responsible for the inhibition of histamine release, released by mast cells previously stimulated with DSCG, was produced when the cells were again treated with the same drug. Nevertheless, the presence of such a factor(s) has not been confirmed in lung preparations, although tachyphylaxis to DSCG was clearly demonstrable in this system. In contrast, Taylor et al. (28) pointed out that DSCG's tachyphylaxis resulted from a compensatory activation of one mast cell phosphodiesterase isoenzyme which is insensitive to DSCG through the inhibition of another isoenzyme which is sensitive to DSCG. Finally, and most importantly, Marshall et al. (29) and Sung et al. (20) suggested that tachyphylaxis observed in mast cells might be due to physical modification of DSCG-specific binding sites. In fact, the DSCG molecule was shown to bind to a specific site expressed on mast cells in the presence of extracellular Ca2+ (30). However, it should be stressed that such tachyphylaxis was demonstrable only in the rat system as described by Thomson and Evans (22). Therefore, the development of tachyphylaxis to TBX, which was observed in rat PCA, may not be so important in the clinical field; indeed, tachyphylaxis to antiallergic drugs such as DSCG and tranilast has not been clinically reported.

In summary, TBX that has no antagonistic actions on chemical mediators is an orally effective antiallergic drug capable of inhibiting both rat and guinea pig homologous PCAs mediated by IgE or IgG antibody, possibly by preventing chemical mediator release from skin mast cells. In addition, the antiallergic properties of TBX are suggested to be different from those of DSCG which exerts weak inhibitory influence on guinea pig PCA and exhibits no cross-tachyphylaxis with TBX.

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