Tachykinins in endocrine tumors and the carcinoid syndrome

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Abstract

Objective: A new antibody, active against the common tachykinin (TK) C-terminal, was used to study TK expression in patients with endocrine tumors and a possible association between plasma-TK levels and symptoms of diarrhea and flush in patients with metastasizing ileocecal serotonin-producing carcinoid tumors (MSPCs).

Method: TK, serotonin and chromogranin A (CgA) immunoreactivity (IR) was studied by immunohistochemistry in tissue samples from 33 midgut carcinoids and 72 other endocrine tumors. Circulating TK (P-TK) and urinary-5 hydroxyindoleacetic acid (U-5HIAA) concentrations were measured in 42 patients with MSPCs before treatment and related to symptoms in patients with the carcinoid syndrome. Circulating CgA concentrations were also measured in 39 out of the 42 patients.

Results: All MSPCs displayed serotonin and strong TK expression. TK-IR was also seen in all serotonin-producing lung and appendix carcinoids. None of the other tumors examined contained TK-IR cells. Concentrations of P-TK, P-CgA, and U-5HIAA were elevated in patients experiencing daily episodes of either flush or diarrhea, when compared with patients experiencing occasional or none of these symptoms. In a Spearman partial rank test, the correlation of P-TK with daily diarrhea was independent of both U-5HIAA and CgA levels.

Conclusion: We found that TK synthesis occurs in serotonin-IR tumors and that P-TK levels are significantly correlated with symptoms of flush and diarrhea in patients with MSPCs. This is, to our knowledge, the first report demonstrating an independent correlation of P-TKs with carcinoid diarrhea, a symptom that is customarily regarded as serotonin mediated. Further investigations may present opportunities for new therapeutic possibilities.

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Introduction

Patients with metastasizing ileocecal serotonin-producing carcinoid tumors (MSPCs; also called midgut carcinoids and EC-cell carcinoids in the literature) often suffer from the carcinoid syndrome that includes hormone associated symptoms such as diarrhea and flush, carcinoid heart disease, and bronchial constriction.

Tachykinins (TKs) are present in normal enterochromaffin cells in the gastrointestinal (GI) tract and in MSPCs (1–4). TKs have also been found in other endocrine tumors, such as appendix carcinoids (5), serotonin-producing lung carcinoids (6), ovarian carcinoids (7), medullary carcinoma of the thyroid (8, 9), and in pheochromocytomas (10).

Among the members of the TK family are substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), neurokinin B (NKB), and hemokinin-1; these hormones are coded on three genes: TAC1, TAC3, and TAC4 (11). Recent reviews have provided more details (12–14). TKs are biologically active neuropeptide hormones that share a conserved C-terminal sequence, -Phe-Xaa-Gly-Leu-Met-NH2, where Xaa is hydrophobic and either an aromatic or a branched-chain aliphatic amino acid. The C-terminal sequence is important for the activation of each of the three known receptors, NK1, NK2, and NK3. The N-terminal sequence differs considerably in both length and amino acid composition. The activation of these receptors has been correlated with a range of biological activities, e.g., smooth-muscle contraction, vasodilatation, pain transmission, activation of the immune system, and stimulation of endocrine gland secretion (15).

The biological action of TKs suggests a role in the development of both flush and diarrhea but few studies correlate TKs with specific symptoms in patients with the carcinoid syndrome. A correlation between TK levels and flush has been described (16, 17), but not between plasma SP concentrations and pentagastrin-induced flush (18). It has also been shown that intraluminal TK concentrations were higher in patients with MSPCs than in healthy control subjects (19). A study in rats showed that serotonin but not SP increased gastric emptying and upper gastrointestinal transit.
times (20). No positive association between carcinoid diarrhea and TKs has been reported, as far as we know.

In this study, a new polyclonal antibody to the common C-terminal of the TK family was used in a RIA and in immunohistochemical analysis (IHC) to study P-TK association with clinical symptoms in MSPCs and to screen other endocrine tumor tissues for the expression of members of the TK peptide family.

Materials and methods

Sample composition

In this retrospective study, a total of 137 patients were included, 65 of whom were diagnosed with MSPCs (tumor tissue available from 33 patients) and 72 with other types of endocrine tumors. Plasma samples from 42 consecutive patients with MSPCs were collected for RIA analysis before treatment was initiated. In the IHC analysis tumor tissues were studied from 33 patients with MSPCs where 10 patients were included in both the RIA and IHC analysis. The IHC analysis also entailed 72 patients with various types of endocrine tumors elsewhere in the GI tract and other organs including: ECLoma type 1 (n=4), ECLoma type 3 (n=2), typical lung carcinoid (n=3), atypical lung carcinoid (n=3), endocrine pancreatic tumor (n=11), poorly differentiated endocrine carcinoid (stomach) (n=3), poorly differentiated endocrine carcinoid (rectum) (n=2), appendix carcinoid (n=4), goblet cell carcinoid (n=2), L-cell rectal carcinoid (n=1) pheochromocytoma (n=5), neuroblastoma (n=2), adrenal cortical adenoma (n=2), adrenal cortical carcinoma (n=7), follicular thyroid adenoma (n=5), follicular thyroid carcinoma (n=2), papillary thyroid carcinoma (n=2), medullary thyroid carcinoma (n=5), parathyroid adenoma (n=4), and parathyroid carcinoma (n=1), simple goiter (n=2) see Table 1. The tumors were diagnosed histopathologically at the Laboratory for Pathology and Cytology and patients with MSPCs were treated at the Department of Endocrine Oncology at The University Hospital, Uppsala. All MSPCs displayed chromogranin A (CgA) immunoreactive (IR) and/or argyrophil (Grimelius) reaction (21); and also showed serotonin and vesicular monoamine transporter 1 IR and/or argentaffin (Masson) reaction (22). At the time of the study, 48 MSPC patients had metastases and the rest of these patients developed metastases during the course of the disease. Tumor diagnosis was based on recommendations in the World Health Organization classification of endocrine tumors (23).

Forty-four of the patients with MSPCs had symptoms of either flush and/or diarrhea. Medical records were reviewed in order to establish the symptoms evident at diagnosis. All patients were asked specifically about symptoms of diarrhea and flush. When symptoms were absent, a negative entry was correspondingly

Table 1 Immunohistochemical detection of tachykinins, serotonin, and chromogranin A in endocrine tumors.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>N</th>
<th>Tachykinin</th>
<th>Serotonin</th>
<th>Chromogranin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foregut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECLoma type 1</td>
<td>4</td>
<td>0</td>
<td>0a</td>
<td>4</td>
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<tr>
<td>ECLoma type 3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Typical lung carcinoid</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Atypical lung carcinoid</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Endocrine pancreatic tumor</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Poorly differentiated endocrine carcinoid (stomach)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3b</td>
</tr>
<tr>
<td>Midgut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileocecal serotonin-producing carcinoids</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Appendix carcinoid</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Goblet cell carcinoid</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Hindgut</td>
<td></td>
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<td>L-cell rectal carcinoid</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Poorly differentiated endocrine carcinoid (rectum)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2b</td>
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<tr>
<td>Other endocrine tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>5</td>
<td>0c</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Neuroblastoma</td>
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<tr>
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<td>Parathyroid adenoma</td>
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<td>2</td>
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<tr>
<td>Parathyroid carcinoma</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Simple goiter</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aFewer than 1% of tumor cells were serotonin immunoreactive.
bChromogranin A expression was seen focally in 20–80% of tumor cells.
cTwo out of five pheochromocytomas contained tachykinin positive nerve cells.
documented. Patients were grouped according to symptom frequency into three groups: no symptoms, occasional symptoms (at least once in a week), and daily symptoms of either flush or diarrhea. Eight patients included in the RIA analysis had symptoms of both diarrhea and flush, 16 only diarrhea, 8 only flush, and 10 did not experience symptoms.

**Antibody construction**

Based on the amino acid sequence of human TKs, a polypeptide was synthesized by a solid-phase system using Fmoc chemistry. The peptide was purified by reverse-phase chromatography and analyzed by plasma desorption mass spectrometry.

The sequence was selected to be specific for the conserved C-terminal of human TKs (Fig. 1). A cysteine residue was added to the N-terminal to facilitate coupling to the carrier protein. To facilitate iodine labeling, a tyrosine residue was added in the position between the cysteine residue and the N-terminal of the peptide.

Before immunization, the peptide was coupled to a carrier protein. One milligram peptide was dissolved in 100 μl DMSO after which, 1 mg Imject maleimide-activated keyhole limpet hemocyanin (Pierce Biotechnology, Rockford, IL, USA) was added. This mixture was allowed to react for 2 h at room temperature.

The coupled peptide was then purified on a PD-10 column (Amersham Biosciences, Freiburg, Germany) with PBS as the moving phase. Aliquots of 200 μg coupled peptide were frozen and stored at −20°C until immunization. The peptide complex was injected into New Zealand white rabbits, using the intradermal injection technique to produce polyclonal antibodies.

**Development of the RIA**

All chemicals used were of pro analysis grade (Merck). Dilutions in the RIA were performed in the assay buffer, a 0.05 M sodium phosphate buffer at pH 7.4, with 0.15 M sodium chloride, 0.02% sodium azide, 0.2% BSA, and 0.5% Tween 20. The antibodies and the synthesized peptide were used to develop a specific RIA. For preparation of the tracer, the peptide was labeled with 125I (Amersham International plc, Amersham) using the chloramine-T method as previously described (24). The assay was devised as follows: standards and unknown samples were incubated with a tracer (30 000 c.p.m./tube) and primary antibodies, at a final dilution of 1/45 000, for 3 days at +4°C. All standards and samples were assayed in duplicate. Antibody-bound radioactivity was separated from free tracer by adding a second antibody, goat anti-rabbit IgG coupled to a solid phase (Decanting suspension 3, Pharmacia Biotech). The antibody-bound radioactivity was then measured in a γ-counter (Autogamma, Wallac, Pharmacia Biotech) and the data were calculated with a logit-log transformation program (Multi-calc, PerkinElmer, Massachusetts, USA).

The cross-reactivity and specificity of the antibodies were tested in the RIA by serial dilutions of the commercial TK peptides: SP1–11, Tyr-SP1–11, SP1–9, SP7–11, SP4–11, NPK, NKA and NKB, and the synthesized peptide (Nova Biochem Darmstadt, Germany and Sigma Chemical Co., LabKemi).

**Collection of plasma samples for TK analysis**

Plasma samples from the 42 consecutive patients were collected after an overnight fast. TK-related IR in plasma samples was measured without prior extraction. Plasma samples were collected in heparinized tubes, stored on ice, and centrifuged within 30 min. The plasma was frozen immediately and stored at −20°C until analysis.

**Measurement of urinary-5 hydroxyindoleacetic acid (U-5HIAA) and P-CgA**

Thirty-seven of the above-mentioned plasma samples, collected after an overnight fast before treatment was initiated, were also analyzed for P-CgA concentrations using a previously described method (25). In all 42 patients, U-5HIAA before treatment start was measured.

Figure 1 The TK antibodies are specific for the TK family common C-terminal, as shown in a competition study where labeled synthesized peptide and unlabeled TK peptides, listed in table below, compete to bind the antibody in a RIA. All peptides with intact C-terminal bound to the antibodies, while the peptide missing two amino acids, Substance P (1–9), did not bind. Thus, an intact C-terminal was mandatory for antibody binding.
using HPLC and calculated as the mean amount (μmol) of two 24 h urine collections (26).

**IHC**

Tumor biopsies were fixed in buffered formalin, dehydrated, and embedded in paraffin wax. Sections, ~4 μm thick, were attached to positively charged glass slides, deparaffinized in xylene, and rehydrated using decreasing concentrations of ethanol. Before immunostaining, the sections were treated in a microwave oven at 700 W for 15 min and 350 W for 10 min in 50 mM Tris buffer (pH 8.0). DAKO EnVision + System-RP was used as a staining technique with diaminobenzidine as chromogen substance. The sections were counterstained with hematoxylin.

A dilution series of the TK antibody was tested on tumor sections from a patient with MSPC known to have elevated plasma levels of NPK. A distinct staining of tumor cells was noted at a dilution of 1:5000. IHC without antigen retrieval and with microwave pre-treatment in citrate buffer pH6 was also tested but gave weaker staining. As a negative control, an absorption treatment in citrate buffer pH6 was also tested but gave without antigen retrieval and with microwave pre-treatment in citrate buffer pH6 was also tested but gave weaker staining. As a negative control, an absorption test was also performed with the synthesized peptide, using the concentrations 10, 1, and 0.1 nmol/ml.

**Statistical analysis**

A possible correlation between the biochemical markers and the presence of daily, occasional or the absence of symptoms at diagnosis and before the initiation of treatment, was tested using a Spearman rank test. A P value less than 0.05 was considered a statistically significant result. In order to analyze the independent effects of the three hormones on the severity of diarrhea, each marker was tested while the others were held constant in a Spearman partial rank test.

**Ethics**

The study was reviewed and approved by the local Medical Ethics Board at Uppsala University Hospital.

**Results**

**Antibody specificity**

Clear IHC staining of tumor cells in a patient with elevated plasma levels of NPK was noted. In the IHC specificity test of anti-TK antibody, a complete elimination of IR was seen when the TK antibody was pre-incubated in a solution containing either 10 or 1 nmol/ml of the synthesized peptide (see inset, Fig. 2B). The specificity of the anti-TK antibody was also tested using TK-peptides in a competitive-binding assay. All TK-peptides with an intact TK C-terminal bound the antibodies competitively in the RIA setting, whereas the peptide lacking two C-terminal amino acids did not (see Fig. 1).

**TK expression in endocrine tumors**

All 33 MSPC specimens included in this study showed intense TK-IR. No difference in staining intensity was noted in patients with when compared to those without the carcinoid syndrome. Furthermore, TK-IR was detected in 2 out of 5 lung carcinoids, 5 out of 6 appendix carcinoids; all TK-IR tumors also displayed serotonin IR. TK expression could not be detected in the other endocrine tumors studied (see Table 1 and Fig. 2).

TKs were, as reported earlier (13), expressed in neuronal tissue. Slender cells surrounding the Bru- ner’s glands showed TK-IR possibly explained by the fact that these glands are innervated by TK signaling neurons (27). TK-IR cells were rarely seen in mucosa from the distal duodenum but were abundant the jejunal/ileal mucosa.

**Hormone markers in relation to the carcinoid syndrome**

A Spearman correlation test showed that P-TK, U-5HIAA, and P-CgA concentrations were significantly correlated with each other in the 42 consecutive patients diagnosed with MSPCs. The correlation coefficient was the highest for P-CgA and U-5HIAA levels (0.93), 0.60 for P-TKs and U-5HIAA levels, and 0.49 for P-TK and CgA. Ten patients, all with high TK-IR in the IHC analysis, displayed P-TK levels ranging from 30 to 186 pmol/l (median 49.5).

The median P-TK level in patients with liver metastases (n=22) was 65.5 pmol/l (range 30–570) in comparison to those without 46 pmol/l (range 30–96). Patients with lymph node metastases had a median of 62 pmol/l (30–570) vs 43 pmol/l (43–186) for those without. In patients where neither liver nor lymph node metastases had yet developed (10) the median was 44 pmol/l (range 30–87). Patients with metastases tended to have higher concentrations of P-TK but this did not reach significance in this study.

Results from a Spearman rank test, performed to determine the association of the hormone levels with the grade of clinical symptoms, are shown in Table 2. All tumor marker concentrations were elevated in patients with daily episodes of flushing. However, the hormone effects were not significant in the partial Spearman Rank test and therefore not independent effects. In patients with daily episodes of flushing at diagnosis (n=8), the median P-TK level was 86.5 pmol/l (range 30–493), for those with occasional flushes (n=8), 53 pmol/l (range 30–186), whereas for patients without flushes (n=26), 44 pmol/l (range 30–570) (see Fig. 3).

All three hormone marker concentrations were significantly elevated in patients with daily episodes of
diarrhea, though the association between P-TK and the severity of diarrhea was independent of both CgA and U-5HIAA concentrations (correlation coefficient: 0.4, $P < 0.01$). In patients with daily episodes of diarrhea at diagnosis ($n = 11$), the median P-TK level was 105 pmol/l (range 30–570), for those with occasional diarrhea ($n = 12$), 49.5 pmol/l (range 30–96), whereas for patients free from diarrhea ($n = 19$), 30 pmol/l (range 30–129) (see Fig. 3).

**Discussion**

The new antibody to the common C-terminal of members of the TK protein family has been designed and raised in our laboratory and is shown to bind specifically to members of the TK family and useful in both RIA and IHC analyses. In the RIA setting, we could show that an intact C-terminal was mandatory for antibody binding. TK expression was seen only in tumors that simultaneously expressed serotonin. All MSPCs studied displayed strong TK-IR, which may be of diagnostic value. Our results corroborate earlier findings indicating that serotonin and TK are often expressed in the same cell (1, 28, 29). Previously, focal SP expression had been described in a minor subset of medullary thyroid carcinoma and pheochromocytomas (8–10, 30). In our study, TK-IR was not found in non-serotonin-producing endocrine tumors; however, our sample population is too small to rule out the existence of other TK-IR endocrine tumors.

In contrast to homogenous TK-IR in tumor tissue variation in P-TK was noted in patients with MSPCs.

**Table 2** Biochemical markers associated with flush and diarrhea in patients with metastasizing ileocecal serotonin-producing carcinoid tumors before treatment start.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Grade of flush$^a$</th>
<th>Grade of flush$^b$</th>
<th>Grade of diarrhea$^a$</th>
<th>Grade of diarrhea$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma-tachykinins</td>
<td>0.3</td>
<td>0.18</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>$P = 0.03$</td>
<td>$P = 0.3$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>U-5 HIAA</td>
<td>0.4</td>
<td>0</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>$P = 0.01$</td>
<td>$P = 0.8$</td>
<td>$P &lt; 0.05$</td>
<td>$P = 0.5$</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P = 0.2$</td>
</tr>
</tbody>
</table>

All markers are elevated in patients with daily episodes of flush and diarrhea when compared with those experiencing occasional or no symptoms. Plasma-tachykinin association with the severity of diarrhea is independent from urinary-5 hydroxyindoleacetic acid (U-5HIAA) and plasma-chromogranin A effects.

$^a$Spearman’s rank correlation ($r$) between measure levels and severity of diarrhea.

$^b$Partial Spearman’s correlation coefficients adjusted for the other two biochemical markers.
This may be due to several factors. Patients with metastases had higher levels when compared with those without. Notably, all patients with P-TK over 100 pmol/l had extensive liver metastases. Other factors, such as the secretory activity of the tumor, the short half-life of TK peptides in circulation, and variation in liver function may also be important.

MSPCs produce many biologically active substances with partially overlapping biological functions. The biological processes underlying the specific symptoms of the carcinoid syndrome are probably multifactorial. In the present study, we could confirm results from earlier studies showing that TK and U-5HIAA levels are elevated in patients with daily episodes of flushing (16, 31). The hormone effects were not mutually independent in this study. It is possible that the development of flushes is the result of multi-hormonal stimulation. Other biologically active substances, such as kallikrein, (32, 33) and prostaglandins (34), may also contribute.

Secretory diarrhea in patients with MSPCs is currently thought to be related to serotonin production based on the functional role of serotonin in the normal gut. However, studies have not shown any significant correlation between U-5HIAA levels and the severity of diarrhea (31, 35). Serotonin receptor antagonists alleviate gastrointestinal symptoms in some but not all patients (18, 35). A more recent study found that wide variation in, rather than the absolute value of, diurnal U-5HIAA concentrations was correlated with the severity of the diarrhea (36). In our study, all biochemical marker concentrations were elevated in patients with daily episodes of diarrhea, though the association between increased P-TK and the severity of diarrhea was independent of both CgA and U-5HIAA concentrations.

There is the evidence of a biological mechanism that could explain this association between TK and carcinoid diarrhea. P-TKS are involved in other diarrheic conditions such as the irritable bowel syndrome (37, 38) and ulcerative colitis (39). In the normal gut, SP and NKA, via TK receptors on muscle cells and secretomotor neurons, have stimulatory effects on gut motility (37, 40). There is also evidence that TK can induce serotonin release from colonic mucosa via NK2 and NK3 receptors (41). P-SP levels have been shown to correlate with the severity of diarrhea in cryptosporidiosis (42). Earlier studies have not shown any significant correlation between individual members in

Figure 3 Plasma tachykinins (P-TK), urinary-5-hydroxyindoleacetic acid (U-5HIAA), and plasma chromogranin A (P-CgA) in relation to flush and diarrhea severity in patients with MSPCs.
Apostolos Tsolakis for help in gathering the tissue
Inger Olsson for their excellent technical assistance,
financial support for this study.

Go¨sta Selanders Fund are acknowledged for providing
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28 patients in whom P-NKA levels continued to rise
A shorter survival, however, was reported for 5 out of

reduced NKA-induced gut motility in healthy volun-
tional modulation of cholinergic motor neuron activity
NK2 receptor antagonists by a mechanism of prejunc-

been speculated that secretory diarrhea in irritable
diarrhea and possible role in the carcinoid flush.
Jaffe BM. The pentagastrin test in the diagnosis of the carcinoid

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