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Abstract

Research in the past on transient receptor potential cation channel subfamily V member 1 (TRPV1) has been limited to mainly nervous tissue TRPV1 because of the channel's role in pain perception. Here, we studied the potential role of TRPV1 in vascular smooth muscle. We have observed that capsaicin, a TRPV1 agonist, induced constriction of the middle meningeal artery (MMA). Our goal was to decipher the mechanism of capsaicin-induced constriction of the MMA. Arterial diameter measurements showed that constriction due to 100 nM capsaicin (65.4% ± 3.7, n=7) was significantly diminished in the presence of the voltage-dependent calcium channel (VDCC) blocker 100 µM diltiazem (43.1% ± 8.1, n=7). Capsaicin-induced constriction was not significantly altered in the presence of the sarco/endoplasmic reticulum calcium transport ATPase (SERCA) inhibitor 30 µM cyclopiazonic acid (63.7 ± 9.0%, n=5) compared to control arteries (58.4 ± 8.6%, n=5). The unaltered capsaicin-induced constriction of the MMA in the presence of a SERCA inhibitor suggests that calcium-induced calcium release does not contribute to the overall calcium influx mechanism within the smooth muscle cells of the MMA. The diminished capsaicin-induced constriction of the MMA in the presence of a VDCC blocker suggests that sodium entry through TRPV1 channels can possibly lead to the membrane potential depolarization and increased activity of VDCCs causing further calcium influx. Furthermore, since the capsaicin effect was not abolished by the blockage of VDCCs, our data suggest that calcium entry through TRPV1 is sufficient to cause approximately 65% of the total constriction of the MMA in response to activation of TRPV1.
Introduction

Transient receptor potential cation channel subfamily V member 1 (TRPV1) belongs to a large family of cation channels of about 50 members, 28 of which are mammalian, found across different species (Vriens et al., 2004a) making the TRP channels one of the largest families of ion channels (Vriens et al., 2009). Some of the better studied ion channels in this family are involved in thermoregulation and pain perception; this includes TRPV1.

TRPV1 is a non-selective cation channel found primarily in nerve cell membranes with a permeability preference to Ca\(^{2+}\). Its main agonists are capsaicin, high heat stimuli, and low pH levels. Besides the “classical” agonists of TRPV1 the channel can also be activated by endogenous compounds such as inflammatory agents bradykinin, serotonin, histamine, or prostaglandins, which stimulate TRPV1 through PKC-dependent, PKA-mediated, and PIP\(_2\)-dependent, and various other pathways. It is composed of four identical subunits that form a pore through which cations can move. Each subunit has six transmembrane domains with the N- and C-termini on the intracellular side and a pore-forming hydrophobic loop between transmembrane domains 5 and 6. TRPV1 channels are distinctive in that their ligand-binding site is on the intracellular portion of the receptor (Vriens et al., 2009). The channel has regulatory domains at various sites throughout the protein. TRPV1 can be phosphorylated by kinases including protein kinase A (PKA; Bhave et al., 2002), which seems to, protein kinase C (PKC; Bhave et al., 2003), Ca\(^{2+}\)/CaM-dependent kinase II (Jung et al., 2004), Src kinase (Jin et al., 2004), and phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), which seems to be play an inhibitory effect on the TRPV1 channel. PKC-dependent phosphorylation of TRPV1 reduces the temperature
threshold for TRPV1 activation (~43°C) and increases the channel open probability at a lower temperature (Vriens et al., 2009).

Over the recent past, TRPV1 has come into focus because of its role in pain perception; this has caused a surge of research in therapeutic targeting of TRPV1 with a number of compounds in Phase III trials (Moran et al., 2011). Because of the focus on pain perception most research has been performed using nervous tissue TRPV1. This manuscript focuses on TRPV1 channels in vascular smooth muscle (SM) cells. Direct capsaicin action on vascular SM cells was first reported in 2008 (Kark et al., 2008) with the results suggested non-neuronal vasoconstriction upon TRPV1 stimulation in the skeletal muscle arterioles. Vasoconstriction is caused by contraction of smooth muscle cells on arteries that experience an increase in intracellular calcium. Free Ca$^{2+}$ binds to a calcium binding protein calmodulin; calcium-calmodulin activates myosin light chain kinase (MLCK), which then phosphorylates myosin light chain (MLC), a 20-kD regulatory subunit found on myosin heads, in the presence of ATP; MLC phosphorylation leads to the interaction, specifically a cross-bridge formation, between the myosin and the actin filaments resulting in SM contraction (Webb, 2003).

Novel data from the Wellman Lab demonstrates constriction of the middle meningeal artery (MMA) when exposed to capsaicin, which suggests the presence of TRPV1 on the SM cells of the MMA. The MMA is located in the dura mater on the surface of the brain and may play an important role in the pathophysiology of migraine.

The vascular theory of migraine, first proposed by Ray and colleagues in the 1940s, contends that dilation of the dural vasculature is involved in pain perception during headache. In a more current model the release of neurotransmitters or neuropeptides
dilate the middle meningeal artery (MMA), causing sensitization of the trigeminal sensory nerve tissue surrounding the artery, and leading to the perception of pain (Goadsby et al. 1993). A clinical study with migraineurs found MMA dilation during migraine attacks. Additionally, the study demonstrated that administration of the anti-migraine drug sumatriptan caused amelioration of the headache and MMA constriction without affecting other vasculature (Asghar et al., 2011). These findings indicate that compounds causing constriction of the MMA may provide therapeutic benefit to people suffering from migraine.

The purpose of this study was to elucidate the mechanism that leads to the capsaicin-induced constriction via enhanced Ca\(^{2+}\) entry into the SM of the MMA. We hypothesized that calcium entry directly through TRPV1 contributes to the constriction response that is observed when the MMA is exposed to capsaicin. Further study of capsaicin-induced MMA constriction may provide insight on the physiological role of SM TRPV1 and present different methods of migraine headache or cluster headache treatment.

**Materials and Methods**

**Isolation of Middle Meningeal Arteries**

All experiments and protocols were conducted in accordance with the Guide for the Care and Use of Laboratory animals (NIH Pub. No. 85-23, revised 1996) and approved by the Institutional Animal Use and Care Committee of the University of Vermont (IACUC NO. 10-032). Male Sprague–Dawley rats (300–350 g; Charles River Laboratories, Saint Constant, QC, Canada) were euthanized by decapitation under deep isoflurane anesthesia. The brain and calvaria were removed and placed in ice-cold aCSF (artificial cerebral spinal fluid) of the following composition (in mM): 120 NaCl, 3 KCl, 23 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 1
MgCl$_2$, 2 CaCl$_2$, 5 glucose. To isolate the MMA, the dura mater was removed and the artery was gently dissected away from the dura while visualized under a high power dissection microscope (Figure 1A).

**Ex Vivo Diameter Measurements**

The following procedures to quantify changes in the diameter of the MMA segments were based on methodology from the Wellman Lab (Syed et al., 2012). MMA artery segments were cannulated on glass micropipettes and mounted onto a 5-mL arteriograph chamber (Figure 1B). Arteries were continuously superfused with warmed aCSF (37°C) aerated with 5% CO$_2$, 20% O$_2$, 75% N$_2$ (pH, 7.30–7.35) and pressurized at 10 mmHg (arteries did not develop myogenic tone at this pressure). Changes in arterial diameter were measured with video edge detection (Living Systems Instrumentation, St Albans, VT) using WinDaq data acquisition software (Dataq Instruments; Akron, OH). Arteries were allowed to equilibrate for at least 30 min followed by a 5 min exposure to 60 mM K$^+$ to test for viability of the artery. Experiments were carried out using only arteries that showed a 50% or greater decrease in diameter when exposed to 60 mM K$^+$. Constriction was analyzed as the decrease in arterial diameter relative to the passive diameter using the following equation: %Constriction = \[
\left[\frac{(DP-DA)}{DP}\right] \times 100
\] where DP is the fully dilated, or passive, diameter of the artery in Ca$^{2+}$-free aCSF containing two vasodilators, 100 μM diltiazem and 1 μM forskolin, and DA is the active diameter of the artery in response to the stimulus in aCSF.

**Statistical Analysis**

Data are expressed as mean ± s.e.m. and analyzed by Student’s paired t-test. Statistical significance was considered at the level of P < 0.05 (*) or P < 0.01 (**).
Results

Capsaicin-induced constriction of the middle meningeal artery (MMA) is abolished by the selective TRPV1 antagonist, capsazepine.

Preliminary experiments in the Wellman laboratory have demonstrated that capsaicin induces a concentration-dependent constriction of isolated pressurized MMA with an EC\textsubscript{50} of approximately 100 nM (Syed, Wellman, Nelson Lab unpublished observations). No constriction was observed in cerebral arteries nor mesenteric arteries when exposed to capsaicin suggesting that the response was unique to certain vascular beds such as the dura mater (Syed and Wellman, unpublished observations). As capsaicin has been widely described as an activator of TRPV1 channels (Vriens et al., 2009), initial experiments were performed to examine the ability of the TRPV1 antagonist, capsazepine (Vriens et al., 2009) to inhibit capsaicin-induced MMA constriction. Figure 2 illustrates that
capsazepine (1 µM) abolished MMA constriction elicited by 100 nM capsaicin. However, capsazepine did not interfere with MMA constriction caused by agents acting independently of TRPV1, i.e. elevated extracellular potassium (60 mM K⁺) or the alpha-1 adrenergic receptor agonist phenylephrine (PE, 3 µM) (Figure 2C). High extracellular potassium causes vasoconstriction through membrane potential depolarization and opening of the VDCCs (Nystoriak et al, 2011), while PE activates Gq-coupled α1-adrenergic receptors ultimately leading to inositol triphosphate (IP3)-mediated release of Ca²⁺ release from the SR stores and PKC-dependent inhibition of K⁺ channels leading to membrane potential depolarization and activation of VDCCs (Jahnel et al., 1991). As protocols in this and following experimental series required that MMAs be exposed to capsaicin, 60 mM K⁺, and PE multiple times (up to 3 times), time control studies were conducted to examine the reproducibility of multiple applications of these vasoconstrictor agents. Figure 3 demonstrates that capsaicin- and PE-induced constriction did not decline after multiple applications. These data indicate that capsaicin-induced MMA constriction is mediated by activation of TRPV1 channels.

**Capsaicin-induced MMA constriction does not involve Ca²⁺ release from the SR.**

Capsaicin-mediated TRPV1 activation may cause MMA constriction via multiple mechanisms including direct Ca²⁺ entry, depolarization-induced VDCC activation and CICR from the SR. Experiments were performed to explore the possibility that the capsaicin-induced constriction involves the release of intracellular Ca²⁺. Cyclopiazonic acid (CPA), an inhibitor of the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA), was used to deplete the SR stores of Ca²⁺ to assess the role of CICR in capsaicin-induced constriction. Arteries were first treated with capsaicin (100 nM) alone, then following capsaicin washout (30
min) and a 15 min treatment with CPA (30 uM), arteries were again treated with 100 nM capsaicin this time in the presence of 30 uM CPA. Constrictions due to capsaicin alone and in the presence of CPA were not significantly different (Figure 4). Cyclopiazonic acid did, however, significantly reduce MMA constrictions in response to 3 µM phenylephrine (PE), consistent with SR Ca\(^{2+}\) store depletion (Figure 4C). These results indicate that the release of intracellular Ca\(^{2+}\) stores does not contribute to TRPV1-mediated constriction of the MMA.

**Block of L-type VDCCs significantly diminished, but did not abolish capsaicin-induced MMA constriction.**

To explore the possibility that Ca\(^{2+}\) entry through VDCCs contributes to capsaicin-induced MMA constriction, diltiazem, a calcium channel blocker, was used. First, the effectiveness of diltiazem to block VDCCs in the MMA was examined by comparing arterial constriction to 60 mM K\(^{+}\) and 60 mM K\(^{+}\) in the presence of 100 µM diltiazem. We hypothesized that diltiazem would block K\(^{+}\)-induced constriction given that 60 mM K\(^{+}\) causes membrane potential depolarization and VDCC activation in other vascular tissue (Nystoriak et al., 2011). As predicted, arterial constriction to 60 mM K\(^{+}\) was significantly reduced in the presence of 100 uM diltiazem (Figure 5A, C) from an average of 65.5% ± 5.3 to 23.5% ± 7.2 (n=7). Experiments were then performed to compare arterial response to 100 nM capsaicin and 100 nM capsaicin in the presence of 100 µM diltiazem. Arterial constriction due to capsaicin was significantly diminished in the presence of 100 µM diltiazem by approximately 34% (Figure 5B, C). Phenylephrine (3 µM) was also examined in the absence and presence of 100 uM diltiazem with the respective average constrictions of 47.7% ± 8.8 and 22.2% ± 6.6 (n=7); differences in constriction were significant.
Interestingly, although diltiazem alone reduced capsaicin-induced constriction, the combination of CPA and diltiazem was without effect. Specifically the average constriction due to 100 nM capsaicin alone was 76.1% ± 4.5 while the constriction due to 100 nM capsaicin in the presence of 100 µM diltiazem and 30 µM CPA was 62.6% ± 10.5. There was no significant difference in the constrictions (n=5).

**Discussion**

**Mechanism of capsaicin-induced MMA constriction**

This set of experiments suggests that that capsaicin-induced MMA constriction involves both TRPV1-mediated Na$^+$ influx, causing membrane potential depolarization and opening of L-type VDCCs and direct Ca$^{2+}$ entry through TRPV1 channels (Figure 6). The opening of these channels contributes to an increase in intracellular Ca$^{2+}$ concentration leading to vasoconstriction. As there was only a diminished MMA constriction due to capsaicin in the presence of diltiazem (100 µM), versus an abolished constriction, this suggests the important finding that Ca$^{2+}$ influx through TRPV1 channels also contributes to MMA constriction (Figure 5). Consistent with direct Ca$^{2+}$ entry through TRPV1 channels, a preliminary experiment performed without extracellular calcium showed that the MMA does not constrict upon TRPV1 activation by capsaicin (data not shown). This provides further evidence that direct Ca$^{2+}$ entry through TRPV1 channels is important in the constriction mechanism.

We observed that the combination of diltiazem and CPA did not have an effect on capsaicin-induced constriction (figure not shown). This was unexpected, since the use of diltiazem alone did cause a significant decrease in arterial constriction. We speculate that this result is due to a rise in PKC activity. Phosphorylation of TRPV1 by PKC has been
shown to reduce temperature threshold for TRPV1 activation and potentiates the capsaicin induced channel open probability (Vriens et al., 2009). In sensory nerves there is evidence that PKC can be Ca\(^{2+}\)-dependent (Bessac et al., 2008) and we can speculate that the same can be said about SM cells. SERCA inhibition by CPA could cause an increase in the intracellular free calcium ion concentration, which would increase PKC activity. An increase in PKC activity would lead to an increase in the phosphorylation of TRPV1 and the exposure of TRPV1 to capsaicin after phosphorylation would increase the channel open probability ultimately leading to a larger influx of calcium through TRPV1 channels and a constriction response comparable to a control capsaicin-induced constriction response. Further experiments would have to be conducted, perhaps with the use of PKC inhibitors, to find evidence to support this hypothesis.

The MMA is surrounded by sensory nerves, therefore, the possibility that capsaicin was sensitizing TRPV1 channels on nervous tissue on the dissected MMA instead of TRPV1 channels in the SM cells of the artery was not ruled out. To explore this possibility, MMA myocytes were enzymatically isolated in the Wellman Lab in order to see the effect of capsaicin directly on MMA SM. The myocytes were isolated from MMA of transgenic mice with SM expressing a green Ca\(^{2+}\)-sensitive protein (GCaMP5) and a red Ca\(^{2+}\)-insensitive protein (mCherry). The fluorescent proteins allowed for the visualization of intracellular calcium increase. Figure 7 shows increased GCAMP5 fluorescence when myocytes are exposed to capsaicin. This exciting data paralleled a paper published in 2011 that showed evidence of TRPV1 expression in the SM cells of arteries in certain vascular beds including the dura mater on the surface of the brain (Cavanaugh et al., 2011). The calcium imaging
data on isolated MMA myocytes provided direct evidence of the presence of TRPV1 channels on the SM cells of the MMA.

**Role of TRPV1 in vascular SM cells**

While it has been established that capsaicin induces vasodilation via the release of neuropeptides when it activates TRPV1 channels on sensory nerves (Griesbacher et al., 1982), capsaicin action on smooth muscle cells and constriction of skeletal muscle arterioles was reported recently (Kark et al., 2008). The role of TRPV1 in vascular smooth muscle cells is not well understood but the channel may play an important balancing function that opposes the vasodilator response to activation of sensory neuron TRPV1 channels. This may be useful in the development of tissue specific drugs to evoke desired responses in arteries, constriction versus dilation, although further study is needed to determine which response dominates in certain tissues.

**Connection of SM TRPV1 of the MMA to migraine**

Migraine is a complex neurological disorder that presents as an intense unilateral headache, which can be triggered by environmental factors and can persist for days. The causes of migraine headache remain a topic of controversy. However, targeting the MMA for constriction may provide therapeutic benefit to people suffering from migraine. As previously mentioned, the vascular theory of migraine contends that pain perception is linked to dilation of brain vasculature.

One neuropeptide that seems to have a prominent role in the pathophysiology of migraine is calcitonin gene related peptide (CGRP), which has been shown to induce migraine symptoms in subjects (Lassen et al., 2002) and has been correlated with vasodilation of the MMA and induced-headache after exogenous administration (Asghar et
There are other factors that could be coactive with CGRP. One such neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP) has also been shown to cause vasodilation in vivo and in isolated MMA (Schytz et al., 2009; Syed et al., 2012). Similarly to the administration of exogenous CGRP, intravenous infusion of PACAP during clinical studies triggers MMA vasodilation and induce headache in control subjects and migraineurs (Amin et al., 2012; Schytz et al., 2009).

This work followed conflicting data involving vasoactive intestinal polypeptide (VIP), a neuropeptide related to PACAP. This neuropeptide induced vasodilation but did not cause migraine (Rahmann et al., 2007). These results dismissed vasodilation as an important factor in the development in migraine symptoms and instead indicated that it was the activated receptor site that was important in migraine headache development. However, more conflicting data was published in 2011 using a novel high-resonance MRA imaging technique to monitor arterial dilation/constriction (Asghar et al., 2011). They found dilation in the MMA and the middle cerebral artery (MCA) during migraine attack (triggered by CGRP). More importantly, they found that administration of an antimigraine drug, sumatriptan, caused amelioration of the headache and specific constriction in the MMA, not the MCA. These results caused a reconsideration of vasodilation and its role in migraine and its symptoms. Although the dilation of the MMA cannot be said to be the cause of migraine in light of conflicting results from other studies (Schoonman et al., 2008; Olesen et al., 1990) important evidence suggests that MMA vasodilation is involved in the perception of pain and that the constriction of vasculature is correlated with the improvement of headache symptoms (Asghar et al., 2011).
Several clinical studies showed intranasal application of capsaicin relieved migraine and cluster headache (Fusco et al., 2003). The mechanism is thought to be through the desensitization of the TRPV1 channels on the trigeminal nerves (Winter et al., 1995). The capsaicin-induced MMA constriction discussed in our research provides a possible parallel mechanism to the improved headache in addition to the desensitization of the trigeminal nerves.

**Future Directions**

In addition to ex vivo experiments with capsaicin as an agonist, we would like to explore the effect of other TRPV1 agonists on the MMA. Preliminary data from the Wellman Lab has shown constriction of the MMA when exposed to temperatures over ~44°C (Manuelyan, Syed, Wellman, unpublished observations). Besides physical factors, we would like to explore the role of endogenous compounds like inflammatory mediators and the possible sensitization of TRPV1 channels by downstream second messenger systems activated by these mediators. The effects of these inflammatory mediators on TRPV1 channels would shed more light on their role in vascular smooth muscle cells. Further, direct measurements of TRPV1 currents in MMA myocytes using patch clamp electrophysiology would be of great value.

**Conclusions**

Our initial observation of capsaicin-induced MMA constriction led to further study of the mechanism of calcium ion increase within the smooth muscle cells of the MMA and the subsequent constriction of the artery. We conclude, based on the gathered data, that the capsaicin-induced constriction is TRPV1-mediated. Further, our evidence indicates that two mechanisms: (1) Na$^+$ entry through TRPV1 leading to membrane potential
depolarization and VDCC activation and (2) direct entry through TRPV1 channels contribute to the capsaicin-mediated constriction of the MMA.
Figure 2: TRPV1-mediated constriction of the rat middle meningeal artery
Summary data of the effect of capsaicin (caps), high extracellular potassium, and phenylephrine (PE) in the presence of the TRPV1 antagonist, 1 µM capsazepine. (a) Control: Measurement of arterial diameter of MMA with application of 100 nM caps for 5 min. (b) 1 µM capsazepine was applied 15 min prior to a 5 min application of 100 nM caps. (c) The average arterial constriction to 100 nM capsaicin was 72.2% ± 5.0 while average constriction to 100 nM capsaicin in the presence of 1 µM capsazepine was 4.6% ± 1.0 (P<0.01, n=5). Constriction to 60 mM K⁺, 75.9% ± 3.5, was not significantly affected in the presence of 1 µM capsazepine, 72.9% ± 4.6 (n=5). Constriction to 3 µM PE, 43.3% ± 7.0, also was not significantly affected in the presence of 1 µM capsazepine, 52.4% ± 5.8 (n=5).
Figure 3: Multiple applications of capsaicin cause reproducible MMA constriction
Time-control experiments were conducted to demonstrate no significant difference in constriction of the MMA upon multiple applications of capsaicin (cap) or phenylephrine (PE). (a) The MMA was equilibrated for 30 min or more prior to each 5 min application of cap. Constrictions due to cap were 72.94%, 78.90%, 78.92% from 1st to 3rd application, respectively. (b) The MMA was equilibrated for 30 min or more prior to each 10 min application of PE. Constriction due to PE during the 1st and 2nd applications were 23.3% ± 9.4 and 45.1% ± 6.8 (N.S., n=3), respectively.
Figure 4: SR store depletion does not alter capsaicin-induced MMA constriction
Summary data of the effect of 30 µM cyclopiazonic acid (CPA) on constriction due to 100 nM caps. (a) Control response to capsaicin. (b) 30 µM CPA was applied 15 min prior to 5 min application of 100 nM capsaicin. (c) Caps-induced constriction (68.5 % ±7.7) was not significantly different in the presence of 30 µM CPA (57.7 % ±14.8). There is a significant decrease in constriction response to 3 µM PE (59.4 % ± 9.8) in the presence of CPA (21.4 % ± 17.2) (n=5).
Figure 5: Diltiazem diminishes capsaicin-induced MMA constriction
Summary data of the effect of 100 µM diltiazem (dilt) on mouse MMA responding caps and high extracellular potassium. (a) MMA was exposed to 60 mM K\(^+\) for 5 min for a control response; 100 µM dilt was applied 15-20 min prior to a 5 min application of 60 mM K\(^+\). (b) MMA was exposed to 100 nM cap for a control response; dilt was applied 15 min in advance prior to a second 5 min application of 100 nM capsaicin. (c) Constriction was significantly reduced in the presence of the dilt with both caps and 60 mM K\(^+\). For high potassium, average constriction in the presence of dilt was reduced to 23.5% \(\pm 7.2\) from the control response of 65.5%\(\pm 5.3\). For caps, average constriction in the presence of dilt was reduced to 43.1% \(\pm 8.1\) from the control response of 65.4 \(\pm 3.7\) (n=7).
Figure 6: Proposed mechanism of TRPV1-mediated MMA constriction
Mechanism of capsaicin-evoked Ca\(^{2+}\) concentration increase within a vascular smooth muscle cell via direct Ca\(^{2+}\) entry through TRPV1 channels and entry through activated L-type Ca\(^{2+}\) channels upon TRPV1- mediated membrane potential depolarization.
Figure 7: Capsaicin increases intracellular Ca\textsuperscript{2+} in isolated MMA myocytes
Enzymatically isolated MMA myocytes from Acta2-GCaMP5-mCherry mouse with Ca\textsuperscript{2+}-insensitive mCherry and with Ca\textsuperscript{2+}-sensitive GCaMP5 gene. HEPES-PSS: the faint green fluorescence is due to intrinsic calcium activity and red fluorescence is due to the mCherry protein. Capsaicin (1 µM): application of capsaicin causes an increase in green fluorescence in myocytes indicating capsaicin-evoked calcium entry. (Koide and Wellman, unpublished observation).
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