Ca^2+ dependent but PKC independent signalling mediates UTP induced contraction of rat mesenteric arteries

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Abstract

Uridine triphosphate (UTP) can be released from damaged cells to cause vasoconstriction. Although UTP is known to act through P2Y receptors and PLC activation in vascular smooth muscle, the role of PKC in generating the response is somewhat unclear. Here we have used Tat-linked membrane permeable peptide inhibitors of PKC to assess the general role of PKC and also of specific isoforms of PKC in the UTP induced contraction of rat mesenteric artery. We examined the effect of PKC inhibition on UTP induced contraction, increased cytoplasmic Ca^2+ and reduction of K^+ currents and found that PKC inhibition caused a relatively small attenuation of contraction but had little effect on changes in cytoplasmic Ca^2+. UTP attenuation of both voltage-gated (K_v) and ATP-dependent (K_ATP) K^+ currents was abolished when intracellular Ca^2+ was decreased from 100 to 20 nM. PKC inhibition reduced slightly the ability of UTP to attenuate K_v currents but had no effect on K_ATP current inhibition. In conclusion, both UTP induced contraction of mesenteric artery and the inhibition of K_v and K_ATP currents of mesenteric artery smooth muscle cells by UTP are relatively independent of PKC activation; furthermore, the inhibition of both K_v and K_ATP currents requires intracellular Ca^2+.

Key words: K_v channel, K_ATP channel, PKC, UTP, calcium

Introduction

Contraction of arterial smooth muscle results from an increase in internal [Ca^{2+}], occurring either by entry through Ca^{2+} permeable channels or by release from intracellular Ca^{2+} stores. Many vasoconstrictors stimulate G_{q} linked receptors activating phospholipase C leading to generation of DAG, IP_3 and subsequent activation of
PKC and Ca$^{2+}$ release. In many instances intracellular Ca$^{2+}$ is raised further through PKC induced reduction of K$^+$ currents causing depolarization and subsequent activation of L-type Ca$^{2+}$ channels (1). For example, we and others have shown that PKC is involved in generating the contraction of arterial smooth muscle in response to both Ang II and ET-1 (2–4). UTP, which is released in response to cellular injury (5), causes contraction of vascular smooth muscle by activating G-protein coupled P2Y receptors (6, 7); but in rat cerebral arteries UTP induced contraction was unaffected by the general PKC inhibitor bisindolylmaleimide and UTP inhibition of voltage-gated K$^+$ channels was not dependent on PKC (8). However, in rat mesenteric artery smooth muscle cells UTP, Ang II and ET-1 all increase intracellular Ca$^{2+}$ and induce the translocation of PKC$\alpha$, PKC$\delta$ and PKC$\varepsilon$ (9). Interestingly, the contraction induced by Ang II works almost exclusively through PKC$\varepsilon$ while ET-1 works through PKC$\alpha$ (4), but whether specific PKC isoforms are involved in generating UTP induced contraction is not known.

In this study we have used myography, Ca$^{2+}$ imaging and electrophysiological techniques to investigate the degree of PKC involvement in the signalling mechanisms of UTP induced contraction of rat mesenteric arteries. In our study we have used cell-permeable general and isoform specific peptide inhibitors of PKC and found that PKC activation has only a minimal role in generating UTP induced contraction. Our data also show that UTP induced attenuation of K$^+$ currents is dependent on intracellular Ca$^{2+}$.

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**Materials and Methods**

**Preparation of arterial smooth muscle cells**

All experiments were carried out on adult male Wistar rats (200–300 g) killed by stunning and cervical dislocation. The care of animals was in accordance with the UK Animals (scientific procedures) Act 1986. Investigations carried out in this study conformed to the Guide for the Care and use of laboratory animals published by the US National Institutes of Health (NIH publications No. 85-23 revised 1996). The procedures used in this study were approved by the University of Leicester Animal Care and Use Committee.

The mesentery was cleaned of fat and connective tissue and mesenteric arteries were either stored in ice cold physiological saline for myography or were immediately subjected to enzymatic digestion to obtain isolated smooth muscle cells as described previously by Rainbow *et al.* (10). Briefly, the arteries were incubated at 35 °C for 31 minutes in a low Ca$^{2+}$ solution comprising (mM): 137 NaCl, 5.4 KCl, 0.42 Na$_2$HPO$_4$, 0.44 NaH$_2$PO$_4$, 1 MgCl$_2$, 0.1 CaCl$_2$, 10 Hepes, 4 glucose and 6 mannitol, adjusted with NaOH to pH7.4 to which was added (mg ml$^{-1}$): 0.9 albumin, 1.4 papain and 0.9 dithioerythritol. This initial digestion was followed by a further digestion for 12.5 minutes in the low Ca$^{2+}$ solution containing (mg ml$^{-1}$): 0.9 albumin, 1.4 collagenase type F and 0.9 hyaluronidase type I-S. Arteries were then washed in low Ca$^{2+}$ solution containing 0.9 mg ml$^{-1}$ albumin. Individual cells were isolated by gentle trituration in low Ca$^{2+}$ solution and stored at 4 °C until used.

**Solutions and chemicals**

For myography experiments the physiological bathing solution comprised (mM): 135 NaCl, 6 KCl, 1.2 MgCl$_2$, 1.8 CaCl$_2$, 4 glucose, 6 mannitol, 10 HEPES, adjusted to pH 7.4. For the 60 mM K$^+$ solution, KCl was increased to 60 and NaCl decreased to 81 mM, other solution changes were made by adding or omitting compounds as necessary. Whole-cell recording was done with pipette solution comprising (mM): 110 KCl, 30 KOH, 1 MgCl$_2$, 1 or 3.9 CaCl$_2$, 1.0 Na$_2$ATP, 0.1 ADP, 0.5 GTP, 10 EGTA, and 10 HEPES adjusted to pH 7.2. The free [Ca$^{2+}$] calculated with the program Maxchelator (http://www.stanford.edu/~cpatton/maxc.html) were 20 and 100 nM for 1 and 3.9 mM CaCl$_2$ respectively. The 6 mM K$^+$ extracellular solution comprised (mM):
135 NaCl, 6 KCl, 1 MgCl₂, 0.1 CaCl₂, 10 HEPES, 4 glucose and 6 mannitol; the 140 mM K⁺ solution contained (mM): 140 KCl and no NaCl. Both solutions were adjusted to pH 7.4. All chemicals were obtained from Sigma-Aldrich. The general and isoform-specific PKC inhibitor peptides were linked to a HIV derived Tat peptide, C-YGRKKRRQRRR, using a disulphide link between N-terminal cysteine residues. This makes the peptides membrane permeable and the disulphide link is cleaved by the reducing environment of the cell. The peptide inhibitors used were: PKC₂₀-₂₈ (N-Myr-F ARKGALRQ); PKCα (SLNPQWNET); PKCβI (KLFIMN); PKCβII (QEVIRN); PKCδ (SFNSYELGSL) and PKCε (EAVSLKPT). Peptides were synthesized by Pepceuticals Ltd. and linking the inhibitory peptides to the Tat peptide was done by Dr R. Norman of the Department of Cardiovascular Sciences, University of Leicester.

Myography

To record contractile force, 2–4 mm segments of third order mesenteric arteries were mounted in a Myo-interface model 500A myograph (JP Trading, Denmark). In addition to the compounds being tested, all bathing solutions contained 20 µM L-NAME to eliminate endogenous nitric oxide synthesis and were added to the bath directly, which was maintained at 37°C.

Ca²⁺ imaging

Intracellular Ca²⁺ was measured in isolated mesenteric arterial smooth muscle cells cultured for 3 days. Cells were pre-loaded with Fura-2-AM (5 μM) for 30 minutes. Fluorescent signals were measured every 3 seconds following alternate excitation with 340 and 380 nm light using a monochromator (deltaRAM, Photon Technology, UK) and 45 second pulses of UTP were applied every 3 minutes to stimulate Ca²⁺ release. Emitted light >520 nm was recorded with a Roper Scientific CCD97 camera and values are expressed as the ratio of light emitted following excitations at 340 and 380 nm respectively. All measurements were made at 30–32 °C.

Electrophysiology

Whole-cell Kᵥ and KᵥATP currents were recorded from single smooth muscle cells using the patch clamp technique. Currents were filtered at 2 kHz (-3 dB) and recorded with an Axopatch 200A amplifier (Molecular Devices) and digitized at 10 kHz. Patch pipettes were made from thick-walled borosilicate glass (Clark Electromedical, Pangbourne, Berks, UK) using a pp-83 vertical puller (Narishige, Tokyo, Japan). Electrode resistances before sealing were 3–5 MΩ and after sealing were >1 GΩ. All experiments were done at 30°C, maintained using a Dagan HW-30 temperature controller.

Analysis

Results are expressed as means ± S.E.M. Intergroup differences were tested by analysis of variance followed by Bonferroni’s post hoc test, P<0.05 was considered statistically significant.

Results

UTP induced contraction depends on Ca²⁺ entry

Under control conditions (6 mM K⁺ and 1.8 mM Ca²⁺) we found that repeated 5 minute applications of UTP (100 µM) with 10 minute wash intervals gave consistent contractions for at least 8 applications. This enabled comparisons of the contractions before and after pharmacological interventions without desensitization of UTP responses being a major factor; thus for all myography experiments described we used 100 µM
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UTP. Fig. 1A shows contractile responses to the application of 60 mM K⁺ or 100 µM UTP in the absence or presence of 50 µM diltiazem as indicated. (B) Example trace showing the effect of U73122 on 100 µM UTP and 60 mM K⁺ induced contractions. (C) Mean contractile responses, normalized to their respective control amplitudes, to 100 µM UTP (n=15) or 60 mM K⁺ (n=15) in the presence of 50 µM diltiazem (n=15), or to 100 µM UTP in the presence of 1 µM U73122 (n=10). (*P<0.05, two-way ANOVA.)

UTP. Fig. 1A shows contractile responses to 60 mM K⁺ and to UTP in the absence and presence of diltiazem (50 µM), an L-type Ca²⁺ channel blocker. As expected, the response to 60 mM K⁺ was completely abolished by diltiazem, indicating that the depolarization induced by 60 mM K⁺ activated L-type channels causing Ca²⁺ entry and thereby contraction. Approximately 50% of the contraction induced by UTP remained in the presence of diltiazem (Fig. 1A & C), suggesting that UTP application triggers additional mechanisms for raising intracellular Ca²⁺. UTP has been shown to activate P2Y receptors which are Gq-linked (6, 7), and in line with this, pre-treatment of arterial segments with the PLC inhibitor U73122 (1 µM) virtually abolished the UTP induced contractions to 4.6 ± 1.9% of control values (n=10, P<0.05).

**UTP induces Ca²⁺ release**

Since blocking Ca²⁺ entry through L-type Ca²⁺ channels only partially reduced UTP induced contraction we examined changes in Ca²⁺ induced fluorescence of Fura-2 by UTP in the presence of 0.1 and 1.3 mM...
As can be seen in Fig. 2A & 2B, in cells bathed with 1.3 mM Ca\(^{2+}\) UTP induced a concentration dependent increase in Fura-2 fluorescence ratio which corresponds to an increase in cytoplasmic Ca\(^{2+}\). In the presence of 0.1 mM Ca\(^{2+}\) there was still a substantial increase in fluorescence ratio (Fig. 2C & 2D). These data suggest that UTP causes Ca\(^{2+}\) release from intracellular stores, as Ca\(^{2+}\) entry in 0.1 mM external Ca\(^{2+}\) would be less than 8% of that in 1.3 mM Ca\(^{2+}\) assuming a similar open probability of Ca\(^{2+}\) permeable ion channels present on the cell membrane.

**PKC inhibitor peptides are relatively ineffective at inhibiting UTP induced responses**

We have shown that the vasoconstrictors AngII, ET-1 and UTP all mobilize PKC\(\alpha\), PKC\(\delta\) and PKC\(\epsilon\) in mesenteric smooth muscle cells (9). We investigated the extent of various PKC isoform involvement in UTP
induced contractions by using Tat-linked membrane permeable peptide inhibitors targeting particular PKC isoforms. Previous experiments in our lab show that ET-1 induced contraction was markedly inhibited by application of Tat-PKCα-IP (4) and we therefore used this as a control to confirm the efficiency of the Tat-linked inhibitors in our current investigation. Inhibition of the ET-1 induced contraction by Tat-PKCα-IP is shown in Fig. 3A. Because ET-1 induces a prolonged contraction we quantified the effect of Tat-PKCα-IP by normalizing responses to the contractions induced by 60 mM K⁺ (Fig. 3B). It is evident that Tat-PKCα-IP inhibited the ET-1 induced contraction significantly and to a similar extent as in our previous work (4). UTP responses could be compared directly (normalized to control UTP induced contractions) and as Fig. 3C shows, in contrast to ET-1, UTP induced contractions were unaffected by Tat-PKCα-IP. None of the PKC isoform-specific peptide blockers affected UTP induced contractions, only the non-specific peptide blocker Tat-PKC²₀-²₈-IP produced a small but significant inhibition of UTP induced contractions (decreased to 82.8 ± 6.8%, P<0.05, n=7). We found that the UTP induced increase in Fura-2 ratio (with 1.3 mM Ca²⁺) was also reduced slightly, but not significantly, by pre-treatment with Tat-PKC²₀-²₈-IP (Fig. 2D).
Whole-cell $K_v$ currents were induced following depolarizing voltage pulses to potentials more positive than about -40 mV from a holding potential of -65 mV. To minimize contamination by BK channel activity, penitrem A (100 nM), a specific BK channel blocker, was included in the external solution (11). $K_v$ current density (normalized to cell capacitance) was quite variable in these cells; for this reason we have plotted currents normalized to control current at +60 mV to enable comparison between cells. The average cell capacitance of mesenteric arterial smooth muscle cells was $13.9 \pm 0.7 \, \text{pF}$ and the average current recorded was $290 \pm 52 \, \text{pA}$ at +60 mV. $K_v$ current densities measured at +60 mV under control conditions (100 nM penitrem A) were $18.7 \pm 2.7 \, (n=9)$ and $21.9 \pm 4.8 \, (n=10) \, \text{pA pF}^{-1}$ where the pipette solution contained 20 and 100 nM free $\text{Ca}^{2+}$ respectively; these values are not statistically different ($P=0.57$, two-tailed $t$-test). UTP has been shown to inhibit $K_v$ currents in rat cerebral arteries in a Rho kinase dependent manner (8). We found UTP (100 µM) inhibited $K_v$ currents of rat mesenteric artery smooth muscle by a similar amount to that reported by Luykenaar et al. (8), provided the pipette solution contained 100 nM free $\text{Ca}^{2+}$ (Fig. 4C & D). Under these conditions $K_v$ current inhibition by UTP was more prevalent at positive potentials (see Fig. 4D); UTP reduced the $K_v$ current recorded at +60 mV to $59.8 \pm 7.3\%$ of the control amplitude ($P<0.05$, $n=9$). However, in recordings made from cells where the pipette solution contained only 20 nM free $\text{Ca}^{2+}$, UTP did not inhibit $K_v$ currents (see Fig. 4A & B).

PKC inhibition reduces but does not abolish UTP modulation of $K_v$ currents

To assess the involvement of PKC in the UTP induced modulation of $K_v$ currents a comparison of the inhibition was made in the absence and presence of Tat-PKC$_{20,28}$-IP, a non-isoform specific inhibitor of PKC. As shown in Fig. 4E & F, UTP inhibition persisted in the presence of Tat-PKC$_{20,28}$-IP, though to a lesser extent than in its absence. Again the inhibition of $K_v$ currents by UTP in the presence of this PKC inhibitor remained more pronounced at positive potentials and was similar in extent to UTP inhibition of $K_v$ currents of rat cerebral arteries in the presence of the non-peptide PKC inhibitors calphostin C and bisindolylmaleimide (8).

$K_{\text{ATP}}$ channels are strongly inhibited by UTP in a $\text{Ca}^{2+}$ dependent manner

To measure $K_{\text{ATP}}$ currents the membrane potential was held at -60 mV and external $K^+$ was raised from 6 to 140 mM, setting $E_K$ at 0 mV and resulting in inward $K_{\text{ATP}}$ currents. At this membrane potential $K_v$ and BK currents were virtually absent. Pinacidil (10 µM) was used to activate $K_{\text{ATP}}$ channels further and their identity was confirmed by applying the $K_{\text{ATP}}$ channel blocker glibenclamide (10 µM). Application of UTP (100 µM) following activation by pinacidil caused a marked reduction in $K_{\text{ATP}}$ current when the pipette solution contained 100 nM free $\text{Ca}^{2+}$, but, similar to the case with $K_v$ currents, UTP inhibition was much less and did not reach statistical significance when the pipette contained only 20 nM free $\text{Ca}^{2+}$ (Fig. 5).

PKC inhibition has little effect on UTP modulation of $K_{\text{ATP}}$ currents

To establish the extent of PKC involvement in the UTP induced inhibition of $K_{\text{ATP}}$ currents cells were pre-exposed to 100 nM Tat-PKC$_{20,28}$-IP for at least 10 minutes before recording. Whole-cell recordings were established with 100 nM $\text{Ca}^{2+}$ in the pipette and a comparison of the UTP inhibition of pinacidil activated $K_{\text{ATP}}$ currents between control cells and pre-treated cells was made. No significant difference between the inhibition of $K_{\text{ATP}}$ current by UTP in control cells and in cells pre-treated with Tat-PKC$_{20,28}$-IP ($n=8$) was observed (Fig. 5C).
In the presence of a functional epithelium, endothelial P2Y1 receptor activation results in mesenteric arteriole dilation (12, 13). However, if the endothelial layer is damaged or, as in our experiments, nitric oxide synthesis is suppressed, UTP causes a potent contraction of rat mesenteric artery segments. This is partly...
dependent on Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels, as indicated by the ability of diltiazem to reduce the contraction to about 50% of control values (see Fig. 1C). UTP induced contraction of rat cerebral arteries was also sensitive to diltiazem but to a somewhat lesser degree (8). UTP is known to activate G\(_q\) linked P2Y1, P2Y2, P2Y4 and P2Y6 receptors on vascular smooth muscle which generate PLC dependent Ca\(^{2+}\) signals (6, 7, 14). In our Ca\(^{2+}\) measurements with 0.1 mM external Ca\(^{2+}\), which would support only a small Ca\(^{2+}\) influx, there was still a substantial increase in intracellular Ca\(^{2+}\) in response to UTP which is consistent with Ca\(^{2+}\) release from intracellular stores. Sanchez-Fernandez et al. (15) show that UTP causes Ca\(^{2+}\) mobilization from intracellular stores of culture bovine aortic cells which persisted in the presence of L-type channel blockers or following removal of extracellular Ca\(^{2+}\). Sugihara et al. (16) reported a dual action of UTP on arterial smooth

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**Fig. 5.** Inhibition of K\(_{ATP}\) currents by UTP is not dependent on PKC activation but does require intracellular Ca\(^{2+}\). (A and B) Representative K\(_{ATP}\) current traces obtained at -60 mV in symmetrical 140 mM K\(^{+}\) following the application of pinacidil (10 \(\mu\)M), UTP (100 \(\mu\)M) and glibenclamide (10 \(\mu\)M) as indicated in the presence of 20 nM (A) or 100 nM (B) free Ca\(^{2+}\) in the patch pipette. The arrow indicates the change from 6 to 140 mM external K\(^{+}\). (C) Mean K\(_{ATP}\) current, normalized to that in the presence of pinacidil, under the conditions indicated. Note that blocking PKC by pre-treatment for over 10 minutes with Tat-PKC\(_{20-28}\)-IP (100 nM) had little effect on the inhibition of the current by UTP. (*\(P<0.05\), two-way ANOVA, \(n=8\)).
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muscle with contributions from both P2X and P2Y receptor signalling. These authors show that Ca\(^{2+}\) entry through L-type channels mediate a phasic contraction while Ca\(^{2+}\) release from endoplasmic reticulum caused tonic contraction of rat aortic rings (16).

We have shown previously that UTP causes mobilization of PKCa, \(\delta\) and \(\epsilon\) in these cells (9). However, examination of the PKC dependence revealed that both contraction and the increased Ca\(^{2+}\) in response to UTP were relatively insensitive to Tat-linked membrane permeable peptide inhibitors of PKC (see Fig. 2D & 3C). The general PKC inhibitor peptide, Tat-PKC\(_{20,28}\)-IP, was the only one to cause a small but significant decrease (17\%) in the contraction; isoform specific inhibitors were without effect, although the ET-1 contraction was strongly inhibited by Tat-PKCa-IP as we have reported previously (4).

Many vasoconstrictors reduce smooth muscle K\(^+\) currents causing depolarization and activation of L-type Ca\(^{2+}\) channels which increases contraction. UTP has been shown to inhibit K\(_{V}\) channels of rat cerebral arteries (8) and K\(_{ATP}\) channels in rat coronary arteries (17). We also found that UTP reduced K\(_{V}\) currents and K\(_{ATP}\) currents of rat mesenteric arteries when the pipette contained 100 nM free Ca\(^{2+}\) (Fig. 4C & D). Of note, however, was that lowering pipette [Ca\(^{2+}\)] from 100 to 20 nM abolished UTP inhibition of both currents (Fig. 4A & B). This is unlikely to result from BK channel inhibition at the higher Ca\(^{2+}\) level as these experiments were done in the presence of the BK channel blocker penitrem A. Furthermore, no difference in current density between cells recorded with 20 or 100 nM Ca\(^{2+}\) in the pipette was observed, indicating that BK current was absent under our recording conditions. In rat cerebral artery UTP still reduced K\(_{V}\) currents in the presence of the PKC inhibitor bisindolylmaleimide, but the reduction was not to the same degree as in its absence (8); we found a similar effect with the more specific peptide PKC inhibitor Tat-PKC\(_{20,28}\)-IP on UTP reduction of mesenteric artery smooth muscle K\(_{V}\) currents (Fig. 4E & F). The inhibition of mesenteric artery K\(_{ATP}\) currents by UTP was considerable, with 100 \(\mu\)M UTP leading to an 85\% reduction in K\(_{ATP}\) current; as was the case with K\(_{V}\) currents, lowering pipette free [Ca\(^{2+}\)] to 20 nM virtually abolished the effect of UTP. The inhibition of K\(_{ATP}\) currents by UTP persisted in cells pre-treated with Tat-PKC\(_{20,28}\)-IP (Fig. 5). It is known that activation of PLC is enhanced by Ca\(^{2+}\) (18), and recently Jones \textit{et al} (19) have shown that Ca\(^{2+}\) entry through P2X receptors can enhance ADP responses acting through P2Y receptors in platelets, possibly by a mechanism that involves enhanced PLC activation. Although the mechanism whereby intracellular Ca\(^{2+}\) appears necessary for UTP signalling to K\(^+\) channels in our experiments is unclear, a reduced PLC activation in experiments with low (20 nM) intracellular Ca\(^{2+}\) is certainly plausible.

We have shown that DiC8, an analogue of DAG, is an effective blocker of K\(_{V}\) and to a lesser extent K\(_{ATP}\) currents in these cells (11). This raises the possibility that DAG produced following PLC activation may contribute to the UTP induced reduction of both K\(_{V}\) and K\(_{ATP}\) currents; it should be noted, however, that another DAG analogue (OAG), did not inhibit K\(_{V}\) currents (11). Furthermore, Luykenaar \textit{et al} (8) have shown that in rat cerebral artery smooth muscle cells the Rho kinase inhibitor Y27632 abolished the inhibition of K\(_{V}\) currents by UTP.

In conclusion, the contraction of rat mesenteric arteries by UTP is rather insensitive to peptide inhibitors of various PKC isoforms. The contraction depends partly on Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels, but Ca\(^{2+}\) release is also important. In addition to initiating contraction, it appears that intracellular Ca\(^{2+}\) is also necessary for UTP signalling, as the reduction in both K\(_{V}\) and K\(_{ATP}\) currents are virtually abolished if free [Ca\(^{2+}\)] is lowered to 20 nM. This raises the interesting possibility that UTP induced vasospasm has a positive feedback component, where raised intracellular Ca\(^{2+}\) increase the ability of UTP to inhibit K\(^+\) currents, thereby leading to increased depolarization and further Ca\(^{2+}\) entry.
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Conflict of interest

The authors declare that they have no conflict of interest.

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