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Original

Ca²⁺ 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 Ca²⁺ 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 Ca²⁺. UTP attenuation of both voltage-gated (K_v) and ATP-dependent (K_{ATP}) K⁺ currents was abolished when intracellular Ca²⁺ 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 Ca²⁺.

Key words: K_v channel, K_{ATP} channel, PKC, UTP, calcium

Introduction

Contraction of arterial smooth muscle results from an increase in internal [Ca²⁺], occurring either by entry through Ca²⁺ permeable channels or by release from intracellular Ca²⁺ stores. Many vasoconstrictors stimulate G_q linked receptors activating phospholipase C leading to generation of DAG, IP₃ and subsequent activation of

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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 PKCα, PKCδ and PKCε (9). Interestingly, the contraction induced by Ang II works almost exclusively through PKCε while ET-1 works through PKCα (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+}.

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_2HPO_4, 0.44 NaH_2PO_4, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 Hepes, 4 glucose and 6 mannitol, adjusted with NaOH to pH 7.4 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_2ATP, 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 $MgCl_2$, 0.1 $CaCl_2$, 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: PKC_{20-28} (N-Myr-FARKGALRQ); 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.

Ca^{2+} imaging

Intracellular Ca^{2+} 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 Ca^{2+} 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 K_v and K_{ATP} 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 Ca^{2+} entry

Under control conditions (6 mM K^+ and 1.8 mM Ca^{2+}) 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
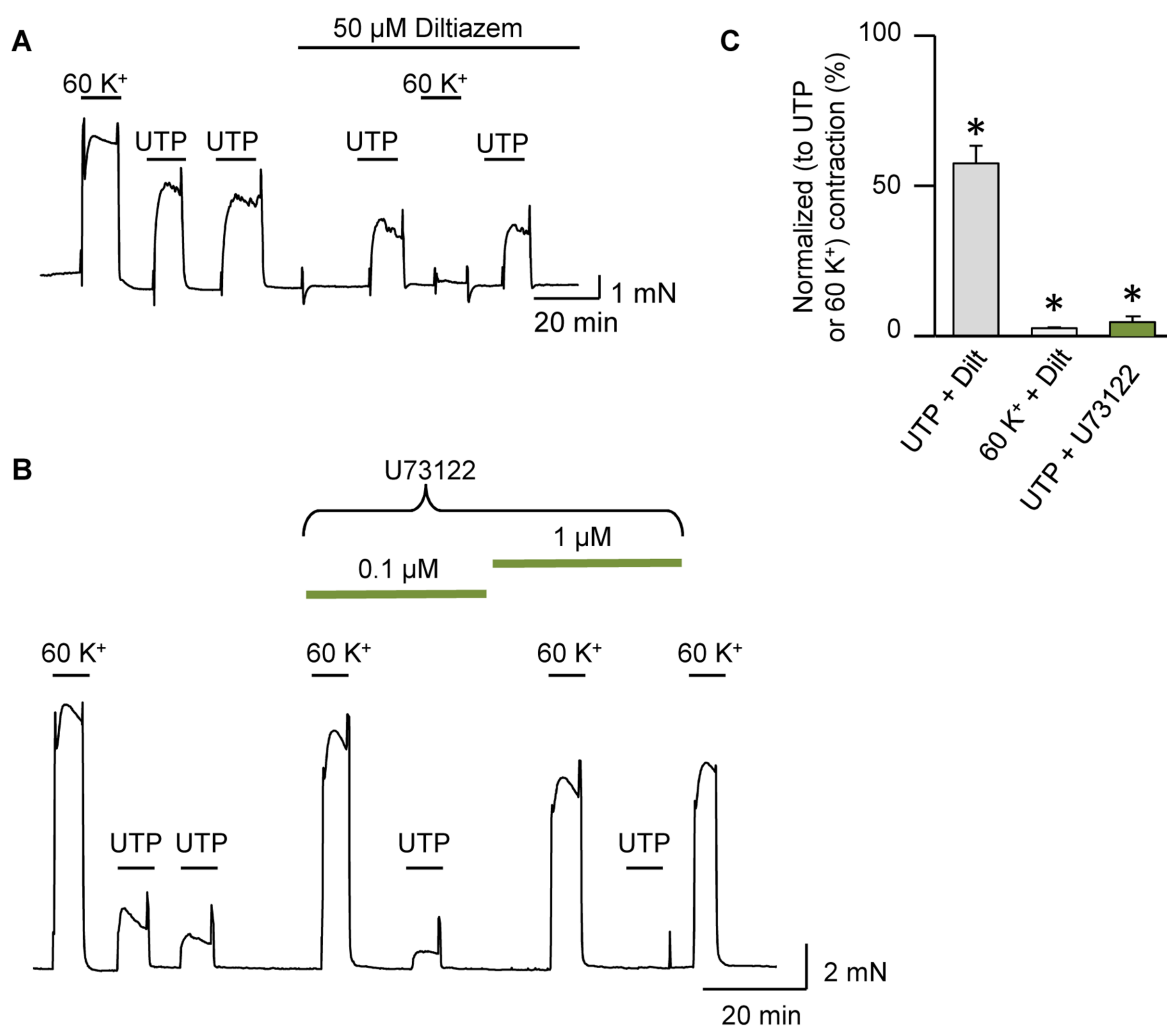
Fig. 1. Contractile responses of rat mesenteric artery. (A) Example trace showing contractile responses to the application of 60 mM K$^+$ or 100 μM UTP in the absence or presence of 50 μM diltiazem as indicated. (B) Example trace showing the effect of U73122 on 100 μM UTP and 60 mM K$^+$ induced contractions. (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 U71322 (*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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}. UTP has been shown to activate P2Y receptors which are G$_q$-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 Ca^{2+} release

Since blocking Ca^{2+} entry through L-type Ca^{2+} channels only partially reduced UTP induced contraction we examined changes in Ca^{2+} induced fluorescence of Fura-2 by UTP in the presence of 0.1 and 1.3 mM

A

B

C

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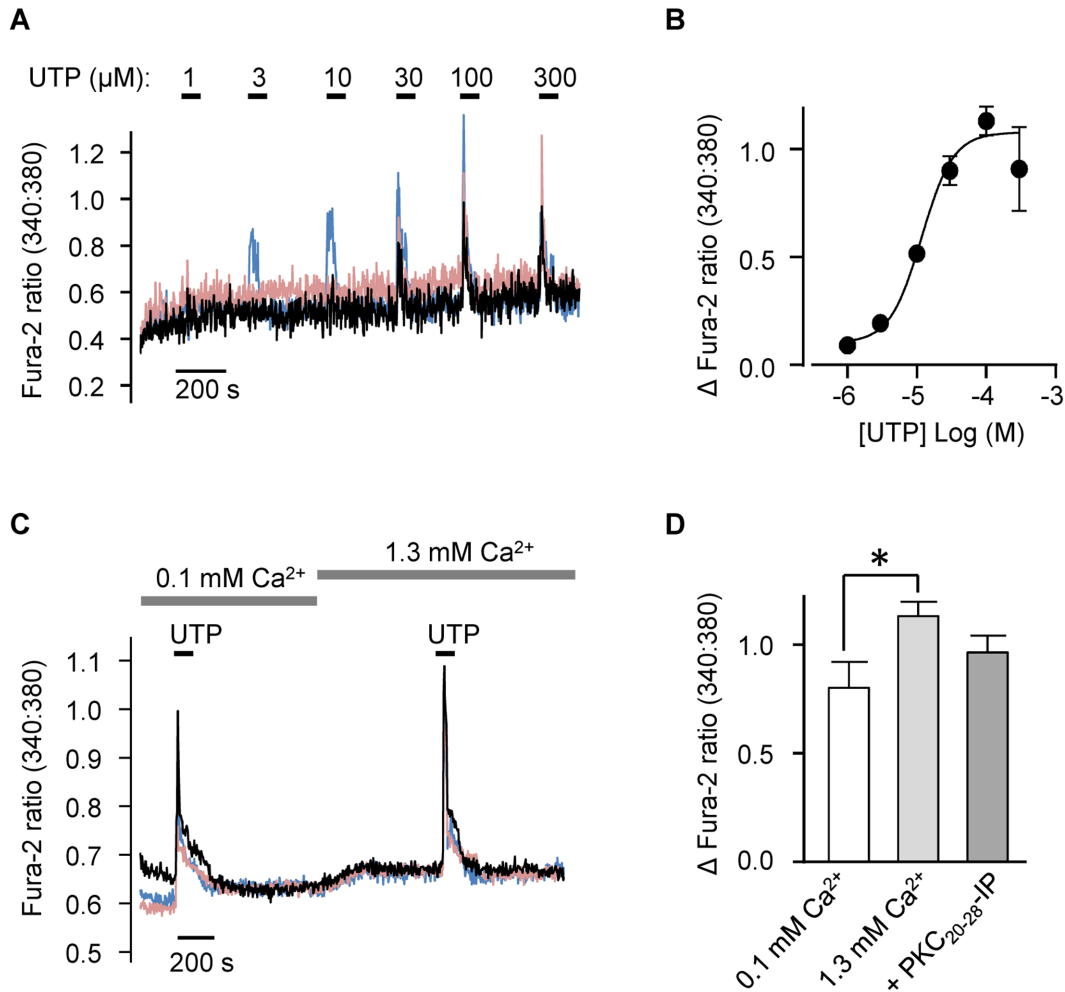
Fig. 2. UTP increases intracellular Ca^{2+} concentration. (A) Overlay of the Fura-2 ratios recorded from 3 cells (different colours) in response to 45 second pulses of UTP at the concentrations (in μM) indicated in the presence of 1.3 mM external Ca^{2+}. (B) Concentration response plot of the change in Fura-2 ratio. Data points are the mean ± s.e.m. of between 65 and 185 cells measured from 3 different preparations, all in 1.3 mM external Ca^{2+}. (C) Overlay of the Fura-2 ratios recorded from 3 cells in response to 45 second pulses of UTP (100 μM) in the presence of 0.1 or 1.3 mM external Ca^{2+} as indicated. (D) Mean changes in Fura-2 ratio in response to 100 μM UTP measured in cells bathed in 0.1 mM Ca^{2+}, 1.3 mM Ca^{2+} or 1.3 mM Ca^{2+} following pre-treatment for 15 minutes with Tat-PKC_{20-28}-IP (100 nM) as indicated. (*$P<0.05$, one-way ANOVA.)

external Ca^{2+}. 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 suggests 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 PKCα, PKCδ and PKCε in mesenteric smooth muscle cells (9). We investigated the extent of various PKC isoform involvement in UTP

A

B

C
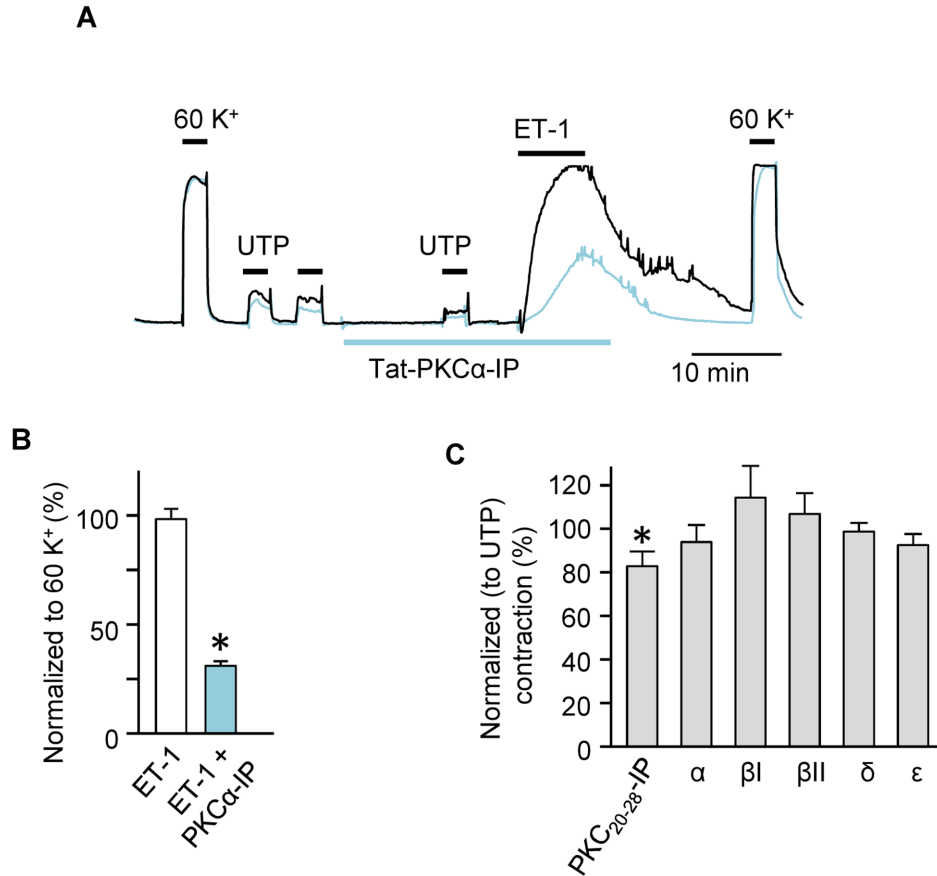
Fig. 3. Effect of PKC inhibitory peptides on ET-1 and UTP induced contractions. (A) Overlaid traces showing contractile responses of two arterial segments to 60 mM K$^+$, 100 µM UTP and 3 nM ET-1. The segment corresponding to the light blue trace was exposed to Tat-PKCα-IP (100 nM) for the duration indicated; for clarity the traces have been scaled to their respective initial responses to 60 mM K$^+$. (B) Mean contractions, normalized to those obtained with 60 K$^+$, to 3 nM ET-1 in the absence and presence of 100 nM Tat-PKCα-IP. (C) Mean UTP (100 µM) induced contractions, normalized to control, in the presence of the Tat-linked peptide PKC inhibitors indicated (100 nM, except Tat-PKCδ-IP at 50 nM). (*$P<0.05$, two-way ANOVA or *t*-test as appropriate.)

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-PKC$_{20-28}$-IP produced a small but significant inhibition of UTP induced contractions (decreased to $82.8 \pm 6.8\%$, $P<0.05$, $n=7$). We found that the UTP induced increase in Fura-2 ratio (with 1.3 mM Ca^{2+}) was also reduced slightly, but not significantly, by pre-treatment with Tat-PKC$_{20-28}$-IP (Fig. 2D).

UTP reduces Kv current in a Ca^{2+}-dependent manner

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 ± 0.7 pF and the average current recorded was 290 ± 52 pA at +60 mV. Kv current densities measured at +60 mV under control conditions (100 nM penitrem A) were 18.7 ± 2.7 ($n=9$) and 21.9 ± 4.8 ($n=10$) pA pF^{-1} where the pipette solution contained 20 and 100 nM free 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 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 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_{ATP} channels are strongly inhibited by UTP in a Ca^{2+} dependent manner

To measure K_{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_{ATP} currents. At this membrane potential K_v and BK currents were virtually absent. Pinacidil (10 µM) was used to activate K_{ATP} channels further and their identity was confirmed by applying the K_{ATP} channel blocker glibenclamide (10 µM). Application of UTP (100 µM) following activation by pinacidil caused a marked reduction in K_{ATP} current when the pipette solution contained 100 nM free 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 Ca^{2+} (Fig. 5).

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

To establish the extent of PKC involvement in the UTP induced inhibition of K_{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 Ca^{2+} in the pipette and a comparison of the UTP inhibition of pinacidil activated K_{ATP} currents between control cells and pre-treated cells was made. No significant difference between the inhibition of K_{ATP} current by UTP in control cells and in cells pre-treated with Tat-PKC$_{20-28}$-IP ($n=8$) was observed (Fig. 5C).
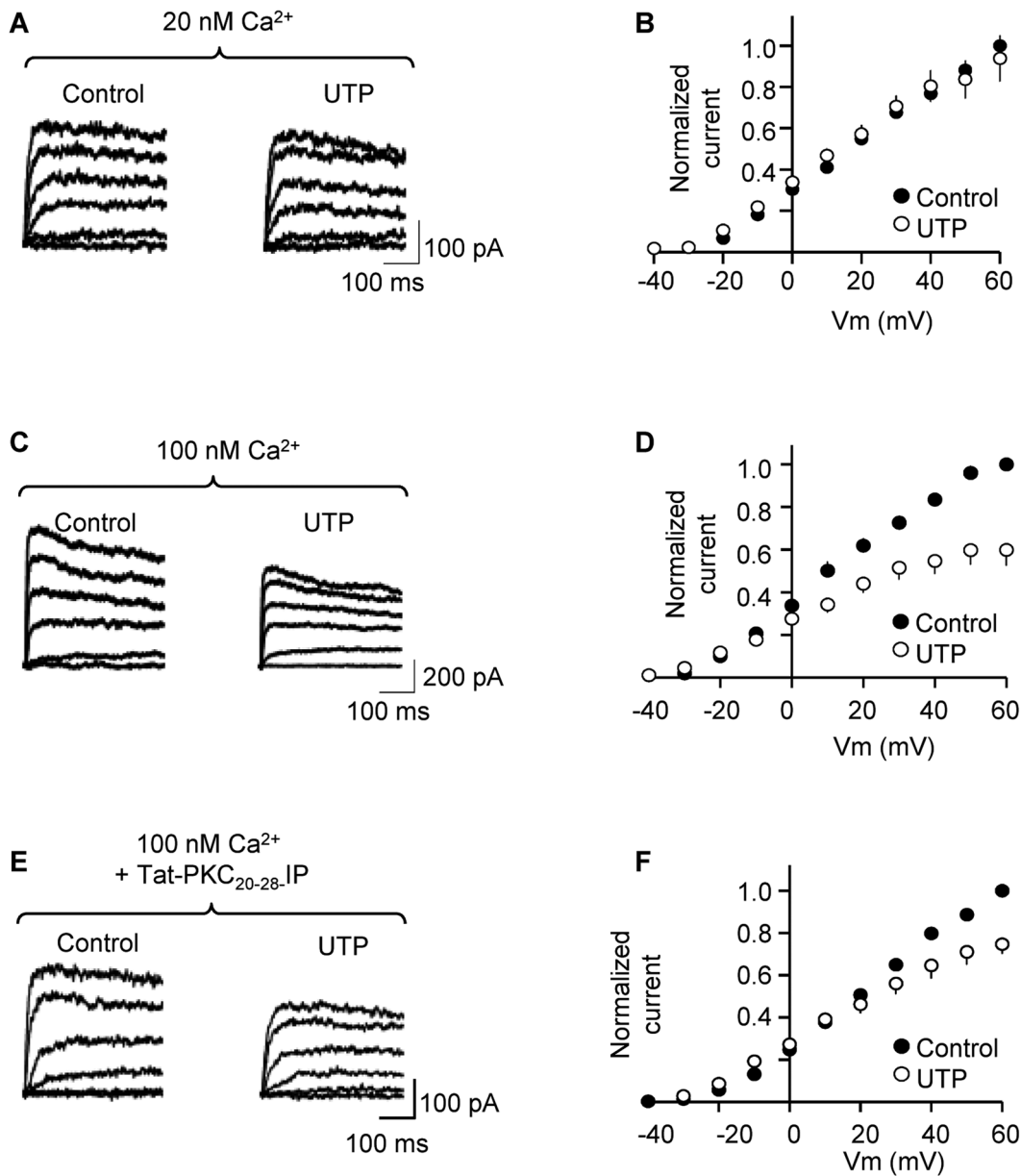
Fig. 4. Inhibition of K_v current by UTP requires intracellular Ca^{2+}. (A) Examples of whole-cell currents under control conditions and 9 minutes after applying UTP (100 μM). These currents were recorded with 20 nM free Ca^{2+} in the patch pipette. (B) Mean I-V plots, normalized to control current at +60 mV, under the conditions of part A (*n*=4). (C) Examples of whole-cell currents under control conditions and 9 minutes after applying UTP (100 μM) in a cell where the pipette contained 100 nM free Ca^{2+}. (D) Mean I-V plots, normalized to control current at +60 mV, under the conditions of part C (*n*=9). (E) Examples of whole-cell currents under control conditions and 9 minutes after applying UTP (100 μM). These currents were recorded from a cell pre-treated for over 10 minutes with Tat-PKC$_{20-28}$-IP (100 nM) and with 100 nM free Ca^{2+} in the patch pipette. (F) Mean I-V plots, normalized to control current at +60 mV, under the conditions of part E (*n*=7).

Discussion

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 supressed, UTP causes a potent contraction of rat mesenteric artery segments. This is partly
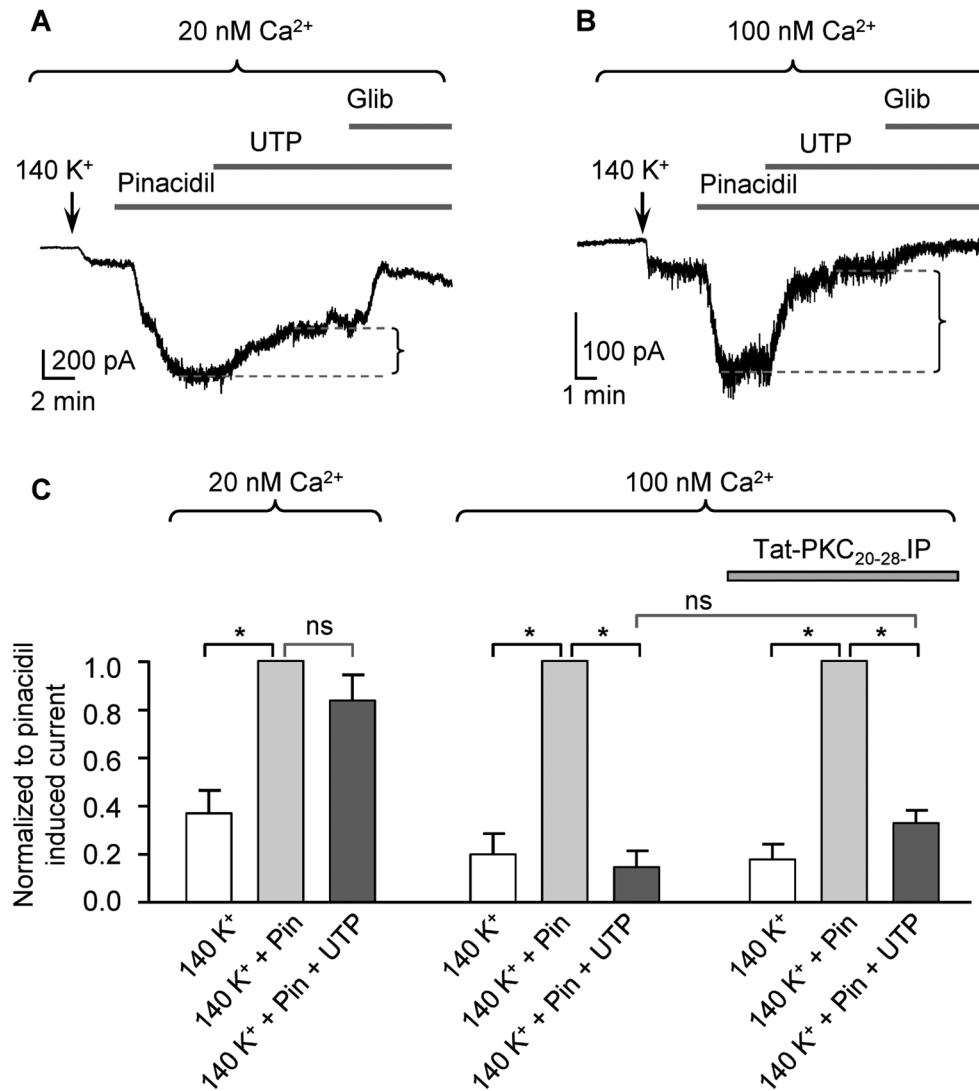
Fig. 5. Inhibition of K_{ATP} currents by UTP is not dependent on PKC activation but does require intracellular Ca^{2+}. (A and B) Representative K_{ATP} current traces obtained at -60 mV in symmetrical 140 mM K^+ following the application of pinacidil (10 μM), UTP (100 μM) and glibenclamide (10 μM) 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).

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

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 PKCα, δ and ε 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-PKCα-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 μM 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 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 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.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Standen NB, Quayle JM. K$^+$ channel modulation in arterial smooth muscle. Acta Physiol Scand. 1998; 164(4): 549–57.

2. Massett MP, Ungvari Z, Csiszar A, Kaley G, Koller A. Different roles of PKC and MAP kinases in arteriolar constrictions to pressure and agonists. Am J Physiol Heart Circ Physiol. 2002; 283(6): H2282–7.

3. McNair LL, Salamanca DA, Khalil RA. Endothelin-1 promotes Ca^{2+} antagonist-insensitive coronary smooth muscle contraction via activation of epsilon-protein kinase C. Hypertension. 2004; 43(4): 897–904.

4. Rainbow RD, Norman RI, Everitt DE, Brignell JL, Davies NW, Standen NB. Endothelin-I and angiotensin II inhibit arterial voltage-gated K$^+$ channels through different protein kinase C isoenzymes. Cardiovasc Res. 2009; 83(3): 493–500.

5. Lazarowski ER, Homolya L, Boucher RC, Harden TK. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. J Biol Chem. 1997; 272(39): 24348–54.

6. Gitterman DP, Evans RJ. Properties of P2X and P2Y receptors are dependent on artery diameter in the rat mesenteric bed. Br J Pharmacol. 2000; 131(8): 1561–8.

7. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006; 58(3): 281–341.

8. Luykenaar KD, Brett SE, Wu BN, Wiehler WB, Welsh DG. Pyrimidine nucleotides suppress K$_{DR}$ currents and depolarize rat cerebral arteries by activating Rho kinase. Am J Physiol Heart Circ Physiol. 2004; 286(3): H1088–100.

9. Nelson CP, Willets JM, Davies NW, Challiss RA, Standen NB. Visualizing the temporal effects of vasoconstrictors on PKC translocation and Ca^{2+} signaling in single resistance arterial smooth muscle cells. Am J Physiol Cell Physiol. 2008; 295(6): C1590–601.

10. Rainbow RD, Hardy ME, Standen NB, Davies NW. Glucose reduces endothelin inhibition of voltage-gated potassium channels in rat arterial smooth muscle cells. J Physiol. 2006; 575(Pt 3): 833–44.

11. Rainbow RD, Parker A, Davies NW. Protein kinase C-independent inhibition of arterial smooth muscle K$^+$ channels by a diacylglycerol analogue. Br J Pharmacol. 2011; 163(4): 845–56.

12. Rodriguez-Rodriguez R, Yarova P, Winter P, Dora KA. Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries. Br J Pharmacol. 2009; 158(6): 1609–20.

13. Winter P, Dora KA. Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. J Physiol. 2007; 582(Pt 1): 335–47.

14. Govindan S, Taylor EJ, Taylor CW. Ca^{2+} signalling by P2Y receptors in cultured rat aortic smooth muscle cells. Br J Pharmacol. 2010; 160(8): 1953–62.

15. Sanchez-Fernandez M, Katz GM, Suarez-Kurtz G, Kaczorowski GJ, Reuben JP. Mobilization of intracellular calcium in cultured vascular smooth muscle cells by uridine triphosphate and the calcium ionophore A23187. J Membr Biol. 1993; 135(3): 273–87.

16. Sugihara M, Morita H, Matsuda M, Umebayashi H, Kajioka S, Ito S, Nishida M, Inoue R, Futatsuki T, Yamazaki J, Mori Y, Inoue R, Ito Y, Abe K, Hirata M. Dual signaling pathways of arterial constriction by extracellular uridine 5'-triphosphate in the rat. J Pharmacol Sci. 2011; 115(3): 293–308.

17. Welsh DG, Brayden JE. Mechanisms of coronary artery depolarization by uridine triphosphate. Am J Physiol Heart Circ Physiol. 2001; 280(6): H2545–53.

18. Eberhard DA, Holz RW. Intracellular Ca^{2+} activates phospholipase C. Trends Neurosci. 1988; 11(12): 517–20.

19. Jones S, Evans RJ, Mahaut-Smith MP. Ca^{2+} influx through P2X1 receptors amplifies P2Y1 receptor-evoked Ca^{2+} signaling and ADP-evoked platelet aggregation. Mol Pharmacol. 2014; 86(3): 243–51.