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Small-Molecule G Protein–Coupled Receptor Kinase Inhibitors Attenuate G Protein–Coupled Receptor Kinase 2–Mediated Desensitization of Vasoconstrictor-Induced Arterial Contractions [S]

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ABSTRACT

Vasoconstrictor-driven G protein–coupled receptor (GPCR)/phospholipase C (PLC) signaling increases intracellular Ca^{2+} concentration to mediate arterial contraction. To counteract vasoconstrictor-induced contraction, GPCR/PLC signaling can be desensitized by G protein–coupled receptor kinases (GRKs), with GRK2 playing a predominant role in isolated arterial smooth muscle cells. In this study, we use an array of GRK2 inhibitors to assess their effects on the desensitization of UTP and angiotensin II (AngII)–mediated arterial contractions. The effects of GRK2 inhibitors on the desensitization of UTP- or AngII-stimulated mesenteric third-order arterial contractions, and PLC activity in isolated mesenteric smooth muscle cells (MSMC), were determined using wire myography and Ca^{2+} imaging, respectively. Applying a stimulation protocol to cause receptor desensitization resulted in reductions in UTP- and AngII-stimulated arterial contractions. Preincubation with the GRK2 inhibitor paroxetine almost completely prevented desensitization of UTP- and attenuated desensitization of AngII-stimulated arterial contractions. In contrast, fluoxetine was ineffective. Preincubation with alternative GRK2 inhibitors (Takeda compound 101 or CCG224063) also attenuated the desensitization of UTP-mediated arterial contractile responses. In isolated MSMC, paroxetine, Takeda compound 101, and CCG224063 also attenuated the desensitization of UTP- and AngII-stimulated increases in Ca^{2+}, whereas fluoxetine did not. In human uterine smooth muscle cells, paroxetine reversed GRK2-mediated histamine H_1 receptor desensitization, but not GRK6-mediated oxytocin receptor desensitization. Utilizing various small-molecule GRK2 inhibitors, we confirm that GRK2 plays a central role in regulating vasoconstrictor-mediated arterial tone, highlighting a potentially novel strategy for blood pressure regulation through targeting GRK2 function.

Introduction

Arterial contractile tone is mediated by a plethora of different inputs, which ultimately regulate the level of intracellular Ca^{2+} and hence the degree of muscle contraction (Hill-Eubanks et al., 2011). The majority of vasoactive agents interact with their cognate G protein–coupled receptors (GPCRs) to promote either smooth muscle contraction or relaxation. Vasodilatory ligands interact with G_s-coupled GPCRs to promote cyclic AMP generation and to activate K^+ channels to promote relaxation, whereas vasoconstrictors interact with G_q-coupled receptors to promote inositol 1,4,5-trisphosphate (IP_3) generation and liberate Ca^{2+} from sarcoplasmic reticular stores and/or to promote opening of plasma membrane Ca^{2+} channels (Brinks and Eckhart, 2010). Vascular tone is highly dependent on smooth muscle cell membrane potential, in which depolarizing stimuli trigger an increase in the voltage-gated Ca^{2+}-window current, resulting in an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and thus vasoconstriction (Nelson et al., 1990). The increase in $[Ca^{2+}]_i$ via depolarization-induced Ca^{2+} influx is antagonized by a variety of K^+ channels, which hyperpolarize the membrane and reduce Ca^{2+} influx. It is well established that the activity of these channels is highly regulated by GPCR signaling, with

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ABBREVIATIONS: AngII, angiotensin II; ANOVA, analysis of variance; AT1, AngII type 1; $[Ca^{2+}]_i$, intracellular Ca^{2+}; eGFP, enhanced green fluorescent protein; GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase; IP_3, inositol 1,4,5-trisphosphate; MSMC, mesenteric smooth muscle cell; $P2Y_2$, purinergic; PH, pleckstrin homology; PKA, protein kinase A; PLC, phospholipase C; SSRI, selective serotonin reuptake inhibitor; VOCC, voltage-operated Ca^{2+} channel.

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vasodilators generally increasing K^+ and inhibiting Ca^{2+} channel activities, and vasoconstrictors inhibiting K^+ and increasing Ca^{2+} influx (Nelson et al., 1990; Hill-Eubanks et al., 2011).

Dysregulation of the balance in vasodilator and vasoconstrictor signaling can lead to changes in vascular tone (Hill-Eubanks et al., 2011). Therefore, understanding how GPCRs that modulate vascular tone are themselves regulated is vital to understanding how vessel tone is maintained and varied. Activation of GPCRs not only initiates intracellular signaling pathways, but also concurrently recruits G protein–coupled receptor kinases (GRKs) to agonist-bound receptors. Once bound to receptor, GRKs phosphorylate serine and/or threonine residues within the third intracellular loop and/or C-terminal tail of a GPCR, bringing about receptor desensitization and terminating G protein signaling through recruitment of an arrestin protein, which sterically excludes further interaction between GPCR and G protein (Pitcher et al., 1998). The GRK family has seven members (Pitcher et al., 1998), of which two are exclusively expressed in rod and cone cells (GRK1 and 7), one has limited expression in the kidney and testes (GRK4), and four are ubiquitously expressed (GRK2, 3, 5, and 6) (Willets et al., 2003), including in vascular smooth muscle (Cohn et al., 2008; Morris et al., 2010).

We have previously shown, in smooth muscle cells isolated from third-order rat mesenteric arteries, that GRK2 is the key regulator of phospholipase C (PLC)/Ca^{2+} signaling induced by the vasoconstrictors endothelin and UTP, via the endothelin A and purinergic ($P2Y_2$) receptors (Morris et al., 2010, 2011, 2012). GRK2 is also reported to regulate signaling by two further vasoconstrictors, angiotensin II (AngII) and noradrenaline, through negative regulation of AngII type 1 (AT1) receptor (Oppermann et al., 1996; Kim et al., 2009) and α_{1D}-adrenoceptor (Cohn et al., 2008) signaling. Together, these data highlight the importance of GRK2 in the regulation of vascular contractile GPCR signaling and suggest that GRK2 might also play a vital role in the regulation of vascular tone. Although we have shown that we can induce desensitization of mesenteric vessel contractions to vasoconstrictors (Morris et al., 2011), the absence of small-molecule GRK2 inhibitors has hindered our ability to investigate further the role that GRK2 plays in the regulation of whole vessel tone. However, the report that the selective serotonin reuptake inhibitor (SSRI) paroxetine can inhibit GRK2 function (Thal et al., 2011) prompted us to examine whether this drug can provide a pharmacological means to delineate a role for GRK2 in vasoconstrictor-mediated vessel desensitization. Furthermore, paroxetine has also been shown to enhance β-adrenoceptor–mediated contraction of cardiomyocytes (Thal et al., 2012), a process regulated by GRK2 (Koch et al., 1995; Williams et al., 2004), and to improve cardiac function postmyocardial infarction (Schumacher et al., 2015).

In this study, we examined whether small-molecule GRK2 inhibitors, including paroxetine, can alter the desensitization of vasoconstrictor-induced arterial contractions using both single-cell imaging and wire myography methods.

Materials and Methods

Materials. AngII, UTP, histamine, and oxytocin were from Sigma-Aldrich (Poole, Dorset, UK). Paroxetine, fluoxetine, and compound 101 were from Tocris (Bristol, UK). Fluo4-AM was from Thermo-Fisher Scientific (Loughborough, UK). The CCG compounds were synthesized at the University of Michigan, as described previously (Waldschmidt et al., 2016). All other chemicals were from Sigma-Aldrich.

Isolation and Culture of Mesenteric Arterial Smooth Muscle Cells. Adult male Wistar rats were killed by stunning and cervical dislocation, a method approved under the UK Animals (Scientific Procedures) Act 1986, Amendment Regulations (SI 2012/3039). Smooth muscle cells were isolated from small branches of mesenteric artery by enzymatic dissociation, as previously described (Hayabuchi et al., 2001; Jackson et al., 2016). Following enzymatic digestion, cells were separated by trituration in 231 medium (Cascade Biologics, Nottingham, UK), containing smooth muscle growth supplement, 100 IU penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. For single-cell imaging experiments, cells were plated onto glass coverslips and maintained in a humidified environment at 37°C and 5% CO_2:air.

ULTR Cell Culture. The immortalized human ULTR myometrial cell line was cultured in Dulbecco's minimal essential medium, supplemented with Glutamax-1, 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml). Cells were maintained under humidified conditions at 37°C, in 5% CO_2:air.

Single-Cell Confocal Imaging. After 4 days in culture, mesenteric smooth muscle cells (MSMC) were loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4 μM) for 30 minutes at room temperature. After loading, MSMC were maintained at 37°C by a Peltier unit and continuously perfused with a modified Krebs–Henseleit buffer (in millimolars: NaCl 134, KCl 6, $MgCl^2$ 1, $CaCl_2$ 1.3, glucose 10, HEPES 10, pH 7.4). Real-time images were taken using an Olympus FV500 laser-scanning confocal IX70 inverted microscope (oil immersion objective 60×). Cells were excited at 488 nm, and emissions were collected at 505–560 nm. Agonists were applied via the perfusion line, and changes in cytosolic fluorescence were represented as the fluorescence emission (F)/initial basal fluorescence (F_0) (F/F_0). Human ULTR cells were seeded onto 25-mm glass coverslips, and when 70% confluent, loaded with Fluo4-AM (4 μM) for 30 minutes at room temperature. After loading, cells were subjected to the same protocols as outlined above for MSMC.

To measure cellular changes in IP_3, cells were transfected with (0.5 μg) of the previously characterized IP_3 biosensor enhanced green fluorescent protein (eGFP)-pleckstrin homology (PH)-$PLC_{\delta 1}$ (the PH domain of $PLC_{\delta 1}$) (Morris et al., 2010, 2011; Willets et al., 2015a,b), using Lipofectamine2000, according to the manufacturer's instructions. After 48 hours, cells were imaged by excitation at 488 nm and emissions were collected at 505–560 nm. Changes in cytosolic eGFP-PH-$PLC_{\delta 1}$ fluorescence are represented as the fluorescence emission (F)/initial basal fluorescence (F_0) (F/F_0).

Myography. Contractile force recordings were made from 1.4-mm ring segments of third-order mesenteric arteries mounted in a Mulvany–Halpern 610 M wire myograph (DMT, Aarhus, Denmark). The bath solution contained (in millimolars): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 5, mannitol 5, HEPES 10, pH 7.4. NaCl was reduced to 81 mM and replaced with 60 mM K^+ for the high K^+ solution additions (see *Results*). All bathing solutions contained L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) (20 μM) to prevent endogenous nitric oxide generation. Solutions and drugs were added directly to the organ bath, maintained at 37°C.

Data and Statistical Analysis. Data presented are from a minimum of three different cell preparations, each obtained from a different rat. Data are expressed as means ± S.D. Parametric data were analyzed using one-way or two-way analysis of variance (ANOVA), as indicated, with appropriate post hoc testing, and outlined in the corresponding figure legends (Prism v7.04; GraphPad, San Diego, CA). Where data were normalized, nonparametric ANOVA analysis using Kruskal–Wallis, Dunn's post hoc test was applied. In all cases, post hoc tests were only applied when initial ANOVA testing revealed a significant ($P < 0.05$) result.

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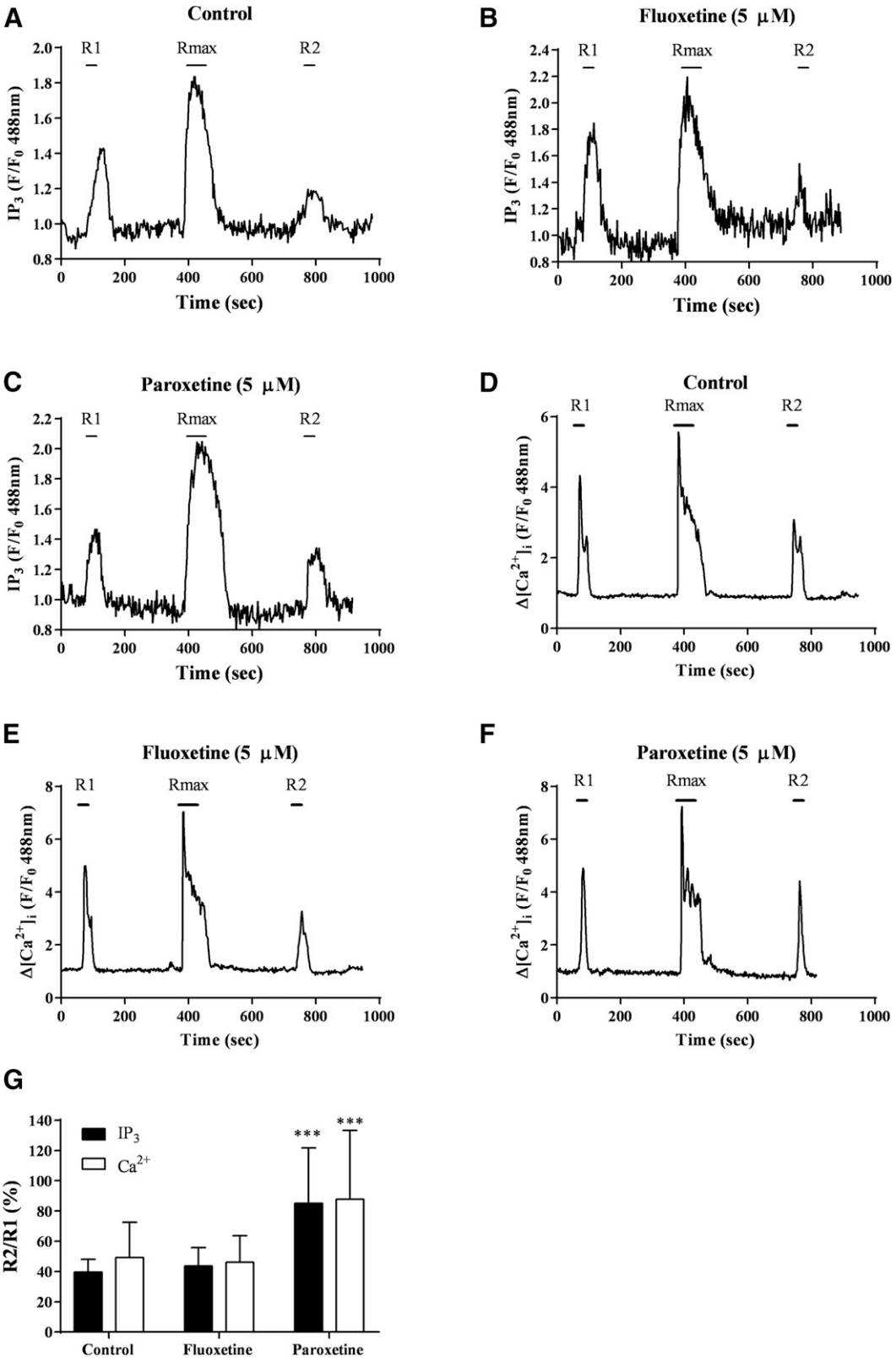

Fig. 1. Paroxetine inhibits desensitization of the $P2Y_2$ receptor in rat MSMC. For IP_3 measurements, cells were transfected with eGFP-PH (as described in *Materials and Methods*). After 48 hours, cells were preincubated with paroxetine or fluoxetine (5 μM, 30 minutes) before being subjected to the following desensitization protocol: cells were challenged with UTP (R1, 10 μM, 30 seconds) for 5 minutes before a high, desensitizing UTP concentration (R_{max} = 100 μM for 60 seconds), and again after a 5-minute washout period (R2, 10 μM, 30 seconds). For Ca^{2+} experiments, cells were loaded with Fluo4-AM (4 μM, 30 minutes) and subjected to a similar desensitization protocol with 1 μM UTP used for R1 and R2 challenges. Representative traces from single cells show the effects of preaddition (30 minutes) of vehicle-control (A and D), or 5 μM fluoxetine (B and E) or paroxetine (C and F) on $P2Y_2$ receptor-stimulated IP_3 and Ca^{2+} signals, respectively. $P2Y_2$ receptor desensitization was determined as the relative (%) change in R2 response compared

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Results

Paroxetine, but Not Fluoxetine, Attenuates $P2Y_2$ Receptor Desensitization. We have previously shown that in isolated rat MSMC UTP-stimulated PLC/Ca^{2+} signaling is mediated by the $P2Y_2$ receptor and that GRK2 is the key kinase that induces receptor desensitization (Morris et al., 2011). Therefore, we examined whether the GRK2 inhibitor paroxetine could prevent UTP-stimulated $P2Y_2$ receptor desensitization in isolated MSMC. A previously characterized desensitization protocol was used (Morris et al., 2011), in which cells are challenged with an approximate EC_{50} concentration of UTP (1 μM for 30 seconds; termed R1) 5 minutes before application of a maximal concentration of UTP (100 μM, termed R_{max}) to induce receptor desensitization. A second EC_{50} concentration of UTP (1 μM, termed R2) was applied 5 minutes after R_{max}. Comparison of the R2 and R1 responses in vehicle-treated MSMC showed a reduction in R2 compared with R1 of approximately 50%, indicative of receptor desensitization (Fig. 1, A and D), which is comparable with our previous findings (Morris et al., 2011). Pretreatment with the SSRI paroxetine (5 μM; 30 minutes) attenuated the reduction in R2 compared with R1, indicating that this agent could largely prevent $P2Y_2$ receptor desensitization (Fig. 1, C and F). Contrastingly, pretreatment (5 μM; 30 minutes) with the structurally distinct SSRI, fluoxetine, did not modify the UTP-induced desensitization (Fig. 1, B and E), indicating that effect of paroxetine is unrelated to its SSRI activity.

Paroxetine Inhibits GRK2 but Not GRK6-Mediated GPCR Desensitization. As GRK2 appears to play a key role in the regulation of all the endogenously expressed G_q/PLC-coupled receptors that we have examined in MSMC (Morris et al., 2010, 2011), we switched our focus to an immortalized human smooth muscle cell line (ULTR) that expresses endogenous PLC-coupled receptors that are exclusively regulated by either GRK2 (H_1 histamine) (Willets et al., 2008) or GRK6 (oxytocin) (Willets et al., 2009). To study H_1 histamine receptor desensitization, we applied a similar R1/R_{max}/R2 protocol to that used above for the $P2Y_2$ receptor; however, in this case, ULTR cells were transfected with the IP_3 biosensor (eGFP-tagged PH domain of $PLC_{\delta 1}$; 0.5 μg, for 48 hours) (Willets et al., 2008, 2009; Morris et al., 2010, 2011). We assessed receptor/PLC activity by assessing IP_3 biosensor translocation, rather than intracellular Ca^{2+} changes; nevertheless, we have previously shown that both outputs are valid and essentially interchangeable readouts of GPCR/PLC activity and receptor desensitization (Willets et al., 2008, 2009; Morris et al., 2010, 2011, 2012). Following a 30-minute preincubation with fluoxetine, the reduction in the R2 relative to R1 was similar to that seen in vehicle-treated cells (R2 expressed as a percentage of R1: vehicle-control, 43.8 ± 6.3, $n = 7$ versus fluoxetine, 47.4 ± 7.9, $n = 5$; mean ± S.E.M.), suggesting that fluoxetine has no effect on H_1 histamine receptor desensitization (Fig. 2, B and G). In contrast, inclusion of paroxetine (30-minute pretreatment) markedly attenuated the reduction in the R2 relative to R1, indicating that paroxetine is able to largely prevent receptor desensitization (Fig. 2, C and G). To induce oxytocin receptor desensitization, we changed the protocol, comparing the responses of two maximal concentrations of oxytocin (R1 and R2) either side of a 5-minute washout period. In this case, neither fluoxetine nor paroxetine altered the observed desensitization of oxytocin receptor–PLC signaling (Fig. 2, D–F), suggesting that these compounds do not inhibit GRK6-mediated receptor desensitization.

Characterization of UTP-Mediated Contractions in Mesenteric Arteries. We have previously characterized UTP-stimulated contraction of third-order mesenteric arteries (Morris et al., 2011). In this work, we found that our concentration–response curves differed slightly from our previous data, with UTP showing slightly reduced potency (Fig. 3A), requiring additions of ≥300 μM UTP to bring about a maximal contraction. In light of these findings, we slightly adjusted our desensitization protocol, applying 100 μM UTP as R1 and R2 additions (and 300 μM UTP as R_{max}; Fig. 3B). We also assessed how the R2 value was affected by the washout time between R_{max} and R2 and observed that a maximal desensitization to UTP was observed with a delay of only 2 minutes (Fig. 3D). Extending the washout period between R_{max} and R2 revealed a time-dependent resensitization of UTP-induced contractions, with the R1/R2 ratio returning to ~1 after 10 minutes (Fig. 3, C and D).

Paroxetine and Fluoxetine Cause a Transient Block of 60 mM K^+-Induced Arterial Contraction. In the absence of SSRIs, K^+ (60 mM) addition induced robust mesenteric artery contraction (Fig. 4). Addition of either fluoxetine or paroxetine prevented contraction on readdition of K^+ (Fig. 4, A and B), likely reflecting a direct inhibition of voltage-operated Ca^{2+} channels (VOCCs) (Stauderman et al., 1992). Interestingly, the SSRI-induced inhibition of depolarization-mediated arterial contraction was transient, as sensitivity to K^+ was restored following a 30-minute washout (Fig. 4C).

SSRIs Do Not Affect Acute Stimulation of GPCR-Stimulated Arterial Contraction. Given that both AngII and UTP act via $G_{q/11}$-coupled receptors, we hypothesized that, despite inhibition of L-type VOCCs in the presence of SSRI, there would still be a substantial IP_3 receptor–dependent increase in intracellular Ca^{2+}, and therefore vasoconstriction. Maximally effective concentrations of AngII (100 nM) and UTP (300 μM) in the presence or absence of fluoxetine or paroxetine were used to test this hypothesis (Fig. 5, A–C). Reassuringly, the contractile responses to vasoconstrictor were indistinguishable in the absence or presence of SSRI (Fig. 5D). Furthermore, inclusion of the L-type VOCC blocker, nifedipine, inhibited K^+-induced mesenteric artery contraction, but did not affect UTP-mediated contractions (Fig. 6). Collectively, these data suggest that AngII and UTP rely primarily on mobilization of intracellular Ca^{2+} stores, rather than VOCC-dependent Ca^{2+} entry, to mediate arterial contractions.

with R1 signals. Cumulative data (G) are presented as means ± S.D. for 8–13 cells for IP_3, and 76–102 cells for Ca^{2+} experiments, respectively, generated from preparations from five different animals. Statistical analysis (one-way ANOVA; Sidak's post hoc test) showed that paroxetine, but not fluoxetine, attenuated receptor $P2Y_2$ receptor desensitization observed as changes in IP_3 or Ca^{2+} responses (***$P < 0.001$).

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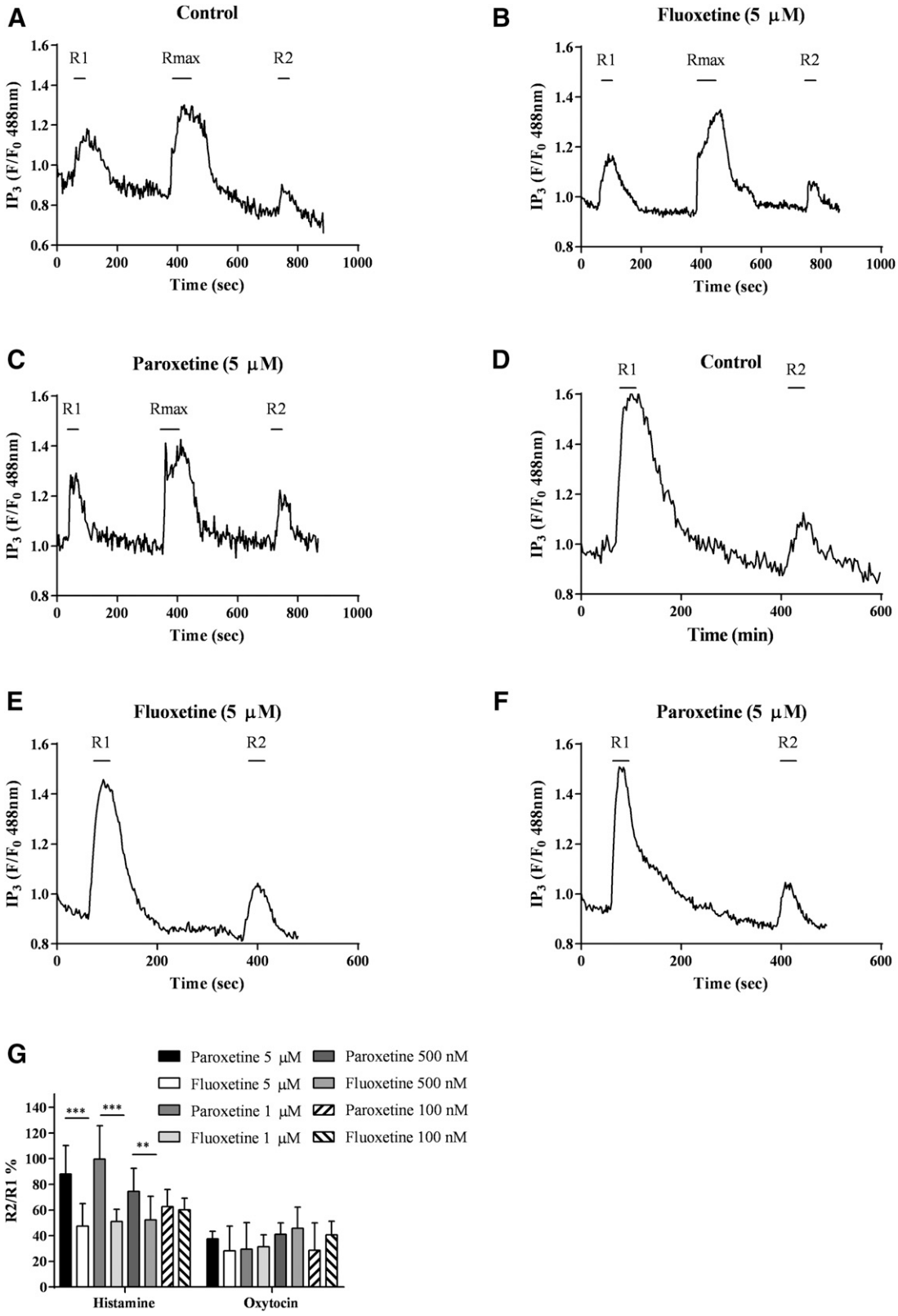

Fig. 2. Paroxetine selectively inhibits desensitization of the H_1 histamine, but not the oxytocin receptor in human ULTR cells. Cells were loaded simultaneously with Fluo4-AM (4 μM), and vehicle, or 5 μM fluoxetine or paroxetine for 30 minutes, before being subjected to a standard desensitization protocol. To assess H_1 receptor-mediated responses (A–C), cells were challenged with histamine (R1, 1 μM, 30 seconds) for 5 minutes before a desensitizing concentration (R_{max} = 100 μM for 60 seconds), and again after a 5-minute washout period (R2, 1 μM, 30 seconds). To determine oxytocin receptor-mediated desensitization (D–F), cells were challenged with a maximal concentration of oxytocin (100 nM; R1) for 30 seconds and washed for 5 minutes before a second 30-second oxytocin (100 nM; R2) challenge. In both cases, receptor desensitization was determined as the relative (%) change in R2 versus R1 signals. Cumulative data (G) show the extent of receptor desensitization (means ± S.D. for 5–19 cells for each treatment group). Statistical analysis (two-way ANOVA; Sidak's post hoc test) shows that paroxetine, but not fluoxetine, attenuates H_1 histamine receptor desensitization (**$P < 0.01$; ***$P < 0.001$), whereas no significant differences were found between treatments with respect to oxytocin receptor desensitization.

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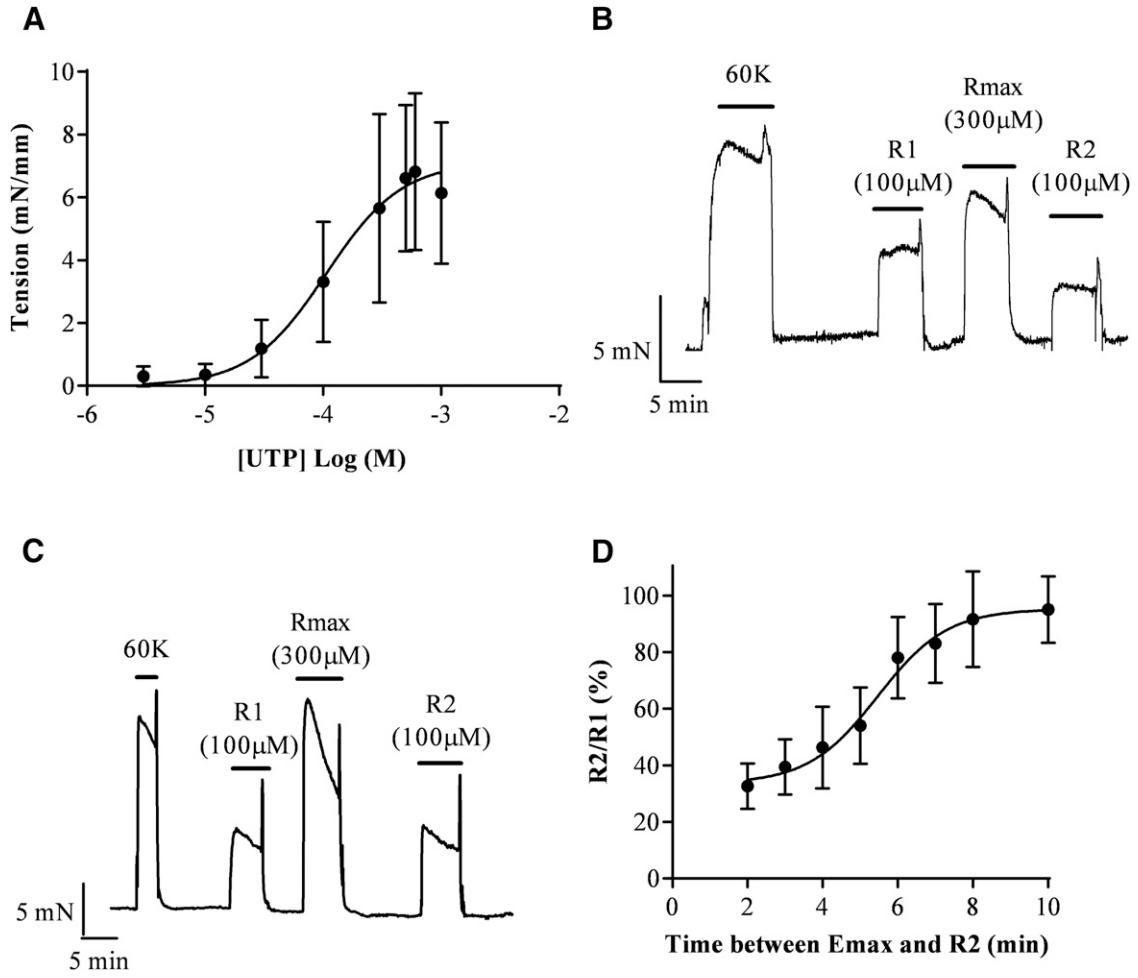

Fig. 3. Desensitization and resensitization kinetics of UTP-stimulated contractions in rat mesenteric artery. Arterial ring preparations were exposed to increasing concentrations of UTP (A), interspersed with 5-minute washout periods between each agonist addition. Data are expressed as means ± S.D. for $n = 35$ vessels from $n \geq 5$ animal preparations to show the concentration–dependency of UTP-induced contractile responses. To examine contractile desensitization/resensitization, arterial rings were exposed to the following protocol: R1 (100 μM UTP for 5 minutes), followed by 5-minute washout, R_{max} (300 μM for 5 minutes), followed by a variable time (2–10 minutes) before R2 (100 μM for 5 minutes). Desensitization was determined as the percentage change in R2 when compared with R1. Representative traces are shown from single arteries with either a 5 (B)- or 10 (C)-minute washout period between R_{max} and R2. The time course of resensitization of the contractile response to UTP (means ± S.D.; arterial preparations from at least seven animals) is also shown (D).

Paroxetine Attenuates the Desensitization of UTP-Stimulated Arterial Contractions. To assess the ability of paroxetine to inhibit the desensitization of UTP-stimulated arterial contractions, mesenteric arterial rings were subjected to the standard R1/R_{max}/R2 desensitization protocol, following a 5-minute preaddition of paroxetine or fluoxetine (5 μM). In the presence of fluoxetine, the reduction in the R2 response relative to R1 was similar to that observed in vehicle-treated arteries (Fig. 7, A and E), indicating that fluoxetine does not affect the desensitization of UTP-induced arterial contractions. Contrastingly, in the presence of paroxetine, the R2 response was comparable to R1, indicating that this SSRI can selectively ablate UTP-induced desensitization (Fig. 7, B and E). In addition, to examine the desensitization of AngII-mediated contractions, vessels were challenged with two maximal AngII (100 nM) additions either side of the UTP desensitization protocol. Comparison of the R1 and R2 responses showed that, in the presence of vehicle or fluoxetine, R2 responses were virtually undetectable, indicating that AngII-stimulated contractions were almost completely desensitized (Fig. 7, C and E). However, although the magnitude of initial (R1) AngII-induced arterial contraction was identical in all treatments, R2 responses were greater following paroxetine pretreatment, suggesting that paroxetine attenuates AngII-stimulated desensitization of arterial contraction (Fig. 7, D and E). These data are similar to AT1 receptor desensitization in isolated MSMC, in which full recovery of AngII Ca^{2+}/PLC signals takes >20 minutes (Supplemental Fig. 1).

To examine the temporal effects of paroxetine on the desensitization of UTP-stimulated arterial contractions, we applied a modified protocol, whereby arteries were pretreated with SSRI (5 μM, for 5 minutes) before vessels were repeatedly (five times) challenged with an EC_{50} concentration of UTP (100 μM) for 5 minutes, interspaced with 5-minute washouts. In the presence of fluoxetine, UTP-induced contractions gradually waned over the time course of the experiment, and a time-dependent desensitization of contractile responses was indicated (Fig. 7, G and H). In the presence of paroxetine, UTP-mediated contractions were

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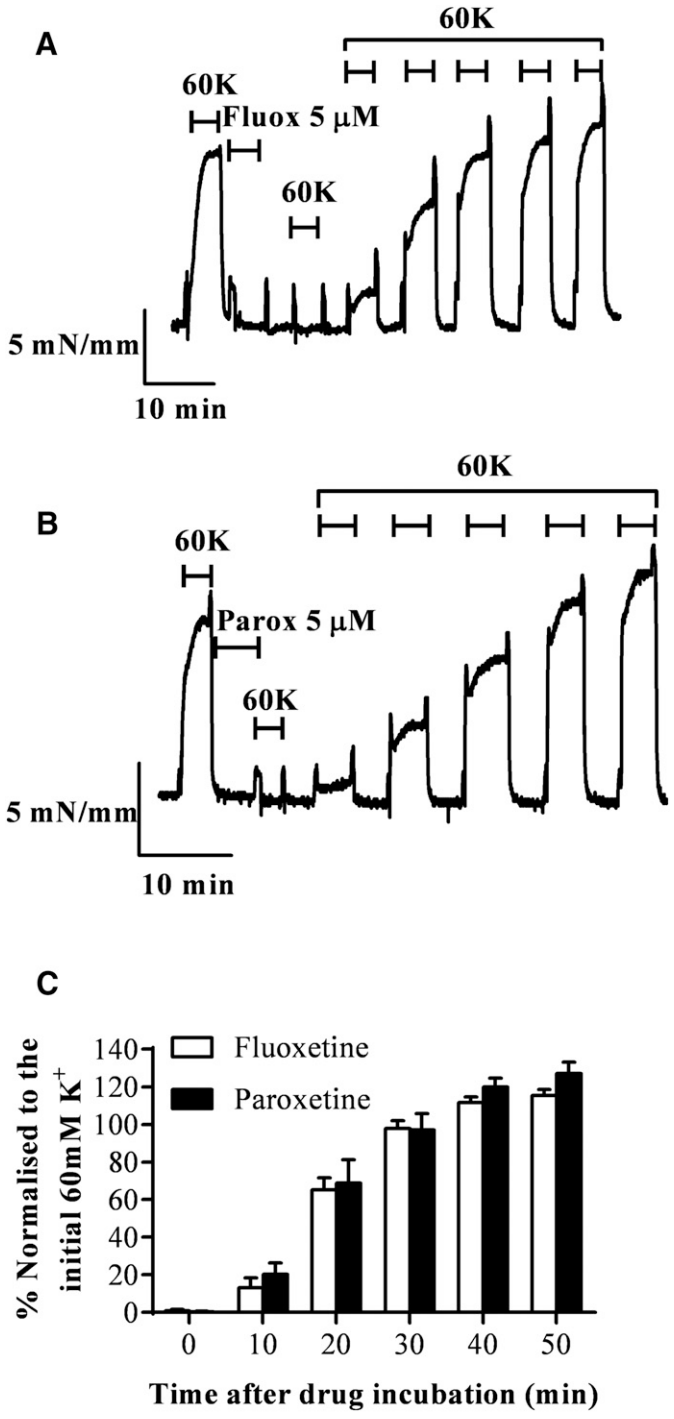

Fig. 4. SSRIs induce a temporary block of K^+-induced arterial contraction. Arterial rings were contracted with K^+ (60 mM) and washed for 5 minutes before incubation with either fluoxetine or paroxetine (5 μM) for an additional 5 minutes. Arteries were then repeatedly challenged with K^+ (60 mM), with 5-minute washouts between exposures. Representative traces show the effects of 5 μM fluoxetine (A) or 5 μM paroxetine (B) on K^+-induced contractions. (C) Cumulative data (means ± S.D.; for n = 3 to 4 arteries from ≥3 animal preparations) show the temporal effects of SSRI treatment on K^+-induced arterial contraction.

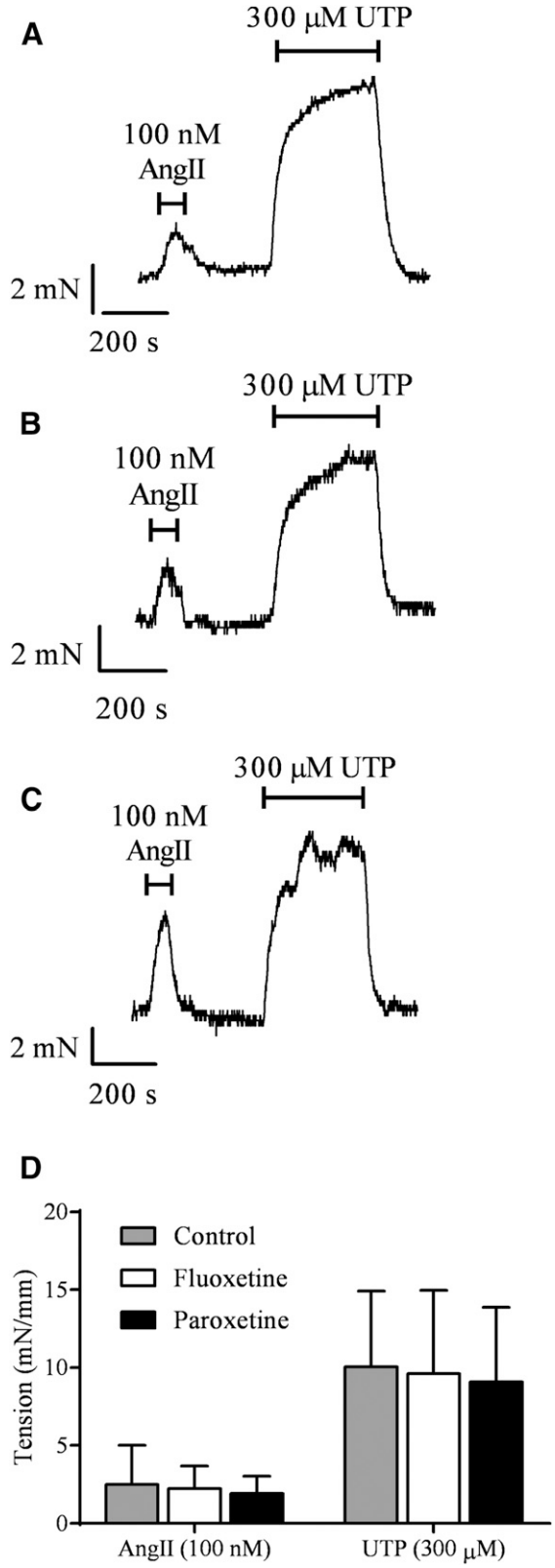

Fig. 5. SSRI-induced block of K^+-driven contraction does not affect agonist-mediated arterial contraction. Representative traces show arterial contractions after acute applications of AngII (100 nM) or UTP (300 μM) following pretreatment (5 minutes) with vehicle (A), fluoxetine (5 μM) (B), or paroxetine (5 μM) (C). Cumulative data (D) show no significant differences in agonist-induced arterial contractions in the absence or presence of SSRIs (means ± S.D.; for n = 6–11 arteries from ≥4 animal preparations; two-way ANOVA).

well maintained throughout the time course, indicating that paroxetine can cause a sustained ablation of the otherwise progressive desensitization of UTP-mediated contractions (Fig. 7, G and H).

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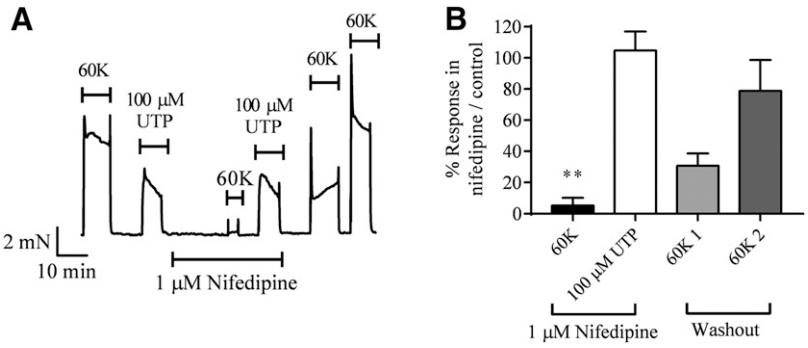

Fig. 6. Nifedipine inhibits K^+, but not UTP-stimulated arterial contractions. (A) Representative trace shows the contraction of mesenteric arterial rings when exposed to K^+ (60 mM) and UTP in the presence and absence of the L-type Ca^{2+}-channel blocker, nifedipine (1 μM). Cumulative data (B) show that K^+-stimulated contractions are blocked by nifedipine, which recovered gradually upon nifedipine washout. In contrast, UTP-stimulated contractions were unaffected by the L-type Ca^{2+}-channel blocker. Data are means ± S.D.; for n = 10 arteries from three animal preparations. $^{**}P < 0.01$ (Kruskal–Wallis, Dunn's post hoc test).

Effects of Other GRK Inhibitors on Desensitization of UTP-Stimulated Arterial Contractions. To further confirm the role of GRK2 in facilitating the desensitization of agonist-driven arterial contractions, we conducted similar desensitization protocols following the preincubation of two alternative GRK small-molecule inhibitors with largely unique chemical structures, CCG215022 and CCG224063. These compounds are 2-pyridylmethyl amide derivatives of GSK180736A (Waldschmidt et al., 2016), with CCG215022 being regarded as a pan GRK inhibitor, with a high degree of selectivity for GRK over protein kinase A (PKA) (Homan et al., 2015), whereas CCG224063 shows high selectivity for GRK2/3 with >100-fold selectivity over GRK5, ROCK1, and PKA. In addition, we examined the ability of the previously characterized GRK2/3 inhibitor, Takeda compound 101, (3-[[[4-methyl-5-(4-pyridyl)-4H-1,2,4-triazole-3-yl]methyl]-amino]-N-[2-(trifuoromethyl)benzyl]benzamide hydrochloride) (Thal et al., 2011; Okawa et al., 2017), to inhibit UTP-mediated desensitization of arterial contractions. Mesenteric arterial rings were subjected to the standard UTP desensitization protocol and then washed extensively and pretreated with inhibitors (10 μM) for 1 hour, before being again subject to the desensitization protocol. In the absence of inhibitors, an approximate 60% decrease in the R2 response versus R1 was observed (Fig. 8, A and E). However, the UTP-induced reduction in the R2/R1 ratio was attenuated in the presence of each of the GRK inhibitors (Fig. 8, B–E), consistent with the need to inhibit GRK2 to attenuate the agonist-induced desensitization.

Compounds CCG215022, CCG224063, and Takeda Compound 101 Inhibit GRK2-Mediated Desensitization of UTP, AngII, or Histamine-Mediated PLC Signaling. To confirm that compounds CCG215022 and CCG224063 inhibit GRK2-mediated desensitization of PLC signaling, we examined their abilities to attenuate the desensitization of $P2Y_2$ and AT1 receptor-mediated PLC signaling in MSMC and H_1 histamine receptor-mediated signaling in ULTR cells. In this study, cells were preincubated with vehicle (control), CCG215022 or CCG224063 (each at 10 μM, for 30 minutes), or Takeda compound 101 (30 μM, for 30 minutes) prior to application of the standard $R1/R_{max}/R2$ desensitization protocol for UTP and histamine. To examine AT1 receptor desensitization, cells were challenged with two maximal concentrations of AngII (100 nM; 30 seconds; termed R1 and R2) either side of a 5-minute washout period. In MSMC, inclusion of each compound attenuated the expected decrease in $[Ca^{2+}]_i$ R2/R1 ratio observed in vehicle-treated cells, suggesting that each agent attenuated $P2Y_2$ receptor desensitization of Ca^{2+} signals (Fig. 9, A and D). In addition, all three compounds attenuated desensitization of AngII-stimulated Ca^{2+} signaling in MSMC (Fig. 9, B and D). Furthermore, in ULTR cells, inclusion of each compound also attenuated the reduction in R2:R1 ratio observed in vehicle-treated cells, in this case utilizing the IP_3 biosensor to observe the functional desensitization (Fig. 9, C and E). Preincubation with the GRK2 inhibitor Takeda compound 101 (30 μM, for 30 minutes) also attenuated $P2Y_2$ (Fig. 9D) and histamine H_1 receptor desensitization (Fig. 9E). To determine whether the concentrations of the GRK2 inhibitors used were maximally effective, we further examined their concentration dependency to inhibit the desensitization of H_1 and $P2Y_2$ receptor-driven PLC/Ca^{2+} activity in ULTR and MSMC. All of the compounds tested produced concentration-dependent inhibition of PLC desensitization with maximal inhibition produced at 10 μM paroxetine, CCG215022 and CCG224063, and 30 μM Takeda compound 101 (Supplemental Fig. 2). IC_{50} values indicate similar potencies for individual compounds in either cell type (Table 1). Moreover, paroxetine, Takeda compound 101, and CCG215022 produced IC_{50} values of 1, 4.4, and 2.95 μM, respectively, whereas CCG224063 was slightly more potent, producing an IC_{50} of 46 nM (in MSMC). These data demonstrate the effectiveness of these GRK2 isoenzyme-selective inhibitors to alter the desensitization of different receptors endogenously expressed in MSMC and ULTR cells.

Discussion

Previous in vitro studies identified GRK2 as the key regulator of contractile GPCR-mediated PLC signaling in arterial smooth muscle cells (Cohn et al., 2008; Morris et al., 2010, 2011, 2012); however, the process of interrogating the role that GRK2 undertakes in the regulation of vascular tone has been difficult. Indeed, homozygous GRK2 knockout animals are nonviable (Jaber et al., 1996), and, although hemizygous knockout animals are viable (Rivas et al., 2013), the remaining GRK expression (50%) could still be adequate to maintain GPCR desensitization capacity. Furthermore, the use of viral and nonviral delivery techniques in an attempt to genetically manipulate GRK2 expression or activity in blood vessels has produced variable degrees of success (unpublished data; Newman et al., 1995; Havenga et al., 2001). Therefore, identification of small-molecule GRK inhibitors is vital to confirm the role that GRKs play in whole-body physiology and develop potential new therapeutic strategies. Because the SSRI paroxetine has been reported to inhibit GRK2 activity not only against in vitro substrates such as tubulin and

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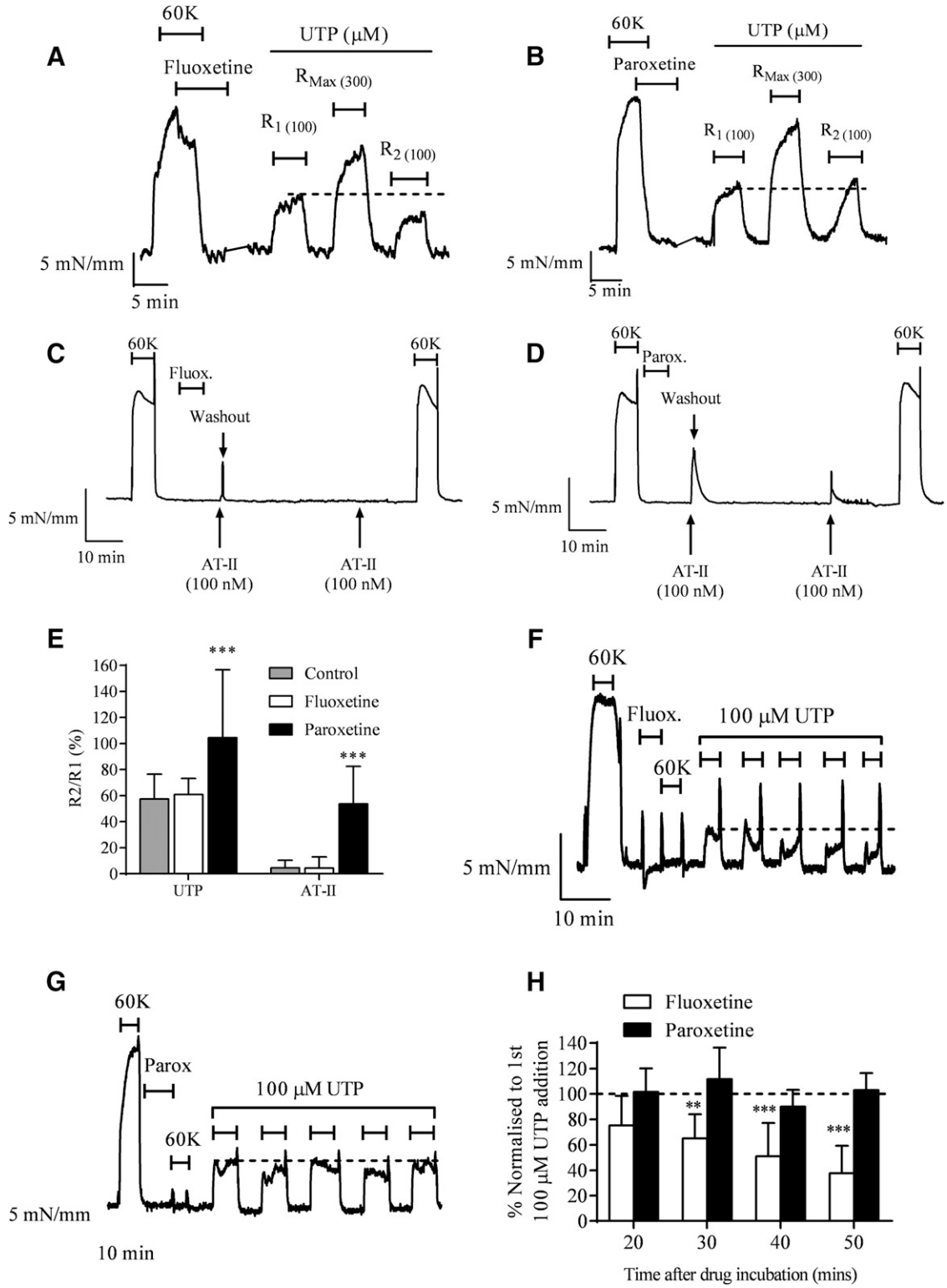

Fig. 7. Paroxetine inhibits the desensitization of UTP-mediated arterial contractions. Mesenteric arterial rings were initially exposed to K^+ (60 mM), before washout and addition of either fluoxetine or paroxetine (5 μM) for 5 minutes. Arteries were then subjected to the standard UTP desensitization protocol, or stimulated with AngII (100 nM, for 5 minutes), washed for 30 minutes, and stimulated a second time with AngII (100 nM). Representative traces show UTP-induced contractions from single arteries treated with either (A) fluoxetine or (B) paroxetine. Representative traces show AngII-induced contractions in single arteries treated with either (C) fluoxetine or (D) paroxetine. Desensitization of contractile responses was determined as the percentage decreased R2 response compared with R1 and is shown in (E). Data are means ± S.D.; for $n = 6$–11 arteries from ≥5 animal preparations (two-way ANOVA, Holm-Sidak post hoc test, $^{***}P < 0.001$). Representative traces show that arterial contractions, mediated by an EC_{50} (100 μM) concentration of UTP, decrease over time following a single 5-minute pretreatment with fluoxetine (F), but are maintained following pretreatment with paroxetine (5 μM) pretreatment (G). Cumulative data (H) show that paroxetine, but not fluoxetine, prevents the loss of arterial contraction to repeated UTP challenge (data are means ± S.D.; for $n = 7$ to 8 arteries from ≥5 animal preparations. $^{**}P < 0.01$; $^{***}P < 0.001$ (two-way ANOVA, Holm-Sidak post hoc test).

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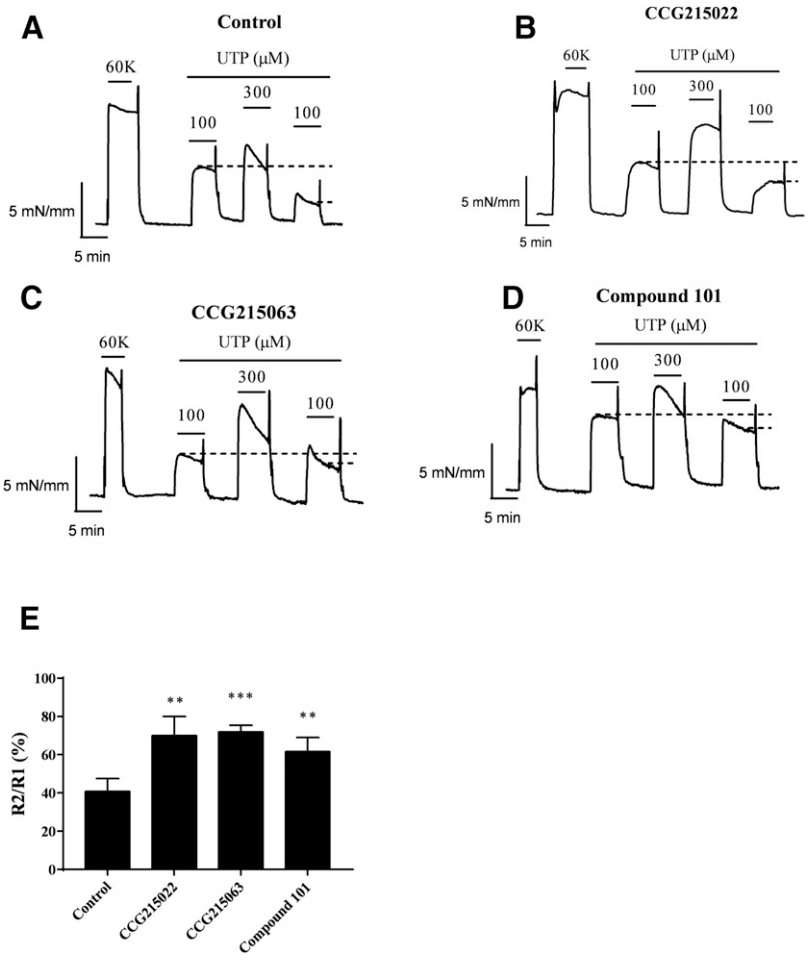

Fig. 8. Desensitization of UTP-stimulated arterial contraction is attenuated following addition of GRK2 inhibitors. Representative traces are shown for arterial rings preincubated for 1 hour with vehicle control (A), CCG215022 [(B), 10 μM], CCG224063 [(C), 10 μM], or Takeda compound 101 [(D), 30 μM] before being subjected to the standard UTP desensitization protocol. Cumulative data (E) show that inclusion of GRK2 inhibitors attenuates UTP-mediated desensitization (data are means ± S.D.; for $n = 8$ arteries from ≥4 animal preparations). $^{**}P < 0.01$; $^{***}P < 0.001$ (one-way ANOVA, Holm–Sidak post hoc test).

isolated rhodopsin, but also in whole-cell systems (Thal et al., 2011; Schumacher et al., 2015), we investigated whether paroxetine could prevent desensitization of GRK2-mediated GPCR activity in arterial rings.

Initially, we examined the effects of paroxetine on UTP/$P2Y_2$-induced PLC signaling in isolated MSMC, and, in agreement with our original findings (Morris et al., 2011), application of our standard desensitization protocol uncovered an approximate 60% and 50% reduction in the R2/R1 ratio for IP_3 and Ca^{2+}, indicative of receptor desensitization (Morris et al., 2010, 2011, 2012). Preincubation with paroxetine attenuated $P2Y_2$ receptor desensitization, yielding comparable results as when GRK2 expression was knocked down (>80%) using small-interfering RNA treatment (Morris et al., 2010, 2011). Moreover, the structurally distinct SSRI fluoxetine was unable to prevent $P2Y_2$ receptor desensitization, which, when combined with previous reports that fluoxetine is unable to inhibit GRK2 activity (Thal et al., 2011), strongly suggests that the effects of paroxetine are on GRK2 rather than alternative off-target SSRI interactions. Furthermore, in ULTR cells paroxetine blocked H_1 receptor desensitization, a GRK2 exclusive process (Willets et al., 2008), whereas the exclusively GRK6-mediated oxytocin receptor desensitization (Willets et al., 2009) was unaffected. In support of these observations, paroxetine has previously been shown to inhibit GRK2-mediated phosphorylation of thyrotropin-releasing hormone receptor with an IC_{50} of 30 μM (Thal et al., 2012) and to enhance βAR-mediated cardiomyocyte contraction (a process negatively regulated by GRK2) (Thal et al., 2012). Recently, paroxetine has been shown to inhibit β_2-receptor desensitization by blocking GRK2-mediated adrenoceptor phosphorylation and arrestin recruitment (Guo et al., 2017). Collectively, these data further support the notion that paroxetine selectively interacts with GRK2 to inhibit its ability to desensitize GPCR signaling.

As our data suggest that paroxetine can inhibit GRK2-mediated desensitization of UTP-driven PLC signaling in isolated MSMC, we examined whether paroxetine could display a similar ability to prevent the desensitization of UTP-induced arterial contractions. Initial exposure to either SSRI caused a transient inhibition of depolarization-induced arterial contractions, which is in agreement with previous reports that highlight fluoxetine and paroxetine as L-type Ca^{2+} channel blockers (e.g., Stauderman et al., 1992). Interestingly, the SSRI-mediated blockade of voltage-operated Ca^{2+}-channel activity was temporary and fully reversed after 30 minutes of repeated washouts, possibly reflective of the extracellular rather than intracellular actions of these SSRI compounds. It is also noteworthy that any SSRI-mediated block of L-type channels would not be obvious in isolated MSMC because L-type channel expression is lost rapidly in culture (Gollasch et al., 1998; Patel et al., 2005). Nevertheless, AngII- or UTP-induced arterial contractions were equivalent when assessed either directly after the addition of the SSRI

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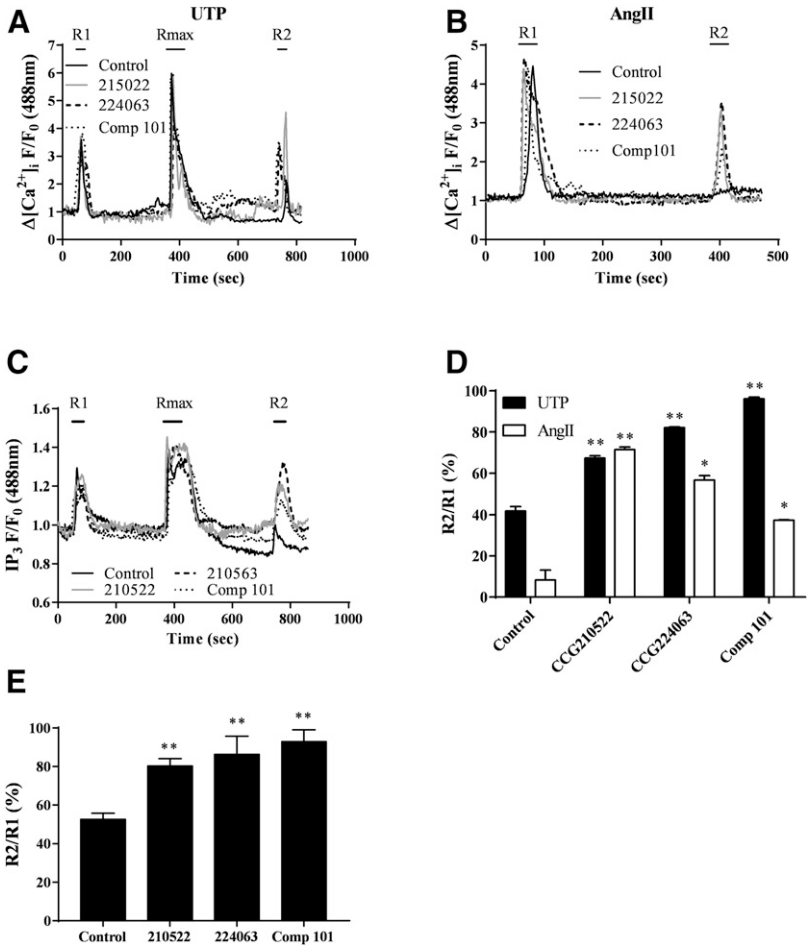

Fig. 9. Effects of GRK inhibitors on $P2Y_2$ and H_1 histamine-mediated PLC signaling in MSMC and ULTR cells. Cells were preincubated with either vehicle or GRK inhibitors for 30 minutes prior to being exposed to the standard desensitization protocols outlined previously. Representative traces show the effects of CCG215022 and CCG224063 (10 μM) and compound 101 (30 μM) on $P2Y_2$ receptor (A) and AT1 receptor desensitization (B) in MSMC. Representative traces show the effects of CCG215022, CCG224063, and Takeda compound 101 on H_1 histamine (C) desensitization in ULTR cells. Cumulative data show that CCG215022, CCG224063, and Takeda compound 101 are able to attenuate the desensitization of agonist-mediated PLC signaling (*$P < 0.05$; **$P < 0.01$, Kruskal–Wallis, Dunn's post hoc test) in MSMC (D) and ULTR cells (E), respectively. Receptor desensitization was determined as the reduction of the R2 response when compared with R1 [means ± S.D. for, n = 11–96 cells, generated from preparations from five different animals for MSMC experiments (D); n = 10–30 cells for ULTR experiments (E)].

(i.e., when voltage-operate channels are inhibited) or in the absence of the SSRI. Together these data suggest that firstly, paroxetine and fluoxetine do not affect acute GPCR-stimulated contractions, and secondly, that AngII- and UTP-stimulated arterial contractions rely on Ca^{2+} release from intracellular stores rather than extracellular Ca^{2+} sources.

Previously, we have shown that by applying a variation of our standard R1/R_{max}/R2 protocol we can measure desensitization of UTP-induced contractions in intact mesenteric vessel rings (Morris et al., 2011). Applying the same protocol in this study, we demonstrate that paroxetine prevented the loss of arterial responsiveness to UTP. Although reduced vessel contractility may reflect a loss in responsiveness of a wide variety of contractile pathways (Brozovich et al., 2016), PLC signaling contributes significantly to this process (Ureña et al., 2013). Moreover, because paroxetine inhibits the desensitization of $P2Y_2$ receptor/PLC signaling in isolated MSMC, a process exclusively regulated by GRK2 (Morris et al., 2011), suggests that paroxetine plays a similar role to prevent the desensitization of $P2Y_2$ activity, which underpins UTP-induced arterial contractions. Bioavailability studies indicate plasma paroxetine concentrations remain steady at around 125 nM (Bourin et al., 2001), which is considerably lower than the concentrations required to block GRK2 function in whole tissues. Nonetheless, paroxetine is known to distribute throughout the body, which, combined with the finding that plasma levels contains <1% of the total ingested drug, suggests that paroxetine is likely to accumulate in tissues where concentrations may also increase. Interestingly, in some ways our data reflect this notion, because the ability of paroxetine to inhibit GRK2-induced desensitization appeared to be prolonged. Indeed, a single application (5 minutes) of 5 μM paroxetine prevented the loss of arterial responsiveness to UTP, even after five agonist challenges over a 50-minute period, suggesting a lack of cellular removal/metabolism and/or prolonged association of paroxetine and GRK2.

AngII is a potent vasoconstrictor known to induce arterial contraction through activation of PLC signaling activating the AT1 receptor (Montezano et al., 2014). In this study, we found

TABLE 1

Data are derived from the concentration–response curves shown in Supplemental Fig. 2, demonstrating IC_{50} values for the abilities of GRK2 inhibitors to prevent the desensitization of H_1 or $P2Y_2$ receptor desensitization of PLC/Ca^{2+} signals in ULTR and MSMC, respectively

Data are expressed as means ± S.E.M. for n = 20–123 cells from at least four separate experiments for each drug concentration.

Compound	pIC_{50}	
	ULTR	MSMC
Paroxetine	6.1 ± 0.2	5.99 ± 0.18
Compound 101	5.35 ± 0.2	5.48 ± 0.18
CCG224063	7.33 ± 0.18	7.35 ± 0.17
CCG215022	5.51 ± 0.19	5.53 ± 0.24

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that AngII-induced arterial contractions were highly susceptible to desensitization and were resistant to resensitization, with little detectable contraction even when measured ~60 minutes after initial agonist application. These findings are similar to AT1 receptor desensitization in isolated MSMC, where full recovery of AngII Ca^{2+}/PLC signals takes >20 minutes, likely reflecting the requirement for AT1 receptor internalization and recycling (Hunyady et al., 2000). The fact that paroxetine is able to partially attenuate the loss of arterial responsiveness to AngII implicates GRK2 in the desensitization of the AT1 receptor PLC/contractile activity in arteries. Indeed, this finding is supported by previous observations that the AT1 receptor is a substrate for GRK2-induced desensitization (Olivares-Reyes et al., 2001; Kim et al., 2005). Interestingly, the inability of paroxetine to completely reverse AT1 receptor desensitization suggests that other kinases such as GRK4 (Chen et al., 2014) might be responsible for the residual loss of AngII-mediated contractile response.

To corroborate our findings, we also examined the effects of three other GRK2 inhibitors on UTP-induced desensitization of arterial contractions. First, we used the Takeda compound 101, which is a highly selective GRK2/3 inhibitor especially against other GRK family members, PKA and protein kinase C (Thal et al., 2011; Lowe et al., 2015). Indeed, at the concentrations used in this study, Takeda compound 101 is unlikely to interact with other kinases that may desensitize UTP- or AngII-mediated contractions (Thal et al., 2011). Furthermore, we and others have shown that arterial smooth muscle cells express little, if any GRK3 (Cohn et al., 2008; Morris et al., 2010), and overexpression of dominant-negative GRK3 in isolated mesenteric arterial cells failed to prevent $P2Y_2$ desensitization (Morris et al., 2011), which implies that Takeda compound 101 is targeting GRK2 to prevent loss of $P2Y_2$ receptor responsiveness. Unlike the SSRIs, the pan GRK inhibitor CCG215022 and the GRK2-selective inhibitor CCG224063 did not affect depolarization-induced contractions, suggesting that neither compound interfered with L-type Ca^{2+}-channel activity. Nevertheless, both compounds appeared slightly less effective inhibitors of GRK2 function than paroxetine in whole tissues because twofold more was required to attenuate UTP-induced desensitization; however, this may reflect lack of tissue penetration as 60 minutes of pretreatment were required to achieve optimal results with CCG215022 and CCG224063. Interestingly, this difference was not evident in isolated cells as the CCG compounds and paroxetine were equally efficacious at blocking desensitization of both the $P2Y_2$ and H_1 receptor-stimulated PLC signaling after only 30-minute pretreatment. Furthermore, our data suggest that both CCG compounds were as effective as small-interfering RNA–mediated GRK2 depletion in preventing GRK2-mediated GPCR desensitization (Willets et al., 2008; Morris et al., 2011).

Previously published data (Thal et al., 2011; Waldschmidt et al., 2016) highlight that the compounds used in this study possess IC_{50} values within the nanomolar range (i.e., Takeda compound 101 54 nM; CCG215022 150 nM; CCG224063 130 nM), which might suggest that the higher micromolar concentrations used in this study may lose selectivity. However, these data are derived from in vitro assays using isolated GRK enzymes and substrates, which do not have the added complications such as membrane permeability, possible cellular metabolism, or nonspecific binding to off-target cellular proteins. Furthermore, all the compounds used are competitive inhibitors of the GRK2 ATP binding site, and, because ATP concentrations are likely higher in cells than in vitro assays, this will potentially underestimate kinase inhibition in whole cells. Therefore, it is not surprising that in cellular systems and tissues higher micromolar concentrations are required to produce maximal effects on kinase inhibition (Lowe et al., 2015). The exception seems to be paroxetine, which inhibits isolated GRK2 with an IC_{50} of 1.38 μM (Waldschmidt et al., 2016), and GRK2-mediated β_2AR phosphorylation in HEK293 cells with an IC_{50} of 5.9 μM (Guo et al., 2017), which is similar to our findings.

Our data confirm that paroxetine functions as a selective inhibitor of GRK2-mediated desensitization of G_q-coupled receptors and PLC signaling. Moreover, because PLC signaling plays a central role in increasing intracellular Ca^{2+} concentration, and thus induces arterial contraction, it appears likely that paroxetine prevents the loss of arterial contractile responsiveness to UTP by inhibiting GRK2-mediated P2Y receptor desensitization. Likewise, if inhibition of GRK2 function attenuates the desensitization of PLC-mediated arterial contractions, one would expect increased GRK2 expression to have an opposite effect. Our previous data support this notion, because the doubling of GRK2 expression observed in mesenteric arteries during the early stages of hypertension results in a twofold enhancement of the desensitization of UTP-stimulated arterial contractions (Willets et al., 2015b). In summary, we have used a variety of small-molecule GRK2 inhibitors to confirm for the first time the central role that GRK2 plays in the regulation of vasoconstrictor-mediated arterial tone, which highlights a potentially novel strategy for blood pressure regulation through targeting GRK2 function. The results also suggest that some of the benefit of applying small-molecule inhibitors of GRK2 systemically (Schumacher et al., 2015) is to improve the hormonal responsiveness of smooth muscle cells in addition to that of cardiac myocytes.

Authorship Contributions

Participated in research design: Rainbow, Challiss, Willets.

Conducted experiments: Rainbow, Brennan, Jackson, Beech, Bengreed, Willets.

Contributed new reagents or analytic tools: Waldschmidt.

Performed data analysis: Rainbow, Brennan, Jackson, Beech, Bengreed, Willets.

Wrote or contributed to writing of the manuscript: Rainbow, Brennan, Waldschmidt, Tesmer, Challiss, Willets.

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