Differential Effects of Selective Cyclooxygenase-2 Inhibitors on Vascular Smooth Muscle Ion Channels May Account for Differences in Cardiovascular Risk Profiles


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ABSTRACT

Celecoxib, rofecoxib, and diclofenac are clinically used cyclooxygenase-2 (COX-2) inhibitors, which have been under intense scrutiny because long-term rofecoxib (Vioxx; Merck, Whitehouse Station, NJ) treatment was found to increase the risk of adverse cardiovascular events. A differential risk profile for these drugs has emerged, but the underlying mechanisms have not been fully elucidated. We investigated the effects of celecoxib, rofecoxib, and diclofenac on ionic currents and calcium signaling in vascular smooth muscle cells (VSMCs) using patch-clamp techniques and fura-2 fluorescence and on arterial constriction using pressure myography. Celecoxib, but not rofecoxib or diclofenac, dramatically enhanced KCNQ (Kv7) potassium currents and suppressed L-type voltage-sensitive calcium currents in A7r5 rat aortic smooth muscle cells (native KCNQ currents or overexpressed human KCNQ5 currents) and freshly isolated rat mesenteric artery myocytes. The effects of celecoxib were concentration-dependent within the therapeutic concentration range, and were reversed on washout. Celecoxib, but not rofecoxib, also inhibited calcium responses to vasopressin in A7r5 cells and dilated intact or endothelium-denuded rat mesenteric arteries. A celecoxib analog, 2,5-dimethyl-celecoxib, which does not inhibit COX-2, mimicked celecoxib in its enhancement of vascular KCNQ5 currents, suppression of L-type calcium currents, and vasodilation. We conclude that celecoxib inhibits calcium responses in VSMCs by enhancing KCNQ5 currents and suppressing L-type calcium currents, which ultimately reduces vascular tone. These effects are independent of its COX-2 inhibitory actions and may explain the differential risk of cardiovascular events in patients taking different drugs of this class.

Celecoxib (Celebrex; Pfizer, New York, NY) and rofecoxib (Vioxx; Merck, Whitehouse Station, NJ) are nonsteroidal anti-inflammatory drugs (NSAIDs) that selectively inhibit cyclooxygenase-2 (COX-2). They were introduced to the market in 1999 and rapidly became the most frequently prescribed new drugs in the United States. These drugs are used clinically to treat pain and inflammation. COX-1 and COX-2 convert arachidonic acid into prostaglandin H₂, which is further converted to a variety of prostanoids, including prostaglandins, thromboxanes, and prostacyclins. Thromboxane A₂, a product of COX-1 activity in platelets, promotes vasoconstriction, smooth muscle proliferation, and platelet aggregation. In contrast, prostacyclin generated by COX-2 in the blood vessel walls promotes vasodilatation and inhibition of platelet aggregation. As analgesic/anti-inflammatory agents, COX-2 inhibitors were considered to be an improvement over less selective COX-1/COX-2 inhibitors because they prevent the generation of prostaglandins involved in inflammation and pain while sparing some beneficial effects of COX-1-generated prostanoids. However, these drugs have been under intense scrutiny since 2004, when Vioxx was voluntarily withdrawn from the market because of a reported increased risk of myocardial infarction and stroke in patients taking the drug for prolonged periods of time (Marnett, 2009).

A systematic review of randomized clinical trials of COX inhibitors revealed that rofecoxib, a highly COX-2-selective agent, and diclofenac, an NSAID with COX-2/COX-1 selectivity similar to that of celecoxib, both significantly increased the risk of cardiovascular events (McGettigan and Henry, 2009).
2006). In contrast, a number of clinical studies failed to demonstrate an increased cardiovascular risk with celecoxib relative to placebo (McGettigan and Henry, 2006; White et al., 2007). The reasons for the differences between celecoxib and other COX-2 inhibitors are still widely debated.

Antihypertensive effects might lead to fewer cardiovascular complications with celecoxib compared with other COX-2 inhibitors. Meta-analysis of results from 19 clinical trials involving COX-2 inhibitors revealed a significantly lower risk for developing hypertension among patients treated with celecoxib compared with rofecoxib (Aw et al., 2005). Likewise, a retrospective analysis of medical records for patients treated with celecoxib or rofecoxib over a 90-day period revealed that, whereas systolic blood pressure was significantly elevated after 90 days among rofecoxib-treated patients, systolic blood pressures decreased slightly among the celecoxib-treated patients (Cho et al., 2003).

Several recent studies have reported vasorelaxant effects of celecoxib (Hermann et al., 2003; Widlansky et al., 2003; Klein et al., 2007), which may account for its differential effects on blood pressure relative to other COX-2 inhibitors (Hermann et al., 2003). In this article, we explored mechanisms that might underlie the vasodilatory actions and lower risk for cardiovascular complications for celecoxib, examining the effects of celecoxib and other NSAIDs on vascular smooth muscle ion channels and physiological functions. We focused our attention on two types of ion channels that are perhaps the most important in determining the contractile state of vascular smooth muscle cells (VSMCs): K+ channels that determine the resting membrane voltage, and L-type voltage-gated Ca2+ channels, the activation of which induces Ca2+ influx, smooth muscle contraction, and vasoconstriction. We provide clear evidence that both types of ion channels are robustly affected by celecoxib but not by rofecoxib or diclofenac at comparable therapeutic concentrations. These ion channel effects have important functional consequences for VSMC Ca2+ signaling and vasomotor tone in resistance arteries.

Materials and Methods

Isolation of Myocytes. All animal studies were approved by the Loyola University Chicago Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (n = 20) were anesthetized by inhalation with isoflurane, and segments of small intestinal mesentery were surgically removed as described previously (Henderson and Byron, 2007). Methods for isolation of mesenteric artery smooth muscle cells (MASMCs) were described previously (Mackie et al., 2008). Freshly isolated MASMCs were kept on ice until use. The cells were then dispensed onto a glass coverslip base of the recording chamber and allowed to adhere for at least 15 min at room temperature.

Cell Culture. A7r5 cells were cultured as described previously (Byron and Taylor, 1998). For KCNQ5 overexpression studies, subcultured A7r5 cells at 50 to 70% confluence were transfected with a FLAG-tagged human KCNQ5 DNA sequence (inserted into a pIREs2-enhanced green fluorescent protein vector) using Lipofectamine (Invitrogen, Carlsbad, CA) transfection reagent according to the manufacturer’s protocol. Confluent subcultures of A7r5 cells were trypsinized and replated on glass coverslips. Green fluorescent protein-expressing cells were used for electrophysiological recording 5 to 10 days after transfection.

Patch Clamp. The whole-cell perforated patch configuration was used to measure membrane currents under voltage-clamp conditions. Amphotericin B (120 μg/ml) in the internal solution was used for membrane patch perforation. All experiments were performed at room temperature with continuous perfusion of bath solution as described previously (Brueggemann et al., 2007; Mackie et al., 2008).

Voltage-clamp command potentials were generated using an Axopatch 200B amplifier under control of pCLAMPS software (Molecular Devices, Sunnyvale, CA). Procedures for recording KCNQ K+ currents and L-type Ca2+ currents were essentially as described previously (Brueggemann et al., 2005, 2007; Mackie et al., 2008). Details of the voltage protocols and recording conditions are provided in the supplemental materials.

[Ca2+]i, Measurements with Fura-2. Essentially as described previously (Mani et al., 2009), confluent monolayers of A7r5 cells cultured in six-well plates were washed twice with control medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2, 11.5 mM glucose, and 11.6 mM HEPES, pH 7.3) and then incubated in the same medium with 1 μM fura-2/acetoxymethyl ester (Invitrogen), 0.1% bovine serum albumin, and 0.02% Pluronic F127 detergent for 60 min at room temperature (22–25°C) in the dark. Fura-2 fluorescence was measured using a Biotek Synergy HT plate reader (340- and 380-nm excitation, 510-nm emission) (BioTek Instruments, Winooski, VT). All experiments were performed at room temperature. Frequency of spiking was calculated as the number of spikes per minute from the time of onset of repetitive Ca2+ spiking. Each “n” represents the mean of triplicate wells.

Pressure Myography. Methods used for isolated artery pressure myography have been described previously (Henderson and Byron, 2007). For some experiments, after dissection of a mesenteric artery, endothelial denudation was performed by gently rubbing the arterial lumen with a human hair. A small amount of air (~0.5 ml) was then passed through the lumen to further disrupt the endothelium, followed by physiological saline to remove the endothelial cells. To confirm attenuation of endothelial function, endothelium-dependent vasodilation was evaluated after preconditioning of the arteries with 100 pM [Arg9]vasopressin (AVP). After the development of a stable level of constriction, increasing concentrations of the endothelial-dependent vasodilator carbachol were administered. In intact arteries, 10 μM carbachol induced 97 ± 1.2% dilation (n = 7), but in denuded arteries, the same concentration induced significantly less dilation (49 ± 14%, n = 6, p < 0.005, Student’s t test; Supplemental Fig. 3). After the carbachol dose-response evaluation, atropine (100 μM) was administered to reverse the effects of carbachol. When AVP-induced constriction was restored to its original level, celecoxib was administered (see Results; Fig. 5A).

Statistics. SigmaStat (Systat Software, Inc., Point Richmond, CA) was used for all statistical analyses. Fairly Student’s t test was used for comparisons of parameters measured before and after treatments. Comparisons among multiple treatment groups were evaluated by analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Cumulative concentration-response data were analyzed by repeated-measures ANOVA and post hoc Holm-Sidak test. Differences associated with p ≤ 0.05 were considered statistically significant.

Materials. Cell culture media and antibiotics were from Invitrogen or MediaTech (Herndon, VA). Lipofectamine reagent was from Invitrogen. Celecoxib and rofecoxib were from LKT Laboratories, Inc. (St. Paul, MN). Linopirdine, flupirtine, diclofenac sodium salt, collagenase, elastase, [Arg9]-vasopressin, carbachol, atropine, and verapamil were from Sigma-Aldrich (St. Louis, MO). Amphotericin B was from Calbiochem (San Diego, CA). cDNA encoding FLAG-tagged human KCNQ5 was generously provided by Professor Thomas Jentsch (Leibniz-Institut fuer Molekulare Pharmakologie, Berlin, Germany). 2,5-Dimethyl-celecoxib was generously provided by Dr. Axel Schöntahl (University of Southern California, Los Angeles, CA).
Results

COX-2-Independent Effects of Celecoxib on Vascular Smooth Muscle Cell Ion Channels. K⁺ and Ca²⁺ currents in A7r5 rat aortic vascular smooth muscle cells were recorded simultaneously under approximately physiological ionic conditions, as described previously (Brueggemann et al., 2007). Inward Ca²⁺ currents were recorded at the beginning of 5-s voltage steps, and steady-state K⁺ currents were recorded at the ends of the voltage steps (Fig. 1A). Application of 10 μM celecoxib dramatically enhanced outward K⁺ currents (by 2.3-±0.3-fold at -20 mV; Fig. 1, B–D; Supplemental Table 1) and abolished the inward Ca²⁺ current (Fig. 1, A and E). Inhibition of Ca²⁺ currents reproducibly preceded enhancement of K⁺ currents (Fig. 1C). Both effects were reversible on washout of celecoxib (Supplemental Table 1).

The same set of experiments was repeated with the highly selective COX-2 inhibitor rofecoxib (Patrono et al., 2001) and with diclofenac, which exhibits a COX-2/COX-1 selectivity profile similar to that of celecoxib (Patrono et al., 2001). Neither rofecoxib (10 μM) nor diclofenac (10 μM) significantly affected Ca²⁺ or K⁺ currents (Fig. 1, D and E). However, application of 10 μM celecoxib after washout of diclofe-
nac or rofecoxib still robustly enhanced K⁺ currents and inhibited Ca²⁺ currents (Supplemental Tables 2 and 3). A celecoxib analog, 2,5-dimethylcelecoxib (DMC, 10 μM), which does not inhibit COX-2 (IC₅₀ > 100 μM) (Schönthal et al., 2008), mimicked both effects of celecoxib: enhancement of K⁺ currents, and inhibition of Ca²⁺ currents (Fig. 1, D and E; Supplemental Tables 1 and 2). Flupirtine (10 μM), an activator of KCNQ K⁺ channels, mimicked only the enhancement of K⁺ currents, whereas verapamil (10 μM), a known blocker of L-type Ca²⁺ currents, abolished the inward Ca²⁺ currents and partially inhibited the outward K⁺ currents (Fig. 1, D and E; Supplemental Table 3).

**Celecoxib Effects on KCNQ5 Currents.** Outward K⁺ currents measured in A7r5 cells at membrane potentials ≤−20 mV have previously been attributed to Kᵥ7.5 (KCNQ5) channels. We also measured the effects of celecoxib on overexpressed human KCNQ5 channels using the A7r5 cells as an expression system. Because the exogenous channels produce resting currents that are approximately 2 orders of magnitude larger than the native currents (compare scales in Fig. 2, A and E), recordings reflect predominantly the activity of the human channels with little contribution of the native channels. We found that celecoxib robustly enhanced human KCNQ5 currents in MASMCs (Supplemental Fig. 1, C and D).

Neither rofecoxib (10 μM) nor diclofenac (10 μM) affected the KCNQ currents in MASMCs (Supplemental Fig. 1, C and D). As reported previously (Mackie et al., 2008; Schoenthal et al., 2008), under these recording conditions, outward currents measured at voltages ≤−20 mV were completely abolished by the selective KCNQ channel blocker linopirdine (Fig. 2C).

To further characterize the effects of celecoxib, we performed cumulative dose-response experiments with 10 nM−10 μM celecoxib on hKCNQ5 currents recorded at −20 mV in a single A7r5 cell (C = 43 pF) before and during application of 10 μM celecoxib (white bar). E, current-voltage relationships measured in A7r5 cells overexpressing human KCNQ5 channels; control (●); 10 μM celecoxib (○); and after washout of celecoxib (▲), *, significant difference from control (n = 5, p < 0.05, paired Student’s t test). F, representative time course of hKCNQ5 current recorded at −20 mV in a single A7r5 cell (C = 83 pF) before and during application of 10 μM celecoxib (white bar).
KCNQ5 currents, and this effect was completely reversed after washout of celecoxib (Fig. 2, E and F).

**Celecoxib Effects on L-Type Ca2+ Channels.** Using conditions to record L-type Ca2+ currents in isolation, we observed a concentration-dependent and reversible suppression of the currents by celecoxib (IC50 = 8.3 ± 1.3 µM; Fig. 3, A and B). Celecoxib (10 µM) induced a pronounced inhibition of Ca2+ current amplitude, along with a significant positive shift of the activation curve (~12 mV) (Fig. 3C). A similar effect was observed when Ba2+ was used as the charge carrier (Fig. 3D). In both cases, the shift in activation was reversed after washout of celecoxib (data not shown).

L-type Ca2+ currents were also measured in freshly isolated mesenteric artery myocytes. Using Ba2+ as a charge carrier, we found that L-type currents were significantly suppressed by 10 µM celecoxib (Fig. 3, E and F). With 2 mM Ca2+ as charge carrier, the L-type currents were smaller, but the effect of 10 µM celecoxib was similar (data not shown).

**Functional Effects of KCNQ5 Channel Activation and/or Inhibition of L-Type Ca2+ Channels.** Both KCNQ5 channels and L-type Ca2+ channels are important for functional responses of VSMCs. We have demonstrated previously the involvement of both channel types in the stimulation of repetitive Ca2+ spiking in A7r5 cells treated with a physiological concentration of AVP (Byron, 1996; Bruegge-...
not rofecoxib (10 μM) completely abolished AVP-stimulated Ca^{2+} spiking when added at the same time as 25 pM AVP (Fig. 4), and spiking ceased when celecoxib (but not rofecoxib) was added after achieving a sustained Ca^{2+} spiking response to 25 pM AVP (Supplemental Fig. 2).

We also examined the functional effects of celecoxib on vasoconstrictor responses of pressurized rat mesenteric arteries. Celecoxib induced concentration-dependent dilation of mesenteric arteries preconstricted with 100 pM AVP (Fig. 5A). We found that disruption of the endothelium (Supplemental Fig. 3) did not reduce celecoxib-induced vasodilation, suggesting that the response was mediated at the level of the smooth muscle cells (Fig. 5B). The EC_{50} values (11.2 ± 1.1 μM for intact arteries, 8.9 ± 0.8 μM for denuded arteries) were not significantly different. In a separate set of experiments, rofecoxib or diclofenac (both at 20 μM) induced very modest dilation (11.5 ± 2.5 and 5.8 ± 2.0%, respectively) of arteries preconstricted with 100 pM AVP, whereas celecoxib at the same concentration fully dilated the same arteries (to ~98% maximal outer diameter; Fig. 5C). DMC was also very effective as a vasodilator: arteries constricted by 100 pM AVP were relaxed to 99.9 ± 0.1% (n = 3) of their original diameter in the presence of 20 μM DMC (Fig. 5D).

The vasodilatory actions of celecoxib may be due to activation of KCNQ K^+ channels or inhibition of L-type Ca^{2+} channels, either of which we have shown previously can reverse AVP-induced vasoconstriction in rat mesenteric arteries (Henderson and Byron, 2007; Mackie et al., 2008). To evaluate whether the Ca^{2+}-channel blocking actions of celecoxib are sufficient to induce dilation, we treated mesenteric arteries with a maximal vasoconstrictor concentration of the KCNQ channel blocker linopirdine and then added 20 μM celecoxib (in patch-clamp studies, we verified that celecoxib cannot reverse the inhibitory effects of linopirdine on KCNQ currents in MASMCs; Supplemental Fig. 1F). Even though activation of vascular KCNQ channels was prevented by linopirdine, celecoxib produced near full relaxation of all arteries tested (95 ± 1.4% maximal dilation, n = 3; a representative recording is shown in Fig. 5E).

**Discussion**

Our findings may help to explain why celecoxib is a safer drug in terms of cardiovascular complications compared with rofecoxib or diclofenac. We found that, unlike rofecoxib or diclofenac, celecoxib potently enhances KCNQ potassium channel activity.
Fig. 5. Celecoxib dilates preconstricted mesenteric arteries. A, representative recording of outer vessel diameter demonstrating dose-dependent dilation of a mesenteric artery treated with increasing concentrations of celecoxib. Atropine (see Materials and Methods) was used to restore 100 pM AVP-induced constriction after treatment with the endothelium-dependent dilator carbachol (see Supplemental Fig. 3). B, celecoxib dose-response relationship for intact and endothelium-denuded arteries. C, representative traces (left) illustrating the inability of 20 µM rofecoxib (top) and 20 µM diclofenac (bottom) to dilate arteries preconstricted with 100 pM AVP. Celecoxib (20 µM) fully dilated the same arteries when added after either rofecoxib or diclofenac. The bar graph at the right of each representative trace illustrates the mean dilation caused by each drug. *, significantly less dilation than celecoxib, p < 0.001, Student’s t test. D, representative recording demonstrating that DMC (20 µM) produced full dilation of arteries preconstricted with 100 pM AVP; representative of three similar experiments. E, representative recording demonstrating that celecoxib (20 µM) also produced full dilation of arteries preconstricted with the selective KCNQ channel blocker linopirdine (10 µM); representative of three similar experiments.
current and inhibits L-type calcium current in VSMCs, resulting in marked dilation of intact arteries. These COX-2-independent actions may offset what would otherwise be a detrimental increase in vasoconstriction mediated by COX-2 inhibition. In contrast, for rofecoxib and diclofenac, which do not exhibit this ion channel-mediated protective effect, the COX-2 inhibition-mediated vasoconstrictor effects are unopposed, perhaps accounting for their increased cardiovascular complications.

The measured peak concentrations of celecoxib in the plasma of patients taking the drug for pain and/or inflammation generally average between 1 and 3 μM, whereas therapeutic concentrations of rofecoxib and diclofenac are slightly lower (Hinz et al., 2006). Considerably higher plasma concentrations of celecoxib may be achieved in patients with slower metabolism (Lundblad et al., 2006; Shi and Klotz, 2008) or when higher doses are given (e.g., as an anticancer therapy) (Sauter et al., 2008). Significant effects of celecoxib on VSMC KCNQ5 currents and L-type Ca\(^{2+}\) currents were apparent within the range of clinically achieved concentrations, whereas neither rofecoxib nor diclofenac exhibited these effects, even at concentrations in excess of therapeutic plasma levels. At supratherapeutic concentrations, additional effects of celecoxib may affect the ionic currents and account for a somewhat bell-shaped dose-response relationship for activation of KCNQ5. For example, celecoxib has been reported to induce endoplasmic reticulum stress at concentrations ≥40 μM (Pyrko et al., 2007), an effect associated with elevated cytosolic [Ca\(^{2+}\)] that might directly or indirectly inhibit KCNQ channels (Delmas and Brown, 2005). A reduction in KCNQ5 current when celecoxib concentration was increased from 20 to 30 μM in VSMCs (Fig. 3B; Supplemental Fig. 1E) was also observed in human embryonic kidney 293T cells overexpressing human KCNQ5 (results not shown), suggesting that KCNQ5 is a specific target for both the positive and negative actions of celecoxib.

Although celecoxib potently inhibits COX-2, with an IC\(_{50}\) of 0.87 μM (Mardini and FitzGerald, 2001), the mechanism of ion channel modulation is unlikely to involve COX-2 inhibition. Other selective COX-2 inhibitors, rofecoxib and diclofenac, did not mimic celecoxib in its ion-channel modulatory effects, and DMC, a celecoxib analog that does not inhibit COX-2, was as effective as celecoxib in both enhancement of KCNQ5 currents and suppression of L-type Ca\(^{2+}\) currents.

It remains to be determined whether the effects of celecoxib on KCNQ5 currents and L-type Ca\(^{2+}\) channels are due to direct interactions with the respective channel proteins or indirect (e.g., involving signal transduction intermediates). Other known activators of KCNQ5, such as retigabine and flupirtine, are believed to bind directly to the channel to stabilize its open state (Lange et al., 2009). These compounds also shift the voltage dependence of activation of KCNQ channels to more negative potentials (Wickenden et al., 2000; Schenzer et al., 2005; Wladyka and Kunze, 2006), whereas we found no shift in the voltage dependence of activation with celecoxib.

The effects of celecoxib on KCNQ5 channels and L-type Ca\(^{2+}\) channels are probably independent. The effects were exerted with slightly different time courses—both the onset of effect and the reversal after washout were temporally distinct for inhibition of L-type currents compared with enhancement of KCNQ5 currents. Furthermore, the KCNQ channel activator flupirtine enhanced KCNQ5 currents without inhibiting L-type Ca\(^{2+}\) currents, and the Ca\(^{2+}\)-channel blocker verapamil was able to inhibit Ca\(^{2+}\) currents without enhancing KCNQ5 currents. Although these results suggest that the activities of these channel types are not inextricably linked, our results do not rule out the possibility of a common signaling intermediate upstream of either effect of celecoxib.

We found previously that either activation of KCNQ channels (with flupirtine) or inhibition of L-type Ca\(^{2+}\) channels (with verapamil) was sufficient to fully dilate rat mesenteric arteries preconstricted with AVP (Henderson and Byron, 2007; Mackie et al., 2008). The observations reported here, that celecoxib can elicit both of these ion channel effects, support the hypothesis that the vasodilatory actions of celecoxib, and hence its lower propensity to induce cardiovascular side effects, result from these actions. It nonetheless remains possible that effects on other ion channels or unrelated effects of celecoxib also contribute to vasodilation or other cardiovascular protective actions.

Celecoxib has not been reported previously to activate KCNQ channels, but diclofenac, an NSAID that is widely used to treat inflammation and pain, has been reported to activate KCNQ2-KCNQ3 (K\(_{V}7.2–7.3\)) heteromeric channels (Peretz et al., 2005). KCNQ2-KCNQ3 channels are found in neurons, in which they mediate the well known “M currents” that regulate neuronal excitation (Jentsch, 2000). The finding that vascular KCNQ5 currents are not enhanced by diclofenac may suggest selectivity in the actions of this drug, allowing it to distinguish among the different KCNQ channels found in different tissues. Many of the known KCNQ channel activators, such as retigabine and flupirtine, can activate either neuronal or vascular KCNQ channels (Mackie and Byron, 2008). Structural analogs of diclofenac were recently identified in which the COX inhibitory activity could be dissociated from the activation of KCNQ2-KCNQ3 channels, suggesting that these activities involve different moieties within the drug structure (Peretz et al., 2007).

Celecoxib has been found previously to inhibit voltage-gated K\(^{+}\) (K\(_{V}\)) channels in several other cell types, including rat retinal neurons (Frolov et al., 2008b) and cardiac myocytes (Frolov et al., 2008a). Although we found that celecoxib enhanced KCNQ currents in MASMCs, we also found that celecoxib suppressed the much larger K\(_{V}\) currents that activate at more positive potentials (Supplemental Fig. 1E), consistent with the effects observed in neurons and cardiac myocytes. In vascular myocytes, suppression of K\(^{+}\) currents typically leads to Ca\(^{2+}\) influx and vasoconstriction, whereas an increase in K\(^{+}\) channel activity is typically associated with vasodilation (Standen and Quayle, 1998). Celecoxib induced dilation of mesenteric arteries, suggesting that the enhancement of KCNQ current and/or the suppression of L-type Ca\(^{2+}\) currents outweigh the inhibitory effect on K\(_{V}\) currents in MASMCs.

Although previous studies have implicated the effects on endothelial function to explain the antihypertensive and vasodilatory actions of celecoxib (Hermann et al., 2003; Widlansky et al., 2003; Klein et al., 2007), we observed concentration-dependent vasodilatory effects of celecoxib on pressurized rat mesenteric arteries that were not attenuated when the endothelium was disrupted. This is consistent with a more direct role of vascular smooth muscle ion channels in the vasodilatory and antihypertensive actions of celecoxib.
Celecoxib (but not rofecoxib) was found previously to inhibit L-type voltage-gated Ca\(^{2+}\) channels in PC12 pheochromocytoma cells (Zhang et al., 2007). Calcium-channel blockers, such as verapamil, diltiazem, and nifedipine, are clinically used antihypertensive agents that selectively inhibit L-type Ca\(^{2+}\) channels in vascular myocytes and consequently dilate arteries. We report here for the first time that celecoxib acts as a calcium-channel blocker in vascular myocytes. The inhibition of L-type Ca\(^{2+}\) channels in VSMCs may account for most of the vasodilatory actions of celecoxib on pressurized mesenteric arteries, considering that maximal dilation was achieved even when KCNQ channel activation was blocked by linopirdine. However, at submaximal concentrations of celecoxib, L-type Ca\(^{2+}\) channel inhibition is combined with enhancement of KCNQ5 K\(^+\) currents. The latter effect should stabilize the membrane potential at more negative voltages. Celecoxib also shifts the threshold for voltage-dependent activation of L-type channels to more positive voltages; therefore, their open probability would be further reduced and the vasodilatory effect enhanced. At concentrations of celecoxib achieved clinically, the combined effects we observed on L-type currents and KCNQ5 currents may represent important cardiovascular side effects that might account for the observed vasodilation and may result in a reduction in blood pressure among patients taking celecoxib compared with those taking rofecoxib (Cho et al., 2003; Aw et al., 2005; Sowers et al., 2005).

In summary, celecoxib, and its analog DMCT, are potent modulators of vascular KCNQ K\(^+\) and L-type Ca\(^{2+}\) channels. These effects are manifested by suppression of VSMC Ca\(^{2+}\) signaling and vasorelaxation, even in the absence of an intact endothelium. These potentially protective effects are not induced by other members of this class of NSAIDs, such as rofecoxib and diclofenac, that have been found to increase the risk of cardiovascular complications. The COX-2-independent ion channel modulatory actions of celecoxib may account for the lower risk of cardiovascular events in patients treated with celecoxib. Understanding the effects of celecoxib or its analogs on vascular ion channels may help to predict potential adverse or beneficial cardiovascular side effects and may lead to alternative uses of these drugs in the treatment of cardiovascular diseases.

References

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SUPPLEMENTAL MATERIAL:

Detailed Methods for patch clamp recording of K⁺ and Ca²⁺ currents:

The standard bath solution for A7r5 cells contained (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 CaCl₂, 1.2 MgCl₂, 5 glucose, pH 7.3. Standard internal (pipette) solution for A7r5 cells contained (in mM): 110 K gluconate, 30 KCl, 5 HEPES, 1 K₂EGTA, pH 7.2. Osmolality was adjusted to 268 mOsm/l with D-glucose. The standard bath solution for MASMCs contained (in mM): 140 NaCl, 5.36 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-Glucose, pH 7.3, 298 mOsm/l. Standard internal (pipette) solution for MASMCs contained (in mM): 135 KCl, 5 NaCl, 10 HEPES, 0.05 K₂EGTA, 1 MgCl₂, 20 D-Glucose, pH 7.2, 298 mOsm/l. To isolate KCNQ currents, 100 μM GdCl₃ (sufficient to block L- and T-type Ca²⁺ channels, non-selective cation channels, and to shift activation of 4-AP-sensitive Kv channels to more positive potentials) was added to external solutions.

To record L-type Ca²⁺ currents in isolation, a Cs⁺-containing internal solution was used. For A7r5 cells, the internal solution contained (in mM): 110 Cs aspartate, 30 CsCl, 5 HEPES, 1 Cs₂EGTA, pH 7.2. Osmolality was adjusted to 268 mOsm/l with D-glucose. For MASMCs internal solution contained (in mM): 135 CsCl, 10 HEPES, 10 Cs₂EGTA, 2.5 MgCl₂, 10 D-Glucose, pH 7.2, 298 mOsm/l. In A7r5 cells L-type Ca²⁺ currents were recorded either in standard bath solution containing 2 mM Ca²⁺ as a charge carrier or in external solution containing 10 mM Ba²⁺ as a charge carrier (in mM): 120 Tris-MES, 10 BaCl₂, 5 HEPES, 1 MgCl₂, pH 7.3, 268 mOsm/l. In MASMC L-type currents were recorded in bath solution containing 10 mM Ba²⁺ as a charge carrier (in mM): 140 NaCl, 2.7 KCl, 10 BaCl₂, 10 HEPES, pH 7.3, 298 mOsm/l.

To record current-voltage (I-V) relationships for Ca²⁺ and K⁺ currents in A7r5 cells simultaneously, or for KCNQ5 current in isolation, 5 s voltage steps from a -74 mV holding potential to test potentials ranging from -94 mV to +36 mV were applied. KCNQ currents in MASMCs were recorded by application of 5 s voltage steps from a -4 mV holding potential to test potentials ranging from -84 to +16 mV. 4-AP-sensitive Kv currents in MASMCs were recorded in the absence of GdCl₃, with 500 ms voltage steps from a -74 mV holding potential to test potentials ranging from -84 to +46 mV. Isolated Ca²⁺ currents in both A7r5 cells and MASMCs were recorded with a 300 ms voltage step protocol from -90 mV holding potential.

Whole-cell Ca²⁺ currents were digitized at 10 kHz and filtered at 5 kHz; peak inward current was measured and normalized by cell capacitance. Whole-cell K⁺ currents or combined K⁺ and Ca²⁺ currents were digitized at 2 kHz and filtered at 200 Hz. The last 2000 points recorded during each voltage step (corresponding to 1000 ms recording time) were averaged and normalized by cell capacitance to obtain end pulse
steady-state $K^+$ current. Stable currents were recorded for at least 15 min prior to drug application.

Time courses of drug application in A7r5 cells (shown in Fig. 1C of the main article) were recorded with a protocol combining 100 ms voltage ramps (from a -74 mV holding potential to +36 mV) followed by 5 sec voltage steps to -20 mV, applied every 15 s. After recording the control current-voltage relationship (with the voltage step protocol described above), the time course protocol was applied for 5 min under control conditions to verify stable currents, and then each drug was applied for 10 min (15-20 min for celecoxib and DMC due to longer time to reach steady-state effect). Leak subtraction was performed by extrapolating the linear portion of the I-V curve at voltages negative to the range where the L-type currents begin to activate (based on linear regression of 19 points measured between -64 mV and -54 mV) (Passmore et al., 2003). Time courses of enhancement of isolated KCNQ currents by celecoxib in both A7r5 cells and MASMCs were recorded by continuous measurement of outward current at -20 mV holding potential.

Whole-cell capacitance was compensated. Cells with series resistance below 30 MΩ were used for recording of endogenous $K^+$ and $Ca^{2+}$ currents at physiological ionic conditions. Cells with series resistance below 15 MΩ were used for recording of overexpressed KCNQ5 currents or $Ca^{2+}$ currents; series resistance was not compensated. With 10 mM $Ba^{2+}$ as a charge carrier, 40% series resistance compensation was used. Cells with abrupt changes in series resistance were discarded. Leak subtraction was performed for some experiments (see figure legends) by extrapolation of the linear portion of the current-voltage (I-V) curve negative to -69 mV as described by Passmore et al. (Passmore et al., 2003). Liquid junction potentials were calculated using Junction Potential Calculator provided by PCLAMP8 software and subtracted off-line.

To analyze the voltage-dependence of channel activation, the conductance was calculated from peak $Ca^{2+}$ currents or steady-state $K^+$ currents and normalized by maximum conductance for each experiment, according to the equation $G=I/(V-E_{rev})$, where $I$ is the steady-state current, $V$ is the step potential and $E_{rev}$ is the reversal potential (Wickenden et al., 2001). $E_{rev}$ for potassium was calculated to be -86 mV using the Nernst equation; $E_{rev}$ for $Ca^{2+}$ current was measured from I-V curves and the averaged value at +40 mV used to calculate conductance for all experiments. Normalized conductance was fitted by a Boltzmann distribution: $G/G_{max}=1/[1+exp(V_{0.5}-V)/s]$, where $G/G_{max}$ is fractional maximal conductance, $V_{0.5}$ is the voltage of half-maximal activation and $s$ is the slope factor.

COX inhibitors were dissolved in dimethylsulfoxide to prepare stock solutions at a concentration of 100 mM. We found that, in aqueous solutions used for recording currents, celecoxib tended to precipitate at concentrations greater than 40 µM, limiting evaluation of concentration-dependent effects to concentrations below 40 µM.

We also found that celecoxib and verapamil were not easily washed out of the perfusion system and recording chamber, requiring an extensive series of washes between experiments to eliminate residual effects of prior drug use: 60 ml of hot detergent solution, followed by 120 ml of hot distilled water, 30 ml of ethanol, and 180 ml of hot water were run through the perfusion system between experiments.
Supplemental Figure 1. **Coxib effects on K⁺ currents in VSMCs.**

A. Endogenous KCNQ5 current was recorded in a single A7r5 cell at -20 mV holding potential. Enhancement of KCNQ5 current by celecoxib reached its maximum effect at 20 µM and started to decline with 30 µM celecoxib (representative of 3 similar experiments). White bars indicate celecoxib application. A break in the recording, for approximately 10 min, is indicated by parallel lines. Dotted line indicates zero current level. B. Celecoxib (10 µM) does not shift the activation curve of endogenous KCNQ5 current in A7r5 cells.

Normalized fractional conductance plots fitted to a Boltzmann distribution; control (filled circles, Vₐ₅₀ = -46.4 ± 0.3 mV, n= 8); 10 µM celecoxib (open circles, Vₐ₅₀ = -43.2 ± 0.5 mV, n= 8). C. Rofecoxib (10 µM) had no effect on endogenous KCNQ current in MASMCs. Mean current-voltage relationships of endogenous KCNQ current isolated and recorded in MASMCs (n=3); control (black circles); 10 µM rofecoxib (white circles); 10 µM linopirdine (black diamonds). D. Diclofenac (10 µM) had no effect on endogenous KCNQ current in MASMCs. Mean current-voltage relationships of endogenous KCNQ currents in MASMCs (n=3); control (black circles); 10 µM diclofenac (white circles); 10 µM linopirdine (black diamonds). E. Delayed rectifier Kᵥ currents in MASMCs were reversibly inhibited by 10 µM celecoxib. Mean current-voltage relationships of delayed rectifier Kᵥ currents (n=3) recorded as described in the Methods section; control (black circles); 10 µM celecoxib (white circles); after washout of celecoxib (black up triangles). F. Celecoxib can not reverse linopirdine-induced inhibition of KCNQ currents. Representative current-voltage relationship of endogenous KCNQ currents recorded in a MASMC; control (black circles); 10µM celecoxib (white circles); 10 µM linopirdine (black up triangles); 10 µM linopirdine together with 20 µM celecoxib (white up triangles); representative of two similar experiments.
Supplemental Figure 2. **Celecoxib but not rofecoxib inhibits AVP-induced Ca\(^{2+}\) spiking in A7r5 cells.**

**A.** Repetitive recordings of Ca\(^{2+}\) spiking following addition of 25 pM AVP measured in confluent fura-2-loaded A7r5 cells plated on 6-well plates. Vehicle, 10 µM rofecoxib, or 10 µM celecoxib was added 20 minutes after addition of 25 pM AVP. AVP-induced Ca\(^{2+}\) spiking was attenuated following the addition of celecoxib (middle panel) but not following the addition of vehicle (top panel) or rofecoxib (bottom panel).

**B.** Bar graph shows that the frequency of AVP-induced Ca\(^{2+}\) spiking was significantly reduced 10 min after addition of celecoxib (n= 3), while vehicle (n= 6) or rofecoxib (n= 3) had no effect during the same time (* p<0.01; paired Student’s t-test, comparing 10 min before treatment with 10 min after treatment in the same wells).
Supplemental Figure 3. Validation of endothelial disruption technique: reduced carbachol-induced dilation of arteries following endothelial denudation.

A. Representative recordings of outer vessel diameter showing dose-dependent dilation of intact and denuded arteries in response to the endothelium-dependent vasodilator, carbachol. In intact arteries, 10 µM carbachol produced nearly complete dilation in all arteries whereas denuded arteries failed to dilate fully even at 100 µM carbachol. B. Summarized carbachol dose-response relationship.
TABLE S1. Enhancement of K⁺ current by celecoxib and DMC in A7r5 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( I_{K, \text{at -20mV}} ) pA/pF</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.27 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>10 ( \mu )M celecoxib</td>
<td>0.68 ± 0.20*</td>
<td>5</td>
</tr>
<tr>
<td>washout</td>
<td>0.32 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0.31 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>10 ( \mu )M DMC</td>
<td>0.62 ± 0.13*</td>
<td>4</td>
</tr>
<tr>
<td>washout</td>
<td>0.55 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

* statistically significant difference from control, \( P < 0.05 \), paired Student's t-test
TABLE S2. Effects of various drugs on L-type Ca\(^{2+}\) current in A7r5 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak of I(_{\text{Ca}}), pA/pF</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-0.43 ± 0.13</td>
</tr>
<tr>
<td>10 µM celecoxib</td>
<td>#</td>
</tr>
<tr>
<td>washout</td>
<td>-0.49 ± 0.21</td>
</tr>
<tr>
<td>control</td>
<td>-0.50 ± 0.21</td>
</tr>
<tr>
<td>10 µM DMC</td>
<td>#</td>
</tr>
<tr>
<td>washout</td>
<td>-0.14 ± 0.07</td>
</tr>
<tr>
<td>control</td>
<td>-0.47 ± 0.09</td>
</tr>
<tr>
<td>10 µM rofecoxib</td>
<td>-0.52 ± 0.09</td>
</tr>
<tr>
<td>control</td>
<td>-0.61 ± 0.12</td>
</tr>
<tr>
<td>10 µM diclofenac</td>
<td>-0.66 ± 0.16</td>
</tr>
<tr>
<td>control</td>
<td>-0.76 ± 0.20</td>
</tr>
<tr>
<td>10 µM flupirtine</td>
<td>-0.66 ± 0.20</td>
</tr>
<tr>
<td>control</td>
<td>-0.85 ± 0.22</td>
</tr>
<tr>
<td>10 µM verapamil</td>
<td>#</td>
</tr>
</tbody>
</table>

# undetectible inward Ca\(^{2+}\) current
TABLE S3. Celecoxib enhances K⁺ current after application of other drugs that inhibit or enhance the current.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{K_{-20mV}}$ pA/pF control</th>
<th>$I_{K_{-20mV}}$ pA/pF during treatment</th>
<th>$I_{K_{-20mV}}$ pA/pF in the presence of celecoxib (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM rofecoxib</td>
<td>0.28±0.08</td>
<td>0.25±0.08</td>
<td>0.59±0.14* n=5</td>
</tr>
<tr>
<td>10 μM diclofenac</td>
<td>0.24±0.03</td>
<td>0.23±0.03</td>
<td>0.61±0.14* n=5</td>
</tr>
<tr>
<td>10 μM flupirtine</td>
<td>0.34±0.07</td>
<td>0.47±0.10*</td>
<td>0.65±0.14* n=5</td>
</tr>
<tr>
<td>10 μM verapamil</td>
<td>0.30±0.06</td>
<td>0.22±0.04*</td>
<td>0.55±0.22 n=5</td>
</tr>
</tbody>
</table>

* statistically significant difference from control, P<0.05, paired Student's t-test

Currents were recorded as described in Figure 1 legend. Treatments were applied for 20 min followed by washout for 20 min and then application of celecoxib for 25 min.