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

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Running title: Celecoxib effects on vascular Ca^{2+} and K^+ channels.

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Nonstandard abbreviations:
AVP, [Arg^8]-vasopressin;
COX, cyclooxygenase;
DMC, 2,5-dimethyl-celecoxib;
MASMC, mesenteric artery smooth muscle cell;
NSAIDs, nonsteroidal anti-inflammatory drugs;
VSMC, vascular smooth muscle cell;

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ABSTRACT

Celecoxib, rofecoxib, and diclofenac are clinically used cyclooxygenase-2 (COX-2) inhibitors, which have been under intense scrutiny since chronic rofecoxib (Vioxx®) 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.

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INTRODUCTION

Celebrex® (celecoxib) and Vioxx® (rofecoxib) are nonsteroidal anti-inflammatory drugs (NSAIDs) which 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. Cyclooxygenase-1 (COX-1) and COX-2 convert arachidonic acid into prostaglandin H_2, which is further converted to a variety of prostanoids, including prostaglandins, thromboxanes, and prostacyclins. Thromboxane A_2, 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 celecoxib, both significantly increased the risk of cardiovascular events (McGettigan and Henry, 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.

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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). Similarly, 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; Klein et al., 2007; Widlansky et al., 2003), which may account for its differential effects on blood pressure relative to other COX-2 inhibitors (Hermann et al., 2003). In this paper 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 Ca^{2+} channels, activation of which induces Ca^{2+} 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 Ca^{2+} signaling and vasomotor tone in resistance arteries.

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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, 1993). For KCNQ5 overexpression studies, subcultured A7r5 cells at 50-70% confluence were transfected with a FLAG-tagged human KCNQ5 DNA sequence (inserted into a pIRES2-EGFP vector) using Lipofectamine® transfection reagent according to the manufacturer's protocol. Confluent subcultures of A7r5 cells were trypsinized and replated on glass coverslips. GFP fluorescent 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. 120 μg/ml Amphotericin B 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).

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Voltage-clamp command potentials were generated using an Axopatch 200B amplifier under control of PCLAMP8 software. Procedures for recording KCNQ K^+ currents and L-type Ca^{2+} currents were essentially as described previously (Brueggemann et al., 2005; Brueggemann et al., 2007; Mackie et al., 2008). Details of the voltage protocols and recording conditions are provided in the Supplemental Materials.

$[Ca^{2+}]_i$ measurements with fura-2—Essentially as described previously (Mani et al., 2009), confluent monolayers of A7r5 cells cultured in 6-well plates were washed twice with control medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM glucose, 11.6 mM HEPES, pH 7.3) and then incubated in the same medium with 1 μM fura-2-AM (Invitrogen/Molecular Probes), 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). All experiments were performed at room temperature. Frequency of spiking was calculated as the number of spikes per min from the time of onset of repetitive Ca^{2+} 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, following 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

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evaluated after pre-constriction of the arteries with 100 pM [Arg^8]-vasopressin (AVP). Following 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). Following 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. Paired 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 values ≤ 0.05 were considered statistically significant.

Materials—Cell culture media were from Gibco-BRL (Gaithersburg, MD) or MediaTech (Herndon, VA). Lipofectamine® reagent was from Invitrogen (Carlsbad, CA). Celecoxib and rofecoxib were from LKT Laboratories, Inc (Saint Paul, MN). Linopirdine, flupirtine, diclofenac sodium salt, collagenase, elastase, [Arg^8]-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

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Jentsch (Leibniz-Institut fuer Molekulare Pharmakologie, Berlin, Germany). 2,5-dimethyl-celecoxib was generously provided by Dr. Axel Schönthal (University of Southern California).

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RESULTS

COX-2-independent effects of celecoxib on vascular smooth muscle cell ion channels

K^+ and Ca^{2+} currents in A7r5 rat aortic vascular smooth muscle cells were recorded simultaneously under approximately physiological ionic conditions, as previously described (Brueggemann et al., 2007). Inward Ca^{2+} currents were recorded at the beginning of 5 s voltage steps, and steady-state K^+ currents 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. 1B-D and Supplemental Table 1) and abolished the inward Ca^{2+} current (Fig. 1A,E). Inhibition of Ca^{2+} 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 celecoxib (Patrono et al., 2001). Neither rofecoxib (10 μM) nor diclofenac (10 μM) significantly affected Ca^{2+} or K^+ currents (Fig1 D,E). However, application of 10 μM celecoxib after washout of diclofenac or rofecoxib still robustly enhanced K^+ currents and inhibited Ca^{2+} currents (Supplemental Tables 2 and 3). A celecoxib analog, 2,5-dimethylcelecoxib (DMC, 10 μM), which does not inhibit COX-2 (IC_{50} > 100 μM (Schönthal et al., 2008)), mimicked both effects of celecoxib: enhancement of K^+ currents and inhibition of Ca^{2+} currents (Fig. 1 D,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 Ca^{2+} currents, abolished the inward Ca^{2+} currents, and also partially inhibited the outward K^+ currents (Fig. 1 D,E and Supplemental Table 3).

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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) channel activity (Brueggemann et al., 2007; Mani et al., 2009). Using conditions to record KCNQ5 currents in isolation, we evaluated the cumulative dose-response relationship for celecoxib (Fig.2 A,B). At concentrations ≥ 1 μM, celecoxib significantly and robustly enhanced native KCNQ5 currents, with a maximal effect (> 3-fold enhancement) at 20 μM celecoxib (Fig. 2B and Supplemental Fig. 1A). The enhancement of KCNQ5 current amplitude by 10 μM celecoxib was not associated with a shift in the voltage-dependence of activation (Supplemental Fig. 1B).

KCNQ5 channels are also expressed in arterial myocytes and KCNQ currents have recently been found to contribute to resting membrane potential in rat mesenteric artery smooth muscle cells (MASMCs) (Mackie et al., 2008). We found that KCNQ currents in MASMCs were robustly enhanced by 10 μM celecoxib. Following acute treatment with 10 μM celecoxib, the KCNQ currents were stably enhanced, and this effect was reversed following washout of celecoxib (Fig. 2 C,D). As reported previously (Mackie 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). Neither rofecoxib (10 μM) nor diclofenac (10 μM) affected the KCNQ currents in MASMCs (Supplemental Fig. 1 C,D).

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 two orders of magnitude larger than the native currents (compare scales in Figs. 2A and 2E), recordings reflect predominantly the activity of the human channels

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with little contribution of the native channels. We found that celecoxib robustly enhanced human KCNQ5 currents, and this effect was completely reversed following washout of celecoxib (Fig 2 E,F).

Celecoxib effects on L-type Ca^{2+} channels

Using conditions to record L-type Ca^{2+} currents in isolation, we observed a concentration-dependent and reversible suppression of the currents by celecoxib (IC_{50} = 8.3 ± 1.3 μM, Fig. 3 A,B). Celecoxib (10 μM) induced a pronounced inhibition of Ca^{2+} current amplitude, along with a significant positive shift of the activation curve (~12 mV) (Fig. 3C). A similar effect was observed when Ba^{2+} was used as the charge carrier (Fig. 3D). In both cases the shift in activation was reversed following washout of celecoxib (data not shown).

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

Functional effects of KCNQ5 channel activation and/or inhibition of L-type Ca^{2+} channels

Both KCNQ5 channels and L-type Ca^{2+} channels are important for functional responses of VSMCs. We have previously demonstrated involvement of both channel types in the stimulation of repetitive Ca^{2+} spiking in A7r5 cells treated with a physiological concentration of arginine vasopressin (AVP) (Brueggemann et al., 2007; Byron, 1996; Mani et al., 2009). Celecoxib (10 μM), but 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

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(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% of maximum outer diameter; Figure 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 previously shown 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

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prevented by linopirdine, celecoxib produced near full relaxation of all arteries tested (95 ± 1.4% of maximal dilation, n=3; a representative recording is shown in Figure 5E).

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DISCUSSION

Our findings may help explain why celecoxib is a safer drug in terms of cardiovascular complications when compared with rofecoxib or diclofenac. We found that, unlike rofecoxib or diclofenac, celecoxib potently enhances KCNQ potassium 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 anti-cancer 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 impact on 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

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from 20 μM to 30 μM in VSMCs (Fig. 3B and Supplemental Fig. 1A) was also observed in HEK293T 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 or DMC on KCNQ5 or 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 thought 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 (Schenzer et al., 2005; Wickenden et al., 2000; 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.

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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 previously found 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 pre-constricted 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 previously been reported to activate KCNQ channels, but diclofenac, an NSAID that is widely used to treat inflammation and pain, has been reported to activate KCNQ2-KCNQ3 (Kv7.2-7.3) heteromeric channels (Peretz et al., 2005). KCNQ2-KCNQ3 channels are found in neurons where 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).

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Celecoxib has previously been found 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 effects on endothelial function to explain the antihypertensive and vasodilatory actions of celecoxib (Hermann et al., 2003; Klein et al., 2007; Widlansky et al., 2003), 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 celecoxib's vasodilatory and antihypertensive actions. Celecoxib (but not rofecoxib), was previously found 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 which 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

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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 result in a reduction in blood pressure among patients taking celecoxib compared with those taking rofecoxib (Aw et al., 2005; Cho et al., 2003; Sowers et al., 2005).

In summary, celecoxib, and its analog DMC, 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.

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FOOTNOTE

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FIGURE LEGENDS

Figure 1. **Enhancement of K^+ current and inhibition of Ca^{2+} current by celecoxib and DMC, but not rofecoxib or diclofenac, in A7r5 cells.**

A. Representative traces of whole cell K^+ and Ca^{2+} currents measured in a single A7r5 cell (capacitance (C) = 215 pF); control (i) and in the presence of 10μM celecoxib (ii). Inward Ca^{2+} currents, activated at the beginning of the voltage steps, are shown in insets on an expanded scale for clarity. **B**. I-V curves, corresponding to traces in panel A, for steady-state K^+ current (filled symbols) and peak inward Ca^{2+} current (open symbols) in control (circles), in the presence of 10 μM celecoxib (up triangles), and after washout of celecoxib (down triangles). Leak subtraction was performed. **C**. Corresponding time course of inhibition of the peak inward Ca^{2+} current measured with 100 msec voltage ramps from -74 mV to +36 mV (open circles) and K^+ current activation (filled circles) measured as average currents at the ends of 5 sec voltage steps to -20 mV. Open bar indicates application of 10 μM celecoxib. A 10 min break in the time course recording is indicated. Leak subtraction was performed. Inset shows initial changes in the currents upon celecoxib application on an expanded scale. **D & E**. K^+ current densities at -20 mV and peak inward Ca^{2+} current densities, respectively, recorded in the presence of 10 μM celecoxib (CXB, n=5) 10 μM dimethylcelecoxib (DMC, n=4), 10 μM rofecoxib (R, n=5), 10 μM diclofenac (D, n=5), 10 μM flupirtine (F, n=6) and 10 μM verapamil (V, n=5). * significant difference from control ($p<0.05$, paired Student's t-test). # absence of detectable inward current. Outward K^+ currents were measured at the ends of 5s voltage steps to -20 mV, whereas the L-type currents were measured as the peak inward current during the voltage step that yielded the maximal inward current (voltage steps ranging from -74 to +36 mV). In both cases, leak

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subtraction was applied and measurements were made after the effects of the treatments had stabilized (between 10 and 20 min, depending on the treatment).

Figure 2. **Enhancement of KCNQ currents in A7r5 cells and freshly isolated mesenteric artery myocytes.**

A. I-V relationships of isolated endogenous KCNQ5 current in A7r5 cells. Control (black circles, n=14); 1 μM celecoxib (dark gray circles, n=9); 10 μM celecoxib (light gray up triangles, n=9); 20 μM celecoxib (white down triangles, n=8). Leak subtraction was performed. **B**. Cumulative celecoxib dose-response: KCNQ5 current densities measured at -20 mV normalized to control current densities at -20 mV. * significant difference from control (1 μM celecoxib, $p<0.05$, n=9; 5 μM celecoxib, $p<0.05$, n=5; 10 μM celecoxib, $p<0.001$, n=9; 20 μM celecoxib, $p<0.001$, n=8; 30 μM celecoxib, $p<0.001$, n=3; paired Student's t-test). **C**. I-V relationships of KCNQ currents in MASMCs; control (black circles); 10 μM celecoxib (white circles); after washout of celecoxib (black up triangles); 10 μM linopirdine (black diamonds). * significant difference from control (n=5, $p<0.05$, paired Student's t-test). **D**. Representative time course of KCNQ current recorded at -20 mV in a single MASMC (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 (black circles); 10 μM celecoxib (white circles); after washout of celecoxib (black up triangles). * 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).

Figure 3. **Celecoxib inhibits L-type Ca^{2+} currents in A7r5 cells and MASMCs.**

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A. Representative I-V curves of Ca^{2+} currents recorded in a single A7r5 cell (2 mM Ca^{2+} as a charge carrier; C = 140 pF); control (black circles); 1 μM celecoxib (white circles), 3 μM celecoxib (white up triangles), 10 μM celecoxib (white down triangles), 30 μM celecoxib (white diamonds). **B**. Cumulative dose-response plot of peak inward Ca^{2+} currents, normalized to control Ca^{2+} current, and fit to a Hill equation (IC_{50} = 8.3 ± 1.3 μM). * significant difference from control (n= 3-12, $p<0.001$, one-way ANOVA followed by pair-wise comparison with Holm-Sidak method). **C & D**. Normalized fractional conductance measured in A7r5 cells with 2 mM Ca^{2+} (n=11) and 10 mM Ba^{2+} (n=5), respectively, fitted to a Boltzmann distribution; control (filled circles, $V_{0.5}$ = -18.8 ± 0.2 mV in Ca^{2+}; $V_{0.5}$ = -20.9 ± 0.3 mV in Ba^{2+}); 10 μM celecoxib (open circles, $V_{0.5}$ = -6.8 ± 0.3 mV in Ca^{2+}; $V_{0.5}$ = -12.1 ± 0.7 mV in Ba^{2+}). **E**. Mean I-V curves: L-type Ca^{2+} currents recorded in MASMCs (n= 3) with 10 mM Ba^{2+} as a charge carrier; control (filled circles); 10 μM celecoxib (open circles); after washout of celecoxib (filled up triangles). **F**. Normalized fractional conductance measured in MASMCs, fitted to a Boltzmann distribution; control (filled circles, $V_{0.5}$ = -6.5 ± 0.3 mV, n= 3); 10 μM celecoxib (open circles, $V_{0.5}$ = -2.0 ± 0.4 mV, n= 3).

Figure 4. **Celecoxib, but not rofecoxib, abolishes AVP-induced Ca^{2+} spiking in A7r5 cells.**

A. Ca^{2+} spiking in a confluent monolayer of fura-2-loaded A7r5 cells treated with 25 pM AVP. Representative traces show absence of AVP-induced Ca^{2+} spiking with simultaneous addition of celecoxib (10 μM, middle panel) but not with addition of vehicle (top panel) or rofecoxib (10 μM, bottom panel). AVP-stimulated Ca^{2+} spiking was absent in 8 out of the 9 wells treated with celecoxib, whereas all the wells treated with vehicle or rofecoxib spiked robustly. **B**. Bar graph shows significantly reduced frequency of AVP-induced Ca^{2+} spiking in the presence of celecoxib

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(n=3) compared to control (n=6) or rofecoxib (n=3) (* $p < 0.05$; one-way ANOVA followed by pair-wise comparison with Holm-Sidak method).

Figure 5. **Celecoxib dilates pre-constricted 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 Methods) was used to restore 100 pM AVP-induced constriction following treatment with the endothelium-dependent dilator carbachol (see Supplemental Figure 3). **B**. Celecoxib dose-response relationship for intact and endothelium-denuded arteries. **C**. Representative traces (on the left) illustrating the inability of 20 μM rofecoxib (top) and 20 μM diclofenac (bottom) to dilate arteries pre-constricted with 100 pM AVP. Celecoxib (20 μM) fully dilated the same arteries when added after either rofecoxib or diclofenac. The bar graph to 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 pre-constricted with 100 pM AVP; representative of 3 similar experiments. **E**. Representative recording demonstrating that celecoxib (20 μM) also produced full dilation of arteries pre-constricted with the selective KCNQ channel blocker linopirdine (10 μM); representative of 3 similar experiments.

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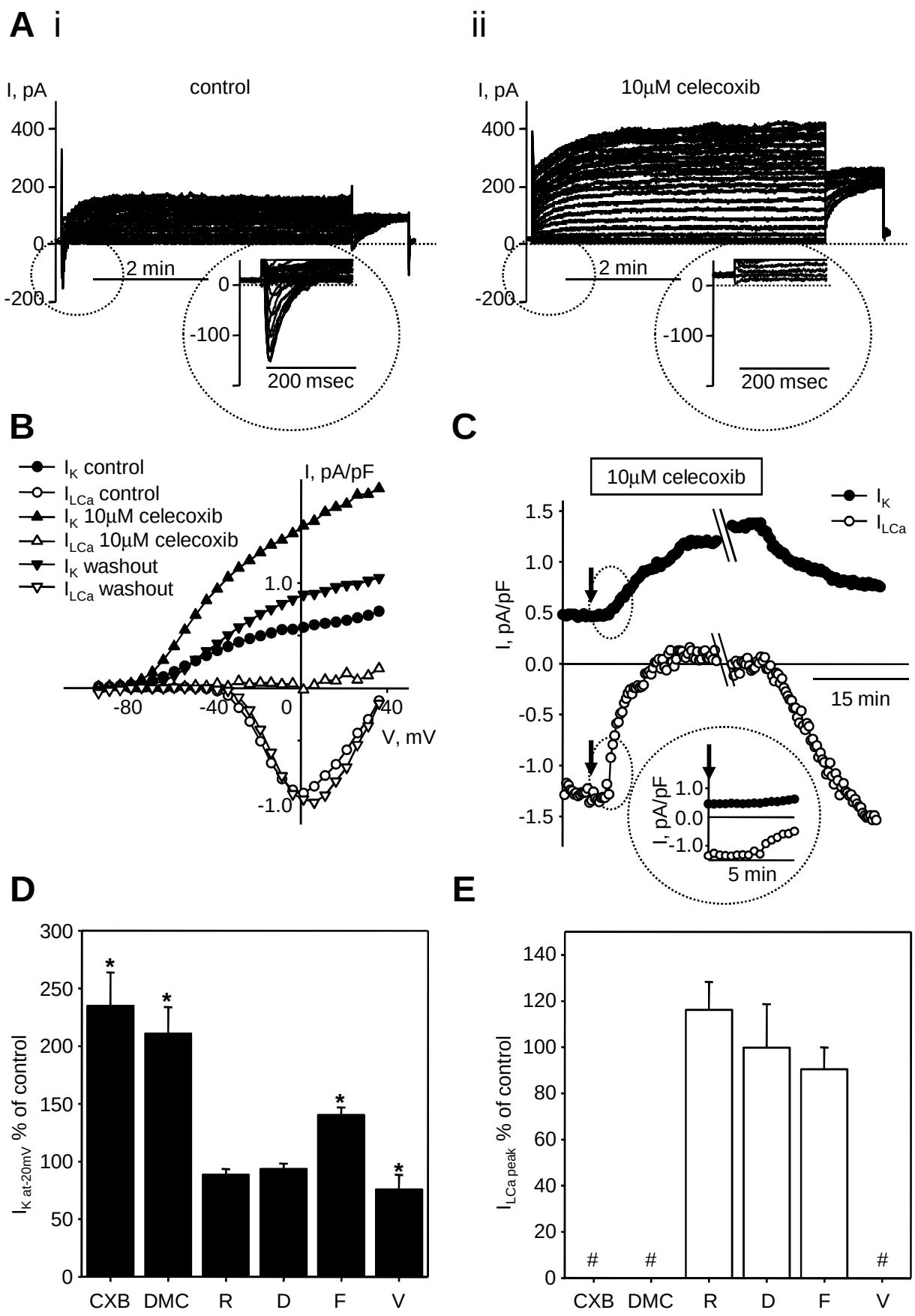

Figure 1

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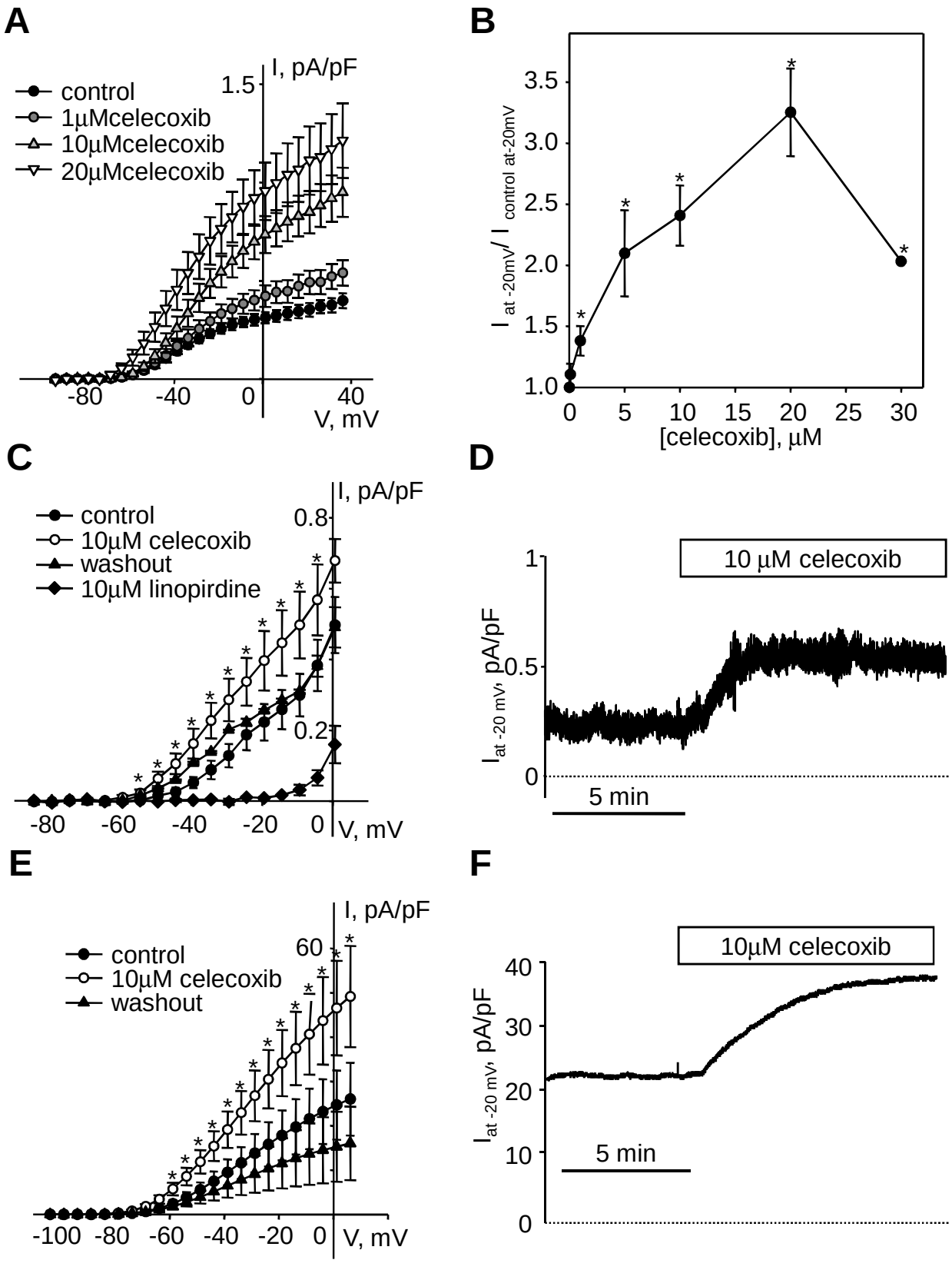

Figure 2

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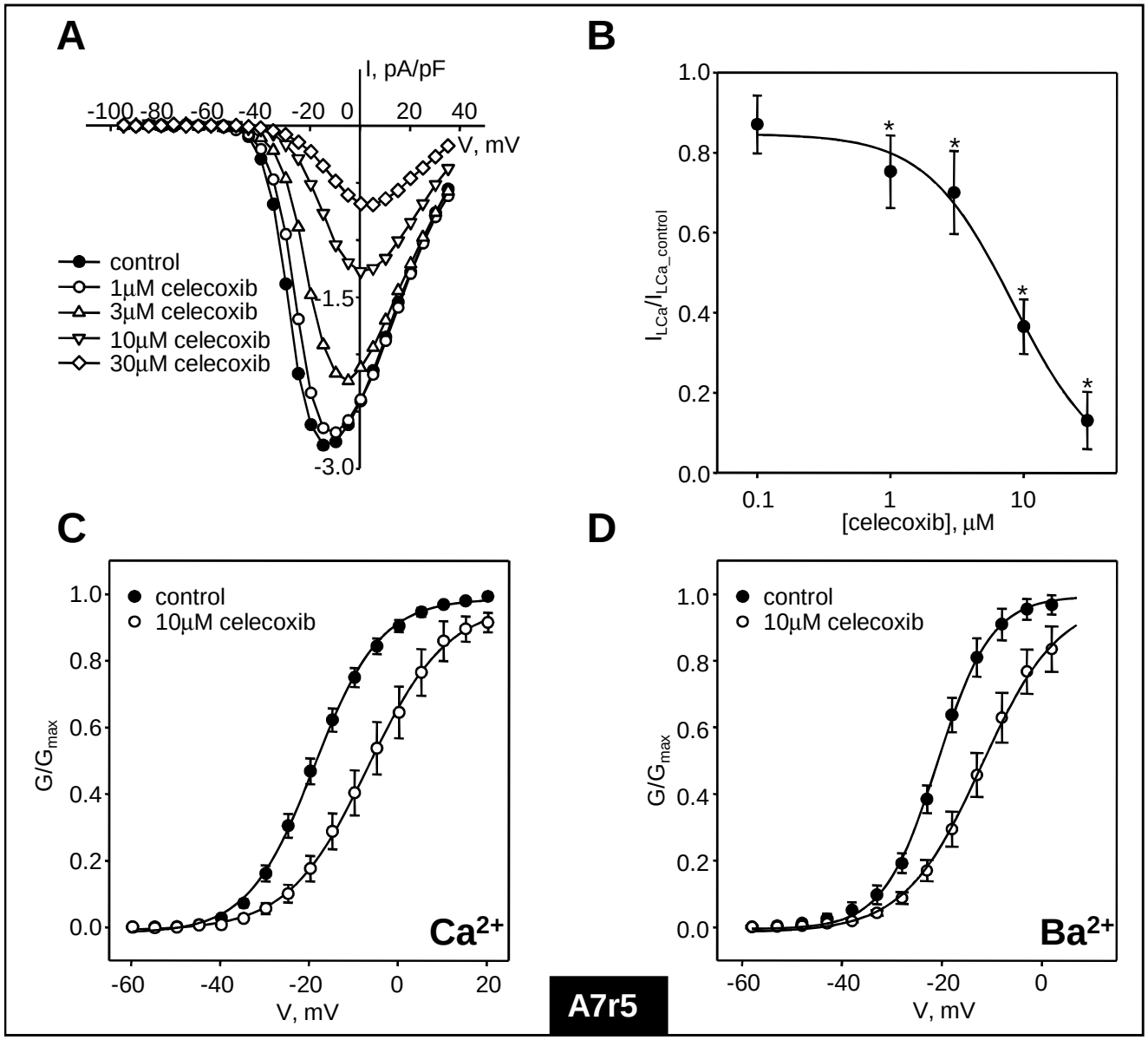

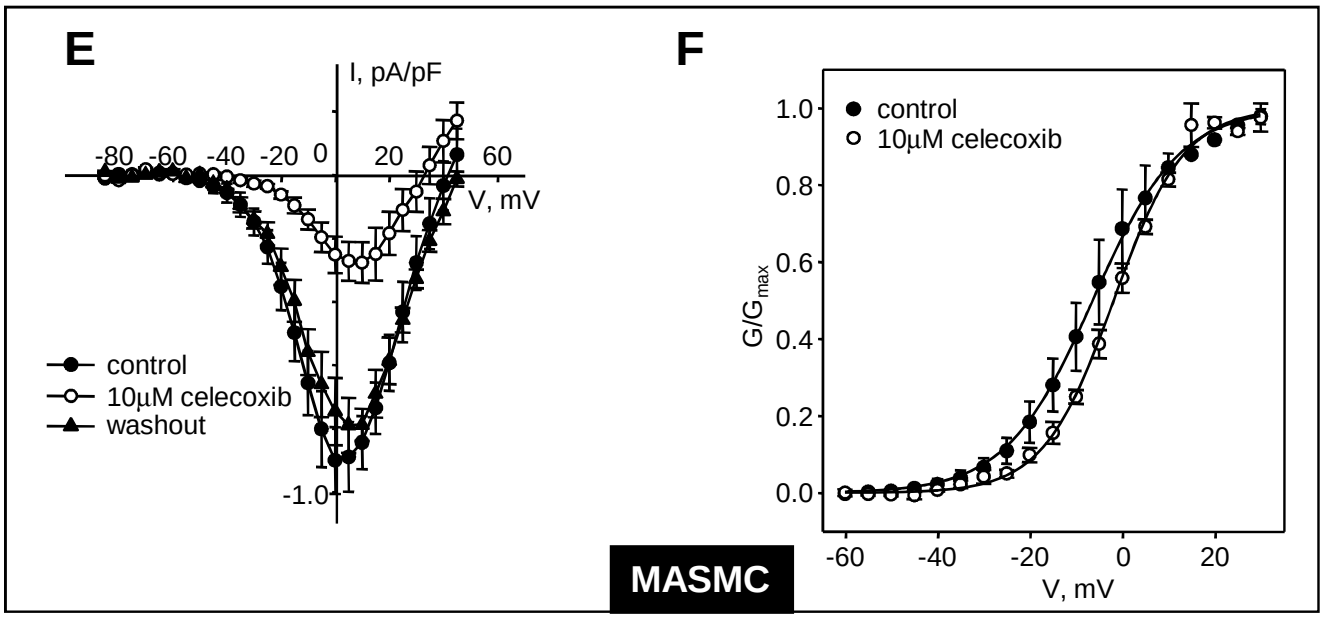

Figure 3

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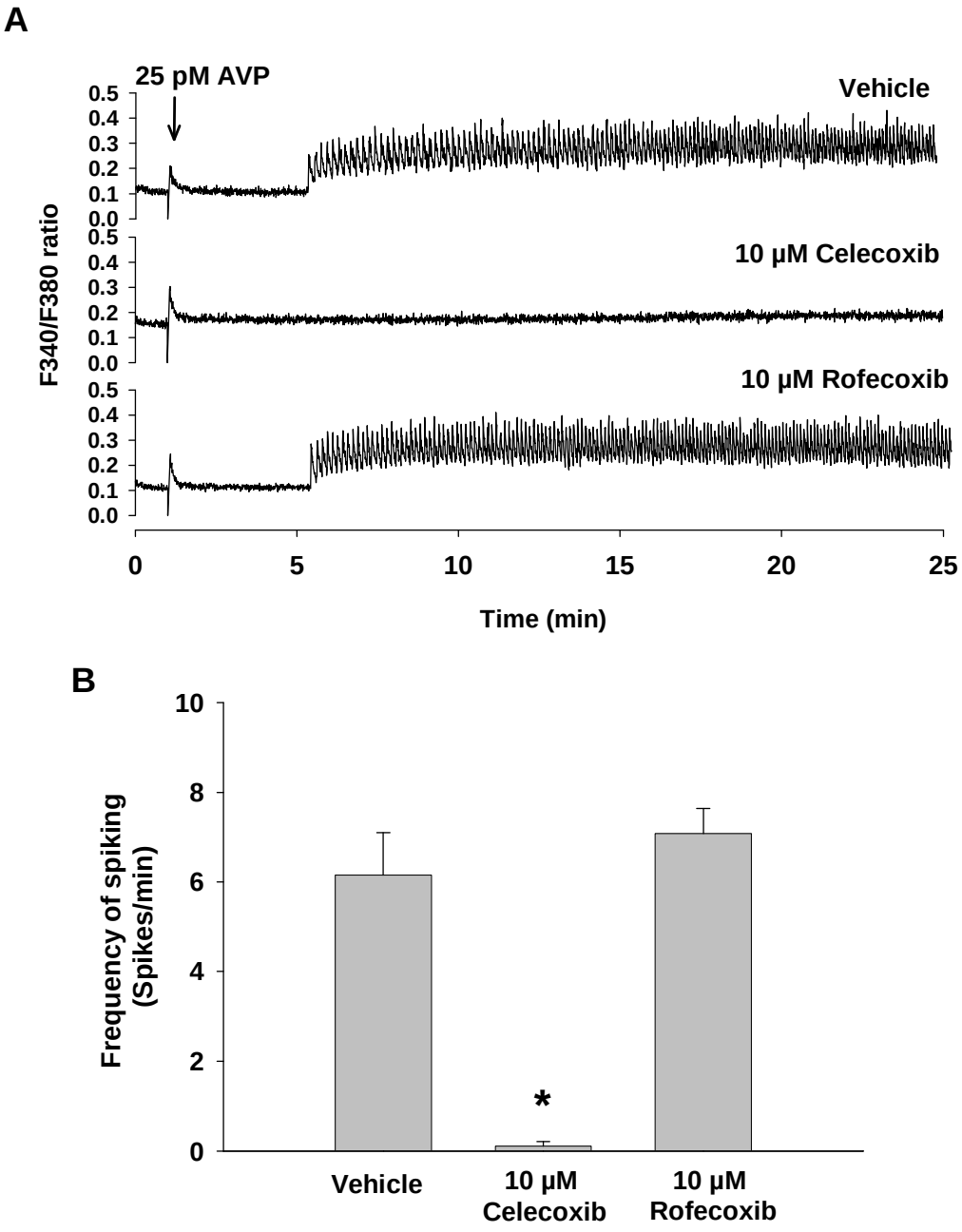

Figure 4

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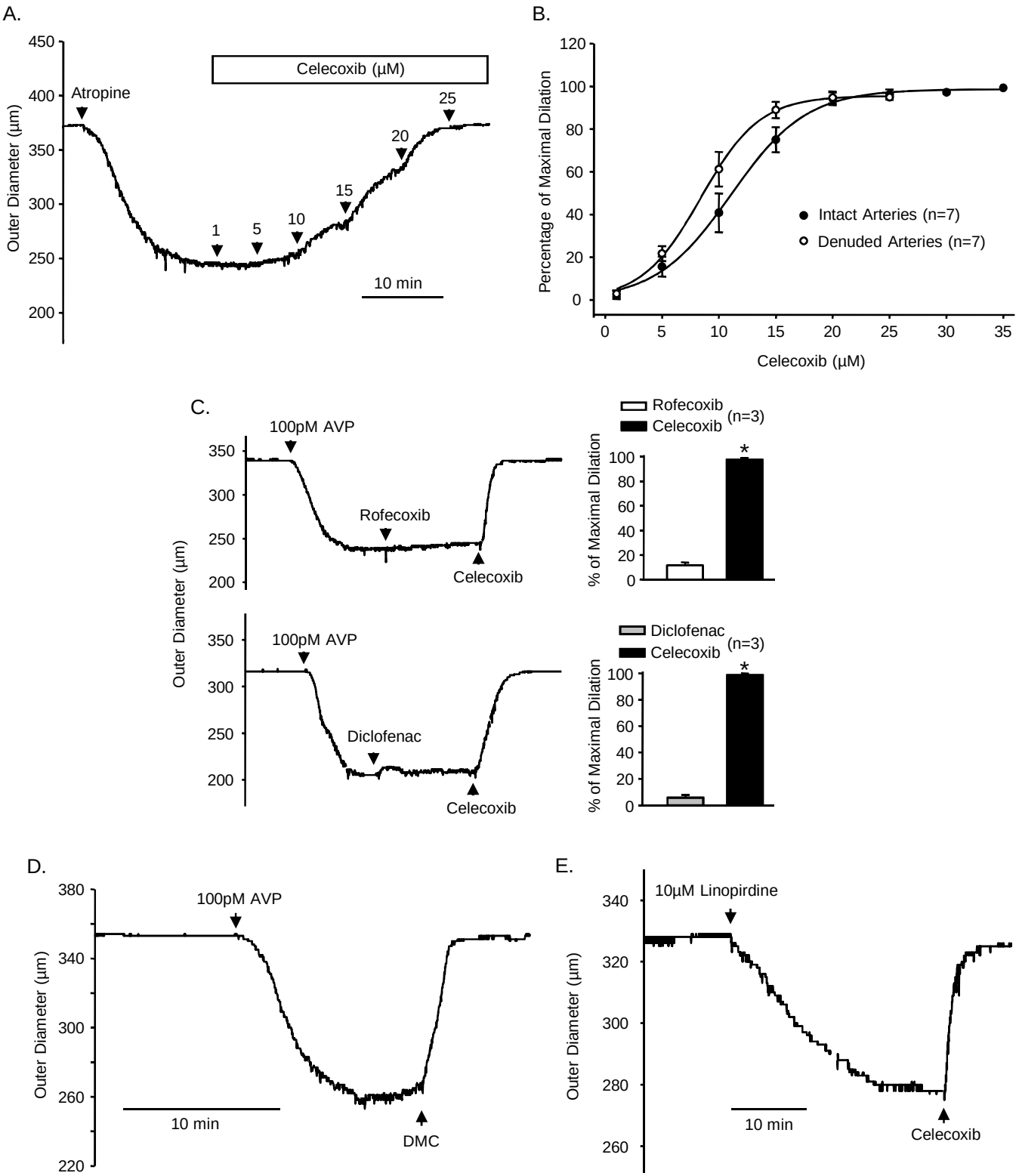

Figure 5