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Multiple mechanisms of vascular smooth muscle relaxation by the activation of Proteinase-Activated Receptor 2 in mouse mesenteric arterioles

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1 Activation of PAR2 in second-order mesenteric arteriole (MA) rings from C57BL/6J, NOS3 (−/−) and PAR2 (−/−) mice was assessed for the contributions of NO, cyclo-oxygenases, guanylyl cyclase, adenylyl cyclase, and of K^+ channel activation to vascular smooth muscle relaxation.

2 PAR2 agonist, SLIGRL-NH_2 (0.1 to 30 μM), induced relaxation of cirazoline-precontracted MA from C57BL/6J and NOS3 (−/−), but not PAR2 (−/−) mice. Maximal relaxation (E_{max}) was partially reduced by a combination of L-^{G}N-nitroarginine methyl ester (L-NAME), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and indomethacin. An ODQ/L-NAME/indomethacin resistant relaxation was also caused by trypsin (30 nM) in PAR2 (+/+), but not in PAR2 (−/−) mice. Relaxation was endothelium-dependent and inhibited by either 30 mM KCl-precontraction, or pretreatment with apamin, charybdotoxin, and their combination; iberiotoxin did not substitute for charybdotoxin nor did scyllatoxin substitute fully for apamin.

3 Tetraethylammonium (TEA), glibenclamide, tetrodotoxin, 17-octadecynoic acid, carboxy-2-phenyl-4,4,5,5,-tetramethyl-imidazoline-1-oxyl-3-oxide, SQ22536, carbenoxolone, arachidonyl trifluoromethyl ketone, 7-nitroindazole, N-(3-(aminomethyl)benzyl)acetamidine (1400W), N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398) and propanolol did not inhibit relaxation. 4-aminopyridine significantly increased the potency of SLIGRL-NH_2. A combination of 30 μM $BaCl_2$ and 10 μM ouabain significantly reduced the potency for relaxation, and in the presence of L-NAME, ODQ and indomethacin, E_{max} was reduced.

4 We conclude PAR2-mediated relaxation of mouse MA utilizes multiple mechanisms that are both NO-cGMP-dependent, and -independent. The data are also consistent with a role for endothelium-dependent hyperpolarization of vascular smooth muscle that involves the activation of an apamin/charybdotoxin-sensitive K^+ channel(s) and, in part, may be mediated by K^+.

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Abbreviations: 4-AP, 4-aminopyridine; 7-NI, 7-nitroindazole; 17-ODYA, 17-octadecynoic acid; 1400W, N-(3-(aminomethyl)-benzyl)acetamidine; AACOCF3, arachidonyl trifluoromethyl ketone; BK_{Ca}, large (Big) conductance Ca^{2+}-activated K^+ channels; Carboxy-PTIO, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; ChTX, charybdotoxin; CYP, cytochrome P450s; EDH, endothelium-dependent hyperpolarization; EDHF, endothelium-derived hyperpolarization factor; EET, epoxyeicosatrienoic acids; IbTX, iberiotoxin; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channels; K_{ATP}, ATP-sensitive K^+ channels; K_{ir}, inwardly-rectifying K^+ channels; K_V, voltage-dependent K^+ channels; L-NAME, L-^{G}N-nitroarginine methyl ester; MA, second-order mesenteric arterioles; NOS1, NOS isoform 1; neuronal NOS; NOS2, NOS isoform 2; inducible NOS; NOS3, NOS isoform 3; endothelial NOS; NOS3 (−/−), NOS3 transgenic deletion; eNOS-gene 'knockout'; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; PAR2 (−/−), PAR2 transgenic deletion; PAR2-gene 'knockout'; ScTX, scyllatoxin; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channels; TEA, tetraethylammonium; TTX, tetrodotoxin

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Introduction

Proteinase-activated receptors (PARs) are members of the heptahelical family of G-protein coupled receptors that contain seven putative transmembrane domains. Their activation results in an increased intracellular calcium concentration $[Ca^{2+}]_i$ (Rasmussen *et al*., 1991; Nystedt *et al*., 1994; 1995; Molino *et al*., 1997). Proteolysis of the extracellular N-terminus of PARs by serine proteases, including thrombin and/or trypsin, reveals a characteristic tethered-ligand peptide sequence that is believed to interact with the extracellular loop 2 of the receptor to initiate receptor activation (Vu *et al*., 1991; Coughlin *et al*., 1992). Specific tethered-ligand sequences have been identified for each of the four PAR family members (PAR1, PAR2, PAR3, PAR4). Synthetic peptides based on the N-terminal proteolytically revealed tethered ligand sequences have been shown to be specific agonists for PAR1, PAR2, and PAR4 (Nystedt *et al*., 1994; Blackhart *et al*., 1996; Hollenberg *et al*., 1997; Xu *et al*., 1998; Kahn *et al*., 1998; Kawabata *et al*., 1999; Hollenberg & Saifeddine, 2001).

In whole tissues, PAR2-activating peptides (PAR2-APs) cause endothelium-dependent relaxation of blood vessels principally *via* activation of the nitric oxide synthase (NOS) expressed by endothelial cells (NOS3), presumably a consequence of elevated $[Ca^{2+}]_i$ in these cells (Saifeddine *et al*., 1996; Moffatt & Cocks, 1998; Sobey *et al*., 1999). However, both endothelium-dependent contraction (rat pulmonary artery, and human umbilical vein), and endothelium-independent contraction (mouse renal arteries) have been reported as vascular responses to SLIGRL-NH_2; the former *via* a putative non-PAR2 mechanism (Saifeddine *et al*., 1998; Roy *et al*., 1998), and the latter, supposedly, *via* PAR2 localized to vascular smooth muscle (Moffatt & Cocks, 1998). With respect to haemodynamics *in vivo*, pretreatment of anaesthetized wild-type PAR2 mice with a NOS inhibitor reduced the duration of the hypotension produced by SLIGRL (Damiano *et al*., 1999).

The vasodilator activity of endogenous peptides, bradykinin and Substance P and of acetylcholine (ACh) are mediated by endothelium-dependent mechanisms (Furchgott & Zawadzki, 1980; De Mey *et al*., 1982; Cherry *et al*., 1982; Regoli *et al*., 1984) and both NO/guanosine 3′5′ cyclic monophosphate (cyclic GMP)/PGI_2/adenosine 3′5′ cyclic monophosphate-dependent or -independent mechanisms have been described (Rapoport & Murad, 1983; Ignarro *et al*., 1987; Illiano *et al*., 1992; Enokibori *et al*., 1994; You *et al*., 1995). Under *in vitro* conditions wherein NO/cyclic GMP and COX signal transduction pathways are inhibited, endothelium-dependent vascular smooth muscle relaxation by these agonists has been associated with vascular smooth muscle membrane hyperpolarization (Chen *et al*., 1988). The factor(s) that mediate these hyperpolarizing activities have been referred to as endothelium-derived hyperpolarizing factors (EDHF) and have been the subject of many recent reviews (Quilley & McGiff, 2000; Hecker, 2000; Ding *et al*., 2000a; McGuire *et al*., 2001).

Epoxyeicosatrienoic acids (EETs), products of arachidonic acid metabolism by cytochrome P450s (CYPs), are candidates for being an EDHF (Popp *et al*., 1996; Dong *et al*., 1997; Fisslthaler *et al*., 1999). Endothelial- released K^+ also has the characteristics of an EDHF as it has been demonstrated to activate K^+ inward rectifying channels, and the Na^+,K^+ ATPase to initiate hyperpolarization (Edwards *et al*., 1998). Other mechanisms that have been proposed to mediate the EDHF phenomenon include electrical coupling of the endothelium and vascular smooth muscle through myoendothelial gap junctions (Chaytor *et al*., 1998), and a 'residual NO effect' from incomplete inhibition of guanylyl cyclase (sGC) (i.e. with 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)) and NOS (Kemp & Cocks, 1997; Cohen *et al*., 1997; Vanheel & Van de Voorde, 2000; Ge *et al*., 2000). EDHFs, by definition, cause membrane hyperpolarization. Inhibition of relaxation by raising extracellular K^+ (from 4.7 mM by an additional 30 to 60 mM) or, treatment with a combination of apamin and charybdotoxin, to inhibit small, intermediate and large conductance Ca^{2+} activated K^+ channels, support the role of K^+ channels mediating relaxation by vascular smooth muscle hyperpolarization (Adeagbo & Triggle, 1993; Waldron & Garland, 1994; Hashitani & Suzuki, 1997; Edwards *et al*., 1998; Doughty *et al*., 1999; Quignard *et al*., 2000).

In mice containing a disrupted gene for NOS3 (NOS3 −/−), ACh mediates the endothelium-dependent relaxation of mesenteric arterioles and saphenous arteries through NOS- and COX- inhibitor-insensitive mechanisms and these relaxant effects are greater than found in wild-type NOS3 gene expressing mice (Waldron *et al*., 1999; Ding *et al*., 2000b). The hypothesis underlying the work we report here is that the activation of PAR2 also relaxes mouse mesenteric arterioles by a mechanism that is insensitive to inhibitors of NOS, sGC and COX, and has the characteristics of a relaxant effect mediated by endothelium-dependent hyperpolarization (EDH).

Methods

Materials

Peptides were synthesized by the Peptide Synthesis Core Facility (University of Calgary) and were purified by preparatory HPLC (>95% purity). Stock solutions of all peptides, trypsin and their dilutions were made in 25 mM N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) buffer pH 7.4. The concentration of peptide and purity of all peptide stock solutions were verified by quantitative amino acid analysis and mass spectrometry. Trypsin from Bovine pancreas (Type III EC3.4.21.4; 10,400 u mg^{-1} protein) was purchased from Sigma. The molar concentrations of trypsin solutions were estimated by a conversion factor of 1 u ml^{-1} = 2 nM. 4-aminopyridine, 1400W (N-(3-(aminomethyl)benzyl)acetamidine), apamin, arachidonyl trifluoromethyl ketone (AACOCF3), atropine, $BaCl_2$, carbenoxolone, carboxy-2-phenyl-4,4,5,5,-tetramethyl-imidazoline-l-oxyl-3-oxide (carboxy-PTIO), charybdotoxin, chloropheniramine, glibenclamide, iberiotoxin, indomethacin, L-NAME, 7-nitroindazole (7-NI), NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide), 17-octadecynoic acid (17-ODYA), ouabain, 1-H-[1,2,4]-oxadiazolo [4,3,-a] quinoxalin-1-one (ODQ), propanolol, SQ22536 (9-(tetrahydro-2-furamyl)-9H-purin-6-amine), and tetraethylammonium (TEA) were purchased from Sigma. Tetrodotoxin (TTX) (without citrate) was purchased from Alamone (U.K.). Scyllatoxin (Leiurotoxin I) was purchased from Alexis Biochemicals (San Diego, CA, U.S.A.). SR140333 and

SR48968 were gifts from Sanofi Research Institute (France) to Dr Nathalie Vergnolle (University of Calgary).

Animal sources

Wild-type genetic background strains (C57BL/6J; The Jackson Laboratory, Bar Harbor, ME, U.S.A.) were used as control animals and were matched for sizes when experiments were conducted. Protocols for using animals were approved by the Animal Resources Committee at the University of Calgary and were in accordance with the guidelines of the Canadian Council for Animal Care in Research. NOS3 (−/−) mice were purchased from Jackson Laboratories. PAR2 (−/−), in a C57BL/6 background, were obtained through the courtesy of Dr Patricia Andrade-Gordon (R. W. Johnson Pharmaceutical Research Institute, Spring House, PA, U.S.A.). Initial studies with the PAR2 (−/−) genetic background strain of mice demonstrated pharmacological profiles (cirazoline, KCl, ACh, peptides) that were the same as those obtained with C57BL/6J mice.

Bioassay preparation

Male mice (8 to 14 weeks of age; 20–40 g) were sacrificed by cervical dislocation and then the intestinal tissues were removed, and placed in ice-cooled modified Kreb's buffer. With the aid of a dissection light microscope, the second-order arterioles (arteriole segments separated by two branch points from the superior mesenteric artery) were carefully isolated. Modified Kreb's-bicarbonate solution was gassed with 95% O_2/5% CO_2 (pH 7.4 at 37°C) and had the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 0.87, KH_2PO_4 0.86, $CaCl_2$ 2.5, D-glucose 10, and $NaHCO_3$ 25. Four to eight rings (1–2 mm lengths) were cut from isolated blood vessels to be used for isometric tension measurements. Rings were suspended between a micropositioner and force transducer with gold-plated tungsten wires (20 μm diameter) in a Mulvany-Halpern style organ bath (3–5 ml volume; 610 multi-myograph system; J.P. Trading, Copenhagen, Denmark). Isometric tension was recorded online *via* serial connection to a computer hard drive at a rate of 1 Hz. Resting tension (1 mN) was fixed for an initial equilibration period of 1 h. Software for data acquisition and analysis (Myodaq 2.01/Myodata 2.02) were designed by J.P Trading for the 610 multi-myograph system.

Bioassay protocols

Tissues were routinely contracted with 60–120 mM KCl to determine their viability. Then tissues were submaximally (50–75% of E_{Max}) precontracted with cirazoline (0.1 μM) and the response to either a single dose (10 μM) or a cumulative concentration range (1 nM to 10 μM) was determined for ACh to assess the responsiveness of the endothelium. Tissues from wild-type animals and PAR2 (−/−) mice typically responded to ACh with >80% reversal of precontracted tension whereas maximal relaxation to ACh in MA from NOS3 (−/−) mice was about 60% (data not shown). As part of a separate study, the precontracted aortae from NOS3 (−/−) animals failed to relax when treated with ACh, and thus, confirmed the NOS3-deficient phenotype. In experiments designed to determine the role of the endothelium in the relaxation effects, a stainless steel 40 μm diameter wire was used to rub the interior of a mounted ring. These tissues were deemed to be endothelium-denuded only if there was absolutely no immediate relaxation response to the application of 10 μM ACh. Equilibration periods between treatments and the incubation of inhibitors with tissues were 20 min each. Relaxation activity was determined by the reversal of blood vessel pre-contraction induced by either 0.1 μM cirazoline or 30 mM KCl. Contractions by cirazoline were <75% of a maximal response, as determined by 10 μM cirazoline application to non-pretreated MA, and contractions by 30 mM KCl were about 60% of maximum contraction response caused by 120 mM KCl. The isometric tension (mN) produced by 0.1 μM cirazoline and 30 mM KCl were not significantly different (data not shown). Either single doses (10 μM) or cumulative concentration-response relationships were determined for SLIGRL-NH_2 and the reversed-sequence control peptide LRGILS-NH_2.

Data analysis and statistics

Arteriole ring relaxant responses are reported as a percentage of the initial tension (% initial tension) generated by either 0.1 μM cirazoline or 30 mM KCl. The pD_2 values from SLIGRL-NH_2 induced relaxation were determined from individual concentration-response relationships by manual graph interpolation. Values represent the means ± s.e.mean (error bars) for 3–16 animals with 2–4 measurements per animal. The comparisons of mean values for each parameter were made using analysis of variance calculations (ANOVA) and were followed by Student-Newman Keuls *post-hoc* tests (GraphPad Instat 2.01). Differences between means were considered significant if the Student-Newman Keuls *post-hoc* test indicated a P value less than 0.05.

Results

Activation of endothelial PAR2 causes relaxation of second-order mesenteric arterioles via *a mechanism that is insensitive to inhibitors of NOS, sGC, and COX in NOS3 +/+ mice*

The contributions of NOS, sGC, and COX to the endothelium-dependent vasodilators in mouse second-order mesenteric arterioles (MA) precontracted with cirazoline were assessed by measuring the responses of endothelium-intact and -denuded MA rings in the presence and absence of pharmacological inhibitors. Acetylcholine (10 μM) caused the relaxation of second-order mesenteric arterioles (MA) with an intact endothelium (Figure 1A,B,D) from C57BL/6J mice. This relaxant effect was nearly completely inhibited by the inclusion of L-NAME, ODQ and indomethacin (Figure 1A) and was abolished by removal of the endothelium (Figure 1C). SLIGRL-NH_2 (10 μM; a dose resulting in maximum relaxation: see below) caused the relaxation of endothelium-intact MA (Figure 1B) and this relaxant effect was only slightly inhibited by inclusion of L-NAME, ODQ and indomethacin (Figure 1B), but was abolished by the removal of the endothelium (Figure 1C). SLIGRL-NH_2 did not cause the relaxation of MA from PAR2 −/− mice (Figure 1D). The reversed-sequence control peptide, LRGILS-NH_2 (0.1 to

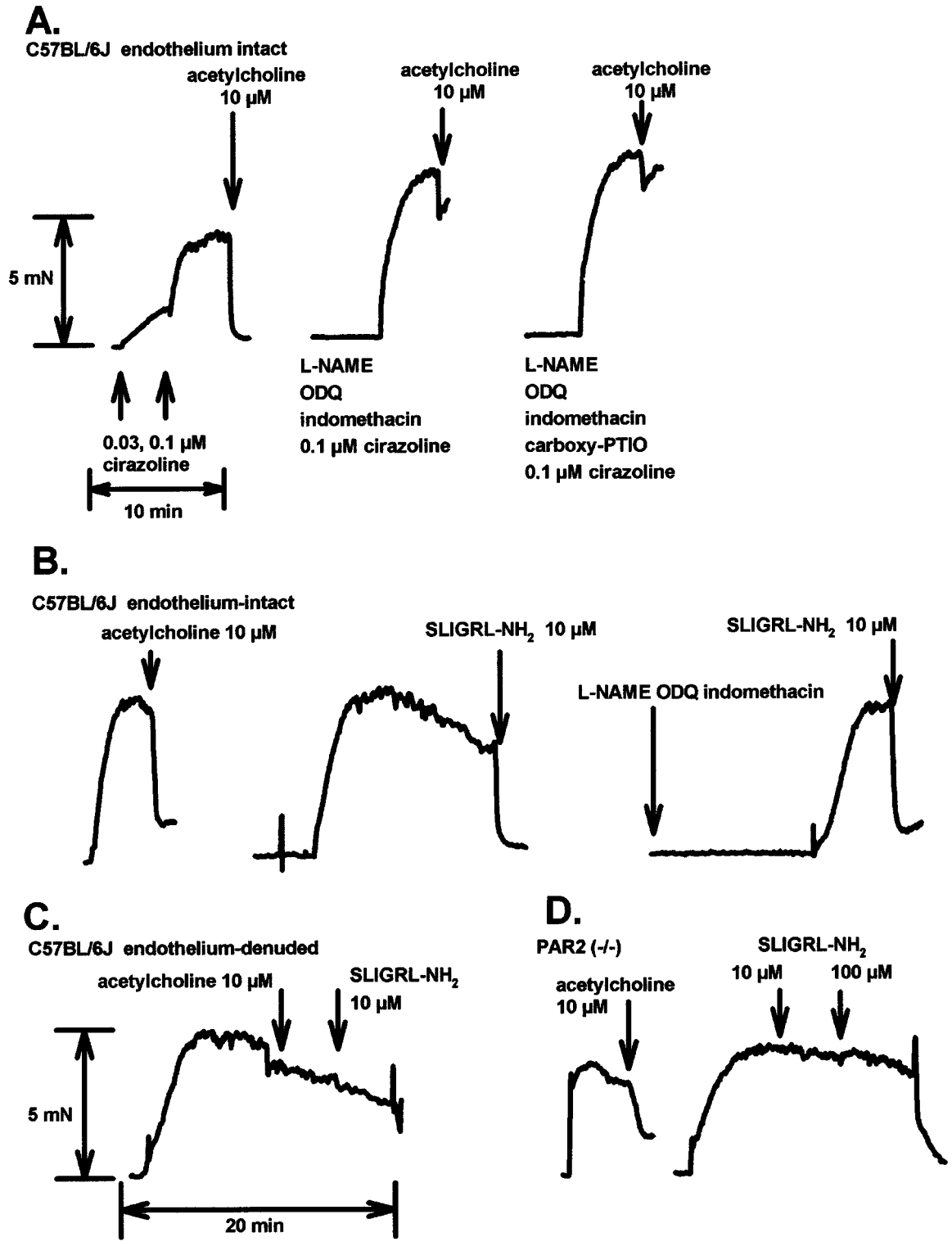

Figure 1 Endothelial PAR2 mediated relaxation of mouse second-order mesenteric arterioles. Online tension recordings were obtained as described in the Methods. Mesenteric arterioles from C57BL/6J mice were tested for the effects of NOS/COX/sGC inhibition on bolus applications of (A) acetylcholine and (B) SLIGRL-NH_2 after 0.1 μM cirazoline precontraction. The requirements for (C) an intact endothelium and (D) PAR2 expression for SLIGRL-NH_2-induced relaxation were tested by rubbing the lumen of the rings from C57BL/6J mice with a wire, and by harvesting tissues from PAR2 (−/−) mice, respectively.

100 μM) did not relax precontracted murine MA (data not shown). The local release of ACh, bradykinin, histamine and Substance P were tested for contributions to SLIGRL-NH_2-mediated relaxation by pretreatment with their receptor antagonists (1 μM atropine, 1 μM D-Arg, [Hyp^3, D-Phe^7]-bradykinin, 10 μM chlorpheniramine, 100 nM SR140333, 100 nM SR48968); these antagonists had no effect on the relaxation response to SLIGRL-NH_3 (data not shown).

Concentration-dependent activation of PAR2 causes the relaxation of second-order mesenteric arterioles from NOS3 −/− mice and is partially sensitive to inhibitors of NOS, sGC and COX

To evaluate the role of NOS3 in SLIGRL-NH_3-induced relaxation of MA, the concentration-dependent effects of PAR2 activation were compared in C57BL/6J (NOS3 +/+) and NOS3 (−/−) mice. SLIGRL-NH_2 (0.1–30 μM) induced a concentration-dependent relaxation of cirazoline-precontracted mesenteric arterioles from C57BL/6J, and NOS3 (−/−) mice (Figures 2A and 3A). The maximal relaxation (E_{max}) response to SLIGRL-NH_2 was partially inhibited by pretreatment of mesenteric arterioles from either C57BL/6J or NOS3 (−/−) mice with a combination of 100 μM L-NAME, 10 μM ODQ and 10 μM indomethacin (Figures 1–3; Table 1). Pretreatment of MA from C57BL/6J mice solely with a nonselective NOS inhibitor (100 μM L-NAME), a neuronal NOS inhibitor (100 μM 7-NI), a selective NOS2 inhibitor (100 μM 1400 W), sGC inhibitor (10 μM ODQ), nonselective COX inhibitor (indomethacin) or a selective COX-2 inhibitor (10 μM NS-398) had no effect on the relaxation response to SLIGRL-NH_2 (Table 1). In NOS3 (−/−) mice, the addition of indomethacin caused no greater effect than L-NAME and ODQ pretreatment alone (Figure 3A; Table 1). The pD_2 values for SLIGRL-NH_2 induced relaxation were not significantly different for tissues treated or not with L-NAME + ODQ + indomethacin (Table 1) from either C57BL/6J or NOS3 (−/−) mice. Further, the E_{max} value for SLIGRL-NH_2-induced relaxation of arterioles from C57BL/6J treated with L-NAME, ODQ plus indomethacin was not significantly different than that from NOS3 (−/−) mice that had not been treated with such reagents (Table 1).

Contributions of K^+ channels to PAR2-mediated relaxation of mouse mesenteric arterioles

If an endothelium-dependent hyperpolarizing (EDH) mechanism that was resistant to inhibitors of NOS, sGC and COX initiated relaxation to SLIGRL-NH_2 in the MA, then either increasing extracellular K^+ or the addition of inhibitors of K^+ channels would be expected to inhibit this relaxation as these treatments inhibit outwardly-rectifying K^+ (membrane hyperpolarizing) currents. Precontraction of mesenteric arterioles from C57BL/6J by addition of 30 mM K^+ reduced E_{max} and caused a rightward shift of the pD_2 value for SLIGRL-NH_2-mediated relaxation compared to cirazoline-precontracted tissues (Figure 2; Table 1). Additional pretreatment with L-NAME + ODQ + indomethacin completely inhibited the relaxation of K^+-precontracted MA to SLIGRL-NH_2 (Figure 2B; Table 1).

For cirazoline-treated tissues, contracted in the presence of L-NAME, ODQ plus indomethacin, neither the addition of 1 mM TEA, a concentration that inhibits selectively K_{Ca} channels, nor 1 μM glibenclamide, a K_{ATP} inhibitor, resulted in additional inhibitory effects (Figure 4A; Table 1). However, the addition of 1 mM 4-AP, a concentration that inhibits selectively K_V channels, to L-NAME/ODQ/indomethacin-treated tissues caused a significant increase in the potency of SLIGRL-NH_2 and enhanced E_{max} relative to

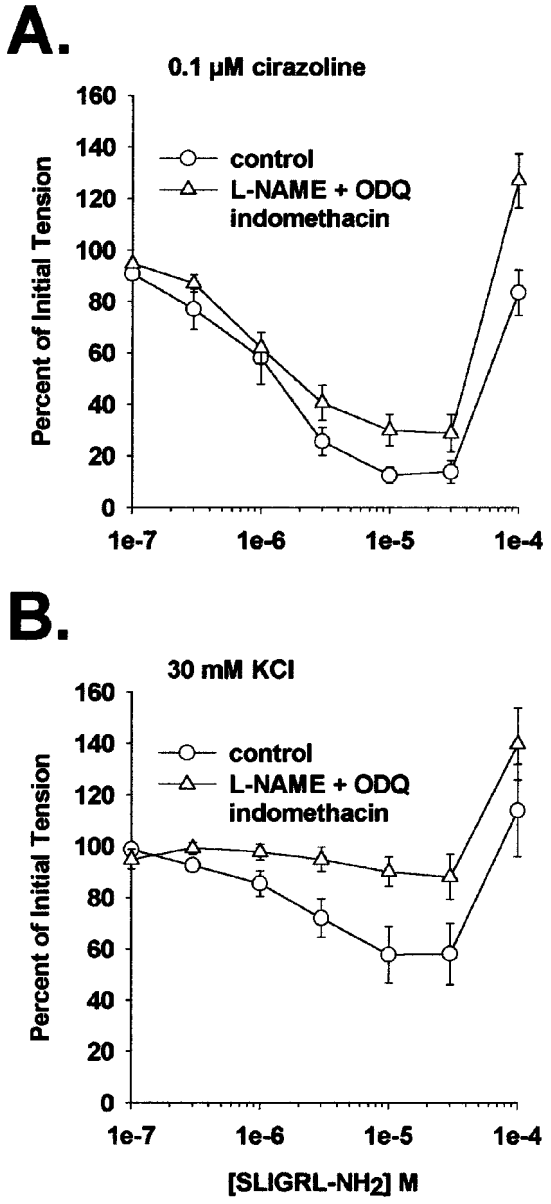

Figure 2 SLIGRL-NH_2 induced relaxation of C57BL/6J mouse second-order mesenteric arterioles in the presence of NOS, sGC and COX inhibitors. Arterioles were pretreated for 20 min with inhibitors and then pre-contracted with either (A) 0.1 μM cirazoline or (B) 30 mM KCl prior to obtaining cumulative concentration-response relationships for SLIGRL-NH_2.

tissue treated with L-NAME/ODQ/indomethacin alone (Figure 4B; Table 1), and this effect was eliminated by a combination of 1 mM 4-AP with either 1 mM TEA or 100 μM carbenoxolone, inhibitor of gap junction communication (Figure 4C,D; Table 1). Pretreatment with either 1 μM apamin, a SK_{Ca} inhibitor, or 0.1 μM charybdotoxin, an inhibitor of both IK_{Ca} and BK_{Ca}, significantly reduced the potency of SLIGRL-NH_2 in tissues from C57BL/6J mice concurrently treated with L-NAME + ODQ + indomethacin (Figure 5A,B; Table 1).

Pretreatment of mesenteric arterioles from either C57BL/6J or NOS3 (−/−) mice with a combination of 1 μM apamin and 0.1 μM charybdotoxin (in the combined presence of L-

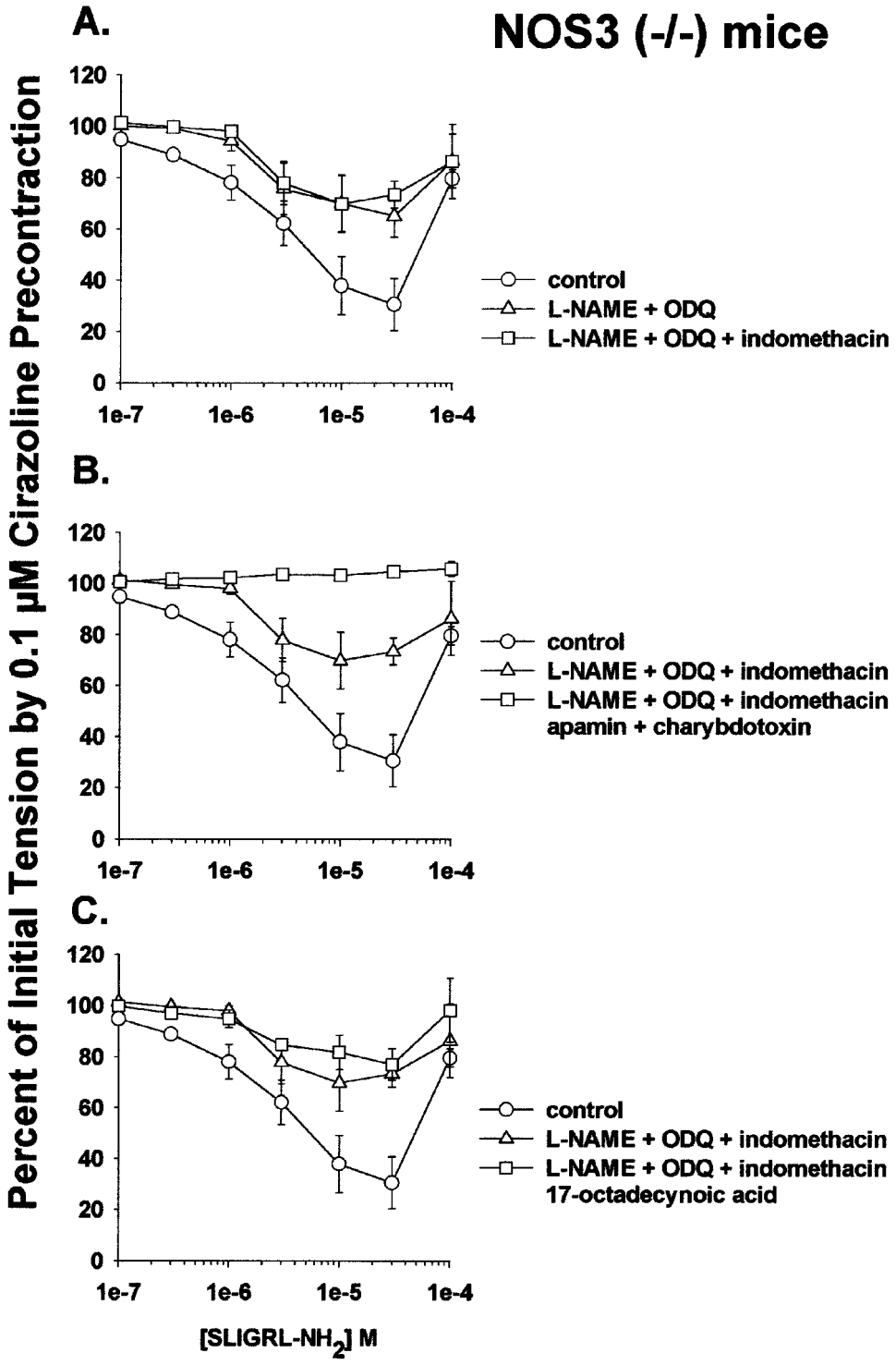

Figure 3 SLIGRL-NH_2 induced relaxation of NOS3 (−/−) mouse second-order mesenteric arterioles. Arterioles were pretreated for 20 min with inhibitors and then pre-contracted with 0.1 μM cirazoline prior to obtaining cumulative concentration-response relationships for SLIGRL-NH_2. Drugs utilized included inhibitors of (A) NOS/COX/sGC (B) SK_{Ca}, IK_{Ca}, and BK_{Ca} (C) cytochrome P450s.

NAME/ODQ/indomethacin) completely inhibited SLIGRL-NH_2-induced relaxation (Figures 3B and 5C; Table 1). In tissues from C57BL/6J mice, relaxation after treatment with 0.1 μM iberiotoxin, an IK_{Ca} inhibitor, plus 1 μM apamin was not significantly different than apamin alone (Figure 5D). Scyllatoxin (1 μM), an inhibitor of SK_{Ca}, substituted for apamin, in combination with charybdotoxin, produced a minor, but statistically significant greater inhibition than charybdotoxin alone with respect to E_{max}, but not pD_2 value (Figure 5E). This minor inhibitory effect was significantly less than inhibition by charybdotoxin plus apamin (compare Figure 5C,E; Table 1).

Table 1 Parameters for SLIGRL-NH_2 induced relaxation of mesenteric arterioles

Mouse strain	*Treatment*	n	*E_{max} (% of initial tension)*	*pD_2 (M)*	*Treatment*	n	*E_{max} (% of initial tension)*	*Pd_2 (M)*
C57BL/6J	Cirazoline +:				Cirazoline + L-NAME + ODQ + indomethacin +:			
	Control	16	10±3	5.9±0.1	Control	15	24±5*$^+$	5.9±0.1
	L-NAME	3	5.8±0.1	6.1±0.2	TEA	8	19±4	6.2±0.1
	7-NI	4	5±2	6.1±0.1	Glibenclamide	5	19±7	5.9±0.2
	1400W	3	5±4	6.1±0.2	4-AP	4	10±5†	6.3±0.1*
	ODQ	5	10±4	6.0±0.2	4-AP + TEA	4	16±4	6.1±0.1‡
	Indomethacin	4	4±1	6.3±0.2	4-AP + carbenoxolone	4	19±9	5.6±0.1‡
	NS-398	4	8±2	5.9±0.3	Apamin	6	37±6	5.3±0.1*
	$BaCl_2$	6	17±9	5.8±0.1	ChTX	6	36±12	5.4±0.1*
	Ouabain	5	11±5	5.7±0.2	Apamin + ChTX	3	85±3*	N.D.
	$BaCl_2$ + ouabain	7	11±2	5.4±0.1*	Apamin + IbTX	4	29±5$^+$	5.4±0.1*
	TTX	6	6±2	6.0±0.2	ChTX + ScTX	5	49±2*$^+$†‡	5.2±0.1*
	Carboxy-PTIO	6	5±1	6.0±0.1	$BaCl_2$ + ouabain	6	44±11*†	5.4±0.1*
	Carbenoxolone	4	12±3	5.8±0.1	TTX	5	34±13	6.0±0.3
	SQ22536	6	9±3	6.1±0.2	Carboxy-PTIO	4	27±2	5.8±0.3
	Propanolol	3	8±2	6.2±0.3	Carbenoxolone	6	26±5	6.0±0.2
					SQ22536	4	15±3	6.2±0.2
					AACOCF3	3	12±7	5.6±0.4
					17-ODYA	5	22±7	5.9±0.2
					propanolol	6	9±5	5.9±0.1
	30 mM KCl precontraction	5	54±11*	5.8±0.1	30 mM KCl precontraction	4	82±3*	N.D.
NOS3 (−/−)								
	Cirazoline +:				Cirazoline + L-NAME + ODQ + indomethacin +:			
	Control	4	30±10$^+$	5.8±0.1	Control	4	61±7*$^+$	5.4±0.2
	L-NAME + ODQ	4	56±6*$^+$	5.4±0.3	Apamin + ChTX	4	100±2*	N.D.
					17-ODYA	4	69±3*$^+$	5.9±0.3

Parameters (mean±s.e.mean) were calculated from each cumulative concentration-response curve for SLIGRL-NH_2 induced relaxation. E_{max} indicates the maximum relaxation effect, and is expressed as % of initial tension. pD_2 values for control-treated mouse strains were not significantly different (ANOVA), $P>0.05$). E_{max} was significantly reduced in NOS3 (−/−) compared to C57BL/6J controls. *$P<0.05$, compared to control; $^+P<0.05$, compared to L-NAME + ODQ + indomethacin + apamin + ChTX; †$P<0.05$ compared to L-NAME + ODQ + indomethacin; ‡$P<0.05$ compared to L-NAME + ODQ + indomethacin + 4-AP. §$P<0.05$ compared to L-NAME + ODQ + indomethacin + ChTX. N.D. indicates the value was not determined. Statistical comparisons were made by ANOVA followed by Student-Newman Keul *post-hoc* test.

The nature of PAR2-mediated relaxation of mesenteric arterioles

Based on our observations that an EDH-like mechanism caused the SLIGRL-NH_2-induced relaxation of MA, we tested whether the activation of the ouabain-sensitive Na^+, K^+, ATPase and $BaCl_2$-sensitive inwardly-rectifying K^+ channel (K_{ir}) could contribute to these effects. Treatment with 30 μM $BaCl_2$ or 10 μM ouabain alone did not significantly affect either the E_{max} or pD_2 values compared to (control) untreated tissues (Figure 6A,B; Table 1). However, the pD_2 value following the pretreatment with a combination of $BaCl_2$ + ouabain was significantly reduced, whereas E_{max} was not significantly different than controls (Figure 6C; Table 1). In the presence of L-NAME, ODQ and indomethacin, the combination of $BaCl_2$ plus ouabain reduced both pD_2 and E_{max} (Figure 6D; Table 1).

If neuronal activation, 'residual NO', gap junction communication, adenylyl cyclase (AC), phospholipase A_2, β-adrenoceptors or CYP epoxygenase reactions mediated SLIGRL-NH_2-induced relaxation then pharmacological inhibitors of these mechanisms would be expected to inhibit relaxation. Pretreatment of MA with either 10 μM TTX, 100 μM carboxy-PTIO, 100 μM carbenoxolone, 10 μM SQ22536, 10 μM AACOCF3 or 1 μM propanolol failed to cause significant changes to the concentration-response relationships with regards to either E_{max} or pD_2 values in either the presence or absence of L-NAME, ODQ and indomethacin (Table 1). E_{max} and pD_2 values for SLIGRL-NH_2 induced relaxation of MA from either C57BL/6J (Table 1) or NOS3 −/− mice (Figure 3C) pretreated with a combination of 10 μM 17-ODYA, L-NAME, ODQ and indomethacin were also not significantly different than tissues treated with L-NAME, ODQ and indomethacin alone without the addition of 17-ODYA.

High concentration SLIGRL-NH_2 induced contraction of mouse mesenteric arterioles

At 30 to 100 μM SLIGRL-NH_2, the relaxation-phase reversed and tension increased in both cirazoline- and 30 mM KCl-treated tissues (Figures 2–6). These contractions (absolute tension changes, data not shown) were not significantly affected by either the strain of mouse or by any of the inhibitors tested ($P>0.05$, ANOVA) with the exception of AACOCF3, that also reduced the initial contractions to cirazoline by >65% (control ($n=15$), 3.2±0.3 mN compared to AACOCF3 ($n=3$), 1.0±0.1 mN; $P<0.05$, ANOVA and Student-Newman Keuls *post-hoc*). Initial contractions after pretreatment with carbenoxolone ($n=4$, 2.2±0.2 mN) or L-NAME, ODQ, indomethacin plus carbenoxolone ($n=6$, 2.1±0.2 mN) were also reduced and the combination of charybdotoxin and scyllatoxin ($n=5$, 7.0±1.3 mN) significantly enhanced contractions compared to their controls (L-

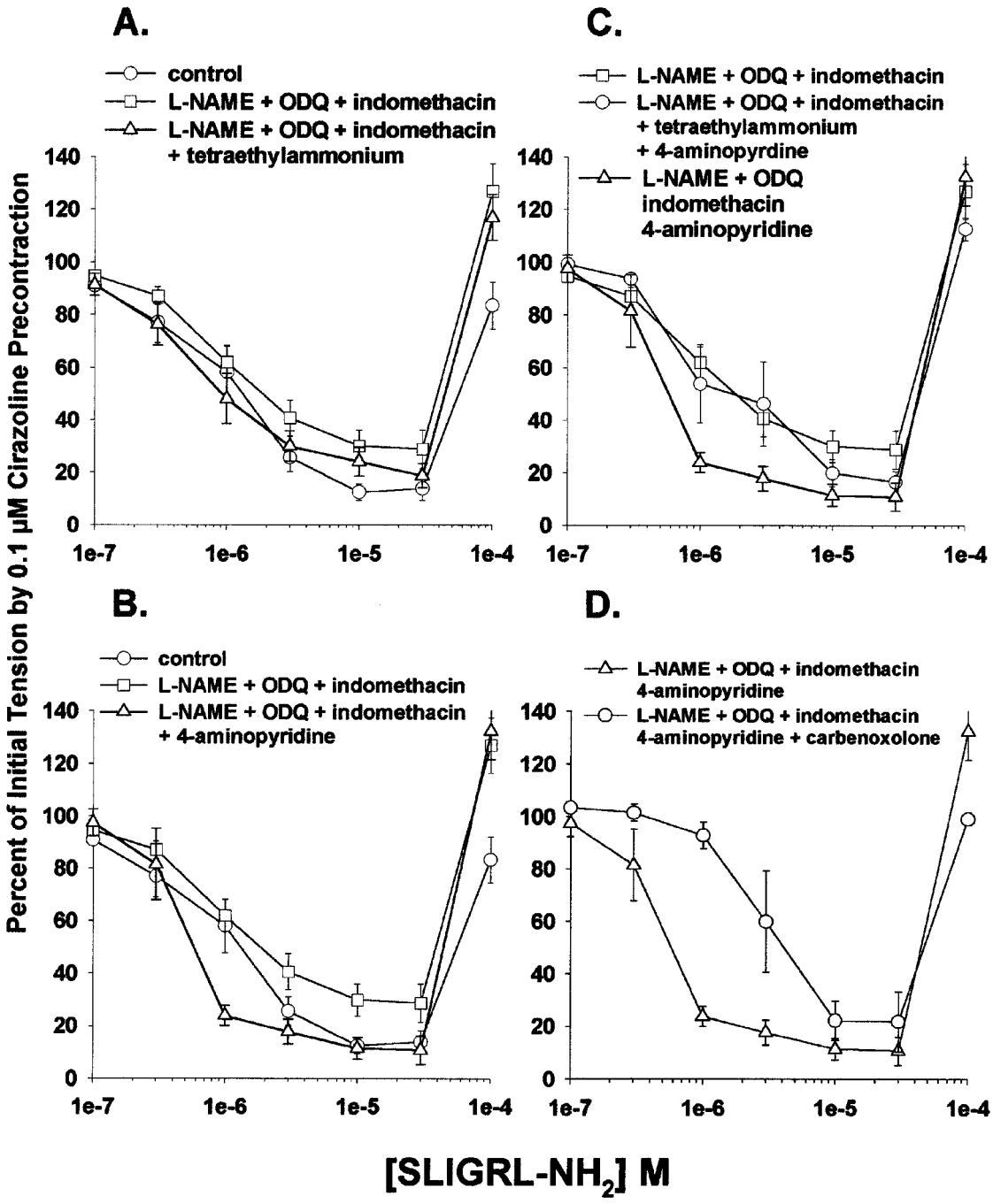

Figure 4 Effects of inhibitors of calcium-activated and voltage-sensitive K^+ channels on SLIGRL-NH_2 induced relaxation of C57BL/6J mouse second-order mesenteric arterioles. Arterioles were pretreated for 20 min with inhibitors and then pre-contracted with 0.1 μM cirazoline prior to obtaining cumulative concentration-response relationships for SLIGRL-NH_2. (A) pretreatment with 1 mM TEA, to inhibit BK_{Ca}, (B) 1 mM 4-aminopyridine, to inhibit K_v channels and (C) TEA + 4-AP, to inhibit both BK_{Ca} and K_v channels. (D) Effects of carbenoxolone (100 μM), to inhibit gap junction communication, on 4-AP enhancement of relaxation.

NAME, ODQ plus indomethacin ($n=15$), 3.7 ± 0.5 mN; $P<0.05$ ANOVA and Student-Newman Keuls *post-hoc*); otherwise contractions after pretreatment were not significantly different ($P>0.05$, ANOVA). SLIGRL-NH_2-induced contractile activity was not observed under baseline tension conditions (data not shown); nor was it found in precontracted MA from PAR2 (−/−) mice (Figure 1D). LRGILS-NH_2 did not contract MA under either condition (data not shown).

Proteinase-activation of PAR2 in mesenteric arterioles

In MA from C57BL/6J mice, the application of trypsin (1 to 300 nM) caused a concentration-dependent relaxation (Figure 7). The concentration-response curve for trypsin was significantly shifted rightward and the E_{max} was reduced in MA from PAR2 (−/−) mice (Figure 7). In comparison, the relaxation caused by trypsin in the PAR2 (+/+) mice was resistant to L-NAME + ODQ + indomethacin (Figure 7). Important to note

was that trypsin, at a concentration (30 nM) that can selectively activate PAR2 without affecting PAR1 (Vu *et al*., 1991; Kawabata *et al*., 1999), significantly relaxed the MA from PAR2 (+/+) and not PAR2 (−/−) mice.

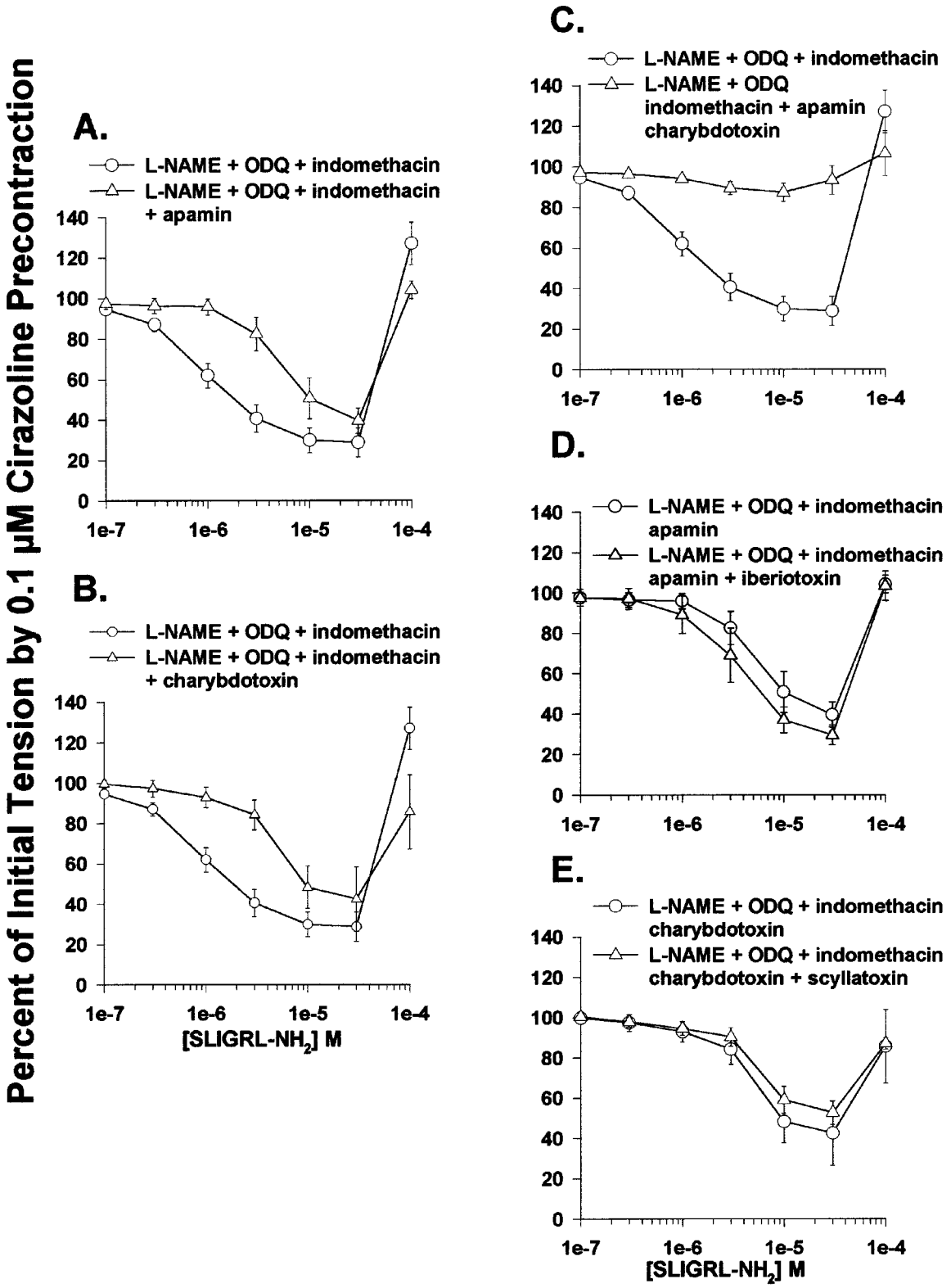

Figure 5 Effects of selective inhibitors of calcium-activated K^+ channels on SLIGRL-NH_2 induced relaxation of C57BL/6J mouse second-order mesenteric arterioles. Arterioles were pretreated for 20 min with inhibitors and then pre-contracted with 0.1 μM cirazoline prior to obtaining cumulative concentration-response relationships for SLIGRL-NH_2. (A) Effects of 1 μM apamin, to inhibit SK_{Ca}, and (B) 0.1 μM charybdotoxin, to inhibit BK_{Ca} and IK_{Ca}. (C) Effects of apamin + charybdotoxin, (D) substitution of 0.1 μM iberiotoxin for charybdotoxin, and (E) substitution of 1 μM scyllatoxin for apamin.

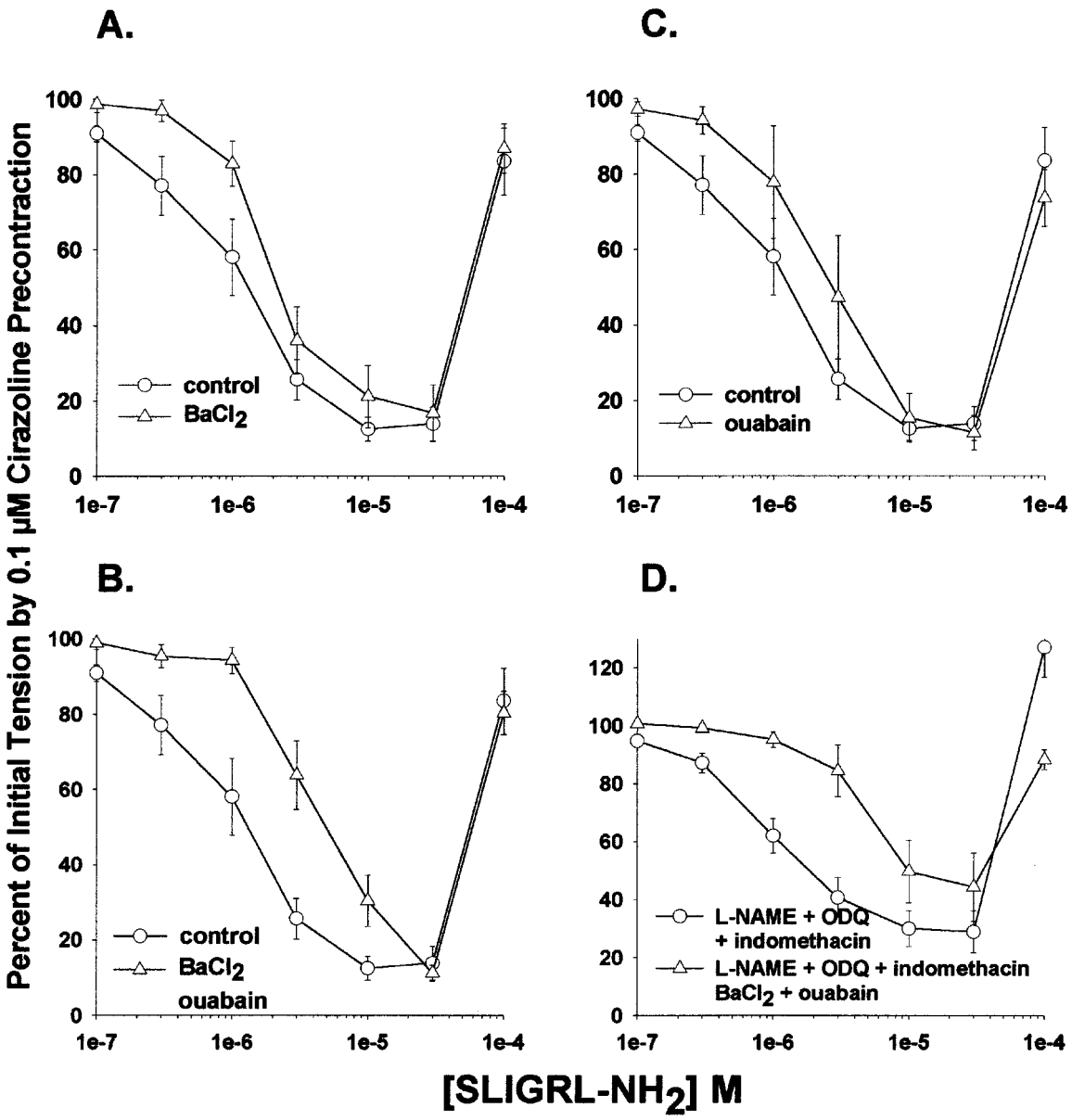

Figure 6 Effects of inhibitors of Na^+, K^+ ATPase and K_{ir} on SLIGRL-NH_2 induced relaxation of C57BL/6J mouse second-order mesenteric arterioles. Arterioles were pretreated for 20 min with inhibitors and then pre-contracted with 0.1 μM cirazoline prior to obtaining cumulative concentration-response relationships for SLIGRL-NH_2. (A) Effects of 30 μM barium to inhibit K_{ir}, (B) 10 μM ouabain, to inhibit Na^+, K^+ ATPase and their combination in the absence (A–C) and presence of L-NAME + ODQ + indomethacin (D).

Discussion

The major finding of our study is that PAR2 activation in isolated second-order mesenteric arterioles leads to a NO/NOS/COX-independent mechanism of murine vascular smooth muscle relaxation. Entirely in keeping with previous reports (Al Ani *et al*., 1995; Saifeddine *et al*., 1996; Emilsson *et al*., 1997; Moffatt & Cocks, 1998) PAR2 activation by the peptide agonist SLIGRL-NH_2 caused an endothelium-dependent relaxation. PAR2-dependency and -selectivity was confirmed by the absence of this relaxation in PAR2 (−/−) mice in response to SLIGRL-NH_2 and to the reversed-sequence peptide, LRGILS-NH_2, in either C57BL/6J (wild-type PAR2) or PAR2 (−/−) mice. However, in contrast with previous observations demonstrating the abrogation of SLIGRL-NH_2-mediated relaxation by L-NAME (Al Ani *et al*., 1995; Saifeddine *et al*., 1996; Emilsson *et al*., 1997; Moffatt & Cocks, 1998), the relaxation caused by SLIGRL-NH_2 in MA rings from wild-type PAR2-expressing mice, treated in the presence of inhibitors of NOS, COX, sGC and AC, was almost as great in the absence of these inhibitors. Observing a comparable relaxation effect (non-NO/non-COX) in NOS3 (−/−) mice bolstered these observations. Therefore, we conclude that PAR2 mediated relaxation of murine MA is both L-NAME/ODQ/indomethacin-sensitive and -insensitive. These results were unexpected, because it has been well documented that PAR2-mediated relaxation of vascular smooth muscle was primarily depen-

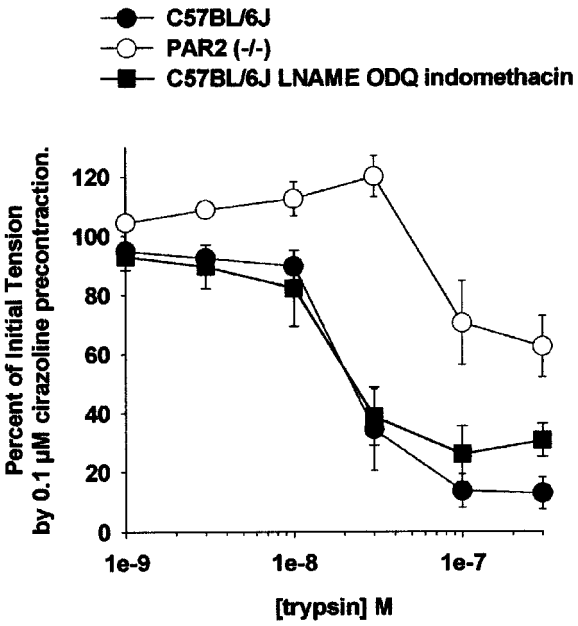

Figure 7 Proteinase-induced relaxation of mouse mesenteric arterioles. Arterioles from C57BL/6J and PAR2 (−/−) mice were precontracted with 0.1 μM cirazoline prior to obtaining cumulative concentration-response relationships for trypsin. For several experiments vessels from C57BL/6J mice were pretreated for 20 min with L-NAME/ODQ/indomethacin prior to precontraction. The pD_2 and E_{max} values for trypsin-induced relaxation were: C57BL/6J control ($n=6$), 7.7 ± 0.1 and 10 ± 5; PAR2 (−/−) ($n=3$), 7.0 ± 0.3 and 61 ± 10; C57BL/6J L-NAME/ODQ/indomethacin ($n=3$), 7.8 ± 0.1 and 24 ± 8.

dent on NOS production of NO (Saifeddine *et al*., 1996; Emilsson *et al*., 1997; Moffatt & Cocks, 1998; Sobey & Cocks, 1998; Hamilton & Cocks, 2000). However, non-NO/non-PGI_2 endothelium-dependent hyperpolarization of vascular smooth muscle is a potential mechanism that has been ascribed to other endothelium-dependent vasodilators such as ACh, Substance P and bradykinin (see Introduction for recent reviews). Indeed, further examination of SLIGRL-NH_2 induced relaxation in the MA revealed that multiple endothelium-dependent mechanisms were likely responsible for this response and involved activation of an apamin/charybdotoxin-sensitive calcium-activated K^+ channel, and possibly the endothelial release of K^+.

Previous studies that utilized first-order mesenteric arterioles from NOS3 (+/+) mice did not describe a NOS/COX/sGC-inhibitors resistant response to ACh, the agonist chosen most often to assess functional endothelial integrity (Waldron *et al*., 1999; Ding *et al*., 2000b). However, these observations may have been dependent on vessel size. With this question in mind, we tested our preparations of second-order mesenteric arterioles for the NOS/COX inhibitor sensitivity of ACh-induced relaxation. However, the inhibition of ACh-induced relaxation by L-NAME/ODQ/indomethacin was about the same (85–90%) as previously reported for the first-order mesenteric arterioles (Waldron *et al*., 1999; Ding *et al*., 2000b). We also took a further measure, and applied the NO scavenger carboxy-PTIO to the preparation to remove 'residual NO', but it too had no significant inhibitory action. It has been previously proposed for studies utilizing NOS3 (−/−) mice that synthesis of an EDHF had been up-regulated because EDHF-like responses were observed in first-order mesenteric arterioles and saphenous arteries for such animals (Waldron *et al*., 1999; Ding *et al*., 2000b). However, a modified interpretation based on our current data is necessary since EDHF-like activity was observed in MA from both the wild-type and NOS3 (−/−) mice. Receptor-selective mechanisms may initiate a common EDHF pathway, for reasons discussed below, and each receptor may have a different intrinsic activity associated with the activation of the pathway. Thus PAR2 activation produced much greater relaxation than did muscarinic receptor activation. Based on this interpretation, NOS3 gene deletion may be partially compensated by enhanced EDHF synthesis, but receptor-dependent connectivity to the EDHF pathway may vary (i.e. muscarinic receptors versus PARs).

In the MA from the NOS3 (−/−) mice, there was a significantly greater contribution of an L-NAME/ODQ/indomethacin-sensitive component to the relaxant effect of SLIGRL-NH_2, than the component we propose as the EDH-mediated effect. The inhibition of SLIGRL-NH_2-induced relaxation of NOS3 (−/−) second-order mesenteric arterioles by L-NAME and ODQ indicated that there may well have been an up-regulation of either NOS1 or NOS2 expression in these vessels or the existence of a non-NOS-derived source of NO. An up-regulation of nNOS (NOS2) in pial vessels from NOS3 (−/−) mice has been reported (Meng *et al*., 1998). We did not test the effects of selective NOS1, NOS2 inhibitors (e.g. 7-nitroindazole, 1400W) nor the sole additions of L-NAME and ODQ separately, and thus, cannot be assured that there was neither NO-dependent nor a non- NO-, but cyclic GMP-dependent response in NOS3 (−/−) mice. Inhibition of relaxation by L-NAME + ODQ was not reported for the mesenteric arterioles from NOS3 (−/−) mice in response to ACh, and thus the observations reported in the current study probably reflect agonist-related differences in the release of endothelium-derived relaxing factors (Waldron *et al*., 1999; Ding *et al*., 2000b). As there was only a partial inhibition of PAR2-mediated relaxation, we interpreted the results to mean that another non-NO factor was also produced by PAR2 activation in NOS3 (−/−) mice. An increased activity of a COX-dependent process in response to ACh was reported for the main branch of the mesenteric artery from NOS3 (−/−) mice (Chataigneau *et al*., 1999). However, based on the results we obtained using either indomethacin or 17-ODYA in the presence of L-NAME + ODQ, it appeared that neither COX- nor CYP-dependent products were responsible for the effects observed in the present study. Therefore, the deletion of the NOS3 gene alters MA signalling to permit PAR2, but not muscarinic receptors, to activate an L-NAME/ODQ-sensitive, but presumably eNOS (NOS3)-independent relaxation mechanism.

Several studies have reported that non-NO/$PG1_2$ EDH-mediated relaxation responses can be reduced by CYP inhibitors (Hecker *et al*., 1994; Fulton *et al*., 1995; 1998; Chen & Cheung, 1996; Dong *et al*., 1997). Cytochrome P450-mediated epoxygenase reaction products of arachidonic acid, epoxyeicosatrienoic acids (EETs), have been proposed as the mediators of EDH (Popp *et al*., 1996; Van de Voorde & Vanheel, 1997; Widmann *et al*., 1998; Fisslthaler *et al*., 1999; Welsh & Segal, 2000; Bolz *et al*., 2000). As of yet, however, CYP metabolism of arachidonic acid has not been shown to

contribute to EDH in mouse mesentery blood vessels. 17-ODYA, a CYP suicide substrate inhibitor, was utilized in our study because it was reported that many of the commonly used CYP inhibitors such as proadifen (SKF525a), and clotrimazole directly inhibit vascular smooth muscle K^+ channels (Zygmunt *et al*., 1996; Van de Voorde & Vanheel, 1997; Waldron *et al*., 1999). In contrast, 17-ODYA has been found to be relatively selective for inhibition of EDH activity attributed to CYPs without directly affecting K^+ channels (Zygmunt *et al*., 1996; Vanheel *et al*., 1999; Welsh & Segal, 2000). It is also important to recognize that hyperpolarization of porcine coronary arterioles by EETs was inhibited by iberiotoxin (Pratt *et al*., 2001), whereas in our preparation iberiotoxin did not affect relaxation. Thus, PAR2 mediated vascular smooth muscle relaxation is unlikely to involve EETs.

Further investigation of SLIGRL-NH_2 induced relaxation of the MA rings from C57BL/6J mice demonstrated characteristics that were consistent with relaxation being mediated by EDHFs. A common protocol for identifying the contribution of an EDHF to vascular smooth muscle relaxation has been to pre-contract a vessel by the addition of a membrane potential depolarizing concentration of KCl (30 to 60 mM) prior to obtaining a concentration-response relationship to the vasodilator (Adeagbo & Triggle, 1993). Indeed, there was a significant inhibition of SLIGRL-NH_2 - induced relaxation under those conditions and thus consistent with a membrane hyperpolarization mechanism mediating vasodilation. Using relatively selective concentrations of inhibitors, we further assessed the contribution of K^+ channels participation. TEA, at 1 mM, was chosen to selectively inhibit large-conductance, BK_{Ca}, Ca^{2+}-activated K^+ channels and 4-AP, at 1 mM, was chosen to inhibit selectively voltage-dependent (K_v) K^+ channels. Since TEA did not affect PAR2-triggered relaxation, it appeared that BK_{Ca} channels were not involved with either the relaxation mediated by or the release of EDHF. The cellular mechanism that mediates the enhanced potency of SLIGRL-NH_2 in the presence of 4-AP is unknown. TEA (1 mM) has previously been reported to inhibit EDHF activity in first-order mouse mesenteric arterioles (Waldron *et al*., 1999; Ding *et al*., 2000b). The combination treatment, TEA plus 4-AP, abolished the 4-AP mediated enhancement of SLIGRL-NH_2-mediated relaxation, but the reason for this was not clear.

Application of either apamin or charybdotoxin partially inhibited, and their combination completely inhibited SLIGRL-NH_2-induced relaxation. These data, together with the lack of inhibitory actions by TEA and iberiotoxin, indicated that small-conductance (SK_{Ca}) and intermediate-conductance (IK_{Ca}) Ca^{2+}-activated K^+ channels regulate EDHF. These observations were similar to those regarding ACh- induced relaxation of NOS3 (−/−) mouse arterioles (Waldron & Garland, 1994; Ding *et al*., 2000b). Our data suggest a selective pharmacology for EDHF pathways (summarized by Triggle, 2001). In contrast to the current study, however, other studies utilizing rabbit mesentery and rat hepatic vessels demonstrated that scyllatoxin, a peptide with selectivity for SK_{Ca}, can substitute fully for apamin (Murphy & Brayden, 1995; Andersson *et al*., 2000). The observation that scyllatoxin did not fully substitute for apamin when applied in combination with charybdotoxin suggests pharmacological differences with the preparations used in those studies (Zygmunt & Hogestatt, 1996).

NO has been demonstrated to elicit vascular smooth muscle hyperpolarization. Hyperpolarization by NO may be mediated either directly on K^+ channels or *via* cyclic nucleotide-dependent mechanisms (Tare *et al*., 1990; Bolotina *et al*., 1994; Carrier *et al*., 1997; Mistry & Garland, 1998). In several preparations that sought to examine NOS-inhibitor insensitive relaxation and hyperpolarization to endothelium-dependent vasodilators, the application of a single NOS inhibitor was insufficient to completely inhibit relaxation, but either additional NOS inhibitors or NO-scavenging compounds, further inhibited or completely blocked relaxation and hyperpolarization, and thus, the EDHF was attributed to a 'residual NO effect' (Cohen *et al*., 1997; Vanheel & Van de Voorde, 2000). We attempted to deal with this issue by additional pretreatment with a NO scavenger, carboxy-PTIO (Chuman *et al*., 1996). We concluded that there was no 'residual NO effect' present in our study because the inclusion of carboxy-PTIO resulted in no additional inhibition to that produced by L-NAME, ODQ plus indomethacin.

K^+ ion has been proposed as an EDHF and small increases in its intercellular concentration by exogenous application or in response to acetylcholine have been demonstrated to produce relaxation of rat hepatic artery, and hypothesized to also contribute to endothelium-dependent relaxation in mesenteric blood vessels from either rat or mouse (Edwards *et al*., 1998; 2000; Ding *et al*., 2000b). The increase in K^+ concentration, assumed to be derived from either endothelial cells or vascular smooth muscle cells, was sensitive to apamin and charybdotoxin, and involved the activation of Ba^{2+}-sensitive inwardly rectifying K^+ channels (K_{ir}) and the activation of ouabain-sensitive Na^+,K^+-ATPase. Our data demonstrated that $BaCl_2$ and ouabain inhibited PAR2 mediated relaxation, and thus, our results are consistent with the hypothesis that an increase in extracellular K^+ may mediate a component of the PAR2-induced relaxation effect. However, there was a substantial relaxation effect that was resistant to $BaCl_2$ and ouabain, thus suggesting that multiple EDHFs and/or cellular mechanisms were involved. It has been reported that EETs produced an endothelium-independent ouabain-sensitive relaxation of bovine coronary arteries (Pratt *et al*., 2001). However, we found that 17-ODYA and iberiotoxin were without effect and we conclude that EETs are unlikely to be involved with PAR2-mediated relaxation in mouse mesenteric arterioles.

The transmission of the EDH signal *via* through gap junctions between endothelial and vascular smooth muscle provides an alternative cellular process to a purely chemical transmission. Possible mechanisms include the transfer of soluble factors (such as cyclic GMP) and the transmission of electrotonic potentials from endothelial cells to vascular smooth muscle, and vice versa (Chaytor *et al*., 1998; Dora *et al*., 2000). Using carbenoxolone, a compound that has been used to investigate intercellular communication *via* gap junctions (Davidson *et al*., 1986), our results indicate that gap junction communication was most likely not involved in PAR2-mediated EDH. Carbenoxolone, however, reversed the potentiation of SLIGRL-NH_2 that was caused by 4-AP, suggesting that gap junctions may be involved in mediating calcium signalling between endothelial and vascular smooth muscle. In other studies, it has been proposed that inhibition

of gap junctions modifies EDHF not by inhibiting chemical transmission, but through their effects on electrotonic potentials, however, non-specific actions by gap junction-targeted reagents on ion channels cannot be ruled out.

An important issue to address was whether the non-NO/non-COX response observed as a result of PAR2 activation with peptide agonist is the same as that caused by proteinase activation (examples trypsin and tryptase). We have found that trypsin, at a concentration selective for PAR2 versus PAR1, indeed produced an ODQ/L-NAME/indomethacin-resistant relaxation equivalent to that caused by SLIGRL-NH_2 (Figure 7). The complete pharmacological profile remains to be determined and may provide clues regarding the pathophysiological significance of this PAR2 signalling pathway.

To summarize, we have described vascular smooth muscle relaxation that was mediated by endothelial PAR2 activation, and was dependent on multiple endothelial-dependent mechanisms that included partial contributions by NO and cyclic GMP. It was concluded that Cytochrome P450s, COX, AC, a neuronal-mediated mechanism, intercellular gap junction communication or a 'residual NO effect' did not contribute to the non-NO mediated relaxation. There was also no evidence of a PAR2 activation that indirectly, at least through activation of NK1/NK2, B_2, $H_{1/2}$, or β-adrenergic receptors, mediated the relaxation, although the contribution of an endothelial release of K^+ and the activation of K_{ir} and Na^+, K^+-ATPase may contribute to the relaxation. The characteristics of the relaxation are consistent with EDHF-mediated mechanisms previously described, and indicate a dependence on activation of an apamin/charybdotoxin-sensitive calcium activated K^+ channel(s). However, electrophysiological studies will be required to further elucidate the putative vascular smooth muscle hyperpolarization mechanism.

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