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Thromboxane A_2 inhibition of SK_{Ca} after NO synthase block in rat middle cerebral artery.

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Running title: Thromboxane A_2 inhibition of SK_{Ca} after NOS block

Background and purpose. NO/prostanoid independent, EDHF-mediated hyperpolarization and dilation in rat middle cerebral arteries is mediated solely by endothelial cell IK_{Ca}. However, when the NO-pathway is also active, both SK_{Ca} and IK_{Ca} contribute to EDHF responses. As the SK_{Ca} component can be inhibited by stimulation of thromboxane A_2 (TxA_2) TP receptors and NO has the potential ability to inhibit thromboxane synthesis, we investigated whether TxA_2 might explain loss of functional input from SK_{Ca} during NOS inhibition in cerebral arteries.

Experimental approach Rat middle cerebral arteries were mounted in a wire myograph. Endothelium-dependent responses to the PAR2 agonist, SLIGRL were assessed as simultaneous changes in smooth muscle membrane potential and tension.

Key results Responses were obtained in the presence of L-NAME as appropriate. Inhibition of TP receptors with either ICI 192,605 or SQ 29,548, did not effect EDHF mediated hyperpolarization and relaxation, but in their presence neither TRAM-34 nor apamin (to block IK_{Ca} and SK_{Ca} respectively) individually affected the EDHF response. However, in combination they virtually abolished it. Similar effects were obtained in the presence of the thromboxane synthase inhibitor, furegrelate, which additionally revealed an iberiotoxin-sensitive residual EDHF hyperpolarization and relaxation in the combined presence of TRAM-34 and apamin.

Conclusions and implications In the rat middle cerebral artery, inhibition of NOS leads to a loss of the SK_{Ca} component of EDHF responses. Either antagonism of TP receptors or block of thromboxane synthase restores an input through SK_{Ca}. These data indicate that NO normally enables SK_{Ca} activity in rat middle cerebral arteries.

Keywords: Thromboxane A2, small conductance calcium activated potassium channel, intermediate conductance calcium activated potassium channel, large conductance calcium activated potassium channel, endothelium derived hyperpolarizing factor, nitric oxide, cerebral artery.

Abbreviations: 20-HETE, 20-Hydroxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid; BK_{Ca}, large conductance calcium activated potassium channel; CYP450, cytochrome P450; EDHF; endothelium-derived hyperpolarizing factor; EETs; epoxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids; IK_{Ca}, intermediate conductance calcium activated potassium channel; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; PKG, protein kinase G; SK_{Ca}, small conductance calcium activated potassium channel; TxA2, thromboxane A_2; TP, thromboxane A_2 receptor;

Introduction

In rat middle cerebral arteries treated with a NO synthase (NOS) inhibitor (L-NAME), blockade of endothelial cell intermediate conductance calcium activated potassium channels (IK_{Ca}) alone is sufficient to block smooth muscle hyperpolarization and relaxation due to endothelium-derived hyperpolarizing factor (EDHF: (McNeish et al., 2005, Marrelli et al., 2003). This is in contrast to peripheral arteries, where EDHF mediated responses are only abolished by the combined inhibition of both small conductance calcium activated potassium channels (SK_{Ca}) and IK_{Ca} (Busse *et al.*, 2002). A number of studies with peripheral arteries have provided functional, electrophysiological and immunohistochemical data showing that SK_{Ca} and IK_{Ca} channels are expressed only within the endothelium (Burnham et al., 2002, Bychkov et al., 2002, Walker et al., 2001). We recently investigated if the apparent solitary role of IK_{Ca} in the EDHF response of rat middle cerebral arteries reflected an absence of SK_{Ca} channels. Surprisingly in the light of the functional data, but in common with peripheral arteries, both IK_{Ca} and SK_{Ca} channels were demonstrated in the endothelium (McNeish *et al.*, 2006).

The relative contribution from SK_{Ca} and IK_{Ca} channels to the EDHF response is altered by arterial stimulation. In rat mesenteric arteries, during smooth muscle contraction evoked by phenylephrine, block of endothelium-dependent hyperpolarization and relaxation requires inhibition of both endothelial SK_{Ca} and IK_{Ca}, but in unstimulated vessels (where there is no contraction to reverse) inhibition of SK_{Ca} alone is sufficient to block EDHF-mediated hyperpolarization (Crane *et al.*, 2003). In contrast to mesenteric arteries, arterial stimulation may not regulate K_{Ca} function in middle cerebral arteries, as increases in stretch-induced tension did not alter SK_{Ca} input (McNeish *et al.*, 2006). However, basal release of NO suppresses myogenic tone and an ability to elaborate NO is associated with maintained SK_{Ca} input to EDHF-evoked hyperpolarization in this artery. It is only after inhibition of NO synthase that the contribution of SK_{Ca} is lost, with EDHF responses becoming entirely reliant on IK_{Ca} (McNeish et al., 2005, McNeish et al., 2006) .

Although NO may suppress EDHF activity under some circumstances, these two separate dilator pathways do operate simultaneously in many arteries, including the rat middle cerebral artery (Zygmunt et al., 1998, Feletou and Vanhoutte, 2006, McNeish et al., 2006), but it is not clear how NO may alter SK_{Ca} activity. In non-vascular smooth muscle from the rat, there is evidence that NO can directly stimulate SK_{Ca} channel function (Geeson *et al.*, 2002). Basal release of NO may also protect SK_{Ca} channel function indirectly. For example, NO may suppress an inhibitory mediator for the SK_{Ca} channel (and thus the EDHF response) such as the potent vasoconstrictor 20-HETE. 20-HETE does inhibit the EDHF response in small porcine coronary arteries (Randriamboavonjy *et al.*, 2005) and NO can inhibit the synthesis of 20-HETE, by binding to the heme group of cytochrome P450 (CYP450) (Minamiyama *et al.*, 1997). Another mediator that could potentially affect the EDHF response and in particular the SK_{Ca} channel is thromboxane A_2 (TxA_2). In rat mesenteric resistance arteries, stimulation of TxA_2 receptors (TP receptors) with U46619 attenuates SK_{Ca} function (Crane and Garland, 2004). NO has also been shown to inhibit the synthesis of TxA_2, again by an interaction with a heme active site, this time in thromboxane synthase (Wade and Fitzpatrick, 1997). Indeed, increased TxA_2 signalling has been reported to contribute to vasoconstriction and the development of vasomotion induced in middle cerebral arteries by NO synthase inhibitors (Lacza et al., 2001, Benyo et al., 1998). Finally, NO may also affect K_{Ca} function via indirect cGMP mediated effects. So, for example, NO/cGMP causes desensitisation of TP receptors via a protein kinase G (PKG) dependent mechanism (Reid and Kinsella, 2003).

Therefore we hypothesised that constriction following inhibition of NOS and the associated loss of the SK_{Ca} component of agonist induced hyperpolarization may be underpinned by an increase in the synthesis and/or function of TxA_2 or 20-HETE in the rat middle cerebral artery. The aim of the present study was therefore to assess the contribution of K_{Ca} channel subtypes to hyperpolarization

and relaxation in rat middle cerebral artery smooth muscle cells by stimulating the endothelium with the PAR2 receptor agonist, SLIGRL; SLIGRL was the only agent used to stimulate endothelium dependent responses as other mediators such as ACh, ADP and bradykinin elicit, at best, a weak EDHF response in middle cerebral arteries (unpublished data). Subsequently we investigated whether inhibiting TP receptors, thromboxane synthesis or synthesis of 20-HETE can restore the SK_{Ca} component of the EDHF response that is lost after inhibition of NOS. The ability of these treatments to reverse the L-NAME induced depolarisation and constriction was also evaluated.

Materials and Methods

The brain from male Wistar rats (200-300g) was removed and immediately placed in ice-cold Krebs solution. Segments of the middle cerebral artery (~2mm long) were dissected and stored in ice-cold Krebs for use within 30 min, with similar size vessels used in all experimental groups

Experimental protocols

Segments of middle cerebral artery (internal diameter ~150 μm) were mounted in a Mulvany-Halpern myograph (model 400A, Danish Myotechnology) in Krebs solution containing (mM): NaCl, 118.0, $NaCO_3$, 24; KCl, 3.6; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 11.0; $CaCl_2$, 2.5; gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. After equilibration for 20min, vessels were tensioned to 1-1.5mN (approximates wall tension at 60mmHg). Smooth muscle tension was recorded with an isometric pressure transducer and Powerlab software (ADI, Australia). Vessel viability was assessed by adding exogenous K^+ (15-55mM, total K^+ concentration); only vessels developing tension of ≥3 mN were used. Endothelial cell viability was assessed by the ability of SLIGRL (20μM) to relax myogenic tone and to hyperpolarize the smooth muscle cell membrane by >15mV.

All EDHF responses to SLIGRL (20 μM) were obtained in the presence of L-NAME (100 μM) to block NO synthase (NOS), unless otherwise stated. EDHF-mediated responses were assessed in the presence of the K_{Ca} channel blockers, apamin (SK_{Ca}, 50 nM), TRAM-34 (IK_{Ca}, 1μM) and iberiotoxin (BK_{Ca}, 100 nM) The effect of K_{Ca} blockers on EDHF mediated responses was also assessed after addition of the TP receptor antagonists SQ 29,548 (10 μM) and ICI 192,605 (100 μM); the TxA2 synthase inhibitor, furegrelate (10 μM); the cyclo-oxygenase inhibitor, indomethacin (10 μM) and the phospholipase A_2 inhibitor, $AACOCF_3$ (10 μM). In some experiments endothelium dependent hyperpolarization was assessed in vessels without L-NAME and still able to synthesise NO. In these experiments, the effect of the K_{Ca} channel blockers was assessed on endothelium-dependent hyperpolarization induced by SLIGRL (20 μM) in the presence of the TP receptor agonist U46619 (5 nM). Papaverine (150 μM) was added at the end of each experiment to assess overall tone. All drugs were allowed to equilibrate for at least 20min before vasodilator responses were stimulated. In most experiments smooth muscle membrane potential (E_m) and tension were measured simultaneously as previously described, using glass microelectrodes (filled with 2M KCl; tip resistance, 80-120MΩ) to measure E_m (Garland and McPherson, 1992).

Data analysis and statistical procedures

Results are expressed as the mean±s.e.mean of *n* animals. Tension values are given in mN (always per 2mm segment) and E_m as mV. Vasodilatation is expressed as percentage reduction of the total vascular tone (myogenic tone plus vasoconstrictor response), quantified by relaxation with papaverine (150μM). Graphs were drawn and comparisons made using one-way ANOVA with Tukeys' post-test (Prism, Graphpad). $P \leq 0.05$ was considered significant.

Drugs, chemicals, reagents and other materials

Exogenous K^+ was added as an isotonic physiological salt solution in which all the NaCl was replaced with an equivalent amount of KCl. Concentrations of K^+ used are expressed as final bath concentration. AACOCF3 (1,1,1-Trifluoromethyl-6,9,12,15-heieicosatetraen-2-one) L-NAME (N^G-nitro-L-arginine methyl ester), papaverine HCl and U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α) were all obtained from Sigma (Poole, U.K.). Apamin and iberotoxin, from Latoxan (Valence, France). ICI 192,605 (4(Z)-6-(2-o-chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid) from Tocris (Nottingham, UK). SLIGRL (serine, leucine, isoleucine, glycine, arginine, leucine) from Auspep (Parkville, Australia). Furegrelate and SQ 29,548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carnonyl]hydrazine]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) from Cayman-Europe (Tallinn, Estonia). TRAM-34 was a generous gift from Dr H. Wulff (University of California, Davis). All stock solutions (100 mM) were prepared in dimethylsulfoxide (DMSO) except L-NAME, apamin, iberiotoxin, papaverine and SLIGRL which were dissolved in 0.9% NaCl and indomethacin which was dissolved in Na_2CO_3 (1 M); vehicle controls were performed when necessary.

Results

We have previously reported myogenic tone equivalent to about 14% of the maximal vasoconstriction induced in rat middle cerebral arteries by raising $[K^+]_o$ to 55 mM, and that the NO synthase inhibitor, L-NAME (100 μM) further contracts the artery to *circa* 70% of this maximum associated with smooth muscle depolarization of 12.8 ± 0.7 mV (McNeish *et al.*, 2005). In the current study, in the presence of L-NAME induced vasoconstriction SLIGRL induced EDHF-mediated hyperpolarization and relaxation of 19.6 ± 3.1 mV and 80.9 ± 6.7 % (n=4, respectively). In agreement with our previous studies, this hyperpolarization and relaxation was abolished by the selective IK_{Ca} channel inhibitor TRAM-34 (to 1.2 ± 0.8 mV and 6.0 ± 1.8%, respectively, n=4).

Effect of inhibiting TP receptors or CYP 450 on L-NAME constriction and EDHF mediated hyperpolarization and relaxation

The TP receptor antagonist ICI 192,605 (100 μM) reversed depolarization and contraction to L-NAME (by 12.9 ± 2.9 mV, n=3 and 67.9 ± 6.3%, n=7, respectively), but did not alter the EDHF-mediated hyperpolarization (of 18.7 ± 3.8 mV, n=7) and relaxation (73.2 ± 8.5%, n=9) to SLIGRL (20 μM). In the presence of ICI 192,605, EDHF responses were now resistant to block of IK_{Ca} with 1 μM TRAM-34 (hyperpolarization of 13.8 ± 2.3 mV and 52.1 ± 8.6% relaxation, respectively n=4) and remained insensitive to blockade of SK_{Ca} (apamin 50 nM, 11.9 ± 3.5 mV and 59.4 ± 6.1 %, respectively, n=5: Figure 1 & 2). However, in combination these blockers markedly attenuated the EDHF response (to only 4.0 ± 1.9 mV and 21.7 ± 7.3 %, respectively, n=10, $P<0.05$: Figure 1 & 2).

The structurally distinct TP receptor antagonist SQ 29,546 (10 μM) did not modify L-NAME induced tone, but did have similar effects to ICI 192,605 against the EDHF response. In the presence of SQ 29,546 EDHF-mediated hyperpolarization (18.0 ± 2.8 mV, n=10) and relaxation (74.2 ± 6.3%, n=10) was not significantly altered (16.2 ±3.2 mV and 63.6 ± 6.2 %, respectively, n=7). Neither apamin (50 nM, 14.7 ± 0.7 mV and 64.1 ± 2.3%, respectively, n=3) nor TRAM-34 (13.2 ± 4.8 mV and 58.1 ± 9.3%, respectively, n=4, $P>0.05$; Figure 2) had a significant effect on the EDHF hyperpolarization and relaxation (Figure 2), but in combination abolished the response (to 0.5 ± 0.9 mV and 5.0 ± 4.8 %, respectively, n=6, $P<0.05$; Figure 2).

The non-selective CYP 450 inhibitor, 17-ODYA (10 μM) did not alter L-NAME induced constriction (total tone 4.2 ± 0.4 mN and 4.0 ± 0.4 mN in the absence and presence of 17-ODYA, respectively, n=6), or EDHF mediated hyperpolarization and relaxation (19.8 ± 4.3 mV and 74.9 ±

5.8 %, versus 19.0 ± 2.3 mV and 74.4 ± 5.5 %, respectively, n=4). Furthermore, in the presence of 17-ODYA, TRAM-34 alone still effectively abolished EDHF responses (residual, 2.2 ± 2.4 mV and 13.1 ± 5.1%, n=4).

Effect of TP receptor stimulation on endothelium dependent hyperpolarization in the absence of L-NAME

With NO (basal) synthesis extant, endothelium dependent hyperpolarization to SLIGRL (20 μM) in cerebral arteries reflects activation of both SK_{Ca} and IK_{Ca} channels (McNeish *et al.*, 2006). In the present study, under similar conditions, the TP receptor agonist U46619 (5 nM) depolarized and contracted the cerebral arteries (by 7.2 ± 2.8 mV and 3.5 ± 0.3 mN; n=5 and n=13, respectively). During constriction with U46619, SLIGRL-induced hyperpolarization (25.6 ± 2.7 mV n=13) was resistant to apamin (25.6 ± 6.7 mV, n=4) but partially inhibited by TRAM-34 (12.0 ± 1.0 mV, $P<0.01$). The inhibitory action of TRAM-34 was not increased by the additional presence of apamin. The remaining, residual hyperpolarization was, however, attenuated by the inhibitor of BK_{Ca}, iberiotoxin (to 4.6 ± 0.6 mV, n=5, $P<0.001$; Figure 3). Apamin and TRAM-34 alone or in combination did not affect relaxation to SLIGRL (Figure 3), reflecting the direct smooth muscle vasodilator action of NO in these vessels. However, a combination of apamin, TRAM-34 and iberiotoxin did significantly inhibit SLIGRL-induced relaxation (Figure 3). This probably reflects block of NO action, as hyperpolarization (McNeish *et al.*, 2006) and relaxation (unpublished observation) to the NO donor DEA-NONOate is inhibited by iberiotoxin in this artery.

Effect of inhibiting TxA_2 synthase, cyclo-oxygenase or phospholipaseA_2 on L-NAME induced tone and EDHF responses

The thromboxane synthase inhibitor, furegrelate (10 μM), did not affect L-NAME induced constriction (5.0 ± 0.4 mN and 5.0 ± 0.4 mN before and after furegrelate, respectively, n=6), EDHF-mediated hyperpolarization (25.7 ± 5.5 mV, n=5) and relaxation (76.1 ± 6.8, n=5) evoked by SLIGRL (20 μM) was also not significantly modified by furegrelate (at 19.1 ± 3.2 mV and 67.8 ± 5.3%, respectively, n=6) and TRAM-34 did not have any additional effect (14.6 ± 5.9 mV and 67.9 ± 8.8%, respectively, n=4). However, in combination apamin and TRAM-34 did attenuate EDHF-mediated hyperpolarization (to 8.2 ± 1.2 mV, n=5, $P<0.05$; figure 4), but without significantly altering relaxation (64.6 ± 6.3 %, n=5). In the additional presence of the BK_{Ca} channel inhibitor, iberiotoxin (100 nM) EDHF responses were blocked (hyperpolarization of 0.8 ± 1.6 mV and relaxation of 6.4 ± 2.4%, n=4, $P<0.001$; figure 4 & 5). N.B. control EDHF responses obtained in the presence of L-NAME alone (hyperpolarization of 26.0 ± 6.0 mV and relaxation of 84.6 ± 4.7%, n=5) were unaffected by the addition of iberiotoxin (hyperpolarization of 21.2 ± 6.3 mV and relaxation 75.8 ± 4.2 %, n=5, respectively).

Similar results were obtained after inhibition of cyclo-oxygenase with 10 μM indomethacin. Indomethacin did not affect L-NAME induced vasoconstriction or the EDHF response (tone of 5.0 ± 0.6 and 4.7 ± 0.4 mN, n=6 and 11, before and after indomethacin, respectively), but when present TRAM-34 alone only slightly depressed EDHF-mediated hyperpolarization and relaxation (12.1 ± 3.2 and 46.6 ± 10.3%, n=10). However, in combination with apamin these responses were significantly attenuated (7.4 ± 2.0 mV and 35.5 ± 9.3%, respectively, n=6, $P<0.05$). The residual response was blocked by the addition of iberiotoxin (1.4 ± 1.8 mV and 11.6 ± 3.7%, respectively, n=3, $P<0.05$; Figure 5).

The PLA_2 inhibitor $AACOCF_3$ (10 μM) reversed L-NAME-induced tone (85.6 ± 3.8%; Figure 6) and inhibited EDHF hyperpolarization and relaxation (27.7 ± 6.0 mV and 72.0 ± 7.4%, n=7, versus

9.4 ± 3.5 mV and 32.7 ± 15.0%, n=6, respectively). The residual EDHF response was blocked by TRAM-34 alone (5.6 ± 2.8 mV and 4.5 ± 6.5%, respectively, n=4, $P<0.05$; Figure 6).

Discussion

These data demonstrate that activation of TxA_2 (TP) receptors in the rat middle cerebral artery can explain the absence of SK_{Ca} input to endothelium dependent hyperpolarization when NO synthesis is inhibited. Furthermore, inhibition of NOS may result in an increase in TxA_2 synthesis, as inhibiting TxA_2 synthesis restores SK_{Ca} input as well as uncovering a previously unrecognised role for large conductance calcium activated potassium channels (BK_{Ca}) in the EDHF response. These results help to explain our previous observation, that NO protects a functional input from SK_{Ca} in the rat middle cerebral artery (McNeish *et al.*, 2006) and provide a link to our demonstration that the stimulation of TP receptors inhibits SK_{Ca} function (Crane and Garland, 2004, Plane and Garland, 1996).

EDHF responses in the rat middle cerebral artery are unusual, in being dependent only on activation of IK_{Ca}. In most vessels that exhibit an EDHF response, inhibition of both SK_{Ca} and IK_{Ca} channels is necessary to block the EDHF response (Busse *et al.*, 2002). Despite this difference, the rat middle cerebral artery does exhibit similar morphological features to other vessels i.e. both IK_{Ca} and SK_{Ca} channels are expressed within the endothelium (McNeish *et al.*, 2006) and the endothelium is coupled to the smooth muscle layer by myo-endothelial gap junctions (McNeish et al., 2006, Sokoya et al., 2006). Furthermore, SK_{Ca} channels can contribute to endothelium dependent hyperpolarization in the middle cerebral artery, but only when the vessels are still able to synthesise NO (McNeish *et al*. 2006). The NO-dependent contribution of SK_{Ca} to endothelium dependent hyperpolarization did not appear to involve a concomitant inhibition of IK_{Ca}, because in the presence of apamin a normal, maximum hyperpolarization and relaxation was still evoked

(McNeish *et al.*, 2006). The mechanism responsible for the apparent ability of NO to protect SK_{Ca} function is, however, unclear and may involve both a direct effect of NO and downstream signalling mediators such as cGMP-linked effects. For example, NO may directly interact with SK_{Ca} channels, as it does in smooth muscle of the rat fundus (Geeson *et al.*, 2002). Alternatively or additionally, as NO readily interacts with other signalling pathways, particularly those involving heme containing enzymes and the metabolism of arachidonic acid, a protective role may reflect an interaction of the NO/cGMP pathway with factors elaborated within the artery wall.

One possibility, is an alteration in the synthesis of 20-HETE a potent vasoconstrictor derived from arachidonic acid by cytochrome P450 (CYP 450)-dependent enzymes. 20-HETE is involved in myogenic constriction/autoregulation in cerebral vessels (Gebremedhin et al., 2000, Harder et al., 1994) and is also known to inhibit EDHF mediated responses by reducing K_{Ca} function in small coronary arteries (Randriamboavonjy *et al.*, 2005). Furthermore, synthesis of 20-HETE can be inhibited by NO binding to the heme active site of its synthetic enzyme (Minamiyama *et al.*, 1997). However, despite the fact that 20-HETE has a role in myogenic tone we failed to demonstrate any input to cerebral constriction after inhibition of NOS. The non-selective CYP 450 inhibitor 17-ODYA, did not alter the L-NAME induced constriction in the middle cerebral artery. Furthermore, 17-ODYA also failed to reveal any functional role for the SK_{Ca} channel in the EDHF response, as TRAM-34 alone was still able to abolish this response. This suggests that NO does not normally protect SK_{Ca} channel function by inhibiting the synthesis/function of 20-HETE or a related metabolite generated by CYP 450-dependent enzymes.

Another autacoid that could affect K_{Ca} channel function is the potent vasoconstrictor and platelet activator TxA_2. As well as being involved in NOS mediated vasoconstriction in rat middle cerebral arteries (Benyo et al., 1998, Lacza et al., 2001, Gonzales et al., 2005), we have previously shown that stimulation of TxA_2 receptors (TP) results in a fairly rapid loss of the SK_{Ca} component

of EDHF hyperpolarization and associated relaxation in peripheral arteries of the rat (Crane and Garland, 2004). NO does inhibit the formation of TxA_2, by binding to the heme active site of TxA_2 synthase (Wade and Fitzpatrick, 1997). It may also inhibit cyclo-oxygenase (Kanner *et al.*, 1992), responsible for synthesising the precursor of TxA_2 (and other prostaglandins), PGH_2. In addition to inhibiting synthesis, NO is also known to desensitise the TP receptor through a PKG-dependent mechanism (Reid and Kinsella, 2003). In the present study, L-NAME induced constriction was significantly reduced by the TP receptor antagonist, ICI 192,605, indicating that receptor activation might account for at least some of the constriction following inhibition of NOS. In contrast, a structurally unrelated TP receptor antagonist SQ 29,548 failed to alter L-NAME induced constriction, suggesting that ICI 192,605 may have been acting non-selectively. Indeed, the concentration of ICI 192,605 used in this study (100 μM) is known to have effects on PGE_2 (EP) receptors, which may provide an explanation (Brewster *et al.*, 1988). However, in vessels pre-treated with L-NAME, both of the TP receptor antagonists uncovered a functional role for the SK_{Ca} channel in the EDHF response. Simultaneous block of both SK_{Ca} and IK_{Ca} channels with apamin and TRAM-34 was necessary to abolish the response as the functional ability of either channel appeared sufficient to elicit adequate hyperpolarization for full EDHF mediated relaxation. Therefore, stimulation of TP receptors could explain the loss of the SK_{Ca} dependent component of endothelium dependent hyperpolarization after inhibition of NOS. This suggestion is supported by the observation that activation of TP receptors with U46619 abolishes the SK_{Ca} component of endothelium dependent hyperpolarization, in arteries still able to synthesise NO. NO dependent inhibition of TP receptors appears to depend on activation of PKG (Reid and Kinsella, 2003), which may explain our previous observation that ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) , an inhibitor of soluble guanylate cyclase, revealed endothelium dependent hyperpolarization mainly dependent upon IK_{Ca} (McNeish *et al.*, 2006). The possible involvement of cGMP mediated effects in the regulation of SK_{Ca} channel function is a subject of ongoing investigation.

As stimulation of TP receptors appeared to account for the loss of SK_{Ca} function in arteries treated with a NOS inhibitor, we investigated if receptor stimulation reflected an increased synthesis of TxA_2 (or its precursor PGH_2, which can also stimulate these receptors) as opposed to an inhibitory action of NO/cGMP dependent signalling on TP receptors. The former appeared to be the case, as inhibition of TxA_2 synthase with furegrelate or inhibition of cyclo-oxygenase (to inhibit synthesis of PGH_2 and thus TxA_2) with indomethacin each revealed a role for SK_{Ca} channels in the EDHF response. Neither treatment had any effect on the L-NAME induced constriction, again indicating that stimulation of TP receptors does not form a major component of this response. Interestingly, pre-treatment with either indomethacin or furegrelate also revealed a role for BK_{Ca} in the EDHF response. The explanation for this unexpected observation is the subject of ongoing investigation. One possibility is that altering the prostanoid profile in the vessel wall uncovers an eicosanoid pathway able directly to activate BK_{Ca} on the smooth muscle cells in this artery (McNeish *et al.*, 2006).

The observation that stimulation of either TP receptors or 20-HETE did not appear to contribute to the contraction following inhibition of NOS was surprising, as both signalling pathways have previously been implicated in this response (Benyo et al., 1998, Lacza et al., 2001, Harder et al., 1994). However, our data do indicate that constriction involves a metabolite of arachidonic acid, as the PLA_2 inhibitor $AACOCF_3$ fully reversed L-NAME-induced constriction. Interestingly, the EDHF response was also attenuated by inhibition of PLA_2, which is consistent with previous observations in the rat middle cerebral artery (You *et al.*, 2002). However, a significant EDHF response remained after treatment with $AACOCF_3$ and was abolished by TRAM-34. Overall, these observations suggest there are at least two components of the EDHF response in the rat middle cerebral artery. One component appears to involve a metabolite of arachidonic acid. A recent study by You *et al.* (2005) appears to rule out the involvement of metabolites of either the lipoxygenase

(EETs) or epoxgenase pathways (HETEs) in rat middle cerebral arteries (You *et al.*, 2005), so the identity of such an active metabolite remains uncertain. Other components of the EDHF response appear to rely solely upon the activation of endothelial K_{Ca} channels, and may lead to smooth muscle hyperpolarization/relaxation through an increase in extracellular $[K^+]$ (McNeish *et al.*, 2005) and/or a direct transfer of hyperpolarization via myoendothelial gap junctions (McNeish et al., 2006, Sokoya et al., 2006).

In summary, inhibition of NO synthase leads to pronounced constriction in cerebral arteries that appears to involve an unidentified metabolite of arachidonic acid. This metabolite does not appear to be either of the potent endogenous vasoconstrictors 20-HETE or TxA_2. However, block of the SK_{Ca}-mediated component of endothelium-dependent (EDHF) hyperpolarization which follows inhibition of NOS, can be reversed by inhibiting TP receptors or by reducing the synthesis of TxA_2. Therefore, increased thromboxane signalling after inhibition of NOS may underlie blockade of a fundamental part of the EDHF response in these arteries. The fact that loss of NO signalling can disrupt the EDHF pathway and associated vasodilatation, through increased activity of the potent vasoconstrictor/platelet activator TxA_2, is likely to be of fundamental relevance in disease states where NO release is known to be compromised.

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Conflicts of interest

None

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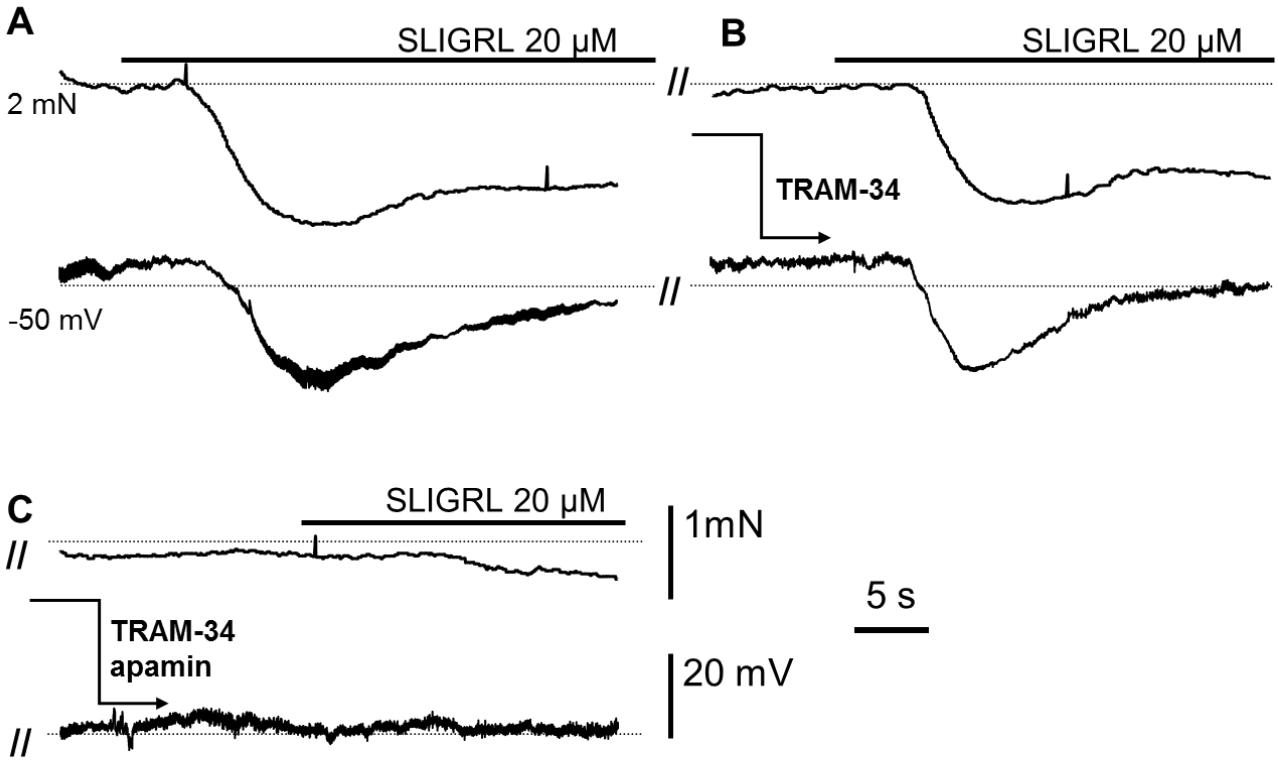
Figure 1 Original recordings of EDHF mediated relaxation (upper trace) and hyperpolarization (lower trace) from a rat middle cerebral artery preconstricted with the NOS inhibitor L-NAME (100 μM) and in the presence of the of the TP receptor inhibitor ICI 192,605 (100 μM; A), also shown is the subsequent effects either of the IK_{Ca} channel inhibitor TRAM-34 (1 μM) alone (B) or the combined blockade of IK_{Ca} and SK_{Ca} channels with TRAM-34 and apamin (50 nM; C) on the EDHF response. Hatched lines represent the control tension and resting membrane potential, respectively. In the presence of ICI 192,605, EDHF responses have a functional input from SK_{Ca} as they were only blocked by the combination of TRAM-34 and apamin. Parallel lines (//) indicate a time break between separate recordings from a single vessel.



L-NAME (100 μM) and ICI 192,605 (100 μM) present throughout

Figure 2 Histograms showing the effect of the structurally distinct TP receptor antagonists ICI 193,605 (100 μM; A) and SQ 29,548 (10 μM; B) on SLIGRL (20 μM)-induced, EDHF mediated hyperpolarization (left panels) and relaxation (right panels) in rat middle cerebral artery preconstricted with the NOS inhibitor L-NAME (100 μM). Also shown is the effect of the IK_{Ca} blocker, TRAM-34 (1 μM) and the SK_{Ca} blocker apamin (50 nM) both alone and in combination. When TP receptors are inhibited, the EDHF response was only blocked by combined incubation of both TRAM-34 and apamin indicating that there is a functional input from SK_{Ca} in this response. **P<0.01 and ***P<0.001 indicate a statistically significant difference from control.

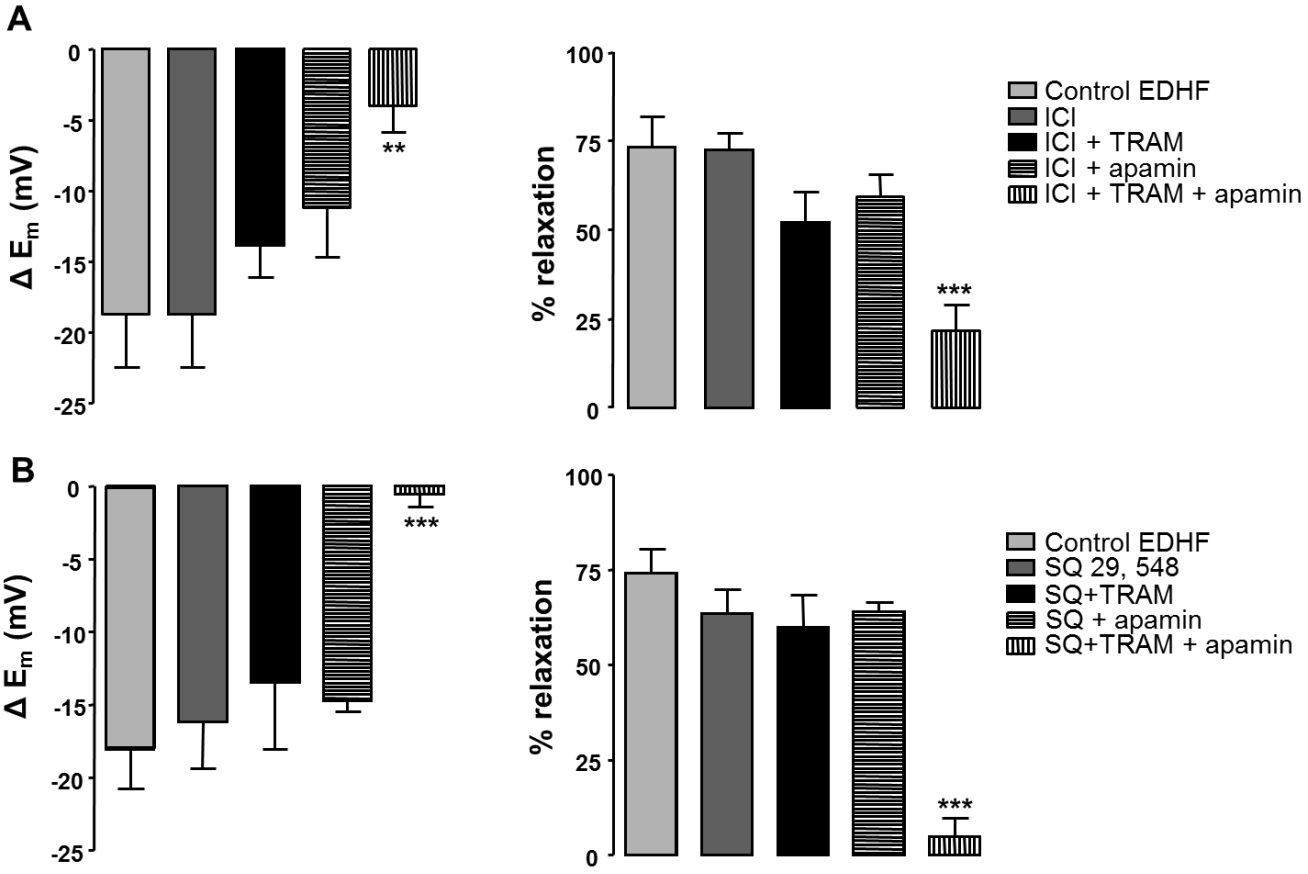


Figure 3 Histograms showing the effect of the TxA_2 mimetic U46619 (5 nM) on hyperpolarization (A) and relaxation (B) produced by SLIGRL (20 μM) in rat middle cerebral arteries that had not been treated with a NOS inhibitor. Also shown are the effects of the IK_{Ca} inhibitor TRAM-34 (1 μM), the SK_{Ca} channel inhibitor, apamin (50 nM) and the BK_{Ca} inhibitor, iberiotoxin (100 nM) on these responses. TRAM-34 alone inhibited the SLIGRL induced hyperpolarization whereas apamin had no effect. Combination of TRAM-34 and apamin had no additional effect when compared to TRAM-34 alone. The combination of apamin, TRAM-34 and iberiotoxin did further attenuate hyperpolarization and relaxation. Relaxations were unaffected by apamin and TRAM-34 alone or in combination with apamin as these vessels are able to synthesise NO. The NO dependent relaxation is affected by iberiotoxin. **P<0.01 and ***P<0.001 indicate a statistically significant difference from control.

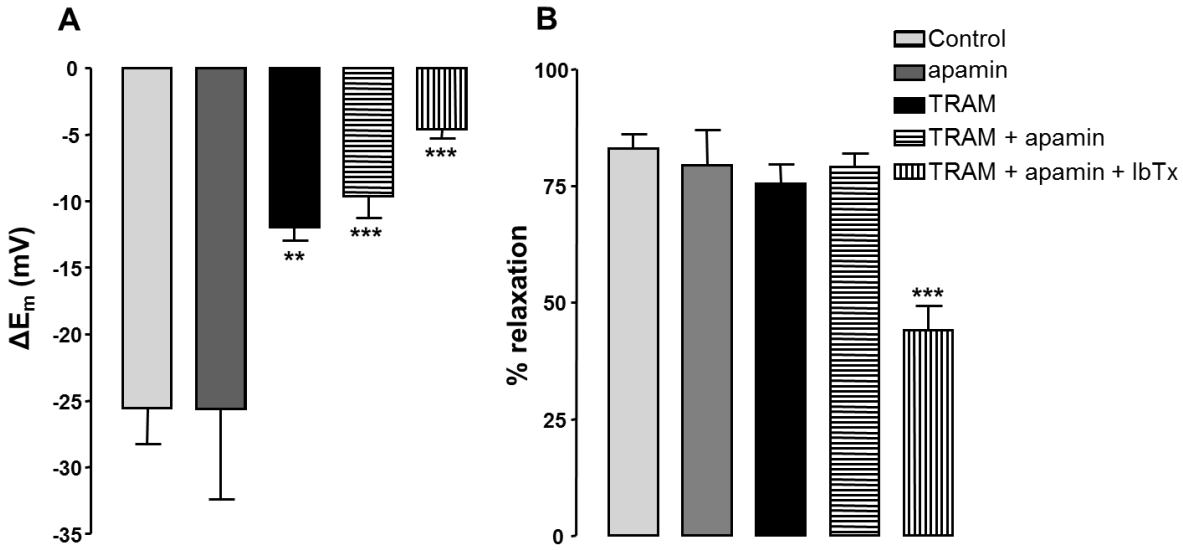
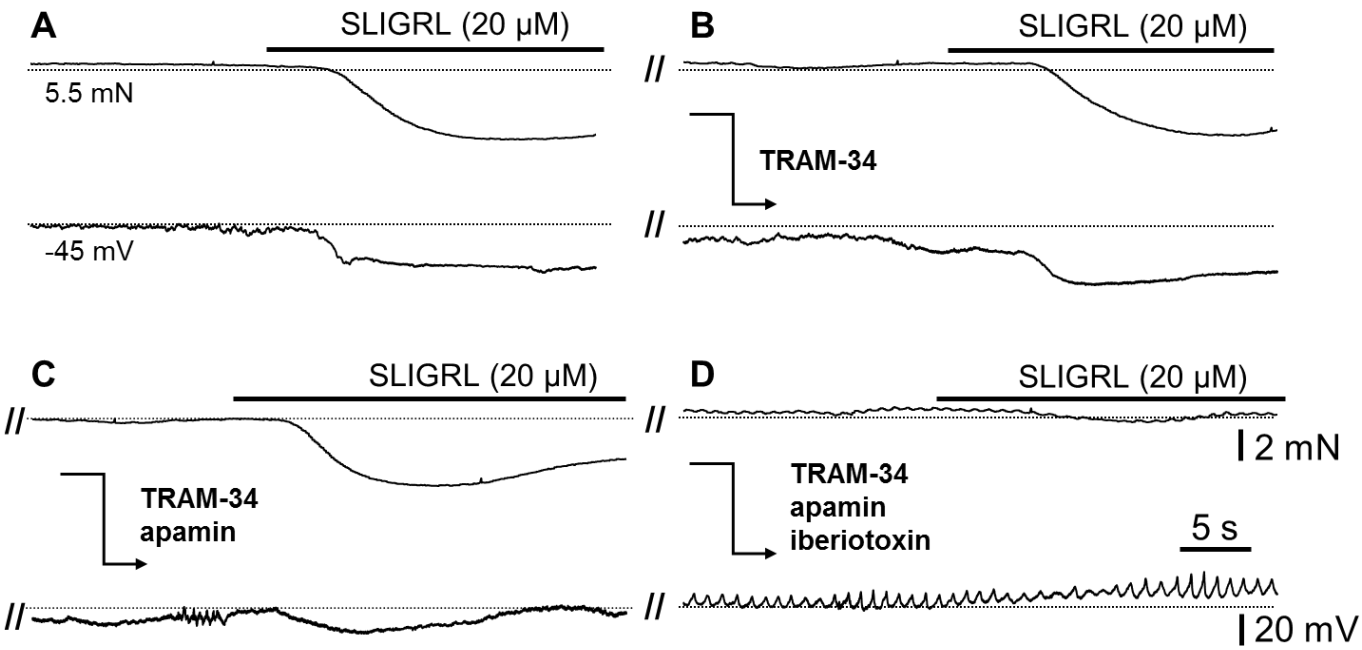


Figure 4 Original traces showing SLIGRL (20 μM)-induced, EDHF mediated relaxation (upper panels) and hyperpolarization (lower panels) in a rat middle cerebral artery treated with the NOS inhibitor L-NAME (100 μM) and the thromboxane synthase inhibitor, furegrelate (10 μM). Also shown is the additional effect of: (B) the IK_{Ca} inhibitor, TRAM-34 (1 μM); (C) the combination of TRAM-34 and the SK_{Ca} inhibitor, apamin (50 nM) and (D) the combination of TRAM-34, apamin and the BK_{Ca} inhibitor, iberiotoxin (100 nM). Hatched lines represent the control tension and membrane potential. Only the combination of apamin, TRAM-34 and iberiotoxin fully blocked the EDHF response indicating that functional inputs from SK_{Ca}, IK_{Ca}, and BK_{Ca} contribute to the EDHF response under these conditions. Parallel lines (//) indicate a time break between separate recordings from a single vessel.



L-NAME (100 μM) and furegrelate (10 μM) present throughout

Figure 5 Histograms showing the EDHF mediated hyperpolarization (left panels) and relaxation (right panels) in the presence of the NO synthase inhibitor L-NAME (100 μM) and either: (A) the thromboxane synthase inhibitor, furegrelate (10 μM), or (B) the cyclo-oxygenase inhibitor indomethacin (10 μM). Also shown are the effects of the K_{Ca} inhibitors, TRAM-34 (IK_{Ca}; 1 μM), apamin (SK_{Ca}; 50 nM) and iberiotoxin (BK_{Ca}; 100 nM). Only combined application of TRAM-34, apamin and iberiotoxin fully blocked the EDHF response in the presence of either furegrelate or indomethacin. *P<0.05, **P<0.01 and ***P<0.001 indicate a statistically significant difference from control.

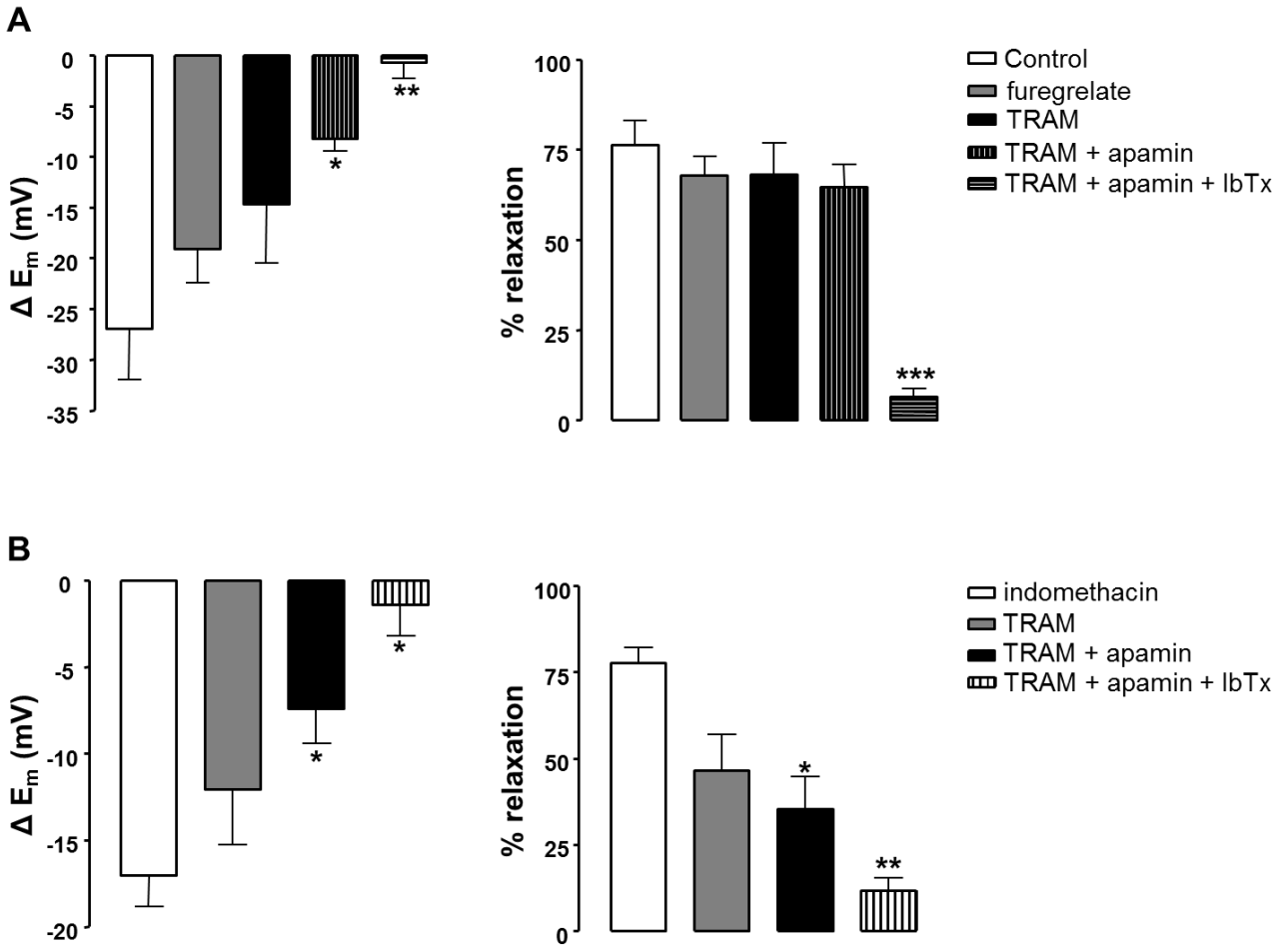
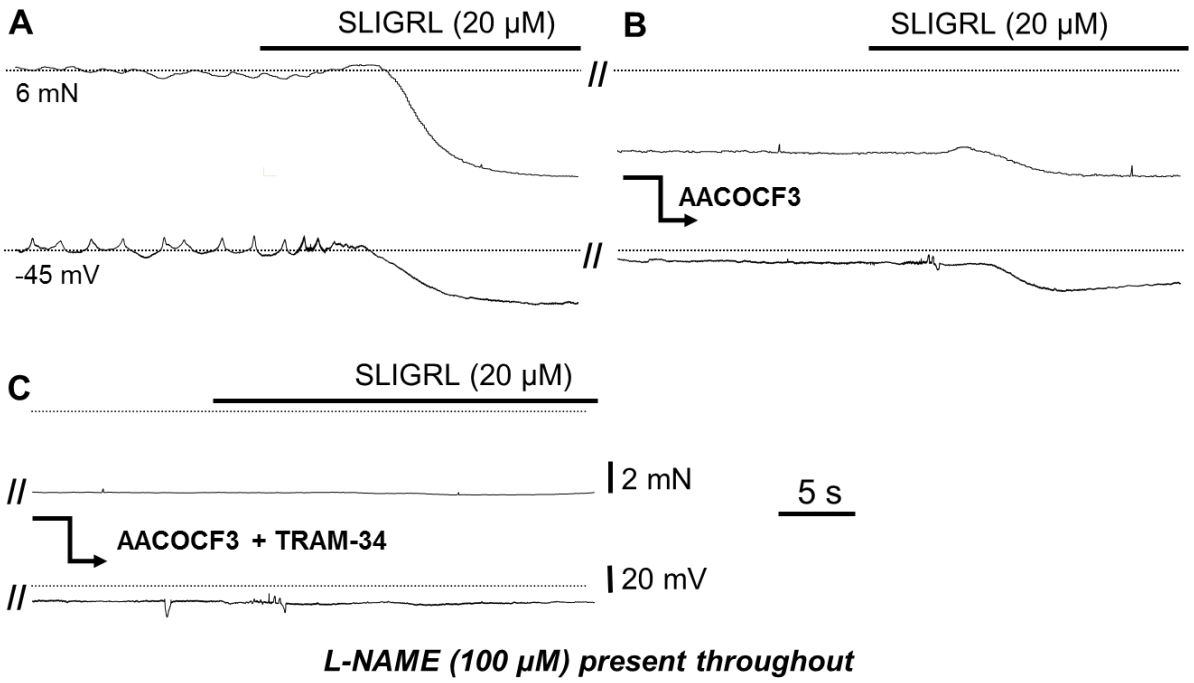



Figure 6 Original traces showing SLIGRL (20 μM)-induced EDHF mediated relaxation (upper traces) and hyperpolarization (lower traces) in a rat middle cerebral artery treated with the NOS inhibitor L-NAME (100 μM; A). Also shown is the effect of the PLA_2 inhibitor AACOCF3 (10 μM) on L-NAME induced tone and the EDHF response (B) and the subsequent effect of IK_{Ca} blocker, TRAM-34 (1 μM) on the residual EDHF response (C). Hatched lines represent the control membrane potential and tension before addition of AACOCF3. AACOCF3 relaxed the L-NAME-induced tone as well as attenuating the EDHF response, the residual EDHF response was completely blocked by TRAM-34 alone. Parallel lines (//) indicate a time break between separate recordings from a single vessel.