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The role of voltage-operated and non-voltage-operated calcium channels in endothelin-induced vasoconstriction of rat cerebral arteries

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ABSTRACT

Endothelin-1 has been identified as a potential mediator in the pathogenesis of ischaemic stroke and cerebral vasospasm. The aim of this study was to analyse the role of voltage-operated calcium channels (VOCC) and non-VOCC in endothelin-1 induced vasoconstriction of rat cerebral arteries. Arterial segments were dissected from different regions of the cerebral circulation and responses assessed using wire myography. Endothelin-1 concentration–contraction curves were constructed in calcium-free medium or in the presence of nifedipine, NNC 55-0396 ((1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride) or SK&F 96365 (1-(2-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenylethyl)-1H-imidazole) to inhibit the L-type VOCC, T-type VOCC and non-VOCC, respectively. Inhibition of the calcium channels or removal of calcium from the medium variably decreased the maximum effects (E_{max}) of endothelin-1, however its potency (pEC_{50}) was unaltered. Endothelin-1 caused a small contraction ($<22\%$) in calcium-free solution. Pre-treatment with nifedipine (1 μM) did not affect responses to low concentrations of endothelin-1 but decreased E_{max}, while NNC 55-0396 (1 μM) and SK&F 96365 (30–100 μM) generally attenuated the endothelin-1-induced contraction. Combination of nifedipine with SK&F 96365 further decreased the E_{max}. The relaxant effect of the calcium channel antagonists was also assessed in pre-contracted arteries. Only nifedipine and SK&F 96365 relaxed the arteries pre-contracted with endothelin-1. In conclusion, VOCC and non-VOCC calcium channels are involved in different phases of the endothelin-1 contraction in rat cerebral vessels. T-type VOCC may be involved in contraction induced by low concentrations of endothelin-1, while L-type VOCC mediate the maintenance phase of contraction. VOCC and non-VOCC may work in concert in mediating contraction induced by endothelin-1.

1. Introduction

Endothelins are vasoactive peptides mainly synthesised and released by endothelial cells (Barton and Yanagisawa, 2008; Yanagisawa et al., 1988). This family of peptides includes endothelin (ET)-1, ET-2 and ET-3, endothelin-1 being the most powerful vasoconstrictor peptide. Endothelins have two distinct receptors – ET_A and ET_B (Davenport, 2002). Endothelin ET_A receptors located on smooth vascular muscle cells mediate constriction while endothelin ET_B receptors located on endothelial cells mediate vasodilatation via the release of nitric oxide. However, endothelin ET_B receptors if located on smooth muscle cells also mediate vasoconstriction (Haynes et al., 1995). Endothelin-1 has been identified as a potential cause of ischaemic stroke and cerebral vasospasm that commonly occurs after subarachnoid haemorrhage. Studies have reported increased concentrations of endothelin-1 in cerebrospinal fluid of subarachnoid haemorrhage patients during cerebral vasospasm (Kästner et al., 2005; Kobayashi et al., 1995). In addition, increased sensitivity of cerebral vessels to endogenous vasoconstrictor agents may also contribute to the pathogenesis of the vasospasm due to upregulation of receptors (Edvinsson and Povlsen, 2011). This indicates that even relatively low concentrations of vasoconstrictor mediators may cause significant vasospasm. The vasoconstrictor action of endothelin-1 depends predominantly on influx of calcium from the extracellular space. It can also cause release of calcium from the sarcoplasmic/endoplasmic reticulum that normally constitutes a

Abbreviations: KPSS, Isotonic potassium physiological salt solution; ROCC, Receptor-operated calcium channels; SOCC, Store-operated calcium channels; VOCC, Voltage-operated calcium channels

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small proportion of the calcium source (Hyvelin et al., 1998; QingHua et al., 2009). Despite initial reports that endothelin-1 activates VOCC in porcine coronary artery smooth muscle cells (Goto et al., 1989), it appears that these channels play a minor role in the vasoconstrictor response since nifedipine, a selective inhibitor of L-type VOCC, did not inhibit calcium influx in this tissue (Kawanabe et al., 2002; Kawanabe and Nauli, 2005). Similarly a nifedipine-resistant response, sensitive to T-type VOCC inhibitors, has been reported in pressurised smaller branches of the rat basilar artery (Kuo et al., 2010). Endothelin-1 has also been reported to enhance calcium entry into cardiac smooth muscle cells via T-type VOCC in rat ventricular myocytes (Furukawa et al., 1992). Non-voltage-operated calcium channels, namely receptor-operated calcium channels (ROCC) and store-operated calcium channels (SOCC), are known to play a major role in the sustained increase of intracellular calcium that maintains the contractile effect of endothelin-1 (Enoki et al., 1995; Iwamuro et al., 1998; Iwamuro et al., 1999; Kawanabe et al., 2002; Zhang et al., 1999, 2000). Understanding the functional relationship between endothelin-1 and calcium channels in the cerebral circulation may help to unravel the mechanism of endothelin-1-induced vasospasm. The aim of this study was to characterize the vasoconstrictor effects of endothelin-1 and the relative roles of L- and T-type VOCC, ROCC and SOCC in arterial segments isolated from different regions of the rat cerebral circulation using wire myography.

2. Materials and methods

2.1. Animals

The University of Melbourne Animal Ethics Committee approved experiments in accordance with the *Australian code for the care and use of animals for scientific purposes* (8th edition, 2013, National Health and Medical Research Council, Canberra). Rats were housed in climate-controlled conditions with 12 h light/dark cycle and had free access to normal pellet diet and drinking water.

2.2. Harvest and set up of cerebral arteries

Male Sprague-Dawley rats (300–350 g) were deeply anaesthetised by spontaneous inhalation of 5% isoflurane (Baxter Healthcare Pty. Ltd.; Toongabbie, NSW, Australia) in 95% O_2 and killed by decapitation. The brains were immediately removed and placed in ice-cold physiological salt solution of the following composition (in mM): NaCl 119, KCl 4.69, $MgSO_4 \cdot 7H_2O$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2 \cdot 6H_2O$ 1.5, EDTA 0.026 and glucose 5.5. In experiments involving calcium-free physiological salt solution, $CaCl_2$ and EDTA were replaced by EGTA (1 mM). The anterior cerebral artery, middle cerebral artery, posterior communicating artery and basilar artery were carefully dissected and cleared of connective tissue. In some protocols mesenteric arteries were dissected and set up to compare effects in the peripheral circulation; the physiological salt solution used for mesenteric artery experiments contained 2.5 mM $CaCl_2$.

The vessels were cut into 2 mm long segments and mounted on two 40 μm stainless steel wires in a myograph chamber (620M; Danish Myo Technology; Aarhus, Denmark). One of the wires was connected to an isometric force transducer while the other was connected to a screw micrometre used for adjustment of passive force by varying the distance between the wires. Measurements of vascular contractile force were recorded on an Apple computer using Chart 5 software (AD Instruments, Pty. Ltd, Bella Vista, NSW, Australia). The arteries were allowed to equilibrate in physiological salt solution at 37 °C (pH 7.4) with continuous aeration by a mixture of 95% O_2 and 5% CO_2. After 20 min, the vessels were gradually stretched to a tension equivalent to a transmural pressure of 100 mmHg, generated by a length-passive tension curve. The vessels were then immediately relaxed to 90% of the internal diameter which was determined from the curve (Mulvany and Halpern, 1977). The viability of the arteries was tested by exposure to isotonic potassium salt solution (K^+ physiological salt solution; KPSS) containing 124 mM K^+ obtained by equimolar substitution of NaCl with KCl in the physiological salt solution. The KPSS-induced contraction was repeated after 5 min and the maximum response of the second contraction was used as a reference for quantification of all vascular responses. Only vessels with a KPSS-induced contraction of 2.5 mN or more were considered acceptable for experimental protocols. The presence of a functional endothelium was tested by the relaxant response to acetylcholine (10 μM) in arteries pre-contracted with 0.1–1 nM vasopressin. The endothelium was considered intact when acetylcholine caused more than 50% relaxation.

2.3. Experimental protocols

The effects of endothelin-1 (0.01–100 nM) were examined by addition of cumulative concentrations in half-$\log_{10}$ increments. Responses were allowed to plateau before addition of the next higher concentration. The functional presence of endothelin ET_B receptors in cerebral arteries was assessed using sarafotoxin S6c, a selective endothelin ET_B receptor agonist. The vessels were pre-contracted to 80–90% of the maximum KPSS-induced contraction using vasopressin (0.1–1 nM), and relaxed with cumulative concentrations of sarafotoxin S6c (0.01–3 nM). Nifedipine (1 μM), NNC 55-0396 ((1S,2S)-2-(2-(N-[(3-benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride) (1 μM) or SK&F 96365(1-(2-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenylethyl)-1 H-imidazole) (10 or 30 μM) were used to assess the roles of L-type VOCC, T-type VOCC or SOCC and ROCC, respectively. Combinations of 1 μM nifedipine with 10 or 30 μM SK&F 96365 were also used to investigate effects of combined blockade of L-type VOCC and non-voltage-operated calcium channels. All calcium channel inhibitors were added 20–25 min before addition of endothelin-1.

In a second series, the ability of these calcium channel antagonists to relax endothelin-1 induced contraction was investigated. The vessels were contracted with endothelin-1 10 nM followed by addition of cumulative concentrations of nifedipine, NNC 55-0396 or SK&F 96365. In a third series, the middle cerebral artery was pre-contracted with endothelin-1 (10 nM) before adding a single concentration of nifedipine (0.01 or 1 μM), SK&F 96365 (1 or 10 μM) or their combinations. Some vessels acted as time controls after the endothelin-1 pre-contraction.

2.4. Statistical analysis

All data were expressed as mean ± S.E.M. Data analysis and sigmoid curve fitting (non-linear regression) were performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA) to derive pEC_{50} and maximum response (E_{max}) values. EC_{50} is defined as the concentration of agonist that evokes the half-maximal response and pEC_{50} is the negative logarithm of the EC_{50}. Statistical comparison between groups of arteries was performed using one-way ANOVA followed by Dunnett's post-hoc test or Bonferroni's post-hoc test between selected pairs. $P < 0.05$ was considered significant in all cases.

2.5. Drugs and chemicals

The drugs used in this study were obtained from the following suppliers: Endothelin-1 (GenScript, Piscataway, NJ, USA), vasopressin and sarafotoxin S6c (AusPep, Parkville, Victoria, Australia), bosentan (gift from Actelion Pharmaceutical Ltd., Allschwil, Switzerland),