Definitive role for natriuretic peptide receptor-C in mediating the vasorelaxant activity of C-type natriuretic peptide and endothelium-derived hyperpolarising factor

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Abstract

Objective: C-type natriuretic peptide (CNP) has recently been suggested to represent an endothelium-derived hyperpolarising factor (EDHF) in the mammalian resistance vasculature and, as such, important in the regulation of local blood flow and systemic blood pressure. Additionally, this peptide has been shown to protect against ischaemia–reperfusion injury and inhibits leukocyte and platelet activation. Herein, we use a novel, selective natriuretic peptide receptor-C (NPR-C) antagonist (M372049) to highlight the pivotal contribution of CNP/NPR-C signalling in the EDHF-dependent regulation of vascular tone and investigate the mechanism(s) underlying the release and biological activity of CNP.

Methods: In vitro pharmacological investigation was conducted in rat (Sprague–Dawley) aorta and mesenteric resistance arteries. Relaxant responses to CNP, atrial natriuretic peptide (ANP), the nitric oxide donor spermine-NONOate (SPER-NO) and the endothelium-dependent vasodilator, acetylcholine (ACh) were examined in the absence and presence of M372049 or inhibitor cocktails shown previously to block endothelium-dependent dilatation in the resistance vasculature. RT-PCR was employed to characterize the expression of NPR subtypes in the vessels studied.

Results: M372049 produced concentration-dependent inhibition of the vasorelaxant activity of CNP in rat isolated mesenteric resistance arteries but not aorta; in contrast, M372049 did not affect relaxations to ANP or SPER-NO in either vessel. M372049 or ouabain alone produced small, significant inhibition of EDHF-dependent relaxations in mesenteric arteries and in combination acted synergistically to abolish such responses. A combination of M372049 with established inhibitors of EDHF-dependent relaxation revealed that multiple, distinct pathways coordinate the bioactivity of EDHF in the resistance vasculature, and that CNP/NPR-C signalling represents a major component.

Conclusions: These data substantiate CNP/NPR-C signalling as a fundamental pathway underlying EDHF-dependent regulation of vascular tone in the rat mesenteric resistance vasculature. An increased understanding of the physiological roles of CNP/NPR-C signalling in the vasculature (now facilitated by the identification of a selective NPR-C antagonist) should aid determination of the (patho)physiological importance of EDHF and might provide the rationale for the design of novel therapeutics.

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Keywords: Blood flow; Natriuretic peptide; Endothelium-derived hyperpolarising factor; Microcirculation; Vasodilation
1. Introduction

C-type natriuretic peptide (CNP) affords a key paracrine element to the biological activity of the natriuretic peptide family of vasoactive mediators that are important in the maintenance of blood volume and blood pressure [1–3]. In contrast to the cardiac-derived, endocrine activity of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), CNP is expressed abundantly in vascular endothelial cells and thought to act in a local fashion to regulate vascular tone and blood flow [4–7]. The importance of endothelium-derived CNP to cardiovascular homeostasis has been highlighted recently by work identifying this peptide as an endothelium-derived hyperpolarising factor (EDHF) in the coronary and mesenteric resistance vasculature [8,9]. These investigations have also elucidated, in part, the signal transduction pathway(s) underlying the vasorelaxant activity of CNP and identified a novel system involving activation of a G-protein-coupled inwardly rectifying K+ channel (GIRK) [8]. Moreover, it has been shown that endothelium-dependent vasorelaxants, such as ACh, release CNP acutely from the vascular endothelium [8,9]. Subsequently, it has been demonstrated that CNP can also procure protection against myocardial ischaemia–reperfusion (I/R) injury [9] and inhibits leukocyte recruitment and platelet aggregation [10]. In summary, these observations suggest that the remit of CNP (and perhaps EDHF) includes regulation of circulating elements (i.e. platelets, leukocytes) in addition to effects on vascular smooth muscle tone; as such, it is likely that CNP maintains an important anti-atherogenic influence of the blood vessel wall akin to endothelium-derived NO. Consequently, interventions that can mimic the effects of CNP may offer therapeutic potential in combating cardiovascular disorders.

The biological activity of CNP is thought to be mediated exclusively via activation of natriuretic peptide receptor (NPR)-B, for which CNP represents the sole endogenous ligand [11]. However, CNP (akin to other natriuretic peptides) also binds to NPR-C, which is thought to clear natriuretic peptides from the circulation by binding, internalization and subjecting them to lysosomal degradation [12]. In addition to this ‘clearance’ function, NPR-C also possesses an intracellular G-protein binding domain and can couple to GβG proteins to inhibit adenylyl cyclase activity and activate phospholipase-Cβ3 [13]. This positive signalling role is exemplified by the CNP-dependent inhibition of neuronal catecholamine release [14]. Previous studies have provided indirect evidence that NPR-C may be important in the vasodilator activity of EDHF in the mesenteric and coronary resistance vasculature [8,9] (in contrast, in conduit arteries CNP elicits a NPR-B, cGMP-dependent reduction in vascular tone [15]). Unfortunately, differentiation between NPR-B, cGMP-dependent and NPR-C, cGMP-independent effects of CNP (and other natriuretic peptides) is difficult due to the lack of selective receptor antagonists. HS-142-1, a polysaccharide derived from Aureobasidium sp., has been used in this regard since it appears to block NPR-A and NPR-B activity [16,17]. However, to provide definitive evidence for a key, positive signalling role for NPR-C in mediating the vasorelaxant (and anti-atherogenic) activity of CNP and EDHF, a selective NPR-C antagonist would be required.

To address this deficit, we used a novel NPR-C selective antagonist, M372049 (Fig. 1). This compound is one member of a family of homologous molecules developed to prevent binding of all natriuretic peptides to NPR-C [18]. Herein, using M372049 we demonstrate that NPR-C is responsible for CNP-dependent and EDHF-dependent dilation in the rat mesenteric resistance vasculature. Hence, we have established NPR-C as an important effector pathway in the regulation of vascular tone, identified CNP as a major component of the EDHF response in this vascular bed and highlighted a selective pharmacological tool for the elucidation of the (patho)physiological significance of CNP.

2. Methods

All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom and conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, 1996).

2.1. Rat isolated mesenteric artery

Rat isolated mesenteric resistance arteries were used as a model of EDHF bioactivity. Male rats (Sprague–Dawley; 200–250 g) were stunned and killed by cervical dislocation. The mesentery was removed and third-order arteries mounted in an automated tension myograph (Danish Myotechnology, Denmark), as previously described [8]. After an equilibration period of 45 min, vessels were normalized according to published protocols and vessel diameter determined [19]. Following normalization, each vessel was contracted repeatedly with the thromboxane A2-mimetic 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin F2α (U46619; 1 μM) until the response was reproducible. The vessels were then washed to restore basal tone before contracting to approximately 50% of the maximum U46619-induced response. Once a stable response to U46619 was achieved, cumulative concentration-response curves were constructed to SPER-NO (0.001–10 μM), ACh (0.001–10 μM), ANP (0.001–
1 μM) or CNP (0.001–1 μM) in the absence or presence of stated interventions. Only one curve to any one agonist was constructed in any single tissue and all experiments were conducted in the presence of L-NAME (300 μM) and indomethacin (5 μM). In certain experiments tissues were exposed to various inhibitors (that have been shown previously to block EDHF-dependent relaxation in the resistance vasculature [20]), either alone or in combination, including the small conductance calcium-activated potassium channel (SKCa) inhibitor apamin (100 nM [21,22]), the intermediate conductance calcium-activated potassium channel (IKCa) inhibitor TRAM-34 (10 μM [23,24]), the inwardly rectifying potassium channel (Kir) blocker Ba2+ (30 μM [22,25]), the Na+/K+-ATPase inhibitor ouabain (1 mM [21,22]) and M372049 (100 nM).

2.2. Membrane potential measurements

In situ membrane potential measurements were recorded in rat isolated mesenteric resistance arteries to link blockade of functional EDHF responses with inhibition of smooth muscle cell hyperpolarisation. Mesenteric resistance arteries were mounted in a tension myograph, normalized and equilibrated using U46619 as described above. Vessels were incubated with L-NAME (300 μM) and indomethacin (5 μM) and membrane potential was measured continuously using aluminum silicate microelectrodes (1 mm in diameter, World Precision Instruments, USA) that had resistances between 50 and 90 MΩ when filled with 2 M KCl. Membrane potential (mV) was measured using an oscilloscope (Gould, UK) connected to an amplifier (Intra 767 electrometer, World Precision Instruments, USA) and recorded on a chart recorder (BBC Goertz Metrawatt). Electrode entry into a vascular smooth muscle cell was determined by an abrupt drop in voltage, followed by a sharp return to baseline on exit, with a minimal change (no more than 10%) in resistance [26].

2.3. Electrophysiological studies

HEK293 cells stably expressing a G-protein-gated inwardly rectifying potassium channel (Kir3.1+Kir 3.2A) and the M₄-muscarinic receptor [27] were employed to ascertain if M372049 was a direct Kir channel blocker. Whole-cell membrane currents were recorded at room temperature with an Axopatch 200B amplifier, and digitised with a Digidata 1322A interface (both Axon Instruments) and analysed with pClamp software (version 8.0; Axon Instruments). Cells were perfused using a gravity-fed bath perfusion system. Pipette solution: 107 mM glutamate,
20 mM KCl, 10 mM NaCl, 1 mM MgCl2, 2 mM EGTA, 10 mM Hepes, 2 mM MgATP, 0.01 mM Na2GTP; KOH to pH 7.3. Bath solution: 120 mM NaCl, 20 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, NaOH to pH 7.4. Cell capacitance was 15–20 pF.

Under voltage-clamp condition, cells were held at 0 mV, stepped to −80 mV for 20 ms and pulsed for 400 ms from −120 mV to +60 mV in 12 mV increments. KIR currents induced by carbachol (10 μM; measured at 120 mV) were compared in the absence and presence of M372049 (10 μM) and Ba2+ (30 μM).

2.4. Rat isolated thoracic aorta

Rat isolated thoracic aortae were employed as a model of endothelium-derived relaxing factor (EDRF) bioactivity and to provide a comparison between the conduit and resistance vasculature in terms of the importance of CNP/NPR-C signalling. Male rats (Sprague–Dawley; 200–250 g) were stunned and killed by cervical dislocation. The thoracic aortae were carefully removed and mounted in organ baths for isometric tension recordings, as described previously [15]. Tissues were then pre-contracted with phenylephrine (PE; EC80). Once a stable response to PE was achieved, cumulative concentration-response curves to SPER-NO (0.001–10 μM), ACh (0.001–1 μM), ANP (0.001–1 μM) or CNP (0.001–1 μM) were constructed in the absence or presence of M372049 (10 μM) and Ba2+ (30 μM).

2.5. NPR mRNA expression

Reverse transcription-polymerase chain reaction (RT-PCR) was used to assess the expression of mRNA for each NPR in aortic and mesenteric vascular smooth muscle following a protocol identical to that we have described previously [28,29]. Thermal cycling conditions for PCR were as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and polymerization at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. RT-PCR products were resolved by agarose gel electrophoresis (2% gel) and stained with ethidium bromide.

Primer sequences were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPR-A sense</td>
<td>CATCCTGGACAACCTGC</td>
<td>714 bp</td>
</tr>
<tr>
<td>NPR-A antisense</td>
<td>TAGGTCGAAACCTTGGCC</td>
<td></td>
</tr>
<tr>
<td>NPR-B sense</td>
<td>CATGCCAGGCAAATCGAACC</td>
<td>720 bp</td>
</tr>
<tr>
<td>NPR-B antisense</td>
<td>TGCTTCGACCCTTGTGATATCG</td>
<td></td>
</tr>
<tr>
<td>NPR-C sense</td>
<td>GGCTCAATGAGGAGGATTACG</td>
<td>554 bp</td>
</tr>
<tr>
<td>NPR-C antisense</td>
<td>AATCTTCCCAGCTCTCGATG</td>
<td></td>
</tr>
</tbody>
</table>

2.6. Materials

All drugs used were obtained from Sigma except ANP (Calbiochem), Apamin (Calbiochem), CNP (Calbiochem), M372049 (kind gift of Dr. C. Veale, AstraZeneca, USA), TRAM-34 (kind gift of Dr. H. Wulff, California, USA) and U-
46619 (Biomol). U-46619 was dissolved in ethanol and then diluted in saline. Indomethacin was dissolved in 5% NaHCO₃ and diluted in saline. TRAM-34 was dissolved in DMSO; all other drugs were prepared using saline (0.9% NaCl).

2.7. Calculations and statistics

Relaxations are expressed as percentage reversal of induced tone. All data are shown as mean±SEM. Tests of significance between curves were conducted using Two-way ANOVA for multiple comparisons or a Students t-test for differences between two data groups, where P<0.05 was considered significant. The n values quoted similarly indicate the number of experiments and animals used.

3. Results

3.1. Characterization of CNP-induced relaxation in rat mesenteric resistance arteries

CNP caused concentration-dependent relaxations of rat mesenteric resistance arteries that were unaffected by endothelium removal or blockade of Na⁺/K⁺-ATPase using ouabain but were suppressed following blockade of KᵢR using Ba²⁺ (Fig. 2). The selective NPR-C antagonist, M372049 (0.1–100 nM), potently inhibited CNP-induced relaxations in a concentration-dependent manner (Fig. 2). M372049 (100 nM) completely abolished responses to CNP and this concentration was therefore used in all further experimentation. The effect of M372049 (100 nM) appeared to be selective for NPR-C since responses to SPER-NO were unaffected (Fig. 2). Whilst ANP had little relaxant activity in control resistance arteries, exposure of these vessels to M372049 resulted in relaxation of arteries at the lower (1 and 10 nM) concentrations of ANP (Fig. 2).

3.2. NPR-C antagonism blocks EDHF responses in rat mesenteric resistance arteries

In the presence of L-NAME and indomethacin, ACh induced concentration-dependent relaxations that have previously been attributed to the activity of EDHF [8,30,31]. These responses were reduced marginally, but significantly, by treatment with M372049 (100 nM) or ouabain (1 mM) alone. However, a combination of M372049 and ouabain abolished responses to ACh (Fig. 3).
3.3. NPR-C antagonism blocks EDHF-mediated smooth muscle hyperpolarisation in rat mesenteric resistance arteries

ACh-mediated vascular smooth muscle hyperpolarisation was examined in rat small mesenteric arteries that were depolarised by 27±1.3 mV (n=6) from a resting membrane potential of −58.4±3.9 mV (n=6) with an approximate EC_{50} concentration of U46619. In the presence of L-NAME (300 μM) and indomethacin (5 μM), a maximum hyperpolarisation of 20.5±0.8 mV (n=3) was obtained with 10 μM ACh. ACh-mediated hyperpolarisation and vasorelaxation were virtually abolished in the presence of M372049 (100 nM) in combination with ouabain (1 mM; Fig. 4). Interestingly, although the hyperpolarisation to ACh was significantly inhibited by M372049 (100 nM), the relaxant response to ACh was only slightly reduced (Fig. 4). Hyperpolarisation and relaxation to the KATP channel opener, levcromakalim (10 μM) was sustained in the presence of M372049 (100 nM) and ouabain (1 mM; data not shown); M372049 (100 nM) alone did not affect resting membrane potential (data not shown).

3.4. NPR-C antagonism does not alter agonist-induced vasorelaxation in rat aorta

M372049 (100 nM) had no inhibitory activity on vasorelaxant responses to CNP, ANP, ACh or SPER-NO in isolated rat aortic rings (Fig. 5). Moreover, combination of M372049 with ouabain, unlike in resistance arteries, had no effect on ACh-induced relaxations (data not shown).

3.5. Combination of IK_{Ca} or SK_{Ca} inhibitors with M372049 inhibits EDHF responses in rat mesenteric resistance arteries

Since we had identified M372049 as a selective antagonist of NPR-C/EDHF responses in rat mesenteric resistance arteries, we proceeded to use this pharmacological tool to dissect the mechanisms involved in EDHF release and smooth muscle activity in this vascular bed. Since only a combination of M372049 plus ouabain abolished EDHF responses, this intimated that the EDHF response in these vessels is comprised of CNP and at least one additional mediator (since M372049 abolished, but ouabain did not affect, CNP-induced relaxations). Thus, we explored whether blockade of NPR-C/KIR in combination with inhibition of the individual pathways involved in EDHF release (i.e. SK_{Ca} and IK_{Ca}) and bioactivity (i.e. KIR and Na^{+}/K^{+}-ATPase) would also result in compete blockade of EDHF and thereby provide insight into the mechanisms governing release of (individual) EDHF(s) in this vascular bed.

Apamin and TRAM-34 had no significant effect on ACh-induced relaxations alone but in combination they abolished EDHF responses (Fig. 6). In the presence of M372049 and
apamin, no additional blockade of EDHF response was observed beyond that achieved with either drug alone (Fig. 6); the same was true of a combination of ouabain plus TRAM-34 (data not shown). However, a combined treatment of tissues with M372049 or Ba^{2+} plus TRAM-34, or ouabain plus apamin, significantly inhibited EDHF responses (Fig. 6).

3.6. RT-PCR analysis of NPR expression in rat mesenteric resistance vessels

All three NPR subtypes (NPR-A, NPR-B and NPR-C) are present (at least at mRNA level) in the rat conduit and resistance vasculature (Fig. 7). It should be noted that both endothelial and smooth muscle cells are present in these preparations so a defined localisation/distribution of each NPR between these two cell types cannot be determined. However, we have found that all NPRs are expressed on primary rat and human aortic vascular smooth muscle cells and human umbilical vein endothelial cells in culture (unpublished observations), suggesting both endothelial and smooth muscle cells express a replete quota of NPR subtypes.

3.7. M372049 does not directly inhibit \( K_{IR} \) channel activity

One explanation for the inhibitory effect of M372049 against CNP- and EDHF-mediated responses is that this compound may act as a direct inhibitor of \( K_{IR} \) channel function. To rule out this possibility we conducted electrophysiological (patch-clamp) studies in a HEK293 cell line expressing a GIRK channel coupled to an M4 receptor [27]. In these cells, addition of carbachol (10 \( \mu M \)) caused a archetypal GIRK-dependent current (control: 188.19±30.58 pA; carbachol: 2863.04±384.85 pA; \( P<0.05 \) versus control) that was abolished in the presence of Ba^{2+} (30 \( \mu M \); 365.02±27.72 pA; \( P<0.05 \) versus carbachol alone) but unaffected in the presence of M372049 (10 \( \mu M \); 3467.96±471.67 pA; \( P>0.05 \) versus carbachol alone; all \( n=8 \)).

4. Discussion

Endothelium-derived mediators including NO and prosta-cyclin play a key role in regulating vascular tone (and the reactivity of circulating immune cells and platelets [32,10]) in resistance vessels, and are therefore thought to contribute significantly to the control of local blood flow, peripheral vascular resistance and systemic blood pressure [33,20]. We have recently published evidence that CNP acts as an EDHF in mesenteric and coronary resistance vessels and also regulates the activation of leukocytes and platelets [8–10]. Moreover, we provided evidence that NPR-C, as opposed to NPR-B, might be the receptor subtype activated by CNP in this context, thereby assigning a key signalling role to this ‘clearance receptor’ in the mammalian resistance vasculature. Investigation of the biological activity of CNP (and other natriuretic peptides) and NPR-C has been hampered by a lack of selective antagonists, limiting delineation of the physiological importance of CNP/NPR-C signalling. Herein, we demonstrate that blockade of NPR-C using the selective antagonist, M372049, inhibits CNP- and EDHF-dependent responses in rat isolated mesenteric arteries, intimating that NPR-C-dependent signalling acts as an important regulatory pathway governing vessel tone and blood flow; moreover, we show that NPR-C antagonism inhibits the smooth muscle hyperpolarisation associated with EDHF-dependent dilation in these arteries and confirm the expression of all three NPR subtypes in resistance vessels. Further, using M372049 and specific inhibitor combinations that block the EDHF-dependent dilation in resistance arteries [20], we further elucidate the mechanism involved in the release and bioactivity of CNP.

The selective NPR-C antagonist, M372049, was first described by Veale et al. [18] as a result of a comprehensive screening exercise identifying antagonists at NPR-C that might be useful in the treatment of heart failure and other disorders where blockade of the removal of circulating natriuretic peptides (via this ‘clearance’ receptor) would be potentially beneficial. Since this compound has not been characterized from a pharmacological perspective in vascular tissue, we initially conducted a number of studies to ascertain the selectivity and potency of M372049 in both conduit and resistance arteries \( \textit{in vitro} \). In rat isolated aorta, M372049 did not alter relaxant responses to CNP, ANP, SPER-NO and ACh. The significance of this finding is three-fold. First, it indicates that the natriuretic peptides are not mediating vascular smooth muscle relaxation \( \textit{via} \) NPR-C in

![Fig. 7. RT-PCR analysis of NPR subtypes present in isolated rat aorta and mesenteric small vessels. Each receptor subtype (i.e. NPR-A, NPR-B & NPR-C) was found in both vessels. Expected product sizes: NPR-A, 714 bp; NPR-B 720 bp; NPR-C, 554 bp. This is representative of 3 separate experiments.](https://academic.oup.com/cardiovascres/article-abstract/74/3/515/367717)
this vessel (this is consistent with an NPR-A or NPR-B-mediated, cGMP-dependent relaxation of this tissue, as we have demonstrated previously [15]). Second, it demonstrates that the in vitro ‘clearance’ activity of NPR-C does not appear to significantly alter the steady-state concentrations, and therefore activity, of natriuretic peptides (otherwise an increase in potency would be expected as ‘clearance’ is blocked by M372049). Third, endothelium-derived vasodilators released by the aortic endothelium do not invoke NPR-C to induce vasorelaxation (consistent with NO acting as the sole endothelium-derived vasodilator in this tissue [34]). In marked contrast, a very different pattern of activity of M372049 was observed in rat isolated mesenteric resistance arteries. M372049 produced a potent and concentration-dependent inhibition of CNP-induced relaxations such that at 100 nM the antagonist abolished CNP-mediated responses. According to ligand-binding data obtained in the characterization of the compound [18], M372049 is a competitive antagonist with a $K_i = 21$ nM. Unfortunately, due to the inability to administer sufficiently high concentrations of CNP to overcome this competitive inhibition in our functional pharmacological investigations, we were unable to obtain a definitive $pA_2$ value. Nonetheless, based on extrapolation of the data sets obtained, an approximate $pA_2$ value of 11.14 ($\sim IC_{30} = 4 \text{ nM}$) was determined. Importantly, patch-clamp studies revealed that M372049 does not inhibit $K_{IR}$ channel activity directly (at a concentration two-orders of magnitude greater than that which completely attenuates functional responses to CNP), intimating this potential mechanism of action is not confounding interpretation.

Interestingly, despite the fact that all natriuretic peptides bind NPR-C [12,35], and that RT-PCR analysis revealed NPR-A, NPR-B and NPR-C mRNA expression in conduit and resistance vessels of the rat, ANP was found to be a very poor relaxant of mesenteric resistance vessels; indeed, at higher concentrations ANP elicited a contraction (as has been reported previously [36]). Relaxant responses observed at lower concentrations of ANP were not blocked by M372049 (indeed, if anything they were enhanced) suggesting that the biological activity of ANP in this resistance vascular bed is dependent on NPR-A activation and, unlike conduit vessels (i.e. aorta), blockade of NPR-C might be affecting the steady-state concentrations of natriuretic peptides. These observations also reveal that binding of ANP and CNP at NPR-C produces a distinct profile of activity; this may be related to the differential binding of these peptides to the receptor [37]. Our data also reveal that CNP mediates idiosyncratic vasorelaxation in conduit and resistance vessels via activation of NPR-B and NPR-C, respectively. Based on pharmacological data obtained with M372049, we postulated that NPR-B expression might be limited to conduit vessels and NPR-C expression to resistance vessels; however, all three NPRs appear to be expressed by both vessel types, intimating that vessel-specific pathways are activated by CNP.

When investigating EDHF-dependent relaxations, M372049 produced a marginal, but significant inhibition. In an analogous fashion, ouabain also caused a small rightward shift in the ACh concentration-response curve. However, in combination M372049 and ouabain completely

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**Fig. 8.** Schematic representation of the proposed multiple EDHF pathways present in rat mesenteric resistance arteries. One ‘arm’ of the EDHF response is triggered by $SK_{Ca}$ channels, is dependent on the release of CNP, activation of NPR-C and opening of a $Ba^{2+}$-sensitive GIRK. An additional component is provided by an as yet unidentified mediator that is released following $IK_{Ca}$ channel activation and is dependent on stimulation of the ouabain-sensitive $Na^{+}/K^{+}$-ATPase.
abolished responses to EDHF. This is in accord with studies in which Ba^{2+} plus ouabain is required to completely attenuate EDHF responses in a number of vascular beds [20]. Since we observed that ouabain alone does not block responses to exogenous CNP in these vessels, such observations give rise to the following thesis: EDHF responses in the rat mesenteric resistance vasculature are comprised of at least two distinct mediators/pathways; one that involves the release of CNP that activates an NPR-C-linked and Ba^{2+}-sensitive K^+ channel (likely a GIRK [KIR3.], as we have described previously [8]) to induce smooth muscle hyperpolarisation; and a second that is CNP-independent but sensitive to blockade by ouabain (Fig. 8).

A key role for CNP/NPR-C signalling in EDHF-mediated relaxations was substantiated in studies exploring the hyperpolarisation associated with EDHF release. ACh caused a marked hyperpolarisation (20–25 mV) of the U46619-depolarised tissues, characteristic of an EDHF response. In accord with the isometric tension recordings, in the presence of M372049 plus ouabain this hyperpolarisation was abolished. Interestingly, M372049 alone had a significant inhibitory effect on EDHF-dependent hyperpolarisation whilst only exerting a marginal effect on smooth muscle tone. This suggests that complete blockade of endothelium-dependent hyperpolarisation is required before the corresponding vasorelaxant activity is impaired significantly. Indeed, this has been demonstrated previously in rat arteries in which barium alone substantially suppresses ACh-induced vascular smooth muscle hyperpolarisation with only a minor effect on relaxation, whereas ouabain alone has little or no effect on EDHF-dependent hyperpolarisation (a combination of the two abolishes both the hyperpolarisation and relaxation) [22].

We confirmed the multiplicity of EDHF responses in the rat mesenteric resistance vessels by using combinations of inhibitors previously characterized to inhibit EDHF responses in a number of vessels in different species [20]. We found that a combination of IK_{Ca} (TRAM-34) and K_{IR} (M372049/Ba^{2+}) or SK_{Ca} (apamin) and Na^{+}/K^{+}-ATPase (ouabain) blockade significantly inhibited EDHF responses (as did the ‘conventional’ cocktails described earlier) whereas any other combination of these selective inhibitors was unable to significantly alter EDHF-induced relaxations. These data have led to the hypothesis and schematic proposed in Fig. 8. Such a mechanism is certainly applicable to the rat mesenteric vasculature, but might hold true for other vessels exhibiting a significant EDHF response which necessitates the use of Ba^{2+} and ouabain in combination to abolish EDHF-dependent relaxation (as is the case for human vessels in vivo [38]); in such cases, it is likely that there are two pathways and (at least) two mediators that constitute the EDHF phenomenon. From our observations we conclude that one pathway, involving the release of CNP is triggered by activation of SK_{Ca} channels on the endothelium and this results in NPR-C activation and hyperpolarisation [8]. The second pathway involves an as yet unidentified mediator/pathway that is initiated via opening of IK_{Ca} on the endothelium and culminates in vasorelaxation via Na^{+}/K^{+}-ATPase activation (i.e. ouabain sensitive). These pathways are redundant in as much as if a single mechanism is blocked only a fraction of the EDHF-dependent dilation is lost and therefore the alternate pathways must act in a compensatory fashion.

The phenomenon of multiple pathways underlying the biological activity of EDHF, inferring the existence of more than one EDHF in a single vascular bed, has been suspected as a result of the heterogeneity of EDHF (in terms of identity and function) across vascular beds and species [20]; the observations contained herein provide clear evidence of such a mechanism. The identity of EDHF appears to vary considerably between vascular beds and the inhibitor combinations we have employed in this study have differing potencies against EDHF responses between species and vessel. For instance, in some vessels ouabain alone can almost abolish EDHF-dependent relaxation [39–41] whereas in other vessels it is the Ba^{2+}-sensitive component that predominates [38,21]. Moreover, the actual identity of EDHF appears to differ between species and vascular bed and this may be explained by the release of more than one EDHF (as we have shown in the mesenteric vasculature); inhibitors of one ‘arm’ of the EDHF response in any given vessel may not be sufficient alone to block endothelium-dependent relaxation and therefore the candidate being investigated may be excluded erroneously. Moreover, the different sensitivity to inhibitor combinations suggests that in different tissues the balance between the two (or more) ‘arms’ of EDHF-mediated effects varies markedly. This latter thesis also gives rise to the possibility that different stimuli can release different EDHFs. For instance, SK_{Ca} channels (including the SK3 subtype that is thought to be expressed on endothelial cells [42,43]) require higher concentrations of calcium (K_{d}=600–700 nM) to open than the IK_{Ca} channels (K_{d}=100–300 nM) [44], suggesting that agonists that induce large, rapid increases in [Ca^{2+}] (e.g. ACh, bradykinin) may activate the SK_{Ca}^+, CNP-dependent pathway preferentially. Indeed, this thesis is supported by a previous report of differential activation of SK_{Ca} and IK_{Ca} in rat mesenteric resistance arteries [45]. This may explain some of the heterogeneity in EDHF responses when different agonists are used to evoke endothelium-dependent relaxation/hyperpolarisation. Moreover, the multiplicity of EDHF responses suggests it may be possible to produce organ- or tissue-specific manipulation of EDHF by targeting the ‘arm’ of the EDHF response that predominates in particular vascular beds.

In summary, we have characterized and employed a selective NPR-C antagonist as a pharmacological tool to inhibit EDHF-dependent relaxations (and hyperpolarisation) in the rat mesenteric resistance vasculature and thereby demonstrated a key role for CNP/NPR-C in the regulation of vascular tone; in so doing, we have identified a unique tool for the study of CNP and EDHF biology. The importance of CNP/NPR-C signalling in EDHF-dependent
hyperpolarisation in rat mesenteric resistance vessels revealed by this study, in combination with previous work by ourselves and others, highlights a novel target for potential therapeutic intervention in the treatment of cardiovascular disorders.

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