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RESEARCH PAPER

Stimulated release of a hyperpolarizing factor (ADHF) from mesenteric artery perivascular adipose tissue: involvement of myocyte BK_{Ca} channels and adiponectin

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BACKGROUND AND PURPOSE

Perivascular adipose tissue (PVAT) releases adipocyte-derived hyperpolarizing factors (ADHFs) that may partly act by opening myocyte K^+ channels. The present study in rat and mouse mesenteric arteries aimed to identify the myocyte K^+ channel activated by PVAT and to determine whether adiponectin contributed to the hyperpolarizing effects of PVAT.

EXPERIMENTAL APPROACH

Myocyte membrane potential was recorded from de-endothelialized, non-contracted rat and mouse mesenteric arteries in the presence and absence of PVAT.

KEY RESULTS

The β_3-adrenoceptor agonist, CL-316,243 (10 μM), generated PVAT-dependent, iberiotoxin-sensitive myocyte hyperpolarizations resulting from BK_{Ca} channel opening and which were partially blocked by L-NMMA (100 μM). Adiponectin (5 $\mu g \cdot mL^{-1}$) also produced iberiotoxin-sensitive hyperpolarizations in PVAT-denuded arterioles. Activation of myocyte AMP-activated protein kinase (AMPK) using 5 μM A-769662 also induced BK_{Ca}-mediated hyperpolarizations. Dorsomorphin abolished hyperpolarizations to CL-316,243, adiponectin and A-769662. In vessels from $Adipo^{-/-}$ mice, hyperpolarizations to CL-316,243 were absent whereas those to A-769662 and adiponectin were normal. In rat vessels, adipocyte-dependent hyperpolarizations were blocked by glibenclamide and clotrimazole but those to NS1619 (33 μM) were unaltered.

CONCLUSIONS AND IMPLICATIONS

Under basal, non-contracted conditions, β_3-adrenoceptor stimulation of PVAT releases an ADHF, which is probably adiponectin. This activates AMPK to open myocyte BK_{Ca} channels indirectly and additionally liberates NO, which also contributes to the observed PVAT-dependent myocyte hyperpolarizations. Clotrimazole and glibenclamide each reversed hyperpolarizations to adiponectin and A-769662, suggesting the involvement of myocyte TRPM4 channels in the ADHF-induced myocyte electrical changes mediated via the opening of BK_{Ca} channels.

Abbreviations

A-769662, 4-hydroxy-3-(2′-hydroxybiphenyl-4-yl)-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-5-carbonitrile; ADHF, adipocyte-derived hyperpolarizing factor; AMPK, AMP-activated kinase; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; CL-316,243, 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]-propyl]-1,3-benzodioxole-2,2-dicarboxylic acid; K_{ATP}, ATP-sensitive K^+ channel; NS1619, 1-(2′-hydroxy-5′ trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone; PVAT, perivascular adipose tissue; TRPM4, transient receptor potential melastatin channel subtype 4

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Introduction

Perivascular adipose tissue (PVAT) exerts anti-contractile influences on the arteries that it surrounds (Dubrovska *et al.*, 2004; Verlohren *et al.*, 2004; Fésüs *et al.*, 2007; Gao *et al.*, 2007; Greenstein *et al.*, 2009). Several reports have concluded that some of the anti-contractile effects of PVAT result from the opening of myocyte K^+ channels (Löhn *et al.*, 2002; Verlohren *et al.*, 2004; Fésüs *et al.*, 2007; Gao *et al.*, 2007; Schleifenbaum *et al.*, 2010), and in this paper the hypothetical factor(s) involved is/are designated adipocyte-derived *hyperpolarizing* factor(s) – ADHF(s).

The possible involvement of any ADHF-induced K^+ channel opening has largely been inferred from myograph experiments in which vessels surrounded by PVAT were simply contracted in the presence or absence of K^+ channel blockers without any measurement of myocyte membrane potential. Because receptors for the contractile agonists used exist not only on myocytes but also on adipocytes (Germack *et al.*, 1997; Kinoshita *et al.*, 2010; Stunes *et al.*, 2011), these agonists could themselves have modified the release of any such ADHFs.

Adiponectin is a peptide secreted almost exclusively by adipocytes (Scherer *et al.*, 1995). Myograph studies using 4-aminopyridine suggested that adiponectin can relax mesenteric artery rings by opening K_V channels (Fésüs *et al.*, 2007), and Greenstein *et al.* (2009) concluded that adiponectin was the anti-contractile adipokine released from rat mesenteric artery PVAT. *In vivo*, serum adiponectin levels are raised by CL-316,243 (Oana *et al.*, 2005; Fu *et al.*, 2008), a selective β_3-adrenoceptor agonist (Bloom *et al.*, 1992; Dolan *et al.*, 1994; Michel *et al.*, 2010) that has been used to investigate their role on adipose tissue (Collins *et al.*, 1997). β_3-adrenoceptors exist on rat adipocytes but are not functionally present on either mesenteric artery myocytes or endothelial cells (Kozlowska *et al.*, 2003; Briones *et al.*, 2005).

Using an electrophysiological approach in the present study, evidence was obtained for the β_3-adrenoceptor-mediated release from PVAT of an ADHF which opens myocyte BK_{Ca} channels in both rat and mouse mesenteric arteries. Authentic adiponectin hyperpolarized mesenteric artery myocytes by opening BK_{Ca} channels by activation of AMP-activated protein kinase (AMPK). Although CL-316,243-stimulated release of adiponectin itself from PVAT could not be detected, this agonist generated PVAT-dependent myocyte hyperpolarizations in vessels from control mice yet had no effect on myocytes from $Adipo^{-/-}$ mice which were unable to synthesize adiponectin.

A preliminary account of some of these findings has been published (Egner *et al.*, 2011).

Methods

Animals

Experiments were performed on second- or third-order mesenteric artery branches dissected from male Sprague–Dawley rats (approximately 250–300 g body weight; bred in-house, The University of Manchester) or on first- or second-order branches from C57BL/6J mice (control; Harlan, UK) or adiponectin-deficient ($Adipo^{-/-}$) mice (B6.129-$adipoq^{tm1Chan}$/J [originally generated by Chan and co-workers (Ma *et al.*, 2002) but purchased from Jackson Laboratory Repository, Bar Harbor, ME, USA], each 10–11 weeks old. The homozygous $Adipo^{-/-}$ mice were confirmed by Jackson Laboratories to lack adiponectin mRNA (adipose tissue) and protein (serum). All animals were killed by cervical dislocation in compliance with Schedule 1 of the UK Animals (Scientific Procedures) Act, 1986. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath *et al.*, 2010).

Electrophysiology

Segments of artery (length 2–3 mm) that, unless otherwise stated, had been de-endothelialized by introducing deionized water into the vessel lumen for approximately 10–15 s were pinned to the Sylgard base of a thermostatically controlled bath and superfused (10 mL·min^{-1}) with a Krebs solution (pH 7.5; 37°C; bubbled with 5% CO_2 in air). Myocytes were impaled through the adventitial surface using 3 M KCl-filled microelectrodes (resistance 40–80 MΩ). About 50 Hz interference at the high impedance amplifier (Intra 767; WPI, Hitchin, UK) output was selectively removed (Humbug; Digitimer, Welwyn Garden City, UK). Signals were digitized and analysed using Chart software (Mac version 5.5.6) in conjunction with a PowerLab 4/SP acquisition system (ADInstruments, Chalgrove, UK).

Adiponectin assay

Segments of rat mesenteric bed (comprising artery, vein and PVAT) were weighed and then incubated in 5 mL Krebs solution (37°C; gassed with 5% CO_2 : 95% air) in the absence (control) or presence of 10 μM CL-316,243. After 30 min of pre-incubation, the total adiponectin incubate concentration was determined (ALPCO Diagnostics Rat Adiponectin Immunoassay Kit; Stratech Scientific Ltd, Newmarket, UK). Reactions were performed in a 96-well plate and absorbance read at 450 nm. The adiponectin concentration was calculated from an adiponectin standard curve.

Statistical analysis

Results were analysed by two-way repeated-measures ANOVA (with Bonferroni *post hoc* test) or Student's *t*-test as appropriate and are expressed as means ± SEM; *n* represents both the number of replications and the number of different animals from which the artery segments were obtained. A *P*-value of ≤0.05 was considered significant.

Drugs, reagents and solutions

A-769662 (4-hydroxy-3-(2′-hydroxybiphenyl-4-yl)-6-oxo-6,7-dihydrothieno[2,3-b]pyridine-5-carbonitrile; Tocris, Bristol, UK), glibenclamide and levcromakalim were dissolved in ethanol. ACh chloride, CL-316,243 (5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]-propyl]-1,3-benzodioxole-2,2-dicarboxylic acid disodium salt; Tocris), dorsomorphin (Tocris; prepared freshly each day) and iberiotoxin (Latoxan, 26000 Valence, France) were each dissolved in deionized water. Recombinant (human) adiponectin (BioVendor, Modrice, Czech Republic) and L-NMMA (N^G-

monomethyl-L-arginine; Tocris) were prepared in Krebs solution and used immediately. Clotrimazole and NS1619 (1-(2′-hydroxy-5′ trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone; Research Biochemicals International, St. Albans, UK) were dissolved in dimethyl sulfoxide. Unless otherwise stated, all drugs and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

ACh, NS1619 and levcromakalim were each added as bolus injections directly into the recording bath in quantities calculated to obtain (transiently) the concentrations indicated. Unless otherwise stated, A-769662, CL-316,243, clotrimazole, dorsomorphin and iberiotoxin were added to the reservoir of Krebs solution superfusing the bath. Due to the expense of adiponectin, Krebs flow was stopped and the peptide was added directly into the recording chamber.

The composition of the Krebs solution was (in mM): NaCl, 118; KCl, 3.4; $CaCl_2$, 1.0; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.

Results

Hyperpolarizing effects of PVAT stimulation in rat mesenteric arteries using CL-316,243: comparison with adiponectin and involvement of myocyte BK_{Ca} channels

In endothelium-denuded vessels (no response to 10 μM ACh) and in the presence of PVAT, the selective β_3-adrenoceptor agonist, CL-316,243 (10 μM), produced myocyte hyperpolarizations of 11.2 ± 1.0 mV (Figure 1A, n = 4), which were abolished (0.2 ± 0.1 mV, n = 4) by the selective BK_{Ca} channel inhibitor, iberiotoxin (100 nM). Hyperpolarizations to the K_{ATP} opener, levcromakalim, were similar before (24.6 ± 0.4 mV, n = 4) and during exposure to iberiotoxin (28.6 ± 1.5 mV, n = 4) (see Figure 1A).

In separate experiments, 10 μM CL-316,243-induced hyperpolarizations (13.4 ± 2.6 mV, n = 4) in the presence of the vascular endothelium were not different (P < 0.05) from those in endothelium-denuded vessels (11.2 ± 1.0 mV, n = 4) but were completely absent after PVAT removal (n = 3; data not shown). Subsequent experiments were therefore conducted in vessels denuded of their vascular endothelium.

Authentic adiponectin (5 $\mu g \cdot mL^{-1}$), like CL-316,243, also evoked iberiotoxin-sensitive myocyte hyperpolarizations in the absence of both PVAT and endothelium (10.3 ± 0.24 mV, n = 4; Figure 1B). Because of the expense of adiponectin, the flow of Krebs over the vessel was stopped immediately before adiponectin exposure (flow off, Figure 1B). In these experiments, hyperpolarizations to the BK_{Ca} channel opener, NS1619 (33 μM; 16.2 ± 2.1 mV, n = 4), were abolished in the presence of 100 nM iberiotoxin, whereas those of the K_{ATP} opener, levcromakalim (10 μM), were still present, indicating both the effectiveness and selectivity of iberiotoxin as a BK_{Ca} channel blocker.

Comparison of hyperpolarizing effects of the AMPK activator, A-769662 with those of CL-316,243 and adiponectin

AMPK is a downstream effector of the actions of adiponectin (Yamauchi *et al.*, 2002; Kadowaki and Yamauchi, 2005). To clarify whether adiponectin-induced myocyte hyperpolarizations resulted from myocyte AMPK activation, the effects of a selective AMPK activator, A-769662 (Cool *et al.*, 2006; Göransson *et al.*, 2007), on membrane potential in the absence of both PVAT and endothelium were investigated. A-769662 (5 μM) hyperpolarized the myocytes by 9.2 ± 0.6 mV (n = 4), an effect reversed by 100 nM iberiotoxin (which also prevented the effects of subsequent exposure to NS1619; Figure 1C).

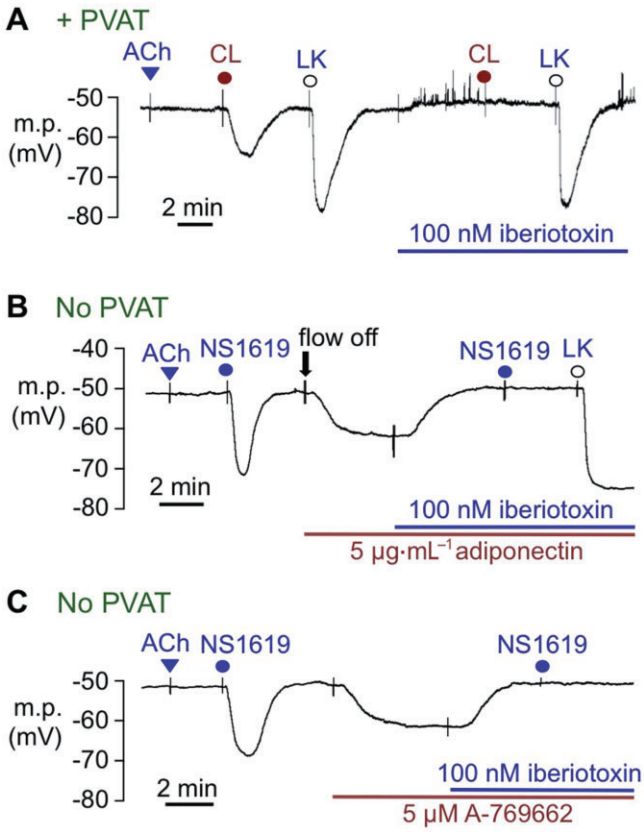

Figure 1

Hyperpolarizing effects of PVAT in rat mesenteric artery segments, all in the absence of the vascular endothelium (no response to 10 μM ACh). (A) In the presence of PVAT (+PVAT), hyperpolarizations induced by 10 μM CL-316,243 (CL) were abolished by prior exposure to 100 nM iberiotoxin, which had no effect on 10 μM levcromakalim (LK)-induced hyperpolarizations. (B) In the absence of PVAT (no PVAT), transient exposure to 33 μM NS1619 produced myocyte hyperpolarizations. After washout, the flow of Krebs was stopped (flow off); exposure to adiponectin hyperpolarized the myocytes, a response that was reversed by 100 nM iberiotoxin. Subsequent exposure to 33 μM NS1619 was without effect whereas 10 μM LK still hyperpolarized myocytes to close to E_K in the continuing presence of iberiotoxin. (C) In the absence of PVAT, the AMPK activator A-769662 evoked hyperpolarizations, which were reversed by 100 nM iberiotoxin. Subsequent exposure to 33 μM NS1619 was also without effect, confirming iberiotoxin-induced blockade of myocyte BK_{Ca} channels.

Hyperpolarizing effects of PVAT stimulation using CL-316,243 are absent in Adipo$^{-/-}$ mice

Although exogenous adiponectin mimicked the hyperpolarizing effects of CL-316,423, the rate of release of adiponectin from PVAT in the presence of CL-316,423 (0.64 ± 0.13 ng·mg·PVAT·h^{-1}; n = 6) was similar to that in its absence (0.63 ± 0.15 ng·mg·PVAT·h^{-1}; n = 6). However, commercially available kits for the quantification of rat adiponectin do not distinguish between the various adiponectin aggregates, which differ in their biological activity (see Brochu-Gaudreau *et al.*, 2010). Thus, our inability to detect any CL-316,243-induced increase in a biologically relevant form of adiponectin could have been due to masking by unimportant aggregates.

Vessels from homozygous, adiponectin-deficient (Adipo$^{-/-}$) mice (B6.129-adipoq^{tm1Chan}/J) which lacked adiponectin mRNA and protein were therefore utilized. Hyperpolarizations to 10 μM CL-316,243 (10.7 ± 0.4 mV, n = 4) in control (C57BL/6J) mouse mesenteric arteries in the presence of PVAT were similar in magnitude (Figure 2A,C) to those in rat vessels under similar conditions (see Figure 1). Mouse myocyte membrane potentials were also increased by 5 μM A-769662 (6.4 ± 0.5 mV; n = 4) and by 5 μg·mL^{-1} adiponectin (9.7 ± 0.6 mV; n = 4). In arteries from Adipo$^{-/-}$ mice (Figure 2B,C), the hyperpolarizations to A-769662 (6.2 ± 0.4 mV; n = 4) and to 5 μg·mL^{-1} adiponectin (9.9 ± 1.1 mV; n = 4) were also not significantly different from those of controls. In marked contrast, however, 10 μM CL-316,243 completely failed to hyperpolarize vessels from Adipo$^{-/-}$ mice in the presence of PVAT (0.3 ± 0.2 mV; n = 4) (Figure 2B,C).

Effects of AMPK inhibition in rat vessels using dorsomorphin

Following 30 min of pre-incubation with 1 μM dorsomorphin (an AMPK inhibitor; Zhou *et al.*, 2001) and in its continued presence, rat myocytes became depolarized (-47.9 ± 0.5 mV, n = 4) relative to controls (–50.3 ± 0.4 mV, n = 8; P = 0.0033, unpaired Student's t-test) and often exhibited spontaneous spike discharges (Figure 3A). In the presence of dorsomorphin, mean hyperpolarizations to 5 μg·mL^{-1} adiponectin (2.6 ± 0.5 mV, n = 4) and to 5 μM A-769662 (2.4 ± 0.3 mV, n = 4) were markedly reduced (P < 0.001; compare Figures 1B,C & 3A), although in the same preparations the hyperpolarizations to 33 μM NS1619 (16.7 ± 0.9 mV, n = 4) were not significantly changed (see Figure 1C and related text above). Responses to 10 μM CL-316,243 in the presence of PVAT (2.0 ± 0.3 mV, n = 4) were similarly reduced by dorsomorphin (P < 0.001; Figure 3B).

Does stimulation of PVAT β_3-adrenoceptors also release NO?

AMPK activates NOS in endothelial cells (Morrow *et al.*, 2003). We therefore speculated that AMPK might enhance NO production from *adipocyte* NOS and contribute to the hyperpolarizing effects of CL-316,243.

L-NMMA (100 μM) did not change myocyte resting membrane potentials (control, –51.0 ± 0.9 mV, n = 4; L-NMMA, –50.1 ± 0.8 mV, n = 4) or NS1619-induced hyperpolarizations (control, 18.1 ± 0.9 mV, n = 4; presence of L-NMMA, 17.5 ± 0.9 mV, n = 4) but partially reduced those generated by

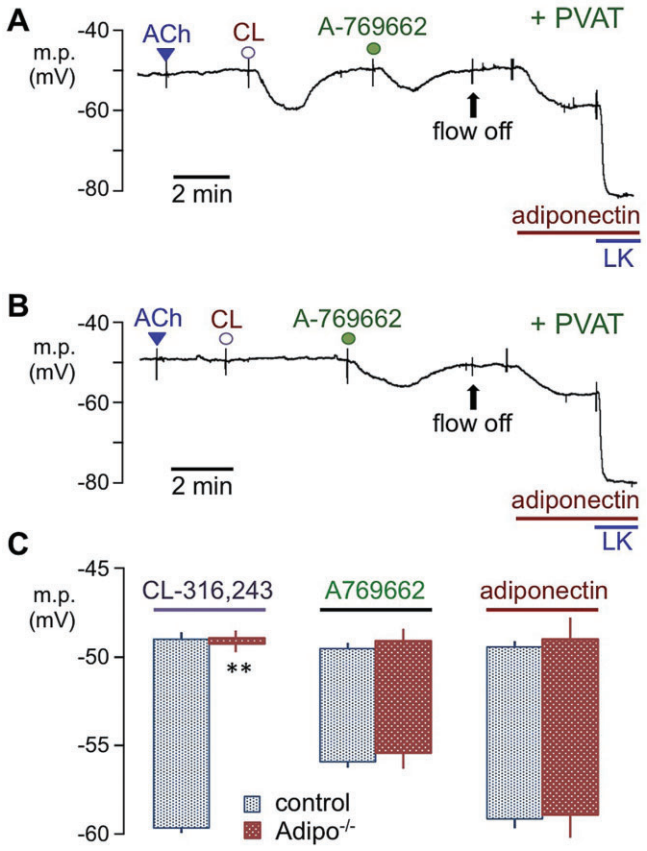

Figure 2

Effects of adiponectin deficiency on responses to CL-316,243, A-769662 and adiponectin in mouse mesenteric arteries. Typical traces showing the hyperpolarizations induced by 10 μM CL-316,243 (CL), 5 μM A-769662 and 5 μg·mL^{-1} adiponectin in PVAT-intact, endothelium-denuded artery segments (no response to 10 μM ACh) from (A) control (wild-type C57BL/6J) or (B) adiponectin-deficient (Adipo$^{-/-}$; B6.129-adipoq^{tm1Chan}/J) mice. In (A) and (B), bolus doses of ACh, CL-316,243 or A-769662 were added to produce, transiently, concentrations of 10, 10 and 5 μM respectively. Flow was stopped (flow off) to minimize the quantity of adiponectin required. Note the complete absence of CL-316,243-induced hyperpolarizations in vessels from Adipo$^{-/-}$ mice whereas authentic adiponectin (5 μg·mL^{-1}) still hyperpolarized the myocytes. Levcromakalim (LK 10 μM, an opener of myocyte ATP-sensitive K$^+$ channels) was added to establish the K$^+$ equilibrium potential. (C) Histograms comparing the mean membrane potential in artery segments from wild-type and adiponectin-deficient (Adipo$^{-/-}$) mice before and after exposure to CL-316,243, A-769662 and adiponectin. Each point represents the mean ± SEM (n = 4).

CL-316,243 in the presence of PVAT (P < 0.0001, two-way ANOVA; Figure 4). Subsequent addition of dorsomorphin in the presence of L-NMMA abolished the residual hyperpolarizations to 10 μM CL-316,243 and repolarized the membrane to control levels (–49.4 ± 0.4 mV, n = 4).

Inhibitory effects of clotrimazole and glibenclamide

The present findings that BK_{Ca} channels in native myocytes were opened by myocyte AMPK activation conflicted with

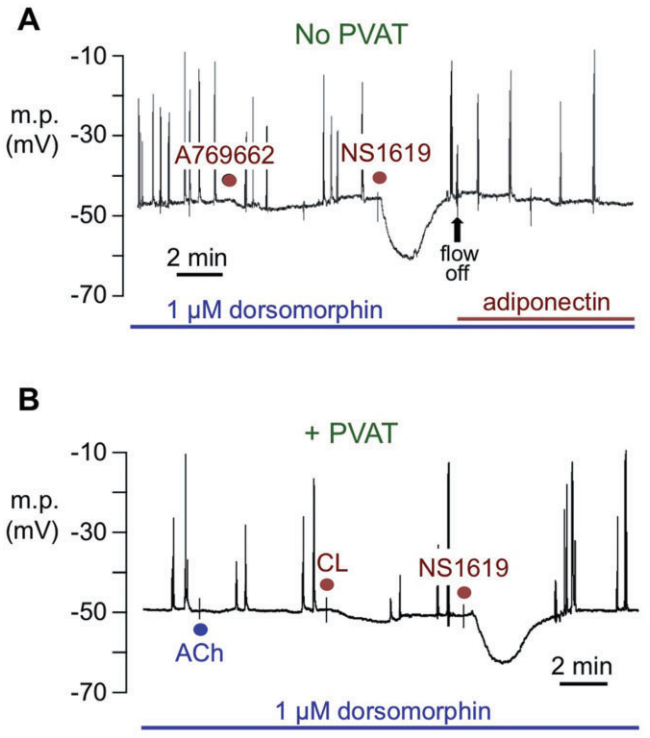

Figure 3

Typical recordings showing the effect of the AMPK inhibitor dorsomorphin on membrane potential in rat mesenteric arteries in the absence of the endothelium (no response to 10 μM ACh). (A) In the absence of PVAT (no PVAT), 30 min of prior exposure to 1 μM dorsomorphin inhibited responses both to 5 μM A-769662 and to 5 mg·mL^{-1} adiponectin, but not those to 33 μM NS1619. (B) In the presence of PVAT (+ PVAT), dorsomorphin inhibited hyperpolarizations to 10 μM CL-316,243 (CL) but not those to the BK_{Ca} channel opener NS1619. In (A), flow was stopped (flow off) to minimize the quantity of adiponectin required; in (B) the tissue was constantly superfused with Krebs to which bolus doses of the drugs were added as previously described.

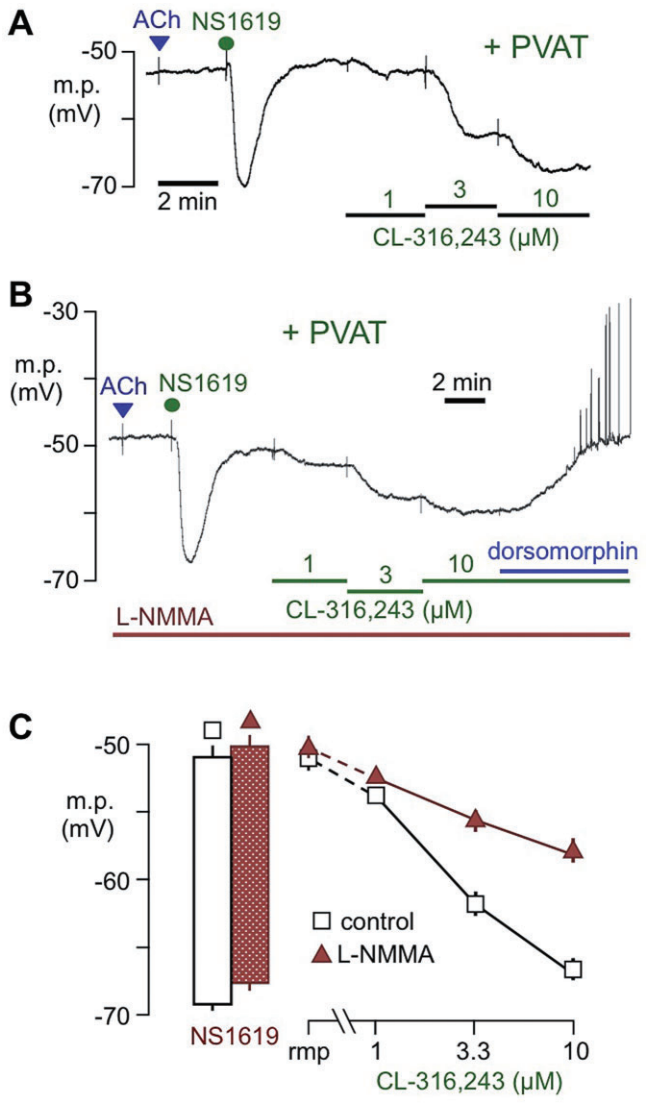

Figure 4

Effects of the NOS inhibitor (L-NMMA) on hyperpolarizations to CL-316,243. Typical traces showing sharp microelectrode recordings from rat mesenteric artery myocytes in the absence of the endothelium (no response to 10 μM ACh) but in the presence of PVAT (+PVAT). The concentration-dependent hyperpolarizations produced by CL-316,243 (A) were reduced by 100 μM L-NMMA (B), although the inhibitor had no effect on hyperpolarizations to 33 μM NS1619. In the presence of L-NMMA, hyperpolarizations to CL-316,243 were reversed by 1 μM dorsomorphin (see text for more details). The line graph in (C) indicates the mean resting membrane potential (rmp) and hyperpolarizations to each concentration of CL-316,243 (increased cumulatively) in the absence (control, open symbols) or presence (filled symbols) of L-NMMA. The histograms to the left depict the membrane potential before and after exposure to 33 μM NS1619 in the absence or presence of L-NMMA. Each point represents the mean ± SEM (n = 4).

the results of Ross *et al.* (2011), who reported that the AMPK activator, aminoimidazole-4-carboxamide-ribonucleoside (AICAR), either had no effect or even inhibited BK_{Ca} channels heterologously expressed in HEK293 cells.

To clarify possible mechanisms involved in BK_{Ca} activation by AMPK in native myocytes following exposure to adiponectin or A-769662, we investigated the effects of clotrimazole, an inhibitor of several types of vascular, non-selective cation channels which could have acted as myocyte calcium-entry pathways. Myocyte hyperpolarizations to CL-316,243 adiponectin and to A-769662 were always reversed by 33 μM clotrimazole (Figure 5A–C). This agent inhibits Na^+- and K^+-permeable TRPM4 channels (Vennekens and Nilius, 2007) that are present in vascular myocytes (Morita *et al.*, 2007; Zholos *et al.*, 2011), an action shared with glibenclamide (Demion *et al.*, 2007), the sulphonylurea previously reported to inhibit the anti-contractile effects of PVAT in rat aorta (Löhn *et al.*, 2002; Fang *et al.*, 2009).

Although 10 μM glibenclamide essentially abolished hyperpolarizations to 5 μM A-769662 (absence: 11.8 ± 1.1 mV; presence: 2.2 ± 0.5 mV, each n = 4), those due to direct opening of BK_{Ca} with 33 μM NS1619 were unaffected (absence: 14.6 ± 0.5 mV; presence: 14.3 ± 1.1 mV, each n = 4; Figure 5D), an indication that these actions of glibenclamide were not due to BK_{Ca} blockade.

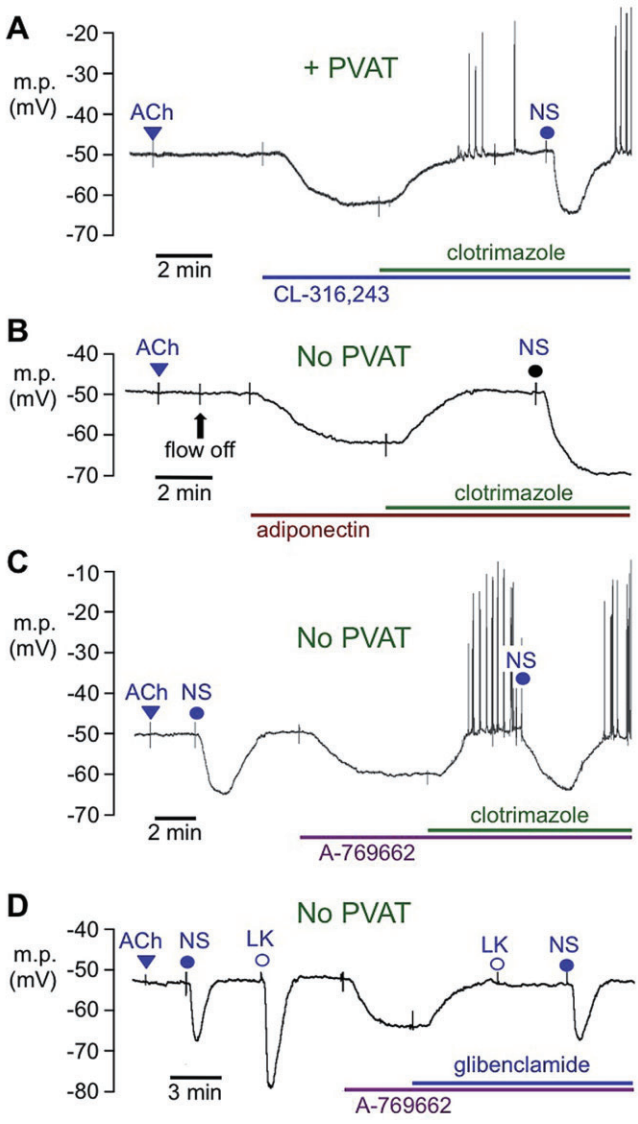

Figure 5

Typical traces showing the inhibitory effects of clotrimazole and glibenclamide on myocyte hyperpolarizations in endothelium-denuded segments (no response to 10 μM ACh) of rat mesenteric artery. In the presence (A; +PVAT) or absence (B and C; no PVAT) of PVAT, hyperpolarizations induced by (A) 10 μM CL-316,243, (B) 5 μM A-769662 or (C) 5 μg·mL^{-1} adiponectin were reversed by 33 μM clotrimazole although 33 μM NS1619 (NS) was subsequently able to induce hyperpolarization. (D) In the absence of PVAT, hyperpolarizations to 5 μM A-769662 were reversed by 10 μM glibenclamide. The hyperpolarizations induced by 10 μM levcromakalim (LK) but not by 33 μM NS1619 were inhibited by glibenclamide. Note that in (A), (C) and (D), the tissue was constantly superfused with Krebs solution to which bolus doses of ACh, NS1619 or LK were added to produce transient responses. Other modulators were added to the superfusate to produce sustained responses; in (B), flow was stopped as indicated (flow off) to minimize the quantity of adiponectin required.

Discussion

Experimental approach: simplify the experimental system

The clarity of the present findings was made possible by simplifying the experimental system in marked contrast to earlier studies. Thus, the vascular endothelium was removed in most experiments and microelectrode techniques allowed assessment of myocyte responses in the absence of spasmogens. Their presence is a powerful modulator of many ion channels, pumps and transporters, and greatly complicates analysis of the results obtained. Consistent with the use of a simplified system, the selective β_3-adrenoceptor agonist CL-316,243 (Bloom *et al.*, 1992; Dolan *et al.*, 1994; Michel *et al.*, 2010) was employed to stimulate adipocytes to release ADHF because these receptors are not functionally present on either rat mesenteric artery myocytes or endothelial cells (Kozlowska *et al.*, 2003; Briones *et al.*, 2005).

The results clearly show that the PVAT surrounding both rat and mouse mesenteric arteries can be stimulated to release an ADHF, which opens myocyte BK_{Ca} channels. The hyperpolarizing effects of the endogenous ADHF on myocyte BK_{Ca} were mimicked by adiponectin, the 30 kDa peptide secreted almost exclusively by adipocytes (Scherer *et al.*, 1995), and proposed by Greenstein *et al.* (2009) to be an adipocyte-derived relaxing factor in rat and human vessels. Powerful evidence was also obtained for the involvement of myocyte AMP kinase, an enzyme known to be activated by adiponectin (Yamauchi *et al.*, 2002; Kadowaki and Yamauchi, 2005) (Figure 6).

Involvement of myocyte BK_{Ca} channels

CL-316,243 always hyperpolarized myocyte in artery segments surrounded by PVAT. Because these electrical changes were abolished by iberiotoxin (a selective BK_{Ca} blocker; Garcia *et al.*, 1991), they were clearly generated by myocyte BK_{Ca} channel opening with no evidence that a voltage-gated K^+ channel such as K_V (as proposed by Verlohren *et al.*, 2004; Fésüs *et al.*, 2007; Schleifenbaum *et al.*, 2010) or indeed any K^+ channel other than BK_{Ca} was involved. In the absence of PVAT, CL-316,243 had no effect on myocyte membrane potentials in segments from the same mesenteric branch (even in vessels with an intact endothelium), confirming that the β_3 agonist exerted its effects on the adipose tissue (Figure 6).

Is adiponectin the CL-316,243-induced ADHF?

In vivo, serum adiponectin levels in obese diabetic mice (db/db and KKAy) are raised by CL-316,243 (Oana *et al.*, 2005; Fu *et al.*, 2008). Exposure of rat mesenteric arteries to authentic adiponectin always hyperpolarized the myocytes in the absence of PVAT or the endothelium, reaching a maximum effect within a few minutes. This action, like the hyperpolarizations induced by CL-316,243 in the presence of PVAT, was also blocked by iberiotoxin, indicating the adiponectin-induced opening of myocyte BK_{Ca} channels (Figure 6).

Although attempts to measure CL-316,243-induced release of adiponectin from PVAT *in vitro* were unsuccessful, adiponectin exists predominantly in trimeric, hexameric and

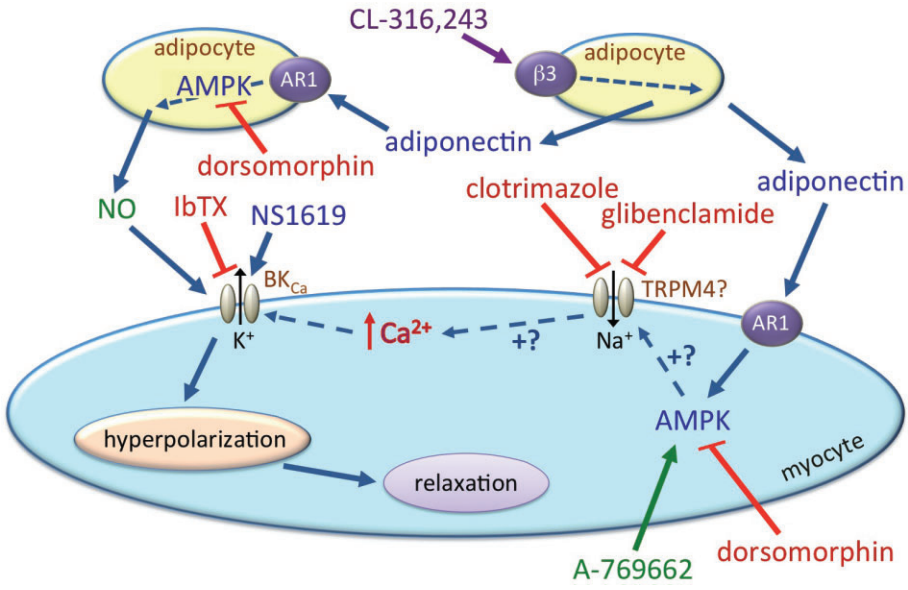

Figure 6

Working model for the stimulated release of a hyperpolarizing factor (ADHF) from mesenteric artery PVAT and involvement of adiponectin and myocyte BK_{Ca} channels. CL-316,243 stimulates the release of adiponectin, which acts through adiponectin receptors (AR1) and AMPK activation both in adipocytes (to release NO) and in myocytes (to open BK_{Ca} channels and induce hyperpolarization sensitive to iberiotoxin – IbTX). All effects of CL-316,243 are blocked by the AMPK inhibitor dorsomorphin. In the absence of PVAT, the hyperpolarizing effects of exogenous adiponectin are mimicked by A-769662, the AMPK activator. Both clotrimazole and glibenclamide (known inhibitors of TRPM4 channels) block the AMPK-induced activation of BK_{Ca} (by A-769662, adiponectin and CL-316,243) but do not modify direct BK_{Ca} activation by NS1619. We speculate that AMPK activation indirectly opens BK_{Ca} by first activating TRPM4 channels; the link between this putative event and BK_{Ca} activation requires further study.

multimeric (>18 adiponectin molecules) aggregates, which differ in their biological activities (see Brochu-Gaudreau *et al.*, 2010). Because commercially available kits for quantification of rat adiponectin do not distinguish between these forms, their presence could have masked any CL-316,243-induced release of a biologically relevant form of the peptide. Thus, although no *direct* evidence of stimulated adiponectin release was obtained, these experiments collectively suggest that the interaction of CL-316,243 with adipocyte β_3-adrenoceptors is likely to release adiponectin which then hyperpolarizes the mesenteric vessel myocytes by activating BK_{Ca} channels (Figure 6).

A component of CL-316,243-induced hyperpolarizations was blocked by L-NMMA. If adiponectin is the ADHF involved, we propose that this peptide can activate adipocyte NOS to release NO. All the effects of CL-316,243 were dorsomorphin sensitive, an indication that the postulated adipocyte NO pathways also involve AMPK activation (Figure 6).

Experiments with vessels from Adipo$^{-/-}$ mice: supportive evidence of adiponectin release from PVAT

Exposure of mouse control (C57BL/6J) mesenteric vessels to CL-316,243 in the presence of PVAT generated iberiotoxin-sensitive hyperpolarizations, which were similar to those observed in rat vessels. Such changes were, however, absent in vessels from Adipo$^{-/-}$ mice, the strain (B6.129-Adipoq^{tm1Chan}/J; Ma *et al.*, 2002) engineered to be adiponectin deficient. This strongly suggests that CL-316,243-induced activation of β_3-adrenoceptors on mouse (and by extrapolation, rat) PVAT can generate adiponectin-mediated myocyte hyperpolarization involving BK_{Ca} activation. This is strongly supported by observations that adiponectin-induced hyperpolarizations are absent from Slo$^{-/-}$ mice mesenteric artery myocytes, which lack BK_{Ca} pore-forming α-subunits (Lynch *et al.*, 2013).

Our findings suggest that adiponectin can fully explain the hyperpolarizing effect of PVAT stimulation in non-contracted vessels. However, in a previous study, Fésüs *et al.* (2007) found that 4-aminopyridine (a relatively non-selective blocker of delayed rectifier K^+ channels, K_V) produced a PVAT-dependent increase in perfusion pressure in mouse-isolated mesenteric bed preparations in the presence or absence of a spasmogen (5-hydroxytryptamine). This effect, which was not modified by adiponectin gene deletion, was interpreted as an indication that the relaxant factor released by PVAT acted by opening myocyte K_V channels. However, little consideration was paid to the possibility that the enhanced contractile effect of 4-aminopyridine following PVAT removal would have occurred irrespective of the mechanism of action of the PVAT-derived factor. It is well established that the vascular endothelium releases several relaxant factors in response to a single stimulus (reviewed by Triggle *et al.*, 2010), and Gollasch (2012) has summarized the various agents, in addition to adiponectin, which can be released from PVAT. Our inability to detect evidence of a role for factors other than adiponectin in our electrophysiological experiments in non-contracted vessels suggests that such additional factors

are released from PVAT by relatively high spasmogen concentrations and/or that their actions are not exerted through myocyte hyperpolarization.

The AMP kinase pathway and ADHF

To obtain further information about the possible role of adiponectin as the mediator of PVAT-dependent myocyte hyperpolarizations, experiments were conducted to determine whether the hyperpolarizing effect of CL-316,243 in the presence of PVAT was associated with modulation of AMPK, the major downstream signalling target activated by adiponectin following its interaction with AdipoR1 receptors (Yamauchi *et al.*, 2007; reviewed by Xu *et al.*, 2010). AMPK activity was first stimulated using A-769662 that, like AMP itself, acts both as an allosteric activator and inhibits the dephosphorylation and consequent inactivation of the enzyme (Göransson *et al.*, 2007). However, unlike other AMPK activators such as the widely employed AICAR, A-769662 does not mimic AMP. Therefore, it neither stimulates glycogen phosphorylase nor inhibits fructose-1,6-bisphosphatase (Göransson *et al.*, 2007), and so is a more selective AMPK activator than AICAR. Although off-target effects for A-769662 have been reported (Moreno *et al.*, 2008; Scott *et al.*, 2008; Treebak *et al.*, 2009), these are associated with concentrations (100 μM–1 mM) that are significantly higher than that (5 μM) employed in the present study.

In strong support of a role for myocyte AMPK in the BK_{Ca}-opening actions of both CL-316,243 and exogenous adiponectin, A-769662 itself generated marked myocyte hyperpolarizations (in the absence of PVAT), which were inhibited not only by iberiotoxin but also by the AMPK inhibitor, dorsomorphin. Furthermore, this inhibitor also abolished the myocyte-hyperpolarizing, BK_{Ca}-opening effects of both CL-316,243 and authentic adiponectin (Figure 6).

Inhibitory actions of glibenclamide and clotrimazole – some speculations

A striking finding was the pivotal involvement of myocyte BK_{Ca} channels in the hyperpolarizing actions of adiponectin and A-769662 and in those of the ADHF liberated from PVAT by CL-316,243. Previous myograph studies (Löhn *et al.*, 2002; Fang *et al.*, 2009) have simplistically concluded that 'ADHF' was an opener of myocyte K_{ATP} channels because the anti-contractile effects of PVAT in rat aorta were inhibited by glibenclamide, the widely used blocker of K_{ATP}. However, direct measurements of membrane potential in the present study have clearly shown that ADHF opens BK_{Ca} channels.

Because 10 μM glibenclamide, the concentration employed by Löhn *et al.* (2002) and Fang *et al.* (2009), also inhibits the cation channel TRPM4 (Demion *et al.*, 2007), we reasoned that myocyte AMPK activation (by an endogenous ADHF) might allow Na^+ entry through TRPM4 channels. As reviewed by Guinamard *et al.* (2010), TRPM4 activation provides an important driving force for Ca^{2+} entry into a variety of cell types, an event that would increase the open probability of a Ca^{2+}-sensitive K^+ channel like BK_{Ca}. If correct, iberiotoxin should block BK_{Ca} opening induced by both A-769662 and NS1619, whereas glibenclamide should only inhibit any 'indirect' effect of A-769662 mediated by hypothetical AMPK-induced activation of Ca^{2+} entry via TRPM4. Consistent with this hypothesis, glibenclamide did inhibit iberiotoxin-sensitive hyperpolarizations induced by the AMPK activator, A-769662, without affecting direct BK_{Ca} activation by NS1619.

In addition to their glibenclamide sensitivity (Demion *et al.*, 2007), TRPM4 cation channels are also inhibited by clotrimazole (Vennekens and Nilius, 2007), which has no effect on NS1619-induced BK_{Ca} currents (Edwards *et al.*, 1996). In the present study, clotrimazole abolished PVAT-dependent hyperpolarizations not only to CL-316,243 but also to A-769664 and adiponectin, with no effect on responses to NS1619 (Figure 6).

Collectively, therefore, the hyperpolarizations generated by ADHF (i.e. by CL-316,243 in the presence of PVAT), A-769664 and adiponectin are consistent with their ability to activate myocyte BK_{Ca} channels indirectly. Experiments to investigate these possibilities further and to explore how the opening of a non-selective, poorly Ca^{2+}-permeable cation channel like TRPM4 might be involved in the activation of myocyte BK_{Ca} channels are currently in progress.

Conclusions

Activation of adipocyte β_3-adrenoceptors on PVAT releases an ADHF, which is likely to be adiponectin. In non-contracted vessels, this adipokine indirectly opens myocyte BK_{Ca} channels, an effect that involves AMPK since it can be mimicked by the AMPK activator, A-769662, and blocked by the kinase inhibitor, dorsomorphin (Figure 6). The ability of glibenclamide and clotrimazole to block adipocyte-dependent myocyte hyperpolarizations but not responses to a BK_{Ca} channel opener, NS1619, suggests that AMPK does not activate BK_{Ca} directly but may involve a cation channel such as TRPM4. Some of the effects of the liberated ADHF are L-NMMA sensitive, a possible indication of adipocyte NO-synthase activation also involving an AMPK-dependent pathway (Figure 6). The PVAT-dependent, BK_{Ca}-mediated hyperpolarizing effects of adiponectin on vascular myocytes may have a variety of long-term consequences *in vivo*, and form the basis of the complex role of adiponectin in cardiovascular disease, including the effects of this adipokine on systolic blood pressure (Avery *et al.*, 2011).

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Conflicts of interest

None.

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