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The quest for endothelial atypical cannabinoid receptor: BK_{Ca} channels act as cellular sensors for cannabinoids in in vitro and in situ endothelial cells

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Abstract

Endothelium-dependent component of cannabinoid-induced vasodilation has been postulated to require G-protein-coupled non-CB₁/CB₂ endothelial cannabinoid (eCB) receptor. GPR18 was proposed as a candidate for eCB. To address the hypothesis that the effects attributed to eCB are mediated by G-protein-coupled receptor (GPCR)-independent targets, we studied the electrical responses in endothelial cells, focusing on BK_{Ca} channels. In patches excised from endothelial-derived EA.hy926 cells, *N*-arachidonoyl glycine (NAGly) and abnormal cannabidiol (abn-cbd), prototypical agonists for eCB receptor, stimulate single BK_{Ca} activity in a concentration- and Ca²⁺-dependent manner. The postulated eCB receptor inhibitors rimonabant and AM251 were found to inhibit basal and stimulated by NAGly- and abn-cbd BK_{Ca} activity in cell-free patches. In isolated mice aortas, abn-cbd and NAGly produced endothelial cell hyperpolarization that was sensitive to paxilline, a selective BK_{Ca} inhibitor, but not to GPR18 antibody, and mimicked by NS1619, a direct BK_{Ca} opener. In excised patches from mice aortic endothelium, single channel activity with characteristics similar to BK_{Ca} was established by the addition of abn-cbd and NAGly. We conclude that the two cannabinoids abn-cbd and NAGly initiate a GPR18-independent activation of BK_{Ca} channels in mice aortic endothelial cells that might contribute to vasodilation to cannabinoids.

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Conflict of interest

None.

Keywords

N-arachidonoyl glycine; Abnormal cannabidiol; Rimonabant; Mouse aorta; Endothelial cells; BK_{Ca} channels

1 Introduction

While it is well known that one of the pharmacological actions of cannabinoids is regulation of vascular function, which includes endothelium-dependent vasodilation and a sustained decrease in arterial blood pressure [1,2], the underlying mechanisms of these effects are not well understood and our current knowledge on them is rather controversial [3]. Historically, sensitivity of mesenteric vasodilation induced by anandamide to the CB₁ receptor inhibitor SR141716A (rimonabant) and persistence of the relaxation in CB₁ knock-out mice has been interpreted as evidence to suggest the existence of the novel endothelially located G-protein coupled non-CB₁/CB₂ receptor [2,4,5], also referred in literature as “endothelial anandamide receptor”, “abn-cbd receptor” or “endothelial atypical cannabinoid (eCB) receptor”. The relaxation commonly attributed to eCB receptor is also antagonized by high concentrations of the CB₁ receptor antagonist AM251, the iodinated analogue of rimonabant [2,6–8], and cannabidiol analogue O-1918 [1,4]. The latter has been concluded to represent a selective antagonist, while abnormal-cannabidiol (abn-cbd), a synthetic analogue of the plant-derived cannabinoid cannabidiol, was introduced as a selective ligand for the novel endothelial receptor [9]. These conclusions fuelled a large number of peer-reviewed publications, in which sensitivities of cannabinoid-induced vasodilation to O-1918, AM251 and rimonabant both in micro- and macrovessels have been interpreted as an evidence for engagement of eCB receptor in the responses [1,6,10–15]. The molecular identity of the postulated receptor has drawn considerable attention and intense research as the receptor may represent a promising target for drug development and therapy of conditions accompanied by endothelial dysfunction without affecting psychoactivity. A number of published reports link the eCB receptor with GPR18 [10,16–18], ascribing the latter as a third cannabinoid receptor. In view of the known sequences of the human genome, GPR18 shows little homology to CB₁ or CB₂ receptor and can not be considered as a true cannabinoid receptor. Nevertheless, due to pharmacological profile overlapping with CB₁ and CB₂ receptors, there is a possibility that the effect of cannabinoids in the vasculature requires GPR18 or other G-protein-coupled receptor (GPCR) with cannabinoid-like pharmacology.

The receptor is thought to be selectively activated by abn-cbd [9–11,19], endogenous anandamide derivative *N*-arachidonoyl glycine (NAGly) [12,20] and a synthetic cannabinoid-like compound VSN16R [6]. In addition to rimonabant, AM251 and O-1918, the vasodilation to cannabinoids is sensitive to the blockers of Ca²⁺-activated K⁺ channels of large conductance (BK_{Ca}) [9,12,21]. Based on these observations it was concluded that BK_{Ca} channels are activated downstream of the receptor stimulation [11,12].

While it has been known that beyond their receptor-mediated effects, endocannabinoids directly alter the function of a number of ion channels [22], an increased understanding on the mechanisms of action of endocannabinoids and cannabinoid-related compounds on

endothelial cells came from studies utilizing patch-clamp electro-physiology, which show direct, GPCR-independent targeting a number of ion-transport systems controlling endothelium-dependent vasodilation [23–27]. We have recently demonstrated that in endothelial-derived cell line EA.hy926, GPCRs are not required for BK_{Ca} potentiation by a cannabinoid-like compound VSN16R [28] and anandamide [29]. Utilizing the same cell line, we have also shown that NAGly concentration-dependently inhibits endothelial Na⁺-Ca²⁺ exchanger (NCX) and at 3 μ M stimulates BK_{Ca} activity in inside-out patches exposed to 300 nM free Ca²⁺ at a holding potential + 40 mV [30]. BK_{Ca} channels are both Ca²⁺ and voltage-dependent and, depending on basal Po level, may respond to some lipids [23,31] and non-lipid compounds [32,33] by either facilitation or inhibition of the channel activity. It is still unclear whether the NAGly action on BK_{Ca} channels is bidirectional in nature and depends on Ca²⁺ and voltage.

Another important finding that advances our understanding of the mechanisms of vascular cannabinoid signaling is the ability of O-1918 to directly inhibit NCX [23] and the BK_{Ca} activity in EA.hy926 cells endogenously expressing BK_{Ca} channel [29] and human embryonic kidney cells (HEK-293) stably transfected with the α -subunit of the human BK_{Ca} channel [34]. These results disregard O-1918 as a selective eCB receptor antagonist, weakening previous conclusions on the eCB receptor engagement based on pharmacological inhibition of the relaxation by this compound [1,6,9,11,12,15,17]. Whether other CB₁ receptor antagonists commonly used to suppress the eCB receptor-attributed effects (rimonabant and AM251) directly influence BK_{Ca} channel function is currently unknown and needs to be clarified. These recent findings, derived mostly from the endothelial cell line, question, but do not negate the possibility of existence of specific cannabinoid receptor on vascular endothelial cells involved in endothelium dependent relaxation. To clarify the mechanisms of action of endocannabinoids on endothelial cells and the role of GPR18, if any, it is essential to perform additional electrophysiological studies on intact, enzymatically untreated endothelium, i.e. under conditions that exclude possible alteration in protein expression profile/cell signaling due to cell isolation or culture [35]. Considering that the presence of BK_{Ca} channels in vascular endothelium is controversial and rather relevant to cultured cells [35], a key question that arises is why then endothelium-dependent vasodilation to cannabinoids is BK_{Ca}-dependent.

In light of this background, the present study further addressed the hypothesis that vascular effect of cannabinoids commonly attributed to stimulation of elusive eCB receptor does not require GPCR. The data presented here identify BK_{Ca} channels as cellular sensor for cannabinoids in in vitro and in situ endothelial cells, urging a re-evaluation of the hypothesis that novel endothelial G-protein-coupled endothelial receptor is required for CB₁/CB₂ receptor-independent vasodilation induced by cannabinoids.

2 Methods

2.1 Cell culture

The human umbilical vein-derived endothelial cell line, EA.hy926 [36] at passage > 40 was grown in DMEM containing 10% FCS and 1% HAT (5 mM hypoxanthin, 20 μ M

aminopterin, 0.8 mM thymidine) and were maintained in an incubator at 37 °C in 5% CO₂ atmosphere. For electrophysiological experiments, cells were plated on glass coverslips.

2.2 Patch-clamp recordings from EA.hy926 cells

Single-channel recordings were obtained from excised inside-out and outside-out membrane patches in symmetrical solutions using the patch-clamp technique. For inside-out configuration, the pipettes were filled with a solution containing (in mM): 135 KCl, 10 HEPES, 1 MgCl₂, 3 EGTA, 2.96 CaCl₂. The free Ca²⁺ concentration in this solution amounted 10 μM according to a program CaBuf, developed by G. Droogmans, Leuven, Belgium. pH was adjusted to 7.2 by adding KOH. Cells were superfused with a bath solution containing (in mM): 145 NaCl, 5 KCl, 1.2 MgCl₂, 5 HEPES, 10 glucose, 2.4 CaCl₂. Following gigaseal formation, bath solution was switched to the following (in mM): 135 KCl, 10 HEPES, 1 MgCl₂, 3 EGTA and a desired free Ca²⁺ concentration which was adjusted by adding different amounts of CaCl₂ calculated by the program CaBuf. pH was adjusted to 7.2 by adding KOH. For outside-out configuration, free Ca²⁺ in the KCl-containing pipette solution was adjusted to 300 nM. Single-channel activity was obtained from > 30 s of continuous recording under each experimental condition.

2.3 Patch-clamp recordings from the endothelium in situ

All animal care and experimental studies were carried out in accordance with the ARRIVE guidelines [37]. Preparation of arterial segments and patch-clamp recordings were essentially performed as previously described [38]. Briefly, C57Bl/6 mice of 8–20 weeks old of both sexes were sacrificed by cervical dislocation. The thoracic aorta was excised, cleaned and placed a solution containing (in mM): 145 NaCl, 5 KCl, 1 NaH₂PO₄, 1.8 CaCl₂, 1.2 MgSO₄, 10 glucose, 5 HEPES. pH was adjusted to 7.4 with NaOH. The aorta was then cut into ring segments of 2–3 mm in width with minimum damage to the endothelium. The segment was cut open and pinned to the bottom of the recording chamber. The membrane potential of endothelial cells was recorded using a perforated patch-clamp technique with escin as a perforating agent. For studies of single channel activity in inside-out configuration, a patch was excised to the bath solution containing: 135 KCl, 10 HEPES, 1 MgCl₂, 3 EGTA and 2.85 mM CaCl₂ (free Ca²⁺ concentration 3 μM calculated by the program CaBuf). pH was adjusted to 7.2 by KOH. Composition of the pipette solution was the same used for respective inside-out experiments with EA.hy926 cells.

2.4 Contractile recording experiments

Vascular contractility experiments were performed as described by us earlier [30]. Briefly, vascular rings were mounted isometrically in a tissue bath between a stationary stainless steel hook and an isometric transducer coupled to a polygraph. The rings were mounted with a resting tension of 1 g. Vascular preparations were superfused with modified Krebs bicarbonate buffer solution gassed with 5% CO₂ in O₂. After the equilibration period, arteries were precontracted with 10 μM norepinephrine and when a stable level of contraction was established, 5 μM NAGly was applied to observe relaxation. Contractile experiments were conducted at 37 °C.

2.5 Chemicals

NAGly and abn-cbd were purchased from Abcam and dissolved in ethanol. Paxilline and TRAM-34 were purchased from Alomone Labs. GPR18 polyclonal antibody (internal region, CSB PA008914) was supplied by Cusabio and diluted to 1:500 in the pipette solution according to the manufacturer's instruction.

2.6 Statistical analysis

Experimental data are expressed as mean $\pm$ SEM. Student's *t*-test was used to compare results, with $P < 0.05$ taken as the level of significance. In experiments with EA.hy926 cells, *n* denotes the number of cells studied and in experiments with isolated aortas, *n* denotes the number of animal used.

3 Results

3.1 NAGly and abn-cbd increase the activity of BK_{Ca} channels in patches excised from EA.hy926 cells

In order to test the hypothesis that one of the molecular targets for NAGly and abn-cbd in endothelial cells is BK_{Ca} channel, we first explored whether NAGly is able to facilitate single BK_{Ca} activity. Therefore, we employed the inside-out patch-clamp configuration obtained from EA.hy926 cells, a suitable endothelial-derived cell model for studying BK_{Ca} channel modulation [24,27–29], and tested a wide concentration range of NAGly at various Ca²⁺ levels and voltages. This configuration was specifically chosen as it allows focusing on the direct pharmacological targeting of the channel of interest, avoiding potential interference originated from cellular metabolites and GPCR stimulation. To determine concentration-dependency of the effect of NAGly on BK_{Ca} activity, single channel activity was recorded in inside-out configuration at a fixed potential +40 mV in high K⁺ solution containing 300 nM free Ca²⁺. Following patch excision, high conductance (~ 300 pS) single channel activity was detected in the majority of patches. Administration of six incremental concentrations of NAGly (0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M and 30 μ M) consistently elicited a significant increase in single channel activity (NPo) in a concentration-dependent manner (Fig. 1A). When 300 nM NAGly was exposed to the cytosolic surface of the membrane patch, NPo increased by $60 \pm 24\%$ of the pre-NAGly levels, and was further increased to $238 \pm 67\%$ and $304 \pm 60\%$ upon addition of 1 μ M and 3 μ M NAGly, respectively. 30 μ M NAGly, the maximal concentration tested, produced a 14.3 ± 4.7 fold increase in the channel activity (Fig. 1A, B). The EC₅₀ value was not possible to determine, since the effect did not reach the plateau. The effect was sustained and slowly reversible after wash out (Fig. 1C). Administration of abn-cbd (1 μ M) to the cytosolic face of the membrane resulted in a robust increase in NPo in all patches tested (Fig.1D).

Consistent with the BK_{Ca} intrinsic properties, the activity of the channels was strictly dependent on the Ca²⁺ concentration at the cytosolic face of the membrane (Fig. 2) as well as the voltage that was applied to the patch (Supplementary Fig. S1A, B). To address how the action of NAGly is related to cytosolic Ca²⁺ level and applied voltage, NAGly was again probed in inside-out configuration over a wide range of bath Ca²⁺ concentrations and various voltages. The stimulatory effect of NAGly on BK_{Ca} activity was observed at a wide

range (0.1–10 μM) of physiological Ca^{2+} levels (Fig. 2A). Notably, the strongest NPo amplification by 3 μM NAGly was observed at 100 nM Ca^{2+} at cytosolic face of the membrane where this compound induced an app. 7-fold NPo increase, whereas in the presence of 10 μM Ca^{2+} , the same concentration of NAGly potentiated NPo < 2-fold (Fig. 2B).

In two patches containing one active channel with very high basal Po levels 0.86 and 0.76, NAGly failed to considerably increase the Po levels (3 μM NAGly: Po = 0.90 and 0.81, respectively). This was somewhat expected, since channels with basal Po level close to 1 unable to considerably further increase the Po level due to the ceiling effect. However, an important observation is that even at very high basal Po levels, NAGly had no inhibitory effect on the channel activity, an observation that is contrasted with the bidirectional effect of LPI on BK_{Ca} channel reported by us earlier [23]. The effect was observed at a wide range of holding voltages and the degree of the NPo potentiation was poorly dependent on the voltage applied (Supplementary Fig. S1A, B). In order to exclude the possibility that NAGly influences the BK_{Ca} function by reaching recognition site exclusively from the inner surface of the membrane, in a set of experiments, NAGly was administered to the outer leaflet of the membrane (i.e. outside-out configuration). In these experiments performed in the presence of 0.3 μM free Ca^{2+} in the pipette solution, bath administration of 3 μM NAGly produced a 9.7 ± 2.1 fold NPo increase, a value significantly ($p < 0.05$) greater than that attained in inside-out configuration (NPo increase: 4.0 ± 0.8) (Fig. 2C, D).

3.2 Rimonabant and AM251 inhibit BK_{Ca} channel activity in a voltage-independent manner

To address whether the eCB receptor antagonists rimonabant and AM251 exert receptor-independent effect on BK_{Ca} function, we again employed inside-out configuration utilizing EA.hy926 cells as a model, thus avoiding GPCR targeting. In the presence of 1 μM free Ca^{2+} in the bath to yield considerable channel activation, administration of 5 μM rimonabant to the cytosolic surface of the membranes suppressed NPo by $74.2 \pm 6.0\%$ (Fig. 3A, B), indicating that the effect does not require rimonabant binding to cannabinoid receptors. Probing rimonabant at different voltages revealed that the degree of BK_{Ca} inhibition was poorly dependent on the voltage applied (Supplementary Fig. S2A, B). The inhibition was also observed when the BK_{Ca} activity was pre-stimulated with NAGly (Fig. 3C, D) or abn-cbd (Fig. 3E). To determine whether changes in the channel activity in excised patches evoked by abn-cbd alone and in combination with rimonabant are translated into membrane potential shifts, additional experiments in current-clamp mode were conducted. In these experiments, abn-cbd (10 μM) elicited a sustained hyperpolarization of EA.hy926 cells with an amplitude 11.7 ± 1.8 mV ($n = 4$, Fig. 3F) and this hyperpolarization was reversed by addition of 3 μM rimonabant ($n = 3$, Fig. 3G). Superfusion of EA.hy926 cells with a bath solution containing 5 μM rimonabant strongly inhibited basal whole-cell BK_{Ca} current elicited by voltage ramps within 10 min (Fig. 3H, I).

When AM251 (5 μM) was exposed to the cytosolic surface of the patches in the presence of 1 μM Ca^{2+} , the BK_{Ca} channel activity was inhibited by $52 \pm 11\%$ ($n = 5$, Fig. 4A). At 10 μM , AM251 also exhibited a strong (~10 times) inhibitory effect when BK_{Ca} channels were

pre-stimulated with 3 μM NAGly in the presence of 0.3 μM Ca^{2+} at cytosolic face of the membrane (Fig. 4B). When 5 μM AM251 was administered to the outer surface of the patch (outside-out configuration), the inhibitory effect was significantly ($P < 0.05$) stronger ($83\% \pm 17\%$, $n = 5$) than that observed in inside-out experiments (Fig. 4C, D). Altogether, these results indicate that at concentrations commonly used to inhibit the eCB receptor-attributed relaxation, rimonabant and AM251 directly inhibit BK_{Ca} channel activity independently of cell integrity, cytosolic messengers and cannabinoid receptors and this effect is accompanied by inhibition of cell hyperpolarization elicited by abn-cbd.

3.3 Cholesterol depletion with methyl- β -cyclodextrin eliminates the BK_{Ca} facilitation and vascular relaxation in response to NAGly

Cholesterol, a critical component of mammalian cell membranes, has been shown to regulate the function of a number of ion channels, including BK_{Ca} channels [39]. Cholesterol-rich microdomains of the plasma membrane are located in caveola, spherical invaginations of the plasma membrane. Taking into account our finding that the site of action of NAGly is likely to be located on the outer surface of the membrane, we next evaluated whether membrane cholesterol and intact caveola are required for the NAGly-evoked BK_{Ca} potentiation and vasodilation. In patches excised from EA.hy926 cells pre-treated for 1 h with the cholesterol-reducing agent methyl- β -cyclodextrin (MCD, 1 mM), NAGly (3 μM) failed to modify NPo level (Fig. 5A). To address the functional relevance of inability of NAGly to affect BK_{Ca} activity in cholesterol-depleted cell membranes, we tested whether cholesterol depletion affects the relaxation of aortic rings by NAGly. Administration of 3 μM NAGly relaxed a norepinephrine-induced contraction by $38.5 \pm 1.9\%$ ($n = 5$) (Fig. 5B, *upper panel*). Administration of 3 μM NAGly to MCD-treated aortic rings pre-contracted with norepinephrine resulted in further constriction (by $2.6 \pm 1.4\%$, $n = 4$) (Fig. 5B, *lower panel*).

3.4 In excised aorta, NAGly and abn-cbd produce BK_{Ca} -dependent endothelial cells hyperpolarization independently of gap junctions and GPR18

To directly address the remaining possibility that activation of specific GPCR is required for electrical responses of intact vascular endothelium to NAGly and abn-cbd, further experiments were conducted on in situ endothelial cells. Mice aortic preparations were chosen to minimize the possibility of transmission of the hyperpolarizing response to the endothelium from the underlying smooth muscle cells via myo-endothelial gap junctions. An ultrastructural analysis of isolated adult mice aortas showed a lack of myo-endothelial gap junctions [40], making this vascular bed a useful model for studying electrical signaling originated within the endothelium itself. In mice aortas, the resting membrane potential of endothelial cells measured with the perforated patch-clamp method averaged -38.2 ± 1.8 mV (49 observations from $n = 21$). To confirm a lack of myo-endothelial electrical communication in mouse aorta, the effects of phenylephrine, a selective agonist of α_1 -adrenergic receptor, and angiotensin II on endothelial membrane potential were examined. Neither phenylephrine (1 μM , $n = 4$, Fig. 6A), nor angiotensin II (100 nM, $n = 3$, not shown) affected the endothelial membrane potential. These observations are consistent with a lack of functional myo-endothelial electrical coupling in this vascular bed. In contrast, in rat aorta, where myo-endothelial gap junctions are present, phenylephrine elicited endothelial cell depolarization accompanied by membrane potential oscillations (Fig. 6B). Superfusion of

mice aortic strips with a solution containing 10 μM NAGly produced a slowly developing sustained endothelial cell hyperpolarization with the mean amplitude 6.9 ± 2.1 mV ($n = 5$, Fig. 6C). The hyperpolarization was increased to 13.3 ± 1.9 mV ($n = 4$) following administration of 30 μM NAGly (Fig. 6D). Exposure to paxilline (1 μM) in the continued presence of NAGly reversed the hyperpolarization ($n = 3$, Fig. 6E). Administration of 10 μM abn-cbd elicited a sustained endothelial hyperpolarization (7.9 ± 1.7 mV), which was sensitive to paxilline ($n = 3$), but not TRAM-34 (1 μM), a selective IK_{Ca} inhibitor ($n = 4$, Fig. 6F).

To assess possible contribution of GPR18 to the NAGly-evoked endothelial cell hyperpolarization in intact mice aortas, a GPR18-specific antibody against cytosolic epitope (obtained from Cusabio, 1:500 v/v) was infused into endothelial cells via a patch pipette, an approach that has been used previously for studying contribution of α subunits of different G-proteins to muscarinic receptor-operated cation currents in guinea pig ileal smooth muscle cells [41]. To minimize signal transmission via gap junctions from neighbouring endothelial cells, bath solution was supplemented with glycyrrhethinic acid (100 μM), a gap junction inhibitor. Intracellular perfusion with the GPR18 antibody failed to significantly modify the amplitude of the hyperpolarization induced by NAGly (Fig. 6G, H). We also examined the impact of a direct BK_{Ca} opener NS1619 on the membrane potential of cells of an intact endothelium of mice aortas. NS1619 (10 and 30 μM) elicited a hyperpolarizing response with the amplitude 6.7 ± 1.9 mV ($n = 6$) and 9.8 ± 2.2 mV ($n = 5$), respectively (Fig. 6I, J), the values compatible with that evoked by NAGly and abn-cbd. Altogether, these data indicate that NAGly and abn-cbd hyperpolarize mouse aortic endothelial cells via GPR18-independent activation of BK_{Ca} channels.

3.5 In patches excised from in situ endothelium, abn-cbd and NAGly trigger single channel activity with characteristics similar to BK_{Ca}

To confirm the presence of BK_{Ca} channels in mice aortic endothelium and further substantiate our hypothesis that endothelial BK_{Ca} channels act as cannabinoids sensor, we next sought to examine the impact of abn-cbd and NAGly on single channel activity in patches excised from in situ endothelium of mouse aorta. Following patch excision and exposure the cytosolic face of the patch to high K^+ solution containing 1–3 μM Ca^{2+} , no BK_{Ca} single channel activity was detected (78 observations from $n = 14$, Fig. 7A). However, exposure to 3 μM abn-cbd triggered single channel activity in 8 out of 41 patches (19.5% of observations) with the mean channel conductance 294 ± 15 pS ($n = 5$, Fig. 7A). Under symmetrical K^+ conditions, unitary currents reversed near 0 mV (Fig. 7B), and the channel activity increased with membrane depolarization (Fig. 7A, C).

In excised patches of endothelial cells from intact mouse aortic endothelium, single channel activity of big (~ 300 pS) conductance was also triggered when 3 μM NAGly was administered to the cytosolic surface of the patch ($n = 6$, Fig. 8). The channel activity following NAGly exposure was observed in 6 out of 37 patches studied. Single channel activity was detected both at positive (Fig. 8A, B) and physiological negative potentials (Fig. 8B, C). The channel activity was observed also in excised patches ($n = 4$) from the intact endothelium of the NAGly-pre-exposed strips (Fig. 8C, D). The activity of the channel was

suppressed by paxilline (2 μ M, Fig. 8D). These characteristics are consistent with identification of this channel as BK_{Ca} channel.

4 Discussion

In the present study, the mechanisms of endothelial electrical signaling accompanying the vasodilation to cannabinoid-like compounds occurred independently of CB₁/CB₂ receptors have been investigated. Considering that endothelial hyperpolarization is a key signaling event in initiating endothelium-dependent relaxation, we explored the respective electrical responses in endothelial cell line and in the in situ endothelium of mice aorta. NAGly and abn-cbd were specifically chosen as agonists, as the vasodilator action of both compounds has been assigned by previous wire myography studies [2,9,11,12,17] exclusively to stimulation of putative G-protein coupled non-CB₁/CB₂ cannabinoid receptor expressed in endothelial cells.

We show here that in inside-out and outside-out patches excised from endothelial cell line EA.hy926, NAGly and abn-cbd strongly potentiate single BK_{Ca} activity, indicating that the effect does not require GPCR or signaling pathways. We explored the effect of NAGly on BK_{Ca} function within wide range of voltages, Ca²⁺ levels and concentration range 0.1–30 μ M, expanding our previous observation [30]. Higher concentrations of NAGly have not been tested due to an increased risk of micelle formation at concentrations higher than 30 μ M [25,42]. The effective concentrations of NAGly appeared to be very close to the respective values characterizing endothelium-dependent relaxation to NAGly [12,43], but far exceed the reported EC₅₀ value for GPR18 (44.5 nM), suggesting that direct activation of endothelial BK_{Ca} channels, but not GPR18, by NAGly accounts for the endothelium-dependent vasodilation. The effect of NAGly on BK_{Ca} activity was sustained, partially reversible and observed within Ca²⁺ range 0.1–10 μ M with the maximum peak at 0.1 μ M free Ca²⁺, a concentration corresponding to the resting Ca²⁺ level in the aortic endothelium [44]. Considering that in pre-contracted smooth muscle cells, intracellular Ca²⁺ level reaches micromolar concentrations, it is reasonable to suggest that in pre-contracted arteries the effect of NAGly on smooth muscle BK_{Ca} channels is likely to be weaker than the respective effect on endothelial BK_{Ca} channels. NAGly was found to increase NPo when applied both to the cytosolic and external face of the membrane. In outside-out configuration, the effect was significantly stronger, suggesting that the site of action is likely to be located on the outer surface of the membrane.

Rimonabant, AM251 and O-1918 have widely been employed as selective blockers of eCB receptor and the sensitivity of cannabinoid-induced vasodilation to these inhibitors has commonly been taken as evidence to suggest that the response requires eCB receptor [2,6,9,11]. We found here that, when applied to the inner surface of the membrane, rimonabant and AM251 exert an inhibitory effect on basal BK_{Ca} activity and oppose BK_{Ca} potentiation elicited by abn-cbd or NAGly. These results show for the first time that rimonabant and AM251 counteract the stimulatory effect of abn-cbd and NAGly via a GPCR-independent mechanism. Taking into account earlier identification of GPCR-independent inhibitory action of O-1918 on heterologously expressed *Slor* BK_{Ca} channels [34] as well as native BK_{Ca} channels [29], our current observations bring into question the

concept of existence of novel G-protein coupled cannabinoid receptor expressed by endothelial cells that is involved in CB₁/CB₂ receptor-independent BK_{Ca}-dependent cannabinoid-induced vasodilation. In the present study, the inhibitors were tested at concentration 3–5 μ M, which we found to be effective when single BK_{Ca} activity was pre-stimulated either by elevated free Ca²⁺, abn-cbd or NAGly. Similar to the effect of NAGly, AM251 was found to be more effective when applied to the outer face of the membrane, pointing that the site of action is more easily reachable from the outer membrane surface. While the precise location(s) of the binding site(s) of NAGly and AM251/rimonabant remains unidentified, higher efficacy of NAGly and AM251 in BK_{Ca} modulation when administered from the outer face of the membrane, and the reversal of the NAGly- and abn-cbd-induced BK_{Ca} potentiation by rimonabant may indicate that the compounds act through the same site located on the outer surface of the membrane. Considering that cholesterol located in caveolae regulates the function of a number of ion channels, including BK_{Ca} [39] and demonstrations of BK_{Ca} unresponsiveness to NAGly (current study), anandamide [29] and *N*-arachidonoyl serine [34] following cholesterol depletion, it is reasonable to suggest that the action of cannabinoids and cannabinoid-like compounds on endothelial cells is accompanied or at least partially underpinned by modulation of cholesterol level in caveolae. This suggestion is substantiated by our demonstration that NAGly no longer produces the vasodilation in MCD-treated aortic preparations.

Previous studies showed some level of inconsistency in the efficacy of different CB₁ antagonists to antagonize the eCB receptor-attributed vasodilation. A lack of the inhibitory effect may be partially attributed to variable concentrations of antagonists and agonists used. Thus, submaximal concentrations of NAGly and abn-cbd (up to 30 μ M) may results in very high level of BK_{Ca} activity that is only partially sensitive to low concentrations of the inhibitors. Thus, the vasodilation to 30 μ M NAGly was shown to be little affected by 1 μ M AM251 and 3 μ M rimonabant, but strongly reduced by 10 μ M O-1918, whereas 3 μ M rimonabant significantly suppressed the vasodilation to 10 μ M NAGly [43]. The other study reported that O-1918 at 3 μ M significantly inhibits mesenteric vasodilation to 0.3–10 μ M NAGly, but is ineffective when probed against 30 μ M NAGly [12]. Another possible explanation in the reported variability in the sensitivity of the vasodilation to the eCB receptor and BK_{Ca} inhibitors [43] is an engagement of additional mechanisms, such as Na⁺-Ca²⁺ exchanger (NCX) [30,43] and TRPV4 channel [26].

While AM251 is generally considered to be a selective CB₁ receptor antagonist with K_i value < 10 nM, at low micromolar concentrations AM251 inhibits the relaxation to abn-cbd [7,45]. Rimonabant was shown to inhibit the endothelium-dependent component of vasodilation to anandamide and its stable analogue methanandamide in pre-contracted rat mesenteric artery [4] and rabbit aortic rings [13]. The effect was ascribed to inhibition of rimonabant-sensitive eCB receptor. Several GPCR-independent effects were described for AM251 and rimonabant. AM251 and rimonabant were recently shown to potentiate GABA_A-mediated currents [46]. Rimonabant at 10 μ M was shown to inhibit Ca²⁺-induced relaxation [47] and the relaxation to K_{ATP} channel openers and BK_{Ca} opener NS1619 [48], an observation that is in agreement with the present study.

We found that in mouse aorta, abn-cbd and NAGly evoke endothelial cell hyperpolarization sensitive to paxilline, pointing for engagement of BK_{Ca} channels in the response. Our observations showing inability of phenylephrine and angiotensin II to modify endothelial cell membrane potential in isolated mice aortas are in agreement with a previous report showing a lack of ultrastructural evidence for myoendothelial communication in this vascular bed [40], thus ruling out the major role of myo-endothelial electrical coupling in signal propagation from smooth muscle cells to endothelial cell monolayer and indicating that the response to NAGly and abn-cbd we measured from endothelial cells was generated mainly within endothelial cells.

Here we report that in isolated mouse aorta exposed to NAGly, endothelial cell hyperpolarization, a critical signaling event in initiating endothelium-dependent relaxation, was independent on GPR18 and reproduced by a direct BK_{Ca} opener NS1619. Taking into account these findings and observations that the proposed eCB receptor antagonists act as BK_{Ca} inhibitors, it is reasonable to conclude that the BK_{Ca}-dependent action of putative eCB receptor agonists does not require GPCR. This conclusion was further supported by detection of single BK_{Ca}-like activity in excised patches exposed to abn-cbd or NAGly. While GPR18 is considered to be the main candidate for eCB receptor [10,17], GPR18-dependent signaling in vascular endothelium has not been described so far [3] and the role of GPR18 in regulation of vascular function is still questionable. GPR18 as a receptor for abn-cbd and NAGly has been introduced from studies performed on non-vascular tissues [16]. However, this interaction has not been confirmed by others [49–51]. Noteworthy, delta (9)-tetrahydrocannabinol (THC), the primary active component of cannabis, was shown to bind to GPR18 [20,52], but, unlike abn-cbd and NAGly, devoid CB₁/CB₂ receptor-independent mesenteric vasodilator activity [4], further questioning the relevance of GPR18 in endothelium-dependent vasodilation.

BK_{Ca} channels regulate many fundamental cellular functions. They are detected both on plasma membrane and intracellularly, including nucleus, endoplasmic reticulum, Golgi apparatus and the inner mitochondrial membrane [53]. In vascular endothelial cells, BK_{Ca} expression is largely associated with pathological conditions [35]. However, in some vascular beds, for example, in porcine renal artery, the BK_{Ca} activity is detected in vascular endothelium under control conditions [54]. While functional BK_{Ca} channels in plasma membrane of vascular endothelial cells are not widely detected, the mounting evidences have been accumulated that BK_{Ca} channels do control endothelial cell function. Thus, in various vascular beds, including rat aorta, it has been shown that the BK_{Ca} channel openers capable to elicit endothelium-dependent relaxation in a concentration-dependent manner [55–58] and a significant increase in intracellular Ca²⁺ level in endothelial cells [55,59]. An increase in Ca²⁺ in response to BK_{Ca} openers was shown to be accompanied by an enhanced NO production [55,59]. Interestingly, the effects of BK_{Ca} openers are accompanied by phosphorylation of p42/p44 MAP kinase in endothelial cells [59], a primary downstream signaling pathway activated by abn-cbd and NAGly and ascribed to stimulation of non-CB₁/CB₂ receptor [16,60]. Our observation that NAGly and abn-cbd act as direct BK_{Ca} openers eliciting hyperpolarization of in vitro and in situ endothelial cells, a mechanism that increases the driving force for Ca²⁺ entry and enhances NO production, provides an alternative mechanistic explanation for the relaxation classically attributed by scientific

community to stimulation of putative G-protein coupled endothelial non-CB₁/CB₂ cannabinoid receptor. To minimize contribution of cytosolic factors and the possibility of GPCR-independent G-protein activation, excised inside-out patches in our experiments were extensively washed-out by bath solution. Moreover, BK_{Ca} activation by *N*-arachidonoyl serine was detected in outside-out patches obtained from HEK-293 cells stably transfected with the α subunit of BK_{Ca} (*Slo1*) channel, i.e. in the absence of expression of additional G-proteins [34], suggesting endocannabinoid interaction with *Slo1* channel protein.

Conclusively, the present study provides electrophysiological evidence for a lack of functional GPCR requirement for endothelial cell BK_{Ca} stimulation and inhibition by putative eCBR agonists and antagonists, respectively, challenging the existing hypothesis on the presence of a third type G-protein coupled cannabinoid receptor in endothelial cells, stimulation of which produces endothelium-dependent relaxation sensitive to BK_{Ca} inhibitors. The results presented herein identify endothelial BK_{Ca} channels as cellular sensors for abn-cbd and NAGly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

Abn-cbd	abnormal cannabidiol
BK_{Ca}	high-conductance Ca ²⁺ -activated K ⁺ channel
CB₁R	cannabinoid receptor type 1
eCB receptor	receptor endothelial atypical cannabinoid receptor
GPCR	G-protein coupled receptor
GPR18	GPCR18
NAGly	<i>N</i> -arachidonoyl glycine
NCX	plasma membrane Na ⁺ -Ca ²⁺ exchanger

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Fig. 1. NAGly and abn-cbd directly activate native BK_{ca} channels in a concentration-dependent manner.

(A) Representative recording in inside-out configuration showing concentration-dependent facilitation of BK_{ca} activity by 0.3–30 μM NAGly. The patch was kept at 40 mV and exposed to 300 nM free Ca^{2+} . Channel openings are shown as upward deflections. Single channel activity was recorded before (control) and after addition 0.3, 1, 3, 10 and 30 μM NAGly to the bath solution. (B) Summary data showing the effects of 0.1–30 μM NAGly on BK_{ca} activity. Recordings from inside-out configuration at holding voltage 40 mV and in the presence of 300 nM free Ca^{2+} . Numbers in brackets indicate the number of respective

experiments. (C) Single BK_{Ca} channel recording showing that the effect of 10 μM NAGly is sustained and only partially reversible after 5 min wash out ($n = 6$). (D) Representative single channel recording showing facilitation of BK_{Ca} activity by 1 μM abn-cbd. Abn-cbd was administered to the inside-out patch exposed to 300 nM free Ca^{2+} at +40 mV ($n = 5$).

Fig. 2. The effect of NAGly on BK_{ca} is Ca²⁺-dependent and stronger in outside-out configuration.

(A) Representative single BK_{ca} recording in outside-out configuration showing the impact of incremental concentrations of free Ca²⁺ in the bath on the channel activity before (left) and after (right) addition of 3 μ M NAGly. Holding voltage is +40 mV. (B) Statistical representation showing the effect of 3 μ M NAGly on NPo increase measured in inside-out configuration in the presence of 0.1–10 μ M free Ca²⁺ in the bath. Numbers on columns indicate the number of patches tested in respective experiments. (C) Representative recording of BK_{ca} activity in outside-out configuration before (control) and after administration of 3 μ M NAGly. Patch pipette solution contained 0.3 μ M free Ca²⁺. Holding voltage is +40 mV. (D) Statistical representation of the effect of 3 μ M NAGly on potentiation of the BK_{ca} single channel activity (NPo) under different recording modes at V_m = +40 mV and in the presence of 0.3 μ M free Ca²⁺ at the inner surface of the membrane. O/O-outside-out configuration (n = 8), I/O-inside-out configuration (n = 11).

Fig. 3. Rimonabant suppresses endothelial hyperpolarization to NAGly and reversibly inhibits BK_{Ca} channels independently of GPCR.

(A) Single BK_{Ca} channel recordings in inside-out patches excised from EA.hy926 cells exposed to a solution containing 1 μ M free Ca^{2+} before (Control) and following exposure to 5 μ M rimonabant to the inner surface of the membrane. $V_m = 40$ mV. (B) Statistical representation of mean NPo values of BK_{Ca} activity under control conditions (1 μ M free Ca^{2+} at $V_m = 40$ mV) and in the presence of 5 μ M rimonabant ($n = 7$). (C) Representative single BK_{Ca} channel recordings under control conditions (Control), in the presence of 3 μ M NAGly (3 μ M NAGly), 3 μ M rimonabant in the continued presence of 3 μ M NAGly (3 μ M

rimonabant + 3 μM NAGly) and following wash-out rimonabant in the presence of NAGly. Inside-out configuration, 0.3 μM free Ca^{2+} , $V_m = 60$ mV. (D) Statistical representation of mean NPo values under control conditions (0.3 μM free Ca^{2+} , $V_m = 60$ mV), in the presence of 3 μM NAGly and combined presence of 3 μM NAGly and 3 μM rimonabant. (E) Single BK_{ca} channel recordings in inside-out configuration in the presence 0.3 μM free Ca^{2+} under control conditions (Control), following administration of 3 μM abn-cbd alone (3 μM abn-cbd) and in combination with either 3 μM rimonabant (3 μM rimonabant+ 3 μM abn-cbd) or 10 μM rimonabant (10 μM rimonabant+ 3 μM abn-cbd). Patch was exposed to 0.3 μM free Ca^{2+} and maintained at $V_m = 80$ mV. (F) Effect of abn-cbd (10 μM) on the membrane potential of EA.hy926 cells. (G) Rimonabant (3 μM) reverses the abn-cbd-induced hyperpolarization. (H) Effect of 5 μM rimonabant on the time course of whole-cell currents elicited by voltage ramps. Peak amplitudes were taken at voltages indicated. (I) Corresponding whole-cell current traces elicited by voltage ramps under control conditions (control) and in the presence of 5 μM rimonabant. Pipette solution contained 1 μM free Ca^{2+} to increase basal current.

Fig. 4. AM251 inhibits single BK_{Ca} channel activity in cell-free patches.

(A) Single BK_{Ca} channel recording in inside-out configuration under control conditions (Control) and following administration 5 μ M AM251 to the inner surface of the patch excised from EA.hy926 cells. $V_m = -60$ mV and 3 μ M free Ca²⁺ in the bath to increase the channel activity at negative voltages. Channel openings are shown as downward deflections. (B) Single channel recordings in inside-out configuration showing the inhibitory effect of 10 μ M AM251 on BK_{Ca} activity pre-stimulated with NAGly (3 μ M). Patch was exposed to 0.3 μ M free Ca²⁺ at $V_m = 40$ mV. (C) Single channel BK_{Ca} activity recorded in outside-out configuration before (control) and after administration of 5 μ M AM251 to the outer face of the membrane. The patch pipette contained 1 μ M free Ca²⁺ and V_m was set at 40 mV under

symmetrical KCl conditions. (D) Statistical representation of the inhibitory effect of 5 μ M AM251 in outside-out ($n = 5$) and inside-out configuration ($n = 5$).

Fig. 5. Cholesterol scavenging with methyl- β -cyclodextrin (MCD) abolishes the NAGly-evoked BK_{Ca} potentiation and relaxation of precontracted rat aorta.

(A) Recoding of single BK_{Ca} channel activity in inside out patches excised from EA.hy926 cells pre-treated with 1 mM MCD for 1 h. (B) Typical traces showing the relaxation of rat aortic rings to NAGly (3 μ M) under control conditions (*upper panel*) and after pre-treatment of aortic rings with 1 mM MCD for 1 h.

Fig. 6. NAGly and abn-cbd induce BK_{ca}-dependent endothelial cell hyperpolarization in mouse aorta independently of gap junctions and GPR18.

(A) A lack of effect of phenylephrine (1 μ M) on the membrane potential of endothelial cells in isolated mouse aorta. Note that subsequent administration of Ach produces a hyperpolarization. (B) In the endothelium of isolated rat aorta, phenylephrine produces depolarization with membrane potential oscillations. (C) Effect of 10 μ M NAGly (n = 5) on endothelial membrane potential in isolated mouse aorta. (D) Effect of 30 μ M NAGly on endothelial membrane potential in isolated mouse aorta (n = 4). (E) Membrane potential recording showing the effect of paxilline (1 μ M) on the hyperpolarization induced by NAGly

(n = 3). (F) Membrane potential recording showing a failure of TRAM-34 to suppress the hyperpolarization to 10 μ M abn-cbd in excised mouse aorta. (G) Intracellular perfusion with GPR18 antibody (1:500) fails to prevent endothelial cell hyperpolarization to 10 μ M NAGly in isolated mouse aorta (n = 4). Recordings were commenced in the presence of 100 μ M glycyrrhetic acid, a gap junction inhibitor. (H) Bars showing the magnitude of shifts in endothelial membrane potential in isolated mice aorta induced by NAGly (10 μ M and 30 μ M) alone and combination of 10 μ M NAGly and paxilline (1 μ M) and GPR18 antibody. (I) Effect of NS1619 on the membrane potential of endothelial cells in excised mouse aorta. (J) Bars showing mean values of endothelial cell hyperpolarization to 10 μ M (n = 6) and 30 μ M NS1619 (n = 5).

Fig. 7. Abn-cbd elicits single channel activity of high conductance in patches excised from mice aortic endothelium.

(A) Exemplary traces showing single channel activity recording from a patch excised from the endothelium of mouse aorta before (Control) and after exposure to 3 μ M abn-cbd at voltages indicated. Dashed line shows zero current level. (B) Corresponding single channel current amplitudes as a function of voltage. (C) Plots of NP_o values in the presence of 3 μ M abn-cbd as a function of voltage.

Fig. 8. Single channel activity of high conductance elicited by NAGly in patches excised from mice aortic endothelium.

(A) Exemplary traces showing single channel activity recording from a patch excised from the endothelium of mouse aorta before (Control) and after exposure to 3 μ M NAGly.

Outside-out configuration. Bath and pipette solution contained 3 μ M free Ca^{2+} and 140 mM KCl. $V_m = +40$ mV. (B) Current traces elicited by voltage ramps from -100 to 100 mV in

outside-out patch excised from mouse aortic endothelium and exposed to 3 μ M NAGly. (C) Single channel activity from a patch excised from the endothelium of a NAGly-pre-treated

strip at physiological voltages (-40 mV and -60 mV) and in the presence of 3 μ M free

strip at physiological voltages (-40 mV and -60 mV) and in the presence of 3 μ M free

strip at physiological voltages (-40 mV and -60 mV) and in the presence of 3 μ M free

Ca^{2+} . Channel openings are shown as downward deflections. (D) Single channel activity from inside-out patch excised from the endothelium of NAGly (3 μM)-pretreated aortic strip before (control) and after exposure to paxilline (2 μM , $n = 3$). $V_m = +60$ mV, 3 μM free Ca^{2+} .