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Direct activation of Ca²⁺ and voltage-gated potassium channels of large conductance by anandamide in endothelial cells does not support the presence of endothelial atypical cannabinoid receptor

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Abstract

Endocannabinoid anandamide induces endothelium-dependent relaxation commonly attributed to stimulation of the G-protein coupled endothelial anandamide receptor. The study addressed the receptor-independent effect of anandamide on large conductance Ca²⁺-dependent K⁺ channels expressed in endothelial cell line EA.hy926. Under resting conditions, 10 μM anandamide did not significantly influence the resting membrane potential. In a Ca²⁺-free solution the cells were depolarized by ~10 mV. Further administration of 10 μM anandamide hyperpolarized the cells by ~8 mV. In voltage-clamp mode, anandamide elicited the outwardly rectifying whole-cell current sensitive to paxilline but insensitive to GDPβS, a G-protein inhibitor. Administration of 70 μM Mn²⁺, an agent used to promote integrin clustering, reversibly stimulated whole-cell current, but failed to further facilitate the anandamide-stimulated current. In an inside-out configuration, anandamide (0.1–30 μM) facilitated single BK_{Ca} channel activity in a concentration-dependent manner within a physiological Ca²⁺ range and a wide range of voltages, mainly by reducing mean closed time. The effect is essentially eliminated following chelation of Ca²⁺ from the cytosolic face and pre-exposure to cholesterol-reducing agent methyl-β-cyclodextrin. O-1918 (3 μM), a cannabidiol analog used as a selective antagonist of endothelial anandamide receptor, reduced BK_{Ca} channel activity in inside-out patches. These results do not support the existence of endothelial cannabinoid receptor and indicate that anandamide acts as a direct BK_{Ca} opener. The action does not require cell integrity or integrins and is caused by direct modification of BK_{Ca} channel activity.

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Keywords

Anandamide; Endothelial cannabinoid receptor; Integrins; BK_{Ca} channels

1 Introduction

Endocannabinoid anandamide (N-arachidonylethanolamine, AEA), an endogenous ligand for specific G-protein-coupled cannabinoid receptors type 1 (CB₁) and type 2 (CB₂), exerts a broad spectrum of biological actions, including psychoactive and antinociceptive effects, immunoregulation and neuroprotection (Maccarrone et al., 2011; Pandey et al., 2009; Pertwee, 2005). In the cardiovascular system, anandamide-mediated signaling has both physiological and pathophysiological relevance (Hoyer et al., 2011; Montecucco et al., 2009). Vasomotor responses to cannabinoids are thought to require a yet unidentified G_{i/o}-protein coupled receptor (GPCR) located on endothelial cells, activation of which results in BK_{Ca}- and nitric oxide (NO)-dependent vasodilation (Baranowska-Kuczeko et al., 2012; MacIntyre et al., 2014; Offertaler et al., 2003; Parmar and Ho, 2010; Wagner et al., 1999), so called endothelial anandamide receptor. The mechanisms of action of anandamide on endothelial cells are still controversial. In the EA.hy926 human endothelial-derived cell line, which was reported to lack CB₁ receptor (Liu et al., 2000), anandamide evokes Ca²⁺ mobilization independently of CB₁ receptor (Mombouli et al., 1999). A more recent study on the same cell line showed that although anandamide binds CB₁ receptor, it actually fails to evoke Ca²⁺ elevation, which was unmasked following external Ca²⁺ chelation (Waldeck-Weiermair et al., 2008). The latter phenomenon was attributed to anandamide binding to GPR55 receptor, which becomes available following integrin clustering in the absence of external Ca²⁺.

In addition to binding to CB₁/CB₂/GPR55 receptors, anandamide targets multiple ion transporting systems independently of GPCRs. The anandamide actions include activation of TRPV1 (Ross, 2003), inhibition of the Na⁺-Ca²⁺ exchanger (Al Kury et al., 2014b; Bondarenko et al., 2013), voltage-dependent Na⁺, Ca²⁺ channels (Al Kury et al., 2014a), and K⁺ channels (Amoros et al., 2010; Barana et al., 2010; Bol et al., 2012). In HEK293 cells expressing BK_{Ca} channels, anandamide were shown to activate whole-cell and single channel BK_{Ca} currents in a cell-attached configuration (Sade et al., 2006). However, the effect was lost following patch excision, suggesting engagement of some unidentified cytosolic factor (Sade et al., 2006). In endothelial cells, the GPCR-independent targets for anandamide and cannabinoids are much less explored and, hence, require special attention, especially in view of recent evidences on the involvement of membrane lipids in regulating the function of ion channels and GPCRs, including cannabinoid receptors (Bukiya et al., 2011; Gasperi et al., 2013).

In the present study we addressed the mechanism of action of anandamide and analyzed the impact of anandamide on the membrane potential and BK_{Ca} activity in EA.hy926 cells. We show that, while under resting conditions anandamide fails to significantly alter the membrane potential, the hyperpolarizing effect becomes evident at membrane depolarizing conditions. In cell-free patches, anandamide concentration-dependent increases the activity

of BK_{Ca} channels mainly by reducing mean closed time. The effect requires permissive Ca²⁺ at cytosolic face of the membrane and is eliminated following pre-incubation with the cholesterol-depleting agent methyl- β -cyclodextrin. Our identification of anandamide as a direct BK_{Ca} opener challenges the concept of the existence of endothelial anandamide receptor involved in endothelium-dependent relaxation.

2 Materials and methods

2.1 Cell culture

Endothelial cells from the human umbilical vein endothelial cell-derived cell line EA.hy926 (Edgell et al., 1983) at passages 45–85 were used in this study. Cells were plated on glass coverslips in DMEM supplemented with 10% fetal calf serum, 1% HAT (5 mM hypoxanthin, 20 μ M aminopterin, and 0.8 mM thymidine), 50 units/ml of penicillin, 50 μ g/ml of streptomycin and maintained in a humidified atmosphere (5% CO₂ at 37 °C). Before electrophysiological experiments, the glass on which the cells were plated was transferred to an experimental recording chamber.

2.2 Electrophysiology

Single-channel recordings were obtained from excised inside-out membrane patches in symmetrical solutions using the patch-clamp technique. For the inside-out configuration, pipettes were filled with (in mM) 140 KCl, 10 HEPES, 1 MgCl₂, 5 EGTA, 4.931 CaCl₂ with pH 7.2 by adding KOH (10 μ M free Ca²⁺ according to a program Cabuf, (G. Droogmans, KU Leuven, Belgium)). Cells were initially superfused with a bath solution containing (in mM) 140 NaCl, 5 KCl, 1.2 MgCl₂, 10 HEPES, 10 glucose, 2.4 CaCl₂. Following gigaseal formation, the bath solution was switched to the following (in mM): 135 KCl, 10 HEPES, 1 MgCl₂, 5 EGTA and a desired free Ca²⁺ concentration which was adjusted by adding different amounts of CaCl₂, as calculated by the program CaBuf. pH was adjusted to 7.2 by adding KOH. Single-channel activity was obtained from > 30 s of continuous recording under each experimental condition. For current- and voltage-clamp whole-cell recordings, the pipette solution contained (in mM) 140 KCl, 1 MgCl₂, 10 HEPES, 3 EGTA and a free Ca²⁺ concentration was adjusted to 300 nM by adding the required amount of CaCl₂, as calculated by the program CaBuf. Data were acquired by EPC7 and Model 2400 (A-M systems) patch-clamp amplifiers using Clampex 8.2 (Molecular Devices, USA) and WinWCP (John Dempster, Strathclyde University, UK) acquisition softwares, respectively.

2.3 Modification of cholesterol level

For cholesterol depletion, EA.hy926 cells were pre-incubated with 0.5% methyl- β -cyclodextrin for 1 h at 37 °C before use in electrophysiological experiments.

2.4 Statistical analysis

Experimental data are expressed as mean $\pm$ S.E.M and n is the number of cells tested. Student's *t*-test (paired or unpaired where appropriate) was used to compare results, with *P* < 0.05 taken as the level of significance. The single-channel recording data were analyzed with Clampfit 9.2 software.

3 Results

3.1 Endothelial cell depolarization uncovers the anandamide-evoked hyperpolarization independently of GPCRs

In a standard bath solution, the resting membrane potential of endothelial EA.hy926 cells amounted -33.1 ± 2.0 (n=21). Bath perfusion with 10 μ M anandamide did not significantly influence the resting membrane potential (control: -32.3 ± 0.8 mV, anandamide: -33.6 ± 0.8 mV, n=11) producing either no changes in the membrane potential (n=5) or a weak hyperpolarization 2.7 ± 0.9 mV (n=6, Fig. 1A). Increasing anandamide concentration to 20 μ M produced a sustained hyperpolarization of higher amplitude (7.1 ± 1.6 mV, n=4). The hyperpolarization was reversed by administration of tetraethyl ammonium (TEA, 2 mM) (Fig. 1B), which at these concentrations is as effective as 100 nM iberiotoxin in inhibition of BK_{Ca} channels (Dimitropoulou et al., 2002). Notably, even at 10 mM TEA has a minor influence (26% inhibition) on IK_{Ca}-mediated current in endothelial cells (Ahn et al., 2004).

A lack of hyperpolarization to 10 μ M anandamide may be determined by the status of integrins, which, when unclustered, are thought to physically prevent anandamide binding to GPR55, resulting in a reduced intracellular Ca²⁺ rise (Waldeck-Weiermair et al., 2008). For this reason, we tested the effect of anandamide in a Ca²⁺-free solution, a condition under which integrins are clustered and anandamide is able to elicit more pronounced intracellular Ca²⁺ rise presumably because of binding to GPR55 (Waldeck-Weiermair et al., 2008). Superfusion of the cells with the Ca²⁺-free solution (1 mM EGTA) produced a gradual depolarization by 10.3 ± 1.8 mV (n=7), an observation similar to that reported earlier (Bondarenko, 2004; Bondarenko et al., 2010), and allowed the appearance of enhanced hyperpolarization with the amplitude of 7.7 ± 2.0 mV, n=9, (Fig. 1C) in response to 10 μ M anandamide.

To understand whether the absence of Ca²⁺ itself or rather the resulting membrane depolarization triggers the response to anandamide, we assessed whether membrane depolarizing agents trigger the hyperpolarization to anandamide in the presence of bath Ca²⁺. Hence, in current-clamp recordings, we manipulated the endothelial membrane potential by two alternative mechanisms, namely by cell superfusion with Ni²⁺-containing solution and by blocking Na⁺-K⁺ ATPase by superfusion with a K⁺-free solution. Both maneuvers depolarize endothelial cells to nearly the same extent (10–12 mV) as superfusion with Ca²⁺-free solution (Bondarenko, 2004; Bondarenko and Sagach, 2006; Marchenko and Sage, 1993). Again, further administration of 10 μ M anandamide produced a significantly increased hyperpolarization ($P < 0.05$) (Fig. 1 D-F). These results demonstrate that anandamide (10 μ M) is most active at membrane-depolarizing conditions and the presence of external Ca²⁺ is not a limiting factor in the development of the hyperpolarization to anandamide.

We have shown recently that the anandamide derivative N-arachidonoyl glycine (NAGly) stimulates voltage and Ca²⁺-dependent BK_{Ca} channels independently of GPCRs (Bondarenko et al., 2013). If the same mechanism is shared by anandamide, then membrane depolarization observed in Ca²⁺-free, K⁺-free- and Ni²⁺-containing solutions can simply enhance activation of Ca²⁺ and voltage-dependent BK_{Ca} channels producing the

hyperpolarizing effect. To test this, we next explored the effect of anandamide on whole-cell currents elicited by voltage ramps. Applying voltage ramps elicited the outwardly rectifying current. Administration of anandamide during voltage ramps in a Ca^{2+} -containing solution gradually potentiated an outwardly rectifying current that was inhibited by paxilline, a selective BK_{Ca} channel blocker ($n=9$, Fig. 2A, B). This observation indicates that the current stimulated by anandamide under these conditions is elicited predominantly by BK_{Ca} channel activation. Incorporation into the pipette solution of $\text{GDP}\beta\text{S}$ (0.5 mM), which impairs G-protein functioning, does not prevent the current potentiation ($n=4$, not shown). These results indicate that anandamide facilitates BK_{Ca} channels independently of classical CB receptors and other GPCRs.

To further address the role of CB receptors and integrins in BK_{Ca} current development initiated by anandamide, Mn^{2+} (70 μM), an agent which has been used at this concentration to promote integrin clustering (Waldeck-Weiermair et al., 2008), was administered in the continued presence of anandamide once the current reached a plateau (Fig. 2C, D). This maneuver negligibly affected the current amplitude ($n=5$). In contrast, in the absence of anandamide exposure, the administration of 70 μM Mn^{2+} during voltage ramps reversibly facilitated the development of an outwardly rectifying current (Fig. 2E, F, $n=4$). This observation is consistent with stimulation of BK_{Ca} channels by Mn^{2+} .

3.2 Anandamide activates BK_{Ca} channels in cell-free patches within physiological ranges of Ca^{2+} and membrane voltage

To further investigate the mechanisms of the anandamide action, the BK_{Ca} single channel recordings were conducted in an inside-out configuration in the presence of physiological levels of Ca^{2+} and Mg^{2+} . Fig. 3A illustrates BK_{Ca} single channel activities recorded in excised inside-out patches maintained at 60 mV and exposed to 1 μM free Ca^{2+} before and after the addition of 0.3–30 μM anandamide. Anandamide reversibly increased NPo in a concentration-dependent manner: $\text{EC}_{50}=1.1$ μM , $\text{E}_{\text{max}}=10$ μM (Fig. 3B). At 10 μM anandamide, NPo reached 400% of control. Hence, these results indicate that the action of anandamide does not require cannabinoid receptors, integrins and cell integrity and is caused by direct modulation of BK_{Ca} channel function.

Because the activity of BK_{Ca} channels is controlled both by Ca^{2+} and voltage, we next explored whether the anandamide-induced increase in NPo is dependent on the cytosolic Ca^{2+} level. The single-channel activity was recorded at a holding voltage 60 mV, and membrane patches were exposed to solutions containing 0.1, 0.3, 1, 3 and 10 μM Ca^{2+} . Anandamide at a fixed concentration 3 μM potentiated single BK_{Ca} channel activity when inside-out patches were exposed to 0.1–10 μM free Ca^{2+} (Fig. 4A–B). Anandamide appeared to be most effective in the presence of 0.3 μM Ca^{2+} at cytosolic face of the membrane (Fig. 4C). Under these conditions, the maximum NPo increase upon administration of 3 μM anandamide reached 3.59 ± 0.57 times ($n=21$) from the basal NPo level 0.107 ± 0.02 ($n=23$). In the presence of 10 μM Ca^{2+} , anandamide was least effective and an increase in NPo was twice less (1.56 ± 0.12 times, $n=11$, from the basal NPo level 0.70 ± 0.12) compared to that observed in the presence of 0.3 μM free Ca^{2+} . Remarkably, in patches with only one active channel, even at the very high basal Po level attained in the presence of 10 μM free Ca^{2+} and

positive voltages, administration of anandamide further increased BK_{Ca} channel activity (Fig. 4B). Notably, when excised inside-out patches were exposed to 5 mM EGTA with no added Ca²⁺, further administration of anandamide to cytosolic face of the membrane failed to affect NPo (n=5, Fig. 4D), suggesting that permissive cytosolic Ca²⁺ is required for anandamide to be effective.

An increase in NPo may result from an increase in the number of active channels pro patch (N), an increase in Po, or both parameters simultaneously. In patches with only one active channel, we could derive useful information about modulation of the channel's gating properties by anandamide. Dwell-time analysis of the BK_{Ca} activity of the patch containing only one active channel (Fig. 5A) showed that anandamide moderately increases (by 16%) the average duration the channel dwells in the open state. At the same time, the action of anandamide is accompanied by a drastic reduction (by 64%) of the average duration the channel dwells in the closed state (Fig. 5B). Thus, anandamide-evoked increase in channel Po stems primarily from destabilization of the channel's closed states. Analysis of single channel BK_{Ca} activity in patches exposed to 10 μM Ca²⁺ and held at positive voltages (to maximally increase NPo) showed that anandamide is able to increase the number active channel pro patch (N) by recruiting silent channels (Fig. 5C). On the other hand, the single channel amplitude was unaffected by anandamide, as evident from the amplitude histogram (Fig. 5D) constructed from original recordings of single channel activity presented in Fig. 5C. Thus, a modification of BK_{Ca} channel function by anandamide is not limited by alterations in channel gating, and includes the recruitment of silent channels. However, single channel conductance is not altered by anandamide.

To gain additional insight into the mechanisms of BK_{Ca} channel activation by anandamide, we addressed how its action is related to applied voltage. Accordingly, we next studied the impact of anandamide on single BK_{Ca} activity under fixed Ca²⁺ and different voltages. Consistent with voltage-dependent BK_{Ca} activation, an increase in holding voltage resulted in NPo increase (Fig. 6A). In the presence of 0.3 μM free Ca²⁺ in the bath, anandamide administered to the inner surface of the patch stimulated the BK_{Ca} single channel activity within a voltage ranging between 20 and 80 mV. The degree of stimulation was poorly dependent on applied voltage (Fig. 6A,B). A stimulatory effect was observed also at -40 mV (n=3, Fig. 6C), that is close to membrane potential of unstimulated endothelial cells. These data suggest that anandamide effectively facilitates the activity of the BK_{Ca} channel without affecting the voltage sensor.

3.3 O-1918 inhibits BK_{Ca} activity in cell-free membrane patches

Being inactive against CB₁ and CB₂ receptors, the cannabidiol analog O-1918 inhibits the vasodilation to anandamide and has been introduced as a selective antagonist of endothelial anandamide receptor (Offertaler et al., 2003). The inhibition of cannabinoid-induced vasodilation by O-1918 (Al Suleimani et al., 2015; Baranowska-Kuczko et al., 2012; Caldwell et al., 2013; Ho and Yeung, 2009; Jarai et al., 1999; Parmar and Ho, 2010; Su et al., 2015; Wagner et al., 1999; Zakrzeska et al., 2010) has let to a wide support of the existence of this receptor and its involvement in regulation of relaxation and blood pressure. To investigate the receptor-independent effect of O-1918 on BK_{Ca} function, we probed

O-1918 on BK_{Ca} single channel activity in inside-out configuration. Following exposure to O-1918 (3 μ M), the BK_{Ca} single channel activity was strongly declined (n=4, Fig. 7A,B), indicating for a direct receptor-independent targeting of BK_{Ca} function.

3.4 Membrane cholesterol determines the stimulatory effect of anandamide on BK_{Ca} activity

Cholesterol is one of the major lipid components of the plasma membrane of mammalian cells. Cholesterol-rich microdomains of the plasma membrane are located in caveolae, which apart from endothelial nitric oxide synthase (NOS) and prostacyclin synthase, host CB₁, but not CB₂ receptors (Maccarrone, 2010). Acute pharmacological disruption of caveolae by cholesterol-binding agents was shown to impair NO synthesis (Xu et al., 2008) and CB₁, but not CB₂, signaling (Bari et al., 2006; Maccarrone, 2010). Taking into account differential sensitivities of CB₁ and CB₂ receptor signaling to cholesterol depletion, we next evaluated whether intact caveolae and membrane cholesterol is implicated in anandamide sensing by BK_{Ca} channels. To this end, we examined the impact of pre-incubation of EA.hy926 cells with the cholesterol-reducing agent methyl- β -cyclodextrin, that is widely used to disrupt the integrity of lipid rafts (Mahammad and Parmryd, 2015) on the anandamide-evoked BK_{Ca} potentiation. Pre-treatment of the cells with 0.5% methyl- β -cyclodextrin resulted in a reduced whole-cell current in response to voltage ramps and eliminated the anandamide-evoked BK_{Ca}-attributable current (Fig. 8A, n=4). In addition, pre-treatment with methyl- β -cyclodextrin resulted in a significantly ($P < 0.05$) reduced basal NPo of BK_{Ca} channels measured in inside-out configuration at $V_m=60$ mV in the presence of 1 μ M free Ca²⁺ (methyl- β -cyclodextrin-treated: NPo=0.04 $\pm$ 0.01, n=5; control: NPo=0.39 $\pm$ 0.05, n=25) and prevented the anandamide-induced facilitation of single BK_{Ca} activity (n=5, Fig. 8B,C) even when patches were held at high voltage (+60 mV). These observations indicate that caveolae-residing cholesterol is required for anandamide sensing of BK_{Ca}.

4 Discussion

We show here that anandamide directly stimulates BK_{Ca} channels natively expressed in the endothelial cell line and this effect occurs independently of CB receptors, G-proteins and cell integrity. The effect is not limited by the presence of bath Ca²⁺ and integrins, but requires permissive cytosolic Ca²⁺. The action is accompanied by an increase in single channel activity (open probability) without affecting unitary conductance. The effect was observed within a wide range of voltages and physiological Ca²⁺ concentrations facing the inner surface of the membrane, being most pronounced at 300 nM Ca²⁺, a concentration close to that reached in endothelial cells during mild stimulation with acetylcholine. While marginally affecting the membrane potential under resting conditions, anandamide potentiated paxilline-sensitive outwardly rectifying current during direct cell depolarization by voltage ramps, indicating that BK_{Ca} channel is the main contributor to the development of whole-cell current during cell stimulation with anandamide. The development of a paxilline-sensitive current was not prevented by intracellular perfusion with GDP β S, a G protein inhibitor. Recordings of unitary BK_{Ca} activity in an inside-out configuration further indicates that the BK_{Ca} stimulation by anandamide does not require CB receptors, G-proteins and results from direct modulation by anandamide of BK_{Ca} function.

Endothelial membrane potential has long been recognized as a modulator of the free intracellular Ca^{2+} concentration in endothelial cells and endothelial cell function (Kamouchi et al., 1999). The mean resting membrane potential in the present study was ~ -33 mV. This value is close to those reported earlier for the membrane potential of endothelial cells of the same cell line (Bondarenko et al., 2011a, 2011b; Kamouchi et al., 1999; Sheng et al., 2009) and human umbilical vein endothelial cells (Adams et al., 1989), but rather depolarized compared to the membrane potential values of smooth muscle cells. Values reported for membrane potential of in situ endothelial cells range from -58 mV (Marchenko and Sage, 1993) to -15 to -18 mV (Kohler et al., 2001; Yamamoto and Suzuki, 2010). It is difficult to explain this variability, but it can be due to either specific experimental conditions or interspecies variations.

Despite negligibly affecting the resting membrane potential, we have shown that anandamide elicits hyperpolarizing responses when cells were preliminary depolarized either by the removal of bath Ca^{2+} or K^{+} or the addition of Ni^{2+} into the bath solution, indicating that anandamide is most active at membrane depolarizing conditions. This observation is consistent with potentiation of voltage- and Ca^{2+} -dependent BK_{Ca} channel. While depolarizing effects of Ni^{2+} and Ca^{2+} -free solution on endothelial cells have been documented earlier (Bondarenko, 2004; Bondarenko et al., 2010, 2017; Marchenko and Sage, 1993; Ohashi et al., 1999), the underlying mechanism is not entirely clear, and likely involves inhibition of the basal Na^{+} - Ca^{2+} exchanger-mediated current. In support of this mechanism, chelation of intracellular Ca^{2+} with BAPTA-AM, a procedure that facilitates the reversal of Na^{+} - Ca^{2+} exchanger, was shown to enhance the depolarization that is mimicked by inhibitors of Na^{+} - Ca^{2+} exchanger (Bondarenko et al., 2017). In addition, contribution of Ni^{2+} -sensitive Cav3.1 channel, which, at least in some vascular beds was recently shown to be involved in NO-dependent vasodilation (Svenningsen et al., 2014) can not be entirely excluded.

According to the recently proposed mechanism (Waldeck-Weiermair et al., 2008), a negligible rise in intracellular Ca^{2+} concentration in response to anandamide in a Ca^{2+} -containing solution is due to anandamide binding to CB_1 receptor, while a more pronounced Ca^{2+} elevation observed in the absence of external Ca^{2+} is attributed to anandamide binding to GPR55, which becomes available following integrin clustering either in Mn^{2+} -containing or Ca^{2+} -free solution. Our present data does not support such a mechanism and demonstrate that the presence of Ca^{2+} in the bath is not a limiting factor in the development of the BK_{Ca} -dependent response to anandamide. In fact, our single channel recordings in an inside-out configuration show that permissive cytosolic Ca^{2+} is required for potentiation of BK_{Ca} activity by anandamide, and the effect is lost once “internal” Ca^{2+} is chelated with 5 mM EGTA. In addition, Mn^{2+} at concentrations used to promote integrin clustering and, hence, anandamide binding to GPR55 (Waldeck-Weiermair et al., 2008), fails to modify whole-cell BK_{Ca} current stimulated by anandamide, but promotes the development of BK_{Ca} current in the absence of anandamide exposure. The most straightforward interpretation of these data is that integrins are not involved in the modification of the anandamide-evoked BK_{Ca} current, but rather Mn^{2+} is capable to mildly stimulate BK_{Ca} channels. A latter conclusion is consistent with earlier findings showing modulation of BK_{Ca} channels through Mn^{2+} binding to one of the three Ca^{2+} binding sites of the BK_{Ca} channel (Zeng et al., 2005).

Noteworthy, several TRP channels including TRPC3-7, TRPM2,7,8 and TRPV4-6 permeate Mn^{2+} (Bouron et al., 2015) allowing access of Mn^{2+} to intracellular Ca^{2+} binding sites of the BK_{Ca} channel.

While not supporting the involvement of endothelial anandamide receptor in the action of anandamide on a human endothelial cell line, our data implicate the membrane cholesterol content as the main determinant of the anandamide effect on BK_{Ca} channel activity. Cholesterol-dependent modulation of GPCRs and distinct ion transport systems has been documented previously (Bergdahl et al., 2003; Dainese et al., 2010; Singh et al., 2011). The mechanisms underlying cholesterol modulation of BK_{Ca} channels are not completely understood and were reported to include direct interaction of cholesterol with several BK_{Ca} channel pore forming protein recognition sites (Singh et al., 2011) and indirect interaction via alteration of the lipid bilayer surrounding the protein (Yuan et al., 2011). Hypercholesterolaemia, a condition defined by an elevated serum cholesterol level (> 240 mg/dL) is linked to a number cardiovascular disorders including atherosclerosis, hypertension or stroke (Kitayama et al., 2007; Montecucco et al., 2009). A high cholesterol diet is directly linked to hypercholesterolaemia and the extent of atherosclerosis (Montecucco et al., 2016). We show here that pre-incubation with cholesterol-depleting methyl- β -cyclodextrin eliminates the sensitivity of BK_{Ca} channel to anandamide, indicating that membrane cholesterol is a prerequisite for BK_{Ca} modulation by anandamide.

Our current identification of anandamide as a BK_{Ca} opener and O-1918 as the channel inhibitor provides further support of the recently raised criticism (Bondarenko, 2014) around the concept of existence of endothelial anandamide receptor, a non- CB_1 /non- CB_2 G-protein coupled CB receptor located on endothelial cells. The idea of the existence of a novel endothelial cannabinoid receptor is fueled mostly by vascular relaxation studies showing that endocannabinoid anandamide, its derivative NAGly and synthetic abnormal cannabidiol (abn-cbd), a behaviorally inactive synthetic cannabinoid, produce endothelium-dependent relaxation likely through binding to a third type cannabinoid receptor located on endothelial cells (Ho and Hiley, 2004; Jarai et al., 1999; Parmar and Ho, 2010; Penumarti and Abdel-Rahman, 2014; Zakrzaska et al., 2010), presumably GPR18 (MacIntyre et al., 2014; Penumarti and Abdel-Rahman, 2014) or, as suggested earlier, GPR55 (Ryberg et al., 2007; Waldeck-Weiermair et al., 2008). While limitations in our cell culture model do not permit to directly negate the existence of anandamide receptor expressed in intact endothelium, our current study does provide evidence of an alternative mechanism of endothelial anandamide receptor-attributable relaxation, thereby raising further concerns around the interpretation of data obtained in the relaxation studies.

Endothelial membrane potential has long been recognized as a modulator of Ca^{2+} entry and intracellular Ca^{2+} concentration, a dynamic equilibrium between Ca^{2+} entry, Ca^{2+} extrusion at the plasma membrane, Ca^{2+} release and sequestration by internal stores. Accumulated evidence indicates that BK_{Ca} openers affect many, if not all, of these processes. In EA.hy926 cells, the BK_{Ca} channel opener CGS7184 causes elevation of intracellular Ca^{2+} and stimulated NO production (Wrzosek et al., 2009). In addition, CGS7184 activates caffeine-sensitive Ca^{2+} permeable ryanodin receptor channels present in sarcoplasmic reticulum and the inner mitochondrial membrane (Wrzosek et al., 2012). As BK_{Ca} channels are present in

the inner mitochondria membrane of EA.hy926 cells (Bednarczyk et al., 2013), activation of mitochondrial BK_{Ca} channels and the associated mitochondria depolarization (Wrzosek et al., 2009) would inevitably result in a reduced transmembrane Ca²⁺ current (Bondarenko et al., 2014) and a decreased capacity of mitochondria to sequester Ca²⁺, leading to an increase in intracellular Ca²⁺ level and Ca²⁺-dependent processes. In precontracted vascular preparations, BK_{Ca} openers cause endothelium-dependent relaxation (Wrzosek et al., 2009). Considering these recent findings, our identification of anandamide as a direct BK_{Ca} opener introduces an alternative mechanism of endothelium-dependent relaxation to cannabinoids, thereby challenging the concept of existence of an endothelial anandamide receptor.

In conclusion, we provide new information regarding the mechanism of action of anandamide on endothelial cells. The mechanism does not engage CB₁, CB₂ or non-CB₁/CB₂ receptors, but involves direct modulation of the BK_{Ca} channel gating without modification of unitary conductance. The action depends on the membrane cholesterol level, intracellular Ca²⁺ concentration and the membrane potential. Our results imply that anandamide plays an important physiological and pathophysiological role in the regulation of vascular function via its direct modulatory effect on BK_{Ca} function.

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Fig. 1. Effect of anandamide on the membrane potential of endothelial cells.

(A) Original recording showing the effect of 10 μM anandamide (AEA) on the membrane potential in normal bath solution. (B) Original recording showing the effect of 20 μM anandamide (AEA) on the membrane potential in normal bath solution. (C) Original recording of the effect of 10 μM anandamide (AEA) on the membrane potential in Ca^{2+} -free bath solution. (D) Original recording of the effect of 10 μM anandamide (AEA) on the membrane potential in K^+ -free solution. (E) Effect of 10 μM anandamide (AEA) on the membrane potential in Ni^{2+} -containing solution. (F) Bars showing the mean values of hyperpolarization in response to bath administration of 10 μM anandamide under control conditions and in the absence of Ca^{2+} , K^+ or the presence of 2 mM Ni^{2+} in the bath solution. $n=4$ to 8 experiments.

Fig. 2. Anandamide stimulates whole-cell BK_{Ca} currents independently of GPCRs.

(A) Time course of the peak current amplitudes at -80 and $+80$ mV in response to $10\ \mu\text{M}$ anandamide either alone or in combination with $1\ \mu\text{M}$ paxilline. Currents were elicited by voltage ramps. (B) Corresponding current traces to voltage ramps before (control), during $10\ \mu\text{M}$ anandamide (AEA) and in the combined presence of anandamide and paxilline. (C) Time course of peak current amplitudes at voltages indicated showing a lack of stimulatory effect of $70\ \mu\text{M}$ Mn^{2+} on anandamide-potentiated paxilline-sensitive current. (D) Corresponding whole-cell current traces elicited by voltage ramps showing a lack of stimulatory effect of $70\ \mu\text{M}$ Mn^{2+} on anandamide ($10\ \mu\text{M}$)-prestimulated currents. (E) Time

course of the peak current amplitudes before and after addition of 70 μM Mn^{2+} to the bath solution. (F) Corresponding whole-cell current traces elicited by voltage ramps showing the effect of 70 μM Mn^{2+} in the absence of anandamide exposure.

Fig. 3. Anandamide stimulates BK_{Ca} activity in cell-free patches in a concentration-dependent manner.

(A) Representative recording in inside-out configuration showing the effect of 0.3, 1 and 10 μ M anandamide on activity of single BK_{Ca} channels. Patch was kept at 40 mV and exposed to 1 μ M free Ca^{2+} before (control) and after addition 0.3, 1 and 10 μ M anandamide (AEA).

(B) Graph showing concentration-dependent effect of anandamide (AEA) on single BK_{Ca} activity in inside-out configuration (n=7-12).

Fig. 4. The effect of anandamide (AEA) on single BK_{Ca} activity is Ca²⁺-dependent.

(A) Single channel recording of BK_{Ca} activity showing the effect of 3 μM anandamide in the presence of 0.1–3 μM free Ca²⁺. Holding voltage is 60 mV. (B) Single-channel recording in inside-out patch containing one active channel showing stimulatory effect of anandamide even at high basal Po level. Patch was exposed to 10 μM free Ca²⁺ and held at 60 mV. (C) Graph showing the effect of 3 μM anandamide on single BK_{Ca} activity at different Ca²⁺ concentrations. The number of patches studied is shown on respective bars. (D) Recording

of single BK_{Ca} activity in inside-out configuration showing a failure of anandamide to increase NPo when trace Ca^{2+} was chelated by 5 mM EGTA. $V_m=60$ mV.

Fig. 5. Anandamide increases BK_{Ca} unitary currents by increasing both P_o and N .

(A) BK_{Ca} current records from inside-out patch containing one active channel before (left) and after (right) administration of 3 μ M anandamide. The patch was exposed to 0.3 μ M free Ca^{2+} at $V_m=40$ mV. (B) The table shows the average duration of the channel open and closed times before (control) and after addition of 3 μ M anandamide. (C) BK_{Ca} current records from inside-out patch showing an increase in the number of active channels (N) following anandamide exposure. To increase the P_o level, the patch was exposed to 10 μ M free Ca^{2+} at $V_m=+60$ mV. Bars on the left of the traces indicate closed states. (D) The

amplitude histograms constructed from the BK_{Ca} activity before and after addition of 3 μM anandamide presented in Fig. 5C.

Fig. 6. Anandamide barely affects voltage-sensitivity of BK_{Ca} channel.

(A) Single BK_{Ca} channel activity before and after addition of 3 μM anandamide (AEA) at different voltages indicated. Inside-out patch was exposed to 0.3 μM free Ca^{2+} under symmetrical K^+ conditions. (B) Graphical representation showing plots of mean NPo values as a function of voltage in the absence and presence of 3 μM anandamide. Patches were exposed to 300 nM Ca^{2+} . (C) Anandamide (AEA) has sustained stimulatory effect on BK_{Ca} activity at physiological voltages. BK_{Ca} current records from inside-out patch held at -40 mV and exposed to 1 μM free Ca^{2+} .

Fig. 7. O-1918 inhibits BK_{Ca} activity independently of GPCRs.

(A) Single BK_{Ca} channel activity in inside-out configuration before and after addition of 3 μM O-1918. The patch was exposed to high K^+ solution containing 1 μM free Ca^{2+} and maintained at 40 mV. (B) Summary data of the inhibitory effect of 3 μM O-1918 on BK_{Ca} channel activity in patches exposed to 1 μM Ca^{2+} at $V_m = +40 \text{ mV}$.

Fig. 8. Membrane cholesterol determines the stimulatory effect of anandamide on BKCa activity.

(A) Anandamide (AEA, 3 μ M) fails to evoke whole-cell current following pretreatment of the cells with methyl- β -cyclodextrin (MCD). (B) Anandamide (3 μ M) fails to stimulate single channel activity following pretreatment of the cells with MCD. Inside-out configuration, $V_m=60$ mV, 1 μ M free Ca^{2+} . (C) Graphical representation showing normalized increase in NPo relative to the basal NPo level in response to 3 μ M anandamide in control cells and after pretreatment with MCD.