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RESEARCH PAPER

N-arachidonoyl glycine suppresses Na^+/Ca^{2+} exchanger-mediated Ca^{2+} entry into endothelial cells and activates BK_{Ca} channels independently of GPCRs

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BACKGROUND AND PURPOSE
N-arachidonoyl glycine (NAGly) is a lipoamino acid with vasorelaxant properties. We aimed to explore the mechanisms of NAGly's action on unstimulated and agonist-stimulated endothelial cells.

EXPERIMENTAL APPROACH
The effects of NAGly on endothelial electrical signalling were studied in combination with vascular reactivity.

KEY RESULTS
In EA.hy926 cells, the sustained hyperpolarization to histamine was inhibited by the non-selective Na^+/Ca^{2+} exchanger (NCX) inhibitor bepridil and by an inhibitor of reversed mode NCX, KB-R7943. In cells dialysed with Cs^+-based Na^+-containing solution, the outwardly rectifying current with typical characteristics of NCX was augmented following histamine exposure, further increased upon external Na^+ withdrawal and inhibited by bepridil. NAGly (0.3–30 μM) suppressed NCX currents in a URB597- and guanosine 5′-O-(2-thiodiphosphate) (GDPβS)-insensitive manner, $[Ca^{2+}]_i$ elevation evoked by Na^+ removal and the hyperpolarization to histamine. In rat aorta, NAGly opposed the endothelial hyperpolarization and relaxation response to ACh. In unstimulated EA.hy926 cells, NAGly potentiated the whole-cell current attributable to large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in a GDPβS-insensitive, paxilline-sensitive manner and produced a sustained hyperpolarization. In cell-free inside-out patches, NAGly stimulated single BK_{Ca} channel activity.

CONCLUSION AND IMPLICATIONS
Our data showed that NCX is a Ca^{2+} entry pathway in endothelial cells and that NAGly is a potent G-protein-independent modulator of endothelial electrical signalling and has a dual effect on endothelial electrical responses. In agonist pre-stimulated cells, NAGly opposes hyperpolarization and relaxation via inhibition of NCX-mediated Ca^{2+} entry, while in unstimulated cells, it promotes hyperpolarization via receptor-independent activation of BK_{Ca} channels.

Abbreviations
BK_{Ca}, high-conductance Ca^{2+}-activated K^+ channel; CB_1 receptor, cannabinoid receptor type 1; CB_2 receptor, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; GDPβS, guanosine 5′-O-(2-thiodiphosphate); GPR18, GPCR 18; GPR92, GPCR 92; NAGly, N-arachidonoyl glycyne; NCX_{pm}, plasma membrane Na^+-Ca^{2+} exchanger; NMDG, *N*-methyl-*D*-glucamine; O-1918, 1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene; RACE, receptor-activated Ca^{2+} entry; SOCE, store-operated Ca^{2+} entry; STIM1, stromal interacting molecule 1; TRPV1, transient receptor potential cation channel V1

Introduction

The plasma membrane Na^+/Ca^{2+} exchanger (NCX_{pm}) is a bidirectional electrogenic transporter that, depending on the transmembrane gradient of substrate ions and membrane potential, transports Ca^{2+} either out of cells or into cells in exchange for three ions of Na^+. In many cell types, this transporter is the principle mechanism of extrusion of Ca^{2+} (forward mode) under conditions of stimulated Ca^{2+} entry. In addition, Ca^{2+} entry via NCX_{pm} operating in reverse mode contributes to intracellular Ca^{2+} overload under pathological conditions such as myocardial ischaemia and reperfusion (Lee *et al.*, 2005). Experimentally, such conditions can be mimicked in other cell types by exposure to hypoxic, acidic, ion-shifted Ringer followed by return to normal solution. In primary astrocyte cultures, such manoeuvres result in widespread cell loss (Bondarenko and Chesler, 2001) due to massive Ca^{2+} influx at the onset of reperfusion (Bondarenko *et al.*, 2005).

NCX_{pm} is also present in endothelial cells, and reports regarding its functional role vary. In experiments using the excised rat aorta, it was found that the Na^+ influx into endothelial cells induced by stimulation with ACh was sufficient to trigger Ca^{2+} inflow via NCX_{pm} (Bondarenko, 2004). Further, the reversed mode of NCX_{pm} following ACh exposure was shown to sustain endothelium-dependent relaxation and NO release (Schneider *et al.*, 2002), indicating that in the vascular endothelium the NCX_{pm} may have an important physiological and pathophysiological role. Given the functional importance of Ca^{2+} entry into endothelial cells, exogenous compounds and endogenous signalling molecules that affect the activity of the NCX_{pm} may have a profound impact on endothelial cell signalling and function.

Over the past years, a number of endogenous compounds consisting of arachidonic acid conjugated with amino acids, including N-arachidonyl glycine (NAGly), have emerged as powerful modulators of pain (Vuong *et al.*, 2008), inflammation (Burstein *et al.*, 2011) and vascular function (O'Sullivan *et al.*, 2005; Milman *et al.*, 2006; Parmar and Ho, 2010). NAGly is an endogenous, enzymatically-oxygenated metabolite of the endocannabinoid anandamide (N-arachidonoyl ethanolamide) (Bradshaw *et al.*, 2009; McHugh, 2012). NAGly is produced from anandamide via two distinct pathways including oxidative metabolism of the ethanolamine moiety of anandamide and conjugation of glycine to arachidonic acid, which is released during anandamide hydrolysis by fatty acid amide hydrolase (FAAH; Bradshaw *et al.*, 2009). Unlike anandamide, NAGly has no effects on cannabinoid receptors, CB_1 and CB_2 (Huang *et al.*, 2001) and has low affinity for the vanniloid transient receptor potential cation channel V1, TRPV1 (Sheskin *et al.*, 1997). Recently, NAGly was demonstrated to be an endogenous ligand for GPCR 18 (GPR18; Kohno *et al.*, 2006; McHugh *et al.*, 2010; Takenouchi *et al.*, 2012) and GPCR 92 (GPR92; Oh *et al.*, 2008). In a microglial cell line (BV-2), human endometrial cell line (HEC-1B) and GPR18-transfected HEK-293 cells, NAGly was shown to control cell migration in a GPR18-dependent manner (McHugh *et al.*, 2010; McHugh, 2012; McHugh *et al.*, 2012). Consequently, GPR18 has been proposed to represent the putative abnormal cannabidiol receptor (McHugh *et al.*, 2010; McHugh, 2012). However, given the discrepancies between the levels of expression of GPR18 and the levels of NAGly in different tissues (Alexander, 2012), the association between GPR18 and NAGly is not straightforward. Indeed, activation of GPR18 by NAGly was not observed in a recent study employing high-throughput beta-arrestin-based screen (Yin *et al.*, 2009). In addition, the concept that NAGly acts as an agonist for GPR18 was further challenged recently when it was found that, in GPR18-expressing rat sympathetic neurons, NAGly failed to induce any GPR18-mediated inhibition of N-type ($Ca_v2.2$) Ca^{2+} channel, a primary downstream effector of Gi/o (Lu *et al.*, 2013). The authors concluded that NAGly is either not an agonist for GPR18 or that GPR18 signalling involves a non-canonical pathway.

Importantly, endocannabinoids and cannabinoid-like lipid compounds are able to modulate the activity of a number of ion channels independently of G-proteins (Oz, 2006; Barana *et al.*, 2010; Bondarenko *et al.*, 2011a,b). In rat odontoblasts, cannabinoid-induced Ca^{2+} influx through TRPV1 was recently shown to be functionally coupled to NCX_{pm}-mediated Ca^{2+} extrusion (Tsumura *et al.*, 2012). Remarkably, NAGly exhibits a number of G-protein-independent effects, including inhibition of T-type ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$) Ca^{2+} channels (Barbara *et al.*, 2009; Ross *et al.*, 2009). NAGly was also shown to enhance inhibitory glycinergic synaptic transmission by blocking glycine uptake and inhibiting excitatory NMDA-mediated synaptic transmission (Jeong *et al.*, 2010). In the vasculature, NAGly (Parmar and Ho, 2010) and its parent molecule anandamide (Jarai *et al.*, 1999; McCollum *et al.*, 2007) act as vasorelaxants, supposedly via stimulation of a putative G-protein coupled non-CB_1 or CB_2 cannabinoid receptor expressed in vascular endothelium, so-called anandamide or abnormal cannabidiol receptor coupled to NO production (Wagner *et al.*, 1999; Herradon *et al.*, 2007; McCollum *et al.*, 2007). However, whether and how NAGly affects NCX_{pm}-mediated endothelial electrical signalling and, if so, whether GPCRs are required for the observed effects is not clear.

Previously, it was shown that stimulation of a human umbilical vein-derived cell line (EA.hy926) with histamine in rather artificial nominally Ca^{2+}-free conditions (no added Ca^{2+}, no EGTA) induces Ca^{2+} oscillations due to a coordinated interplay between reversed NCX_{pm} operation and Ca^{2+} sequestration by sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (Paltauf-Doburzynska *et al.*, 2000). In the present study, we showed that, in the presence of physiological levels of Ca^{2+}, these cells respond to histamine by a sustained hyperpolarization supported by Ca^{2+} influx via reversed mode NCX. We further demonstrated that NAGly concentration-dependently inhibits this NCX_{pm}-mediated ion current in a G-protein-independent manner and the sustained endothelial hyperpolarization, both in cultured and *in situ* endothelial cells, an effect that underlies the transient inhibition of endothelium-dependent relaxation to ACh. In contrast, in unstimulated cells, NAGly promoted hyperpolarization via direct stimulation of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels.

Methods

Cell culture

The human umbilical vein-derived endothelial cell line, EA.hy926 (Edgell *et al.*, 1983) at passage >45 was grown in DMEM containing 10% fetal calf serum and 1% HAT (5 mM hypoxanthine, 20 μM aminopterin, 0.8 mM thymidine) and cells were maintained in an incubator at 37°C in 5% CO_2 atmosphere. Cells were plated on either 10 mm (for patch-clamp recordings) or 30 mm glass coverslips (for Ca^{2+} measurements).

Ca^{2+} measurements

Cytosolic free Ca^{2+} was measured using Fura-2/AM as previously described (Paltauf-Doburzynska *et al.*, 2000; Bondarenko *et al.*, 2010). Briefly, cells were loaded with 2 μM Fura-2/AM for 45 min at room temperature. Before the experiments, cells were washed and equilibrated for a further 20 min. Subsequently, cells were illuminated on an inverted microscope (Eclipse 300 TE, Nikon Instruments Inc., Melville, NY, USA) alternatively at 340 and 380 nm (filters: 340HTI15 and 380HTI15; Omega Optical, Brattleboro, VT, USA) and emitted light was collected at 510 nm (510WB40 emission; Omega Optical) using a cooled charge-coupled device camera (–30°C; Quantix KAF 1400G2, Roper Scientific, Acton, MA, USA). All Ca^{2+} measurements were performed with a 40 × 1.3 N.A. oil-immersion objective (Plan Fluor, Nikon) at room temperature.

Animals and tissue preparation

All animal studies were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Biomedical Ethics Committee of the A. A. Bogomoletz Institute of Physiology (Kiev, Ukraine). 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). Male Wistar rats (250–300 g body weight) were killed by cervical dislocation. The abdomen was opened by a midline incision, and the aorta was carefully excised and placed in ice-cold modified Krebs bicarbonate buffer solution of the following composition (mM): 118.3 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 10 glucose (pH 7.4). All connective and perivascular adipose tissues were removed. The aorta was then cut into ring segments, 2–3 mm wide, care was taken not to disrupt the endothelium. The rings were stored in bubbled Krebs solution maintained at room temperature before use in electrophysiological and contractile experiments.

Contractile recording experiments

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. Throughout the experiment, vascular preparations were superfused with modified Krebs bicarbonate buffer solution gassed with 5% CO_2 in O_2. After the equilibration period, arteries were precontracted with noradrenaline (NA, 10 μM) and when a stable level of contraction was established, 2 μM ACh was applied to observe relaxation. 5 μM NAGly was applied in addition to ACh when sustained relaxation was developed. Contractile experiments were conducted at 37°C. In some experiments, NAGly was applied to precontracted de-endothelized vessels. Denudation was achieved by an infusion of distilled water for 5–7 min and was confirmed by the inability of ACh to produce relaxation in the presence of NA.

Electrophysiological recordings

The membrane potential of endothelial cells from excised rat aorta was recorded using a perforated patch-clamp technique as described previously (Bondarenko, 2004). Briefly, ring segments were cut open and a strip was pinned to the rubber bottom of the chamber, which was perfused with modified Krebs bicarbonate buffer solution at a rate of 1 $mL \cdot min^{-1}$. The membrane potential of EA.hy926 cells was recorded using a nystatin-perforated patch clamp technique. For membrane potential recordings from EA.hy926 cells, the standard bath solution contained (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, 2.4 $CaCl_2$. For membrane potential recordings from both *in situ* endothelial cells and EA.hy926 cells, patch pipettes were filled with a solution containing (in mM) 140 KCl; 10 NaCl; 0.3 EGTA; 10 HEPES (pH adjusted to 7.2 using KOH). The resistance of the pipettes was 3–5 MΩ. Pharmacological agents were applied to the preparation by bath perfusion. Experiments were conducted at room temperature.

Whole-cell I_{NCX} was recorded from single EA.hy926 cells 24–36 h after plating using a conventional whole cell patch-clamp technique. Membrane currents and potential were recorded using a List EPC7 amplifier (List, Darmstadt, Germany) and pClamp 8.2 software (Axon Instruments from Molecular Devices, Sunnyvale, CA, USA). For recording the reverse (outward) NCX_{pm} current obtained after the addition of Ca^{2+} to a Na^+ free solution, the pipette solution contained (in mM) 125 NaCl, 10 CsCl, 2 $MgCl_2$, 5 EGTA, 4.28 $CaCl_2$ (1 μM free Ca^{2+}), 10 HEPES and the bath solution contained 140 *N*-methyl-*D*-glucamine (NMDG)-Cl, 1 $MgCl_2$ 10 TEA, 10 HEPES, 10 glucose with either 1 EGTA or 2.5 $CaCl_2$. For recording the reverse NCX_{pm} current using voltage ramps, the bath solution contained (in mM) 130 NaCl, 10 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose and the pipette solution contained (in mM): 80 Cs-methanesulfonate, 25 NaCl, 20 CsCl, 3 NaATP, 1 MgCl2, 10 HEPES, 5 EGTA and 1.93 Ca^{2+} to set the free Ca^{2+} concentration to 100 nM. For recordings of both reversed and forward modes of the exchanger using voltage ramps, the bath solution contained (in mM) 140 NaCl, 5 TEACl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose and the pipette solution contained (in mM): 110 Cs-methanesulfonate, 10 NaCl, 20 TEACl, 2 MgATP, 10 HEPES, 5 EGTA, 1.93 $CaCl_2$ (100 nM free Ca^{2+}). Voltage ramps of 1 s duration from –100 mV to +90 mV were delivered every 5 s from the holding potential of –40 mV.

Whole-cell recordings of BK_{Ca} channels were obtained in standard bath solution, the pipette solution contained (in mM) 145 KCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA and free Ca^{2+} concentration was set to 300 nM by adding 3.27 mM $CaCl_2$. The bath and the pipette K^+-based solutions with 300 nM free Ca^{2+} were used for recordings of BK_{Ca} single-channel activity in inside-out configuration.

Statistical analysis

Experimental data are expressed as mean ± 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 vascular tissue, *n* denotes the number of rats used.

The nomenclature used for receptors and ion channels conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Results

Reversed mode of NCX_{pm} contributes to sustained endothelial hyperpolarization to histamine in the human umbilical vein-derived endothelial cell line EA.hy926

In the presence of 2.4 mM bath Ca^{2+}, histamine (10–100 μM) produced a sustained endothelial hyperpolarization with a very low decay (Figure 1A). In order to explore the contribution of NCX_{pm} to endothelial hyperpolarization in response to histamine under physiological, Ca^{2+}-containing conditions, bepridil, a blocker of NCX_{pm}, was administered during the plateau phase of the hyperpolarization. Administration of bepridil (50 μM) in the continued presence of histamine fully and reversibly inhibited ($P < 0.05$) the hyperpolarizing response (Figure 1B). Because NCX_{pm} operating in a reversed mode may contribute to the endothelial hyperpolarization by two mechanisms, that is net efflux of positive charge per cycle and secondary stimulation of Ca^{2+}-activated K^+ channels (Bondarenko, 2004), the sensitivity of the hyperpolarization response to bepridil is indicative of NCX_{pm} operating in its Ca^{2+} entry mode during histamine-evoked hyperpolarization. To test more specifically that the reversed mode of NCX_{pm} was involved in this response, the drug KB-R7943 was used. Similar to bepridil, KB-R7943 (20 μM), an inhibitor of reversed NCX_{pm}, reversibly inhibited ($P < 0.05$) the endothelial hyperpolarization response to histamine (Figure 1C).

The operation of NCX_{pm} in Ca^{2+} entry mode following histamine stimulation is apparently ensured by a pronounced increase in Na^+ influx, which would override the forward mode of the exchanger activated by a rise in intracellular Ca^{2+} concentration. To explore the role of Na^+ entry in the NCX_{pm}-mediated sustained endothelial hyperpolarization to histamine, the Na^+ gradient was reduced during the plateau phase of the hyperpolarization. Although the reduction of external Na^+ is an intervention that increases the driving force for reversed mode NCX_{pm} under a fixed level of intracellular Na^+, this manoeuvre was expected to inhibit NCX_{pm}-mediated Ca^{2+} entry in confluent cells, when Na^+ influx triggers the reversed mode of NCX_{pm}. In these experiments, histamine (10 μM) hyperpolarized endothelial cells from –34.3 ± 2.3 mV to –56 ± 3.6 mV ($n = 6$). A reduction in extracellular Na^+ concentration from 140 mM to 20 mM reversibly inhibited ($P < 0.05$) the sustained component of the hyperpolarization, driving the membrane potential level to –33.7 ± 2.4 mV ($n = 5$; Figure 1D). Collectively, these results indicate that cells from the human umbilical vein endothelial cell-derived cell line express a functional NCX_{pm}, the operation of which, in its Ca^{2+} entry mode, ensures a sustained endothelial hyperpolarization during cell stimulation by histamine.

Reversed mode NCX_{pm} current is enhanced by histamine stimulation

To demonstrate an endogenous NCX_{pm} current in endothelial cells, we drove the transporter backwards by switching the Na^+-free external solution from a Ca^{2+}-free to a 2.5 mM Ca^{2+}-containing solution with 120 mM Na^+ in the pipette at a holding potential 0 mV. Adding Ca^{2+} to the bath solution elicited an outward current that was inhibited by 20 μM

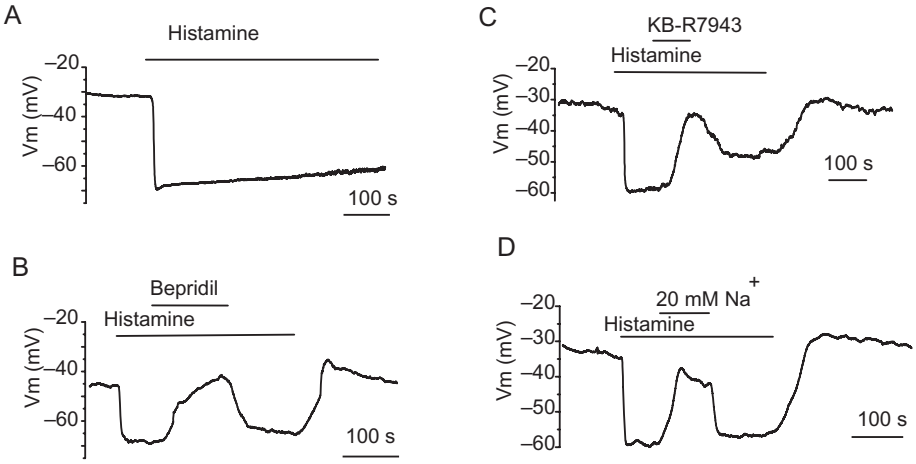

Figure 1

Impact of inhibition of NCX_{pm} on endothelial cell hyperpolarization to histamine. (A) Representative effect of histamine (10 μM) on the membrane potential of EA.hy926 cells. (B) Inhibition of sustained hyperpolarization to histamine (10 μM) by bepridil (50 μM) application during the plateau phase of hyperpolarization. (C) Inhibitory effect of KB-R7943 (20 μM) on sustained endothelial hyperpolarization to histamine. (D) Effect of reduction of external Na^+ concentration to 20 mM on endothelial hyperpolarization to histamine. (A–D) Exemplary traces out of 4–7 individual experiments.

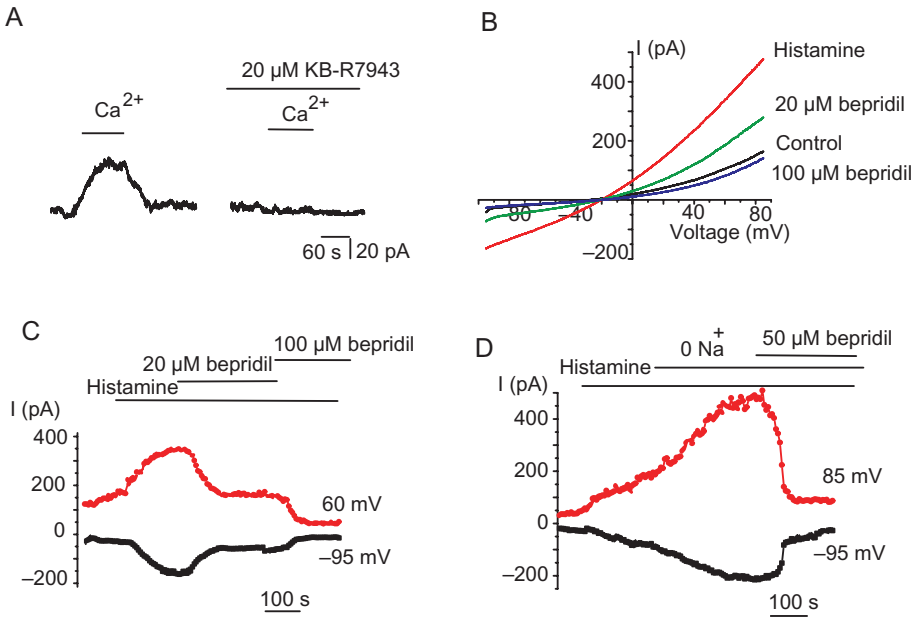

Figure 2

NCX_{pm} is active and is stimulated by histamine. (A) Ca^{2+} addition produces outward KB-R7943-sensitive current in Na^+-loaded endothelial cells. Representative record out of four experiments. Membrane potential was clamped at 0 mV. Pipette was filled with 120 mM NaCl-containing solution. (B) Representative current traces to voltage ramps before (control), during 10 μM histamine exposure (histamine) and in the combined presence of histamine with either 20 μM bepridil or 100 μM bepridil. Internal solution contained 28 mM Na^+ and 100 nM free Ca^{2+}. (C) Corresponding time course of the currents shown in (B) taken at –95 and 60 mV in response to 10 μM histamine and the effects of 20 and 100 μM bepridil. Similar results were obtained in 6 cells. (D) Na^+ removal amplifies histamine-potentiated current. Representative (n = 5) time course of the current response to histamine application followed by Na^+ removal taken at –95 and 85 mV and inhibitory effect of bepridil (50 μM).

KB-R7943 (Figure 2A), demonstrating that these cells develop significant NCX_{pm} currents.

We next examined the role of NCX_{pm} in histamine-evoked whole-cell currents. For this purpose, we applied voltage ramps to Na^+ loaded (28 mM Na^+ in the pipette solution) cells, a condition that favours the NCX_{pm} operating in reversed mode. K_{Ca} channels were suppressed by substituting Cs^+ for K^+ in the internal solution and the internal Ca^{2+} was buffered at 100 nM. Under these conditions, the voltage ramps produced an outwardly rectifying current with a typical *I-V* relationship that is characteristic of NCX_{pm} (Figure 2B). Stimulation with 10 μM histamine markedly enhanced the peak currents (by 230%) and subsequent administration of bepridil (20 and 100 μM) strongly suppressed the current amplitude (Figure 2B,C). In another set of experiments, histamine exposure was followed by cell superfusion with Na^+-free external solution in the continued presence of Ca^{2+} and histamine to boost the reversal of the exchanger. This manoeuvre strongly increased both the outward and inward currents (Figure 2D). Because under these experimental ionic conditions, the driving force of the reversed NCX_{pm} is limitless, the inward current observed at negative potentials is highly unlikely to reflect the forward mode of the exchanger. Nevertheless, subsequent addition of 50 μM bepridil suppressed the outwardly rectifying current (Figure 2D). Taken together, these observations indicate that in endothelial cells under our experimental conditions the basal NCX_{pm} currents are enhanced by histamine exposure.

NAGly inhibits NCX_{pm}-mediated ion currents stimulated by histamine

Next, we assessed the effect of NAGly on NCX_{pm}-mediated whole-cell currents. When cells were dialysed with the Cs^+-based intracellular solution containing low Ca^{2+} (100 nM) and high Na^+ (28 mM), a condition which favours reversed NCX_{pm}, voltage ramps from –100 to 85 mV revealed an outwardly rectifying current that met the characteristics of NCX_{pm}. This outwardly rectifying current was amplified by histamine and was further potentiated by substitution of external Na^+ for $NMDG^+$ (Figure 3A,B). Administration of 10 μM NAGly evoked a gradual suppression of the current (Figure 3A,B), which in some cells was preceded by transient potentiation of current amplitude (Figure 3A). When NAGly (10 μM) was applied to histamine-stimulated cells without prior exposure to Na^+-free solution, it abolished the outwardly rectifying current stimulated by histamine and strongly suppressed basal NCX_{pm} currents (Figure 3C,D).

NAGly inhibits NCX_{pm}-mediated ion currents and intracellular Ca^{2+} rise independently of store depletion

The next question we addressed is whether cell stimulation with histamine and the associated store depletion and/or initiation of store-operated or receptor-activated Na^+ and Ca^{2+} influx is required for the inhibitory effect of NAGly on reversed mode NCX_{pm}. To explore the role of the cell signal-

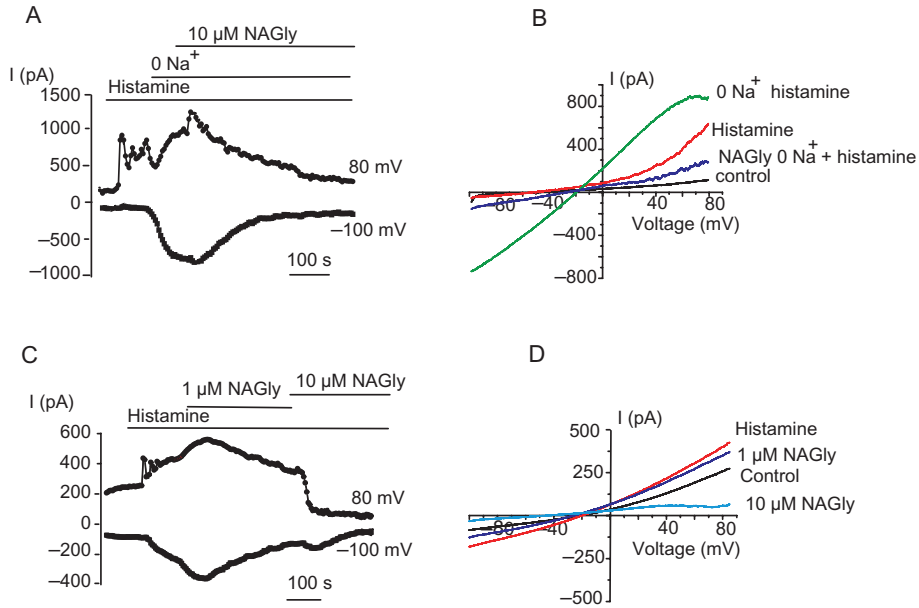

Figure 3

NAGly inhibits NCX_{pm} currents. (A) The time course of inhibition NCX_{pm} currents by 10 μM NAGly. NCX currents were maximally pre-stimulated with histamine and Na^+ removal. (B) Representative NCX currents evoked by voltage ramps from the time course shown in (A) before (control), in the presence of 10 μM histamine (histamine) and following Na^+ removal (0 Na^+ histamine) and the effect of 10 μM NAGly on NCX_{pm}-potentiated currents. Similar results were obtained in six cells. (C, D) The time course of inhibition of NCX_{pm} current amplitude by NAGly (1 and 10 μM) preliminary augmented with histamine (10 μM; C) and corresponding current traces (D) in response to voltage ramps before (control), during 10 μM histamine exposure (histamine) and in the combined presence of histamine with either 1 or 10 μM NAGly. Similar results were obtained in six cells.

ling cascade initiated by histamine in NAGly-evoked inhibition of I_{NCX}, we avoided cell stimulation with histamine and simply drove the transporter backwards by switching to a Na^+-free Ca^{2+}-containing solution while delivering voltage ramp pulses. Substitution of external Na^+ for $NMDG^+$ strongly augmented the outwardly rectifying current (Figure 4A,B), an observation consistent with increased activity of reversed NCX_{pm}. Again, this outwardly rectifying current was inhibited upon NAGly administration, indicating that cell stimulation with an IP_3-generating agonist, such as histamine, is not required for NAGly-induced inhibition of NCX_{pm}. Support for this notion was further obtained by measuring the reversed NCX-driven rise in intracellular Ca^{2+}. Figure 4C shows an exemplary analogous fluorescence trace from a Fura-2-loaded EA.hy926 cell. Replacement of external Na^+ with an equimolar amount of $NMDG^+$ induced an increase in $[Ca^{2+}]_i$, which was largely (by 42%) suppressed by subsequent exposure to 3 μM NAGly. Similar results were obtained in a total of 18 cells from four coverslips. These findings indicate that NAGly inhibits the reversed NCX_{pm} independently of endothelial cell stimulation with agonists.

NAGly reversibly inhibits both reversed and forward mode of NCX_{pm} in a concentration-dependent manner

The above voltage clamp experiments were performed by using pipette solution containing 28 mM Na^+ and 100 nM Ca^{2+}, a condition which favours the reverse mode of NCX_{pm} along the membrane potentials tested. To determine whether NAGly exclusively suppresses the reversed mode of the exchanger, in experiments described below cells were dialysed with internal solution containing 10 mM Na^+ and 100 nM free Ca^{2+} in the presence of 2.4 mM bath Ca^{2+}, a condition which allows the recording of both forward and reversed modes of the exchanger depending on the membrane voltage applied. Forward and reverse mode NCX_{pm} currents were estimated at –95 mV and +85 respectively. Representative currents in response to voltage ramps from –100 to 90 mV before and after NAGly administration are shown in Figure 5 A,B. Both outward and inward currents were inhibited by 0.3 μM NAGly with a similar time course (Figure 5A). At a concentration of 0.3 μM, NAGly was slow to exert its full effect on NCX_{pm} currents, the degree of inhibition increased with time and a steady-state value was reached over 200–250 s after initial application (Figure 5A). The effect of NAGly on NCX_{pm} currents was reversible; restoration of I_{NCX} occurred within 250–300 s following 0.3 μM NAGly washout. Corresponding *I-V* curves before application of 0.3 μM NAGly, during the plateau of the response and following washout of the compound are shown in Figure 5B.

Inhibition of I_{NCX} by NAGly was concentration-dependent within the concentration range tested (0.3–30 μM). Increasing the NAGly concentration from 0.3 to 10 μM produced a stronger inhibitory effect on I_{NCX} and reduced the time to reach the steady-state inhibition of I_{NCX} to 120–150 s (Figure 5C). Cumulative addition of increasing concentrations of NAGly into the bath solution caused a gradually

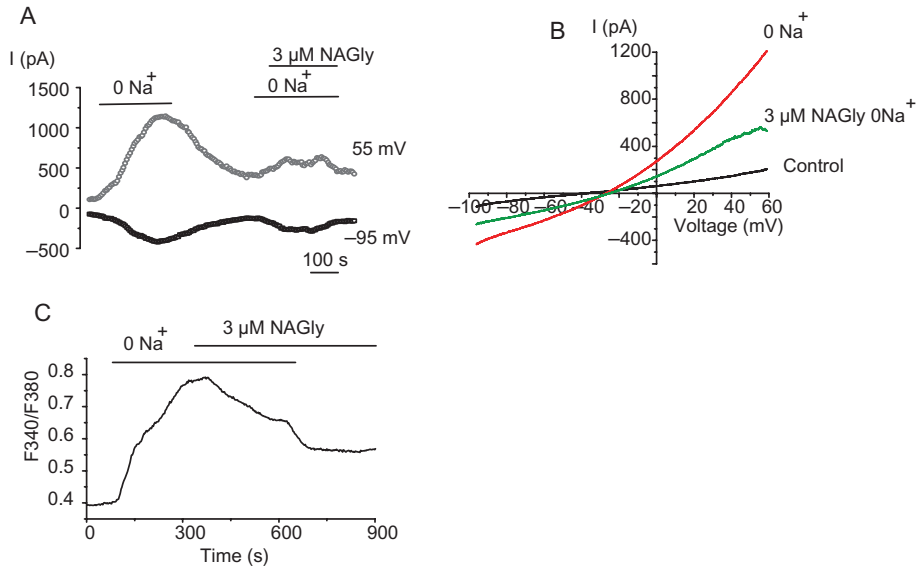

Figure 4

Histamine pre-stimulation is not required for NCX_{pm} inhibition by NAGly. (A, B) Representative time course at voltages indicated (A) and the voltage dependency (B) of NCX_{pm} currents potentiated by Na^+ removal in a Ca^{2+}-containing solution and the effect of NAGly (3 μM), (C) Representative effect of 5 μM NAGly on intracellular free Ca^{2+} rise produced by Na^+ removal in Ca^{2+}-containing solution (n = 18).

developing inhibition of the current amplitude (Figure 5D,E). At the end of experiment, the cells were exposed to bepridil (50 μM). The bepridil-sensitive current, which represents only the NCX current, was calculated by subtracting the current remaining after 50 μM bepridil exposure from the total measured current at each NAGly concentration tested. A similar degree of suppression of both the outward and inward current amplitudes was observed at a particular concentration of NAGly that inhibited the reverse and forward modes of NCX activity at an IC_{50} of 1.22 μM and 1.09 μM respectively (Figure 5F,G). The Hill coefficient was 1.09 for the forward and 1.18 for the reversed mode of the exchanger, suggesting one binding site for NAGly. These experiments indicate that inhibition of NCX_{pm} by NAGly appears to be non-mode selective.

NAGly suppresses NCX currents independently of GPCRs

To examine whether GPCRs mediate the effect of NAGly on I_{NCX}, we next tested the effect of the synthetic cannabinoid analogue O-1918 on I_{NCX}. It was presumed O-1918 is an antagonist of a putative endothelial abnormal cannabidiol receptor activated by NAGly and anandamide (McHugh *et al.*, 2010; Parmar and Ho, 2010). Exposure to O-1918 (10 μM) gradually suppressed (up to 82%) both the basal I_{NCX} (Figure 6A) and that pre-stimulated by low (20 mM) Na^+ (switch from 140 mM $[Na^+]_o$ to 20 mM $[Na^+]_o$) (Figure 6B,C). Administration of 10 μM NAGly in the continued presence of 10 μM O-1918 further suppressed the remaining portion of the current. Supplementation of the patch pipette solution with guanosine 5′-O-(2-thiodiphosphate) (GDPβS; 0.5–1 mM), a potent and irreversible G-protein inhibitor, was also tested to examine whether GPCRs are required for suppression of I_{NCX} by NAGly. Under these conditions, administration of 10 μM NAGly to the external solution inhibited the currents (n = 7) to the same extent as in the absence of GDPβS (Figure 6D,E). Because NAGly is susceptible to metabolic degradation via FAAH (Bradshaw *et al.*, 2009), we explored whether it is actually NAGly that is causing the effect and not arachidonic acid or glycine generated by NAGly hydrolysis. Pre-incubation and continuous incubation of 1 μM URB597, a FAAH inhibitor (Ho and Randall, 2007; Bradshaw *et al.*, 2009), was unable to prevent inhibition of I_{NCX} following NAGly application (Figure 6F,G). When 10 μM NAGly was introduced into the patch pipette, a manoeuvre, which allows NAGly administration to the inner surface of the cell avoiding binding to GPCRs, I_{NCX} was gradual suppressed within 200–250 s (Figure 6H,I) However, the mean suppressive effect on I_{NCX} induced by intracellular NAGly administration was significantly less than that observed induced by external NAGly application (Figure 6J). The fractional inhibition of I_{NCX} by 10 μM of internal NAGly at +85 mV was 55.9 ± 4.2% (n = 4), while external NAGly administration produced a fractional inhibition of I_{NCX} of 75.0 ± 7.1% (n = 9). Taken together, these data strongly indicate that the endogenous lipoamino acid NAGly inhibits NCX_{pm}-mediated currents in a GPCR-independent manner.

Anandamide suppresses NCX currents independently of G-proteins

Unlike NAGly, extracellular administration of anandamide, a parent and structurally similar molecule, at a concentration of 3 μM only marginally suppressed I_{NCX}. Increasing the anandamide concentration to 10 μM strongly (up to 80%) inhibited I_{NCX} (Figure 7A–C; n = 3), suggesting that anandamide inhibits I_{NCX} with the potency lower than that for NAGly. Inclusion of 0.5 mM GDPβS into the patch pipette failed to

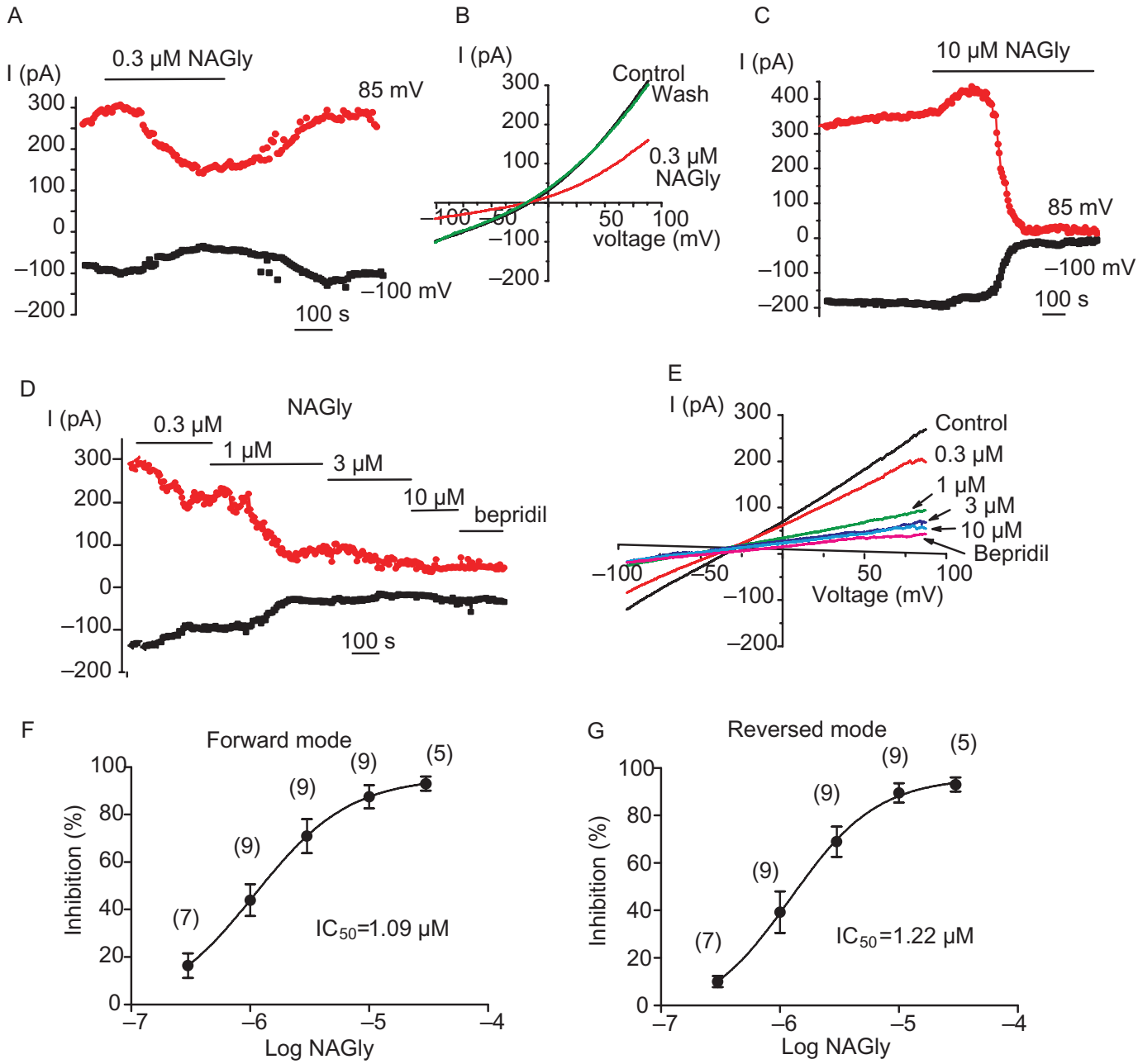

Figure 5

Inhibitory effect of NAGly on NCX_{pm} currents is reversible, concentration-dependent and non-mode selective. (A, B) Representative time course (A) and NCX_{pm} currents elicited by voltage ramps (B) before (control), during 0.3 μM NAGly administration (NAGly) and following NAGly washout. Similar results were obtained in four cells. (C, D). Representative time course (C) and NCX_{pm} currents elicited by voltage ramps showing concentration-dependent inhibition of current responses by NAGly. Bepridil (50 μM) was administered at the end of the protocol. (D, E) Concentration-dependent inhibition of forward (D) and inward (E) NCX_{pm} currents by NAGly. In parentheses are the numbers of individual cells treated with different concentrations of NAGly.

abolish the anandamide effect on both basal (not shown, n = 3) and pre-stimulated I_{NCX} by low (20 mM) Na^+ (n = 4; Figure 7D,E).

Dual effect of NAGly on endothelial membrane potential

We examined the effect of NAGly on the membrane potential of unstimulated and histamine-stimulated EA.hy926 cells. NAGly was administered either before or during the plateau phase of the hyperpolarization to histamine. NAGly (3 μM) induced a slowly developing long-lasting hyperpolarization with an amplitude of 8.9 ± 2.1 mV (n = 8). This hyperpolarization was reversed by iberiotoxin (400 nM), an inhibitor of BK_{Ca} channels (Figure 8A). When histamine was administered in the continued presence of NAGly, only transient hyperpolarization with a reduced amplitude (7.3 ± 0.9 mV, n = 4,

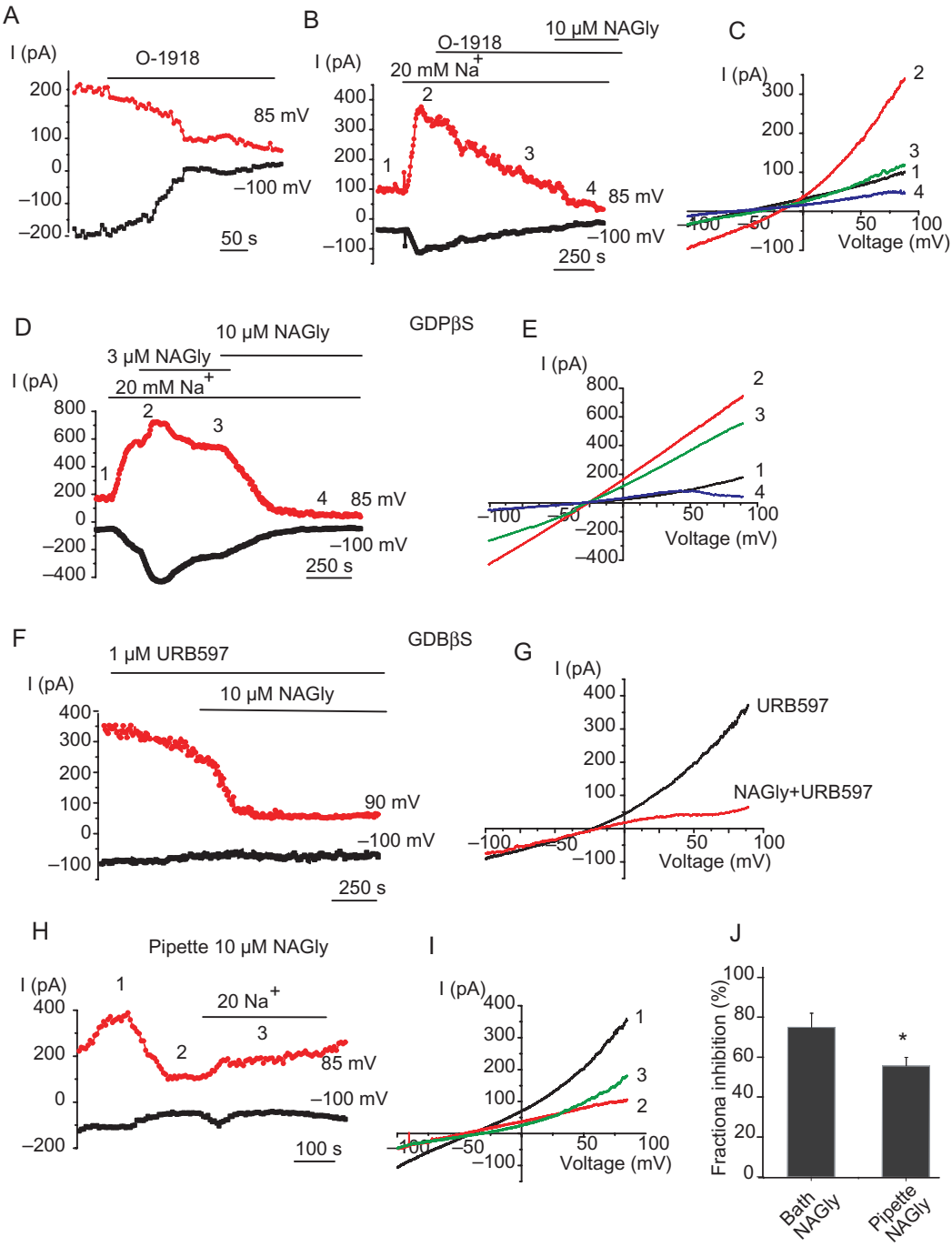

Figure 6

NAGly suppresses NCX currents independently of G-proteins. (A) Representative time course of changes in NCX current amplitudes at –100 and +85 mV during voltage ramps before and during application of 10 μM O-1918. (B) O-1918 (10 μM) suppresses NCX_{pm} current pre-stimulated with a decrease in bath Na^+ to 20 mM. The time course of the response. (C) Corresponding current traces elicited by voltage ramps at time points indicated in (B). (D) Representative time course of changes in the NCX_{pm} current amplitudes to voltage ramps at voltages indicated in the presence of GDPβS in the patch pipette under control conditions (1), in the presence of 20 mM Na^+ in the bath (2) and in the presence of either 3 μM (3) or 10 μM (4) of bath NAGly in low Na^+ solution. (E) Corresponding current traces at time points shown in (D). (F) Representative time course of changes in current amplitudes at –100 and +85 mV during voltage ramps before and during application of 10 μM NAGly in the continued presence of URB597. Pipette solution was supplemented with 0.5 mM GDPβS. (G) Corresponding I-V relationship. (H) Representative time course of the changes in current amplitude during intracellular dialysis of EA.hy926 cell with 10 μM NAGly before and after a decrease in bath Na^+, (I) corresponding I-V relationship at time points indicated in (A). (J) Mean fractional inhibition of I_{NCX} at +85 mV by extracellular and intracellular NAGly (10 μM). Fractional inhibition was obtained using the function: fractional inhibition = $(I_{control} - I_{NAGly})/I_{control} \times 100$.

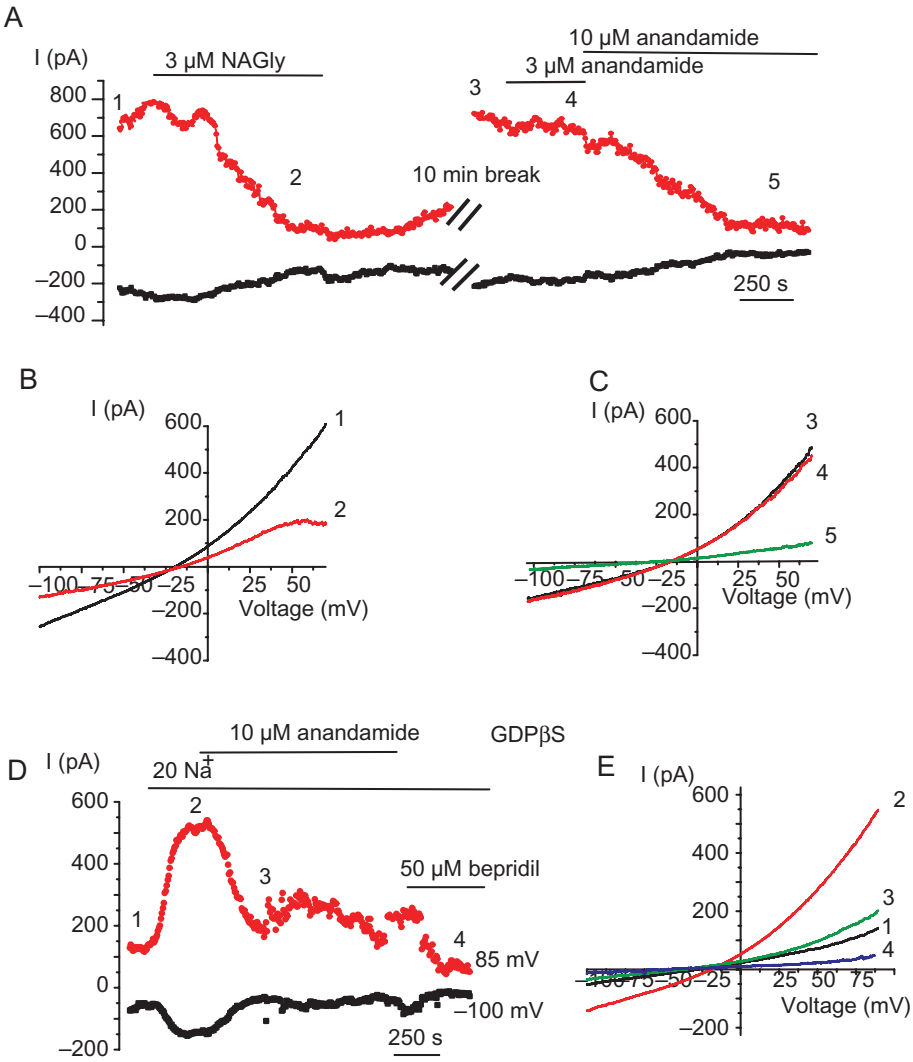

Figure 7

Anandamide suppresses NCX currents independently of G-proteins. (A) Representative time course of changes in current amplitudes at –100 and +85 mV during voltage ramps in response to 3 μM NAGly followed by washout and administration of 3 and 10 μM anandamide. (B, C) Corresponding current traces elicited by voltage ramps at time points indicated in (A). (D) The time course of the changes in NCX_{pm} current amplitude augmented by a decrease in external Na^+ concentration to 20 mM followed by administration of 10 μM anandamide and 50 μM bepridil. (E) Corresponding current traces elicited by voltage ramps at time points indicated in (D). Pipette solution was supplemented with 0.5 mM GDPβS.

$P < 0.05$) was produced (Figure 8B). Administration of 3 μM NAGly during the plateau phase of the hyperpolarization to histamine (10 μM) strongly and reversibly inhibited the hyperpolarization within 4 min from 25.2 ± 3.2 mV to 11.0 ± 3.1 mV ($n = 4$; Figure 8C). Administration of 10 μM NAGly fully inhibited the hyperpolarization to 10 μM histamine (Figure 8D). Upon NAGly washout, the time course of the hyperpolarization was gradually restored within 7–8 min. These results show that NAGly directly affects the endothelial membrane potential.

G-proteins are not required for stimulation of BK_{Ca} channels by NAGly

Because NAGly produced iberiotoxin-sensitive sustained hyperpolarization in EA.hy926 cells, we next assessed the effect of NAGly on whole-cell potassium currents and whether G-proteins are required for this effect. When the pipettes were filled with KCl-based solution containing 300 nM free Ca^{2+}, voltage ramps from –80 to 80 mV revealed an outwardly rectifying current. External NAGly (3 and 10 μM) strongly potentiated the current with characteristics typical of the BK_{Ca} channel present in these cells (Bondarenko *et al.*, 2010; 2011a). The effect of NAGly was completely abolished by 1 μM paxilline ($n = 4$; Figure 8E), a BK_{Ca} channel inhibitor, but not by intracellular dialysis with the irreversible G-protein inhibitor GDPβS (1 mM; $n = 4$, Figure 8F). To explore whether NAGly affects BK_{Ca} channels when applied to the cytosolic face of excised membrane, the BK_{Ca} single-channel activity was recorded in inside-out patches under symmetrical K^+ conditions and in the presence of 300 nM free

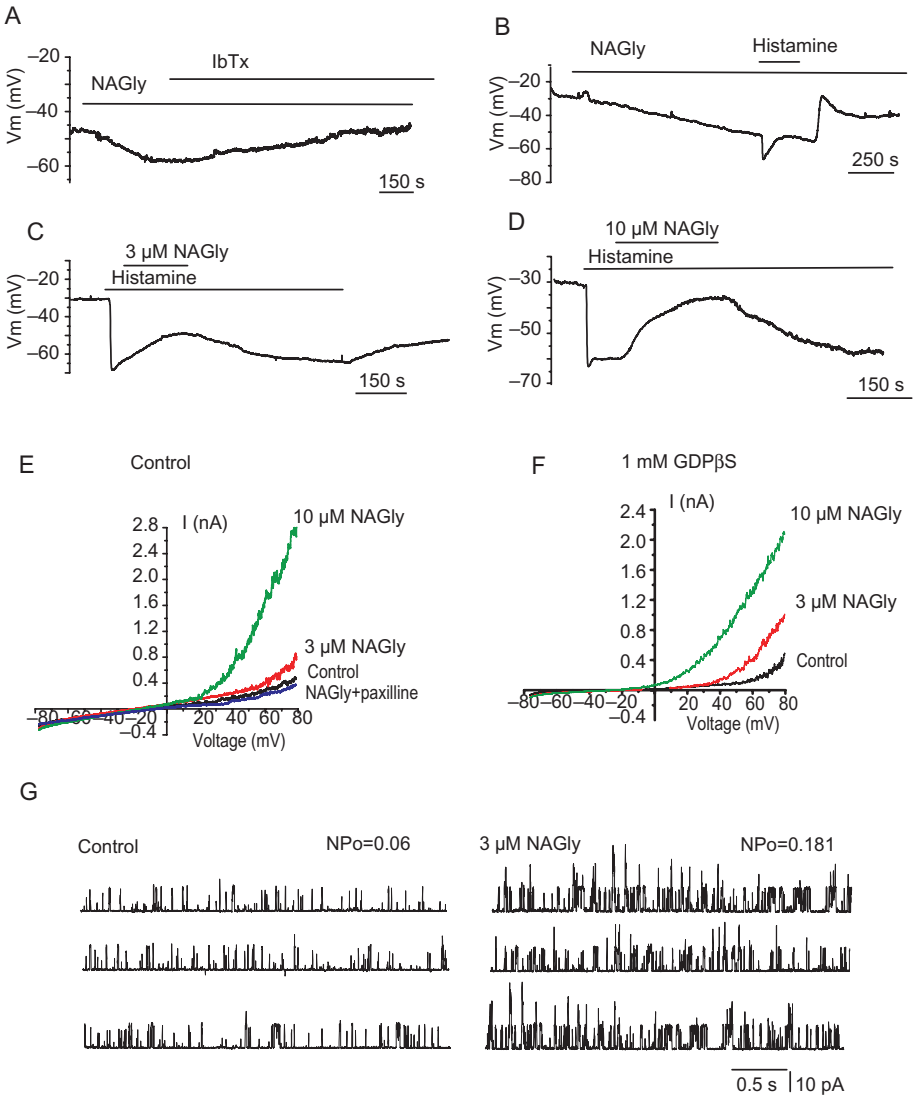

Figure 8

Dual effect of NAGly on the membrane potential of endothelial cells. (A) Representative effect of NAGly (3 μM) and subsequent iberiotoxin (400 nM) administration on the membrane potential of EA.hy926 cells (n = 4). (B) Representative effect of NAGly (3 μM) and subsequent administration of histamine (10 μM) on the membrane potential of EA.hy926 cells (n = 4). (C, D) Effect of 3 μM (C; n = 4) and 10 μM (D) NAGly (n = 6) on histamine (10 μM)-induced sustained hyperpolarization of EA.hy926 cells. (E) Whole cell BK_{Ca} currents in response to voltage ramps from −80 to +80 mV in the absence (control), and presence of either 3 or 10 μM NAGly and in the combined presence of 10 μM NAGly and 1 μM paxilline. (F) The same like in (E) but in the presence of 1 mM GDPβS in the pipette solution. (G) Representative recordings of single BK_{Ca} channel activity in inside-out configuration in the absence (left) and presence (right) of 3 μM NAGly. Recording were performed at +40 mV and in the presence of 300 nM free Ca^{2+}.

bath Ca^{2+}. Under these conditions, 3 μM NAGly potentiated BK_{Ca} channel activity (NPo) from 0.046 ± 0.008 to 0.139 ± 0.025 (n = 11), while the single-channel amplitude was unaffected in the presence of NAGly.

Vascular tissue experiments

To determine whether NAGly-induced inhibition of NCX_{pm} and corresponding suppression of the hyperpolarization to histamine was a phenomenon unique to cell culture, experiments were performed on *in situ* endothelium of excised rat aorta, where NCX_{pm} operating in reverse mode is critical for sustained endothelial cell hyperpolarization (Bondarenko, 2004). When the membrane potential was recorded from *in situ* endothelium, administration of NAGly (3 μM) in the continued presence of ACh strongly inhibited the hyperpolarization (Figure 9A).

To address the functional relevance of the reduction of endothelial hyperpolarization by NAGly, we tested the effect of NAGly on endothelium-dependent relaxation of rat aortic rings produced by ACh (2 μM). Once the relaxation was developed, 5 μM NAGly produced a significant transient reduction of the relaxation (up to 70%) from the pre-NAGly levels (Figure 9B). However, this NAGly-induced inhibition of

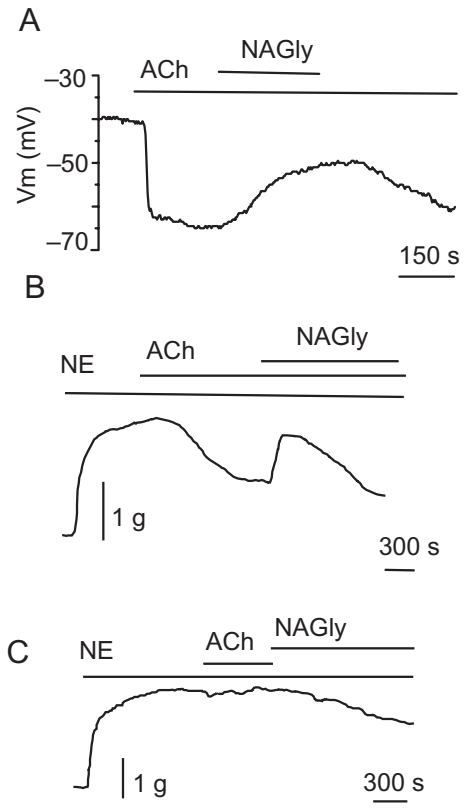

Figure 9

Effects of NAGly on endothelial hyperpolarization and endothelium-dependent relaxation to Ach in excised rat aorta. (A) Representative recording showing the effect of NAGly (3 μM) on the hyperpolarizing response to Ach (2 μM) in rat aortic endothelium (n = 3). (B) Representative recording showing the effect 5 μM NAGly on the relaxation of rat aortic rings produced by Ach (2 μM; n = 4). (C) Representative recording showing the effect of NAGly on norepinephrine-induced contraction of de-endothelized rat aortic ring (n = 4).

the relaxation was not persistent and in the continued presence of ACh and NAGly, the relaxation was slowly (within 5–7 min) restored.

Considering that NCX is highly expressed in vascular smooth muscle cells and partially mediates vascular smooth muscle contraction to phenylephrine (Zhang *et al.*, 2010) and noradrenaline (Lagaud *et al.*, 1999), we next explored the effect of NAGly on NA-induced constriction. In de-endothelialized aortic rings, NAGly (5 μM) attenuated the NA-induced contraction by 19 ± 3.8% (n = 4; Figure 9C).

Discussion

In the present study, we demonstrated that in a human umbilical vein endothelial cell-derived cell line, the sustained hyperpolarization to histamine is sensitive to bepridil, the NCX_{pm} blocker, which inhibits both forward and reversed modes of NCX_{pm}, KB-R7943, which preferentially inhibits NCX_{pm} in reversed mode, and to a decrease in Na^+ gradient, indicating that in these cells, NCX_{pm} operates in a Ca^{2+} influx mode during exposure to histamine and this contributes to cell hyperpolarization. Our membrane potential measurements are substantiated by whole-cell current recordings under experimental conditions adjusted to suppress K^+ conductance with physiological levels of intracellular Na^+. The outwardly rectifying current with typical characteristics of NCX_{pm} observed under basal conditions was potentiated by histamine and further greatly enhanced by Na^+ withdrawal. These outwardly rectifying currents were suppressed by bepridil, further demonstrating that the current potentiated by histamine can be attributed to NCX_{pm}. Our electrophysiological data, obtained from ion substitution and pharmacological experiments, together with the typical *I-V* characteristics of the current indicate that the major mechanism of Ca^{2+} inflow into EA.hy926 cells following stimulation with histamine is the reversed mode NCX_{pm}. The function of NCX_{pm} as a pathway for Ca^{2+} entry has also been demonstrated previously in experiments on the endothelium of excised rat aorta exposed to Ach (Bondarenko, 2004), and mechanical injury (Berra-Romani *et al.*, 2012), emphasizing the importance of the transport in regulation of Ca^{2+}-dependent processes.

Recent findings indicate that in endothelium from rat aorta exposed to ATP, NCX_{pm} operates in the forward mode contributing to the restoration of internal Ca^{2+} (Berra-Romani *et al.*, 2010), suggesting that the mode of NCX_{pm} operation is agonist-specific. Given that NCX_{pm} is an electrogenic transporter, NCX_{pm} operating in the forward mode contributes to a faster decay of the hyperpolarization and intracellular Ca^{2+} rise. Obviously, NCX_{pm} operation in response to the action of specific agonists not only provides an additional pathway for Ca^{2+} entry or extrusion, but also shapes the electrical responses ensuring a characteristic 'fingerprint' typical for a particular agonist. Thus, the hyperpolarization to ATP (Bondarenko and Sagach, 1996) and ionomycin (Bondarenko and Sagach, 2006) in *in situ* aortic endothelium is more transient and is followed by the depolarization phase, while ACh produces a long-lasting hyperpolarization, which in addition to reversed NCX_{pm} is supported by Na^+ pump stimulation (Bondarenko and Sagach, 2006). The results of the current study show that the EA.hy926 cell line may serve as a convenient model for studying the mechanisms of regulation and modulation of NCX_{pm}-mediated Ca^{2+} entry in endothelial cells.

Using the same endothelial cell line, it was demonstrated previously that in rather artificial, nominal Ca^{2+}-free conditions (no added Ca^{2+} no EGTA), histamine evokes oscillations in intracellular Ca^{2+} concentration due to coordinated interplay between reversed NCX_{pm} operation and Ca^{2+} sequestration by sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (Paltauf-Doburzynska *et al.*, 2000). The oscillations were not seen in the presence of physiological levels of bath Ca^{2+}. However, it was not clear from that study whether histamine exposure in the presence of physiological levels of Ca^{2+} results in the NCX_{pm}-mediated Ca^{2+} entry. In fact, in follow up studies, Ca^{2+} entry into EA.hy926 cells stimulated with histamine has widely been attributed to the store-operated Ca^{2+} entry (SOCE; Malli *et al.*, 2003; Naghdi *et al.*, 2010), or receptor-activated Ca^{2+} entry (RACE; Jousset *et al.*, 2008). In experiments with cytosolic and endoplasmic reticulum (ER) Ca^{2+} measurements and with the use of 10 μM La^{3+} as a dis-

criminating compound, the RACE pathway accompanied by very moderate store depletion was suggested to be mainly responsible for histamine-induced Ca^{2+} entry into EA.hy926 cells (Jousset *et al.*, 2008), which questions the relevance of SOCE.

Like in other non-excitable cells, Ca^{2+} influx into endothelial cells upon stimulation by vasoactive compounds is believed to be mainly induced by either the SOCE pathway or receptor-activated non-selective Ca^{2+}-permeable cation channels (Nilius and Droogmans, 2001), an influx pathway(s) activated independently of depletion of intracellular Ca^{2+} stores. A common experimental approach used for SOCE characterization in fluorescent Ca^{2+} measurements is a classical Ca^{2+} re-addition protocol. Ca^{2+} re-addition after a period of exposure to Ca^{2+}-free medium, during which the IP_3-generating agonists or inhibitors of sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase (SERCA) are applied, is associated with a large increase in fluorescence, which is commonly interpreted as a Ca^{2+} influx via store-operated channels (Malli *et al.*, 2003; Naghdi *et al.*, 2010). However, an increase in intracellular Ca^{2+} due to store depletion in a Ca^{2+}-free bath solution inevitably results in Ca^{2+} extrusion in exchange for Na^+ and, consequently, to Na^+ loading. The accompanying $[Ca^{2+}]_o$ and $[Na^+]_i$ shifts aid the reversal of NCX_{pm} upon Ca^{2+} re-addition. Hence, the classical Ca^{2+} re-addition protocol does not allow one to fully discriminate between the SOCE and NCX_{pm}. By using an electrophysiological approach, our results confirm the functional expression of NCX_{pm} in EA.hy926 cells and demonstrate that during stimulation with histamine in the presence of a physiological level of external Ca^{2+}, the exchanger operates in the reverse mode (Girardin *et al.*, 2010) ensuring sustained Ca^{2+} inflow and membrane hyperpolarization. Remarkably, in endothelial cells, despite a robust increase in Ca^{2+} following Ca^{2+} re-addition, whole-cell Ca^{2+}-release-activated inward current (I_{CRAC}) following passive store depletion could only be convincingly shown in the absence of divalent cations (Abdullaev *et al.*, 2008; Beech, 2009; Girardin *et al.*, 2010). This obvious discrepancy may indicate that a mechanism(s) other then SOCE largely support Ca^{2+} entry following stimulation of endothelial cells by vasoactive agonists. Our results suggest that NCX_{pm} is the Ca^{2+} influx pathway involved following stimulation of endothelial cells with either histamine or ACh.

Modulation of stimulated Ca^{2+} entry is an important aspect of endothelial cell (patho)physiology, which influences numerous Ca^{2+}-dependent processes. In the present study, we showed, for the first time, that NAGly, the recently identified endogenous lipoamino acid that acts as a ligand for GPR18/GPR92 (Kohno *et al.*, 2006; Oh *et al.*, 2008), affects endothelial electrical responses in both unstimulated and agonist-stimulated cells. In histamine- and ACh-stimulated cells, NAGly effectively inhibited membrane hyperpolarization via suppression of NCX_{pm}-mediated Ca^{2+} inflow, while in unstimulated cells NAGly produced hyperpolarization via direct stimulation of BK_{Ca} channels. The mechanism of I_{NCX} suppression by NAGly appears to be independent of recruitment of GPCRs, since the effect was unaffected by intracellular dialysis with GDPβS, a potent and irreversible inhibitor of all G-proteins. Moreover, in NAGly-dialysed cells, I_{NCX} was gradually inhibited, although to a lesser extent than that observed during external NAGly application, further indicating that the action of NAGly involves a receptor-independent mechanism and occurs via a mechanism targeting the external face of the membrane leaflet. The role of GPR18 was addressed and ruled out by investigating the effect of the synthetic cannabinoid antagonist, O-1918, on I_{NCX}–induced suppression by NAGly. We found that O-1918 itself acts as an inhibitor of NCX_{pm} eliminating the effect of NAGly. Anandamide, a lipid mediator and a parent molecule for NAGly, also suppressed I_{NCX}, although higher concentrations were required to observe the inhibition. I_{NCX} inhibition by NAGly was independent of FAAH, indicating that it is actually NAGly that is causing the effect but not arachidonic acids or glycine, the products of NAGly degradation via FAAH. Taken together, these observations indicate that the effect of NAGly on NCX_{pm} is either a consequence of NAGly interacting with the NCX protein itself or modifications of the lipid bilayer, an important regulator of imbedded ion transport proteins (Crowley *et al.*, 2003) including NCX (Kuszczak *et al.*, 2011).

The inhibitory effect of NAGly on endothelial hyperpolarization does not appear to be unique for cell culture since NAGly effectively suppressed the endothelial hyperpolarization response to ACh in excised rat aorta. In this vascular bed, ACh produces a sustained endothelial hyperpolarization supported by Ca^{2+} inflow via the reverse mode of NCX_{pm} (Bondarenko, 2004) ensuring a sustained NO release and endothelium-dependent relaxation (Schneider *et al.*, 2002). The inhibitory effect of NAGly on endothelial cell hyperpolarization occurs at low micromolar concentrations, which were previously shown to induce endothelium-dependent component of relaxation, presumably by binding to putative non-CB_1/non-CB_2 endothelial cannabinoid receptors (Parmar and Ho, 2010). In excised rat aorta, the reduced hyperpolarization was accompanied by a transient suppression of the endothelium-dependent relaxation to ACh, demonstrating a functional link between reduced endothelial hyperpolarization and altered endothelium-dependent relaxation induced by NAGly.

It is noteworthy that NAGly exhibited an inhibitory effect on both the forward and reversed NCX_{pm} and taking into account that the mode of NCX_{pm} operation in vascular endothelium may depend on incoming stimuli (Berra-Romani *et al.*, 2010) and vary between different vascular beds, it is tempting to speculate that in certain vascular beds, which respond to a specific agonist by stimulation of NCX_{pm}-mediated Ca^{2+} extrusion from the endothelium, NAGly may promote endothelium-dependent vasodilatation via NCX_{pm} inhibition. Importantly, NAGly appears to have complex effects on different key ion transport systems expressed in endothelial cells. Recently we showed that in cells overexpressing the key molecular constituents of SOCE, the stromal interacting molecule 1 (STIM1) and the pore-forming subunit of SOCE channels, Orai1, NAGly hampers SOCE primarily by uncoupling STIM1 from Orai1 (Deak *et al.*, 2013). Accordingly, that and our current data indicate that endogenous lipoaminoacid NAGly fine-tunes the interplay between crucial molecular players of cellular signalling. Recently, evidence has been obtained indicating that lipoamino acids including NAGly potently inhibit T-type Ca^{2+} channels (Barbara *et al.*, 2009; Ross *et al.*, 2009). Although endothelial cells commonly lack voltage-gated Ca^{2+} channels, some

reports do suggest the functional presence of these channels in endothelial cells. Hence, we performed additional experiments designed to exclude the possibility that the action of NAGly on NCX in our cell model is influenced by voltage-gated Ca^{2+} channels. Depolarizing voltage steps failed to induce inward currents in EA.hy926 cells.

Our data indicate that the inhibitory effect of NAGly on NCX_{pm} is not restricted to endothelial cells. Indeed, we showed here that in de-endothelialized NA-precontracted rat aortic segments, NAGly opposes the contraction, which is partially controlled by NCX_{pm}-mediated Ca^{2+} entry (Lagaud *et al.*, 1999; Zhang *et al.*, 2010). Therefore, it is likely that both NCX_{pm} inhibition and BK_{Ca} stimulation may account for the endothelium-independent component of relaxation. Although our findings indicate that the mechanism of the NAGly action on endothelial cells is not dependent on G-proteins and highlight a receptor-independent action, they do not rule out the possible involvement of as yet unidentified GPCRs sensitive to endocannabinoids in the endothelium-dependent component of the vascular relaxation reported earlier in a number of studies (Jarai *et al.*, 1999; Mukhopadhyay *et al.*, 2002; Offertaler *et al.*, 2003; Begg *et al.*, 2005). Because endothelial cells undergo significant changes in culture, some aspects of endothelial cell physiology, including expression of ion channels and receptors may be altered in cultured cells (Marchenko and Sage, 1993; Sandow and Grayson, 2009). These alterations may be relevant for endocannabinoid signalling mechanisms. More extensive electrophysiological studies using *in situ* vascular endothelium are required to resolve the enigma of the existence of still unidentified cannabinoid receptors in the vascular endothelium. Inasmuch as NCX_{pm} is implicated in ischaemic/reperfusion injury, we hypothesize that this endogenous lipid may exhibit cardio- and neuroprotection against myocardial ischaemia or brain insults. This still unrecognized feature of NAGly and possible alterations of endogenous levels of NAGly in ischaemic tissues warrant further investigation.

Collectively, the results of the present study demonstrate a previously unrecognized inhibitory effect of NAGly on NCX_{pm} activity, which largely controls endothelial cell function. In the vasculature, the action of NAGly does not appear to be limited to promoting endothelial hyperpolarization and vasodilatation, but it may also inhibit agonist-stimulated endothelial hyperpolarization induced by reversed NCX_{pm}. This effect on endothelial electrical signalling is accompanied by a transient reversal of endothelium-dependent vasodilatation. A better understanding of the role of NAGly and related lipid compounds in the mechanisms involved in the regulation and modulation of cardiovascular function in health and disease is important for the development of novel effective approaches for the management of cardiovascular disorders.

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Conflicts of interest

None to declare.

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