NAADP links histamine H1 receptors to secretion of von Willebrand factor in human endothelial cells

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Running Title: NAADP is crucial for histamine H1R Ca\textsuperscript{2+} signalling

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ABSTRACT

A variety of endothelial agonist-induced responses are mediated by rises in intracellular Ca\(^{2+}\), suggesting that different Ca\(^{2+}\) signatures could fine-tune specific inflammatory and thrombotic activities. In search of new intracellular mechanisms modulating endothelial effector functions, we identified NAADP as a crucial second messenger in histamine-induced Ca\(^{2+}\) release via H\(_1\) receptors (H\(_1\)R). NAADP is a potent intracellular messenger mobilizing Ca\(^{2+}\) from lysosome-like acidic compartments, functionally coupled to the endoplasmic reticulum. Using human EA.hy926 endothelial cell line and primary human umbilical vein endothelial cells (HUVEC) we show that selective H\(_1\)R activation increases intracellular NAADP levels and that H\(_1\)R-induced calcium release involves both acidic organelles and endoplasmic reticulum. To assess that NAADP links H\(_1\)R to Ca\(^{2+}\)-signalling we used both microinjection of self-inactivating concentrations of NAADP and the specific NAADP receptor antagonist, Ned-19, both of which completely abolished H\(_1\)R-but not thrombin-induced Ca\(^{2+}\) mobilization. Interestingly, H\(_1\)R mediated von Willebrand factor (vWF) secretion was completely inhibited by treatment with Ned-19 and by siRNA knockdown of two-pore channels NAADP receptors, while thrombin-induced vWF secretion failed to be affected. These findings demonstrate a novel and specific Ca\(^{2+}\)-signalling mechanism activated through H\(_1\)R in human endothelial cells revealing an obligatory role of NAADP in the control of vWF secretion.
INTRODUCTION

Endothelial cells represent the main regulator of vascular homeostasis, are involved in a variety of rapid and persistent inflammatory responses, such as expression of adhesion molecules and other singular biological processes (e.g. exocytosis, angiogenesis, nitric oxide release, membrane permeability) and respond to numerous hormonal and chemical signals as well as mechanical stimuli by releasing a variety of regulatory factors. Histamine, secreted by mast cells and basophils, represents an important regulator of many endothelial functions among which the inflammatory response, in particular of type I endothelial cell activation resulting in acute inflammation. The pleiotropic effects of histamine are triggered by activating one or more subtypes of receptor present in specific combinations in different cells. Four subtypes of G-protein-coupled receptors (H1, H2, H3, H4) are known, acting through different intracellular second messengers; in particular H1 is considered the most important in endothelial cells, regulating vascular permeability and exocytosis of Weibel-Palade bodies (WPBs). WPB, originally defined as an intracellular stores for von Willebrand factor, has been recently shown to contain an increasing number of other molecules, implicating a key role for WPBs exocytosis in inflammation, angiogenesis, hemostasis and vascular tone. It has been established that histamine binding to Gq-coupled H1 receptor activates phospholipase C-beta and increases intracellular calcium but limited information is available concerning the mechanisms underlying these processes in endothelium. In endothelial cells, the rise in intracellular Ca^{2+} induced by HRs mediates histamine inflammatory responses such as the increase of blood flow. These effects result from Ca^{2+}-mediated activation of: 1. phospholipase A2 and production of NO; 2. the localized vascular leakiness of plasma proteins; 3. the exocytosis of WPBs, following the activation of myosin-light-chain kinase (MLCK) by Ca^{2+}-calmodulin complex and phosphorylation of myosin light chain (MLC). Ca^{2+}, released from internal stores, is necessary and sufficient to trigger these histamine-mediated responses.

Ca^{2+} is a ubiquitous signal able to activate a variety of intracellular pathways and to mediate specific cellular responses. Release of Ca^{2+} from internal stores is controlled by various second messengers and by Ca^{2+} itself by a mechanism of Ca^{2+}-induced Ca^{2+} release. The best known second messengers able to regulate the release of Ca^{2+} from intracellular stores are inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR), and nicotinic acid-adenine dinucleotide phosphate (NAADP). In mammals, two different ectoenzymes known as CD38 and CD157, have been characterized, which are able to generate both cADPR and NAADP. However, in some cell types an alternative enzymatic activity has been demonstrated.
NAADP was first identified as a potent Ca\(^{2+}\)-mobilizing agent in sea urchin eggs and more recently in different mammalian cell types\(^\text{10,11}\). Ca\(^{2+}\) mobilization induced by NAADP elicits a variety of biological processes such as lymphocyte activation, exocytosis, platelet activation and neuronal growth and differentiation\(^\text{12-17}\). Interestingly, the Ca\(^{2+}\) stores operated by NAADP seem to be distinct from sarcoplasmic/endoplasmic reticulum and increasing evidence now suggests that NAADP targets lysosome-like acidic compartments\(^\text{18-21}\). Recently two-pore channels (TPCs) have been identified as NAADP receptors releasing Ca\(^{2+}\) from acidic organelles\(^\text{22,23}\), even though different NAADP receptors have been suggested, as the type 1 ryanodine receptors itself\(^\text{24}\) and the transient receptor potential mucolipin 1 channel (TRP-ML1), which both could function as NAADP-sensitive Ca\(^{2+}\) channels\(^\text{25}\).

It has been suggested that NAADP may serve as a universal cell Ca\(^{2+}\) trigger inducing an initial release of Ca\(^{2+}\), which is then amplified by Ca\(^{2+}\)-induced Ca\(^{2+}\) release. In pancreatic acinar cells NAADP induces Ca\(^{2+}\) release from both ER and acidic stores in the secretory granule area\(^\text{20}\) and interaction between these different compartments is required for the response to different agonists\(^\text{26}\). NAADP elicits Ca\(^{2+}\) responses in pancreatic and arterial smooth muscle cells via a two-pool mechanism\(^\text{27,28}\). Specifically, an initial Ca\(^{2+}\) burst is amplified by subsequent Ca\(^{2+}\) release mediated by ryanodine receptors (RyRs) and IP\(_3\) receptors from the ER\(^\text{18,28}\).

The role of NAADP signalling has so far been studied in a limited number of cellular systems where some extracellular stimuli, traditionally described coupled to the production of IP\(_3\), are related to NAADP-induced Ca\(^{2+}\) release from acidic stores. NAADP has been described as an important second messenger for different agonists such as cholecystokinin\(^\text{29}\), T-cell receptor agonist\(^\text{15}\), endothelin-1\(^\text{30}\), histamine\(^\text{9}\), glucagon-like peptide-1\(^\text{31}\), glutamate in the brain\(^\text{32}\) and more recently acetylcholine in endothelium\(^\text{33}\).

In the present work we demonstrate that NAADP is involved in histamine-induced Ca\(^{2+}\) release via H1 receptor in human endothelial cells. We show that stimulation of endothelial cells with the specific histamine H1 receptor agonist, 2-((3-Trifluoromethyl)phenyl)histamine dimaleate (TMPH), leads to an increase of intracellular NAADP levels. Moreover, we observed that H1R-induced calcium release involves both acidic organelles and ER. To assess the contribution of NAADP-induced calcium release to histamine signalling we used the specific NAADP receptor antagonist, Ned-1934, which completely abolished both TMPH-induced calcium release and the secretion of von Willebrand factor induced by TMPH but fails to affect thrombin-induced Ca\(^{2+}\) release and vWF secretion. Notably, double knockdown of TPC1 and TPC2 was found to abrogate vWF secretion following H1R stimulation. These data identify a novel pathway for H1 receptor signalling whereby receptor activation leads to intracellular Ca\(^{2+}\) release directly and specifically stimulated by
NAADP, which in turn regulates the exocytosis of von Willebrand factor from WPBs. These findings demonstrate for the first time the direct relationship between NAADP-mediated calcium release and the signalling mechanism underlying endothelium activation mediated by histamine.
METHODS

Materials

All materials were obtained from Sigma Aldrich (St. Louis, MO) except where it is stated otherwise below.

Cell culture

EA.hy926, human umbilical vein endothelial cells fused with a human pulmonary epithelial cell line (A549) from ATCC (Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS), 2% (w/v) hypoxanthine/aminopterin/thymidine (HAT media supplement), 200 μM l-glutamine, 100 U/ml penicillin/streptomycin (Life Technologies, Gaithersburg, MD). Primary cultures of human umbilical vein endothelial cells (HUVEC) (Lonza, Switzerland) were cultured in EBM-2 medium with a bullet kit (Lonza, Switzerland). Both cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Ca²⁺ imaging

EA.hy926 endothelial cell line cultured on 35-mm dishes were incubated in culture medium containing 3.5 μM fura-2-AM (Invitrogen Corporation, CA) for 1 h at 37°C, and then rinsed with Krebs-Henseleit-Hepes (KHH) buffer (140 mM Na⁺, 5.3 mM K⁺, 132.4 mM Cl⁻, 0.98 mM PO₄²⁻, 1.25 mM Ca²⁺, 0.81 mM Mg²⁺, 5.5 mM glucose and 20 mM Hepes) supplemented with 0.2% fatty acid free BSA or with Hanks’ balanced salt solution (HBSS). Each dish was placed into a culture chamber at 37°C on the stage of an inverted fluorescence microscope (Nikon, TE2000E), connected to a cooled CCD camera (512B Cascade, Roper Scientific, Tucson, AZ). Samples were illuminated alternately at 340 and 380 nm using a random access monochromator (Photon Technology International, NJ) and emission was detected using a 510 nm emission filter. Images were acquired (1 ratio image/s) using Metafluor software (Universal Imaging Corporation, Downingtown, PA). Calibration was obtained at the end of each experiment by maximally increasing intracellular Ca²⁺-dependent fura-2-AM fluorescence with 5 μM ionomycin, followed by recording minimal fluorescence in Ca²⁺-free medium. [Ca²⁺]ᵢ was calculated according to the previously described formulae.


Microinjection of high concentration of NAADP

Micropipettes were pulled from capillary glass with an internal filament and backfilled. The pipettes were then mounted in the electrode holder of an Injectman pressure injection system (Eppendorf, Hamburg, Germany) used at typical pressures of 200 hPa for 2 s, which produces ~1% injection volumes. Self-inactivating concentrations of NAADP were prepared at 50 mM, 100 times the final required concentration. Endothelial cells were then stimulated with 100 μM TMPH or 2 U/ml thrombin and Ca\(^{2+}\) release was measured using fluo-3-AM or fura-2-AM (Invitrogen Corporation, CA).

Acid extraction of NAADP from EA.hy926 and NAADP measurements

Cells cultured in 60-mm dishes in standard medium, were washed two times with HBSS and incubated for the appropriate time at room temperature with 50 μM BAPTA-AM and either 100 μM histamine or 100 μM TMPH. All liquid was removed and ice-cold 1.5 M HClO\(_4\) was added to stop reactions. Sonication was carried out to disrupt the cells and then all samples were centrifuged at 15,000 x g for 10 min at 4°C. Cellular pellet was stored at -80°C for later analysis, the supernatant was neutralized with an equal volume of 2 M KHCO\(_3\), and then centrifuged at 15,000 x g for 10 min at 4°C to remove the KClO\(_4\) precipitate. The resulting supernatant was stored at -80°C for radioreceptor assay analysis. NAADP levels were determined as previously described\(^37\). Briefly, standard or sample was incubated with L. pictus egg homogenate in intracellular medium for 10 min. Then, \(^{[32P]}\)NAADP was added to give approximately 50,000 scintillation counts per tube, with a final homogenate concentration of 0.5% (v/v). After a further incubation for 10 min, the reaction was separated by filtration on Whatmann GF/B filter papers using a Brandell cell harvester. Radioactivity was determined by storage phosphor detection, and sample concentrations interpolated from the standard curve.

Lysosomes and Ned-19 staining

Cells were incubated with 200 ng/ml LysoTracker Red (Invitrogen Corporation, CA) for 30 min and with 100 μM Ned-19.

Cells were viewed with a Zeiss 510 META confocal microscope, in multitrack mode to reduce bleed-through, using the following excitation/emission parameters (nm): Ned-19 (364/385-490), LTR (543/>560).
ELISA for von Willebrand Factor

An immunobind ELISA assay (American Diagnostic Inc., Stamford, CT) was performed to measure levels of vWF released in culture medium after stimulation with 100 μM TMPH, 100 μM histamine and 2 U/ml thrombin (Calbiochem, Merck, Germany). Confluent monolayer of HUVEC, grown in 6-well plates, were washed in HBSS and then incubated in OPTI-MEM I (Invitrogen Corporation, CA) in the presence or absence of the reported agonists for 20 min. Supernatants were collected and 100 μl of each sample was tested. vWF ELISA assay was performed using the same number of cells.

Design and transfection of TPC1 and TPC2 siRNA duplexes.
Small interfering RNA (siRNA) duplex oligonucleotides against the coding sequence of human TPC1 and TPC2 cDNA were designed and purchased from Integrated DNA Technologies (Coralville, IA). We selected two target sequences respectively for TPC1 and TPC2: 5’-rCrCrA rGrGrA rCrUrC rGrGrA rArGrU rUrGrA rUrGrG rUrGG C-3’ (sense), rGrCrC rArCrC rArUrC rArArC rUrUrC rCrGrA rArCrC rArArG rArUrG rArUrC rCrCrA rArUrG rArUrG rGrCrA (antisense) and 5’-rCrCrA rUrCrA rUrUrG rGrGrA rUrCrA rArCrU rUrGrU rUrUA G-3’ (sense), rCrUrA rArArC rArArG rUrUrG rArUrC rCrCrA rArUrG rArUrG rGrCrA (antisense). Transfection with 40nM of each siRNA in HUVEC was carried out by using 0.2% v/v Oligofectamine (Invitrogen), according to the manufacturer’s instructions. Fresh medium was added 4 h after transfection and experiments were conducted for 48 h. Non-targeting control siRNA-A (Santa Cruz, CA) was used as control.

Statistical analysis

Data are presented as the mean ± SEM of results from at least three independent experiments. A Student’s t test was used for statistical comparison between means where applicable.
RESULTS

Evaluation of histamine-induced Ca\textsuperscript{2+} release via H1 receptors

Stimulation with histamine is known to enhance accumulation of inositol phosphates and elevate intracellular Ca\textsuperscript{2+} concentration in different cell types\textsuperscript{2,38}. Four subtypes of surface receptors are known to mediate responses to histamine, all of which are G-protein-coupled receptors\textsuperscript{39}. As already described in literature\textsuperscript{40}, only mRNAs for H1 and H2 receptors are present in EA.hy926 and in HUVEC cells. We confirmed the expression of only H1 and H2, but not H3 and H4 through RT-PCR in EA.hy926 (data not shown). In order to characterize the contribution of the different histamine receptors to Ca\textsuperscript{2+} mobilization, we have stimulated our endothelial cells with specific agonists. Trifluoromethyl-phenyl-histamine dimaleate (TMPH), amthamine dihydrobromide, immethridine hydrobromide and clobenpropit dihydrobromide, which are specific agonists for H1 receptors, H2 receptors, H3 receptors and H4 receptors, respectively, were used. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} after stimulation with each agonist (Fig. 1A-B) shows that only TMPH, a selective agonist for H1 receptors, was able to trigger Ca\textsuperscript{2+} mobilization. Moreover, we observed that the selective H1 receptor antagonist, mepyramine, totally inhibits histamine-induced Ca\textsuperscript{2+} release. The calcium response to TMPH was found to be dose-dependent (data not shown), and the submaximal concentration of 100 \textmu M was chosen for subsequent experiments.

Involvement of NAADP-sensitive Ca\textsuperscript{2+} stores in H1 receptor signalling

To evaluate the involvement of different intracellular compartments in the Ca\textsuperscript{2+} response to TMPH, we adopted a pharmacological approach. Bafilomycin A1 is known to inhibit the vacuolar-type H\textsuperscript{+}-ATPase pump of acidic organelles, preventing the acidification of these compartments by blocking the Ca\textsuperscript{2+} reuptake into acidic stores via the Ca\textsuperscript{2+}/H\textsuperscript{+}-exchanger\textsuperscript{41}. Cells were treated with 500 nM bafilomycin A1, or with vehicle alone, and then stimulated with 100 \textmu M TMPH (Fig. 2A-C). As shown, bafilomycin A1 partially, but significantly, impaired TMPH-induced Ca\textsuperscript{2+} release, suggesting the involvement of acidic organelles in TMPH-induced Ca\textsuperscript{2+} release. Furthermore, treatment with 1 \textmu M thapsigargin (Fig. 2B), which functions by inhibiting SERCA, completely blocked TMPH-induced Ca\textsuperscript{2+} mobilization from the endoplasmic reticulum. As described in other models\textsuperscript{11} also in TMPH-stimulated EA.hy926 endothelial cells Ca\textsuperscript{2+} release requires the integrity of both the endoplasmic reticulum and acidic stores (Fig. 2D). For comparison, parallel experiments with bafilomycin and thapsigargin were performed (in the same conditions as above) in cells
stimulated with 2 U/ml thrombin, a concentration found to be submaximal in dose-response experiments (not shown). As shown in Fig. 2E Ca\textsuperscript{2+} release in response to thrombin involves endoplasmic reticulum stores but not acidic compartment.

**Direct effect of NAADP in [Ca\textsuperscript{2+}]\textsubscript{i} mobilization**

Since cell stimulation with extracellular NAADP failed to induce intracellular Ca\textsuperscript{2+} release (not shown), we used NAADP-AM, a cell-permeant analogue of NAADP known to induce NAADP-mediated Ca\textsuperscript{2+} signalling\textsuperscript{42}. In cells stimulated with NAADP-AM increase in [Ca\textsuperscript{2+}]\textsubscript{i} was induced compared to cells challenged with vehicle alone (Fig. 3A-B).

We further tested the involvement of NAADP in H1 receptor signalling by desensitizing the NAADP receptor by microinjecting high concentrations of NAADP as demonstrated in previous studies\textsuperscript{15,43,44}. As shown in Fig. 3, microinjection of 500 μM NAADP inhibited TMPH- but not thrombin-induced intracellular Ca\textsuperscript{2+} increase, compared to control cells microinjected with the vehicle alone. Figures 3E and 3F show the average ratio F/F₀ of all cells microinjected corresponding to [Ca\textsuperscript{2+}]\textsubscript{i}.

Taken together, these data demonstrate that Ca\textsuperscript{2+} induced by NAADP plays a specific and primary role in endothelial cells histamine H1 receptors signalling.

**TMPH stimulates NAADP production in endothelial cells**

It has been recently demonstrated that in different cell types a variety of agonists can stimulate NAADP production\textsuperscript{32,37}. Generation of NAADP in response to agonist stimulation demonstrates an important role of this second messenger in the release of Ca\textsuperscript{2+} from intracellular stores\textsuperscript{43,45,46}. Figure 4 shows intracellular NAADP measurement in endothelial cells after stimulation with TMPH or with vehicle alone. In this experiment we performed a radioreceptor binding assay as described by Lewis et al.\textsuperscript{37}. As shown, TMPH leads to a time-dependent rise in intracellular NAADP levels, confirming the correlation between H1 receptor and NAADP as specific second messenger. At variance with the kinetics of NAADP increase induced by TMPH, thrombin stimulation resulted in a high but delayed and transient increase in NAADP synthesis with a peak after 1 min and reversion to basal level within 2 min (Supplemental Fig. 1S).
NAADP receptor has a primary role in TMPH- and histamine-induced Ca$^{2+}$ mobilization

By means of a computer-based virtual screening, Naylor and colleagues have recently identified a new compound, Ned-19, which fluorescently labels the NAADP receptor and blocks NAADP-induced Ca$^{2+}$ signalling in intact cells$^{34}$. To date, Ned-19 appears to be an uncompetitive antagonist of NAADP.

We show that pre-treatment of EA.hy926 cells for 20 min with 50 μM Ned-19 blocks both histamine- and TMPH-induced Ca$^{2+}$ release (Figs. 5B and 5D-E). The same experiments performed in HUVEC confirmed the inhibitory effect of Ned-19 on histamine- and TMPH-induced Ca$^{2+}$ mobilization (data not shown). After treatment with Ned-19, cells were still responsive to GPN (Fig. 5B) which proves that the integrity of acidic stores was maintained. To validate the selectivity of Ned-19 in endothelial cells, we stimulated cells with thrombin after treatment with Ned-19. Ned-19 did not affect calcium mobilization induced by submaximal doses of thrombin either in EA.hy926 cells (Fig. 5C) or HUVEC (not shown). The observation that, in both endothelial cell types investigated, inhibition of the NAADP receptor leads to total disappearance of the TMPH-induced intracellular Ca$^{2+}$ rise, but has no effect on thrombin-induced Ca$^{2+}$ mobilization, demonstrates that NAADP is specifically fundamental for H1 receptor signalling.

As Ned-19 is fluorescent$^{34}$, we labelled EA.hy926 cells with Ned-19 and with lysotracker red (Fig. 5F-I). Ned-19 mostly (about 90%) colocalizes with lysotracker red positive organelles but it is possible to observe a population of non acidic organelles displaying Ned-19 fluorescence as well as acidic organelles lacking the NAADP receptor. These data suggest that in endothelial cells the NAADP receptor is expressed in different kinds of intracellular organelles but not all of these organelles are characterized by low pH.

Role of NAADP in regulating vWF secretion

Endothelial exocytosis is one of the earliest responses to vascular damage and plays a pivotal role in thrombosis and inflammation$^{47}$. Von Willebrand factor is the major component inside Weibel-Palade bodies (WPBs), the secretory organelles of endothelial cells that also store other vascular modulators$^{48}$. To evaluate whether NAADP is involved in the exocytosis of vWF mediated by histamine we chose to use primary HUVEC at early passages. The release of vWF, derived by the fusion of WPBs with the plasma membrane, was detected by assaying the culture media. To establish a specific and direct link between the histamine H1R-triggered WPBs exocytosis and the NAADP-mediated Ca$^{2+}$-dependent exocytotic events, we measured the acute release of vWF under
conditions where NAADP action was blocked. Confluent HUVEC monolayers were incubated with 100 μM Ned-19 or with vehicle for 30 min prior to stimulation with either 100 μM histamine, or TMPH, or with 2 U thrombin for 20 min at 37°C. As shown in Fig.6A-B, vWF secretion has been evaluated by ELISA assay on culture medium. Histamine, TMPH and thrombin all induce secretion of vWF in the medium. As shown, TMPH induced-vWF release is completely inhibited by treatment with Ned-19 while Ned-19 fails to affect thrombin-induced vWF secretion. Secretion mediated by histamine is only partially altered by Ned-19 which suggested a NAADP independent contribution from histamine receptors other than H1R. In fact Ned-19 could abolish histamine-induced vWF secretion in cells in which H2R was inhibited by 100 μM cimetidine (Fig. 6B). These data underline a primary role of NAADP as fundamental second messenger specifically involved in H1R signalling in endothelial cells. Recently it has been described that TPC1 and TPC2 represent specific receptors for NAADP. In our cells TPC1:TPC2 relative expression, evaluated by Real Time PCR, was found to be 9:1. To further verify the fundamental role of NAADP in the secretion of vWF stimulated through H1R, we performed experiments of genetic silencing of TPC NAADP receptors followed by evaluation of vWF release. Primary HUVEC were transfected with siRNAs specific for TPC1 and TPC2, and 48h later we measured the acute release of vWF stimulated by 100 μM TMPH for 20 min at 37°C. The efficiency of TPC receptors downregulation, evaluated by qRT-PCR, was found to average about 80% for TPC1 and 40% for TPC2. As shown in Fig. 7, combined down-regulation of TPC1 and TPC2 receptors completely inhibits the secretion of vWF induced by TMPH, which is unaffected in cells transfected with non-targeting control siRNA. The observation that TPCs silencing fails to affect the response to thrombin reinforces our previous evidence and provides more extensive support for a specific link between NAADP and exocytosis of WPBs.
DISCUSSION

In the present study we have focused on endothelial cells activation by histamine signalling as a prototype pathway regulating acute inflammation. The identification and targeting of key signalling factors regulating vascular inflammation which, in turn, contributes to the development of a variety of diseases or pathological processes could in fact represent a promising area of drug development and control of inflammation. It has long been known that histamine plays a fundamental role in driving immune and inflammatory response of endothelial cells. The effects of histamine on endothelial cells, operated through histamine H1 receptors, lead directly to an increase in vascular permeability, increased blood flow and upregulation in adhesion molecules allowing for leukocytes adhesion, rolling and extravasation\(^1\). In addition, the acute release of intracellularly stored factors in WPBs, achieved by secretagogues as histamine and thrombin through intracellular Ca\(^{2+}\)-release, is one of the main mechanisms controlling homeostasis in the vascular system\(^47\).

The aim of the present study was to characterize the calcium signalling pathway activated through histamine H1 receptors stimulation and its mechanistic involvement in the biological functions of endothelial cells. Interestingly, recent data suggest that the calcium-mobilizing second messenger NAADP may function as a universal intracellular Ca\(^{2+}\) trigger of cells and since the rise in intracellular calcium plays a major role in vascular activation mediated by histamine, we investigated its effects in human endothelial cells. Similarly to what was previously described\(^{40}\) we report the expression of both histamine H1 and H2 receptors in EA.hy926 endothelial cell line and primary HUVEC. The current view is that the increase in [Ca\(^{2+}\)]\(_i\), induced by inflammatory agonists such as thrombin and histamine is achieved by the generation of inositol 1,4,5-trisphosphate (IP\(_3\)), activation of IP\(_3\)-receptors (IP\(_3\)-R), release of stored intracellular Ca\(^{2+}\), and Ca\(^{2+}\) entry through plasma membrane channels. We shed new light on this specific signalling pathway and the main finding of this report is that NAADP plays an essential role in histamine H1R-induced Ca\(^{2+}\) release in endothelial cells, thereby regulating endothelial secretory response.

Several lines of evidence support the notion that NAADP represents an H1 second messenger and plays an obligatory role for H1R-mediated calcium signalling in human endothelial cells. First of all, specific H1 stimulation is abrogated by microinjection of inactivating micromolar NAADP concentrations. Secondly, a powerful and recently described chemical probe that specifically blocks NAADP signalling, Ned-1934\(^{34}\), completely inhibited Ca\(^{2+}\) response following the specific stimulation of histamine H1 receptors while failing to affect thrombin-Ca\(^{2+}\) response. Thirdly, NAADP levels are consistently enhanced after challenging endothelial cells with the specific agonist of histamine H1 receptors, TMPH. Moreover, silencing of NAADP receptors obtained by siRNAs knockdown of
TPC1/2 was found to abolish the secretion of vWF following selective histamine H1R activation with TMPH.

The biphasic Ca$^{2+}$ response to NAADP and the dependence of the sustained phase on IP$_3$-R and the ER are regarded as consistent with the idea that NAADP-induced Ca$^{2+}$ signals are small and localized, but able to act as triggers for larger global intracellular Ca$^{2+}$ changes through coupling to ER system$^{11}$. Our data obtained through pharmacological impairment of Ca$^{2+}$ release from different Ca$^{2+}$ store compartments appear to confirm this model. In fact, treatment of cells with thapsigargin (the endoplasmic SERCA inhibitor) as well as with bafilomycin (the inhibitor of vacuolar H$^+$-ATPase), impaired TMPH-induced Ca$^{2+}$ mobilization. Furthermore, consistent with a role for lysosome-like compartments in this process, the vacuolar H$^+$-ATPase inhibitor bafilomycin A1, which inhibited the response to TMPH, failed to affect the increase of intracellular Ca$^{2+}$ induced by thrombin, which directly activates endoplasmic reticulum (ER) Ca$^{2+}$ release. Moreover, our data indicate that NAADP receptors in endothelial cells are mainly localized within acidic organelles, though Ned-19 labels also a small fraction of non acidic organelles suggesting a complex and heterogenous distribution of NAADP receptors within different cytoplasmic vesicles, such as endosomes, reported to represent additional NAADP sensitive stores in pancreatic acinar cells$^{26}$. The fact that the localization is not completely identical demonstrates that this results is not simply due to bleed-through between the two channels used for the different dyes. These data are in agreement with the pharmacology of histamine H1 receptors Ca$^{2+}$ signalling indicating that the integrity of both the endoplasmic reticulum and acidic stores is fundamental for H1R signalling. It remains unclear whether NAADP can release Ca$^{2+}$ from thapsigargin-sensitive ER stores by acting on the same NAADP receptors present on lysosomes or upon a contribution of different receptor type. To this purpose, it is worth to mention that there are also evidence for RyR1 acting as an NAADP-sensitive Ca$^{2+}$ channel in some cell systems.

The increase in intracellular concentration of NAADP in response to agonist stimulation bestows to NAADP a crucial role as second messenger in the release of Ca$^{2+}$ from intracellular stores. By means of a radioreceptor binding assay we demonstrate that stimulation of endothelial cells with TMPH led to a time-dependent rise in intracellular NAADP levels. Our findings show a direct correlation between the H1 receptor engagement and the increase of intracellular NAADP.

In endothelial cells secretory granules known as Weibel-Palade bodies (WPBs) contain pro-inflammatory and pro-thrombotic proteins such as von Willebrand factor (vWF) and other vascular modulators$^4$. These characteristic granules are present in different amounts in established endothelial cell lines, i.e., are particularly abundant in HUVEC and less represented in EA.hy926 cells (not shown). When endothelial cells are activated by appropriate stimuli, the content of WPBs
are released extracellularly\textsuperscript{49}. Proteins contained in WPBs have been shown to promote neutrophil and platelet adhesion to vessel walls as well as vascular inflammation\textsuperscript{47}. Therefore, the inhibition and regulation of endothelial cell exocytosis appear to play a fundamental role in downregulating inflammation and vascular thrombosis. It is known that increased levels of cytosolic free Ca\textsuperscript{2+} concentration are implicated in the mechanism of exocytosis of vWF from WPBs. We provide evidence on HUVEC that secretion of vWF through activation of H1R is completely dependent upon NAADP signalling. Our data show that Ned-19, the specific inhibitor of NAADP receptor, completely blocked TMPH-induced vWF secretion while, conversely, failed to affect the release of vWF stimulated by thrombin. Intriguingly, although the magnitude of the NAADP response was larger with thrombin than TMPH, this delayed and transient increase is neither responsible for calcium response nor for secretion of vWF. This surprising result reveals that it is not just the amount of NAADP produced but also where it is produced that determines the response, in this case the release of vWF, and suggests the involvement of IP3 rather than NAADP in thrombin-mediated stimulation. We observed that Ned-19 could significantly but not completely inhibit the secretory response to histamine, which suggests the engagement of an additional NAADP-independent histamine receptor. In fact following inhibition of H2R, known to be coupled to cAMP, vWF secretion stimulated by histamine was abolished by Ned-19. This observation is in line with previous literature reporting that cAMP, besides Ca\textsuperscript{2+}, is involved in vWF secretion\textsuperscript{50}.

The combined analysis of data from pharmacological NAADP inhibition and from TPCs downregulation demonstrates that NAADP totally and specifically controls calcium signalling and the resulting vWF secretion mediated through H1R but not those elicited by the control agonist thrombin. A side observation from our experiments is that the involvement of TPCs in the secretory response to histamine is not obligatory, suggesting the possible existence of some indirect crosstalk between the signalling pathways linked to H1R and H2R, the latter implying also cAMP production. It could be speculated that histamine engagement of both H1 and H2 receptors through cAMP production possibly results in the recruitment of additional NAADP receptors other than TPCs.

In conclusion, we demonstrate that NAADP is a specific and essential regulator of histamine H1 receptor in endothelial cells. Besides the well studied mechanism of H1R signal transduction via IP\textsubscript{3}, we identified NAADP as novel second messenger in H1R-induced Ca\textsuperscript{2+} release. It is well known that the responses of endothelial cells to histamine span a very wide spectrum, only partially explained by the multiplicity of its known receptors. Our data shed new light on histamine signalling and introduce a new player, hence a new level of complexity, into the specific mechanisms of calcium-regulated activities triggered in endothelial cells by a single histamine.
receptor. They also show that NAADP pathway is involved in a relevant biological function, granule exocytosis evaluated as vWF secretion and, notably, that this pathway is obligatory when H1R activation takes place in conditions of strict selectivity. In our opinion these findings represent a starting point to explore the possible involvement of NAADP in other endothelial functions and, more generally, could open to the stimulating issue of how calcium rises and endothelial cell responses to H1R engagement are finely regulated to result in specifically different cellular responses. Furthermore, histamine driven inflammation following the activation of endothelial cells through an exclusive coupling between H1 receptors and NAADP signalling could possibly be targeted as an experimental new approach for management of vascular diseases, directly focused on downstream signalling for specific histamine-mediated vascular endothelial cell responses.
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AUTHORSHIP CONTRIBUTIONS

BE: designed and performed the experiments, analyzed and collected data, co-wrote the paper; GG: conceived and performed some experiments, analyzed data; AML: performed experiments, analyzed data, performed statistical analysis and contributed to paper writing; FP: analyzed data and contributed to paper writing; AD: performed experiments; LXT: performed selected experiments; AAG: performed experiments and edited the paper; EZ: analyzed data and edited the paper; AG: analyzed data and edited the paper; GCC: designed research, contributed new reagents, analyzed data and edited the paper; AF: designed and supervised research and the experiments, analyzed and interpreted data, co-wrote the paper.

CONFLICT OF INTEREST DISCLOSURES

The authors declare no conflict of interest.
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FIGURE LEGENDS

Figure 1. Ca²⁺ release due to stimulation of different histamine receptors in EA.hy926 endothelial cells. Cells were treated with the following histamine receptor agonists at 100 μM: trifluoromethyl-phenyl-histamine (TMPH; H1 selective), amthamine dihydrobromide (H2 selective), immethridine dihydrobromide (H3 selective), clobenpropit dihydrobromide (H4 selective), and histamine in the presence of mepyramine, a selective H1 antagonist. (A) Bars show the increase in [Ca²⁺]i after stimulation with each agonist. Error bars represent SEM; n = 3 independent experiments. (B) Representative calcium traces of experiment shown in (A).

Figure 2. Role of NAADP-sensitive Ca²⁺ stores in H1 receptor signalling. Ca²⁺ release in cells stimulated with 100 μM TMPH following treatment with (A) vehicle alone (control); (B) 1 μM thapsigargin for 15 min; and (C) 500 nM bafilomycin A1 for 1 h. Maximum Ca²⁺ concentrations following 100μM TMPH stimulation (D) and 2U/ml thrombin (E) are summarized; error bars represent SEM; n= 3; *student’s t-test p < 0.05.

Figure 3. Direct effect of NAADP on [Ca²⁺] mobilization. (A) Representative plot of NAADP-AM-induced Ca²⁺ mobilization in EA.hy926 cells. (B) Increase in F/F₀ in cells stimulated with NAADP-AM compared to cells stimulated with vehicle alone (control). Cells were stimulated with 100 μM TMPH following microinjection with (C) vehicle alone or (D) inactivating concentrations of NAADP. Increase in F/F₀ in response to TMPH (E) and 2 U/ml thrombin (F) in cells microinjected with the inactivating concentration of 500 μM NAADP compared to cells microinjected with vehicle alone. Where applicable error bars represent SEM; n = 3 independent experiments; * student’s t-test p < 0.05.

Figure 4. NAADP levels. NAADP levels measured over time in EA.hy926 endothelial cells stimulated with 100 μM TMPH and control cells. The absolute resting concentration of NAADP was 1.77488+/-0.65 pmol/mg protein. Where applicable error bars represent SEM; n = 2-3 independent experiments; *student’s t-test p < 0.05.

Figure 5. Inhibition of the NAADP receptor using the selective antagonist Ned-19. (A) EA.hy926 cells were treated with vehicle alone for 20 min and then stimulated with TMPH followed by histamine (representative trace). (B) Cells were treated with 50 μM Ned-19 for 20 min followed by stimulation with TMPH, histamine and then 50 μM GPN (representative trace). (C) Cells were treated with 50 μM Ned-19 for 20 min then stimulated or not with 2U/ml thrombin. (D) Summary of responses to TMPH. (E) Summary of responses to histamine. Images show (F) Ned-19 staining, (G) lysotracker red staining, (H) merged image and (I) bright field image.
Cells were viewed on a Zeiss 510 META confocal microscope with a plan apochromat objective 63x oil immersion aperture 1.4, at room temperature in Hank’s buffer, and analyzed with LSM Software.

Where applicable error bars represent SEM; n = 3 independent experiments; *student’s t-test p < 0.05.

**Figure 6. NAADP is essential for vWF release specifically activated through H1R.** Measurement of secreted vWF by ELISA assay in HUVEC. Cells were treated either with vehicle alone or with 100 μM Ned-19 or with 100 μM cimetidine (H2R antagonist), or with 100 μM Ned-19 and 100μM cimetidine for 30 min prior to stimulation with agonists as indicated in (A) and (B); cime stand for cimetidine. Error bars represent SEM; n = 3 independent experiments; * student’s t-test p < 0.05.

**Figure 7. Specific effect of TPC1 and TPC2 receptors silencing on vWF release through H1R.** ELISA assay of secreted vWF by in HUVEC. Cells were treated with non targeting siRNA (black bars) or with TPCs receptors siRNA (white bars) and then stimulated with the indicated agonists. Error bars represent SEM; n=2 independent experiments; * student’s t-test p < 0.05.
Figure 1

A  

\[ \Delta [Ca^{2+}]_i \]

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>TMPH</th>
<th>Amphetamine</th>
<th>Immetridine</th>
<th>Clobenpropit</th>
<th>Histamine</th>
<th>Mepyramine</th>
<th>Histamine</th>
</tr>
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</table>

B  

- **TMPH**: Increase in \([Ca^{2+}]_i\) followed by a decrease.
- **Amphetamine**: No significant change.
- **Immetridine**: No change.
- **Clobenpropit**: No change.
- **Histamine**: Increase in \([Ca^{2+}]_i\) with a peak at 60 seconds.
- **Mepyramine**: No change.

\([Ca^{2+}]_i\) levels measured over time (sec): 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220.
Figure 4

NAADP (fold change) vs. Time (min)

TMPH vs. Control

* denotes statistically significant difference.
Figure 5

A. [Ca^2+], Control, Histamine, TMPH, Time (sec)

B. [Ca^2+], Ned-19, Histamine, TMPH, GPN, Time (sec)

C. Δ[Ca^2+], Vehicle, Thrombin

D. Δ[Ca^2+], Vehicle, Ned-19, TMPH 100μM

E. Δ[Ca^2+], Vehicle, Ned-19, Histamine 100μM

Figure 6

(A) Secreted vWF (mU/ml)

- Control
- TMPH
- Thrombin

(B) Secreted vWF (mU/ml)

- Control
- Histamine

* indicates statistical significance.
Figure 7

Secreted vWF (mU/ml)

Control  |  TMPH  |  Histamine  |  Thrombin

* Significant difference compared to Control
NAADP links histamine H1 receptors to secretion of von Willebrand factor in human endothelial cells

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