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

Bianca Esposito,[1] Guido Gambara,[1] Alexander M. Lewis,[2] Fioretta Palombi,[1] Alessio D'Alessio,[1] Lewis X. Taylor,[2] Armando A. Genazzani,[3] Elio Ziparo,[1] Antony Galione,[2] Grant C. Churchill,[2] and Antonio Filippini[1]

[1]Istituto Pasteur-Fondazione Cenci Bolognetti, Department of Anatomy, Histology, Forensic Medicine and Orthopedic, Section of Histology and Medical Embryology, Sapienza University of Rome, Rome, Italy; [2]Department of Pharmacology, University of Oxford, Oxford, United Kingdom; and [3]Department of Chemical, Food, Pharmaceutical and Pharmacological Sciences and Drug and Food Biotechnology Center, Università del Piemonte Orientale, Novara, Italy

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 nicotinic acid adenine dinucleotide phosphate (NAADP) as a crucial second messenger in histamine-induced Ca^{2+} release via H1 receptors (H1R). NAADP is a potent intracellular messenger mobilizing Ca^{2+} from lysosome-like acidic compartments, functionally coupled to the endoplasmic reticulum. Using the human EA.hy926 endothelial cell line and primary human umbilical vein endothelial cells, we show that selective H1R activation increases intracellular NAADP levels and that H1R-induced calcium release involves both acidic organelles and the endoplasmic reticulum. To assess that NAADP links H1R to Ca^{2+}-signaling we used both microinjection of self-inactivating concentrations of NAADP and the specific NAADP receptor antagonist, Ned-19, both of which completely abolished H1R-induced but not thrombin-induced Ca^{2+} mobilization. Interestingly, H1R-mediated von Willebrand factor (VWF) secretion was completely inhibited by treatment with Ned-19 and by siRNA knockdown of 2-pore channel NAADP receptors, whereas thrombin-induced VWF secretion failed to be affected. These findings demonstrate a novel and specific Ca^{2+}-signaling mechanism activated through H1R in human endothelial cells, which reveals an obligatory role of NAADP in the control of VWF secretion. (*Blood*. 2011;117(18):4968-4977)

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 biologic processes (eg, 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.[1] Histamine, secreted by mast cells and basophils, represents an important regulator of many endothelial functions such as the inflammatory response, in particular the type I endothelial cell activation, which results in acute inflammation.[1] The pleiotropic effects of histamine are triggered by activating one or more subtypes of the 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[2]; in particular H1 is considered the most important in endothelial cells, regulating vascular permeability and exocytosis of Weibel-Palade bodies (WPBs).[3] WPBs, originally defined as intracellular stores for von Willebrand factor (VWF), have 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.[4] It has been established that histamine binding to Gq protein-coupled H1 receptor activates phospholipase C-β and increases intracellular calcium,[5] 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, after 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.[6]

Ca^{2+} is a ubiquitous signal that is able to activate a variety of intracellular pathways and mediate specific cellular responses. Release of Ca^{2+} from internal stores is controlled by various second messengers and by Ca^{2+} itself through 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).[7] In mammals, 2 different ectoenzymes known as CD38 and CD157 have been characterized that are able to generate both cADPR and NAADP.[8] However, in some cell types an alternative enzymatic activity has been demonstrated.[9]

NAADP was first identified as a potent Ca^{2+}-mobilizing agent in sea urchin eggs and more recently in different mammalian cell types.[10,11] Ca^{2+} mobilization induced by NAADP elicits a variety

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of biologic processes such as lymphocyte activation, exocytosis, platelet activation, and neuronal growth and differentiation.[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.[18-21] Recently, 2-pore channels (TPCs) have been identified as NAADP receptors releasing Ca^{2+} from acidic organelles,[22,23] even though different NAADP receptors have been suggested, such as the type 1 ryanodine receptor[24] and the transient receptor potential mucolipin 1 channel (TRP-ML1), which could function as NAADP-sensitive Ca^{2+} channels.[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 the endoplasmic reticulum (ER) and acidic stores in the secretory granule area,[20] and interaction between these different compartments is required for the response to different agonists.[26] NAADP elicits Ca^{2+} responses in pancreatic and arterial smooth muscle cells via a 2-pool mechanism.[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.[18,28]

So far, the role of NAADP signaling has been studied in a limited number of cellular systems where some extracellular stimuli, traditionally described as 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,[29] T-cell receptor agonist,[15] endothelin-1,[30] histamine,[9] glucagon-like peptide-1,[31] glutamate in the brain,[32] and, more recently, acetylcholine in endothelium.[33]

In this work, we demonstrate that NAADP is involved in histamine-induced Ca^{2+} release via the H1R 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 signaling we used the specific NAADP receptor antagonist, Ned-19,[34] which completely abolished both TMPH-induced calcium release and the secretion of VWF 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 after H1R stimulation. These data identify a novel pathway for H1 receptor signaling whereby receptor activation leads to intracellular Ca^{2+} release directly and specifically stimulated by NAADP, which in turn regulates the exocytosis of VWF from WPBs. These findings demonstrate for the first time the direct relationship between NAADP-mediated calcium release and the signaling mechanism underlying endothelium activation mediated by histamine.

Methods

Materials

All materials were obtained from Sigma-Aldrich except where otherwise stated.

Cell culture

The EA.hy926, human umbilical vein endothelial cells (HUVECs) fused with a human pulmonary epithelial cell line (A549)[35] from ATCC, were cultured in DMEM (Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS), 2% (wt/vol) hypoxanthine/aminopterin/thymidine (HAT media supplement), 200μM l-glutamine, 100 U/mL penicillin/streptomycin (Life Technologies). Primary cultures of HUVECs (Lonza) were cultured in EBM-2 medium with a bullet kit (Lonza). Both cell lines were cultured at 37°C in a 5% CO_2-humidified atmosphere.

Ca^{2+} imaging

EA.hy926 endothelial cell line cultured on 35-mm dishes were incubated in culture medium containing 3.5μM fura-2-AM (Invitrogen) for 1 hour at 37°C, and then rinsed with Krebs-Henseleit-Hepes (KHH) buffer (140mM Na^+, 5.3mM K^+, 132.4mM Cl^-, 0.98mM PO_4^{2-}, 1.25mM Ca^{2+}, 0.81mM Mg^{2+}, 5.5mM glucose, and 20mM 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), and connected to a cooled CCD camera (512B Cascade; Roper Scientific). Samples were illuminated alternately at 340 and 380 nm using a random access monochromator (Photon Technology International) and emission was detected using a 510-nm emission filter. Images were acquired (1-ratio image/s) using Metafluor software (Universal Imaging Corporation). Calibration was obtained at the end of each experiment by maximally increasing intracellular Ca^{2+}-dependent fura-2-AM fluorescence with 5μM ionomycin, followed by recording minimal fluorescence in a Ca^{2+}-free medium. $[Ca^{2+}]_i$ was calculated according to the formulas previously described.[36]

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) used at typical pressures of 200 hPa for 2 seconds, which produces ~1% injection volumes. Self-inactivating concentrations of NAADP were prepared at 50mM (100× the final required concentration). Endothelial cells were then stimulated with 100μM TMPH or 2 U/mL thrombin, and Ca^{2+} release was measured using fluo-3-AM or fura-2-AM (Invitrogen).

Acid extraction of NAADP from EA.hy926 and NAADP measurements

Cells cultured in 60-mm dishes in standard medium were washed 2 times with HBSS and incubated for the appropriate time at room temperature with 50μM BAPTA-AM and either 100μM histamine or 100μM TMPH. All liquid was removed and ice-cold 1.5M $HClO_4$ was added to stop reactions. Sonication was carried out to disrupt the cells and then all samples were centrifuged at 15 000*g* for 10 minutes at 4°C. Cellular pellet was stored at −80°C for later analysis, the supernatant was neutralized with an equal volume of 2M $KHCO_3$, and then centrifuged at 15 000*g* for 10 minutes 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 minutes. Then, [^{32}P]NAADP was added to give approximately 50 000 scintillation counts per tube, with a final homogenate concentration of 0.5% (vol/vol). After a further incubation for 10 minutes, 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 (LTR; Invitrogen) for 30 minutes and with 100μM Ned-19. Cells were viewed with on a Zeiss 510 META confocal microscope in multitrack mode to reduce bleed-through, using the excitation/emission parameters 364 nm/385-490 nm for Ned-19 and 543 nm/> 560 nm for LTR.

ELISA for VWF

An immunobind ELISA assay (American Diagnostic Inc) was performed to measure levels of VWF released in culture medium after stimulation with 100μM TMPH, 100μM histamine, and 2 U/mL thrombin (Calbiochem). Confluent monolayers of HUVECs, grown in 6-well plates, were washed in HBSS and then incubated in OPTI-MEM I (Invitrogen) in the presence or absence of the reported agonists for 20 minutes. 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. We selected 2 target sequences for TPC1 and TPC2, respectively: 5′-rCrCrA rGrGrA rCrUrC rGrGrA rArGrU rUrGrA rUrGrG rUrGG C-3′ (sense), rGrCrC rArCrC rArUrC rArArC rUrUrC rCrGrA rGrUrC rCrUrG rGrUrU (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 HUVECs was carried out using 0.2% vol/vol Oligofectamine (Invitrogen) according to the manufacturer's instructions. Fresh medium was added 4 hours after transfection and experiments were conducted for 48 hours. Nontargeting control siRNA-A (Santa Cruz Biotechnology) was used as a control.

Statistical analysis

Data are presented as the mean ± SEM of results from at least 3 independent experiments. A Student *t* test was used for statistical comparison between means where applicable.

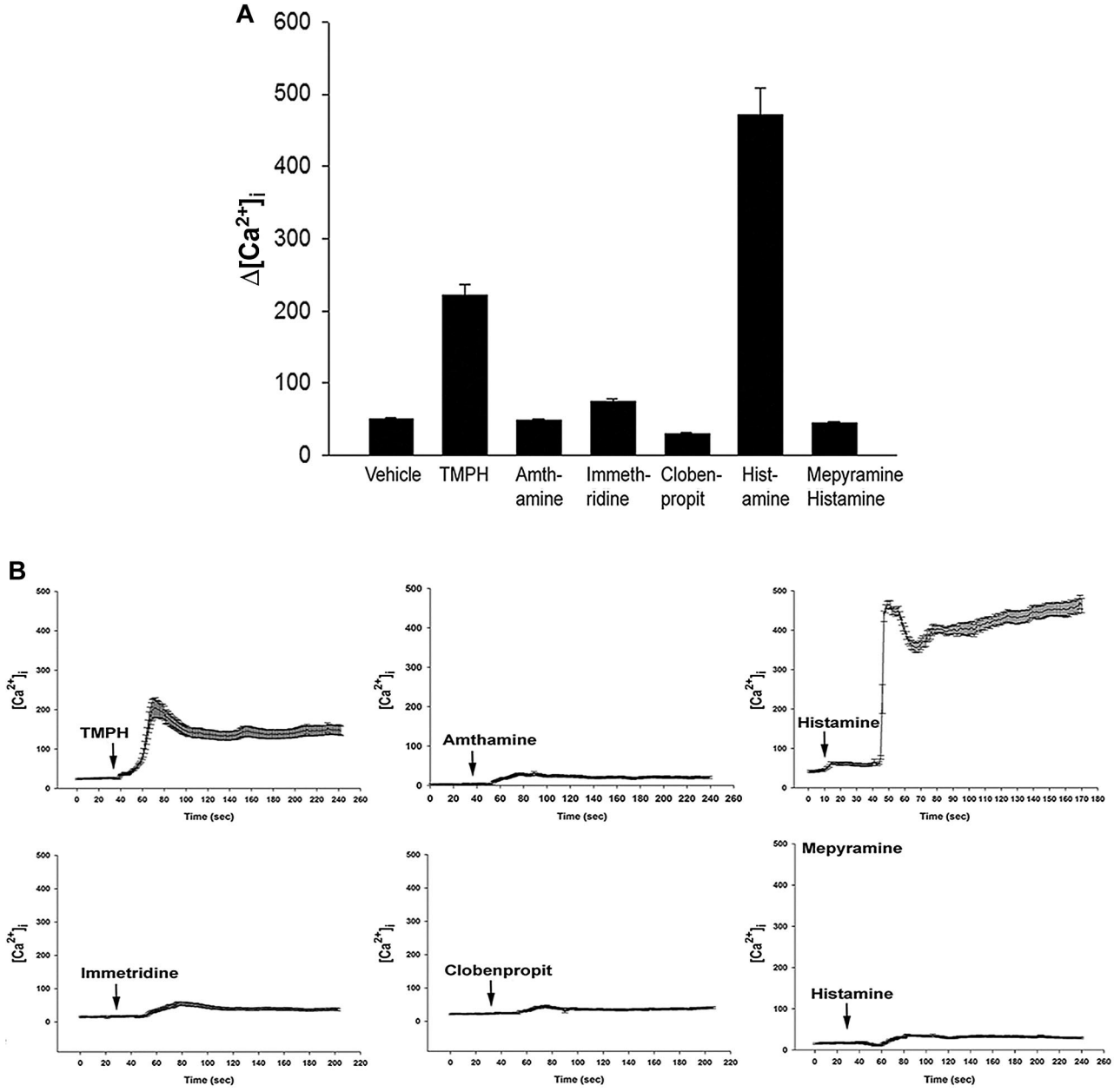

Figure 1. Ca^{2+} release because of stimulation of different histamine receptors in EA.hy926 endothelial cells. Cells were treated with the following histamine receptor agonists at 100μM: trifluoromethyl-phenyl-histamine (H1-selective), amthamine dihydrobromide (H2-selective), immethridine dihydrobromide (H3-selective), clobenpropit dihydrobromide (H4-selective), and histamine in the presence of mepyramine, a selective H1 antagonist. (A) Bars show the increase in $[Ca^{2+}]_i$ after stimulation with each agonist. Error bars represent SEM; n = 3 independent experiments. (B) Representative calcium traces of experiment shown in panel A.

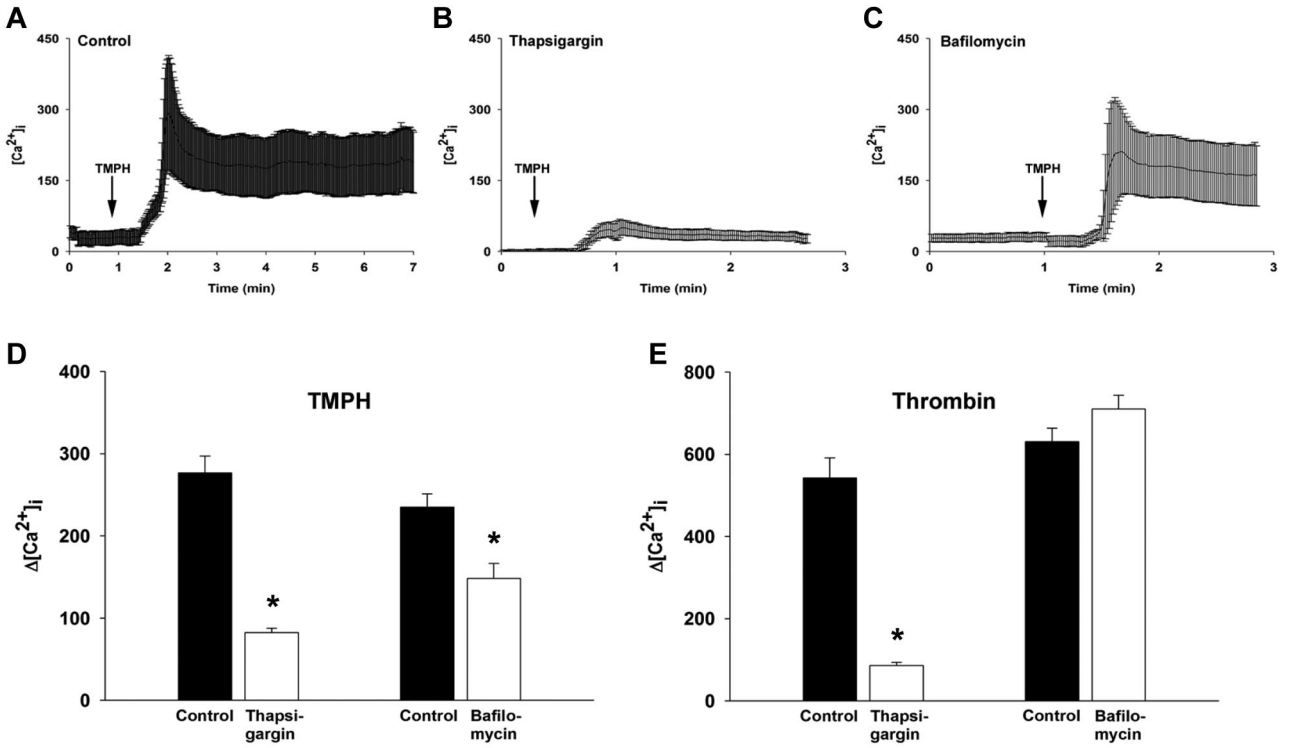

Figure 2. Role of NAADP-sensitive Ca^{2+} stores in H1 receptor signaling. Ca^{2+} release in cells stimulated with 100μM TMPH after treatment with (A) vehicle alone (control); (B) 1μM thapsigargin for 15 minutes; and (C) 500nM bafilomycin A1 for 1 hour. Maximum Ca^{2+} concentrations after 100μM TMPH stimulation (D) and 2 U/mL thrombin (E) are summarized. Error bars represent SEM; n = 3; *$P < .05$ by Student *t* test.

Results

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

Stimulation with histamine is known to enhance accumulation of inositol phosphates and elevate intracellular Ca^{2+} concentration in different cell types.[2,38] Four subtypes of surface receptors are known to mediate responses to histamine, all of which are G protein–coupled receptors.[39] As already described in the literature,[40] only mRNAs for H1 and H2 receptors are present in EA.hy926 and in HUVECs. We confirmed the expression of H1 and H2, but not H3 and H4, through RT-PCR in EA.hy926 (data not shown). To characterize the contribution of the different histamine receptors to Ca^{2+} mobilization, we have stimulated our endothelial cells with specific agonists. TMPH, amthamine dihydrobromide, immethridine hydrobromide, and clobenpropit dihydrobromide were used, which are specific agonists for H1R, H2R, H3R, and H4R, respectively. The increase in $[Ca^{2+}]_i$ after stimulation with each agonist (Figure 1A-B) shows that only TMPH, a selective agonist for H1 receptors, was able to trigger Ca^{2+} mobilization. Moreover, we observed that the selective H1 receptor antagonist, mepyramine, totally inhibits histamine-induced Ca^{2+} release. The calcium response to TMPH was found to be dose-dependent (data not shown), and the submaximal concentration of 100μM was chosen for subsequent experiments.

Involvement of NAADP-sensitive Ca^{2+} stores in H1 receptor signaling

To evaluate the involvement of different intracellular compartments in the Ca^{2+} response to TMPH, we adopted a pharmacologic approach. Bafilomycin A1 is known to inhibit the vacuolar-type H^+-ATPase pump of acidic organelles, preventing the acidification of these compartments by blocking the Ca^{2+} reuptake into acidic stores via the Ca^{2+}/H^+ exchanger.[41] Cells were treated with 500nM bafilomycin A1 or with the vehicle alone, and then stimulated with 100μM TMPH (Figure 2A-C). As shown, bafilomycin A1 partially but significantly impaired TMPH-induced Ca^{2+} release, suggesting the involvement of acidic organelles in TMPH-induced Ca^{2+} release. Furthermore, treatment with 1μM thapsigargin (Figure 2B), which functions by inhibiting sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), completely blocked TMPH-induced Ca^{2+} mobilization from the ER. As described in other models,[11] TMPH-stimulated EA.hy926 endothelial cells Ca^{2+} release requires the integrity of both the ER and acidic stores (Figure 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 Figure 2E, Ca^{2+} release in response to thrombin involves ER stores but not acidic compartment.

Direct effect of NAADP in $[Ca^{2+}]_i$ mobilization

Because cell stimulation with extracellular NAADP failed to induce intracellular Ca^{2+} release (not shown), we used NAADP-AM, a cell-permeant analog of NAADP known to induce NAADP-mediated Ca^{2+} signaling.[42] In cells stimulated with NAADP-AM, increase in $[Ca^{2+}]_i$ was induced compared with cells challenged with the vehicle alone (Figure 3A-B).

We further tested the involvement of NAADP in H1 receptor signaling by desensitizing the NAADP receptor by microinjecting high concentrations of NAADP as demonstrated in previous studies.[15,43,44] As shown in Figure 3, microinjection of 500μM NAADP inhibited TMPH-induced but not thrombin-induced intracellular Ca^{2+} increase, compared with control cells microinjected

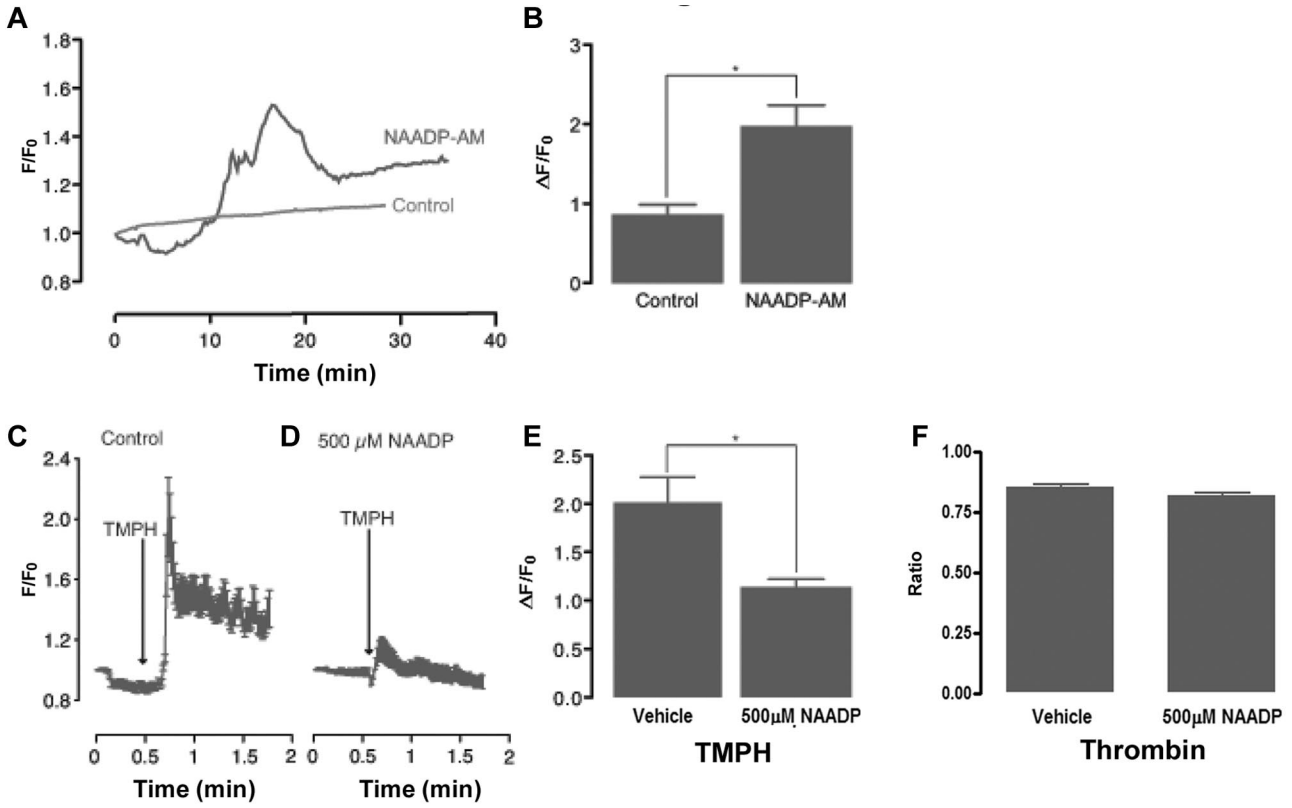

Figure 3. Direct effect of NAADP on $[Ca^{2+}]_i$ mobilization. (A) Representative plot of NAADP-AM–induced Ca^{2+} mobilization in EA.hy926 cells. (B) Increase in F/F_0 in cells stimulated with NAADP-AM compared with cells stimulated with vehicle alone (control). Cells were stimulated with 100μM TMPH after microinjection with (C) vehicle alone or (D) inactivating concentrations of NAADP. Increase in F/F_0 in response to TMPH (E) and 2 U/mL of thrombin (F) in cells microinjected with the inactivating concentration of 500μM NAADP compared with cells microinjected with vehicle alone. Where applicable, error bars represent SEM; n = 3 independent experiments; *$P < .05$ by Student *t* test.

with the vehicle alone. Figure 3E-F shows the average ratio F/F_0 of all cells microinjected corresponding to $[Ca^{2+}]_i$.

Taken together, these data demonstrate that Ca^{2+} induced by NAADP plays a specific and primary role in endothelial cell histamine-H1R signaling.

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.[32,37] Generation of NAADP in response to agonist stimulation demonstrates an important role of this second messenger in the release of Ca^{2+} from intracellular stores.[43,45,46]

Figure 4 shows intracellular NAADP measurement in endothelial cells after stimulation with TMPH or with the vehicle alone. In this experiment we performed a radioreceptor binding assay as described by Lewis et al.[37] As shown, TMPH leads to a time-dependent rise in intracellular NAADP levels, confirming the correlation between the H1R and NAADP as a 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 minute and reversion to basal level within 2 minutes (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

NAADP receptor has a primary role in TMPH-induced 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+} signaling in intact cells.[34] To date, Ned-19 appears to be an uncompetitive antagonist of NAADP.

We show that pretreatment of EA.hy926 cells for 20 minutes with 50μM Ned-19 blocks both histamine-induced and TMPH-induced Ca^{2+} release (Figures 5B,D-E). The same experiments performed in HUVECs confirmed the inhibitory effect of Ned-19 on histamine-induced and TMPH-induced Ca^{2+} mobilization (data not shown). After treatment with Ned-19, cells were still responsive to GPN (Figure 5B), which proves that the integrity of acidic stores was maintained. To validate the selectivity of Ned-19 in

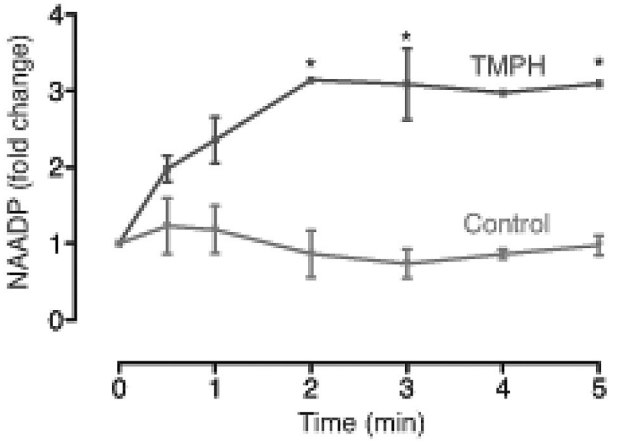

Figure 4. NAADP levels. NAADP levels measured over time in EA.hy926 endothelial cells stimulated with 100μM TMPH and control cells. The absolute resting concentration of NAADP was 1.774 88 ± 0.65 pmol/mg protein. Where applicable, error bars represent SEM; n = 2-3 independent experiments; *$P < .05$ by Student *t* test.

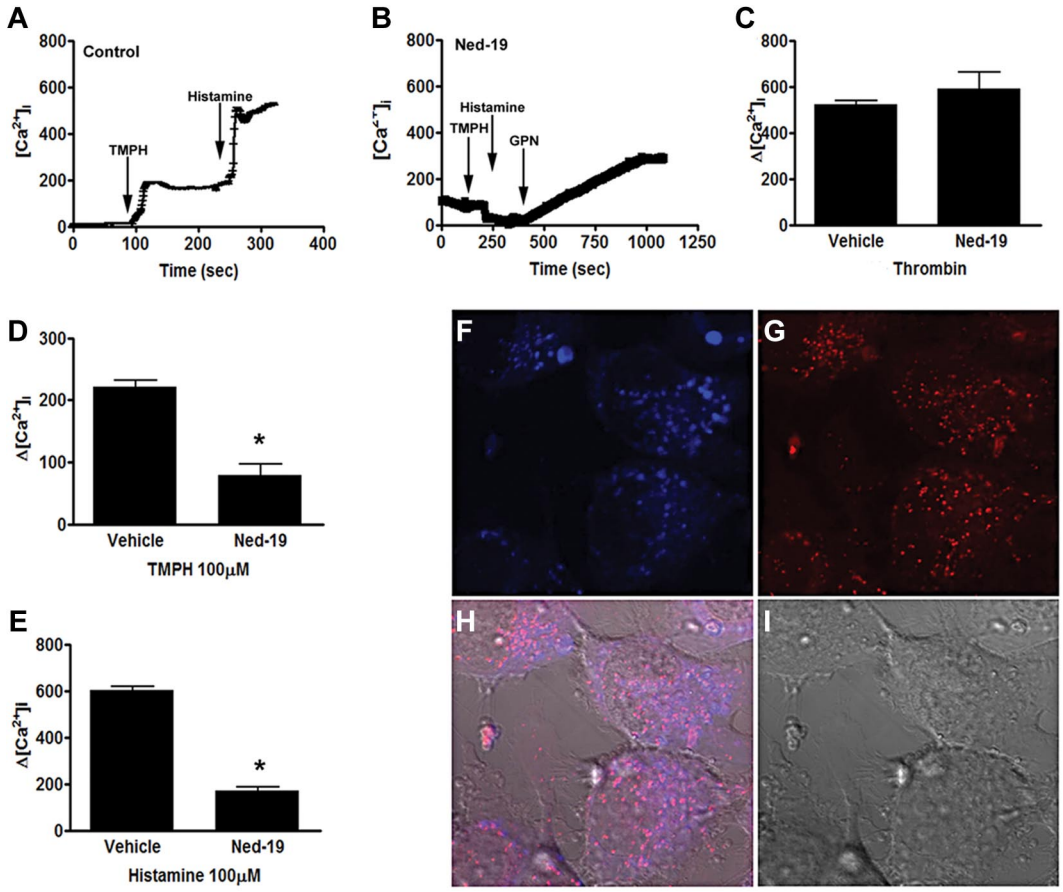

Figure 5. Inhibition of the NAADP receptor using the selective antagonist Ned-19. (A) EA.hy926 cells were treated with vehicle alone for 20 minutes and then stimulated with TMPH followed by histamine (representative trace). (B) Cells were treated with 50μM Ned-19 for 20 minutes followed by stimulation with TMPH, histamine, and then 50μM GPN (representative trace). (C) Cells were treated with 50μM Ned-19 for 20 minutes, then stimulated or not with 2 U/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) brightfield image. Cells were viewed on a Zeiss 510 META confocal microscope with a plan apochromat objective 63× oil immersion aperture 1.4, at room temperature in Hanks buffer, and analyzed with LSM Software. Where applicable, error bars represent SEM; n = 3 independent experiments; *$P < .05$ by Student *t* test.

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 (Figure 5C) or HUVECs (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 signaling.

As Ned-19 is fluorescent,[34] we labeled EA.hy926 cells with Ned-19 and with lysotracker red (Figure 5F-I). Ned-19 mostly (approximately 90%) colocalizes with lysotracker red positive organelles, but it is possible to observe a population of nonacidic 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] VWF is the major component inside 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 HUVECs 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 WPB 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 100μM Ned-19 or with the vehicle for 30 minutes before stimulation with either 100μM histamine, TMPH, or with 2 U thrombin for 20 minutes at 37°C. As shown in Figure 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, whereas 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 100μM cimetidine (Figure 6B). These data underline a primary role of NAADP as a fundamental second messenger specifically involved in H1R signaling in endothelial cells.

Recently it has been described that TPC1 and TPC2 represent specific receptors for NAADP. In our cells, TPC1:TPC2 relative

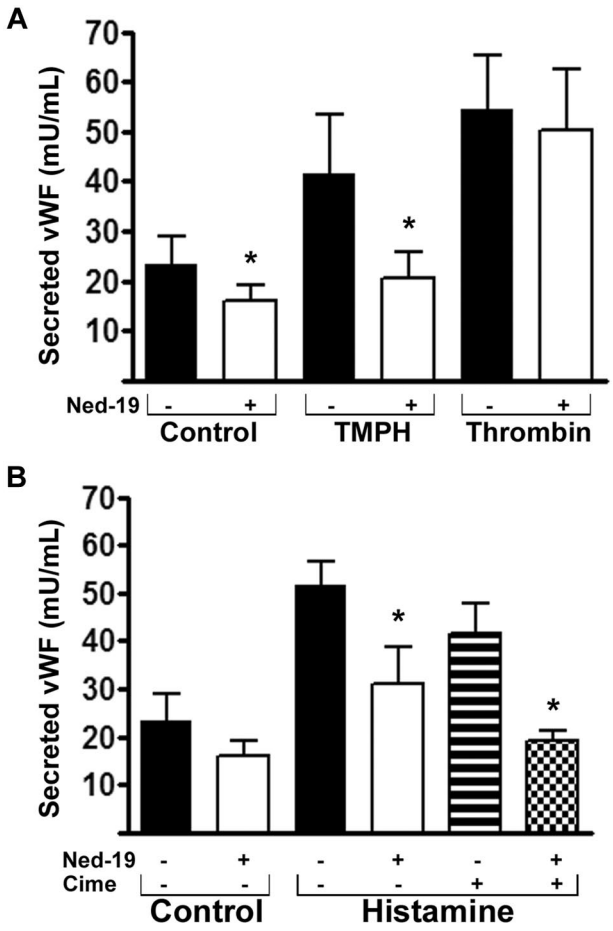

Figure 6. NAADP is essential for VWF release specifically activated through H1R. Measurement of secreted VWF by ELISA assay in HUVECs. Cells were treated either with vehicle alone or with 100μM Ned-19 or with 100μM cimetidine (H2R antagonist), or with 100μM Ned-19 and 100μM cimetidine for 30 minutes before stimulation with agonists as indicated in panels A and B. cime indicates cimetidine. Error bars represent SEM; n = 3 independent experiments; *$P < .05$ by Student *t* test.

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 HUVECs were transfected with siRNAs specific for TPC1 and TPC2, and 48 hours later we measured the acute release of VWF stimulated by 100μM TMPH for 20 minutes at 37°C. The efficiency of TPC receptor down-regulation, evaluated by qRT-PCR, was found to average approximately80% for TPC1 and 40% for TPC2. As shown in Figure 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 nontargeting control siRNA. The observation that TPC 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 cell activation by histamine signaling as a prototype pathway regulating acute inflammation. The identification and targeting of key signaling factors regulating vascular inflammation which, in turn, contributes to the development of a variety of diseases or pathologic 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 H1R, lead directly to an increase in vascular permeability, increased blood flow, and up-regulation in adhesion molecules allowing for leukocyte 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 signaling pathway activated through histamine H1R stimulation and its mechanistic involvement in the biologic 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 because 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 the EA.hy926 endothelial cell line and primary HUVECs. 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 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 signaling 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 signaling 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 signaling, Ned-19,[34] completely inhibited Ca^{2+} response after the specific stimulation of histamine H1R while failing to

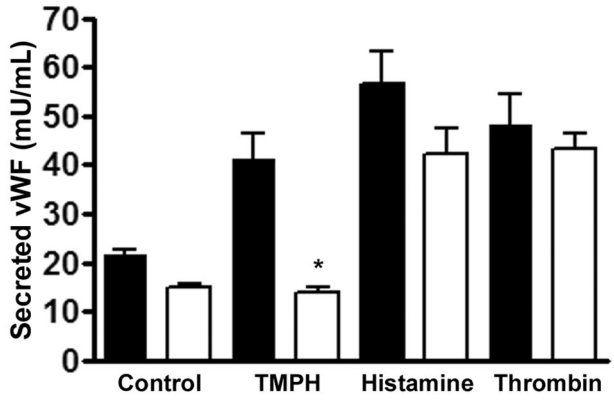

Figure 7. Specific effect of TPC1 and TPC2 receptor silencing on VWF release through H1R. ELISA assay of secreted VWF in HUVECs. Cells were treated with nontargeting siRNA (■) or with TPCs receptors siRNA (□) and then stimulated with the indicated agonists. Error bars represent SEM; n = 2 independent experiments; *$P < .05$ by Student *t* test.

affect thrombin-Ca^{2+} response. Thirdly, NAADP levels are consistently enhanced after challenging endothelial cells with the specific agonist of histamine H1R, TMPH. Moreover, silencing of NAADP receptors obtained by siRNA knockdown of TPC1/2 was found to abolish the secretion of VWF after 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 the ER system.[11] Our data obtained through pharmacologic 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 inhbitor) 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 inihibited the response to TMPH, failed to affect the increase of intracellular Ca^{2+} induced by thrombin, which directly activates ER Ca^{2+} release. Moreover, our data indicate that NAADP receptors in endothelial cells are mainly localized within acidic organelles, though Ned-19 also labels a small fraction of nonacidic 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 result is not simply because of bleed-through between the 2 channels used for the different dyes. These data are in agreement with the pharmacology of histamine H1R Ca^{2+} signaling, indicating that the integrity of both the ER and acidic stores are fundamental for H1R signaling. 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 on a contribution of different receptor types. To this purpose, it is worth mentioning that there is 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 a second messenger in the release of Ca^{2+} from intracellular stores. By means of a radioreceptor binding assay, we demonstrated 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 H1R engagement and the increase of intracellular NAADP.

In endothelial cells secretory granules known as WPBs contain pro-inflammatory and prothrombotic proteins such as VWF, and other vascular modulators.[4] These characteristic granules are present in different amounts in established endo,thelial cell lines, they are particularly abundant in HUVECs and less represented in EA.hy926 cells (not shown). When endothelial cells are activated by appropriate stimuli, the content of WPBs are released extracellularly.[49] Proteins contained in WPBs have been shown to promote neutrophil and platelet adhesion to vessel walls as well as vascular inflammation.[47] Therefore, the inhibition and regulation of endothelial cell exocytosis appears to play a fundamental role in down-regulating inflammation and vascular thrombosis. It is known that increased levels of cytosolic free Ca^{2+} concentration are implicated in the mechanism of exocytosis of VWF from WPBs. We provide evidence on HUVECs that secretion of VWF through activation of H1R is completely dependent on NAADP signaling. Our data show that Ned-19, the specific inhibitor of the NAADP receptor, completely blocked TMPH-induced VWF secretion while, conversely, it 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 IP_3 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, after inhibition of H2R, known to be coupled with cAMP, VWF secretion stimulated by histamine was abolished by Ned-19. This observation is in line with previous literature reporting that besides Ca^{2+}, cAMP is involved in VWF secretion.[50]

The combined analysis of data from pharmacologic NAADP inhibition and from TPC down-regulation demonstrates that NAADP totally and specifically controls calcium signaling 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 signaling pathways linked to H1R and H2R, the latter also implying 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 H1R in endothelial cells. Besides the well studied mechanism of H1R signal transduction via IP_3, we identified NAADP as a novel second messenger in H1R-induced Ca^{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 signaling 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 the NAADP pathway is involved in a relevant biologic 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 the stimulating issue of how calcium rises and endothelial cell response to H1R engagement are finely regulated to result in specifically different cellular responses. Furthermore, histamine driven inflammation after the activation of endothelial cells through an exclusive coupling between H1R and NAADP signaling could possibly be targeted as an experimental new approach for management of vascular diseases, directly focused on downstream signaling for specific histamine-mediated vascular endothelial cell responses.

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Authorship

Contribution: B.E. designed and performed the experiments, analyzed and collected data, cowrote the paper; G.G. conceived and performed some experiments, analyzed data; A.M.L. performed experiments, analyzed data, performed statistical analysis and contributed to paper writing; F.P. analyzed data and contributed to paper writing; A.D. performed experiments; L.X.T. performed selected experiments; A.A.G. performed experiments and edited the paper; E.Z. and A.G. analyzed data and edited the paper; G.C.C. designed research, contributed new reagents, analyzed data and edited the paper; and A.F. designed and supervised research and the experiments, analyzed and interpreted data, cowrote the paper.

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Correspondence: Prof Antonio Filippini, MD, PhD, D.A.H.-F.M.O. Section of Histology and Medical Embryology, Sapienza University of Rome, Via A. Scarpa 16, 00161 Rome, Italy; e-mail: antonio.filippini@uniroma1.it.

References

1. Pober J S, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol.* 2007;7(10):803-815.
2. Parsons ME, Ganellin CR. Histamine and its receptors. *Br J Pharmacol.* 2006;147(suppl 1): S127-S135.
3. Romani de Wit T, Rondaij MG, van Mourik J A. [Weibel-Palade bodies: unique secretory organelles within endothelial cells]. *Ned Tijdschr Geneeskd.* 2004;148(32):1572-1577.
4. Rondaij MG, Bierings R, Kragt A, van Mourik J A, Voorberg J . Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. *Arterioscler Thromb Vasc Biol.* 2006;26(5):1002-1007.
5. MacGlashan D J r. Histamine: A mediator of inflammation. *J Allergy Clin Immunol.* 2003; 112(suppl 4):S53-59.
6. Erent M, Meli A, Moisoi N, et al. Rate, extent and concentration dependence of histamine-evoked Weibel-Palade body exocytosis determined from individual fusion events in human endothelial cells. *J Physiol.* 2007;583(Pt 1):195-212.
7. Berridge MJ , Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol.* 2000;1(1):11-21.
8. Lee HC. A unified mechanism of enzymatic synthesis of two calcium messengers: cyclic ADP-ribose and NAADP. *Biol Chem.* 1999;380(7-8): 785-793.
9. Soares S, Thompson M, White T, et al. NAADP as a second messenger: neither CD38 nor base-exchange reaction are necessary for in vivo generation of NAADP in myometrial cells. *Am J Physiol Cell Physiol.* 2007;292(1):C227-239.
10. Lee HC, Aarhus R. A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. *J Biol Chem.* 1995; 270(5):2152-2157.
11. Galione A. NAADP, a new intracellular messenger that mobilizes Ca2+ from acidic stores. *Biochem Soc Trans.* 2006;34(Pt 5):922-926.
12. Brailoiu E, Miyamoto MD, Dun NJ . Nicotinic acid adenine dinucleotide phosphate enhances quantal neurosecretion at the frog neuromuscular junction: possible action on synaptic vesicles in the releasable pool. *Mol Pharmacol.* 2001;60(4): 718-724.
13. Brailoiu E, Churamani D, Pandey V, et al. Messenger-specific role for nicotinic acid adenine dinucleotide phosphate in neuronal differentiation. *J Biol Chem.* 2006;281(23):15923-15928.
14. Mitchell KJ , Lai FA, Rutter GA. Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca2+ release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). *J Biol Chem.* 2003;278(13): 11057-11064.
15. Berg I, Potter BV, Mayr GW, Guse AH. Nicotinic acid adenine dinucleotide phosphate (NAADP(+)) is an essential regulator of T-lymphocyte Ca(2+)-signaling. *J Cell Biol.* 2000; 150(3):581-588.
16. Lopez J J , Redondo PC, Salido GM, Pariente J A, Rosado J A. Two distinct Ca2+ compartments show differential sensitivity to thrombin, ADP and vasopressin in human platelets. *Cell Signal.* 2006;18(3):373-381.
17. Guse AH. Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP): novel regulators of Ca2+-signaling and cell function. *Curr Mol Med.* 2002;2(3):273-282.
18. Churchill GC, Galione A. NAADP induces Ca2+ oscillations via a two-pool mechanism by priming IP3- and cADPR-sensitive Ca2+ stores. *EMBO J .* 2001;20(11):2666-2671.
19. Churchill GC, Okada Y, Thomas J M, Genazzani AA, Patel S, Galione A. NAADP mobilizes Ca(2+) from reserve granules, lysosome-related organelles, in sea urchin eggs. *Cell.* 2002;111(5):703-708.
20. Gerasimenko J V, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV. NAADP, cADPR and IP3 all release Ca2+ from the endoplasmic reticulum and an acidic store in the secretory granule area. *J Cell Sci.* 2006;119(Pt 2):226-238.
21. Macgregor A, Yamasaki M, Rakovic S, et al. NAADP controls cross-talk between distinct Ca2+ stores in the heart. *J Biol Chem.* 2007; 282(20):15302-15311.
22. Calcraft PJ , Ruas M, Pan Z, et al. NAADP mobilizes calcium from acidic organelles through two-pore channels. *Nature.* 2009;459(7246):596-600.
23. Brailoiu E, Churamani D, Cai X, et al. Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. *J Cell Biol.* 2009; 186(2):201-209.
24. Dammermann W, Zhang B, Nebel M, et al. NAADP-mediated Ca2+ signaling via type 1 ryanodine receptor in T cells revealed by a synthetic NAADP antagonist. *Proc Natl Acad Sci U S A.* 2009;106(26):10678-10683.
25. Zhang F, J in S, Yi F, Li PL. TRP-ML1 functions as a lysosomal NAADP-sensitive Ca2+ release channel in coronary arterial myocytes. *J Cell Mol Med.* 2009;13(9B):3174-3185.
26. Menteyne A, Burdakov A, Charpentier G, Petersen OH, Cancela J M. Generation of specific Ca(2+) signals from Ca(2+) stores and endocytosis by differential coupling to messengers. *Curr Biol.* 2006;16(19):1931-1937.
27. Gerasimenko J V, Flowerdew SE, Voronina SG, et al. Bile acids induce Ca2+ release from both the endoplasmic reticulum and acidic intracellular calcium stores through activation of inositol trisphosphate receptors and ryanodine receptors. *J Biol Chem.* 2006;281(52):40154-40163.
28. Boittin FX, Galione A, Evans AM. Nicotinic acid adenine dinucleotide phosphate mediates Ca2+ signals and contraction in arterial smooth muscle via a two-pool mechanism. *Circ Res.* 2002; 91(12):1168-1175.
29. Cancela J M, Charpentier G, Petersen OH. Coordination of Ca(2+) signalling in mammalian cells by the new Ca(2+)-releasing messenger NAADP. *Pflugers Arch.* 2003;446(3):322-327.
30. Gambara G, Billington RA, Debidda M, et al. NAADP-induced Ca(2+) signaling in response to endothelin is via the receptor subtype B and requires the integrity of lipid rafts/caveolae. *J Cell Physiol.* 2008;216(2):396-404.
31. Kim BJ , Park KH, Yim CY, et al. Generation of nicotinic acid adenine dinucleotide phosphate and cyclic ADP-ribose by glucagon-like peptide-1 evokes Ca2+ signal that is essential for insulin secretion in mouse pancreatic islets. *Diabetes.* 2008;57(4):868-878.
32. Pandey V, Chuang CC, Lewis AM, et al. Recruitment of NAADP-sensitive acidic Ca2+ stores by glutamate. *Biochem J .* 2009;422(3):503-512.
33. Brailoiu GC, Gurzu B, Gao X, et al. Acidic NAADP-sensitive calcium stores in the endothelium: agonist-specific recruitment and role in regulating blood pressure. *J Biol Chem.* 2010; 285(48):37133-37137.
34. Naylor E, Arredouani A, Vasudevan SR, et al. Identification of a chemical probe for NAADP by virtual screening. *Nat Chem Biol.* 2009;5(4):220-226.
35. Edgell CJ , McDonald CC, Graham J B. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A.* 1983;80(12):3734-3737.
36. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985; 260(6):3440-3450.
37. Lewis AM, Masgrau R, Vasudevan SR, et al. Refinement of a radioreceptor binding assay for nicotinic acid adenine dinucleotide phosphate. *Anal Biochem.* 2007;371(1):26-36.
38. J utel M, Blaser K, Akdis CA. Histamine receptors in immune regulation and allergen-specific immunotherapy. *Immunol Allergy Clin North Am.* 2006; 26(2):245-259.
39. Haaksma EE, Leurs R, Timmerman H. Histamine receptors: subclasses and specific ligands. *Pharmacol Ther.* 1990;47(1):73-104.
40. Li H, Burkhardt C, Heinrich UR, Brausch I, Xia N, Forstermann U. Histamine up-regulates gene expression of endothelial nitric oxide synthase in

human vascular endothelial cells. *Circulation.* 2003;107(18):2348-2354.

41. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem.* 1991;266(26):17707-17712.
42. Parkesh R, Lewis AM, Aley PK, et al. Cell-permeant NAADP: a novel chemical tool enabling the study of Ca2+ signalling in intact cells. *Cell Calcium.* 2008;43(6):531-538.
43. Masgrau R, Churchill GC, Morgan AJ , Ashcroft SJ , Galione A. NAADP: a new second messenger for glucose-induced Ca2+ responses in clonal pancreatic beta cells. *Curr Biol.* 2003;13(3):247-251.
44. Cancela J M, Churchill GC, Galione A. Coordination of agonist-induced Ca2+-signalling patterns by NAADP in pancreatic acinar cells. *Nature.* 1999;398(6722):74-76.
45. Churchill GC, O'Neill J S, Masgrau R, et al. Sperm deliver a new second messenger: NAADP. *Curr Biol.* 2003;13(2):125-128.
46. Yamasaki M, Thomas J M, Churchill GC, et al. Role of NAADP and cADPR in the induction and maintenance of agonist-evoked Ca2+ spiking in mouse pancreatic acinar cells. *Curr Biol.* 2005; 15(9):874-878.
47. Goligorsky MS, Patschan D, Kuo MC. Weibel-Palade bodies–sentinels of acute stress. *Nat Rev Nephrol.* 2009;5(7):423-426.
48. Wagner DD, Bonfanti R. von Willebrand factor and the endothelium. *Mayo Clin Proc.* 1991; 66(6):621-627.
49. van Mourik J A, Romani de Wit T, Voorberg J . Biogenesis and exocytosis of Weibel-Palade bodies. *Histochem Cell Biol.* 2002;117(2):113-122.
50. Cleator J H, Zhu WQ, Vaughan DE, Hamm HE. Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. *Blood.* 2006; 107(7):2736-2744.