Transcription of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase type 3 gene, *ATP2A3*, is regulated by the calcineurin/NFAT pathway in endothelial cells

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Histamine, known to induce Ca^{2+} oscillations in endothelial cells, was used to alter Ca^{2+} cycling. Treatment of HUVEC (human umbilical-vein endothelial cell)-derived EA.hy926 cells with histamine for 1–3 days increased the levels of SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase) 3, but not of SERCA 2b, transcripts and proteins. Promoter-reporter gene assays demonstrated that this increase in expression was due to activation of SERCA 3 gene transcription. The effect of histamine was abolished by mepyramine, but not by cimetidine, indicating that the H1 receptor, but not the H2 receptor, was involved. The histamine-induced up-regulation of SERCA 3 was abolished by cyclosporin A and by VIVIT, a peptide that prevents calcineurin and NFAT (nuclear factor of activated T-cells) from interacting, indicating involvement of the calcineurin/NFAT pathway. Histamine also induced the nuclear translocation of NFAT. NFAT did not directly bind to the SERCA 3 promoter, but activated Ets-1 (E twenty-six-1), which drives the expression of the SERCA 3 gene. Finally, cells treated with histamine and loaded with fura 2 exhibited an improved capacity in eliminating high cytosolic Ca^{2+} concentrations, in accordance with an increase in activity of a low-affinity Ca^{2+} -ATPase, like SERCA 3. Thus chronic treatment of endothelial cells with histamine up-regulates SERCA 3 transcription. The effect of histamine is mediated by the H1R (histamine 1 receptor) and involves activation of the calcineurin/NFAT pathway. By increasing the rate of Ca^{2+} sequestration, up-regulation of SERCA 3 counteracts the cytosolic increase in Ca^{2+} concentration.

Key words: calcium, E twenty-six-1 (Ets-1), histamine, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase 3 (SERCA 3), specificity protein 1 (Sp1), transcription factor.

INTRODUCTION

The SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase) is encoded by three different genes *ATP2a1*, *ATP2a2* and *ATP2a3*, each of which gives rise to multiple isoforms by alternative splicing of their 3' end. *ATP2a3* generates at least seven alternatively spliced SERCA 3 isoforms [1]. SERCA 3 is present in specific cell types: endothelial cells in a wide variety of tissues [2,3]; epithelial cells of the trachea, salivary glands and intestine [2,4]; platelets and haematopoietic cells [5]; pancreatic β -cells and the acini of salivary glands [6]; and brain Purkinje cells [4]. SERCA 3 is present in the heart tube early in development. Its expression is subsequently restricted to the dorsal aorta and small foci in the liver [7].

Little is known about the mechanisms that regulate SERCA 3 gene: we have shown previously that basal transcription of the SERCA 3 gene is controlled by Ets-1 (E twenty-six-1) and Sp1 (specificity protein 1) [8]; a few studies found an alteration in the accumulation of the SERCA 3 protein and mRNA. For instance, expression of SERCA 3 was shown to decrease during proliferation of endothelial cells in culture [3]. *Trans*-retinoic acid and cAMP increase SERCA 3 and decrease SERCA 2b transcript levels, whereas phorbol ester enhances the production of both isoforms [9]. There is also some evidence that SERCA expression is

regulated by Ca^{2+} . Indeed, the levels of SERCA 2b and SERCA 3 in cultured VSMCs (vascular smooth muscle cells) are increased by treatment with thapsigargin or the Ca^{2+} ionophore A-23187 [10]. However, there is no indication of how an increase in Ca^{2+} can regulate SERCA 3 expression.

Specific spatio-temporal patterns of Ca^{2+} oscillations differently modulate gene transcription by activating specific transcription factors [11]. In endothelial cells, $[\text{Ca}^{2+}]_i$ (internal Ca^{2+} concentration) oscillation frequency regulates agonist-stimulated NF- κ B (nuclear factor κ B) transcriptional activity [12]. NFAT (nuclear factor of activated T-cells) is another transcription factor regulated by Ca^{2+} oscillation frequency [13,14]. Histamine increases Ca^{2+} release from the ER (endoplasmic reticulum) by activating IP3Rs [Ins(1,4,5) P_3 receptors] and induces $[\text{Ca}^{2+}]_i$ oscillations in endothelial cells [15]. The actions of histamine are mediated by four specific receptors (H1R–H4R): H1R and H2R are expressed in vascular endothelial cells. H1R is coupled to G_{q-11} /PLC (phospholipase C)-dependent breakdown of the phosphoinositides which results in an increase in intracellular $[\text{Ca}^{2+}]_i$, whereas H2R is linked to adenylate cyclase through G_s [16].

We hypothesized that chronic stimulation of endothelial cells with histamine could modify intracellular Ca^{2+} homeostasis and, consequently, the activity of Ca^{2+} -regulated transcription factors, thereby contributing to the establishment of a new pattern of gene

Abbreviations used: Ad, adenoviral; $[\text{Ca}^{2+}]_i$, internal Ca^{2+} concentration; CRE, cAMP-response element; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle's medium; EBS, E twenty-six-1-binding protein; eGFP, enhanced green fluorescent protein; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; Ets-1, E twenty-six-1; FCS, foetal calf serum; H1R, histamine 1 receptor; H2R, histamine 2 receptor; IP3R, Ins(1,4,5) P_3 receptor; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor κ B; pfu, plaque-forming units; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+} -ATPase; RT, reverse transcriptase; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase; Sp1, specificity protein 1; VSMC, vascular smooth muscle cell.

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expression. As the spatio-temporal pattern of Ca^{2+} oscillations is mainly controlled by SERCA, we wondered whether chronic stimulation with histamine would modify the expression of this Ca^{2+} -ATPase. In the present paper, we show that chronic treatment of endothelial cells EA.hy926 with histamine up-regulates SERCA 3 expression by activating the calcineurin/NFAT pathway. The effect of histamine is mediated through H1R. This increase in SERCA 3 expression is associated with an increased rate in Ca^{2+} transient decay in endothelial cells, especially at high Ca^{2+} concentrations.

MATERIALS AND METHODS

Cell culture

HUVEC (human umbilical-vein endothelial cell)-derived EA.hy926 cells [17] were cultured in DMEM (Dulbecco's modified Eagle's medium) (Gibco) supplemented with 10% (v/v) FCS (foetal calf serum), 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 units/ml penicillin, 1 mM L-glutamine and 2% HAT (hypoxanthine/aminopterin/thymidine). Cells were grown in the presence of 10% (v/v) FCS for 24 h, then deprived of serum and incubated with histamine in serum-free DMEM at different concentrations and for different periods of time. Antagonists of the histamine receptors, mepyramine and cimetidine (Sigma–Aldrich) were used at a concentration of 1 μM . CsA (cyclosporin A) (Sigma–Aldrich) was used at 10 μM for 24 h. In several experiments, cells were infected with AdVIVIT [100 pfu (plaque-forming units)/cell] for 48 h before stimulation with histamine.

RT (reverse transcriptase)-PCR

Total RNA was prepared using RNA NOWTM (Ozyme) and 1 μg was reverse-transcribed using a standard protocol. One-third of the resulting cDNA was amplified by 40 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by a final amplification at 72°C for 7 min using 2.5 units of Amplitaq (Invitrogen) and 200 pmol each of the following primers: human SERCA 3 sense primer, 5'-GAGTCACGCTTCCACCACC-3', and antisense primer, 5'-GCCTGTCAATTTATCCGGCG-3'; human SERCA 2b sense primer, 5'-TCATCTTCCAGATCACACCGCT-3', and antisense primer, 5'-TCAAGACCAGAACATATCGC-3'; human β -actin sense primer, 5'-GGGTCAAGGATTCTTA-3', and antisense primer, 5'-GGTCTCAAACATCATCTGGG-3'. The ratio RT-PCR assay was based on the simultaneous amplification of two SERCA isoforms by using identical primer template sequences, as described in [3]. The amplified products were discriminated by XbaI digestion. cDNA was amplified by 40 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C using 200 pmol each of the following primers: human SERCA 3/SERCA2b sense primer, 5'-TGCCTGGTAGAGAAGATGAA-3', and antisense primer, 5'-CCCTTCACAAACATCTTGCT-3'. After the primary PCR, a radioactive labelling was performed by adding four additional PCR cycles to a 20-fold dilution of the reaction product in fresh amplification mixture containing 15 nCi/ μl of [α -³²P]dCTP (Amersham Biosciences). The labelled amplification products and their restriction fragments were separated on 8% polyacrylamide gel and quantified using a PhosphorImager (STORM 840, Molecular Dynamics). The intensity of the bands was corrected for the CG content of the amplified sequences for the calculation of isoform ratios.

Plasmids and Ad (adenoviral) constructs

The SERCA 3 promoter constructs were described previously [8]. The EBS (Ets-1-binding protein) and Sp1 mutant constructs

were generated using the QuikChangeTM (Stratagene) site-directed mutagenesis kit and the following mutated EBS and Sp1 oligonucleotides (sense strand): EBS mut, 5'-GGCTGCTAT-TGCGGCTATCAGGGGTGTGGGTGC-3'; Sp1 mut, 5'-GGTG-CAGGGCTAGTCCCGCGCCGCTATGCCAGAGGACGG-3' (mutagenic sites are indicated in bold). The eGFP (enhanced green fluorescent protein)–VIVIT DNA fragment was excised from pGFP-VIVIT [18], subcloned in the Ad vector to produce and pAd-eGFP-VIVIT. The eGFP–AdVIVIT construct was provided by Susan Kraner and Chris Norris (University of Kentucky, Lexington, KY, U.S.A.). The viruses, named AdVIVIT, were amplified in HEK-293 (human embryonic kidney) cells and purified by CsCl gradient ultracentrifugation using standard protocols.

Transient transfection and reporter gene assay

Cells were co-transfected with the different SERCA promoter–luciferase constructs and a construct containing the β -galactosidase gene driven by the CMV (cytomegalovirus) promoter (pCMV β , Clontech) for normalization. Constructs containing the luciferase reporter gene driven by four NFAT consensus sites (Stratagene), CRE (cAMP-response element) [19] or six NF- κ B binding sites [20] were also used. Cells were grown in the presence of 10% (v/v) FCS for 24 h, then deprived of serum and incubated with histamine alone or in combination with antagonists or CsA for a further 24 h. In several experiments, cells were infected with AdVIVIT (100 pfu/cell) for 48 h before transfection. Luciferase activity was normalized to the β -galactosidase activity and is expressed either as a percentage of the control or as fold induction over the activity of pGL3-basic. The results are means for at least three independent experiments performed in triplicate.

Immunofluorescence and immunoblot

Cells, plated on glass coverslips in the presence of 10% (v/v) FCS for 24 h, were then deprived of serum and treated with CsA or infected with AdVIVIT (100 pfu/cell) for 48 h. Nuclear NFAT translocation was analysed at 30 min–4 h after stimulation with histamine (1 μM) by immunolabelling of methanol-fixed cells with an anti-NFATc1 antibody (1:200, K18; Santa Cruz Biotechnology). Proteins were visualized using biotin/streptavidin–Texas Red-conjugated amplification (Amersham Biosciences). For immunolocalization anti-(SERCA 3a) antibody [21] was revealed with a biotinylated anti-rabbit antibody streptavidin–TRITC (tetramethylrhodamine β -isothiocyanate) conjugate, and SERCA 2b was visualized with the anti-pan-hSERCA2 (h is human) monoclonal antibody IID8 (1:500; Abcam) and anti-mouse-FITC secondary antibody. Slides were examined using a Zeiss LSM-510 confocal laser-scanning microscope equipped with a 25 mW argon laser and a 1 mW helium–neon laser, using a Plan Apochromat 63 $\times$ objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505–550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.4–0.6 μm along the z-axis. Membrane proteins were isolated by scraping the cells in 10 mM KCl, 10 mM Hepes, pH 7, 50 mM EGTA, 50 μM EDTA, 50 $\mu\text{g}/\text{ml}$ soya bean trypsin inhibitor, 40 $\mu\text{g}/\text{ml}$ Bowman–Birk trypsin–chymotrypsin inhibitor, 0.1 mM dithiothreitol, 25 μM PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin and 5 $\mu\text{g}/\text{ml}$ pepstatin A. The lysate was centrifuged at 7000 g for 15 min at 4°C. The supernatant was centrifuged at 100 000 g for 1 h at 4°C, and the pellet was resuspended in a buffer containing 160 mM KCl, 17 mM Hepes, pH 7, and 0.1 mM dithiothreitol. Proteins

(100 μ g) were separated by SDS/7.5% PAGE, blotted on Hybond-C membrane (Amersham Biosciences) and incubated with the anti-pan-hSERCA2 monoclonal antibody IID8 (1:2500; Abcam), the anti-pan-hSERCA 3 monoclonal antibody PLIM/430 (1:50; Research Diagnostic) and the anti-pan-PMCA (plasma membrane Ca^{2+} -ATPase) monoclonal antibody (1:1000, clone 5F10; Abcam). Immunoreactive proteins were visualized using an ECL[®] (enhanced chemiluminescence) detection system (Amersham Biosciences).

Identification of DNA-binding proteins

Nuclear extracts were prepared as described in [22]. DNA-binding proteins were isolated using streptavidin-agarose beads coated with biotinylated double-stranded EBS or SP1 oligonucleotides as described [8]. Proteins were subjected to SDS/PAGE, transferred on to Hybond C membranes and probed with anti-NFAT (1:200, K18), anti-Ets-1 (1:1000, E34620; Transduction Laboratories) and anti-Sp1 (1:500; Sigma-Aldrich) antibodies and revealed as described above.

Fura 2 loading and Ca^{2+} imaging

EA.hy926 cells were plated at a density of 70000 cells/35-mm diameter dish and treated with histamine in FCS-free medium for 3 days, in the absence or presence of antagonists. Cells were washed in buffer A containing in mM: 116 NaCl, 5.6 KCl, 1.8 CaCl_2 , 1.2 MgCl_2 , 5 NaHCO_3 , 1 NaH_2PO_4 and 20 Hepes, pH 7.4, and then loaded for 30 min at 25 °C with 4 μM fura 2/AM (fura 2 acetoxymethyl ester). Ca^{2+} transient imaging was performed as described previously [23]. Each fluorescent image was the average of 16 images to improve the signal/noise ratio. One averaged image was recorded every 3 s. Tracings of fluorescence ratio (F_{360}/F_{380}) are representative of at least 15 cells, and were collected from at least two different cell preparations. The ATP-induced Ca^{2+} responses (10 μM ATP) were evaluated in the absence of extracellular Ca^{2+} by adding 100 μM EGTA to Ca^{2+} -free buffer A. It is noteworthy that, in order to accurately compare the different cell culture conditions, all experiments were performed in parallel, using the same Fura 2 loading conditions and the same camera settings.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. An unpaired Student *t* test was used to compare means. Data were analysed by using the StatView software. Differences were considered significant at $P < 0.05$.

RESULTS

Histamine increases SERCA 3 expression

RT-PCR analysis showed that serum-deprived EA.hy926 cells contain mainly SERCA 2b (95.79 $\pm$ 0.72 %) and only low levels of SERCA 3 mRNA (4.37 $\pm$ 0.93 %) (Figure 1A). Chronic treatment with histamine did not change the amount of SERCA 2b mRNA. In contrast, histamine increased the amount of SERCA 3 mRNA in a concentration- and time-dependent manner (Figures 1B and 1C). The increase in SERCA 3 mRNA level was evident after 48 h of treatment, and remained stable for up to 72 h. The proportion of SERCA 3 mRNA over total SERCA mRNA was 13.66 $\pm$ 3.48 %, $P < 0.05$ compared with non-treated, $n = 4$ (Figure 1A). The induction of SERCA 3 expression was also confirmed by Western blot. SERCA 3 was present at low level in cells cultured in the absence of serum. Treatment for 72 h (corresponding to the time necessary for synthesis of SERCA)

Figure 1 Histamine increases SERCA 3 mRNA and protein levels in human endothelial cells

(A) Representative PhosphorImage of RT-PCR using a common primer to amplify SERCA 3 and SERCA 2. nd, non-digested DNA; X1, DNA digested with XbaI. Cells were cultured in absence of serum (ctr) or treated with histamine (Hist) for 48 h. Sizes are indicated in bp. (B) Representative results of RT-PCR analysis of SERCA 3 and SERCA 2b in EA.hy926 cells treated with the indicated concentrations of histamine for 48 h. ctr, untreated control. Sizes are indicated in bp. (C) Time-dependency of histamine-induced SERCA 3 expression. β -Actin was used as an internal control. Sizes are indicated in bp. (D) Upper panel: representative Western blot of membrane proteins from cells treated with the indicated concentrations of histamine for 72 h. Expression of SERCA 3, SERCA 2 and PMCA was revealed with PLIM/430, IID8 and anti-pan-PMCA respectively. Lower panel: relative ratio of SERCA 3/SERCA 2b (closed bars) obtained from four independent Western blots, relative ratios of SERCA 3/PMCA (grey bars) and SERCA 2b/PMCA (open bars) determined from three independent experiments. The values were expressed as a percentage of the value obtained for the control in the same blot. * $P < 0.05$, *** $P < 0.001$ compared with each control (ctr). (E) Immunofluorescence of EA.hy926 cells with anti-(SERCA 3a) (red) and IID8 (green). The images represent individual optical slices of 0.6 μm through the nucleus.

with histamine resulted in a dose-dependent increase in SERCA 3 protein levels, whereas SERCA 2b and PMCA levels were unchanged (Figure 1D, upper panel). Thus the relative level of SERCA 3 over SERCA 2b or PMCA was increased by treatment with histamine, whereas the level of SERCA 2b over PMCA was constant (Figure 1D, lower panel). Immunofluorescence of EA.hy926 cells indicated that the two SERCA isoforms are not co-localized. SERCA 2b is more internal and perinuclear, whereas SERCA 3a, the major SERCA 3 isoform expressed in these cells, is spread over the entire cytosol (Figure 1E).

Histamine enhances the activity of the SERCA 3 promoter and the histamine-induced increase in SERCA 3 expression is mediated by the H1R

EA.hy926 cells were transiently transfected with various SERCA 3 promoter-luciferase reporter gene constructs (Figure 2A). Incubation with 1 μM histamine for 24 h significantly increased the

Figure 2 Histamine enhances the activity of the SERCA 3 promoter and the histamine-induced increase in SERCA 3 expression is mediated by H1R

(A) EA.hy926 cells, transfected with various constructs of the mouse SERCA 3 promoter and the pCMV β -gal normalization plasmid for 24 h, were treated with (closed bars) or without (open bars) 1 μ M histamine for 24 h in the absence of FCS. Luciferase (Luc) activity was normalized to β -galactosidase activity and expressed, in relative luciferase units (RLU), as fold induction compared with the promoterless construct, OpGL3. Results are means $\pm$ S.E.M. for three experiments in triplicate; $^{***}P < 0.01$. (B) After 24 h of transfection with the -97pGL3-luciferase construct in 10% (v/v) FCS, EA.hy926 cells were treated with selective antagonists (1 μ M each) for the H1R (mepyramine; Mepy) or for the H2R (cimetidine; Cime) for 24 h in serum-free medium. The antagonists were used alone (open bars) or in combination with histamine (1 μ M) (closed bars). Results are means $\pm$ S.E.M. for five experiments in triplicate; $^{*}P < 0.05$, $^{***}P < 0.001$. (C) Representative Western blot showing the effect of mepyramine (Mepy) and cimetidine (Cime) on accumulation of SERCA 3, SERCA 2b and PMCA in cells treated with 0.1 μ M histamine for 3 days. ctr, untreated control.

basal promoter activity (-97pGL3) ($P < 0.01$). Histamine could not overcome the inhibitory effect that was observed previously with longer promoter constructs (up to -1341) (Figure 2A) [8]. Thus the minimal promoter was used in subsequent studies.

EA.hy926 cells normally express both H1R and H2R [24]. We used selective antagonists of H1R (mepyramine) and H2R (cimetidine) to determine which of these receptors was involved in the histamine-induced up-regulation of SERCA 3. Mepyramine, but not cimetidine, completely abolished the effect of histamine (Figure 2B). Mepyramine alone slightly increased the activity of the promoter in the absence of histamine ($P < 0.05$), but this remains unexplained. Mepyramine abolished accumulation of SERCA 3 protein without interfering with SERCA 2b or PMCA expression, whereas cimetidine was without effect on SERCA 3, SERCA 2b or PMCA protein levels (Figure 2C).

Histamine activates the calcineurin/NFAT pathway

The effect of histamine on SERCA 3 promoter activity was blocked by CsA (Figure 3A), suggesting that the calcineurin path-

Figure 3 The histamine-induced increase in SERCA 3 expression is mediated by the calcineurin/NFAT pathway

(A) The effect of histamine on SERCA 3 expression is abolished by CsA or adVIVIT. Cells were transfected with the -97pGL3-luciferase construct for 24 h in serum-containing medium (basal) and then treated with (closed bars) or without (open bars) histamine (1 μ M for 24 h) in serum-free medium. CsA (10 μ M) was added 24 h after transfection in serum-free medium in the presence (closed bars) or absence (open bars) of histamine (1 μ M for 24 h). In other experiments, adVIVIT (100 pfu/cell) was added 48 h before transfection. Results are means $\pm$ S.E.M. for four (CsA) or three (VIVIT) experiments in triplicate; $^{**}P < 0.01$. RLU, relative luciferase units. (B) Induction of an NFAT-responsive luciferase reporter gene by histamine. Cells were transfected with an NFAT-, CRE- or NF- κ B-luciferase reporter construct for 24 h and treated with histamine (1 μ M for 24 h) alone or in combination with mepyramine (Mepy, 1 μ M), cimetidine (Cime, 1 μ M) or CsA (10 μ M). Luciferase activity, in relative luciferase units (RLU), was normalized to β -galactosidase activity and expressed as a percentage of the value obtained for -97pGL3 in the control condition (0% FCS). Results are means $\pm$ S.E.M. for three experiments in triplicate; $^{**}P < 0.01$, $^{***}P < 0.001$. Open bars represent cells incubated without histamine and closed bars represent cells treated with histamine. (C) Histamine induces NFAT nuclear translocation. Cells were treated for 24 h with CsA (10 μ M) or infected with AdVIVIT for 48 h. Then they were incubated with or without histamine (1 μ M) for 4 h in serum-free medium. NFAT was visualized by an anti-NFATc1 antibody (a-NFAT). The images represent individual optical slices of 0.43 μ m through the nucleus. Scale bar, 20 μ m.

way is involved in the up-regulation of the SERCA 3 gene. To confirm this further, we infected cells with AdVIVIT, an Ad vector specific for the calcineurin/NFAT inhibitory peptide, before histamine treatment. VIVIT also completely abolished the effect of histamine (Figure 3A).

To determine whether histamine activates NFAT transcriptional activity specifically, we transfected cells with an NFAT-, NF- κ B- or CRE-luciferase reporter construct (Figure 3B). The activity of the NFAT-luciferase construct was clearly increased in the presence of histamine, but not that of CRE and NF- κ B reporter constructs. The activity of the NFAT reporter construct was abolished by CsA. Furthermore, mepyramine, but not cimetidine, abolished the induction of NFAT-luciferase activity by histamine.

To analyse further the mechanism of histamine-induced activation of NFAT, we studied the distribution of NFAT by immunofluorescence. A single NFAT isoform, NFATc3, was detected by RT-PCR in total RNA from EA.hy926 (results not shown). NFAT was found mainly in the cytosol of untreated cells. Treatment with 1 μ M histamine induced the nuclear translocation of NFAT within 30 min (results not shown). NFAT was still found in the nucleus after 4 h (Figure 3C). After pre-treatment of the cells with CsA, or infection with AdVIVIT before stimulation with histamine (1 μ M), NFAT was located mainly in the cytosol (Figure 3C).

Figure 4 NFAT induces SERCA 3 transcription by enhancing expression of Ets-1 transcription factor

(A) EA.hy926 cells transfected with -97pGL3-luciferase construct, or with the same vector where the EBS or Sp1 sites were mutated (EBS mut and Sp1 mut), were treated with (closed bars) or without (open bars) 1 μ M histamine for 24 h. Activities obtained with the EBS mut and Sp1 mut constructs are expressed as the percentage of the values obtained with the control -97pGL3-luciferase construct. (B) Effect of CsA, or AdVIVIT, on the amount of Ets-1, Sp1 and NFAT, measured by Western blotting, in nuclear extracts. Molecular-mass sizes are given in kDa. a-Sp1, anti-Sp1; a-Ets-1, anti-Ets-1; a-NFAT, anti-NFAT.

NFAT induces SERCA 3 transcription by enhancing Ets-1 expression

The fact that NFAT is involved in the activation of the basal SERCA 3 promoter is surprising because there is no clear NFAT-binding element in this region. We have shown previously that activation of SERCA 3 gene transcription is controlled by Ets-1 and Sp1 binding to the proximal region of the promoter [8]. Figure 4(A) demonstrates that mutation of Ets-1- and Sp1-binding sites prevents induction by histamine, suggesting involvement of these regions. In other systems, NFAT activates transcription by binding to Sp1/Sp3 [25,26]. Alternatively, NFAT could act indirectly by enhancing the expression of Ets-1 or Sp1. We performed DNA-binding protein assays to test whether NFAT binds to Ets-1- and Sp1-binding elements. We were unable to detect any binding of NFAT on these two elements (results not shown). We then performed Western blot experiments on nuclear extracts from cells treated with histamine in the presence or absence of CsA or AdVIVIT (Figure 4B). Ets-1 is present in control cells, and treatment with histamine enhanced its expression. This up-regulation was blocked by both CsA and VIVIT. The level of Ets-1 was correlated with that of nuclear NFAT. The level of Sp1 was similar in all conditions.

Histamine-treated cells display an increased rate of initial Ca^{2+} decay following stimulation of intracellular Ca^{2+} release by ATP

SERCA activity plays a powerful and dynamic role in regulating Ca^{2+} responses in endothelial cells. In addition to modulating the phases of Ca^{2+} release, SERCA activity is responsible for the falling phase of Ca^{2+} spikes [15,27]. To determine the functional consequence of histamine-induced SERCA 3 up-regulation, we used ATP to trigger the release of Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pools in EA.hy926 cells and compared the time taken for 80%, 50% and 20% Ca^{2+} decay in histamine-treated cells and control cells in the absence and presence of cimetidine or mepyramine. To

Figure 5 Chronic stimulation of H1Rs with histamine modifies ATP-induced Ca^{2+} responses in EA.hy926 cells and increases the rate of initial Ca^{2+} decay

Cells were cultured for 3 days with or without histamine (1 μ M), in the absence or in the presence of mepyramine (Mepy) or cimetidine (Cime). ATP-induced Ca^{2+} responses (10 μ M ATP) were then evaluated in the absence of extracellular Ca^{2+} in cells loaded with fura 2 acetoxymethyl ester. (A) Typical traces are represented and the t20%, t50% and t80% values, i.e. the time necessary for the initial Ca^{2+} peak to decrease by 20, 50 and 80% respectively were determined. (B)–(D) Mean values of t20%, t50% and t80% respectively. (E) Mean values of basal fura 2 fluorescence ratio. (F) Mean values of maximal ATP-induced increases in fura 2 fluorescence ratio; * $P < 0.05$.

overcome the impact of Ca^{2+} influx pathways, experiments were performed in the absence of extracellular Ca^{2+} . Application of 10 μ M ATP triggered a unique Ca^{2+} spike (Figure 5A). Note that all examined cells presented a similar basal fura 2 fluorescence ratio (Figure 5E) as well as a similar ATP-induced maximal fura 2 fluorescence ratio (Figure 5F), suggesting both comparable basal $[\text{Ca}^{2+}]_i$ and intracellular ATP-releasable Ca^{2+} pools. Histamine increased the rate at which $[\text{Ca}^{2+}]_i$ declined as seen by the reduced times for $[\text{Ca}^{2+}]_i$ to fall by 20% (Figure 5B, t20%) or 50% (Figure 5C, t50%) from its peak. There was no change in Ca^{2+} decays by 80% from its peak (Figure 5D, t80%). The effect of chronic histamine treatment on the ATP-induced Ca^{2+} response was neutralized by mepyramine, but not by cimetidine

(Figures 5B and 5C). Thus H1R, but not H2R, relays histamine-induced modifications in Ca^{2+} handling in EA.hy926 cells.

DISCUSSION

In the EA.hy926 cell line, SERCA 3 represents only a minor proportion of total SERCA, with SERCA 2b being the predominant isoform in agreement with results obtained in other endothelial cell types [3]. SERCA 3 represents a substantial proportion of SERCA in freshly dissociated human or rat endothelial cells, but disappears in culture and is replaced by SERCA 2b [3]. The fact that SERCA 3 represents only a minor proportion of SERCA does not mean that it does not contribute significantly to the activity of the cell. First, in endothelial cells, as well as in other cell types, SERCA 3 and SERCA 2 are differently located in the cells (Figure 1E) [6,28], suggesting that they are involved in restoring basal Ca^{2+} in specific subcellular compartments. Secondly, the K_m for Ca^{2+} of SERCA 3 is much higher than that of SERCA 2b, suggesting that it functions only at high $[\text{Ca}^{2+}]$ [29,30]. In pancreatic β -cells, Ca^{2+} uptake to the ER at basal cytosolic $[\text{Ca}^{2+}]$ has been attributed to SERCA 2b, whereas SERCA 3 becomes operative when cytosolic $[\text{Ca}^{2+}]$ is elevated [31]. In the present paper, we demonstrate that histamine selectively up-regulates the SERCA 3 gene without affecting SERCA 2b expression. The up-regulation of SERCA 3 expression can thus be considered as a compensatory mechanism that counteracts increases in $[\text{Ca}^{2+}]_i$ by accelerating the rate of ER Ca^{2+} uptake. We cannot exclude the possibility that histamine also affects other components of the Ca^{2+} cycling such as $\text{Ins}(1,4,5)\text{P}_3$ production, IP3R expression and activity. It could also affect Ca^{2+} influx or the activity of PMCA, but expression of PMCA was unaltered.

Our results demonstrate that histamine up-regulates SERCA 3 expression upon binding to the H1R; an effect abolished by the selective H1 antagonist mepyramine. Histamine also induces nitric oxide (NO) synthesis, partly by activating the eNOS (endothelial NO synthase) gene [24]. The effects of histamine on the expression of cytokines, adhesion molecules and eNOS are all mediated through H1R [24,32]. The H2R is coupled to the Gs and protein kinase A pathway. This pathway does not seem to affect expression of the SERCA 3 gene or to relay histamine-induced modifications in Ca^{2+} handling. The H1R is known to activate the PLC/ $\text{Ins}(1,4,5)\text{P}_3$ pathway and thus to induce oscillatory increases in the intracellular Ca^{2+} levels [33], suggesting that alteration in Ca^{2+} signalling might be involved in the process.

This suggestion is reinforced by the demonstration that up-regulation of the SERCA 3 gene is mediated by the Ca^{2+} -regulated calcineurin/NFAT pathway. Histamine induced nuclear translocation of NFAT and specifically activated the NFAT-luciferase reporter construct. Histamine was unable to activate the transcription of a reporter gene driven by an NF- κ B consensus sequence, unlike what was observed in human aortic endothelial cells [34]. More importantly, up-regulation of the SERCA 3 gene by histamine was abolished by CsA and AdVIVIT, confirming that the calcineurin/NFAT pathway mediates the effect of histamine. NFAT is involved in histamine-induced up-regulation of the interleukin-8 gene in human endothelial cells [32]. It is also involved in the induction of the expression of the tissue factor [35] and cyclo-oxygenase 2 [36] genes by the vascular endothelial factor and in the stimulation of tissue factor gene expression by oxidized phospholipids [37]. In VSMCs, NFAT is activated under the influence of Gq-coupled receptor agonists such as endothelin 1 [38] and angiotensin 2 [39]. Mitogenic factors such as PDGF (platelet-derived growth factor) [40,41] or VLDLs (very-low-density lipoproteins) [42] also exert their proliferative effect through activation of NFAT.

Surprisingly, we found that the SERCA 3 basal promoter does not contain any NFAT consensus sequences and that NFAT does not bind directly to the promoter. Histamine enhanced the expression of SERCA 3 through NFAT-mediated activation of Ets-1. Both Ets-1 and Sp1 are necessary for the induction of SERCA 3 [8], but we found that histamine selectively increased the expression of Ets-1. Ets-1 also regulates normal and tumour angiogenesis through the induction of angiogenic growth factors [VEGF (vascular endothelial growth factor) and HGF (hepatocyte growth factor)] [43].

We have shown that histamine up-regulates SERCA 3 at the transcriptional level. Little is known about the mechanisms that regulate SERCA 3 gene, but we have shown previously that basal transcription of the SERCA 3 gene is dependent on binding of the transcription factors Ets-1 and Sp1 on the proximal region of the promoter. There is also some evidence that SERCA expression is regulated by Ca^{2+} . Indeed, the levels of SERCA 2b and SERCA 3 in cultured VSMCs are increased by treatment with thapsigargin or the Ca^{2+} ionophore A-23187 [10]. The presence of SERCA 3 in VSMCs is rather surprising, as SERCA 3 has been detected in the endothelial layer of the vessel [2,7]. Contamination of the culture with endothelial cells may explain this result and suggest that SERCA 3 in endothelial cells is regulated by Ca^{2+} . However, there is no indication of how an increase in Ca^{2+} can regulate SERCA 3 expression. We provide the first evidence that a physiological agonist acting through a Gq-coupled receptor regulates SERCA 3 gene expression at the transcriptional level.

In conclusion, chronic stimulation of endothelial cells with a physiological G-protein-coupled receptor agonist, such as histamine, results in an up-regulation of SERCA 3, which is normally activated at high cytosolic Ca^{2+} level, to compensate for the increase in cytosolic Ca^{2+} cycling. Up-regulation of SERCA 3 by histamine is mediated by the H1R and involves activation of the calcineurin/NFAT pathway.

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