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Regulation of Nuclear Factor of Activated T Cells (NFAT) in Vascular Endothelial Cells

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Abstract

Proteins of the NFAT family (Nuclear Factor of Activated T-Cells) are Ca^{2+} -sensitive transcription factors, which are involved in hypertrophic cardiovascular remodeling. Activation and nuclear translocation is mediated by dephosphorylation by the Ca^{2+} -sensitive phosphatase calcineurin (CaN). We identified Ca^{2+} signals that induced nuclear translocation of NFAT in cultured calf pulmonary artery endothelial (CPAE) cells using confocal fluorescence microscopy to measure simultaneously $[\text{Ca}^{2+}]_i$ and subcellular localization of NFAT-GFP (isoforms NFATc1 and NFATc3). The vasoactive agonists ATP (5 μM) or bradykinin (20 μM) in the presence of 2 mM extracellular Ca^{2+} induced Ca^{2+} release from the endoplasmic reticulum (ER) and activated capacitative Ca^{2+} entry (CCE), which caused robust translocation of NFAT to the nucleus. This effect was sensitive to the CaN-inhibitor Cyclosporin A (1 μM). Influx of extracellular Ca^{2+} via CCE, but not ER Ca^{2+} release was identified as the activating Ca^{2+} source. NFAT was also activated by Ca^{2+} influx induced by cell swelling, reverse mode Na/Ca exchange or ionomycin treatment. NFAT regulation was isoform-specific. Whereas activation of NFATc1-GFP by ATP resulted in persistent nuclear localization, NFATc3-GFP was only transiently imported into the nucleus, followed by rapid export back to the cytoplasm. Inhibition of nuclear kinases, which mediate export of NFAT via phosphorylation, or direct block of nuclear export (Leptomycin B) resulted in stable nuclear localization of NFATc3. These data demonstrate that extracellular Ca^{2+} entry mediates NFAT activation. Furthermore, the regulation of nuclear localization of NFAT is isoform-specific and dependent on nuclear export processes.

Keywords

Nuclear factor of activated T-cells; vascular endothelium; Ca^{2+} influx; CCE; NFATc1; NFATc3

Introduction

Proteins of the NFAT family are Ca^{2+} -sensitive transcription factors that regulate gene transcription in response to intracellular Ca^{2+} signals. They are present in numerous cell types,

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Disclosures

None.

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including endothelial cells, skeletal muscle cells, smooth muscle cells, cardiomyocytes and neurons [1]. Activity of NFAT is regulated by phosphorylation. Inactive NFAT is highly phosphorylated and localized in the cytoplasm. Intracellular Ca^{2+} signals activate the calmodulin-dependent serine/threonine phosphatase calcineurin (CaN), which dephosphorylates NFAT and induces translocation to the nucleus. To control gene transcription, NFAT interacts with other transcription factors (e.g. AP-1, MEF-2 or GATA-4) to bind DNA [2,3], which may also stabilize its nuclear localization. Activity of nuclear NFAT is terminated by intracellular kinases (e.g. p38, JNK, GSK3) and rephosphorylation of NFAT results in transport back to the cytoplasm via an export pathway that involves the transport protein Crm1 (exportin 1) [4,5].

The molecular mechanisms that increase intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) leading to NFAT activation vary with cell type and tissue. Nuclear translocation of NFAT has been observed after activation of store-operated Ca^{2+} entry (SOCE) [6], IP_3 -mediated Ca^{2+} release [7] and Ca^{2+} entry via voltage-operated Ca^{2+} channels in a frequency-dependent manner [8,9]. Thus, the specific spatio-temporal organization of the Ca^{2+} signal that activates NFAT varies and does not seem to follow a general pattern. For many cell and tissue types the specific Ca^{2+} signal is unknown, including in the vascular endothelium.

Five isoforms of NFAT (NFATc1 to NFATc5) have been cloned to date [10], with NFATc1 to NFATc4 being expressed in the cardiovascular system. The isoforms NFATc3 and NFATc4 are active during pathophysiological conditions that affect the cardiovascular system, including atrial fibrillation [11,12] and hypertrophy [13]. In the vascular system NFAT (including the isoforms c1 and c3) contributes to cell growth, remodeling of smooth muscle cells [14–16], controls vascular development and angiogenesis [17–19] and is activated in response to inflammatory processes [20] and high intravascular pressure [21]. In the endothelium, NFAT controls gene expression during remodeling and is activated by growth factors [22,23] or histamine [24]. However, the precise physiological or pathophysiological Ca^{2+} signals that cause activation of NFAT in endothelial cells have not been determined.

Endothelial cells (ECs) respond to extracellular stimuli such as neurotransmitters, hormones and mechanical or osmotic stress with a rise in $[\text{Ca}^{2+}]_i$, a signal that regulates numerous cellular functions [25]. Changes in endothelial $[\text{Ca}^{2+}]_i$ depend on two main Ca^{2+} sources, release of Ca^{2+} from intracellular Ca^{2+} stores (endoplasmic reticulum, ER) and activity of Ca^{2+} -permeable ion channels in the plasma membrane [26]. ER Ca^{2+} release is mediated primarily by the activity of ligand-operated Ca^{2+} release channels for Inositol 1,4,5-trisphosphate (IP_3 receptors, IP_3Rs) after stimulation of phospholipase C, which produces the second messengers IP_3 and diacylglycerol (DAG) that activates protein kinase C (PKC) [27].

A typical intracellular Ca^{2+} transient elicited by activation of Gq protein-coupled receptors after exposure to vasoactive agonists (e.g. ATP, bradykinin, histamine) consists of two phases that are due to activity of each Ca^{2+} source: An initial transient ER Ca^{2+} release of large amplitude, followed by a sustained elevation of the Ca^{2+} signal with lower amplitude, which depends on $[\text{Ca}^{2+}]_o$ (see also Figure 1B). The latter is referred to as SOCE or capacitative Ca^{2+} entry (CCE) [28, 29]. The molecular properties of CCE have been investigated in many studies over the past years [28, 30–33] and CCE has been shown to regulate various cellular functions, including its primary task of replenishing of intracellular Ca^{2+} stores, but also secretion, activation of intracellular enzymes and gene transcription [34]. Endothelial cells express numerous transcription factors, including members of the NFAT family [35], however the mechanism of Ca^{2+} -dependent activation of NFAT in endothelial cells has remained elusive. Thus, the present study was designed to investigate the Ca^{2+} -sensitive mechanisms and the specific Ca^{2+} signals and sources that activate NFAT in endothelial cells. Furthermore,

the study revealed isoform-specific differences in the activation and nuclear localization of the isoforms NFATc1 and NFATc3.

Part of this work has been published in abstract form [36,37].

Material and Methods

Cell Culture and Adenoviral Gene Transfer

Calf pulmonary artery endothelial (CPAE) cells were obtained at passage No.15 from the American Type Culture Collection (ATCC CCL-209; Manassas, VA, USA). CPAE cells were cultured using Eagle's minimum essential medium (MEM, 2 mM L-glutamine; 10% FBS and 1% penicillin/streptomycin; Mediatech, Herndon, VA, USA) and passaged onto sterile glass coverslips before experiments were performed. To maintain a uniform phenotype of the cells, no more than six passages were used for experiments. Cells were infected with adenoviruses encoding for NFATc1-GFP or NFATc3-GFP and measurements were taken 24 h to 48 h after infection at ambient temperature using single, non-confluent CPAE cells. Recombinant adenoviruses were kindly provided by Dr. Jeffery D. Molkentin, Cincinnati Children's Hospital Medical Center, Cincinnati, OH.

Solutions and Chemicals

During all experiments, cells were superfused with an extracellular HEPES-buffered solution (HBS) containing in mM: 135 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/NaOH, pH = 7.3. In some experiments, CaCl₂ was omitted (nominally Ca²⁺-free HBS), NaCl was reduced to 67.5 mM (hypo-osmotic HBS, hHBS) or NaCl was substituted using N-methyl-D-glucamine (NMDG; Na⁺-free HBS). Agonists and antagonists were prepared in HBS and were applied acutely (e.g. ATP, bradykinin) or incubated for 60 min before experiments were performed (e.g. cyclosporin A (CsA) or Leptomycin B (LB)). Substances that were applied for short-term incubation (2 h) were added to the culture medium directly. Unless stated otherwise, all standard chemicals, agonists or inhibitors were purchased from Sigma (St. Louis, MO, USA).

Fluorescence Measurements

[Ca²⁺]_i measurements—Changes in [Ca²⁺]_i from single endothelial cells were measured with fluorescence laser scanning confocal microscopy (Biorad Radiance 2000/MP), using the membrane-permeable acetoxymethyl ester of the Ca²⁺-sensitive dye rhod-2 (rhod-2/AM, Invitrogen/Molecular Probes, Carlsbad, CA, USA). This particular dye was chosen because it allows for simultaneous measurements of [Ca²⁺]_i and GFP emission (see below). Cells were loaded using an HBS solution containing 5 μM rhod-2/AM and 5 μM Pluronic F-127 (Pluronic stock solution: 0.2 g ml⁻¹ in DMSO) for 20 min at room temperature, followed by a wash in HBS for 20 min. Rhod-2 was excited with the 543 nm line of a HeNe laser and emitted fluorescence was recorded at λ ≥ 570 nm. Ca²⁺-induced changes in rhod-2 fluorescence (F) were normalized to the level of fluorescence prior to stimulation (F₀).

Dynamic measurements of NFAT-GFP—To visualize NFAT-GFP with confocal microscopy (Biorad Radiance 2000/MP), cells were excited using the 488 nm line of an argon ion laser and cellular emission was recorded between 500 nm and 520 nm. Subcellular distribution of NFAT-GFP was quantified as the ratio NFAT_{NUC}/NFAT_{CYT} using a region of interest (ROI) that covered the area of the nucleus (NFAT_{NUC}) and a cytoplasmic ROI (NFAT_{CYT}) of the same size (number of pixels). The mean fluorescence of a particular ROI was analyzed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD). Unless otherwise stated, the individual cellular ratio NFAT_{NUC}/NFAT_{CYT} was measured 20 minutes after stimuli were applied, and normalized to the initial ratio of the same cell before

stimulus application. For simultaneous measurements of $[Ca^{2+}]_i$ and subcellular localization of NFAT-GFP, cells were excited at 543 and 488 nm, and changes of both emission signals over time were assessed using identical ROIs covering the nucleus and a cytoplasmic region of the same size.

Staining of cell nuclei—To identify nuclei, CPAE cells were loaded for 20 min with 5 μ M of the membrane-permeable nucleic acid dye SYTO 59 (Invitrogen/Molecular Probes, Carlsbad, CA, USA). For confocal imaging, cells were excited at 637 nm (red diode laser) and SYTO 59 fluorescence was collected at $\lambda > 660$ nm. Dyes of the SYTO family allow simultaneous imaging of GFP, and SYTO 59 staining was used to identify nuclear localization of NFAT-GFP (Fig. 1F).

Immunocytochemistry—The preparation and immunofluorescence staining of CPAE cells were carried out as previously described [38]. Cells were incubated with a mouse monoclonal antibody against NFATc1 (sc-7294; Santa Cruz Biotechnology) using a 1:100 dilution. For visualization, an AlexaFluor 488 donkey anti-mouse secondary antibody (A-21202; Invitrogen) was used at a 1:800 dilution. Fluoromount G with DAPI (SouthernBiotech; excitation 405 nm) was used as a mounting medium and to stain the nucleus.

Statistical Analysis

Data are presented as individual observations or as mean $\pm$ S.E.M. and were analyzed using Student's t-test. n represents the number of individual cells. Differences were considered statistically significant at $P < 0.05$.

Results

ATP- and bradykinin-induced elevation of intracellular Ca^{2+} resulted in nuclear translocation of NFATc1

The subcellular distribution of NFATc1-GFP was analyzed with confocal microscopy 24 to 48 h after infections. In non-stimulated CPAE cells, NFATc1-GFP was localized to the cytoplasm of the cells (Figure 1A, upper panel) with an average NFAT_{NUC}/NFAT_{CYT} ratio of 0.55 ± 0.04 (n=50) under control conditions ($[Ca^{2+}]_o = 2$ mM). We tested two physiologically important G_q protein-coupled vasoactive agonists (ATP and bradykinin) that mobilize Ca^{2+} from intracellular IP_3 -dependent Ca^{2+} stores. Stimulation of cells with ATP (5 μ M, 5 min, $[Ca^{2+}]_o = 2$ mM) induced translocation of NFATc1-GFP to a subcellular region defined as the nucleus (Figure 1A lower panel and Figure 1F, see below). The degree of nuclear import was quantified as a 10-fold increase in the NFAT_{NUC}/NFAT_{CYT} ratio (Figure 1D). Translocation of NFATc1-GFP was prevented by the calcineurin inhibitor CsA (1 μ M), which indicates specific activation of the CaN/NFAT-pathway.

Application of ATP in the absence of extracellular Ca^{2+} (nominally Ca^{2+} -free HBS) failed to induce translocation of NFATc1-GFP (Figures 1C, 1D), even though ATP stimulation elicited a large transient increase of $[Ca^{2+}]_i$. To analyze the corresponding intracellular Ca^{2+} signals under both conditions, $[Ca^{2+}]_i$ (rhod-2 signal) and nuclear translocation of NFAT-GFP (ratio NFAT_{NUC}/NFAT_{CYT}) were measured simultaneously using confocal microscopy. We identified different intracellular Ca^{2+} signals for each type of stimulation: Application of 5 μ M ATP with 2 mM $[Ca^{2+}]_o$ (Figure 1B) resulted in IP_3 -mediated Ca^{2+} release from the ER (grey trace), followed by sustained elevation of the Ca^{2+} signal due to activation of CCE [28]. This Ca^{2+} signal caused nuclear translocation of NFAT, as indicated by a 6-fold increase in NFAT_{NUC}/NFAT_{CYT} ratio (black trace). An increase of the NFAT_{NUC}/NFAT_{CYT} ratio was detected rapidly after the rise of $[Ca^{2+}]_i$ and began to level off after ~20 minutes. While CsA prevented the nuclear translocation of NFAT (Figure 1C), it did not affect the ATP-induced

Ca^{2+} signal (data not shown), suggesting that the inhibitory effect of CsA on calcineurin was Ca^{2+} -independent. In cells where ATP was applied in the absence of extracellular Ca^{2+} , a different intracellular Ca^{2+} signal was observed: ER Ca^{2+} release was comparable with regard to amplitude and duration, but CCE was not activated (i.e. the sustained component of the signal, due to Ca^{2+} influx from the extracellular space, was absent). In the absence of extracellular Ca^{2+} , exposure to ATP failed to induce nuclear translocation of NFATc1-GFP (Figure 1C), suggesting an important role of CCE (or Ca^{2+} influx) in NFAT activation and nuclear translocation. To analyze the role of the diacylglycerol (DAG)/protein kinase C (PKC)-pathway, ATP was applied in the presence of the PKC inhibitor BIS VII (Bisindolylmaleimide VII; 100 nM). Under these conditions, nuclear import of NFATc1-GFP was reduced by ~50% (see Figure 1D and discussion). Consistent with this observation BIS VII also significantly reduced the sustained Ca^{2+} influx component (data not shown). To exclude effects of ATP that are not related to PLC stimulation we applied bradykinin (BK, 20 μM for 5 min), a different agonist that activates CCE following IP_3 -mediated Ca^{2+} release in CPAE cells [39], using otherwise the same experimental conditions (Figure 1E). Application of BK in the presence of extracellular Ca^{2+} resulted in a 5-fold increase in the $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio. As shown for ATP, translocation of NFAT to the nucleus was prevented by CsA ($[\text{Ca}^{2+}]_o = 2 \text{ mM}$) or by removing extracellular Ca^{2+} .

To identify the organelle that imports NFAT-GFP as the nucleus, we stained nuclei with dye SYTO 59 in cells that also expressed NFAT-GFP (Figure 1F). In non-stimulated cells, the NFAT-GFP signal was cytoplasmic (Figure 1Fa) and did not overlap with the fluorescence signal from the nucleus (SYTO 59 signal and corresponding line profiles, Figures 1Fb and 1Fc). In case of stimulated cells, both fluorescence signals did overlap, indicating localization of NFAT-GFP to the nucleus (Figures 1F, d–f).

Ca^{2+} influx from the extracellular space is required to activate NFATc1 in endothelial cells

To test whether extracellular Ca^{2+} represents a source that is sufficient to activate NFAT in the vascular endothelium, we experimentally separated intracellular Ca^{2+} release and extracellular Ca^{2+} influx and analyzed the impact of each on NFAT activation. CPAE cells expressing NFATc1-GFP were treated with thapsigargin (10 μM , 5 min in Ca^{2+} -free HBS), an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), to obtain complete depletion of the ER. Under these conditions, influx of extracellular Ca^{2+} via CCE can be activated and precisely controlled by adding Ca^{2+} back to the extracellular solution [28,33]. Figure 2A shows a representative CCE transient (grey trace) induced by exposure to 2 mM $[\text{Ca}^{2+}]_o$ after store depletion with thapsigargin (not shown). Application of 2 mM extracellular Ca^{2+} for 5 min resulted in rapid Ca^{2+} influx, which caused nuclear translocation of NFATc1-GFP (black trace). This translocation was dependent on the duration of CCE (Figure 2B, black columns) and quantified as a 4- to 5-fold increase in $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio (5 min CCE). Nuclear translocation of NFAT was never observed when CCE was activated in the presence of CsA (1 μM) or after incubation with thapsigargin alone (i.e. after store depletion in the absence of extracellular Ca^{2+} ; Figure 2B, grey columns).

CCE can be inhibited pharmacologically by La^{3+} ions [34] (Figure 3A, right trace). To further underscore the requirement of Ca^{2+} influx for NFAT activation during agonist application, we applied ATP in the presence of 100 μM La^{3+} . This concentration of La^{3+} was chosen because it causes CCE inhibition without affecting plasmalemmal Ca^{2+} ATPases in CPAE cells [40]. In comparison to a normal ATP-induced Ca^{2+} transient, La^{3+} blocked the sustained component of the transient (i.e. CCE). Under these conditions, the Ca^{2+} transient was similar in amplitude and duration to a typical Ca^{2+} transient observed under Ca^{2+} -free conditions (Figure 3B) and nuclear translocation of NFATc1-GFP was substantially decreased (Figure 3C). These results

provide evidence that nuclear translocation of NFAT in endothelial cells is directly controlled by influx of Ca^{2+} from the extracellular space without contribution of ER Ca release.

Under the premise that Ca^{2+} influx per se represents the predominant Ca^{2+} source to activate NFAT in CPAE cells, it should be independent from the molecular mechanism and/or pathway that transports Ca^{2+} across the plasma membrane. To address this issue, cells were treated with ionomycin, an ionophore that generates Ca^{2+} -permeable pores in the plasma membrane [41, 42]. CPAE cells were treated with ionomycin (1 μM for 5 min in Ca^{2+} -free HBS) to allow for Ca^{2+} pore formation and Ca^{2+} -influx was experimentally controlled by changing $[\text{Ca}^{2+}]_o$. Exposure of cells to 2 mM $[\text{Ca}^{2+}]_o$ for 5 min resulted in an elevation of $[\text{Ca}^{2+}]_i$ (Figure 4A) that was sufficient to induce nuclear translocation of NFATc1-GFP (Figure 4B). In comparison to the degree of translocation observed after ATP- or BK-application (5- to 10-fold increase of $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio; Figure 1D and 1E), direct influx of Ca^{2+} caused only a modest translocation (2- to 3-fold increase in $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio; Figure 4B). This rise of $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio could be prevented by CsA. Nevertheless, these data demonstrate that extracellular Ca^{2+} influx is required to activate nuclear translocation of NFAT in CPAE cells.

Cell swelling represents an additional stimulus that causes elevation of $[\text{Ca}^{2+}]_i$ in endothelial cells (presumably through opening of stretch-activated Ca^{2+} -permeable channels [43] or release of vasoactive agonists [44]) and has been implicated in gene transcription and remodeling [45,46]. Therefore, we tested if cell swelling activates NFAT in CPAE cells. Cell swelling was induced by exposure to a hypoosmotic extracellular solution (hHBS), which contained 67.5 mM NaCl instead of 135 mM NaCl. Exposure of CPAE cells to Ca^{2+} -free hHBS did indeed cause cell swelling, as indicated by a decrease in absolute rhod-2 fluorescence (dilution of the Ca^{2+} -sensitive dye), a decrease of the rhod-2 F/F_0 ratio signal (presumably due to dilution of $[\text{Ca}^{2+}]_i$; Figure 5A) or movement and change of the shape of the cells. Subsequent addition of extracellular Ca^{2+} caused influx of Ca^{2+} and elevation of $[\text{Ca}^{2+}]_i$. As shown in Figure 5B, the Ca^{2+} influx induced by cell swelling caused robust translocation of NFATc1-GFP to the nucleus (summarized data Figure 5C). This nuclear import was absent when Na^+ was reduced to 67.5 mM and the HBS was osmotically balanced with 67.5 mM NMDG. Nuclear translocation of NFAT was sensitive to CsA and could be prevented by blocking Ca^{2+} influx using La^{3+} or by omitting Ca^{2+} in the extracellular solution (Figure 5C). Thus, cell swelling caused an influx of extracellular Ca^{2+} which was sufficient to induce activation of NFAT. Furthermore, moderate translocation of NFAT was observed when Na^+ was substituted completely with 135 mM NMDG (3-fold increase in $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ -ratio, Figure 5C), presumably due to increasing $[\text{Ca}^{2+}]_i$ through reverse mode Na/Ca exchange [47]. These data indicate that Ca^{2+} influx mechanisms distinct from ion channels can contribute to NFAT activation in endothelial cells.

Dynamic regulation of nuclear import and export of NFATc1 and NFATc3

The nuclear-cytosolic distribution of NFAT is also influenced by the activity of nuclear export [48,49]. To investigate the impact of nuclear export on the subcellular localization of NFAT we compared the isoforms NFATc1 and NFATc3 and used pharmacological inhibition of the NFAT export pathway. To analyze NFAT export dynamics, NFAT was translocated to the nucleus first by ATP application as described above (Figure 1). Maximum nuclear accumulation of NFATc1 and NFATc3 was achieved by stimulation with 5 μM ATP in $[\text{Ca}^{2+}]_o = 2 \text{ mM}$ (Figure 6B). Nuclear accumulation of NFATc1 and NFATc3 was quantified 20 min after ATP application. Similar to NFATc1-GFP, the isoform NFATc3-GFP showed cytoplasmic distribution in non-stimulated cells with an average ratio $\text{NFATc3}_{\text{NUC}}/\text{NFATc3}_{\text{CYT}} = 0.57 \pm 0.04$ (n=50), which increased substantially 20 min after application of

ATP ($\text{NFATc3}_{\text{NUC}}/\text{NFATc3}_{\text{CYT}} = 4.89 \pm 0.61$; $n=50$). This approximately 9-fold increase in $\text{NFATc3}_{\text{NUC}}/\text{NFATc3}_{\text{CYT}}$ ratio was similar to the increase seen for NFATc1 (Figure 1C).

After maximum nuclear accumulation of both isoforms induced by ATP stimulation was observed (20 min after ATP application, Figure 6B middle panel), $[\text{Ca}^{2+}]_i$ was reduced by incubation in Ca^{2+} -free HBS for 30 min. As shown in Figure 6A incubation in Ca^{2+} -free HBS resulted in a decrease in $[\text{Ca}^{2+}]_i$. This substantial reduction in $[\text{Ca}^{2+}]_i$ caused a redistribution of both isoforms to the cytoplasm (Figure 6B, lower panel), which was significantly more pronounced in case of NFATc3 (summarized in Figure 6C, grey columns) as compared to NFATc1 (black columns). The underlying mechanism of this redistribution was identified as nuclear export due to its sensitivity to Leptomycin B (40 nM), a selective inhibitor of the export protein Crm1 [50] (Figure 6C).

Figures 6D and 6E show that nuclear export of NFATc1 is highly active under basal, unstimulated conditions. When Leptomycin B was applied alone in the presence of 2 mM $[\text{Ca}^{2+}]_o$ (i.e. block of nuclear export while basal $[\text{Ca}^{2+}]_i$ and import rates remain intact), a robust increase of the $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio was observed (Figure 6Da, summary data Figure 6Db). This suggests efficient nuclear export under basal conditions, which prevents nuclear accumulation of NFATc1 and keeps transcriptional activity low.

The rate of nuclear export of NFAT is determined by phosphorylation of NFAT, thus inhibition of underlying nuclear kinases should facilitate accumulation of NFAT in the nucleus. Figure 6E shows an increase of the $\text{NFATc1}_{\text{NUC}}/\text{NFATc1}_{\text{CYT}}$ ratio in CPAE cells when glycogen synthase kinase 3 (GSK3), a key enzyme for NFAT phosphorylation [51] was inhibited by adding LiCl_2 (20 mM) or alsterpaullone (1 μM) to the culture medium of the cells for 2 hours.

The profound regulation of NFATc3 by nuclear export was further analyzed under normal Ca^{2+} conditions ($[\text{Ca}^{2+}]_o = 2 \text{ mM}$) and compared to the nucleo-cytoplasmic shutteling of the isoform NFATc1. Application of ATP for 5 min, a stimulus that resulted in stable and persistent nuclear localization of NFATc1 (Figure 7A, black trace), caused only transient nuclear localization of NFATc3 that was followed by complete export of NFATc3 back to the cytoplasm (grey trace). The summary data (Figure 7B) show that the degree of peak nuclear localization of NFATc3 (grey column, 15 min after ATP application) was comparable to that of NFATc1 after 30 min (ratio $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}} > 5$). Nuclear NFATc3 translocation reversed within 30 min due to export back to the cytoplasm, however when nuclear export was reduced by inhibition of intracellular kinases (GSK3, JNK2) or blocked using 40 nM Leptomycin B, application of ATP for 5 min resulted in stable accumulation of NFATc3 in the nucleus (Figure 7C). Given this dependence on nuclear export, brief application of ATP (5 min) might be too short to generate a nuclear import rate that is sufficient to exceed nuclear export. Indeed, longer application of ATP (20 min) resulted in robust translocation of NFATc3-GFP to the nucleus (13-fold increase of $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio, Figure 7C, grey column). These data indicate that nuclear localization of NFAT is not only determined by intracellular Ca^{2+} signals, but also regulated by nuclear export. This dependence on nuclear export is isoform-specific, allowing fine tuning of activation between different NFAT isoforms.

Nuclear translocation of endogenous NFAT

Endothelial cells express endogenous NFAT isoforms [35]. We tested whether extracellular Ca^{2+} also represents the Ca^{2+} source that activates endogenous NFAT in endothelial cells. To address this issue, cells were exposed to ATP as described above and fixed 20 min after agonist application. Subcellular distribution of endogenous NFAT was analyzed with immunostainings using an antibody against NFATc1 combined with a fluorescent (AlexaFluor 488) secondary antibody. This particular isoform was chosen, since we observed robust nuclear translocation of NFATc1-GFP in CPAE cells (see above). Consistent with our experiments

above, endogenous NFATc1 was localized predominantly cytoplasmic in non-stimulated cells (Figure 8A, i). Application of ATP induced nuclear translocation (Figure 8A, ii), which was sensitive to CsA (2 μ M) and required extracellular Ca^{2+} (summary data, Figure 8B). In addition, endogenous NFATc1 also displayed basal activity: inhibition of nuclear export with LB (40 nM) for 30 min resulted in substantial nuclear accumulation of endogenous NFATc1. Thus, endogenous NFAT and the adenovirally expressed NFAT-GFP fusion proteins behaved similarly, confirming that the latter are valuable tools to study NFAT regulation.

Discussion

Although Ca^{2+} signaling in the endothelium is well defined, little is known how Ca^{2+} regulates NFAT in endothelial cells. Early studies on NFAT activation using Luciferase-based reporter assays or immunohistochemistry showed that NFAT activation depends on a G_q protein-coupled pathway that involves phospholipase C (PLC) [52]. In some studies using endothelial cells, nuclear localization of NFAT and transcriptional activity of NFAT were induced using vascular endothelial growth factor or histamine as stimulating agonists [24,53]. It has also been demonstrated that NFAT localized in the nucleus is transcriptionally active, regulating interleukin or SERCA gene expression [23,24]. Distinct from ER Ca^{2+} release, the intracellular Ca^{2+} response elicited by agonists such as histamine typically occurs in form of Ca^{2+} oscillations, which critically depend on extracellular Ca^{2+} [54].

The aim of the present study was to identify the intracellular Ca^{2+} signal that activates the CaN/NFAT-pathway and to obtain insight into NFAT regulation in endothelial cells. To address these issues, we used NFAT-GFP fusion proteins to monitor nuclear translocation of NFAT with confocal microscopy and relate it to changes in $[\text{Ca}^{2+}]_i$ using the Ca^{2+} -sensitive dye rhod-2. NFAT-GFP fusion proteins are ideal for this purpose since they behave similarly to endogenous proteins (Figure 8; cf. also reference [55]) and have been used in several studies [8,56–58].

One of the striking results of this study was the novel observation that extracellular Ca^{2+} influx was an absolute requirement for NFAT activation and translocation, whereas even large amplitude Ca^{2+} signals generated by intracellular Ca^{2+} release were essentially ineffective. We used the physiological agonists ATP and bradykinin [28,30,39,40,59] to induce activation of PLC and subsequent IP_3 -mediated elevations in $[\text{Ca}^{2+}]_i$. Application of ATP and bradykinin in the presence of 2 mM extracellular Ca^{2+} induced substantial translocation of NFAT to the nucleus of the cells, which was identified independently with SYTO 59 nuclear stainings (Figure 1F). The underlying biphasic Ca^{2+} transient consisted of IP_3 -mediated Ca^{2+} release from the ER and capacitative Ca^{2+} entry (CCE) [59,60]. Stimulation of PLC via G_q -coupled receptors does not only produce IP_3 , but also generates DAG. Therefore, we tested the impact of the DAG/PKC pathway on NFAT-activation by inhibiting PKC with BIS VII. Application of ATP under these conditions resulted in a 50% reduction of nuclear NFAT translocation as compared to control cells. This is in line with the observation that BIS VII reduced the Ca^{2+} influx (CCE) component of the Ca^{2+} signal as well as with early studies of NFAT in T-cells, where nuclear localization of NFAT was enhanced by a synergistic effect of $[\text{Ca}^{2+}]_i$ and PKC activity. Activation of PKC and downstream pathways may result in the presence of nuclear binding partners of NFAT, which stabilize its nuclear localization [5,61]. An important result of the present study is the dominant role of extracellular Ca^{2+} in the activation of NFAT in CPAE cells, with CCE representing the major influx pathway under physiological conditions. Application of ATP or BK in the absence of extracellular Ca^{2+} (i.e. ER Ca^{2+} release alone) did not induce nuclear translocation of NFAT. Furthermore, CCE activation after store depletion with thapsigargin was capable of inducing robust nuclear translocation of NFATc1-GFP, whereas ER depletion alone (treatment with thapsigargin in Ca^{2+} -free Tyrode) failed to induce nuclear translocation.

Moreover, removal of the CCE component of the ATP-induced Ca^{2+} transients using La^{3+} ions suppressed nuclear translocation of NFATc1-GFP, without affecting ER Ca^{2+} load. Lanthanum also inhibits plasma membrane Ca^{2+} ATPases (PMCA) [62], thus raising the question whether a La^{3+} -PMCA interaction might influence NFAT activation in our experiments. Previous work from our group showed that in CPAE cells extrusion of Ca^{2+} via PMCA represents the major pathway for the rapid decrease of elevated $[\text{Ca}^{2+}]_i$ after activation of CCE [40]. Complete inhibition of PMCA (which can be achieved in CPAE cells with La^{3+} concentrations in the low mM range [40]) slowed Ca^{2+} extrusion kinetics significantly (however Ca^{2+} continues to be extruded through Na/Ca exchange). The La^{3+} concentrations used in the present study (10–100 μM), however, did not alter the decline of the Ca^{2+} transient (TG-induced CCE) as compared to control conditions (Figure 3A). Therefore, it is unlikely that PMCA was substantially inhibited under our experimental conditions.

Influx of extracellular Ca^{2+} via SOCE has been shown to be essential for the control of NFAT activity in other non-excitable cells. Pathological disruption of SOCE blunted the activation of NFAT in lymphocytes, where the proteins Stim1 and Orai1 have been identified as molecular correlates for CRAC channels and SOCE [63,64]. However, molecular influx pathways generating CCE and SOCE vary with the cell type under study, with Stim1, Orai1 and transient receptor potential (trp) channels being discussed as underlying proteins. Briefly, it has remained a matter of controversy whether SOCE is due to Stim1 and Orai1 with no contribution of trp channels [65], or whether SOCE requires Stim1, Orai1 and trpc1 [66]. In case of CPAE cells, it has been demonstrated that overexpression of trp channels was sufficient to increase the amplitude of the CCE component during agonist-induced Ca^{2+} transients [67,68]. To our best knowledge, involvement of Stim1 and Orai1 during CCE has not been studied yet in this particular cell type. While the molecular mechanisms of CCE in CPAE cells will require further clarification (which is beyond the scope of the present NFAT study) our study clearly demonstrates that functionally CCE represents an efficient Ca^{2+} source for the nuclear translocation of NFAT.

The role of extracellular Ca^{2+} in nuclear translocation of NFAT was further confirmed in experiments using conditions that generate Ca^{2+} influx into the cytoplasm independent of IP_3 -dependent ER Ca^{2+} release. In cells treated with ionomycin, elevation of $[\text{Ca}^{2+}]_i$ by adding Ca^{2+} to the extracellular solution caused nuclear translocation of NFAT as well as an increase in $[\text{Ca}^{2+}]_i$. Ionomycin increases $[\text{Ca}^{2+}]_i$ either by generating Ca^{2+} -permeable pores in the plasma membrane [41,42] or via a store-regulated Ca^{2+} influx mechanism [69]. In addition, swelling of CPAE cells using hypoosmotic solutions caused elevation of $[\text{Ca}^{2+}]_i$ and robust translocation of NFAT to the nucleus due to Ca^{2+} influx from the extracellular space, which was dependent on $[\text{Ca}^{2+}]_o$ and sensitive to La^{3+} -ions (Figure 5). Furthermore, Ca^{2+} entry via reverse mode Na/Ca exchange can potentially contribute to the Ca^{2+} signal required for NFAT translocation.

A common feature of all experimental conditions that caused NFAT translocation described above is to generate sustained $[\text{Ca}^{2+}]_i$ elevations. The data suggest that aside the source of Ca (influx vs. release) the duration of the Ca signal rather than its amplitude determines NFAT activation. For example, the degree of NFAT translocation correlated with the duration of the CCE signal (Figure 2B), whereas a high-amplitude but short Ca spike failed to activate NFAT (Figure 1C). This is consistent with the notion that sustained $[\text{Ca}^{2+}]_i$ elevations stimulate CaN more efficiently than the large amplitude transient Ca^{2+} peaks observed during ER Ca^{2+} release [48].

We also tested the impact of nuclear export on the nuclear-cytosolic distribution of NFATc1 and NFATc3 and identified isoform-specific differences. Lowering $[\text{Ca}^{2+}]_i$ in cells where NFAT was located initially to the nucleus induced nuclear export and resulted in transport of

NFAT back to the cytoplasm (Figure 6B). In comparison, the isoform NFATc3 was more affected by nuclear export than NFATc1. These differences were also present in experiments using normal Ca^{2+} conditions ($[\text{Ca}^{2+}]_o = 2 \text{ mM}$). Application of ATP for 5 min in the presence of 2 mM $[\text{Ca}^{2+}]_o$ resulted in persistent nuclear accumulation of NFATc1, but only transient nuclear import of NFATc3 (Figure 7), a result that is consistent with recent work on skeletal muscle cells [58]. Persistent nuclear localization of NFATc3 in CPAE could be induced when intracellular kinases were inhibited, when export was completely blocked using Leptomycin B or when nuclear import rates were enhanced by longer application of ATP (20 min). As shown in Figures 6D and 6E, inhibition of GSK3 or application of Leptomycin B under basal conditions caused a massive nuclear translocation of NFATc1 (nearly 6- and 25-fold increase of $\text{NFAT}_{\text{NUC}}/\text{NFAT}_{\text{CYT}}$ ratio, respectively) suggesting that a tightly regulated export pathway for NFAT is responsible for keeping NFAT out of the nucleus, which thereby prevents transcriptional activity.

There is growing evidence that NFAT activation is related to cardiovascular pathologies. In vascular smooth muscle cells (VSMCs), NFATc3 was activated upon application of high intravascular pressure to intact arteries [21], which was due to influx of extracellular Ca^{2+} and also negatively regulated by the intracellular kinase JNK2. In agonist-induced hypertrophy (Ang II), NFATc3 was translocated to the nucleus in VSMCs via facilitation of L-type Ca^{2+} channel activity. Interestingly, this facilitation was dependent on PKC phosphorylation of the channel and involved the scaffolding protein AKAP150 and CaN to generate a signaling unit in the plasma membrane that controls NFAT signaling via Ca^{2+} influx independent from $[\text{Ca}^{2+}]_i$ [70,71]. NFATc1 has been shown to be activated in response to injury of VSMCs and was related to damage repair and proliferation [72]. Consistent with data from the present work, activity of NFATc1 was negatively regulated by GSK3 in these cells.

We conclude that activation and nuclear translocation of NFAT in the endothelium is induced by extracellular Ca^{2+} with negligible contribution of ER Ca^{2+} release. In addition to its activating Ca^{2+} signal, nuclear localization of NFAT is also influenced by activity of nuclear kinases, depends on nuclear export processes, and reveals profound NFAT isoform-specific differences.

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Figure 1. The vasoactive agonists ATP and bradykinin induce translocation of NFATc1-GFP to the nucleus of CPAE cells

A: NFATc1-GFP was localized to the cytoplasm of CPAE cells (upper panel). Application of extracellular ATP (5 μ M, 5 min) caused nuclear translocation of NFAT in the same cells (lower panel, image recorded 20 min after ATP-application). **B:** Simultaneous measurements of $[\text{Ca}^{2+}]_i$ (rhod-2, grey trace) and nuclear NFAT translocation (black trace) in response to ATP stimulation ($[\text{Ca}^{2+}]_o = 2$ mM). NFAT translocation was quantified as the ratio NFAT_{NUC}/NFAT_{CYT}, normalized to the nuclear-cytosolic distribution of NFAT under basal conditions. **C:** Nuclear translocation was absent when ATP was applied in the absence of extracellular Ca^{2+} (ER Ca^{2+} release alone). **D:** Summarized data showing that nuclear translocation was

dependent on $[Ca^{2+}]_o$, sensitive to CsA (1 μ M) and PKC inhibitor BIS VII (Bisindolylmaleimide VII). **E:** Nuclear translocation of NFAT after application of bradykinin (BK, 20 μ M) under the same experimental conditions. **F:** Staining of the nucleus using SYTO 59 identified cytoplasmic localization of NFAT-GFP in non-stimulated cells (a through c) and nuclear localization of NFAT-GFP in stimulated (5 μ M ATP, $[Ca^{2+}]_o = 2$ mM) cells (d through f). Traces represent raw fluorescence intensity profiles of GFP and SYTO 59 fluorescence across the nucleus and adjacent cytoplasmic regions, normalized to maximum fluorescence. CCE: capacitative Ca^{2+} entry; CsA: cyclosporin A; ER: endoplasmic reticulum. *, statistically significant ($P < 0.05$) compared to control. Here and elsewhere: numbers in parentheses indicate number of individual cells tested.

Figure 2. Capacitative Ca^{2+} entry (CCE) controls nuclear translocation of NFATc1 after store depletion with thapsigargin

A: Representative CCE trace induced by 2 mM $[\text{Ca}^{2+}]_o$ in CPAE cells with depleted Ca^{2+} stores. Cells were pre-treated with 10 μM thapsigargin (TG) under Ca^{2+} -free conditions to deplete the ER of Ca^{2+} (not shown). Application of 2 mM $[\text{Ca}^{2+}]_o$ for 5 min resulted in Ca^{2+} -influx (grey trace), which induced nuclear translocation of NFATc1 (black trace). **B:** Summary data: CCE alone was sufficient to induce nuclear translocation of NFATc1-GFP in CPAE cells. This effect was dependent on CCE duration (black columns). CsA inhibited CCE-induced nuclear translocation of NFAT. Store depletion alone (TG, $[\text{Ca}^{2+}]_o = 0$) without CCE failed to

initiate NFAT translocation (grey columns). *, statistically significant ($P < 0.05$) compared to control.

Figure 3. ATP-induced nuclear translocation of NFATc1 is sensitive to La^{3+} and extracellular Ca^{2+}

A: Pharmacological inhibition of CCE by La^{3+} ions. TG-induced CCE (control, left trace) was blocked in the presence of La^{3+} -ions in the extracellular solution (right trace). **B:** Representative recordings of ATP-induced Ca^{2+} transients showed that La^{3+} or the absence of extracellular Ca^{2+} prevented the sustained component of the Ca^{2+} transient (CCE). **C:** Summary data: Nuclear translocation of NFATc1-GFP in response to ATP was suppressed by La^{3+} ions. *, statistically significant ($P < 0.05$) compared to control.

Figure 4. Extracellular Ca^{2+} induces nuclear translocation of NFATc1 in ionomycin-treated CPAE cells

A: Application of 2 mM extracellular Ca^{2+} resulted in a substantial elevation of $[Ca^{2+}]_i$ in ionomycin-treated (5 min) CPAE cells. **B:** Summary data: Direct influx of extracellular Ca^{2+} induced moderate nuclear translocation of NFATc1-GFP that was blocked by CsA. *, statistically significant ($P < 0.05$) compared to control.

Figure 5. Cell swelling and reverse mode Na/Ca exchange activate nuclear translocation of NFATc1-GFP in CPAE cells

A: Exposure to a hypoosmotic solution resulted in cell swelling (indicated by a decrease in rhod-2 F/F_0 -ratio signal) and reversible Ca^{2+} influx from the extracellular space. **B:** Cell swelling in the continuous presence of extracellular Ca^{2+} resulted in Ca^{2+} -influx (grey trace) and translocation of NFATc1-GFP to the nucleus (black trace). Inset: Images of subcellular NFAT distribution before (a) and after (b) cell swelling. **C:** Summary data. Swelling induced nuclear import of NFAT was absent when extracellular Ca^{2+} was omitted or La^{3+} -ions were present in the extracellular solution. Complete substitution of $[\text{Na}^+]_o$ with NMDG resulted in moderate nuclear import of NFATc1-GFP (see text for details), presumably due to Ca^{2+} influx via reverse mode Na/Ca exchange. NMDG: N-methyl-D-glucamine. *, statistically significant ($P < 0.05$) compared to control.

Figure 6. Nuclear localization of NFAT-GFP is influenced by nuclear export mechanisms
A: Incubation in Ca^{2+} -free HBS decreased $[Ca^{2+}]_i$ in CPAE cells. **B:** Application of ATP induced nuclear import of NFATc1-GFP and NFATc3-GFP in CPAE cells (middle images acquired 20 min after ATP application). Subsequent incubation in Ca^{2+} -free HBS (30 min) resulted in export of both isoforms. **C:** Summary data: NFATc3-GFP was more affected by nuclear export than NFATc1-GFP. Incubation with Leptomycin B inhibited nuclear export back to the cytoplasm for both isoforms. Note that in these experiments NFAT_{NUC}/NFAT_{CYT} was normalized to the fluorescence level (ratio) of maximal nuclear translocation induced by ATP. *, difference statistically significant at $P < 0.05$. **D:** Inhibition of nuclear export by Leptomycin B under basal conditions ($[Ca^{2+}]_o = 2$ mM) facilitated nuclear

localization of NFATc1-GFP within 30 min. **E:** Short-term inhibition (2 h) of glycogen synthase kinase 3 (GSK3) by adding LiCl (20 mM) or alsterpaullone (alsterp, 1 μ M) to the culture medium resulted in nuclear accumulation of NFATc1.

Figure 7. NFATc3 is strongly regulated by nuclear export

A: Application of ATP for 5 min (5 μ M, [Ca²⁺]_o = 2 mM) induced persistent nuclear translocation of NFATc1, but only transient nuclear translocation of NFATc3. **B:** Summary data: Initial translocation of NFATc3 (15 min after application of ATP) was completely reversed 30 min after ATP application (grey columns), while NFATc1 remained in the nucleus (black column). *, difference statistically significant at $P < 0.05$. **C:** Pharmacological inhibition of nuclear kinases or block of nuclear export with Leptomycin B (40 nM) resulted in persistent nuclear localization of NFATc3 (application of ATP for 5 min, ratio NFAT_{NUC}/NFAT_{CYT} was analyzed 30 min after application). Grey column: longer application of ATP (20 min) resulted in robust nuclear accumulation of NFATc3. LB: Leptomycin B (40 nM): inhibitor of nuclear export; SB 202190 (1 μ M): inhibitor of kinase p38; LiCl (20 mM) and alsterpaullone (1 μ M): inhibitor of GSK3; SP 600125 (1 μ M): inhibitor of JNK2.

Figure 8. Nuclear translocation of endogenous NFATc1 in CPAE cells

A: Immunostaining using an antibody against NFATc1 displays cytoplasmic distribution of NFATc1 in non-stimulated cells (i). Application of ATP (5 μ M, 5 min; [Ca²⁺]_o = 2 mM) resulted in nuclear translocation of NFATc1 (ii). Localization of nuclei was identified with simultaneous DAPI staining (data not shown). **B:** Summary data. Nuclear translocation of NFATc1 was sensitive to cyclosporin A (CsA) and extracellular Ca²⁺. In addition, block of nuclear export with 40 nM Leptomycin B (LB) for 30 min resulted in robust nuclear accumulation of NFATc1. Ab, antibody directed against NFATc1. *, difference statistically significant at P < 0.05 compared to control.