Am J Physiol Heart Circ Physiol 298: H2001–H2009, 2010.
First published March 19, 2010; doi:10.1152/ajpheart.01072.2009.

Isoform- and tissue-specific regulation of the Ca^{2+}-sensitive transcription factor NFAT in cardiac myocytes and heart failure

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Submitted 11 November 2009; accepted in final form 15 March 2010

Rinne A, Kapur N, Molkentin JD, Pogwizd SM, Bers DM, Banach K, Blatter LA. Isoform- and tissue-specific regulation of the Ca^{2+}-sensitive transcription factor NFAT in cardiac myocytes and heart failure. *Am J Physiol Heart Circ Physiol* 298: H2001–H2009, 2010. First published March 19, 2010; doi:10.1152/ajpheart.01072.2009.—Nuclear factors of activated T cells (NFATs) are Ca^{2+}-sensitive transcription factors that have been implicated in hypertrophy, heart failure (HF), and arrhythmias. Cytosolic NFAT is activated by dephosphorylation by the Ca^{2+}-sensitive phosphatase calcineurin, resulting in translocation to the nucleus, which is opposed by kinase activity, rephosphorylation, and nuclear export. Four different NFAT isoforms are expressed in the heart. The activation and regulation of NFAT in adult cardiac myocytes, which may depend on the NFAT isoform and cell type, are not fully understood. This study compared basal localization, import, and export of NFATc1 and NFATc3 in adult atrial and ventricular myocytes to identify isoform- and tissue-specific regulatory mechanisms of NFAT activation under physiological conditions and in HF. NFAT-green fluorescent protein fusion proteins and NFAT immunocytochemistry were used to analyze NFAT regulation in adult cat and rabbit myocytes. NFATc1 displayed basal nuclear localization in atrial and ventricular myocytes, an effect that was attenuated by reducing intracellular Ca^{2+} concentration and inhibiting calcineurin, and enhanced by the inhibition of nuclear export. In contrast, NFATc3 was localized to the cytoplasm but could be driven to the nucleus by angiotensin II and endothelin-1 stimulation in atrial, but not ventricular, cells. Inhibition of nuclear export (by leptomycin B) facilitated nuclear localization in both cell types. Ventricular myocytes from HF rabbits showed increased basal nuclear localization of endogenous NFATc3 and reduced responsiveness of NFAT translocation to phenylephrine stimulation. In control myocytes, Ca^{2+} overload, leading to spontaneous Ca^{2+} waves, induced substantial translocation of NFATc3 to the nucleus. We conclude that the activation of NFAT in adult cardiomyocytes is isoform and tissue specific and is tightly controlled by nuclear export. NFAT is activated in myocytes from HF animals and may be secondary to Ca^{2+} overload.

nuclear factor of activated T cells; intracellular Ca^{2+} concentration; calcineurin; nuclear translocation

DURING PATHOLOGICAL REMODELING associated with hypertension, hypertrophy, heart failure (HF), and arrhythmias, the cardiovascular system experiences changes in gene transcription and protein expression. Nuclear factor of activated T cells (NFAT) transcription factors play a key role in cellular remodeling, integrating intracellular Ca^{2+} signals and gene transcription. NFAT is controlled by phosphorylation [e.g., by the kinases glycogen synthase kinase (GSK)3β, p38, and JNK], and its phosphorylation status regulates its translocation into the nucleus. Intracellular Ca^{2+} signals stimulate the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (CaN), which dephosphorylates NFAT, causing translocation to the nucleus, where it regulates gene transcription (4, 43, 59). Rephosphorylation of NFAT causes NFAT nuclear export and relieves its transcriptional effect. Cardiac myocytes display large rhythmic changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with every heart beat (5). This raises the question of how NFAT is activated in a Ca^{2+}-dependent fashion either normally or during pathological situations (5, 35, 57). Several mechanisms have been hypothesized to selectively activate CaN and NFAT dephosphorylation [e.g., Ca^{2+} fluxes through voltage-gated L-type Ca channels and Ca^{2+}-induced Ca^{2+} release (CICR) from ryanodine receptor (RyR) sarcoplasmic reticulum (SR) Ca^{2+}-release channels, Ca^{2+} entry via T-type Ca^{2+} channels, store-operated Ca^{2+} entry, and inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} release; for a review, see Ref. 58]. However, none of these pathways has been demonstrated experimentally in conclusive and unequivocal ways. Nonetheless, several recent reports have shed new light on two Ca^{2+} signaling mechanisms that appear to be involved in the activation of NFAT in diseased cardiomyocytes: first, under conditions of tachycardia, the high frequency of action potentials can cause a net intracellular Ca^{2+} gain that increases $[Ca^{2+}]_i$ and can activate the CaN/NFAT pathway (30). Two more recent studies (42, 61) have revealed a potential link between NFAT activation and remodeling of ion channels [L-type Ca^{2+} channels and voltage-gated K^+ channels (transient outward K^+ current)] in canine myocytes in response to high-frequency pacing. Second, chronic activation of G_q protein-coupled receptors by neurohumoral agonists [e.g., angiotensin (ANG) II and endothelin (ET)-1] may represent an alternative mechanism for NFAT activation observed during hypertrophy (32, 36). G_q proteins stimulate phospholipase C, which generates the second messengers IP_3 and diacylglycerol. IP_3 promotes Ca^{2+} release from intracellular stores by activating specific Ca^{2+}-release channels of the SR (IP_3 receptors). IP_3-mediated Ca^{2+} release could either act as a Ca^{2+} source independent from RyR-mediated Ca^{2+} release or modulate RyR-dependent CICR, resulting in arrhythmogenic Ca^{2+} release and elevated systolic $[Ca^{2+}]_i$ (63). The application of ET-1 has been shown to generate cytoplasmic Ca^{2+} signals that affect the nucleus and activate NFAT, thereby inducing hypertrophy in ventricular myocytes (23). In addition, IP_3-

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dependent Ca^{2+} signaling also occurs locally at the nuclear envelope and affects the nuclear Ca^{2+} concentration, which, in turn, controls many cellular functions in cardiac cells (22, 27), including the regulation of transcription factors (60).

The mammalian heart expresses four different NFAT isoforms (NFATc1, NFATc2, NFATc3, and NFATc4), which are regulated by $[Ca^{2+}]_i$ via CaN (54). Open questions regarding our understanding of NFAT regulation in adult cardiac myocytes are: Is there a common mechanism that activates all NFAT isoforms in cardiac cells, or are there isoform-specific or even tissue-specific (atrium vs. ventricle) differences? Is NFAT solely regulated via the balance of cytosolic phosphatase (CaN) and kinase activity, or are regulated nuclear export pathways and nuclear kinases involved in NFAT activation/deactivation?

The present study analyzed basal and agonist-induced (ET-1 and ANG II) activation of NFATc1 and NFATc3 in quiescent myocytes (measured as nuclear localization of NFAT) using NFAT-green fluorescent protein (GFP) fusion proteins in conjunction with confocal microscopy. We found isoform- and tissue-specific differences in the activation of NFATc1 and NFATc3 in adult cardiac myocytes as well as increased basal activity of NFATc3 in myocytes from failing hearts.

Part of this work has been previously published in abstract form (45, 47).

MATERIALS AND METHODS

Isolation, Cell Culture, and Viral Transduction of Cardiac Myocytes

Atrial and ventricular myocytes were isolated from cat or rabbit hearts as previously described (26, 50). The procedure for cell isolation was approved by the Institutional Animal Care and Use Committees. Briefly, atrial and ventricular myocytes were isolated from cat or rabbit hearts using animals of either sex. Animals were anesthetized with thiopental sodium (35 mg/kg ip). After a thoracotomy, the hearts were quickly excised, mounted on a Langendorff apparatus, and retrogradely perfused via the aorta. After an initial washing step with an oxygenated Ca^{2+}-free solution [which contained (in mM) 137 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 12 $NaHCO_3$, 0.6 NaH_2PO_4, and 11 glucose], the heart was perfused with oxygenated HEPES-buffered saline solution (HBSS) containing 36 μM Ca^{2+} and collagenase at 37°C (0.06% collagenase type II, Worthington Biochemical, Freehold, NJ) to obtain single myocytes. Isolated cells were adapted to the final Ca^{2+} concentration of the medium (1.8 mM) over a period of 2 h and plated on sterile, laminin-coated glass coverslips. Myocytes were cultured using serum-free medium 199, which was supplemented with 25 μg/ml gentamycin and 25 μg/ml kanamycin (all from Mediatech, Hernon, VA). One day after isolation, cells were infected with adenoviruses encoding for NFATc1-GFP and NFATc3-GFP, and experiments were performed 24 h (ventricular myocytes) or 48 h (atrial myocytes) after infections. In addition, ventricular myocytes were isolated from rabbits with nonischemic HF induced by combined aortic insufficiency and stenosis (38, 39). This HF model is characterized by the combination of the gradual development of hypertrophy and HF (over 4–9 mo, as monitored by serial echocardiography), depressed systolic function, and arrhythmogenesis in an animal that has human-like Ca^{2+} handling and cellular electrophysiological properties. The echocardiographic index of severe left ventricular dysfunction is a left ventricular end-systolic dimension of >1.4 cm (>40% increase). This model has been well characterized on structural, biochemical, molecular, Ca^{2+} handling, and electrophysiological levels (14, 15, 38, 40, 41, 49).

Solutions and Chemicals

During all experiments, cells were bathed in an extracellular solution (HBSS), which contained (in mmol/l) 135 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). Agonists or antagonists were prepared in HBSS for acute application or added to the medium for longer incubations. Unless otherwise stated, all chemicals, agonists, or inhibitors were purchased from Sigma (St. Louis, MO) or Tocris (Ellisville, MO).

Fluorescence Measurements

Measurements of NFAT-GFP. To analyze the subcellular localization of NFAT-GFP with confocal microscopy (Biorad Radiance 2000/MP) in virally transduced myocytes, we measured the mean fluorescence of a region of interest (ROI) covering the nucleus ($NFAT_{nuc}$) and a cytoplasmic ROI ($NFAT_{cyt}$) of the same size. The subcellular distribution of NFAT was then quantified as the ratio of $NFAT_{nuc}$ to $NFAT_{cyt}$. In some experiments, where agonists or antagonists were applied acutely, this ratio was normalized to the ratio measured from the same cell before the stimulus application. GFP was excited with an argon ion laser (wavelength: 488 nm), and emitted fluorescence was collected at 500–520 nm.

Intracellular Ca^{2+} measurements. Intracellular Ca^{2+} transients from single myocytes evoked by electrical field stimulation (0.5 Hz) or $[Ca^{2+}]_i$ signals evoked by Ca^{2+} overload were measured with rhod-2 and confocal microscopy in the line-scan mode (3 ms/line, pixel size: 1.5 μm). Briefly, cells were loaded for 20 min at room temperature with 5 μM rhod-2 AM and 5 μM Pluronic F-127 in HBSS (Pluronic stock solution: 0.2 g/ml DMSO). After the removal of excessive dye, rhod-2 was excited at 543 nm (green He-Ne laser), and emitted fluorescence was recorded at a wavelength of ≥570 nm. Changes in rhod-2 fluorescence were normalized to the level of fluorescence before stimulus application. Rhod-2 was chosen as the Ca^{2+}-sensitive dye because it allows for measurements of $[Ca^{2+}]_i$ in the presence of GFP (46).

Immunocytochemistry. The preparation and immunofluorescence staining of rabbit ventricular myocytes were carried out as previously described (46). Myocytes were plated on laminin-coated coverslips and washed with *solution A* (450 mM NaCl, 20 mM phosphate buffer, pH 7.2). Cells were fixed for 30 min using 4% (wt/vol) paraformaldehyde in 200 mM phosphate buffer (pH 7.4). Excessive paraformaldehyde was washed out using *solution A*, and cells were permeabilized using *solution B* (0.3% Triton X-100, 450 mM NaCl, and 20 mM phosphate buffer; pH 7.2) supplemented with 1% goat serum. Cells were then incubated with goat polyclonal antibody C-20 against NFATc3 (sc-1152, Santa Cruz Biotechnology, Santa Cruz, CA) using a 1:300 dilution. For visualization, an Alexa fluor488 donkey anti-goat secondary antibody (A11055, Invitrogen, Carlsbad, CA) was used at a 1:500 dilution. Fluoromount G was used as a mounting medium.

Data Analysis and Presentation

Data are presented as individual observations or as means ± SE and were analyzed using a Student's *t*-test. *n* represents the number of individual cells, and differences were considered significant at $P < 0.05$.

RESULTS

Localization of NFAT in Resting Myocytes Is Isoform Specific

The subcellular distribution of NFAT-GFP in resting (extracellular Ca^{2+} concentration: 2 mmol/l) adult cat myocytes was analyzed with confocal microscopy 24–48 h after infections. NFATc1-GFP was predominantly localized to the nucleus of

atrial and ventricular cells (Fig. 1, *A*,*a* and *A*,*c*). Average $NFATc1_{nuc}$-to-$NFATc1_{cyt}$ ratios were 1.71 ± 0.31 (n = 31) for atrial myocytes and 2.50 ± 0.12 (n = 22) for ventricular myocytes. In contrast, NFATc3-GFP displayed cytoplasmic localization under basal conditions, as indicated by lower average $NFATc3_{nuc}$-to-$NFATc3_{cyt}$ ratios of 0.54 ± 0.05 (n = 17) and 0.51 ± 0.04 (n = 34) for atrial myocytes (Fig. 1*A*,*b*) and ventricular (Fig. 1*A*,*d*) myocytes, respectively.

Adult myocytes in culture may be subject to morphological and functional changes that are characterized by the loss of t-tubules and insensitivity to electrical excitability (20, 33, 34, 55) and may affect Ca^{2+} handling. To test for functional changes of excitation-contraction coupling and CICR, we measured action potential-evoked intracellular Ca^{2+} transients from myocytes expressing NFATc1-GFP using the Ca^{2+}-sensitive dye rhod-2. Cultured myocytes responded to electrical field stimulation (0.5 Hz) with Ca^{2+} transients that were typical for atrial cells (Fig. 1*B*, *top*) or ventricular cells (Fig. 1*B*, *bottom*), indicating no significant functional changes due to time in culture.

Pharmacological Manipulation of NFATc1 in Cardiac Myocytes

If the nuclear localization of NFATc1 is due to basal Ca^{2+}-dependent CaN activity, then reduction of $[Ca^{2+}]_i$ or inhibition of CaN with cyclosporin A (CsA) would be expected to shift NFATc1 toward the cytoplasm (i.e., decrease the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio). Indeed, the acute application of CsA (1 μmol/l) in Ca^{2+}-free HBSS for 60 min resulted in the redistribution of NFATc1 to the cytoplasm in atrial cells (Fig. 2, *A*,*c* and *A*,*d*), which was quantified as a decrease in the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio by up to 50% of the initial level (summary data shown in Fig. 2*A*,*e*); however, NFATc1 retained the preferential nuclear localization.

The observation that nuclear localization of NFAT is reversible indicates the involvement of a nuclear export process for NFAT. It has been suggested that the nuclear export of NFAT involves the transport protein Crm1 (exportin 1), which can be inhibited by leptomycin B (LB) (28). The application of LB for 60 min resulted in an even higher $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio for NFATc1 (Fig. 2, *A*,*a*, *A*,*b*, and *A*,*e*). This indicates that in the basal state there is some dynamic balance of NFATc1 import (driven by CaN activity) and export via a Crm1-dependent pathway (as opposed to a maximal nuclear concentration).

These observations were not restricted to atrial tissue, since LB also increased the steady state ratio by a factor of ~1.8 (Fig. 2, *B*,*a* and *B*,*b*), and CaN inhibition (and reduction of $[Ca]_i$) caused nearly a 50% reduction in the ratio (Fig. 2, *B*,*c* and *B*,*d*) in cat ventricular myocytes.

Kinase activity and rephosphorylation of NFAT can also influence NFAT localization in ventricular myocytes (4, 9, 53). When we inhibited the cellular kinases GSK3β with 1 μmol/l alsterpaullone or JNK2 using 1 μmol/l SP-600125 in ventricular cells by incubations overnight, there was a substantial nuclear accumulation of NFATc1, as indicated by an increase in the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio from 2.10 ± 0.08 (control, n = 34) to 4.17 ± 0.32 (SP-600125, n = 20) and 5.28 ± 0.36 (alsterpaullone, n = 24).

These data suggest that the CaN/NFATc1 pathway has a high basal activity in resting atrial myocytes and even more so in ventricular myocytes. Nonetheless, nuclear kinase activity and export processes can shift the nuclear/cytosolic localization of NFATc1 in adult cardiac myocytes.

Activation of NFATc3 by Neurohumoral Stimuli

The NFATc3 isoform has been shown to play an important role in hypertrophy and HF-related remodeling in the cardio-

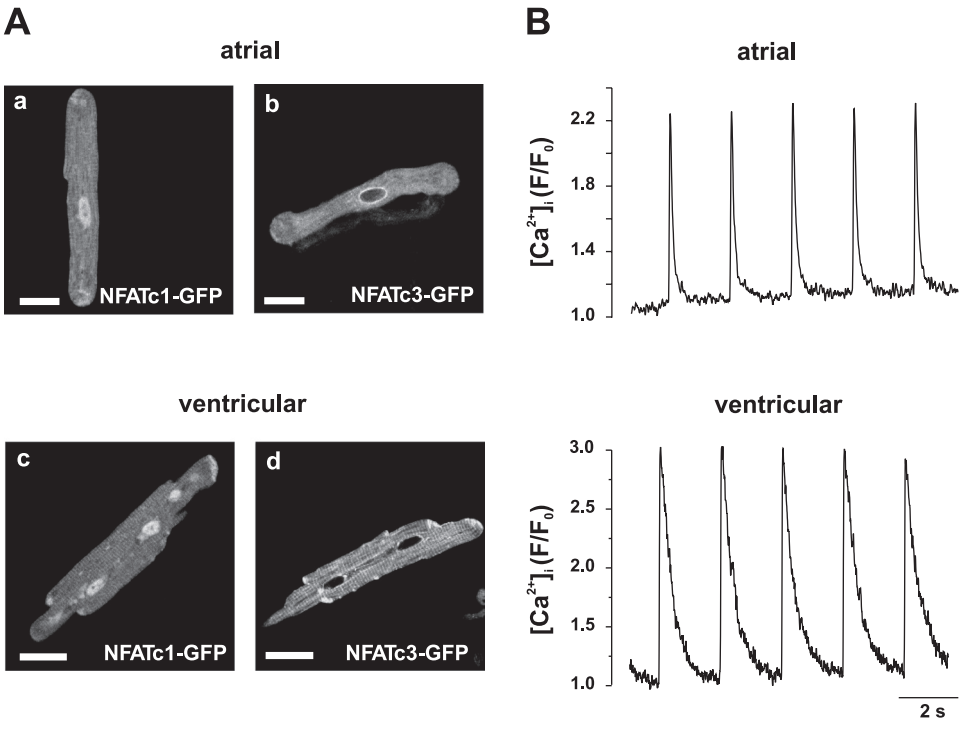

Fig. 1. Isoform-specific subcellular localization of nuclear factors of activated T cells (NFATs) in adult cardiomyocytes. *A*: representative confocal images showing the basal nuclear localization of NFATc1-green fluorescent protein (GFP) in atrial (*a*) and ventricular (*c*) myocytes. In contrast, NFATc3-GFP was localized to the cytoplasm in cardiac myocytes [atrial cells (*A*,*b*) and ventricular cells (*A*,*d*)]. Images were taken 24 h (ventricular cells) or 48 h (atrial cells) after adenoviral transduction. *B*: representative intracellular Ca^{2+} transients from adult cultured myocytes expressing NFATc1-GFP in response to electrical stimulation (0.5 Hz). Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured with confocal laser scanning microscopy in the line-scan mode using the Ca^{2+}-sensitive dye rhod-2. Changes in rhod-2 fluorescence (F) were normalized to the level of fluorescence before stimulus application (F_0). Scale bars = 30 μm.

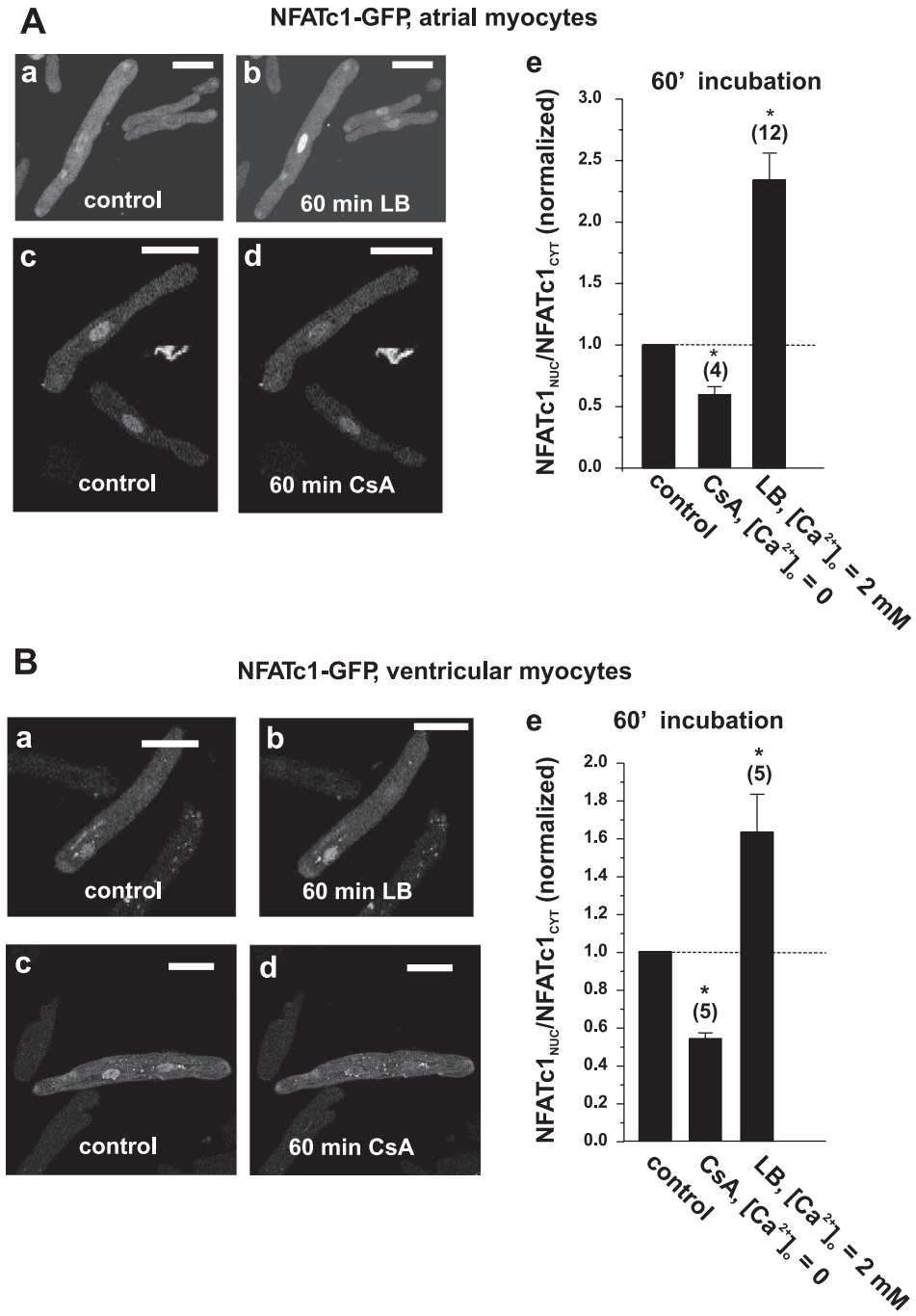

Fig. 2. Pharmacological manipulation of NFATc1 basal activity. *A*: nuclear localization of NFATc1 in atrial cells was sensitive to $[Ca^{2+}]_i$, calcineurin (CaN) activity, and the nuclear export processes. Short-term incubation (60 min) of atrial cells with 40 nM leptomycin B (LB) caused further accumulation of NFATc1 in the nucleus (*A*,*a* and *A*,*b*), whereas the inhibition of CaN, induced by an incubation with nominally Ca^{2+}-free extracellular solution containing cyclosporin A (CsA; 1 μmol/l), resulted in the export of NFATc1 back to the cytoplasm (*A*,*c* and *A*,*d*). *A*,*e*: summary data from *A*,*a*–*A*,*d*. *B*: as in atrial cells, basal nuclear localization in ventricular cells was also sensitive to CaN activity (*B*,*a*, *B*,*b*, and *B*,*e*) and enhanced when nuclear export was inhibited with LB (*B*,*c*–*B*,*e*). Nuclear-to-cytoplasmic NFAT ratios ($NFAT_{nuc}/NFAT_{cyt}$ ratios) were normalized to the control ratio at an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) of 2 mmol/l before incubation. Numbers in parentheses indicate the number of individual cells tested. Scale bars = 30 μm. *Significantly different from control at $P < 0.05$.

vascular system (11, 19, 48, 61). In contrast to NFATc1, we found NFATc3 to be localized to the cytoplasm of resting atrial and ventricular myocytes (cf. Fig. 1*A*) under basal conditions. We tested whether the hypertrophy-related extracellular agonists ANG II or ET-1 were capable of inducing the translocation of NFATc3 to the nucleus. These agonists activate the G_q protein/IP_3 pathway and liberate Ca^{2+} from the SR via IP_3 receptor Ca^{2+}-release channels (44, 63), a pathway that has been linked to the activation of transcription factors in myocytes (22, 60). Atrial myocytes expressing NFATc3-GFP were incubated overnight in medium containing 2 μmol/l ANG II or 100 nmol/l ET-1. Both agonists induced the nuclear translocation of NFATc3, which was quantified as a two- to threefold increase in the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio (representative images are shown in Fig. 3*A*; summary data are shown in Fig. 3*B*). The degree of agonist-induced translocation was comparable with nuclear accumulation induced by LB (Fig. 3*B*). The combination of agonist stimulation and block of nuclear export further enhanced the nuclear accumulation of NFAT (4- to 5-fold increase of the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio compared with control). Shorter incubation times (up to 4 h) did not result in detectable changes in the subcellular distribution of NFATc3 (data not shown).

In striking contrast to atrial myocytes, stimulation with ANG II and ET-1 failed to induce the nuclear accumulation of NFATc3 in ventricular myocyes (Fig. 4, *A* and *B*). However,

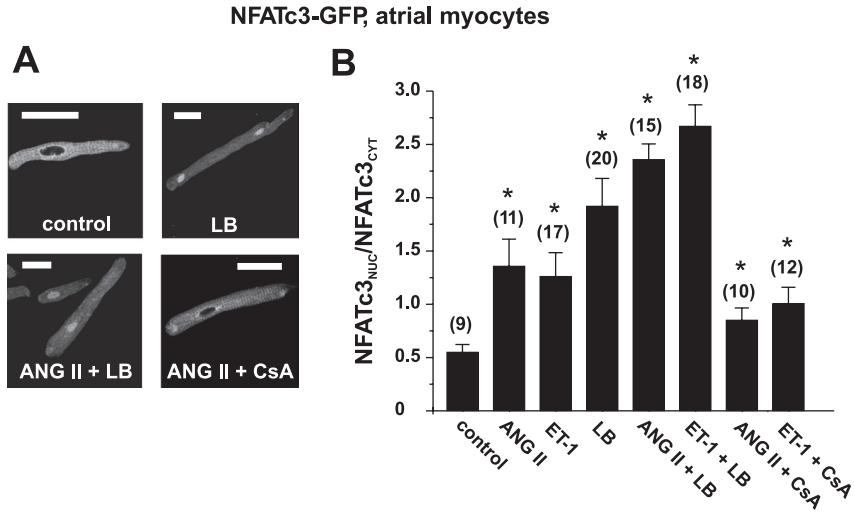

Fig. 3. Nuclear translocation of NFATc3 is induced by angiotensin (ANG) II and endothelin (ET)-1 in adult atrial myocytes. *A* and *B*: overnight incubation of atrial myocytes with ANG II (2 μmol/l), ET-1 (100 nmol/l), or LB (40 nmol/L) resulted in the nuclear translocation of NFATc3-GFP, which was sensitive to CsA (1 μmol/l). This effect could be enhanced when agonist stimulation was combined with LB incubation. Numbers in parentheses indicate the number of individual cells tested. Scale bars = 30 μm. *Significantly different from control at $P < 0.05$.

the inhibition of nuclear export (40 nmol/l LB) resulted in the substantial nuclear accumulation of NFATc3-GFP in time-matched parallel cultures (Fig. 4*B*). Combined incubation with ANG II and LB did not induce further nuclear accumulation of ventricular NFATc3, in contrast to observations in atrial cells (compare Fig. 3*B*).

Furthermore, the inhibition of cellular kinases (with alsterpaullone or SP-600125), an experimental intervention that induced the nuclear accumulation of NFATc1 in ventricular cells (see above), did not induce nuclear localization of NFATc3 in ventricular myocytes [$NFAT_{nuc}$-to-$NFAT_{cyt}$ ratios: control, 0.66 ± 0.04 (n = 51); alsterpaullone, 0.65 ± 0.06 (n = 12); and SP-600125, 0.63 ± 0.07 (n = 13)].

Another notable observation was a strong fluorescence signal of NFATc3-GFP around the nucleus (Fig. 4*C*; see also Fig. 1*A*,*a* for atrial cells). As shown for ventricular cells at two different magnifications, NFATc3 did accumulate around the nucleus in nonstimulated cells (Fig. 4*C*). This finding might support the hypothesis that NFATc3 is regulated locally by IP_3-dependent nuclear Ca^{2+} signals (see the DISCUSSION) (27).

Our data suggest that the activation and regulation of NFATc3 are different in atrial cells compared with ventricular cells. The activation of atrial (but not ventricular) IP_3 pathways by hypertrophic, neurohumoral agonists appears to be capable of inducing the nuclear translocation of NFATc3. Comparable with the NFATc1 isoform above, nuclear NFATc3 accumulation is also limited by dynamic nuclear export mechanisms under basal conditions.

HF and Ca^{2+} Overload Induce Nuclear Localization of NFATc3

HF is characterized by profound changes in Ca^{2+} handling (38) and gene transcription (7). Since the activation of CaN depends on the sustained elevation of $[Ca^{2+}]_i$ (59), a mechanism that results in Ca^{2+} overload would favor the activation of NFAT transcription factors under pathological conditions. NFATc3, in particular, is active during pathological situations, including atrial and ventricular fibrillation or the presence of hypertrophic extracellular agonists (23, 42, 61). We analyzed the nuclear localization of endogenous NFATc3 in ventricular cells from a chronic rabbit HF model (38) using immunocytochemistry (NFATc3-specific antibody). In ventricular myocytes from HF rabbits, nuclear localization of NFATc3 was en-

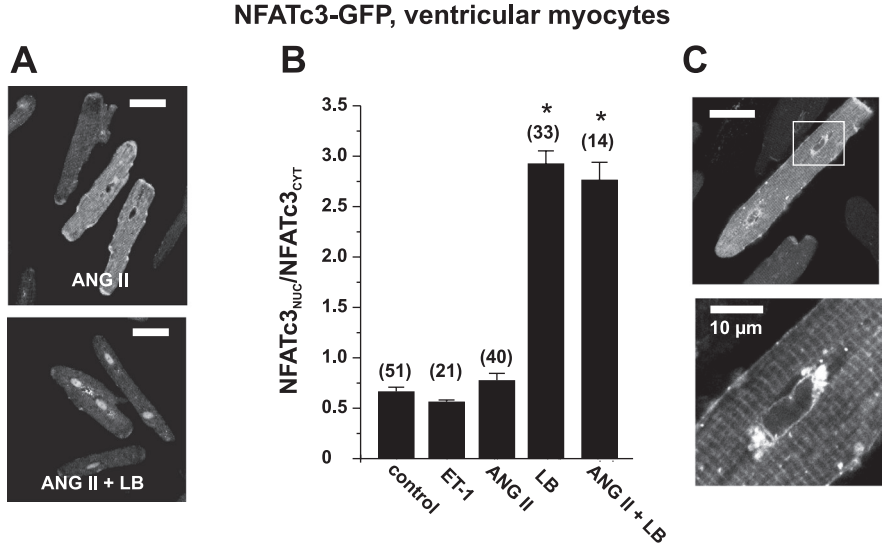

Fig. 4. Regulation of NFATc3 is tissue specific. *A* and *B*: agonists ANG II and ET-1 failed to induce the nuclear translocation of NFATc3 in ventricular myocytes. In contrast, inhibition of nuclear export with LB caused substantial nuclear accumulation of NFATc3, which was not further enhanced by ANG II (compare with Fig. 3*B*). *C*: NFATc3-GFP accumulated in a region surrounding the nucleus in resting myocytes. Numbers in parentheses indicate the number of individual cells tested. Scale bars = 30 μm except for *C*, *bottom*, where the scale bar = 10 μm. *Significantly different from control at $P < 0.05$.

hanced compared with myocytes from normal rabbits [$NFAT_{nuc}$-to-$NFAT_{cyt}$ ratios: 0.49 ± 0.014 (n = 37) vs. 0.40 ± 0.013 (n = 22), $P < 0.002$; Fig. 5*A*]. The application of phenylephrine (100 μmol/l) resulted in further nuclear accumulation of NFATc3 in ventricular cells from control rabbits but failed to facilitate nuclear localization in myocytes from HF animals (Fig. 5*B*). Similar to cat ventricular myocytes, ET-1 and ANG II, however, failed to induce translocation in rabbit ventricular cells (data not shown). Phenylephrine application in the presence of CsA prevented the agonist-induced nuclear translocation in control cells and reduced the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio below control levels in HF myocytes. These data suggest that the Ca^{2+}/CaN pathway may be basally activated in ventricular myocytes from HF animals.

Cardiac myocytes from failing hearts develop irreversible changes in Ca^{2+} homeostasis due to altered function and/or the expression of several Ca^{2+}-handling proteins, among them, the Na^+/Ca^{2+} exchanger (NCX). In HF rabbits, alterations in $[Ca^{2+}]_i$ contribute to systolic dysfunction and arrhythmogenesis (39, 40) and may also contribute to altered transcriptional regulation (7). To test whether diastolic $[Ca^{2+}]_i$ elevation (e.g., at a high heart rate in HF) activates NFATc3 in ventricular myocytes from normal rabbits, we induced Ca^{2+} overload in myocytes expressing NFATc3-GFP by Na^+,K^+-ATPase inhibition (K^+-free solution), which elevates the intracellular Na^+ concentration and, in turn, $[Ca^{2+}]_i$ [via NCX, leading to SR Ca^{2+} overload (18)]. Figure 6*A*,*a* shows the sustained elevation of $[Ca^{2+}]_i$ in K^+-free solution, which caused the translocation of NFATc3 to the nucleus (over 2 h) to a similar extent as LB in normal HBSS (Fig. 6*A*,*b*). This NFATc3 translocation was prevented by either blocking CaN with CsA or removing extracellular Ca^{2+} (Fig. 6*A*,*b*) and further enhanced by 40 nM LB. The further analysis of $[Ca^{2+}]_i$ signals under Ca^{2+} overload conditions using higher temporal resolution (confocal line scan mode) indicated that these cells developed spontaneous Ca^{2+} release in the form of spontaneous Ca^{2+} waves and a net increase in diastolic $[Ca^{2+}]_i$ (see Fig. 6*B*,*a*). In contrast, control cells did not show comparable changes in $[Ca^{2+}]_i$ (control myocytes; Fig. 6*B*,*b*).

These data indicate that the nuclear localization of NFATc3 is regulated by a dynamic balance between import and export rates. Under normal conditions, a net nuclear export rate prevents the nuclear localization of NFATc3. A pathological Ca^{2+} signal (e.g., Ca^{2+} waves and elevated diastolic $[Ca^{2+}]_i$, as in Fig. 6) changes this balance to a net nuclear import, thereby stabilizing the nuclear localization of NFATc3 in cardiomyocytes.

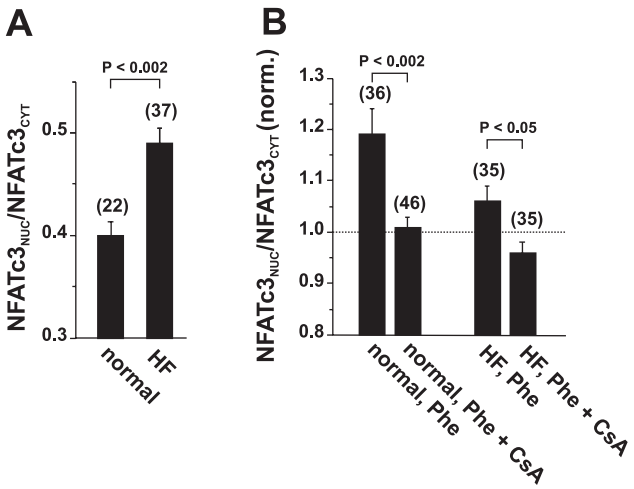

Fig. 5. NFATc3 is activated in ventricular myocytes from rabbits with heart failure (HF). *A*: basal nuclear localization of endogenous NFATc3 (NFATc3 antibody) was enhanced in ventricular myocytes from HF rabbits compared with control rabbit myocytes. *B*: stimulation with phenylephrine (Phe; 100 μmol/l) induced further nuclear accumulation of NFATc3 in control myocytes but not in HF myocytes. $NFAT_{nuc}/NFAT_{cyt}$ ratios were normalized to the respective level of unstimulated cells as shown in *A* (dashed line). In *A* and *B*, endogenous NFAT was detected by immunocytochemistry using a specific antibody against NFATc3. Numbers in parentheses indicate the number of individual cells tested.

DISCUSSION

Transcription factors of the NFAT family are involved in the pathological remodeling of cardiac myocytes (42, 61). The mammalian heart expresses four different Ca^{2+}-sensitive isoforms of NFAT (NFATc1, NFATc2, NFATc3, and NFATc4) (54). Although Ca^{2+}-dependent activation and regulation of NFAT have been investigated in detail in many cell types (10, 21, 59), it is not fully understood how NFAT is regulated in adult cardiac myocytes, which experience large changes in $[Ca^{2+}]_i$ during every heart beat (5, 35, 57). Several Ca^{2+}-dependent signals for the activation of NFAT have been proposed for neonatal and adult cardiac tissue, including contributions of extracellular Ca^{2+} (i.e., Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels) (24, 42, 56) or local spatially restricted Ca^{2+} signals, such as nuclear Ca^{2+} and IP_3-mediated Ca^{2+} release (27). The activation of NFAT in adult myocytes has been observed during high-frequency pacing (42, 61) or the application of neurohumoral stimuli (ET-1) that activate the IP_3 pathway (23). In addition, recent evidence has suggested an upstream regulatory function of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) during NFAT activation by means of CaN phosphorylation (31, 61); however, the details of this type of regulation remain to be substantiated. The studies mentioned above have also indicated that the activation of NFAT is tissue specific and may also be regulated through pathways other than the Ca^{2+}-dependent activation of CaN alone.

The present study focused on the analysis of basal and agonist-induced activation of NFAT (measured as nuclear translocation of NFAT) in adult myocytes. To visualize and analyze the subcellular localization of NFAT, we used NFAT-GFP fusion proteins (NFATc1 and NFATc3 isoforms) and quantified the subcellular localization of NFAT as the $NFAT_{nuc}$-to-$NFAT_{cyt}$ ratio. NFAT-GFP fusion proteins are widely used to study NFAT in living cells because they behave similarly to endogenous proteins (13, 17, 25, 31, 46, 51).

We found that the NFATc1 isoform, but not the NFATc3 isoform, displayed nuclear localization in resting myocytes. To our best knowledge, this is the first study to compare these isoforms in adult cardiac myocytes. Consistent with the notion of the high activity of NFATc1 compared with other isoforms, a recent study on skeletal muscle cells has described the robust nuclear translocation of NFATc1 but only the transient nuclear localization of NFATc3 in response to electrical stimulation. In those cells, as well as in endothelial cells, it was found that

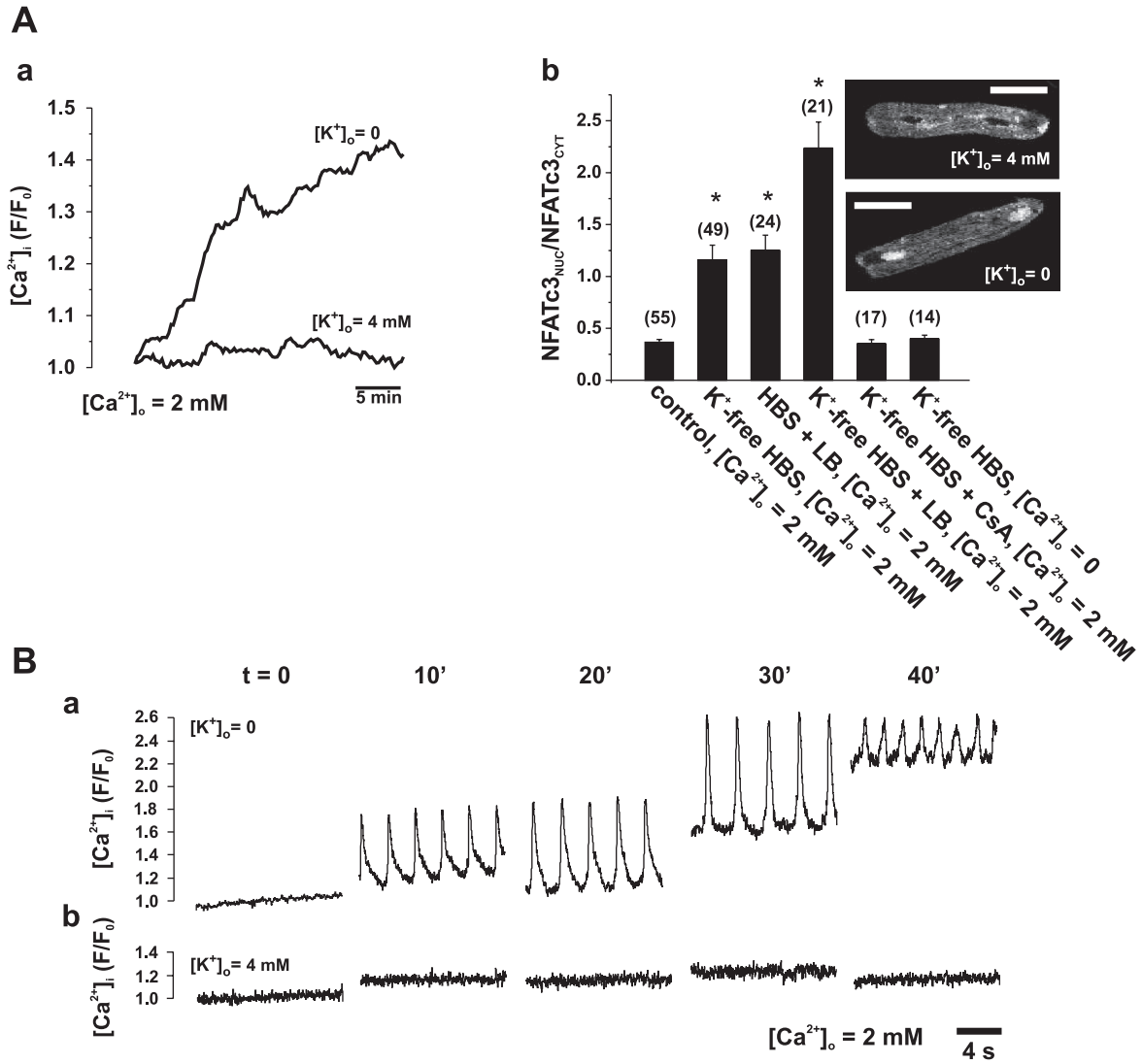

Fig. 6. Ca^{2+} overload caused the nuclear translocation of NFATc3 in ventricular myocytes from normal rabbits. *A*,*a*: removal of external K^+ caused an increase of $[Ca^{2+}]_i$. *A*,*b*: removal of external K^+ caused the nuclear translocation of NFATc3-GFP in control myocytes (*inset*). The summary data show that nuclear translocation after incubation in K^+-free HEPES-buffered saline (HBS) solution for 2 h was sensitive to CsA (1 μmol/l) and dependent on $[Ca^{2+}]_o$. Inhibition of nuclear export with LB (40 nmol/l, 2-h incubation) further facilitated the nuclear accumulation of NFATc3. *B*: $[Ca^{2+}]_i$ profiles derived from confocal line-scan images indicating that Ca^{2+} overload resulted in spontaneous Ca^{2+} release and Ca^{2+} waves in an extracellular K^+ concentration ($[K^+]_o$) of 0 mmol/l (*a*) but not of 4 mmol/l (*b*). All experiments were performed in the presence of 10 mmol/l 2,3-butanedione monoxime to prevent contractions. Numbers in parentheses indicate the number of individual cells tested. Scale bars = 30 μm. *Significantly different from control at $P < 0.05$.

NFATc3, but not NFATc1, was tightly controlled by nuclear export processes (46, 51).

The molecular mechanism for the basal nuclear localization of NFATc1 is not well understood. Transcriptional activity and the localization of NFAT in the nucleus are determined by binding DNA and other accessory transcription factors in the nucleus (52, 59). This mechanism would provide another layer of (isoform-specific) regulation of NFAT in the nucleus. Cardiac NFATc1 controls valve formation and morphogenesis of the heart, and disruption of this isoform results in lethal defects (12). Thus, the basal activity of NFATc1 in cardiac myocytes is likely to be required to maintain the differentiated phenotype of adult myocytes. This idea is supported by a more recent study that analyzed the role of different NFAT isoforms for the differentiation of skeletal muscle fibers. The study (8) demonstrated that the activation of a single NFAT isoform or the concerted activation of up to four NFAT isoforms controls the differentiation of skeletal muscle fibers into slow or fast fibers. However, it remains to be determined how exactly specific patterns of NFAT isoforms are activated differentially. It is conceivable that NFAT isoforms reside in distinct cytosolic domains and restricted Ca^{2+} signals activate CaN locally, that the spatiotemporal organization of the Ca^{2+} signal (e.g., steady-state elevation vs. oscillatory changes) is responsible for the activation of a specific isoform, or that different NFAT isoforms have different levels of sensitivity for dephosphorylation by CaN.

Another striking result of the present study is the specific activation of NFATc3 by the neurohumoral agonists ANG II and ET-1 in atrial, but not ventricular, myocytes from the cat. Our laboratory has previously demonstrated that the density of IP_3 receptors is higher in atrial than ventricular myocytes (16)

and that IP_3 signaling strongly influences Ca^{2+} handling, excitation-contraction coupling, and arrhythmogenesis in atrial myocytes (29, 62, 63). While the more pronounced IP_3-dependent Ca^{2+} release in atrial cells would support the hypothesis that this specific source of Ca^{2+} preferentially activates NFAT, further investigation will be required to clarify whether this difference in IP_3 receptor signaling indeed represents the molecular mechanism responsible for the cellular differences in NFAT regulation or if there are other mechanisms involved, such as different expression levels of membrane surface receptors for these agonists.

We also observed a concentrated localization of NFATc3 around the nucleus. This observation supports the notion that cardiac NFAT and other Ca^{2+}-dependent transcription factors are regulated by local, nuclear Ca^{2+} signals, independent from normal "beat-to-beat" Ca^{2+} (22, 27, 60). In support of this idea are the observations that the nuclear envelope is well equipped with IP_3 receptors (3) and that IP_3 can release Ca^{2+} from the cytosolic side of the nuclear envelope as well as into the nucleoplasm via IP_3 receptors located in the inner membrane of the nuclear envelope (62). Thus, IP_3-mediated Ca^{2+} release can act locally around the nucleus and affect nuclear envelope $[Ca^{2+}]$ and the nuclear Ca^{2+} concentration (62, 63), which could represent the basic elements of a mechanism for NFAT activation independent from global cytoplasmic Ca^{2+} signals. Thus, the perinuclear region could reflect a local reserve of NFAT that is poised for shuttling in and out of the nucleus when local $[Ca^{2+}]_i$ is elevated.

In ventricular myocytes from HF rabbits, basal nuclear localization of NFATc3 was increased compared with control cells (Fig. 5*A*). HF myocytes are characterized by increased expression of IP_3 receptors and increased diastolic Ca^{2+} release (1) as well as calmodulin/CaMKII-dependent nuclear export of HDAC5 (another transcriptional regulator) (7).

The higher circulating levels of neurohumoral factors in HF, which trigger IP_3 signaling (e.g., ET-1 and ANG II), may contribute to the enhanced IP_3-dependent NFAT nuclear import seen here and may reinforce the HF phenotype. In HF, the intracellular Na^+ concentration is increased (2, 6, 37) and can increase $[Ca^{2+}]_i$ and spontaneous SR Ca^{2+} release (especially at higher heart rates). This could also contribute to enhanced CaN activity and nuclear translocation of NFATc3-GFP in HF, as seen here in Ca^{2+}-overloaded control ventricular myocytes (Fig. 6).

The fact that there are substantial basal levels of nuclear NFATc3 (and especially NFATc1) that are acutely increased by block of nuclear export implies that there is a significant rate of basal CaN-dependent import and export of NFAT that results in the steady-state distribution. Moreover, this may poise this system for dynamic manipulation by cytosolic or perinuclear Ca^{2+} signals.

We conclude that the regulation of NFAT in adult cardiac myocytes is isoform specific and differs among atrial and ventricular tissue. Nuclear localization of NFAT is regulated by both the Ca^{2+} signal, which activates CaN, but also by nuclear NFAT kinases and the nuclear export machinery.

GRANTS

This work was supported by grants National Heart, Lung, and Blood Institute Grants HL-62231 and HL-80101 (to L. A. Blatter), HL-089617 (to K. Banach), HL-30077 and HL-80101 (to D. M. Bers), HL-62927 (to J. D. Molkentin), and HL-46929 and HL-73966 (to S. M. Pogwizd) and by American Heart Association Grant 0820080Z (to A. Rinne).

DISCLOSURES

No conflicts of interest are declared by the author(s).

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