# Pacing-induced calcineurin activation controls cardiac $Ca^{2+}$ signalling and gene expression

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**Calcineurin, a $Ca^{2+}$–calmodulin-dependent protein phosphatase (PP2B) is one of the links between $Ca^{2+}$ signals and regulation of gene transcription in cardiac muscle. We studied the $Ca^{2+}$ signal specificity of calcineurin activation experimentally and with modelling. In the rat atrial preparation, an increase in pacing frequency increased nuclear activity of the calcineurin-sensitive transcription factor, nuclear factor of activated T-cells (NFAT), 2-fold in a cyclosporin A (CsA)-sensitive manner. In line with this, modelling results predicted that the frequency of cardiac $Ca^{2+}$ transients encodes the stimulus for calcineurin activation. We further observed experimentally that calcineurin inhibition by CsA modulated $Ca^{2+}$ release in a $Ca^{2+}$-dependent manner. CsA had no effect on $[Ca^{2+}]_i$ at a pacing frequency of 1 Hz but it significantly suppressed the amplitude of $Ca^{2+}$ transients, systolic $[Ca^{2+}]_i$ and time averaged $[Ca^{2+}]_i$ at 6 Hz. Calcineurin had a differential role in the expression of immediate-early genes B-type natriuretic peptide (BNP) and *c-fos*. CsA inhibited the pacing-induced BNP gene expression, whereas pacing alone had no effect on the expression of *c-fos*. However, in the presence of CsA, *c-fos* mRNA levels were significantly augmented by increased pacing frequency. These results show that frequency-dependent calcineurin activation has a specific role in $[Ca^{2+}]_i$ regulation and gene expression, constantly recruited by varying cardiac $Ca^{2+}$ signals.**

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Muscle cells are capable of changing their structure and function in response to altered activity. Research in recent years shows that $Ca^{2+}$ has a central role in this adaptation process (for review see Molkentin, 2000; Wilkins & Molkentin, 2002; Frey & Olson, 2003). One suggested link between the free myoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and altered gene expression is the $Ca^{2+}$–calmodulin-dependent protein phosphatase-2B, calcineurin (Timmerman *et al.* 1996; for review see Rusnak & Mertz, 2000). Activated calcineurin mediates nuclear translocation of cytosolic nuclear factor of activated T-cells (NFAT), which controls transcription co-operatively with other transcription factors, e.g. activator protein-1 (AP-1), myocyte enhancer factor and GATA-4 (Molkentin *et al.* 1998; Wu *et al.* 2000; Macian *et al.* 2000; for review see Rao *et al.* 1997).

Originally it was shown that transgenic mice with constitutively active calcineurin develop cardiac hypertrophy and express fetal cardiac genes (Molkentin *et al.* 1998), making the calcineurin–NFAT pathway an attractive candidate for coupling of $Ca^{2+}$ signals to cardiac gene expression. Moreover, overexpression of an endogenous calcineurin inhibitory protein, modulatory calcineurin-interacting protein-1 (MCIP1), inhibits hypertrophy induced by overexpression of constitutively active calcineurin or chronic administration of $\beta$-adrenoreceptor agonist (Rothermel *et al.* 2001), indicating that activation of calcineurin is critical for the development of the hypertrophy. However, pharmacological inhibition of calcineurin in a variety of rodent models of heart disease has produced controversial results. Calcineurin inhibitors (CsA or FK506) have been reported to inhibit the load-induced hypertrophy (Shimoyama *et al.* 1999; Zou *et al.* 2000) or to have no effect (Ding *et al.* 1999). CsA has even been found to escalate the development of the mouse cardiomyopathy induced by a myosin heavy chain mutation (Fatkin *et al.* 2000). Furthermore, calcineurin inhibition is not

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selective to the pathological hypertrophy, since both CsA (Eto *et al.* 2000) and MCIP1 (Rothermel *et al.* 2001) also suppress the favourable hypertrophic adaptation to exercise. One possible explanation for these conflicting results could be that the effect of calcineurin inhibition depends on the hypertrophic model used. For example, lack of MCIP1 in mice heart results in an increased hypertrophic response to overexpression of constitutively active calcineurin, but a reduced hypertrophic response to pressure overload and to adrenergic stimulation (Vega *et al.* 2003), suggesting that calcineurin may have a different role depending on the stimulus inducing the hypertrophy.

While there is strong evidence from genetic mouse models to support the involvement of calcineurin in the development of various forms of hypertrophy (for reviews see Molkentin, 2000; Wilkins & Molkentin, 2002; Frey & Olson, 2003), very little is known about the normal $Ca^{2+}$ activation of calcineurin and its immediate functional implications. Because the frequency of $Ca^{2+}$ transients encodes an adequate stimulus for calcineurin activation in skeletal muscle (Liu *et al.* 2001; Kubis *et al.* 2002), we first examined if cardiac calcineurin is activated by pacing-induced $[Ca^{2+}]_i$ changes in rat atrium. Secondly, since it was reported that calcineurin inhibitors (McCall *et al.* 1996; Janssen *et al.* 2000) and/or activated calcineurin (Bandyopadhyay *et al.* 2000; Münch *et al.* 2002) regulate the $[Ca^{2+}]_i$ balance in cardiac myocytes, we studied the $Ca^{2+}$-dependent effects of CsA on the myocyte $Ca^{2+}$ signalling. Thirdly, the findings that in several cell types the calcineurin–NFAT cascade has been implicated in both activation (Rao *et al.* 1997; Molkentin *et al.* 1998; Macian *et al.* 2000) and suppression of the expression of immediate genes (Su *et al.* 1996; Bito *et al.* 1996; Schaefer *et al.* 1998) led us to examine if the calcineurin-dependent transcriptional pathway shows dual effects with respect to the expression of immediate-early genes.

## Methods

### Preparation and perfusion of rat atrial appendix

The rat atrial appendix preparation was set up and perfused as previously described (Tavi *et al.* 1999). The Animal Use and Care Committee of the University of Oulu approved the experimental design. Male Sprague-Dawley rats weighing 290–400 g were used. The rats were decapitated, and the hearts were rapidly removed and placed in oxygenated (*ca* 10°C) Tyrode buffer solution (mM): 113.8 NaCl; 17.6 $NaHCO_3$; 4.7 KCl; 2.0 $CaCl_2$; 1.1 $MgSO_4$; 1.2 $KH_2PO_4$; 11.0 glucose; and 10 $\mu$IU $ml^{-1}$ insulin; pH was 7.4 when bubbled with 5% $CO_2$–95% $O_2$ gas. The same solution was used for superfusion of the atrium (2.5 ml $min^{-1}$) at 37°C. The atria were paced with a field stimulus (1 ms, 50% over threshold) by two platinum electrodes located inside the perfusion chamber. CsA (1 $\mu$M, Sigma-Aldrich) was applied to the perfusion medium 30 min before the change of the pacing frequency. For mRNA measurements the atria were preincubated for 50 min with 1 Hz pacing, followed by 30 min pacing at 1, 4, 5, or 6 Hz. The tissue was frozen in liquid nitrogen and stored at –70°C.

### $[Ca^{2+}]_i$ measurements

For indo-1AM loading, the atria were superfused for 25–40 min at 25–32°C (flow 7 ml $min^{-1}$) with Tyrode solution (volume 4 ml) containing (mM): 103.8 NaCl; 17.6 $NaHCO_3$; 4.7 KCl; 2.0 $CaCl_2$; 1.1 $MgSO_4$; 1.2 $KH_2PO_4$; 10 glucose; 9.1 sodium pyryvate. Indo-1 AM (Molecular Probes, Europe) was dissolved in DMSO with 20% Pluronic. The final concentration of indo-1 AM was 20 $\mu$M and the amount of DMSO/Pluronic was 25 $\mu$l $ml^{-1}$ Tyrode solution. The solution was bubbled with 5% $CO_2$–95% $O_2$ (pH 7.4). The $Ca^{2+}$ measurement setup has been previously described (Tavi *et al.* 1998, 1999). The Indo-1 emission ratio (405 nm/485 nm) was acquired at 200 Hz with two photomultiplier tubes (Hamamatsu, Japan) and filtered at 100 Hz. Both excitation and emission were guided to and from the tissue with a quartz fibre optic cable (diam. 1 mm). The distance between the tissue and the cable head was constant (~3 mm) and the illuminated area covered 100% of the tissue area facing the cable. Therefore the amount of the Indo-1 molecules within the illuminated area was constant. For calibration of the measured signal, $R_{min}$ and $R_{max}$ were determined at the end of each experiment. To get $R_{min}$ the atria were perfused with $Ca^{2+}$-free buffer (0 $Ca^{2+}$, 0.5 $Mg^{2+}$, 5 mM EGTA) containing 10 $\mu$M of the $Ca^{2+}$ ionophore 4-Br-A23187 (Molecular Probes) until a steady state fluorescence signal was obtained (10–12 min). This was followed by perfusion with high $Ca^{2+}$ solution (15 mM) with 10 $\mu$M $Ca^{2+}$ ionophore. The atria was then exposed to high frequency stimulation (8–10 Hz), resulting in a tetanic-like contraction within 10 min and a steady-state value of $R_{max}$. To evaluate the degree of loading of the non-cytosolic compartments, 1 mM of $Mn^{2+}$ was applied at the end of calibration procedure. The residual fluorescence after $Mn^{2+}$ was 6 ± 2% ($n = 6$) representing the maximal error caused by non-cytosolic fluorescence.

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To estimate the non-myocyte fluorescence, we induced $Ca^{2+}$ release in endothelial cells with 10 $\mu$M bradykinin (Field *et al.* 1994), which caused no detectable change in the fluorescence ($n = 4$), indicating that either the tissue does not contain endothelial cells or these cells were not loaded with indo-1. The $[Ca^{2+}]_i$ was calculated by using Grynkiewicz formalism:

$$[\mathrm{Ca}^{2+}]_i = K_d\beta\left[\frac{(R - R_{min})}{(R_{max} - R)}\right] \quad (1)$$

where $K_d$ is the dissociation constant for indo-1 and $\beta$ is the ratio of the free/bound indo-fluorescence at 485 nm from measured $R_{min}$ and $R_{max}$. We used a $K_d$ value of 844 nM determined in cellular environment of cardiac myocytes (Bassani *et al.* 1995).

### Isolation of mRNA and quantitative PCR

Total RNA was prepared from rat atria and used as a template for the cDNA first strand synthesized by M-MuLV reverse transcriptase. The quantitative PCR reactions were performed with an ABI 7700 Sequence Detection System using the TaqMan chemistry. The forward and reverse primers for rat b-type natriuretic peptide (BNP) mRNA detection were TGGGCAGAAGATAGACCGGA and ACAACCTCAGCCCGTCACAG, and for *c-fos* GGCTGAACCCTTTGATGACTTC and GGGCAGTCT-CCGAGCCA, respectively. The bifunctional fluorogenic probes for BNP and *c-fos* were 5′-Fam-CCAAGCGACT-GACTGCGCCG-Tamra-3′ and 5′-Fam-TGTTTCCGG-CATCATCTAGGC-Tamra-3′, respectively. The results were normalized to 18S RNA quantified from the same samples as previously described (Majalahti-Palviainen *et al.* 2000).

### Oligonucleotides and EMSA

All oligonucleotides were purchased from Sigma Chemical Co. For electrophoretic mobility shift assay (EMSA), the NFAT binding element located at –927 bp BNP promoter (NFAT-BNP, Molkentin *et al.* 1998) was used as probe and intact and mutated NFAT binding elements of the interleukin-2 promoter (NFAT-IL-2 and NFATmut-IL-2, respectively, Northrop *et al.* 1994) were used as unlabelled competitor oligonucleotides (coding strand shown, point mutations in bold and 5′-overhangs in italics): NFAT-BNP, 5′-*AGAG*CTATCCTTTTGTTTTCCATCCTG*GCCC*-3′; NFAT-IL-2, 5′-*AGAG*CGCCCAAAGAGGAAAATTTGT-TTCATA*GCCC*-3′ and NFATmut-IL-2: 5′-*AGAG*CG-CCCAAAGCTTAAAATTTGTTTCATA*GCCC*-3′. The sense and corresponding antisense oligonucleotides were annealed to generate double-stranded oligonucleotides. NFAT-BNP was sticky-end-labelled with [$^{32}$P]-dCTP by Klenow enzyme. Nuclear extracts from frozen auricular tissue were prepared (Hautala *et al.* 2001) and protein concentration from each sample was colourimetrically determined (Bio-Rad Laboratories). For each reaction mixture, 12 $\mu$g of nuclear protein and 3 $\mu$g of poly(dI-dC) were used in a buffer containing 10 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.025% NP-40, 0.25 mM PMSF and 1 $\mu$g $ml^{-1}$ each of leupeptin, pepstatin and aprotinin. Reaction mixtures were incubated with a labelled probe for 15 min followed by non-denaturating gel electrophoresis on 5% polyacrylamide gel. Subsequently, gels were dried and exposed in a PhosphorImager screen and analysed with ImageQuant (Molecular Dynamics, Amersham Biosciences, CA, USA). To confirm DNA sequence specificity of the protein–DNA complex formation, competition experiments with 100 M excesses of unlabelled oligonucleotides with intact or mutated NFAT binding sites were performed. Competitor oligonucleotides were added to the reaction mixture 15 min before the labelled probe.

### Model for calcineurin $Ca^{2+}$ activation

We utilized a previously published reaction scheme for $Ca^{2+}$- and calmodulin-dependent activation of calcineurin (CaN) (Bhalla & Iyengar, 1999), where parameters have been adjusted to better meet the characteristics of cardiac myocytes. The reactions and the corresponding rate constants of the model are shown in Fig. 1*A*. All the reactions were incorporated into Mathlab (Mathworks, USA) as time-dependent differential equations of concentration and they were solved using the well-stirred assumption, which states that each molecule has equal access to each other in a single compartment. As initial concentrations we have used 6 $\mu$M for calmodulin ([CaM]) (Shannon *et al.* 2000) and 1 $\mu$M for calcineurin ([CaN]) (Bhalla & Iyengar, 1999; Crabtree, 1999) while the other concentrations being zero at the beginning. The reaction rates were taken initially from the previous model (Bhalla & Iyengar, 1999) and the equilibrated CaN-activation was calculated at different $Ca^{2+}$ concentrations, which allowed plotting of the CaN activation against $[Ca^{2+}]$ (Fig. 1*B*) and data points were fitted to the Hill equation:

$$\mathrm{CaN}_{activity} = V_{Max}\frac{[\mathrm{Ca}^{2+}]_i^n}{K_d^n + [\mathrm{Ca}^{2+}]_i^n} \quad (2)$$

We compared the parameters of our fitted Hill equation with the published experiments ($n = 3$, $K_d = 0.6$ $\mu$M,

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Stemmer & Klee, 1994) and adjusted the reaction rate constants (see Fig. 1*A*) by re-running the simulations until the Hill coefficients matched. After adjusting the parameters, the responses to three $Ca^{2+}$ concentration steps of different magnitude were simulated and the time constants of each calcineurin activation/inactivation were determined (Fig. 1*C* and *D*). Thereafter, experimentally determined $Ca^{2+}$ signals (sampling interval 0.005 s) were used as stimuli for the model. The signals were first smoothed with a three-point moving average filter, after which linear interpolation was used to provide continuous signals as input. To solve the differential equations, we used the variable-step Mathlab routine 'ode45', based on a fourth order explicit Runge-Kutta formula (Dormand & Prince, 1980) with an average time step of 0.003 s.

Artificial $Ca^{2+}$ signals were constructed to study the frequency and base-line dependence of the CaN-activation. The experimentally determined 1, 4, 5 and 6 Hz signals were used to determine the shape of an average $Ca^{2+}$ transient for each frequency. These average transients were then repeated a number of times to form oscillatory signals, which were finally scaled to the same amplitude.

### Statistical testing

Results are expressed as mean ± S.E.M. The statistical testing was done with one-way and two-way ANOVA. Pairwise comparisons between different groups were done by Student–Newman-Keuls method (SigmaStat, USA). For comparison between multiple groups, Bonferroni correction was applied. *P* values smaller than 0.05 were

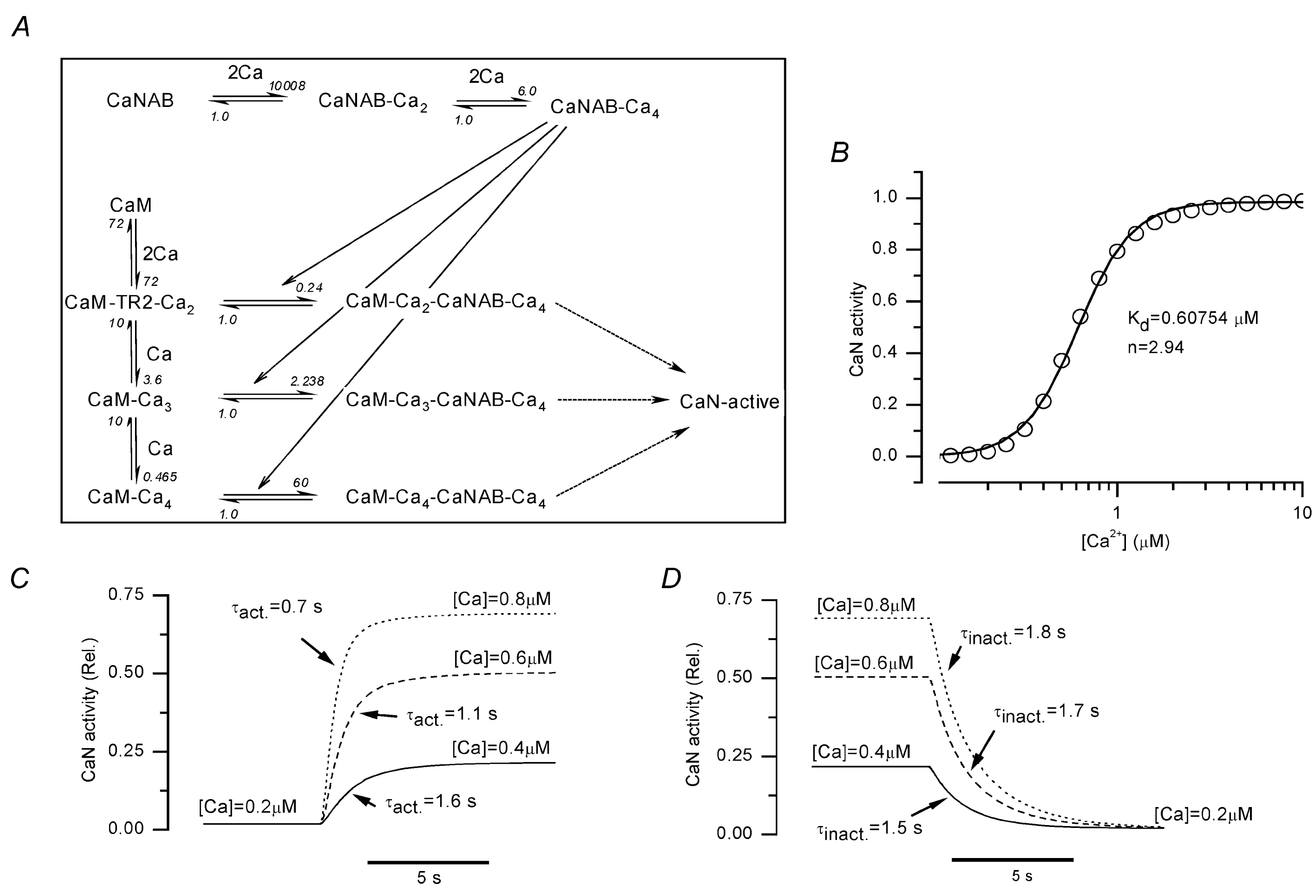


**Figure 1. Characteristics of the calcineurin model**

*A*, reactions with forward and reverse rates included into the model. CaNAB represents calcineurin A and B subunits, which are always assumed to be bound under physiological conditions. The four-$Ca^{2+}$-bound form of calcineurin (CaNAB-$Ca_4$) has a small activity, but it is negligible compared to the CaM-bound forms. For clarity, $Ca^{2+}$ is represented multiple times in the scheme, but it is modelled only as a single reactant. All the CaM-bound forms of calcineurin have been assumed to have the same enzymatic activity, thus the CaN activity can be obtained by simply summing the concentrations of each form. *B*, steady-state activation of the model. The Hill equation (continuous line) has been fitted to simulated steady-state levels of active calcineurin at different [$Ca^{2+}$] (○). *C*, activation time constants of calcineurin in the model upon steps (from 0.2 $\mu$M to 0.4, 0.6 or 0.80 $\mu$M [$Ca^{2+}$]. *D*, deactivation time constants by corresponding $Ca^{2+}$ stimuli (as in *C*).

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considered statistically significant. The data analysis was done with Origin 6.1 (OriginLab) and equations were solved with Mathlab.

## Results

### Pacing-induced changes in $[Ca^{2+}]_i$

Increase of the pacing frequency in different types of cardiac preparations causes a substantial $Ca^{2+}$ accumulation, manifested as an elevation of diastolic $[Ca^{2+}]_i$. This has been reported in rat ventricular trebeculae (Layland & Kentish, 1999; Brandes & Bers, 2002) and in isolated cardiac myocytes of mouse (Antoons *et al.* 2002; Knollmann *et al.* 2003), rabbit (Chudin *et al.* 1999) and cat (Wang *et al.* 2001). Because both the sustained component of the $Ca^{2+}$ signal, like the diastolic $Ca^{2+}$ accumulation, and the frequency of $Ca^{2+}$ transients activate calcineurin (Timmerman *et al.* 1996; Rusnak & Mertz, 2000; Kubis *et al.* 2003), we hypothesized that in cardiac myocytes pacing might serve as a physiological stimulus for calcineurin activation. To quantify this, we measured $[Ca^{2+}]_i$ levels from atria paced at different frequencies. At 1 Hz atrial myocytes generated $Ca^{2+}$ transients from a stable diastolic $[Ca^{2+}]_i$ of $200 \pm 7$ nM to a systolic $[Ca^{2+}]_i$ of $802 \pm 18$ nM, resulting in an amplitude of the transients of $600 \pm 21$ nM and an average $[Ca^{2+}]_i$ of $300 \pm 8$ nM ($n = 24$). An increase in the pacing frequency rapidly shifted the diastolic $Ca^{2+}$ to higher levels as shown by the representative recording in Fig. 2*A*. The diastolic $[Ca^{2+}]_i$ was dependent on the frequency of the pacing and increased up to $511 \pm 40$ nM after 10 min at 6 Hz ($n = 6$, Fig. 2*B*). The increase in diastolic $[Ca^{2+}]_i$ was accompanied by a corresponding increase in the systolic and average $[Ca^{2+}]_i$, but there was no change in the amplitude of the $Ca^{2+}$ transients.

### Simulation of calcineurin activation with cardiac $Ca^{2+}$ signals

To explore the dynamics of calcineurin activation induced by pacing, we used a mathematical model to simulate calcineurin activation using the measured $[Ca^{2+}]_i$ signals as input to the model. A data set for each pacing group was selected where $[Ca^{2+}]_i$ was closest to the mean values of systolic, diastolic and average as calculated from six identical experiments. The simulations showed that calcineurin activity is ~10% of maximum when the pacing frequency is 1 Hz. This baseline activity is brought about by the diastolic $[Ca^{2+}]_i$ since $Ca^{2+}$ transients at 1 Hz do not cause any accumulative activation of calcineurin in the simulations. By increasing the pacing frequency, the calcineurin activity is increased to ~50% at 4 Hz, to ~60% at 5 Hz and to ~70% at 6 Hz (Fig. 3*A*). From the simulation with measured $[Ca^{2+}]_i$ as an input it cannot be judged how much of the calcineurin activity is caused by diastolic $[Ca^{2+}]_i$ increase and how much is due to increase of the frequency of the $Ca^{2+}$ transients. To study this we modelled artificial data mimicking the normal $Ca^{2+}$ signals at different frequencies (1, 4, 5 and 6 Hz, Fig. 3*B*), but with fixed baseline (0.2 or 0.4 $\mu$M) and amplitude (0.8 $\mu$M). With baseline $[Ca^{2+}]_i$ of 0.4 $\mu$M, the frequency-dependent calcineurin activity increases from 37% at 1 Hz to 59% at 6 Hz and with baseline $[Ca^{2+}]_i$ of 0.2 $\mu$M the corresponding values were 13% and 36% (Fig. 3*B*), indicating that calcineurin is activated by the frequency of cardiac $Ca^{2+}$ transients.

### Effect of cyclosporin on the frequency-dependent $Ca^{2+}$ changes in the rat atrium

Cyclosporin A has been reported to cause a calcineurin-independent suppression of $Ca^{2+}$ transients with a corresponding negative inotropy in cardiac muscle by acting on the sarcoplasmic reticulum (SR) (Janssen *et al.* 2000). However, active calcineurin may modulate the $Ca^{2+}$

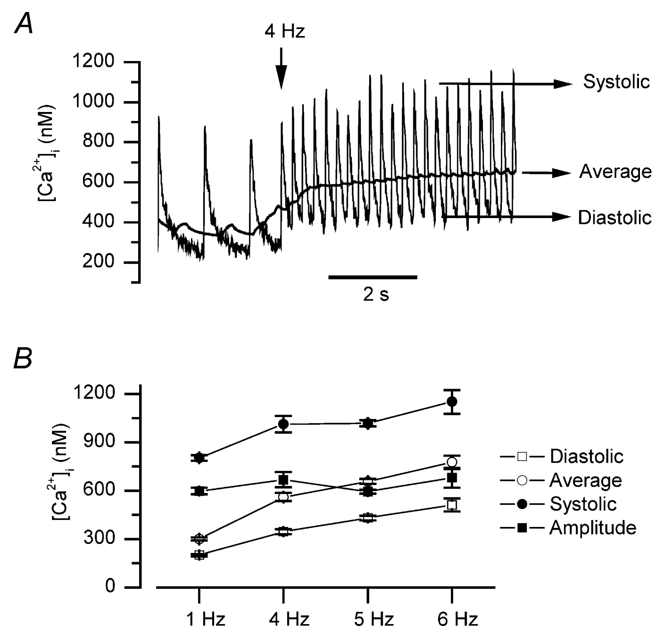


**Figure 2. Pacing increases diastolic, systolic and average $[Ca^{2+}]_i$ but does not change the amplitude of $Ca^{2+}$ transients**
*A*, $[Ca^{2+}]_i$ during change in pacing frequency from 1 to 4 Hz. Arrows indicate systolic, diastolic and average $Ca^{2+}$ (1 s moving average). *B*, pacing-induced changes at the steady-state in different parameters of $[Ca^{2+}]_i$ at different pacing frequencies (1, 4, 5 and 6 Hz, $n = 6$ each). Note that diastolic, systolic and average $[Ca^{2+}]_i$ increase as function of the pacing frequency, whereas the amplitude of $Ca^{2+}$ transients remains virtually constant.

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release in cardiac myocytes by regulating the ryanodine receptors (RyRs) (Bandyopadhyay *et al.* 2000) and reduce the SR $Ca^{2+}$ uptake by dephosphorylating phopholamban, the regulator of cardiac SR $Ca^{2+}$-ATPase (Münch *et al.* 2002). Measuring the $Ca^{2+}$ dependence of the CsA effect can be used to distinguish between the calcineurin-dependent and calcineurin-independent effects of CsA.

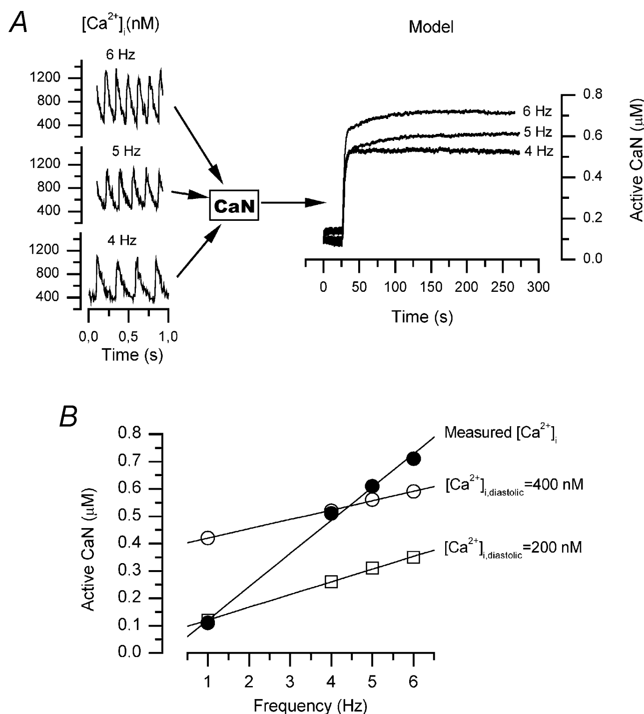


**Figure 3. Simulated calcineurin (CaN) activation by pacing-induced $Ca^{2+}$ changes**

*A*, simulated calcineurin activity produced by representative $[Ca^{2+}]_i$ measurements from rat atrium. Left panel shows samples from the data used at 4, 5 and 6 Hz pacing. *B*, the steady state CaN activity from simulations with artificial $[Ca^{2+}]$ data with 0.4 $\mu$M (○) and 0.2 $\mu$M (□) baseline $[Ca^{2+}]$, respectively, compared with the simulated CaN activity produced by measured $[Ca^{2+}]_i$ changes (●).

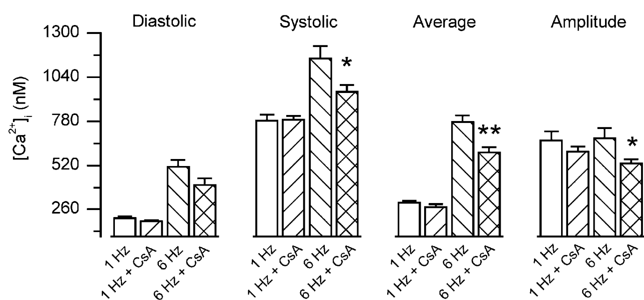


**Figure 4. Cyclosporin A suppresses the pacing-induced $[Ca^{2+}]_i$ changes in rat atrium**

*A*, effect of CsA (1 $\mu$M) and pacing on the diastolic, systolic, average and amplitude of the $Ca^{2+}$ transients in rat atrium. Each bar represents mean ± S.E.M. of 6 separate experiments. * $P < 0.05$ and ** $P < 0.01$.

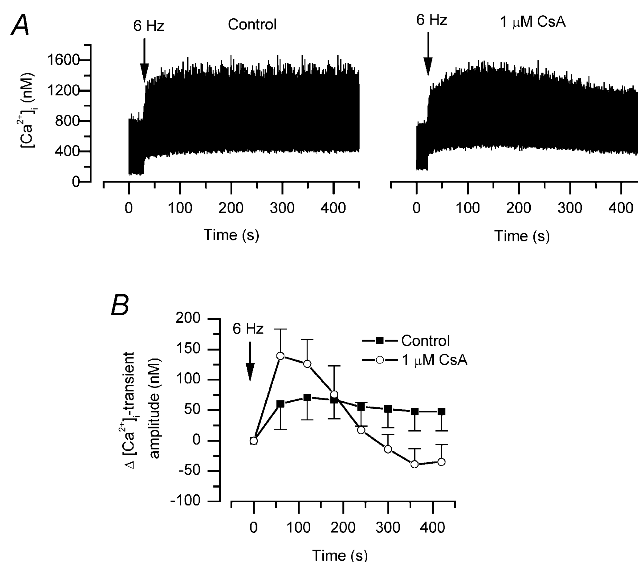


**Figure 5. Cyclosporin A induces biphasic changes in the $Ca^{2+}$ transient amplitude after onset of the pacing**

*A*, after onset of the 6 Hz pacing in rat atrium $[Ca^{2+}]_i$ reaches a steady state within 1 min (left). Pre-incubation of rat atrium with of CsA (1 $\mu$M) promotes biphasic behaviour of $[Ca^{2+}]_i$ when pacing is switched from 1 to 6 Hz (right). *B*, mean change in the amplitude of $Ca^{2+}$-transient in the absence (■) and presence (○) of CsA (1 $\mu$M). Note the biphasic behaviour of transient amplitude with CsA characterized by transient increase of the $Ca^{2+}$ transients before decaying to lower level after 4 min.

When we superfused rat atria paced at 1 Hz with 1 $\mu$M CsA for 15 min, no statistically significant differences in any of the measured parameters of $[Ca^{2+}]_i$ were observed compared to control experiments without CsA (Fig. 4). However, when CsA was applied prior to 6 Hz pacing, $[Ca^{2+}]_i$ was decreased. In six experiments the systolic $[Ca^{2+}]_i$ ($P < 0.05$), the average $[Ca^{2+}]_i$ ($P < 0.01$) and the amplitude of the $Ca^{2+}$ transients ($P < 0.05$) were all significantly suppressed compared to control. It should be noted that CsA affected the $Ca^{2+}$ transients in a biphasic manner. After the onset of 6 Hz pacing the amplitude was transiently increased (Fig. 5*A*), which was then followed by decay to a lower level than in the control (Fig. 5*B*). This suggests that calcineurin has a role in the maintenance of $Ca^{2+}$ release in the face of high levels of $[Ca^{2+}]_i$.

## Effects of calcineurin activation on immediate-early gene expression

To study the immediate effects of calcineurin activation on gene expression, we measured the mRNA levels of two rapidly inducible genes, BNP and *c-fos*. First we measured pacing sensitivity of the expressions of these genes. Atrial tissues were subjected to different pacing

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frequencies (1, 4, 6 and 8 Hz) for 30 min and mRNA levels measured. BNP mRNA level showed near-linear pacing dependence, whereas pacing did not affect the *c-fos* mRNA level (Fig. 6*A*). From the frequencies used here, 8 Hz pacing for 30 min gave the maximal BNP mRNA response of ∼2-fold compared to the baseline expression in 1 Hz. Next we studied the role of calcineurin in these pacing-induced changes in gene expression by exposing atrial tissues to one of the following stimuli for 30 min: 1 Hz pacing, 1 Hz pacing after preincubation (30 min) with 1 $\mu$M CsA, 8 Hz pacing, and 8 Hz pacing with preincubation (30 min) with 1 $\mu$M CsA. Pacing at 8 Hz induced a significant increase in the BNP mRNA levels ($P < 0.05$), which was abolished by pre-exposure of CsA (Fig. 6*B*). It was a surprise to see that while the mRNA levels of *c-fos* were not sensitive to 8 Hz pacing or CsA alone, pacing at 8 Hz increased the *c-fos* mRNA levels when the atria were pre-exposed to CsA ($n = 6$, $P < 0.01$, Fig. 6*C*).

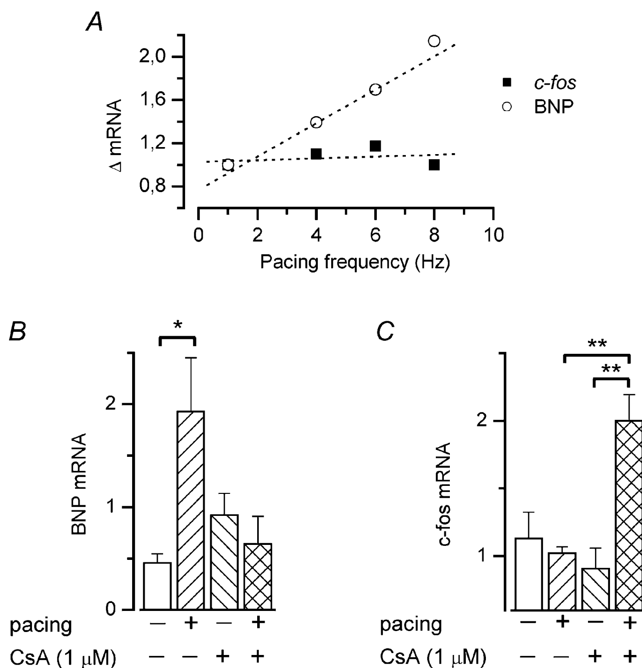


**Figure 6. Cyclosporin A inhibits pacing-induced BNP gene expression while it activates *c-fos* gene expression during pacing**

*A*, relative (1 Hz equals 1) changes in the mRNA ($n = 6$ each) levels of BNP and *c-fos* in rat atria exposed to different pacing frequencies at 30 min as function of given pacing frequency. Apparent slope of the linear fit shows clear pacing sensitivity of BNP mRNA but no pacing sensitivity of the *c-fos* mRNA. The mRNA levels of BNP (*B*) and *c-fos* (*C*) genes from atrial tissues exposed to low pacing 1 Hz (−) or high pacing 8 Hz (+) for 30 min. When indicated, cyclosporin A (CsA, 1 $\mu$M) was included in perfusion medium from start of preincubation BNP and *c-fos* mRNA levels are expressed relative to 18S RNA levels, and each bar represents mean ± S.E.M. of 6 separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

## Pacing-induced increase in NFAT activity

We isolated nuclear fractions from atria exposed to different pacing and/or CsA and from these fractions we measured the NFAT binding activity on the BNP promoter. This step was taken to answer two questions: first, to determine if pacing-induced $Ca^{2+}$ changes are sufficient to activate calcineurin and resulting NFAT nuclear translocation, and second, to study if cyclosporin A inhibits the NFAT activation. This indirect method was chosen because the fast inactivation of calcineurin after $Ca^{2+}$ removal (Stemmer & Klee, 1994) might complicate direct activity measurements from tissue samples, while the calcineurin-induced NFAT nuclear accumulation persists for several minutes (Timmerman *et al.* 1996; Liu *et al.* 2001; Kubis *et al.* 2002). The specificity of NFAT complex formation on BNP promoter was confirmed by the competition analysis for nuclear protein binding with NFAT-BNP as a radioactively labelled probe exposed to 100 M excess of unlabelled competitor

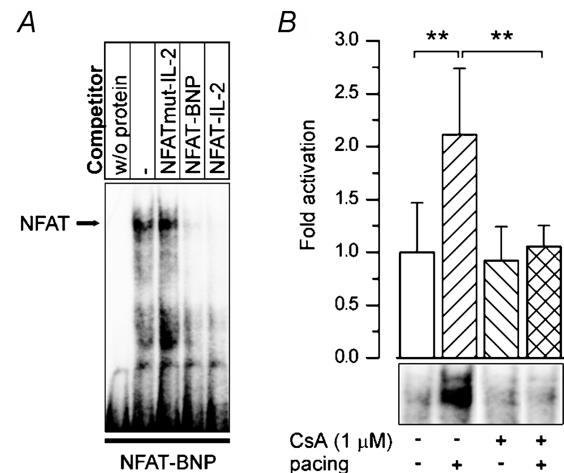


**Figure 7. Pacing activates NFAT in rat atrium with a cyclosporin A-dependent manner**

*A*, specific NFAT binding on BNP promoter (NFAT-BNP). Competition analysis for nuclear protein binding was carried out with NFAT-BNP as a radioactively labelled probe and when designated with 100 M excess of unlabelled competitor oligonucleotides of NFATmut-IL-2, NFAT-BNP and NFAT-IL-2. Nuclear extracts from 8 Hz paced atria were used for competition experiments. *B*, pacing-induced NFAT activation in rat atrium. For binding reaction, 12 $\mu$g of a nuclear protein extract from atrial tissue were used. After preincubation (50 min, 1 Hz), atrial pacing was continued at 1 (−) or 8 Hz (+) for 30 min. When indicated, cyclosporin A (CsA, 1 $\mu$M) was included in perfusion medium from start of preincubation. Each bar represents mean ± S.E.M. of 6 separate experiments. $^{**}P < 0.01$.

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oligonucleotides of NFATmut-IL-2, NFAT-BNP or NFAT-IL-2 (Fig. 7*A*). In rat atrium, 8 Hz pacing for 30 min induced a 2-fold increase in NFAT nuclear activation ($n = 6$, $P < 0.01$, Fig. 7*B*). Application of 1 μM of CsA did not change the NFAT activation at 1 Hz pacing but totally inhibited the activation induced by pacing at 8 Hz.

## Discussion

This is the first report that establishes a relationship between cardiac $Ca^{2+}$ signals and calcineurin activation. Our results show that (1) frequency of cardiac $Ca^{2+}$ transients forms an adequate stimulus for calcineurin activation and corresponding NFAT activation, (2) calcineurin activation regulates $Ca^{2+}$ release in the myocytes, and (3) calcineurin activation results in a dual effect on the immediate gene expression with both augmentation (BNP) and suppression (*c-fos*).

### Calcineurin activation by pacing

The measurements showed a clear CsA-dependent increase in the nuclear NFAT activity when the frequency of atrial pacing was increased. This is in line with the modelling results that predicted that calcineurin activation depends on the frequency of cardiac $Ca^{2+}$ transients. Similarly, in skeletal muscle cells NFAT translocation has been shown to be sensitive to the frequency of stimulation and to CsA (Lui *et al.* 2001; Kubis *et al.* 2002). Although the NFAT translocation is induced by calcineurin activation, the time courses of these two processes are different. The nuclear import of NFAT requires a minimum stimulation time of 1.5–5 min and is complete after 20–30 min (Liu *et al.* 2001; Kubis *et al.* 2002), whereas calcineurin *in vitro* responds to $Ca^{2+}$ on a time scale of seconds (Stemmer & Klee, 1994). This was also seen in the present study where the effects of calcineurin inhibition on $[Ca^{2+}]_i$ were apparent within seconds after increasing the pacing frequency. In our simulations, the activation of calcineurin was fast enough ($\tau_{act} = 0.7$–$1.6$ s) to respond to a brief rise in $[Ca^{2+}]_i$ such as that during the cardiac $Ca^{2+}$ transient. The kinetics of calcineurin deactivation ($\tau_{deact} \sim 1.5$ s) ensures that activity triggered by a single $Ca^{2+}$ transient decays if the frequency is low enough. Upon an increase of frequency, the deactivation of calcineurin becomes incomplete before the next transient and calcineurin activation increases. The frequency-dependent calcineurin activation appears to be prominent at frequencies corresponding to the normal rat heart rate (∼5–8 beats $s^{-1}$) and thus calcineurin is likely to exhibit a significant level of activity even at rest, with rapid responses to both increase *and* decreases of heart rate. It is also interesting to note that although pacing produces an increase in the diastolic $[Ca^{2+}]_i$ that activates calcineurin effectively, our modelling experiments show that changes in the frequency of $Ca^{2+}$ transients alone are sufficient to alter calcineurin activity (see Fig. 3*B*). This supports the idea that calcineurin is part of the normal adaptation mechanism, and as such participates in the maintenance of the normal cardiac phenotype (Eto *et al.* 2000). Analogously, it has been suggested that in slow type skeletal myocytes, calcineurin maintains the slow phenotype because the continuous activity in these cells upholds the calcineurin activity (Chin *et al.* 1998).

### Suppression of pacing-induced $Ca^{2+}$ increase by cyclosporin A

In the present study, we found that that all of the effects of CsA on $[Ca^{2+}]_i$ were present only with high pacing frequency (see Fig. 4). This indicates that the changes were caused by inhibition of $Ca^{2+}$-activated calcineurin. In cardiac myocytes, three different calcineurin targets may be involved in $Ca^{2+}$ regulation. First, the inhibitory regulator of SR $Ca^{2+}$-ATPase, phospholamban, is inhibited by calcineurin in ventricular myocytes (Münch *et al.* 2002) and therefore calcineurin inhibition would have a stimulatory effect on the pump. This sort of $Ca^{2+}$-ATPase stimulation would cause faster $Ca^{2+}$ transient decay, possibly a reduced diastolic $[Ca^{2+}]_i$ and increased SR $Ca^{2+}$ content resulting in an increased $Ca^{2+}$ release and subsequently sustained augmentation of $Ca^{2+}$ transients (Song *et al.* 2003). This is opposite to what we found to be the effect of CsA during pacing where the endpoint was a significant reduction in $Ca^{2+}$ transient amplitude. This finding is not surprising since atrial muscle contains much less phospholamban than ventricular muscle (Koss *et al.* 1995). Second, calcineurin inhibition was recently found to stimulate the L-type calcium current and thereby cause a sustained increase of calcium release in ventricular myocytes (Santana *et al.* 2002). This mechanism had either a transient or a small effect in our atrial cells where the endpoint was a significant reduction in calcium release. Third, calcineurin may act on the $Ca^{2+}$ release channels, i.e. the RyRs (Bandyopadhyay *et al.* 2000) or the $IP_3$ receptors (Cameron *et al.* 1995). The contribution of $IP_3$ receptors to the normal calcium release is small in rat atrial myocytes (Mackenzie *et al.* 2002). Calcineurin regulates cardiac ryanodine receptor via FKBP12.6, which de-sensitizes the channel to $Ca^{2+}$, thereby reducing the $Ca^{2+}$ leak during diastole (Bandyopadhyay *et al.* 2000; Marks, 2003). Calcineurin inhibitors make RyRs more

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sensitive to $Ca^{2+}$ leading to increased RyR openings at lower levels of $Ca^{2+}$ manifested as spontaneous $Ca^{2+}$ release events (McCall *et al.* 1996; Bandyopadhyay *et al.* 2000). This type of RyR sensitization leads first to an increase in the $Ca^{2+}$ release, which in turn leads to a reduction of SR $Ca^{2+}$ content and $Ca^{2+}$ release ensuring that the effect is transient (Trafford *et al.* 2000; Eisner *et al.* 2000). If this is accompanied by even a small increase in the passive $Ca^{2+}$ leak like that with ryanodine (Bers *et al.* 1987) or with CsA (Bandyopadhyay *et al.* 2000), a sustained decrease of $Ca^{2+}$ release can be expected. Altogether this is exactly what we found in the present study (see Fig. 4), which supports the idea that RyR-mediated SR $Ca^{2+}$ release was the main target of CsA.

## Effect of calcineurin inhibition on the BNP and *c-fos* gene expression

Part of the controversy concerning calcineurin signalling in cardiac muscle gene expression and hypertrophy is related to the difficulty in isolating the calcineurin pathway from other hypertrophic pathways. *In vivo* hypertrophic stimuli consist not only of activation of $Ca^{2+}$-dependent processes, but also of hormonal, mechanical and neuronal adaptations with corresponding activation of different hypertrophic signalling cascades (for review see Tavi *et al.* 2001). To study the earliest calcineurin-induced transcriptional events, we measured the effects of altered $[Ca^{2+}]_i$ on the expression of the immediate-early genes *c-fos* and BNP, two rapidly inducible marker genes for load-induced hypertrophy (see, e.g. Tavi *et al.* 2001). We found that the $Ca^{2+}$–calcineurin pathway has opposite effects on the expressions of *c-fos* and BNP. While the BNP expression is part of normal adaptation of heart to increased load (Tavi *et al.* 2001; Tokola *et al.* 2001) and a direct target of calcineurin–NFAT-mediated transcription (Molkentin *et al.* 1998), the *c-fos* induction is additionally associated with pathological developments such as induction of fetal gene expression by mechanical unloading of the heart (Depre *et al.* 1998). More importantly, induction of *c-fos* expression is likely to regulate the impending expression of other genes because it is a part of the heterodimeric transcription factor AP-1. We have shown here that in rat cardiac myocytes, CsA increases the *c-fos* mRNA levels in a $Ca^{2+}$-dependent manner. This seems to be a common feature of many cell types since stimulation of *c-fos* expression by CsA has been demonstrated in T-cell lymphoma cells when exposed to ionomycin (Su *et al.* 1996) and in murine erythroleukaemia cell line CsA stimulates *c-fos* expression in a $Ca^{2+}$-dependent manner in correlation with the inhibition of CaN activity (Schaefer *et al.* 1998). These phenomena are probably not caused by unspecific actions of CsA since in hippocampal neurones FK506 enhances the *c-fos* expression after short bouts of electrical stimulation (Bito *et al.* 1996). These results suggest that the same calcium signal promotes both activation and inhibition of the *c-fos* expression. To explain a similar phenomenon in hippocampal neurones, Bito *et al.* (1996) suggested that calcineurin acts indirectly to de-phosphorylate the CaM kinase-phosphorylated cyclic AMP-responsive element binding protein (CREB) transcription factor, thereby suppressing the CREB-induced *c-fos* expression.

Supporting such a dual role of calcineurin in controlling cardiac gene expression, $MCIP1^{-/-}$ mice have an increased hypertrophic response to constitutively active calcineurin expression but a reduced hypertophic response to pressure overload and to adrenergic stimulation (Vega *et al.* 2003). If the primary role of calcineurin activation is suppression of some genes, like *c-fos*, the inhibition of calcineurin would allow these genes to be expressed and would secondarily change the whole pattern of gene expression. This dualism in the calcineurin-dependent immediate-early gene transcription together with the apparent activity of calcineurin, maintained already by moderate heart rates, suggests that calcineurin may orchestrate the transcriptional signals during both normal adaptation and pathological developments. This is not unexpected since the output of calcineurin-dependent transcription has been shown to depend on other accompanying signal cascades (e.g. Rac, Ras or protein kinase C, for review see Crabtree, 2001) also in cardiac myocytes (Bueno *et al.* 2002; for review see Wilkins & Molkentin, 2002).

## Physiological and pathophysiological implications

On the basis of the modelling in the present study it can be predicted that, in addition to the frequency, other factors that modulate the shape or amplitude of cardiac $Ca^{2+}$ transients and the diastolic $[Ca^{2+}]_i$ may have a substantial impact on calcineurin activity. Increased amplitude of the $Ca^{2+}$ transients, induced by increased SR $Ca^{2+}$ uptake (for example, by phosphorylation of phospholamban), may actually reduce calcineurin activity by shortening the duration of transients and decreasing the diastolic $[Ca^{2+}]_i$. On the other hand, factors that act to lengthen the $Ca^{2+}$ transients or augment $Ca^{2+}$ transients without increasing the rate of $Ca^{2+}$ removal may elevate calcineurin activity as shown previously with endothelin-1 (Zhu *et al.* 2000). This mechanism would provide a novel pathway for communication between different hypertrophic pathways.

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This study shows that calcineurin influences excitation–contraction coupling in the heart by regulating the $Ca^{2+}$ release. This extends the spectrum of calcineurin effects in the heart, but also points to the likely consequences of calcineurin inhibition. The vast majority of the reported effects of calcineurin inhibition on the hypertrophy development appear to be due to inhibition of calcineurin-induced transcription activity. Nevertheless, part of the effects can be due to concomitant suppression of cardiac $Ca^{2+}$ signals, which in turn suppresses many other $Ca^{2+}$-mediated processes in the myocytes.

The extent to which frequency-dependent calcineurin activation, as described in the present study, contributes to the development of left ventricular hypertrophy and cardiac failure cannot be precisely estimated. However, given that the increased sympathetic outflow with associated tachycardia constitutes one of the major risk factors of chronic heart failure mortality (Poole-Wilson *et al.* 2003) and calcineurin activity has been reported to increase in patients with cardiac hypertrophy and failure (Haq *et al.* 2001; Ritter *et al.* 2002), the drugs that reduce the activation of the sympathetic nervous system like $\beta$-receptor blockers may be effective in control of cardiac growth and remodelling (Packer *et al.* 1996).

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