Ca^{2+} oscillation frequency decoding in cardiac cell hypertrophy: Role of calcineurin/NFAT as Ca^{2+} signal integrators

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The role of Ca^{2+} signaling in triggering hypertrophy was investigated in neonatal rat cardiomyocytes *in vitro*. We show that an increase in cell size and sarcomere reorganization were elicited by receptor agonists such as Angiotensin II, aldosterone, and norepinephrine and by a small rise in medium KCl concentration, a treatment devoid of direct effects on receptor functions. All these treatments increased the frequency of spontaneous [Ca^{2+}] transients, caused nuclear translocation of transfected NFAT(GFP), and increased the expression of a NFAT-sensitive reporter gene. There was no increase in Ca^{2+} spark frequency in the whole cell or in the perinuclear region under these conditions. Hypertrophy and NFAT translocation but not the increased frequency of [Ca^{2+}] transients were inhibited by the calcineurin inhibitor cyclosporine A. Hypertrophy by the different stimuli was insensitive to inhibition of myofilament contraction. We concluded that calcineurin–NFAT can act as integrators of the contractile Ca^{2+} signal, and that they can decode alterations in the frequency even of rapid Ca^{2+} oscillations.

Cardiac hypertrophy accompanies many forms of heart pathologies, such as genetic or congenital defects, ischemia, and hypertension. The molecular signaling pathways through which the different hypertrophic stimuli modulate cardiac cell size include mitogen-activated protein kinase, Gp130/Stat3, Calmodulin (CaM)-dependent kinases, and the calcineurin-regulated pathway (1). The latter has recently received much attention, in part because it can be the target of therapeutic intervention with well known drugs. For example, in transgenic mice, it has been demonstrated that overexpression of the Ca^{2+}-dependent phosphatase calcineurin causes a dramatic increase in the size of the heart, inhibited by cyclosporine A (CsA), a calcineurin blocker (2). Along the same line, overexpression of Cain/Cabin (molecules that associate with the calcineurin and inhibit its activity) attenuate cardiac hypertrophy caused not only by calcineurin overexpression but also by pressure overload or β-adrenergic receptor stimulation (3). The effect of CsA on cardiac hypertrophy, however, has led to contradictory results in different model systems (4, 5).

At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis, activation of fetal genes, and cytoskeleton reorganization (4, 5). A number of treatments are known to induce cardiac cell hypertrophy *in vitro*, including angiotensin II (Ang II) (6), catecholamines (7), endothelin (8), and aldosterone (9). Many, but not all, of these hormones are known to be coupled to alterations in Ca^{2+} homeostasis. In particular, (*i*) Ang II (6) and endothelin (10) are coupled to IP_3 generation and Ca^{2+} mobilization from stores; (*ii*) catecholamines, in particular through β1 receptors, are known to induce an increase in the frequency and amplitude of Ca^{2+} spiking in cardiomyocytes (7); and (*iii*) the mechanism of aldosterone-induced hypertrophy *in vitro* has not been clarified yet, but indirect evidence suggests that it may affect Ca^{2+} homeostasis (11, 12). Even admitting that many, if not all, hypertrophic agents modulate cardiac cell size via a modification of cellular Ca^{2+} handling, a question that remains open is how a hypertrophic Ca^{2+} signal can be discriminated from the repetitive Ca^{2+} rises underlying contractions. Differences in the amplitude, frequency, duration, or subcellular localization of the Ca^{2+} signals elicited by the hypertrophic agents may represent the biological code for such an action (13–15).

Here, we have addressed the problem of the molecular signaling pathway through which a variety of treatments induce cardiac cell hypertrophy *in vitro* and, in particular whether they exert their action through a modification of Ca^{2+} homeostasis and calcineurin activation. The capability of calcineurin to decode oscillatory Ca^{2+} signals is still a debated question. It has been shown that Ca^{2+} oscillations of tens of seconds are sufficient to activate calcineurin-dependent gene expression in cell lines (16–18). Regarding faster oscillatory patterns, such as those occurring in striated muscle, the situation is more complex: in skeletal muscle calcineurin-dependent NFAT activation/translocation depends on the frequency of the stimulation pattern (19); similarly, the high-frequency pacing of atrial cardiac preparations can induce calcineurin-dependent gene regulation (20). However, it is unclear whether calcineurin can indeed decode the frequency of Ca^{2+} oscillations or rather is activated by the prolonged increases of intracellular [Ca^{2+}], $[Ca^{2+}]_i$, after high-frequency stimulation. Our data demonstrate that a variety of hypertrophic agents modulate the frequency of spontaneous Ca^{2+} oscillations. By monitoring $[Ca^{2+}]_i$, NFAT translocation and/or activation of a calcineurin gene reporter, we here demonstrate that calcineurin can act as an integrator of the single rapid Ca^{2+} transients occurring in spontaneously beating cardiomyocytes *in vitro*, eventually resulting in the translocation of NFAT into the nucleus and the downstream activation of the hypertrophic program.

Results

Confirming previous results, we found that incubation of neonatal cardiomyocytes in serum-free medium with Ang II caused a time-dependent increase in cell area and a reorganization of the cytoskeleton [supporting information (SI) Fig. 6]. The unsolved and most important question, however, is the signaling pathway through which this and other hypertrophic stimuli exert their effect. A large body of data indicates that with a variety of agents, including Ang II, changes in Ca^{2+} homeostasis are pivotal in the hypertrophy of cardiac cells both *in vitro* and *in vivo* (21).

To measure the dynamics of intracellular Ca^{2+} concentration in the cytosol, we used the classical fluorescent Ca^{2+} indicators fura-2 and fluo-3. As shown in SI Fig. 7, we found that, under standard conditions, both indicators caused clear phototoxic effects in car-

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diomyocytes. Conditions can be found, however, where the light-induced damage can be reduced to a minimum (see *SI Text*), and these conditions were used for all of the after experiments.

In unstimulated cells, two types of $[Ca^{2+}]$ behavior were observed: in >90% of the cells there were spontaneous $[Ca^{2+}]$ transients, accompanied by contractions; in the remaining cells, $[Ca^{2+}]$ was stable and no contractions were visible (Fig. 1*A*). In cells undergoing spontaneous contractions, the Ca^{2+} increase caused by Ang II was small and superimposed on the Ca^{2+} oscillations, whereas in nonoscillating cells the Ca^{2+} peaks were much larger and more prolonged (Fig. 1*A*). The question is whether the nonbeating cells represent contaminating nonmyocytes or cardiomyocytes that exhibit no spontaneous action potentials. The cultures were thus on purpose enriched in fibroblasts and other noncardiomyocytes, loaded with fluo-3 and (*i*) the cells were challenged with high K^+ (40 mM) to depolarize the plasma membrane and thus fully activate voltage-operated Ca^{2+} channels, if present; (*ii*) after stimulation with Ang II the monolayer was fixed and stained with antibodies against cardiac α-actinin; the staining pattern (Fig. 1*C*) was compared with the Ca^{2+} signaling behavior of the individual cells (Fig. 1*B*). The results of these experiments demonstrate that the cells that did not show spontaneous Ca^{2+} oscillations did not show any significant Ca^{2+} increase upon K^+ challenge (unlike beating cells) (data not shown); cells that did not oscillate were also negative for the cardiac isoform of α-actinin (10/10) (Fig. 1 *B* and *C*). In both cell types, the effect of Ang II on $[Ca^{2+}]$ was prevented by ZD7155 (data not shown).

Two types of spontaneous Ca^{2+} oscillation patterns were observed: one regular in terms of frequency (in 79% of the cells analyzed) and the other characterized by bursts of activity followed by periods of rest of variable duration, from 10 s to 1 min (SI Fig. 7 *B* and *C*, respectively). On average, the Ca^{2+} oscillation frequency of control cells was ≈0.3 Hz (0.28 ± 0.06; $n = 22$). Addition of Ang II induced the small transient increase of basal $[Ca^{2+}]$ in 55.15% of the spontaneously oscillating cells (91/165, 19 experiments). A closer look at the effect of Ang II on $[Ca^{2+}]$ dynamics in bona fide cardiomyocytes indicated that the peptide also caused an increase in the frequency of Ca^{2+} oscillations that initially superimposed on the transient increase in diastolic Ca^{2+} (when present). The increase in Ca^{2+} oscillation frequency in some cells continued for the period of observation (>5 min; see for example Fig. 1*A*). In other cells, the Ca^{2+} oscillation frequency returned to the original value after 3–5 min (see for example Fig. 1*B*), and then oscillations stopped. This latter behavior is attributable to photoxicity, because closely located nonilluminated cells continued to beat. The effect of Ang II was prolonged, because an increase in the mean oscillation frequency was observed in cells analyzed after 48 h of continuous treatment with Ang II (Fig. 1*D*). Of note, in oscillating cells that did not show the small transient rise in diastolic $[Ca^{2+}]$, the increase in spike frequency was almost always observed (>90%).

The effect on Ca^{2+} oscillation frequency of two other *in vitro* hypertrophic agents was tested, aldosterone and norepinephrine (NE). NE caused, as expected, a rapid increase in both the frequency and amplitude of Ca^{2+} oscillations, whereas aldosterone had no acute effect on Ca^{2+} dynamics (data not shown). However, prolonged incubation with 1 μM aldosterone (Fig. 1*D*) resulted in a clear increase in oscillation frequency, from 0.28 ± 0.06 ($n = 22$) in controls to 0.62 ± 0.12 ($n = 13$) in cells treated with the hormone ($P < 0.05$). This effect was inhibited not only by a classical mineralcorticoid receptor antagonist (spironolactone), but also by ZD7155, suggesting that somehow aldosterone affects Ca^{2+} homeostasis via the type 1 Ang II receptor, AT1R (data not shown and see Fig. 3).

The question thus arises whether the key event in the hypertrophy induced by Ang II is the transient increase in diastolic $[Ca^{2+}]$, or whether it is due to the prolonged increase in Ca^{2+} oscillation frequency caused by this agent. To address this question, cells were treated with Ang II for 5 min, washed and incubated for 48 h with

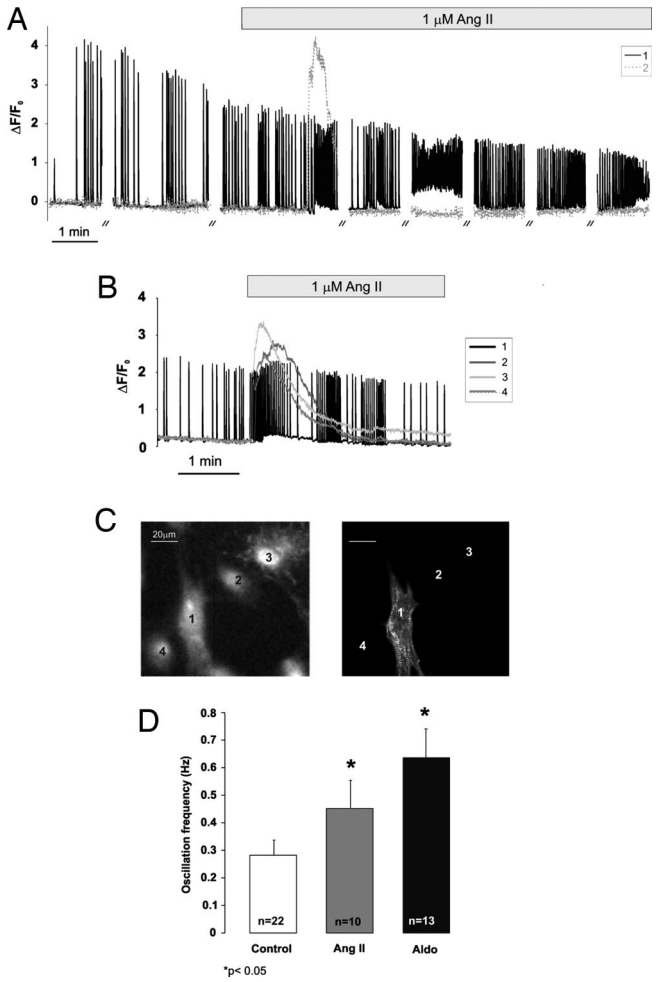

Fig. 1. Acute effects of Ang II and aldosterone on $[Ca^{2+}]_i$. (*A*) The fluorescence changes of two cells, typical of the behavior of many other cells analyzed, loaded with fluo-3 are plotted as $\Delta F/F_0$, where F_0 is the value of fluorescence at time 0, and ΔF is the change in fluorescence intensity at any given time. // indicates an interruption of the illumination for 2 min. (*B*) These four cells were obtained from a culture on purpose enriched in noncardiomyocytes. Where indicated, Ang II (1 μM) was added. Other conditions were as in *A*. (*C*) At the end of the imaging experiment, the cells whose Ca^{2+} changes are shown in *B* were fixed and permeabilized on the microscope stage and then immunostained with an anti-α actinin antibody. (*D*) The mean frequency of spontaneous Ca^{2+} oscillations was monitored in cardiac cells after 48-h incubation in medium supplemented with either 1 μM Ang II or 1 μM aldosterone. Data are means ± SEM of different independent experiments (the number of experiments is indicated in each column); for each experiment, at least 20 randomly chosen single cells were measured. *, $P < 005$ vs. control.

an AT1R antagonist, ZD7155. The area of the cells treated in this way was slightly smaller than that of controls (87.08 ± 2.27%; $n_{exp} = 5$, $n_{cell} = 279$) and the oscillation frequency, measured 10 min or 48 h after Ang II, was indistinguishable from controls.

Hypertrophic agents such as Ang II and NE control complex signal transduction pathways, i.e., G proteins, kinases, and phosphatases or, in the case of aldosterone, directly activate nuclear gene expression. It is possible that downstream effects, other than changes in Ca^{2+} oscillation frequency, may be common among these stimuli and thus responsible for inducing cell hypertrophy. Therefore, we used a protocol that should affect only the frequency of Ca^{2+} oscillations, without perturbing membrane or intracellular receptors. Cells were thus incubated in media where KCl concentration was doubled (to 10 mM), causing a partial depolarization of the plasma membrane and an increase in the frequency of spon-

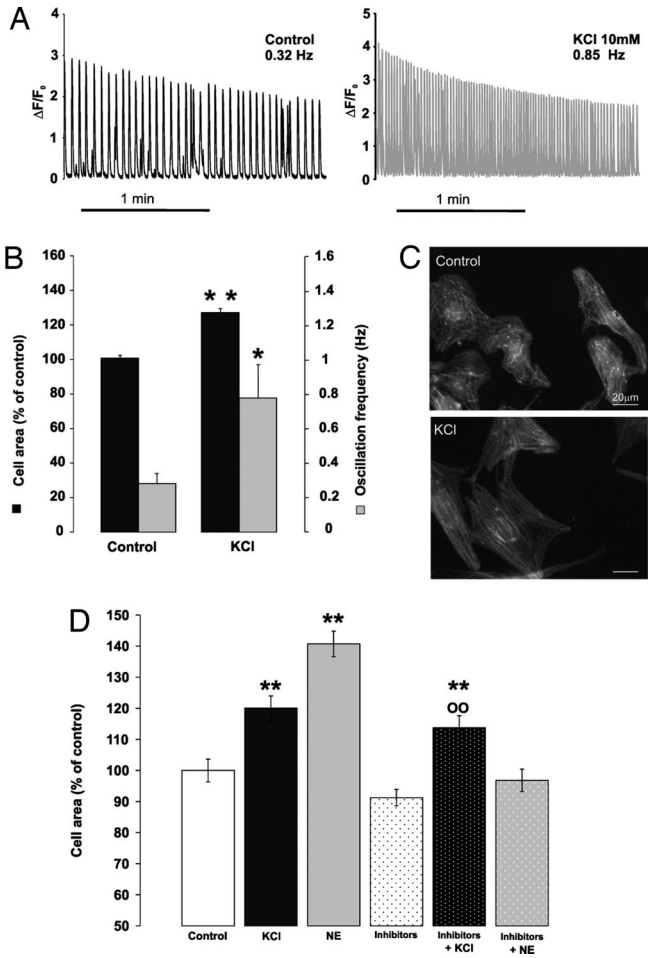

Fig. 2. A rise in extracellular KCl increases Ca^{2+} oscillation frequency and cell size and causes sarcomere reorganization. The incubation medium was supplemented with 5 mM KCl (final concentration 10 mM). (*A*) Other conditions were as in Fig. 1. On the left is the fluorescent trace of a typical control cell; on the right is that of a cell incubated in medium containing 10 mM KCl. (*B*) Mean ± SEM of the oscillation frequency (gray columns; control, $n_{exp} = 22$; KCl, $n_{exp} = 4$, >5 cells analyzed per experiment) and cell area (black columns; control: $n_{exp} = 16$, $n_{cell} = 367$; KCl: $n_{exp} = 14$, $n_{cell} = 294$). *, $P < 0.05$; **, $P < 0.005$ vs. control. (*C*) Representative images of sarcomere organization (labeling with TRITC phalloidin) in controls and cells treated with 10 mM KCl. (Scale bars, 20 μm.) (*D*) Mean ± SEM of cell area of cardiomyocytes incubated for 48 h with KCl (10 mM) or NE (1 μM), alone or preincubated with a mixture of receptor inhibitors: α,β adrenoreceptor inhibitors and AT1 receptors antagonist (10 μM Prazosin, 100 nM ICI118,51, 300 nM CGP20712A, and 10 μM 10 μM ZD7155, respectively) ($n_{exp} = 3$, $n_{cells} = 250$ for each treatment). **, $P < 0.005$ vs. control; ^{00}, $P < 0.005$ vs. inhibitors.

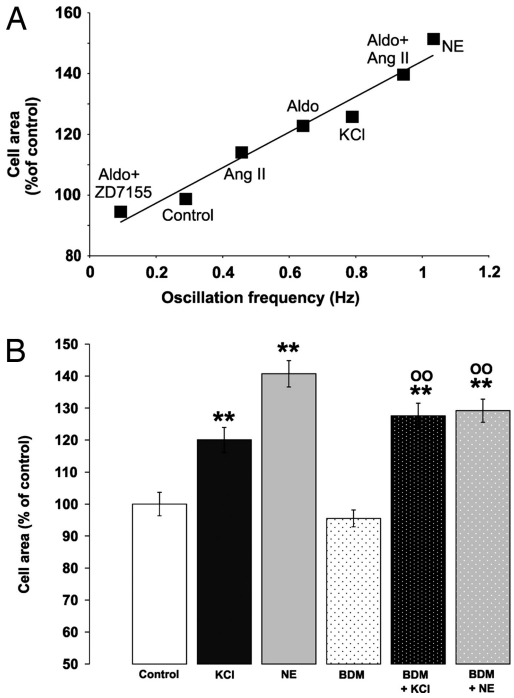

Fig. 3. Correlation between Ca^{2+} oscillation frequency, cell area, and effects of BDM. (*A*) Cells were incubated for 48 h with different stimuli: Ang II (1 μM), aldosterone (1 μM), alone or preincubated with 10 μM ZD7155, KCl (10 mM), and NE (1 μM). For each condition, oscillation frequency and cell area were measured in the same samples. Represented is the mean of at least three independent experiments, with at least 20 cells per experiment. (*B*) Neonatal cardiomyocytes were incubated in serum-free medium supplemented with 5 mM KCl (final concentration, 10 mM) in the presence or absence of 7.5 mM BDM for 48 h. Cells were fixed, permeabilized, stained with TRITC phalloidin, and examined by fluorescence microscopy. The data presented are expressed as percentage of control and are the means ± SEM of three independent experiments ($n_{cells} = 250$ for each treatment). **, $P < 0.005$ vs. control; ^{00}, $P < 0.005$ vs. BDM.

taneous Ca^{2+} oscillations. Measurement of the membrane potential by patch pipette (in the current clamp mode) revealed that the membrane potential of the cells in normal medium (5 mM KCl) was -70 ± 5 mV and decreased to -55 ± 5 mV in a medium containing 10 mM KCl. The incubation of cardiomyocytes in medium containing 10 mM KCl caused a rapid increase in the Ca^{2+} oscillation frequency (>2-fold; Fig. 2 *A* and *B*), and this increase persisted during the whole incubation time. Myocytes treated for 48 h with 10 mM KCl appeared conical-shaped, and their cell areas increased by ≈25% (126.19 ± 1.73%; $n_{cell} = 294$, $n_{exp} = 14$; $P < 0.005$) compared with controls; the myofilaments presented a more regular organization (Fig. 2*C*). The hypertrophic effect of KCl was not modified by a mixture of inhibitors, such as the AT1R inhibitor ZD7155, β and α adrenergic antagonists (Fig. 2*D*), but was completely prevented by the inhibitor of voltage-gated Ca^{2+} channel verapamil (see below).

The hypertrophic effect of NE and its inhibition by β adrenoreceptor antagonists are presented for comparison in Fig. 2*D*. An increase of 5 mM in NaCl concentration had no effect on either Ca^{2+} oscillation frequency or cell size (data not shown). In the graph presented in Fig. 3*A*, we compared the hypertrophic and chronotropic effects of Ang II, NE, aldosterone (with and without ZD7155), aldosterone +Ang II, and KCl. It should be stressed that, despite the very different signaling pathways modulated by these stimuli, a striking linear relationship ($r^2 = 0.9629$) between these two parameters is observed.

It is known that cardiac myocytes show spontaneous local increases in cytoplasmic Ca^{2+}, named Ca^{2+} sparks (22). Wu *et al.* (23) recently suggested that local Ca^{2+} events, in particular those occurring in the perinuclear region, have a primary effect on gene expression in adult cardiomyocytes. However, they did not measure Ca^{2+} sparks directly. We thus used line-scan confocal microscopy to investigate whether hypertrophic stimuli had any effect on the frequency and localization of Ca^{2+} sparks in the neonatal cardiomyocyte preparation. Table 1 shows that the overall frequency of sparks was not significantly affected by Ang II and was actually depressed slightly in the presence of 10 mM KCl. Moreover, there were no significant increases in spark frequency in any of the subcellular regions examined, and the nuclear and perinuclear regions exhibited the lowest spark frequencies under all conditions (Table 1). The intracellular distribution of Ca^{2+} sparks measured from 2D confocal image time series also showed no increase in spark density after Ang II or KCl treatment (SI Fig. 8). These Ca^{2+} spark maps are also consistent with the data of Table 1 in showing

Table 1. Effect of Ang II and elevated extracellular KCl on Ca^{2+} spark frequency

	Spark frequency (no. of sparks per 100 μm/s)				
	Whole cell	Cell periphery	Cytoplasmic	Perinuclear	Nuclear
Control	2.43 ± 0.14	3.87 ± 0.37	2.68 ± 0.25	1.96 ± 0.34	0.59 ± 0.18
Ang II, 1 μM	2.36 ± 0.18	4.13 ± 0.55	2.58 ± 0.27	1.37 ± 0.31	0.22 ± 0.13
KCl, 10 mM	1.78 ± 0.22*	3.25 ± 0.60	2.33 ± 0.57	1.07 ± 0.32	0.07 ± 0.05

Neonatal cardiac myocytes were treated with the indicated agonists for 10 min prior to recording Ca^{2+} sparks from line-scan images. Spark frequency is expressed as the number of sparks normalized to 100-μm scan-line length and 1-s duration. Data are mean ± SEM. Whole cell refers to all sparks recorded in each cell for the following number of cells analyzed: Control, 83; Ang II, 39; KCl, 41. Spark data for subcellular regions were obtained from the same cells with at least 20 cells for each region (scan line did not pass through all regions in all cells). *, $P < 0.05$ vs. control.

higher spark frequency at the cell periphery than in the nuclear and perinuclear regions. Finally, we examined the spatial and temporal properties of the Ca^{2+} sparks and again found no evidence for enhanced signal generation by Ca^{2+} sparks in the presence of the hypertrophic stimuli (SI Table 2).

All of the agents tested, in addition to causing an increase in Ca^{2+} oscillation frequency, also increase either the force and/or frequency of contraction. The possibility that the hypertrophic effect is somehow mediated by a mechanochemical coupling was investigated by treating the cells with 2,3-butanedione monoxime (BDM), an excitation–contraction uncoupler (24). Incubation with 7.5 mM BDM, a dose that inhibits myofilament contraction, while leaving Ca^{2+} oscillations unaffected had no effect on the hypertrophic effects of KCl or NE (Fig. 3*B*).

The Ca^{2+}-dependent phosphatase calcineurin has been at the center of major interest as a key transducer in the Ca^{2+}-activated signaling pathway, particularly in the field of heart hypertrophy (5, 25, 26). A major, although not unique, pathway regulated by calcineurin is that involving the transcription factor NFAT, which, upon dephosphorylation, translocates to the nucleus and eventually results in the transcription of a number of genes. To determine whether calcineurin is involved in the hypertrophic effect of the different treatments described above, cells stimulated with Ang II were treated with CsA, the classical inhibitor of calcineurin. The increase in cell area caused by the stimulus was completely blocked, whereas the frequency of Ca^{2+} oscillations remained comparable to that of cells incubated with Ang II only (Fig. 4). CsA was also able to prevent the hypertrophic effect induced by KCl, NE and aldosterone, again without affecting the changes in Ca^{2+} oscillation frequency induced by these agents (data not shown).

To more directly address the involvement of calcineurin–NFAT, cells were transfected with NFAT-GFP, and its subcellular localization in control and treated cells was compared (SI Fig. 9). Quantitation of such results indicated that the percentage of unstimulated cardiomyocytes with NFAT-positive nuclei was 12% (14/140 cells in six different experiments); these data are in agreement with previous studies (27). In cells treated with aldosterone for 20 h, the number of cells containing NFAT-GFP in the nucleus increased to 59% (142 of 245 cells, in 11 different experiments). Similarly, the number of cells with NFAT-GFP-positive nuclei increased to 47%, 45%, and 58% in cells treated for 4 h with Ang II (1 μM), NE (1 μM), and KCl (10 mM), respectively (average of three to five experiments for each condition). In cells artificially paced for 3 h at a frequency of 3.5 Hz, 72% of the nuclei were positive for NFAT-GFP. Translocation of NFAT-GFP was inhibited by pretreatment with 1 μM CsA (data not shown). The time course of KCl-induced NFAT-GFP translocation was followed in more detail. SI Fig. 10 shows that a significant translocation of NFAT is observed after 1 h of treatment with KCl and increased by almost 3-fold after 5-h treatment.

The final question is whether endogenous NFAT translocation to the nucleus is sufficient to activate the transcription of downstream gene targets. To this end, cells were transfected with the reporter gene luciferase engineered to include an NFAT-sensitive element at the 5′ end (27). Fig. 5 shows that the expression of luciferase was increased on average 2-fold by 10 mM KCl, and this increase was not affected by inhibition of contraction with BDM, whereas it was completely suppressed by an inhibitor of voltage-gated Ca^{2+} channels such as verapamil. The increase in luciferase expression by KCl was unaffected by a mixture of different receptor inhibitors

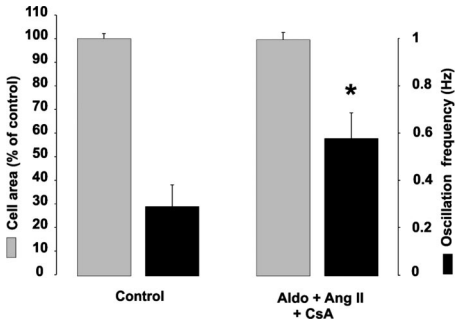

Fig. 4. Translocation of NFATc4-GFP into the nucleus and effects of CsA. Conditions were as in SI Fig. 7. When present, CsA was 1 μM. Other conditions were as in SI Fig. 6. The results are mean ± SEM of independent experiments. Cell area: control, $n_{exp} = 3$, $n_{cell} = 192$; aldosterone + Ang II + CsA: $n_{exp} = 3$, $n_{cell} = 178$. Oscillation frequency: control, $n_{exp} = 5$, $n_{cell} = 5$; aldosterone + Ang II + CsA: $n_{exp} = 9$, $n_{cell} = 9$. *, $P < 0.05$ vs. aldosterone + Ang II.

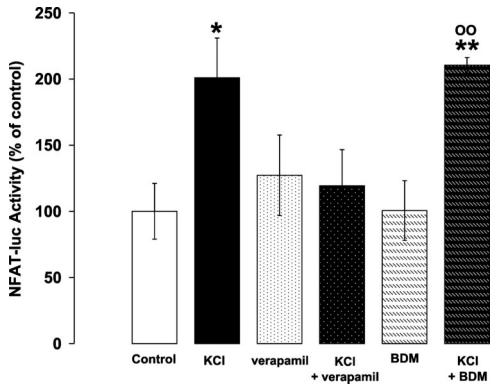

Fig. 5. NFAT transcriptional responses upon treatment with 10 mM KCl. 9xNFAT-TATA luciferase transfected neonatal cardiomyocytes (27) were incubated in serum-free medium for 16 h. Where indicated, the medium was supplemented with 5 mM KCl (final concentration, 10 mM), 10 μM verapamil, or 7.5 mM BDM. NFAT-luciferase expression was measured as described in *Materials and Methods*. The data presented are means ± SEM of at least three independent experiments, each condition tested in triplicate (*, $P < 0.05$; **, $P < 0.001$ vs. control; ^{00}, $P < 0.001$ vs. BDM).

(ZD71055 to inhibit AT1RR; and prazosin, ICI, and CGP to block α, β1, and β2 receptors, respectively). Similar results were obtained with the other hypertrophic stimuli (data not shown).

Discussion

Cardiac hypertrophy is a highly complex phenomenon that can be activated by physiological and pathological conditions. The molecular mechanisms underlying the transformation of physiological into pathological cardiac hypertrophy are the subject of very intense investigation, given that hypertrophy, followed by fibrosis, is the major cause of heart failure in the world.

Results obtained in cardiomyocyte cell cultures from neonatal animals are obviously difficult to extrapolate to the *in vivo* adult situation, because the number, repertoire, and coupling mechanism of different receptors may vary between adult and neonatal cells. The latter model system, however, allows an easier molecular dissection of the mechanism of action of different agents, without interference from effects at the organism level.

Regarding the molecular mechanisms linking Ang II to hypertrophy and cytoskeletal rearrangements, strong evidence supports the idea that changes in cytoplasmic [Ca^{2+}] play an essential role in hypertrophic signaling in the heart (26, 28, 29). Indeed, a fundamental role of extra- and intracellular Ca^{2+} in acute cytoskeleton reorganization by Ang II has been uncovered (30).

Surprisingly, there are few and often contradictory studies on the effects of Ang II on cardiomyocyte Ca^{2+} homeostasis. In particular, some authors have provided evidence that Ang II decreases [Ca^{2+}] transients in cultured neonatal cardiomyocytes (31), whereas others showed intracellular [Ca^{2+}] increases after Ang II stimulation (32). In light of the data presented in Fig. 1 and SI Fig. 7*A*, these contradictory results could be explained by the occurrence of technical problems, such as toxicity of the fluorophore or contaminant nonmyocytes. Differences in culture conditions and/or in the method of Ca^{2+} measurements may explain some discrepancies. We here show unambiguously that, in bona fide neonatal cardiomyocytes in culture, the primary effect of Ang II is a modest rise of the diastolic Ca^{2+} level lasting 1–2 min.

One thus wonders how such a minor effect on [Ca^{2+}], particularly in a cell that continuously undergoes spontaneous large changes in cellular Ca^{2+} levels tens of times per minute, could have such dramatic and long-term effects on cell size (and cytoskeletal organization). Indeed, addition of ZD7155 5–10 min after Ang II resulted in the complete blocking of hypertrophy, indicating that a prolonged occupancy of the receptors is necessary to fully activate the hypertrophic program. However, we found that treatment with Ang II had an unpredicted long-term effect on cardiomyocyte Ca^{2+} handling, i.e., it increased the frequency of their spontaneous Ca^{2+} oscillations. The molecular mechanism of this positive chronotropic effect is presently not yet clear, but it is long-lasting, because it is observable also in cells treated with Ang II for 24/48 h. Other hypertrophic agents, such as NE and aldosterone, also cause an increase in oscillation frequency. Although the positive chronotropic effect of NE has been extensively investigated, the effect of aldosterone was somewhat unexpected. The hormone effect on Ca^{2+} oscillation frequency requires several hours to be appreciated and, based on the inhibitory effect not only of spironolactone but also of ZD7155, it requires the stimulation of both the mineralcorticoid receptor and of AT1R (M.C. and T.P., unpublished results).

The final but key question is the identification of the signaling pathway downstream Ca^{2+} oscillation frequency that leads to activation of the hypertrophy program. In the field of cardiac hypertrophy, much attention has been devoted in the last few years to kinases and phosphatases controlled by CaM. Indeed, overexpression of CaM has been reported to induce hypertrophic growth of cardiomyocytes in transgenic mice (33). The Ca^{2+}/CaM complex exerts its functions through downstream effectors, such as Ca^{2+}/CaM-dependent protein kinases (CaMKs), and calcineurin, a Ca^{2+}/CaM-activated serine-threonine phosphatase. Calcineurin activation by Ca^{2+} results in dephosphorylation of the transcription factor NFAT in the cytosol, followed by its translocation into the nucleus and subsequent transcription of a number of cardiac genes (2, 5, 29). The idea that calcineurin (and NFAT) is primarily involved in cardiac hypertrophy in humans, however, is not shared by all investigators in the field, and this remains a hot topic of debate.

We here addressed the hypothesis that the increased frequency of Ca^{2+} oscillations, if prolonged for hours, is sufficient to substantially activate the calcineurin/NFAT pathway and eventually turns it into a hypertrophic signal. Indeed, not only were all of the hypertrophic effects of Ang II, NE, and KCl inhibited by CsA, but, more importantly, all these stimuli resulted in a substantial, CsA-sensitive, translocation of NFAT into the nucleus. Even simple and direct ways of changing the beating frequency (i.e., pacing of the cells with an extracellular electrode or increases of the extracellular KCl concentration) resulted in an efficient translocation of NFAT. Is it noteworthy that these stimuli activated the expression of a transfected reporter gene under the control of endogenous NFAT, and such an activation was insensitive to the mechanical activity.

The question thus arises of the molecular mechanism of such NFAT activation, because of the very brief duration of each single Ca^{2+} spike in cardiac cells. In previous reports, translocation of NFAT into the nucleus has been obtained by either sustained increases in cellular Ca^{2+} levels (34) or oscillatory Ca^{2+} increases, yet of several tens of seconds duration (16, 17), i.e., 10–100 times more prolonged than that of a single Ca^{2+} oscillation in cardiac cells. More recently, however, it was shown that pacing of atria at high frequency (>4 Hz) results in a substantial activation of NFAT-dependent gene expression (20). Under such conditions, however, the high-frequency pacing resulted in a large increase of diastolic Ca^{2+}. Under our experimental conditions, up to a frequency of 2 Hz, the increase in diastolic Ca^{2+} level is negligible, yet substantial NFAT translocation is observed after a few hours. It can be argued that in the 100/200-ms duration of a single Ca^{2+} oscillation, there is no time for a significant dephosphorylation of NFAT and its translocation into the nucleus. We here propose that calcineurin/NFAT act as integrators of the Ca^{2+} signal. During a single Ca^{2+} oscillation of ≈200-ms duration, one may expect a very minor fraction of NFAT to be dephosphorylated by calcineurin. If the interval between two Ca^{2+} spikes is sufficiently long, this minute dephosphorylated fraction of NFAT will be rephosphorylated during diastole, and no net transfer of NFAT into the nucleus will occur. If the interval between two successive Ca^{2+} spikes is shortened, the balance between phosphorylation/dephosphorylation will be shifted toward the second situation, and dephosphorylated NFAT will tend to slowly accumulate. If such even marginal imbalance is repeated tens of times every minute, eventually, after hours, a significant fraction of NFAT will end up in the nucleus, as observed. This simple kinetic scheme not only has the advantage of explaining NFAT translocation as a function of Ca^{2+} oscillation frequency, but it can also be applied to stimuli that may affect the amplitude rather than the frequency of Ca^{2+} spikes. In this case, a larger Ca^{2+} increase will activate more calcineurin molecules and, even if minute, the shift toward a more dephosphorylated NFAT will tend to accumulate if the episodes occur thousands of times in the same cells.

A similar model, named "integrative tracking," has been proposed by Berridge *et al*. (13–15). The authors proposed that each Ca^{2+} transient has a small effect on itself, but individual transients can be integrated over time to provide a significant change in some cellular process. They identified the NFAT shuttle as a typical example of "integrative tracking." The present data provide experimental support to the model.

An alternative possibility would be that local Ca^{2+} increases, in particular those occurring close to the nucleus, might be part of this integrative tracking system. Wu *et al.* (23) showed that endothelin-1 enhances hypertrophic gene transcription in ventricular myocytes via CaM Kinase II phosphorylation of HDAC. The authors sug-

gested that this effect depends on IP_3-mediated mobilization of Ca^{2+} from the nuclear envelope, although they were unable to detect local nuclear Ca^{2+} increases (23). In our studies, Ca^{2+} sparks were observed throughout the cell during periods between Ca^{2+} transients, including in the perinuclear region, although they were rare inside the nuclear matrix. More importantly, the translocation of NFAT into the nucleus in response to either Ang II or KCl was not associated with any increase in nuclear or perinuclear Ca^{2+} spark frequency, and these agents did not affect the spatial or temporal spreading of Ca^{2+} sparks. Thus, it appears that cytosolic Ca^{2+} transients, rather than localized perinuclear or nuclear Ca^{2+} signals, are responsible for the regulation of NFAT phosphorylation and hence translocation into the nucleus.

It can be argued that the spontaneous activity of the cultures used in this study (0.3–0.5 Hz) is much lower than that of the physiological beating rate of rat heart (3–4 Hz). However, in the kinetic scheme proposed above, it is not difficult to hypothesize that, by adapting the activity and/or level of the NFAT kinases to the activity of calcineurin, cells can adapt to a higher frequency of beating, maintaining NFAT in a largely phosphorylated state under nonstimulated conditions. In addition, the kinetic scheme predicts that short-duration increases in the beating frequency will not result in a substantial translocation of NFAT into the nucleus. The key aspect of the model is that the calcineurin/NFAT pathway has intrinsically the ability to perceive and transduce into NFAT activation the frequency (or amplitude) of Ca^{2+} oscillations, independently of the duration of the single episode of Ca^{2+} increase. A kinetic scheme of this type could, in theory, be hypothesized for other Ca^{2+}-dependent phosphorylation/dephosphorylation processes that involve translocation across the nuclear membrane of transcriptonal regulators, e.g., HDAC and CaM kinases (35, 36), i.e., other signaling pathways that can lead to cardiac cell hypertrophy. Finally, a similar mechanism may apply to the activation of the slow fiber-type program in skeletal muscle (that depends on NFAT translocation to the nucleus) (19), where the single episode of Ca^{2+} rise can be repeated several hundreds/thousands of times, depending on the frequency and pattern of the motor nerve impulse.

Materials and Methods

Cardiomyocyte Culture. Cultures of cardiomyocytes were prepared from ventricles of neonatal Wistar rats (0–2 d after birth), as described (37). On the first day of culture, cells were washed and thereafter maintained in serum-free medium until used.

cDNA Constructs and Transfection. The NFATc4-GFP plasmid, a gift from Chi-Wing Chow (Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY) and 9xNFAT-TATA luciferase plasmid, a kind gift from J. D. Molkentin (27), were transfected on the second day of culture by using the FuGENE 6 Transfection Reagent (Roche), as described in ref. 37. For a 24-mm diameter coverslip, 3 μg of DNA was mixed with 6 μl of FuGENE (37). All experiments were performed 1 d after transfection.

Ca^{2+} Measurements. See *SI Text*.

Statistical Analysis. Data are reported as mean ± SEM of independent experiments or, in cell-area determination, of different cells. Statistical differences were evaluated by one-tailed Student's t test, a P value <0.05 being considered statistically significant.

Materials. All chemicals were of analytical or highest available grade and, unless otherwise stated, were obtained from Sigma. ZD7155 was from Tocris; fura-2 a.m. and fluo-3 a.m. were from Molecular Probes. When dimethyl sulfoxide or ethanol was used as solvents, the final solvent concentration never exceeded 0.01% or 0.1%, respectively.

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