Calcineurin Inhibits Na\(^+\)/Ca\(^{2+}\) Exchange in Phenylephrine-treated Hypertrophic Cardiomyocytes*

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The cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) is the predominant mechanism for the extrusion of Ca\(^{2+}\) from beating cardiomyocytes. The role of protein phosphorylation in the regulation of NCX1 function in normal and diseased hearts remains unclear. In our search for proteins that interact with NCX1 using a yeast two-hybrid screen, we found that the C terminus of calcineurin Aβ containing the autoinhibitory domain, binds to the β1 repeat of the central cytoplasmic loop of NCX1 that presumably constitutes part of the allosteric Ca\(^{2+}\) regulatory site. The association of NCX1 with calcineurin was significantly increased in the B1014.6 cardiomyopathic hamster heart compared with that in the normal control. In hypertrophic neonatal rat cardiomyocytes subjected to chronic phenylephrine treatment, we observed a marked depression of NCX activity measured as the rate of Na\(^+\)/Ca\(^{2+}\) efflux. Depressed NCX activity was partially and independently reversed by the acute inhibition of calcineurin and protein kinase C activities with little effect on myocyte hypertrophic phenotypes. Studies of NCX1 deletion mutants expressed in CCL39 cells were consistent with the view that the β1 repeat is required for the action of endogenous calcineurin and that the large cytoplasmic loop may be required to maintain the interaction of the enzyme with its substrate. Our data suggest that NCX1 is a novel regulatory target for calcineurin and that depressed NCX activity might contribute to the etiology of in vivo cardiac hypertrophy and dysfunction occurring under conditions in which both calcineurin and protein kinase C are chronically activated.

The Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) catalyzes the reversible exchange of Na\(^+\) for Ca\(^{2+}\) across the plasma membrane. In normal cardiac muscle, the primary role of NCX1 (the cardiac isoform of NCX) is to extrude cytoplasmic Ca\(^{2+}\) during myocyte repolarization and diastole, which balances Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels during myocyte depolarization (1, 2). The transport activity of NCX1 is known to be influenced by a variety of factors, including hormones and growth factors, intracellular Na\(^+\) and Ca\(^{2+}\) concentrations, membrane potential, cytoplasmic ATP, and protein and lipid phosphorylation (3). However, information is still limited as to the quantitative aspects of changes in these factors and their consequences on NCX1 activity in normal and diseased cardiomyocytes. For example, in hypertrophic and failing hearts from human patients and animal models, sarcolemmal NCX1 expression has often been shown to be elevated (4–7), which could be compensatory for the reduced ability of the sarcoplasmic reticulum to maintain low diastolic [Ca\(^{2+}\)]i, under these pathological conditions. However, whether increased NCX expression invariably leads to enhanced function under disease conditions is not clear, although enhanced NCX expression and function have been observed in cardiomyocytes isolated from some animal models of cardiac hypertrophy and heart failure (6, 7).

Protein phosphorylation is an important mechanism regulating the functions of many cellular systems. In the case of NCX1, acute treatment with PMA or agonists of Goα-coupled receptors such as phenylephrine (PE) has been shown to enhance NCX activity in isolated cardiomyocytes as well as in cells expressing cloned NCX1 (8–10). Protein kinase A activation has also been reported to stimulate NCX1 activity (11, 12). On the other hand, a protein phosphatase inhibitor, calyculin A, reportedly causes substantial inhibition of NCX activity in cells expressing cloned NCX1 (13). NCX1 stimulation by PMA and agonists of Goα-coupled receptors occurs via a mechanism involving PKC activation and requires the participation of the central cytoplasmic loop of the exchanger (see Fig. 1a) (9). Because these agonist effects did not require the direct phosphorylation of NCX1, the central cytoplasmic loop was considered to serve as an anchor for phosphorylatable regulatory ancillary protein(s) (9).

In this study, we undertook a search for regulatory proteins interacting with the central cytoplasmic loop of NCX1 using a yeast two-hybrid screen. From this search and subsequent analysis, we identified a complex, hitherto unrecognized regulatory mechanism for cardiac NCX1 involving calcineurin and PKC in hypertrophic cardiomyocytes subjected to prolonged PE pretreatment. This mechanism is capable of markedly decreasing NCX1 activity. Because calcineurin acts as a central mediator of transcriptional regulation, this may represent an additional mechanism to down-regulate NCX1 expression and function in pathological conditions.
ator of in vivo cardiac hypertrophy and failure (14–16), NCX might contribute to the etiology of in vivo cardiac dysfunction.

**Experimental Procedures**

**Materials**—FCS, PE, PK506, PMA, calphostin C, 8-bromo-cAMP, Rp-8-CPT-cAMP, 8-bromo-cGMP, Rp-8-CPT-cGMP, and H89 were purchased from Sigma. GF190203X, chelerythrine chloride, KN93, and KN62 were purchased from Calbiochem. Rhodamine-conjugated phalloidin was obtained from Molecular Probes. Antibodies to NCX1 isoforms have been described (8, 9). Rabbit polyclonal anti-pan calcineurin A (CnA) and goat polyclonal anti-CNAb (Santa Cruz Biotechnology), mouse monoclonal anti-CNAb (Upstate Technology), anti-heamagglutinin (Roche Applied Science), rabbit polyclonal anti-atarial natriuretic peptide (anti-ANP; Phoenix Pharmaceuticals), fluorescein isothiocyanate-conjugated anti-rabbit IgG and rhodamine-conjugated anti-goat IgG (ICN/CAPEL), and anti-calmodulin, horseradish peroxidase-conjugated anti-rabbit IgG, and biotin-conjugated anti-mouse IgG (Zymed Laboratories Inc.) were purchased from the sources indicated in parentheses. Horseradish peroxidase-conjugated streptavidin was obtained from Zymed Laboratories Inc..

**Calcineurin—**Purified from rat brain cDNA library fused to the GAL4 activation domain. These bait clones were sequenced using the activation domain sequences primers by the ABI 9600 sequencer. Positive clones were verified by one-on-one transformations and selections by growth on agar plates in -HAlT medium and β-galactosidase assay (see Fig. 1b).

**Immunoprecipitation, Western Blotting, and Immunocytochemical Analyses—**Brain and/or ventricular tissues from rat or hamster and cultured rat cardiomyocytes were homogenized by Hiscorotn (NITI-ON, Funabashi, Japan) in radioimmunoprecipitation assay lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2 µg/ml leupeptin, 1 µg/ml aprotinin, 200 µM phenylmethylsulfonyl fluoride, and 200 µM benzamidine hydrochloride. The lysates were subjected to centrifugation at 100,000 × g for 20 min, and the resultant supernatant (up to 5 mg protein) was pre-cleared with 50 µl of protein A-Sepharose beads for 2 h at 4 °C on a rotator. After centrifugation, the supernatant was incubated with anti-pan CnA for 2 h at 4 °C and then with 50 µl of protein A-Sepharose beads for at least 2 h at 4 °C on a rotator. The beads were washed eight times with ice-cold phosphate-buffered saline. Proteins solubilized from beads by boiling in the Laemlli buffer (20) were subjected to SDS-PAGE on a 5.5% gel and then to immunoblotting with an appropriate antibody. Immunoblot analysis was performed essentially as described previously (21). The immunoblot was visualized using an enhanced chemiluminescence detection system (Amersham Biosciences).

**Immunocytochemistry,** 5-µm-thick sections of normal and BIO14.6 hamster ventricular tissues embedded in OCT compound (Tissue-Tek) were permeabilized with 0.1% Triton X-100 and treated with rabbit polyclonal anti-NCX1 or goat polyclonal anti-CNAb at dilutions of 1:500 and 1:200, respectively. These sections were then treated with fluorescein isothiocyanate-conjugated anti-rabbit IgG or rhodamine-conjugated anti-goat IgG. For immunostaining of rat cardiomyocytes, coverslips were fixed on coverslips glass slides in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and then stained with anti-NCX1, anti-pan CnA, or anti-ANP. For double staining with a combination of polyclonal (rabbit) and monoclonal (mouse) antibodies, fixed and permeabilized myocytes were incubated with a mixture of two primary antibodies and then with a mixture of the fluorescence-labeled anti-mouse and rhodamine-labeled anti-rabbit IgG. Confocal microscopy was performed using an MRC-1024, Bio-Rad mounted on an Olympus AX70WI epifluorescence microscope with a plan-apochromat 60× water immersion objective lens (Olympus).

**Fractionation of Heart Extracts—**Normal and BIO14.6 hamster hearts were homogenized in phosphate-buffered saline using a Hiscorotron homogenizer and centrifuged at 15,000 × g for 15 min. The resulting supernatant was centrifuged at 500,000 g for 1 h to yield supernatant and pellet fractions. Most of the sarcolemmal and sarcoplasmic reticulum membranes were presumably recovered in the pellet fraction. Both the supernant and pellet fractions were then subjected to immunoblot analysis with anti-pan CnA.

**Na⁺/Ca²⁺ Exchange—**To determine the Na⁺/Ca²⁺ exchange activities in control cells not pretreated with PE or other ionophores, Na⁺/Ca²⁺ exchange activities were measured as described previously (8, 9, 22) with slight modifications. Cardiomyocytes or CCL39 cells cultured in 24-well dishes were loaded with Na⁺ by incubating them at 37 °C for 30 min in 0.5 ml of normal BSS (10 mM Hepes/Tris (pH 7.4), 146 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 10 mM glucose, and 0.1% bovine serum albumin) containing 1 mM ouabain and 10 mM monensin. In cardiomyocytes pretreated with PE or FCS, Na⁺ loading was carried out during the last 30 min of such pretreatment. 45Ca²⁺ uptake was then initiated by switching the medium to Na⁺-free BSS containing choline chloride or to normal BSS, both of which contained 370 kg/m3 of 45Ca²⁺ and 1 mM ouabain. After a 30-s incubation, cells were washed with an ice-cold solution containing 10 mM LaCl₃ to stop 45Ca²⁺ uptake. Cells were subsequently solubilized with 0.1 N NaOH, and aliquots were taken for the determination of radioactivity and protein. Na⁺-dependent 45Ca²⁺ uptake was estimated by subtracting 45Ca²⁺ uptake in normal BSS from that in Na⁺-free BSS. To observe the effects of FK506, protein kinase modulators, or thapsigargin, cells were incubated with these substances during the last 15–30 min of Na⁺ loading, except that endogenous PKC was down-regulated by treatment with 0.3 µM PMA for 24 h. The Na⁺-dependent 45Ca²⁺ uptake in cells not pretreated with inhibitors or other agents were as follows: for cardiomyocytes, 12.1 ± 0.5 nmol/mg/30s (n = 39); for CCL39 cells expressing the wild-type NCX1, NCX1L246–672, and NCX1L407–478, 10.4 ± 0.5, 4.2 ± 0.4, and 5.3 ± 0.2 mmol/mg/30s (n = 9), respectively. These values were taken as 100% in Figs. 3d, 4a, 4b, 5, 6, and 7.

To measure 45Ca²⁺ flux, cardiomyocytes in 35-mm dishes were incubated in 1 ml BSS containing 740 kg/m3 of 45Ca²⁺ for the last 2 and
Calcineurin-induced Inhibition of Cardiac NCX1

RESULTS

Isolation of CnAβ as a NCX1-binding Protein and Mapping of the Interacting Site in NCX1—To isolate protein(s) interacting with NCX1, we performed the yeast two-hybrid screen of a human brain cDNA library using various segments of the large central loop of NCX1 as bait (Fig. 1a). From an initial screen in which we used a mixture of yeast populations expressing individual bait sequences, we isolated a positive clone encoding a 100 amino acid C-terminal tail of CnAβ with its autoinhibitory domain (Fig. 1a). We then examined the interaction of individual NCX segments with the CnAβ tail by one-on-one transformations and selection by colony growth and β-galactosidase assays (Fig. 1b). We confirmed that aa 407–478 of the CnAβ tail was required for binding to NCX1 protein, known as the β1 repeat, and other fragments containing this same sequence associate with the CnAβ tail.

We examined whether calcineurin interacts with NCX1 and its isoforms (NCX2 and NCX3) at the protein level. We found that anti-pan CnA co-precipitated proteins reactive with antibody to each isoform from lysates of rat brain and heart, although these proteins were still relatively abundant in the supernatant fractions (Fig. 1c). Thus, at least some calcineurin was physically associated with NCX isoforms, consistent with the fact that the β1 repeat sequence is conserved in these isoforms. Of note, anti-pan CnA immunoprecipitated single major proteins from rat brain and heart (Fig. 1c) and hamster (Fig. 2b) hearts that were recognized by anti-CnAβ, indicating that the antibody predominantly precipitated CnAβ under the conditions used.

Enhanced Association of CnAβ with NCX1 in BIO14.6 Hamster Heart—The BIO14.6 hamsters develop cardiomyopathy and muscular dystrophy due to δ-sarcoglycan deficiency (25, 26). We examined the interaction of NCX1 with calcineurin in the hearts of 120-day-old BIO14.6 hamsters, because our recent study has suggested that [Ca2+]i might be elevated in these hearts of BIO14.6 hamsters. We found that immunoprecipitation of CnAβ with anti-pan CnA or no antibody (IP:control), and post-immunoprecipitation supernatants (Supernatant) were subjected to immunoblot (IB) assays with antibodies to the indicated proteins.

**Fig. 1.** Identification of CnAβ as a NCX1-binding protein. a, domain structures of NCX1 and CnAβ. Segments of the central cytoplasmic loop of NCX1, labeled as indicated (XIP, NCX inhibitory peptide; NT, N terminus; CT, C terminus), were used as bait for the yeast two-hybrid screen. In CnAβ, CnB, CaM, and AI are the calcineurin B binding, the calmodulin binding, and the autoinhibitory domains, respectively. b, colony growth after one-on-one transformation and selection in medium lacking histidine, leucine, and tryptophan (−HALT) or medium lacking histidine, alanine, leucine, and tryptophan (−HLT) or β-galactosidase (β-gal)-positive colonies in −HALT medium. c, co-immunoprecipitation of CnA with NCX isoforms. Lysates from rat brain and heart (Total), materials immunoprecipitated with anti-pan CnA (IP:CnA) or no antibody (IP:control), and post-immunoprecipitation supernatants were subjected to immunoblot (IB) assays with antibodies to the indicated proteins.
protein from BIO14.6 than from normal hearts despite the similar contents of NCX1 and calcineurin in these preparations (Fig. 2b).

Interestingly, calmodulin also was more abundant in the immunoprecipitates from BIO14.6 heart, although total calmodulin was again not different (Fig. 2b), suggesting that calcineurin is activated in the BIO14.6 heart. Immunocytochemistry with anti-CnAβ revealed the presence of calcineurin at the peripheral sarcolemma in BIO14.6 but not in normal cardiomyocytes, although it was detectable in the cell interior and at the intercalated discs in both types of myocytes (Fig. 2a). Striated patterns seen in the cell interior may reflect the presence of calcineurin in the Z-lines (16). Furthermore, calcineurin was more abundant in the membrane versus the cytosolic fraction prepared from the BIO14.6 heart, whereas the opposite was true of normal heart muscle (Fig. 2c). Thus, the association of calcineurin with NCX1 is significantly enhanced in BIO14.6 compared with normal hearts.

NCX Activity Is Regulated by Calcineurin in Rat Cardiomyocytes or CCL39 Cells Expressing Cloned NCX1—To examine the possible effects of calcineurin on NCX activity, we used rat neonatal cardiomyocytes subjected to pretreatment with 10 μM PE for 72 h (see protocol in Fig. 3a). In these myocytes, a prominent increase in cell size with enhanced sarcomere organization and enhanced expression of ANP was observed (Fig. 3b) (14, 17, 28). The NCX1 protein was detectable in the sarcomere, particularly in the intercellular junctions (Fig. 3, b and c), and its expression greatly increased during the PE treatment, although calcineurin expression remained essentially unchanged (Fig. 3d, top). We observed overlapping localization of NCX1 and calcineurin in the sarcolemma of myocytes after a 72-h PE treatment, consistent with the finding that a much larger amount of NCX1 protein was recovered in the anti-CnA immunoprecipitates from the PE-treated versus non-treated myocytes (Fig. 3c). These myocytes did not show a sign of apoptosis, because the number of TUNEL-positive cells was <3% the amount in non-treated control myocytes. Importantly, the rate of Na⁺-dependent 45Ca²⁺ uptake measured as activity per milligram of cell protein was markedly decreased in myocytes after a 72-h PE treatment (Fig. 3d, bottom). The uptake rate in the latter myocytes would be even smaller if it was normalized to cell NCX1 content (see above). On the other hand, the uptake rate was modestly increased in myocytes after a 24-h PE treatment. This uptake increase may be attributable to the increased NCX1 expression seen at this time point (Fig. 3d, top).

Intriguingly, when myocytes were treated with 0.01–1 μM the calcineurin inhibitor FK506 during the last 15–30 min of the 72-h PE treatment, the rate of Na⁺-dependent 45Ca²⁺ uptake increased 2-fold (Fig. 4a). Qualitatively similar results were obtained with myocytes pretreated with 10% FCS (Fig. 4a). Of note, the rate of Na⁺-dependent 45Ca²⁺ uptake also increased by 188 ± 3% (n = 3) when 10 μM cyclosporin A, another calcineurin inhibitor, was added to the PE-treated myocytes. On the other hand, FK506 also enhanced the rate of Na⁺-dependent Ca²⁺ efflux from PE-treated cardiomyocytes (Fig. 4b). In this experiment, we loaded PE-treated and non-treated myocytes with 45Ca²⁺ to equivalent levels of radioactivity, and 45Ca²⁺ efflux was then initiated by acutely raising [Ca²⁺], with thapsigargin under physiological ionic conditions. These data indicate that PE and FK506 regulate both the influx and efflux modes of NCX activity.

To confirm the involvement of calcineurin in NCX regulation, we tested the effect of the adenoviral infection of dominant negative or activated CnA on cardiomyocytes pretreated with PE or FCS for up to 96 h (see Fig. 3a). Like FK506, dominant negative CnA, when infected at 72 h after the start of PE treatment, caused a large increase in the rate of Na⁺-dependent 45Ca²⁺ uptake without an appreciable change in the size of the hypertrophic myocytes (Fig. 5). On the other hand, when infected at 24 h after the start of the 72-h PE or FCS treatment, dominant negative CnA nearly prevented the occurrence of the PE- and FCS-induced uptake inhibition and severely depressed myocyte enlargement, whereas it exerted no effects on controls not pretreated with growth factors (Fig. 5 and data not shown). Under similar conditions, activated CnA significantly increased the size of control myocytes as reported previously (28, 29) and reduced the uptake rate in these cells by ~40% (Fig. 5). Furthermore, activated CnA promoted the FCS-induced uptake inhibition, although it exerted little additional effect on PE-treated myocytes. Thus, a significant portion of NCX inhibition occurring in PE- or FCS-pretreated myocytes is due to the enzymic activity of calcineurin.

We next examined the effects of protein kinase modulators on the rate of Na⁺-dependent Ca²⁺ uptake in chronically PE-treated myocytes. Incubation of myocytes with 0.3 μM PMA during the last 30 min of a 72-h PE treatment caused little effect on the uptake rate (Fig. 6), whereas it produced a 20–30% increase in non PE-pretreated controls (data not shown), consistent with a previous report (9). In contrast, incubation with PMA during the final 24 h of a 72-h PE treatment caused an increase in the uptake rate similar to that seen at 1 μM FK506. The latter PMA effect (PKC down-regulation) was mimicked by a 30-min treatments with PKC inhibitors; the uptake rate in the presence of 0.3 μM calphostin C (Fig. 6), 50
nM GF109203X, or 1 μM chelerythrine increased by 327 ± 4 (n = 3), 300 ± 6 (n = 3), and 296 ± 5% (n = 3), respectively, as compared with that of PE-treated myocytes. The effects of FK506 and 24-h PMA treatment or calphostin C were additive, suggesting that the enzymic activities of calcineurin and PKC contributed independently to the observed reduction of uptake activity in PE-treated myocytes. Under similar conditions, however, the protein kinase A activator 8-bromo-cAMP (100 μM) and the protein kinase A inhibitors Rp-8-CPT-cAMPS (100 μM) and H89 (50 μM), the protein kinase G activator 8-bromo-cGMP (100 μM), the protein kinase G activator inhibitor Rp-8-CPT-cGMPS (100 μM), and the calmodulin-dependent protein kinase II inhibitors KN62 (25 μM) and KN93 (25 μM) did not influence the uptake rate (Fig. 6 and data not shown).

Finally, using CCL39 cells expressing NCX1 variants, we examined the possible role of the central cytoplasmic loop of the exchanger in the reversal of NCX inhibition by the inhibitors of calcineurin and PKC. In cells expressing wild-type NCX1 infected with activated CnA for 48 h, the rate of Na⁺,Ca²⁺ exchange was 40% lower compared with that for control cells in which expression of activated CnA had been suppressed with DOX (Fig. 7a). Importantly, such uptake reduction was reversed by FK506 but significantly promoted by PMA. Thus, uptake inhibitions by calcineurin and PKC were additive in CCL39 cells as in cardiomyocytes. However, in cells expressing an NCX1 mutant lacking most of its central loop (NCX1246–672), the uptake rate was not affected by either FK506 or PMA (Fig. 7a), indicating that the central loop is required for the effects of calcineurin and PKC.

We then used CCL39 cells not infected with activated CnA to examine the possible interaction of endogenous calcineurin with the NCX1 repeat deletion mutant (NCX1407–478). In one series, thapsigargin was added to cells 30 min before the uptake measurement to induce a low but sustained [Ca²⁺]i increase by reducing the Ca²⁺-buffering capacity of the endoplasmic reticulum and, thus, activate endogenous calcineurin (Fig. 7b), whereas it was not added in another series (Fig. 7c). The rate of Na⁺,Ca²⁺ exchange was 50% lower in thapsigargin-treated and non-treated cells, respectively, compared with those in the absence of FK506. In contrast, FK506 produced little effect on cells expressing either NCX1246–478...
Calcineurin-induced Inhibition of Cardiac NCX1

REGULATION OF CARDIAC NCX ACTIVITY BY CALCINEURIN AND PKC—Recent studies have demonstrated that of the three CnA isoforms, CnAα and CnAβ are expressed in cardiomyocytes, with the latter playing a pivotal role in the induction of cardiac hypertrophy (28, 30). Here, we have provided evidence that the carboxyl tail of CnAβ containing an autoinhibitory domain binds to the β1 repeat of NCX1, one of two internal repeat motifs conserved in the central cytoplasmic loop of NCX family members (31). The β1 repeat constitutes part of the putative Ca2+ regulatory site that is responsible for the allosteric regulation of NCX activity by intracellular Ca2+ (see review, Ref. 3). Hence, CnAβ binds to a critically important portion of the exchanger. This inhibitory effect of PE, together with the acute action of endogenous calcineurin. We speculate that the large cytoplasmic loop of NCX1 is required for the effects of calcineurin and PKC. Recent studies (29, 32) have revealed that hypertrophic calcineurin signaling is closely interconnected with activations of PKCα and θ and that PKCα is a necessary mediator of the PE-induced hypertrophy of isolated rat and mouse cardiomyocytes.

We observed that the β1 repeat deletion from NCX1 expressed in CCL39 cells abolished the effect of endogenous calcineurin, whereas the deletion of the large cytoplasmic loop of NCX1 is required for the effects of calcineurin and PKC. Importantly, this same finding strongly argues against the view that the observed NCX inhibition arose secondarily from changes in cellular conditions, such as altered ion distribution across the plasma membrane. We thus consider that PE alters the functional state of the exchanger causing NCX inhibition. This inhibitory effect of PE, together with the acute NCX stimulation by PE or other agonists of G protein-coupled receptors reported earlier (8–10), suggests that there exists a complex regulatory mechanism(s) for cardiac NCX1.

We used neonatal rat cardiomyocytes treated with 10 μM PE or 10% FCS for 72 h as an in vitro hypertrophic model (see Fig. 3a). The PE-treated myocytes exhibited typical hypertrophic responses characterized by increased cell size, enhanced sarcoplasmic organization, and increased ANP expression, although FCS treatment induced less prominent responses (Fig. 3b). Importantly, the NCX activity in these hypertrophic myocytes, which was measured as the rate of Na+,Ca2+-dependent 45Ca2+ uptake or the rate of Na+,Ca2+-dependent Ca2+ influx, was markedly decreased relative to those in non-treated controls (Figs. 3d and 4b). Such depressed activity was partially reversed by FK506, cyclosporin A, or infection with dominant negative CnA (Figs. 4–6 and “Results”). On the other hand, activated CnA caused a significant decrease in NCX activity in non-treated controls or FCS-treated myocytes, although it had little additional effect on PE-treated cells (Fig. 5). Dominant negative CnA nearly prevented the occurrence of both NCX inhibition and cell hypertrophy in PE- or FCS-treated myocytes (Fig. 5 and “Results”). Therefore, we suggest that calcineurin activity is elevated in PE-treated hypertrophic myocytes as reported previously (28) and that this activity causes NCX inhibition.

PKC inhibitors also caused a partial reversal of depressed NCX activity in PE-treated myocytes (Figs. 6 and “Results”). The effects of the inhibitors of calcineurin and PKC were additive, suggesting that the actions of these enzymes are mutually independent. Importantly, FK506 and calphostin C acted acutely with little influence on myocyte hypertrophic phenotypes. Hence, it is likely that these enzyme actions occur via different mechanisms involving distinct substrate proteins. Of note, however, the PKC-dependent NCX inhibition requires prior activation of calcineurin, because prior infection with dominant negative CnA nearly abolished the PE-induced NCX inhibition (Fig. 5) and because the PKC-dependent NCX inhibition occurred only after the infection of CCL39 cells with activated CnA (Fig. 7a). Recent studies (29, 32) have revealed that hypertrophic calcineurin signaling is closely interconnected with activations of PKCα and θ and that PKCα is a necessary mediator of the PE-induced hypertrophy of isolated rat and mouse cardiomyocytes.

The NCX inhibition by calcineurin and PKC seen in PE-treated cardiomyocytes was reproduced in CCL39 cells expressing cloned wild-type NCX1, but not in those expressing NCX1Δ246–672 (Fig. 7a), suggesting that the central cytoplasmic loop of NCX1 is required for the effects of calcineurin and PKC. Importantly, this same finding strongly argues against the view that the observed NCX inhibition arose secondarily from changes in cellular conditions, such as altered ion distribution across the plasma membrane. We thus consider that PE alters the functional state of the exchanger causing NCX inhibition. This inhibitory effect of PE, together with the acute NCX stimulation by PE or other agonists of G protein-coupled receptors reported earlier (8–10), suggests that there exists a complex regulatory mechanism(s) for cardiac NCX1.
recombinant activated CnA overexpressed in myocytes, although this may not happen when the large cytoplasmic loop is deleted. It is noteworthy, however, that activated CnA, lacking its C-terminal tail and the ability to bind to the NCX1 repeat, was able to regulate NCX activity (Fig. 5). Thus, in view of the limitations inherent in these mutation studies, we cannot rule out the possibility that calcineurin may inhibit NCX activity without binding to NCX1. Further studies are required to establish the causal relationship between calcineurin binding and NCX inhibition.

Pathological Relevance of Depressed NCX Function—Myocardial hypertrophy occurs in response to a variety of stimuli, including the chronic activation of Goα class of G-proteins that leads to the activation of calcineurin, PKC, and mitogen-activated protein kinases (15). On the other hand, calcineurin and its primary downstream effector, the nuclear factor of activated T cells (NFAT), have been shown to be important mediators of in vitro and in vivo cardiac hypertrophic responses (see reviews, Refs. 15 and 16). The exact mechanism by which calcineurin promotes pathological hypertrophic responses is currently unknown, as few targets for calcineurin other than the NFATs that contribute to the development of cardiac hypertrophy have been identified. This study has provided evidence suggesting that NCX1 may be one of such targets for calcineurin. The results shown here are in good agreement with the recent report by Wang et al. (33) showing that NCX current density was significantly reduced in hypertrophic cardiomyocytes isolated from aortic banded mice, whereas this did not occur in mice receiving daily cyclosporin A injection during aortic constriction. Because depressed NCX activity is likely to cause the chronic elevation of [Ca2+]i in cardiomyocytes, depressed NCX activity might play an important role in the development of myocardial hypertrophy and the subsequent contractile dysfunction. However, the pathological significance of depressed NCX activity needs to be established in future studies, as enhanced NCX1 expression and function have also

**FIG. 5.** Effect of activated or dominant negative CnA on Na+-dependent 45Ca2+ uptake in cardiomyocytes pretreated with PE or FCS. Myocytes were treated with no growth factor (−PE), 10 μM PE (+PE), or 10% FCS (+FCS) for 72 h. In one series (bar on the extreme right for +PE), PE-treated myocytes were subsequently infected with dominant negative can, and the rate of Na+-dependent 45Ca2+ uptake was measured 24 h later. In all other series, myocytes were infected at 24 h after the start of growth factor treatment either with activated CnA in presence (+DOX/AdCnA) or absence (+AdCnA) of DOX or with dominant negative CnA (+AdD.N.CnA), and uptake rates were measured 48 h later. The uptake rate in the −PE+DOX/AdCnA series was taken as 100%. Data are averages ± S.D. (n = 3).

**FIG. 6.** Effects of FK506, protein kinase modulators and their combinations on Na+-dependent 45Ca2+ uptake into PE-treated cardiomyocytes. Myocytes were incubated with the indicated agents during the last 30 min of a 72-h treatment with 0 or 10 μM PE, and then the rates of Na+-dependent 45Ca2+ uptake were measured. In some series, myocytes were incubated with 0.3 μM PMA during the last 24 h of PE treatment (PMA(24h)). Cal.C, calphostin C. Data are averages ± S.D. (n = 3).
been reported for cardiomyocytes isolated from some animal models of cardiac hypertrophy and heart failure (6, 7).

REFERENCES

1. Bridge, J. H. B., Smolley, J. R., and Spitzer, K. W. (1990) Science 248, 376–378.
2. Bers, D. M. (2000) Circ. Res. 87, 275–281.
3. Shigekawa, M., and Iwamoto, T. (2001) Circ. Res. 88, 864–876.
4. Kent, R. L., Rozich, J. D., McCollam, P. L., McDemott, D. E., Thacker, U. F., Menick, D. R., McDermott, P. J., and Cooper, G., IV (1993) Am. J. Physiol. 265, H1024–H1029.
5. Studer, R., Reinecke, H., Biger, J., Eschenhagen, T., Böhml, M., Hasenfuss, G., Just, H., Holtz, J., and Drexler, H. (1994) Circ. Res. 75, 443–453.
6. Ahmmed, G. U., Dong, P. E., Song, G., Ball, N. A., Xu, Y., Walsh, R. A., and Chiamvimonvat, N. (2000) Circ. Res. 86, 558–570.
7. O’Rourke, B., Kass, D. A., Tomaselli, G. F., Ka¨a¨b, S., Tunin, R., and Marba´n, E. (1999) Circ. Res. 6, 558–570.
8. Iwamoto, T., Pan, Y., Wakabayashi, Y., Imagawa, T., Yamanaka, H. I., and Shigekawa, M. (1999) J. Biol. Chem. 274, 13609–13615.
9. Iwamoto, T., Pan Y., Nakamura, T. Y., Wakabayashi, S., and Shigekawa, M. (1998) Biochemistry 37, 17230–17238.
10. Stengl, M., Muhagawa, K., Carmeliet, E., and Flameng, W. (1998) Cardiovasc. Res. 38, 703–710.
11. Link, B., Qiu, Z., He, Z., Tong, Q., Hilgeman, D.W., and Philipson, K.D. (1998) Am. J. Physiol. 274, C415–C423.
12. Wei, S.-K., Ruknudin, A., Hanlon, S. U., McCurley, J. M., Schuler, D. H., and Haigrey, M. C. P. (2003) Circ. Res. 92, 897–903.
13. Condrescu, M., Hantash, B. M., Fang, Y., and Reeves, J. P. (1999) J. Biol. Chem. 274, 33279–33286.
14. Molken, T. D., Lu, J.-R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olsen, E. N. (1998) Cell 93, 215–228.
15. Molken, T. D., and Dorn, G. W., II (2001) Ann. Rev. Physiol. 63, 391–426.
16. Vega, R. B., Bussel-Duby, R., and Olson, E. N. (2003) J. Biol. Chem. 278, 36981–36984.
17. Simpson, P., McGrath, A., and Savion, S. (1982) Circ. Res. 51, 787–801.
18. Shibasuki, F., and MacKeon, F. (1995) J. Cell Biol. 131, 735–743.
19. Wang, H.-G., Pathan, N., Ethell, I. M., Yamaguchi, Y., Shibasuki, F., McKeon, F., Robo, T., Franko, T. P., and Reed, J. C. (1999) Science 284, 339–343.
20. Laemmli, U. K. (1970) Nature 227, 680–685.
21. Tawada-Iwata, Y., Imagawa, T., Yoshida, A., Takahashi, M., Nakamura, H., and Shigekawa, M. (1993) Am. J. Physiol. 264, H1447–H1453.
22. Pan, Y., Iwamoto, T., Uehara, A., Nakamura, T. Y., Imanaga, I., and Shigekawa, M. (2000) Am. J. Physiol. 279, C393–C402.
23. Furukawa, K., Tawada, Y., and Shigekawa, M. (1988) J. Biol. Chem. 263, 8058–8065.
24. Saito, S., Hiroi, Y., Zou, Y., Aikawa, R., Toko, H., Shibasuki, F., Yazaki, Y., Nagai, R., and Komuro, I. (2000) J. Biol. Chem. 275, 34528–34533.
25. Bajusz, E., Homburger, F., Raker, J. R., and Bogdonoff, P. (1989) Ann. N. Y. Acad. Sci. 156, 396–420.
26. Negro, V., Ozakaki, Y., Belato, A., Piluso, G., Matsuda, Y., Politano, L., Negro, G., Ventura, C., Abbondanza, C., Molinari, A. M., Acampora, D., Nishimura,
Calcineurin-induced Inhibition of Cardiac NCX1

27. Iwata, Y., Katanosaka, Y., Arai, Y., Komamura, K., Miyatake, K., and Shigekawa, M. (2003) J. Cell Biol. 161, 957–967
28. Taigen, T., De Windt, L. J., Lim, H. W., and Molkentin, J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1196–1201
29. De Windt, L. J., Lim, H. W., Haq, S., Force, T., and Molkentin, J. D. (2000) J. Biol. Chem. 275, 13571–13579
30. Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4586–4591
31. Schwarz, E. M., and Benzer, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10249–10254
32. Braz, J. C., Bueno, O. F., De Windt, L. J., and Molkentin, J. D. (2002) J. Cell Biol. 156, 905–919
33. Wang, Z., Nolan, B., Kutschke, W., and Hill, J. A. (2001) J. Biol. Chem. 276, 17706–17711
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