Journal of Cellular Biochemistry 90:987–997 (2003)

Activation of Calcineurin Expression in Ischemia-Reperfused Rat Heart and in Human Ischemic Myocardium

Ashakumary Lakshmikuttyamma,[1] Ponniah Selvakumar,[1] Rakesh Kakkar,[1] Rani Kanthan,[1] Rui Wang,[2] and Rajendra K. Sharma[1]*

[1]Department of Pathology, College of Medicine and Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, University of Saskatchewan, Saskatoon, Canada S7N 4H4
[2]Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Canada S7N 5E4

Abstract Calcineurin (CaN) has been reported as a critical mediator for cardiac hypertrophy and cardiac myocyte apoptosis. In the present study, we investigated the activity and expression of CaN and the effect of calpain in rat heart after ischemia and reperfusion. Rat ischemic heart showed significant increase in CaN activity. Western blot analysis of normal rat heart extract with a polyclonal antibody raised against bovine CaN indicated a prominent immunoreactive band of 60 kDa (CaN A). In ischemic-reperfused hearts, the expression of CaN A was significantly low and immunoreactivity was observed in proteolytic bands of 46 kDa. This may be due to the proteolytic degradation of CaN A in ischemic tissues by *m*-calpain. We also noticed in vitro proteolysis of bovine cardiac CaN A by *m*-calpain. Immunohistochemical studies showed strong staining of immunoreactivity in rat hearts that had gone under 30 min ischemia followed by 30 min reperfusion similar to that found in human ischemic heart. Ischemia is associated with multiple alterations in the extracellular and intracellular signaling of cardiomyocytes and may act as an inducer of apoptosis. The increase in CaN activity and strong immunostaining observed in ischemic/perfused rat heart may be due to the calpain-mediated proteolysis of this phosphatase. J. Cell. Biochem. 90: 987–997, 2003.

Key words: calcineurin; ischemic heart; cardiac hypertrophy; calpain

Calcineurin (CaN) is known as calmodulin (CaM) stimulated phosphatase [Stewart et al., 1982] and protein phosphatase 2B [Ingebritsen et al., 1983], which play a critical role in the coupling of Ca^{2+} signals to cellular responses [Klee et al., 1998; Rusnak and Mertz, 2000]. CaN has been characterized in numerous tissues including brain, heart, kidney, liver, muscle, eye, and T-lymphocytes [Clipstone and Crabtree, 1992; Liu et al., 1992; Jiang et al., 1997; Klee et al., 1998; Rusnak and Mertz, 2000; Seitz et al., 2002]. CaN is a heterodimer consisting of 19 and 57–59 kDa subunits referred to as CaN B and CaN A, respectively [Klee and Krinks, 1978; Sharma et al., 1979]. Three mammalian CaN A genes have been identified (α, β, γ) that share 81% sequence homology across a 350 amino acid stretch that constitutes the catalytic domain. The CaN Aα and Aβ gene products are expressed in a relatively ubiquitous and overlapping pattern throughout the body. CaN Aγ is expressed in a more restricted pattern that includes the testis [Takaishi et al., 1991; Muramatsu et al., 1992; Buttini et al., 1995]. Both CaN Aα and Aβ proteins, are present in cardiomyocytes while CaN Aγ is not [Taigen et al., 2000]. The most clinically significant feature of CaN is its inhibition by the immunosupressant drug cyclosporin A (CsA)-cyclophilin and tarcolimus A. The role of CaN in T cell activation was discovered when

Grant sponsor: The Heart and Stroke foundation of Saskatchewan, Canada.

*Correspondence to: Dr. Rajendra K. Sharma, Department of Pathology, College of Medicine and Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, University of Saskatchewan, Saskatoon, Canada S7N 4H4. E-mail: rsharma@scf.sk.ca

Received 8 September 2003; Accepted 9 September 2003
DOI 10.1002/jcb.10722

these immunosuppressive compounds were found to inhibit Ca^{2+} dependent activation of T cells [Crabtree and Clipstone, 1994].

Recently CaN has attracted great attention as a critical mediator for cardiac hypertrophy [Molkentin, 2000; Wilkins and Molkentin, 2002]. In response to pressure-overload hypertrophy, CaN activity was increased 3.2 fold and the immunosuppressive agent CsA was effective in blocking this process [Lim et al., 2000a]. Endothelin-1 (ET-1) stimulated hypertrophy of cultured cardiomyocytes induced a three fold increase in CaN activity [Zhu et al., 2000]. Pressure overload hypertrophy in aortic-banded rats and exercise-induced cardiac hypertrophy in the rat were each associated with increased CaN activity in the heart [Eto et al., 2000]. These reports demonstrated that CaN activity was upregulated in cardiac hypertrophic conditions. In contrast, no change [Zhang et al., 1999] or a decrease [Ding et al., 1999] in CaN activity has been reported in response to pressure overload hypertrophy. Roles for CaN and nuclear factor of activated T cells (NFAT) as regulators of cardiac hypertrophy were recently identified [Molkentin et al., 1998]. CaN was subsequently shown to operate through NFAT3 in the heart with transgenic mice constitutively expressing a nuclear mutant of NFAT3 also demonstrated in cardiac hypertrophy. The specificity of the transgenes was demonstrated by the observation that CsA inhibited cardiac-hypertrophy in CaN-transgenic mice, but not in NFAT3 transgenic mice [Molkentin et al., 1998; Lim et al., 2000b]. Many contradictory reports are available related to CaN inhibitors and pressure overload hypertrophy. Some studies concluded that CaN inhibitors had no effect in blocking cardiac hypertrophy [Luo et al., 1998; Müller et al., 1998; Ding et al., 1999; Zhang et al., 1999]. Other studies reached the conclusion that CaN inhibitors are effective agents for preventing cardiac hypertrophy [Meguro et al., 1999; Shimoyama et al., 1999; Hill et al., 2000; Lim et al., 2000a]. The reasons behind these divergent conclusions are uncertain.

Apoptotic cell death plays a critical role in a variety of cardiovascular diseases including myocardial infarction, heart failure, and atherosclerosis. An induction of apoptotic cell death was observed in myocardial infarction and in ischemic heart disease. CaN is a potential regulator of stress responses and apoptosis in cardiac myocyte [Molkentin, 2001]. Kakita et al. [2001] identified an anti-apoptotic effect associated with CaN activation in cardiomyocytes and suggested that CaN activation normally antagonized cardiomyocyte apoptosis in response to H_2O_2 injury. This report was supported by the finding of De Windt et al. [2000a] that transgenic mice expressing an activated form of CaN in the heart were largely protected from ischemia-reperfusion induced DNA laddering. In addition, CsA reversed the protective effects of α-adrenergic stimulation in the presence of 2-deoxyglucose treatment in vitro. In contrast to these reports, Saito et al. [2000] found that isoproterenol stimulation of cardiac β-adrenergic receptors promoted myocyte apoptosis by stimulating CaN activity. Recently, we observed that brain CaN is proteolysed by *m*-calpain and, consequently, its phosphatase activity increased nearly 50% (unpublished data). Calpains are Ca^{2+} dependent cysteine protease, which is one of the major mediators of Ca^{2+} signals in many biological systems [Croall and DeMartino, 1991; Sorimachi et al., 1994]. The involvement of calpains has been indicated in myocardial ischemia/reperfusion injury, myocardial stunning, and cardiac hypertrophy. Ischemia is associated with multiple alterations in the extracellular and intracellular milieu of cardiomyocytes that may act as inducers of apoptosis [Haunstetter and Izumo, 1998]. The role of CaN in cardiac hypertrophy and apoptosis in cardiomyocyte is unclear. More knowledge on the cellular signaling network of CaN is needed to elucidate its exact role in cardiomyocyte apoptosis.

In the present work, we examined CaN activity and its protein expression in ischemia-reperfusion damaged rat heart. In addition, the protective effect of cell permeable calpain inhibitor (ALLM, N-Ac-Leu-Leu-methoninal) was assessed during reperfusion myocardial injury. Its effect on CaN was studied to correlate the expression of CaN in ischemia-reperfusion by calpain mediated signaling mechanisms.

MATERIALS AND METHODS

Materials

Bovine heart CaN and bovine brain CaM were purified as described [Sharma et al., 1983, 1984]. Ca^{2+} activated neutral protease (*m*-calpain) from bovine cardiac muscle was purified by the method of Hara et al. [1983]. Nitrocellulose membranes were obtained from BioRad Labora-

tories Ltd. (Mississuaga, Canada). Erythrocyte μ-calpain cell permeable calpain inhibitor ALLM was purchased from Calbiochem (San Diego, CA). Anti-CaN was produced in New Zealand white rabbits as described previously and was demonstrated to be specific for CaN [Seitz et al., 2002]. General laboratory reagents were obtained from Sigma Chemical Co. (Toronto, Canada) or BDH Inc. (Toronto, Canada) and were of analytical grade.

Methods

All procedures for animal experimentation were undertaken according to the guidelines of the Canadian Council for Animal Care. Male Sprague Dawley rats (250–275 g) were anaesthetized with sodium somnitol (0.1 ml/100 g body weight) and heperinized (500 IU) intravenously. The hearts were rapidly excised, washed in ice-cold oxygenated Krebs–Henseleít (KH) buffer solution (pH 7.4); containing (in mM), NaCl 118, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 0.86, KH_2PO_4 1.2, $NaHCO_3$ 2.54, glucose 11.1, EDTA 0.027, and L-ascorbic acid 0.057. The hearts were then mounted on a Langendorff heart perfusion apparatus and perfused at a constant pressure of 10 kPa (100 cm H_2O) at 37°C with KH buffer. The KH buffer was filtered through a 0.8 micron cellulose acetate membrane (to remove particulate contaminants, which otherwise block coronary circulation) and was continuously gassed with a mixture of 95% O_2 and 5% CO_2. Left ventricular pressure (LVP) was monitored by a saline-filled latex balloon inserted into the left ventricle by way of the left atrium and connected to a pressure transducer. Epicardial electrogram (EPI-ECG) was recorded with one electrode positioned to the aorta and another to the apex of the heart. The effluent of the perfusate was collected for the determination of coronary flow (CF). Data acquisition and analysis were done using a BioPac system (BioPac system, Inc., Golato) including TC 100 amplifiers, acknowledge software (3.01), universal modules, and a Macintosh computer.

Experimental Protocol

Regional ischemia was induced according to the procedure of Wang et al. [1999b] by occluding the left anterior descending artery with a ligature positioned around and at a point close to its origin. The ligature was first tied loosely over a piece of plastic tubing and sustained regional ischemia was achieved by tying the ligature tightly around the tube. A successful occlusion was confirmed by 40–50% reduction in LVP and CF, compared to pre-ischemic values. At the end of the ischemic period, reperfusion was achieved by cutting the ligature on the plastic tubing with a scalpel blade and rhythmic disturbances were monitored for the time indicated for the various groups. After an initial 15–20 min of equilibration, the cardiodynamic variables were continuously monitored during the ischemia and reperfusion protocol period. Five experimental groups were studied.

Group 1 (control group): hearts were perfused with KH buffer without interference of ischemia or reperfusion ($n = 5$).

Groups 2 and 3: hearts underwent ischemia for 5 or 30 min followed by 30 min reperfusion ($n = 4$ for each group).

Group 4: hearts were perfused with calpain inhibitor (100 μM dissolved in 0.1% DMSO) for 5 min before 15 min of ischemia and again during 15 min of reperfusion ($n = 5$).

Group 5: hearts were perfused with 0.1% DMSO alone as described in group 4 ($n = 4$).

After completion of the experiment for each heart, a small piece of left ventricle tissue sample was taken and stored in 10% buffered formalin and embedded in paraffin for immunohistochemical studies. The rest of the left ventricle tissue sample was used for biochemical studies and kept at −70°C until use.

Tissue Homogenization

The left ventricle tissue samples were homogenized in 100 mM Tris-HCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, and 0.2 mM phenyl methyl sulfonyl flouride, pH 7.4 with a polytron homegenizer four times each for 30 s at the maximum speed with cooling intervals of 15 s. The homogenates were centrifuged at 1,000*g* for 20 min. The supernatants were further centrifuged at 100,000*g* for 60 min the pellet was discarded and the supernatants were used for various biochemical studies.

Human Tissue Samples

Human heart samples were collected from the Royal University Hospital, Saskatoon, Saskatchewan at autopsy. To minimize the effect of autolysis on post-mortem tissues, we collected samples within 24 h after death, from bodies

kept at 4°C. The storage time for normal and ischemic patient's bodies was the same (within 24 h). The normal control myocardium was taken from patients who had died of non-cardiac causes. The ischemic group contained samples from cases of recent myocardial infarction of 24–48 h clinical duration (age 60 ± 5 years). Ischemic changes took place within 24–48 h according to morphology and histology. Old ischemic lesions were excluded from the study. The heart was cut open from the anterior and septal walls of the left ventricle according to the method described in autopsy procedures. Tissue specimens were a minimum of 2 cm in size and were selected from the left ventricle of both normal and severely affected areas. Normal samples were also taken from the same region of the left ventricle from control patients. The samples were fixed in 10% buffered formalin and embedded in paraffin wax for ultrastructural and immunohistochemical studies.

CaN Assay

CaN activity was assayed using *p*-nitrophenylphosphate (pNPP) as a substrate [Pallen and Wang, 1983]. The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 1 mM Ni^{2+}, 5 μg CaM, 3.4 mM pNPP, and CaN in total volume of 1.0 ml. Ca^{2+}-independent activity of CaN was determined under identical conditions except 1 mM Ni^{2+} was substituted for 5 mM EGTA. The reaction was incubated at 30°C for 30 min. The reaction was initiated by the addition of pNPP and terminated by the addition of 75 mM K_2HPO_4. The pNPP hydrolysis was quantified by the increase of absorbance at 405 nm. One unit of phosphatase activity was defined as the amount of dephosphorylation resulting in an optical density of 0.1 at 30°C after 30 min incubation

Western Blot Analysis

The protein expression level of CaN in different groups was determined by immunoblotting [Towbin et al., 1979]. Equal amounts of proteins from normal and ischemic heart supernatants (100,000*g*) of different groups were separated on 10% SDS–PAGE electroblotted into nitrocellulose membrane and probed with polyclonal antibodies raised against bovine brain CaN (1:1,000). The immunoreactive bands were visualized by chemiluminescence reagent and exposed to Kodak autoradiography film.

Immunohistochemistry

Immunohistochemistry studies were performed on human ischemic myocardium obtained from autopsy materials and also on rat ischemic samples from different groups. Approximately 5 μm thick sections of rat and human hearts were cut and subjected to the avidin–biotin complex method as described previously [Hsu et al., 1981]. CaN polyclonal antibody was used as the primary antibody at a dilution of 1:100.

In Vitro Proteolysis of Bovine Heart CaN

Purified bovine heart CaN (5 μg) was incubated with 3.5 μg of purified heart *m*-calpain in the presence of 1 mM Ca^{2+} for 30 min at 30°C. The reaction was stopped by the addition of sample buffer and subjected to SDS–PAGE followed by Western blotting using CaN antibody as described above.

Other Methods

Protein concentration was measured by the method of Bradford [1976] using bovine serum albumin as the standard.

RESULTS

To examine the role of CaN in ischemic and reperfused rat heart, CaN activity was determined in supernatant of left ventricle tissue. The experimental groups showed a slightly greater increase in CaN activity than the control group except in 5I, 30R (Fig. 1, lane 2). The increase in CaN activity varied between different groups. The enhancement of CaN activity was nearly 165% in 30I, 30R (Fig. 1, lane 3). In group 4 (5CI15I, 15RCI), the ALLM (CI) increased the Ca^{2+}-dependence of CaN activity, whereas Ca^{2+}-independent activity was increased ≅2 fold (Fig. 1, lane 4). The reason for the slightly increased activity in this group was not clear. It was interesting to note that rat hearts perfused with 0.1% DMSO alone (Fig. 1, lane 5) had similar effects on CaN activity. The polyclonal antibody against bovine brain CaN was shown to cross-react with the rat brain CaN (Fig. 2A). We examined levels of protein expression in control and ischemia-reperfusion heart tissue. Western blot analysis of ischemic-reperfusion heart indicated the formation of a 46 kDa immunoreactive fragment from 60 kDa polypeptide of CaN A in experimental groups compared to control (Fig. 2B, lanes 2, 3, 4, 5 vs. lane 1). This suggests that an antigenic epitope was present in 46 kDa

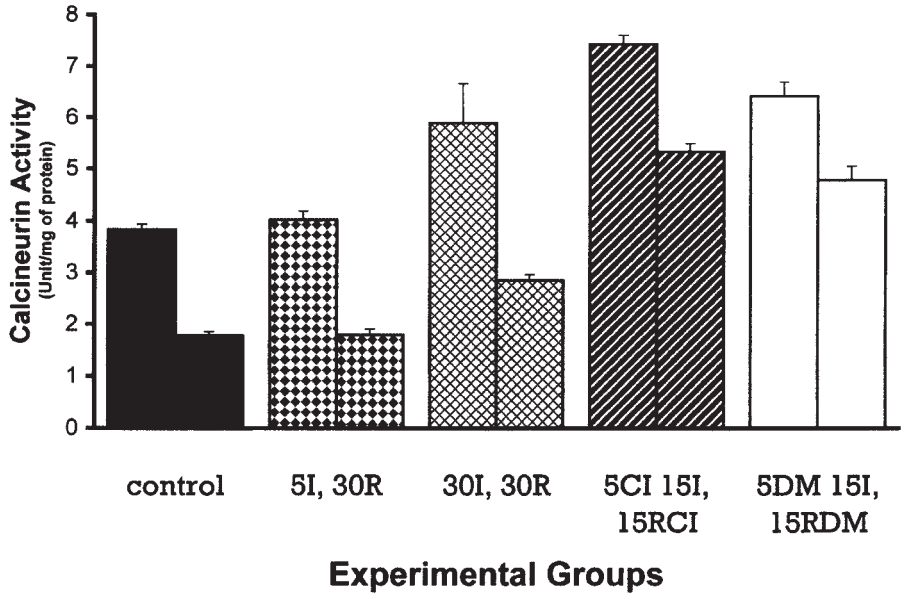

Fig. 1. CaN activity in experimental groups of rat hearts with different ischemia-reperfusion. CaN activity was determined using left ventricle tissue supernatant as described in Materials and Methods. CaN activity was assayed in the presence of (**a**) 5 μg CaM and 1 mM Ni^{2+} (**b**) and 5 mM EGTA. Five ischemia-reperfusion experimental groups were analyzed: ■ group 1, control (no ischemia or reperfusion); ▦ group 2, 5 min ischemia and 30 min reperfusion (5I, 30R); ▩ group 3, 30 min ischemia and 30 min reperfusion (30I, 30R); ▨ group 4, hearts were perfused with 100 μM calpain inhibitor in 0.1% DMSO for 5 min before 15 min of ischemia and again during 15 min reperfusion (5CI15I, 15RCI) (CI, calpain inhibitor (ALLM)) and □, group 5, with 0.1% DMSO alone as described in group 4 (5DM15I, 15RDM) (DM, dimethyl sulfoxide). Values are mean ± SD of four samples from each group.

fragment. Quantitative analysis of the 60 kDa band showed significant decrease in experimental groups compared to control (Fig. 2C). We have not observed any significant change in the expression of CaN B during the course of ischemia-reperfusion (data not shown). Immunohistochemical studies of normal rat cardiac CaN showed weak to moderate staining of myocardial fibers with CaN antibody (Fig. 3A). However, ischemic and reperfused left ventricle tissue (Fig. 3B; 30I, 30R) showed stronger staining with CaN antibody demonstrating higher expression of CaN in ischemia and reperfusion than control cardiac tissue (Fig. 3A). With calpain inhibitor treatment, CaN expression was not altered (Fig. 3C). In addition, DMSO alone (0.1%) showed a similar staining as in control (Fig. 3D vs. A). This increase in CaN expression in ischemic tissue was further confirmed in tissue specimens collected from cardiac patients. The polyclonal antibody raised against bovine CaN cross reacted with CaN from human cardiac tissue (data not shown). The normal human cardiac muscle showed weak staining of myocardial cells with CaN antibody (Fig. 3E), whereas strong staining was observed in ischemic tissue (Fig. 3F).

To study the involvement of calpain in the regulation of CaN activity in heart, purified heart CaN was incubated with *m*-calpain. Western blot analysis indicated that CaN A was degraded by *m*-calpain. In the presence of Ca^{2+} alone, a 46 kDa fragment was produced (Fig. 4, lane 6). However, in the presence of Ca^{2+}/CaM, 48 and 54 kDa fragments were observed (Fig. 4, lane 5). In contrast, CaN B was not affected by *m*-calpain. After proteolysis, CaN activity increased to 40% in both cases (data not shown).

DISCUSSION

The relationship between CaN activation and heart failure has been investigated by various investigators and different signaling pathways were established. CaN is necessary for the NFAT transcription factors which mediate changes in gene expression in response to Ca^{2+} signaling from the T-cell receptor. NFAT3, a member of the NFAT family, interacts with cardiac restricted Zinc finger protein GATA4 [Molkentin et al., 1998]. CaN–GATA4 plays a major role in the stimulation of ET-1 transcription in cardiac myocytes. Upregulated expression of ET-1 in cardiac myocytes has a

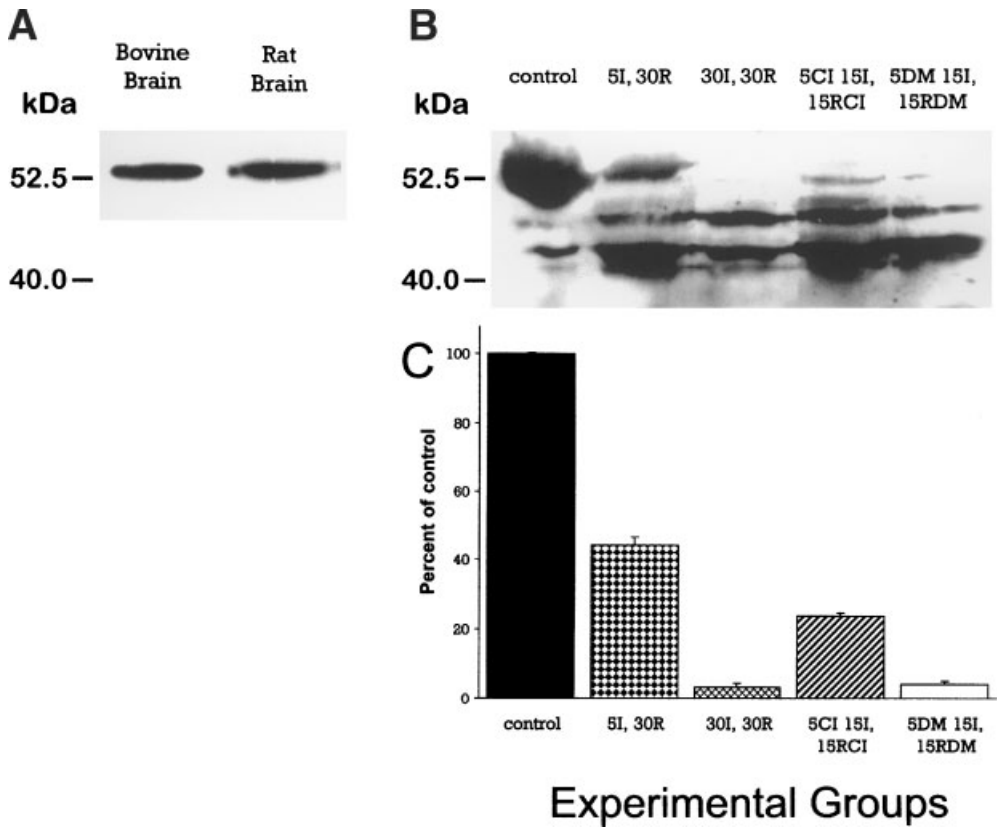

Fig. 2. **A**: Immunological similarity of bovine brain CaN and bovine heart CaN. Purified bovine brain CaN (10 μg , **lane 1**) and normal rat brain supernatant (10 μg, **lane 2**) were subjected to SDS–PAGE, transferred to nitrocellulose membrane, and probed with bovine brain CaN polyclonal antibody as described in Materials and Methods. **B**: Immunoblot of CaN from normal and ischemia-reperfusion injured rat heart tissues. Fifty micrograms of protein was loaded on to each lane, subjected to SDS–PAGE, transferred to nitrocellulose membrane, and blotted with polyclonal antibody of CaN. **Lane 1**: group 1 (control); **lane 2**, group 2 (5I, 30R); **lane 3**, group 3 (30I, 30R); **lane 4**, group 4 (5CI15I, 15RCI); **lane 5**, group 5 (5DM15I, 15 RDM). **C**: Quantitative analysis of 60 kDa band of respective Western blots was carried out using imaging software; (NIH at http://rbs.info.nih.gov/nih-image/down-load.html). The data were expressed as the mean ± SD of three samples in each group.

critical role in the development of heart failure [Morimoto et al., 2001]. Several lines of evidence have suggested the existence of a cross-talk between CaN and mitogen activated protein kinase pathway (MAPK) [De Windt et al., 2000b]. CaN has been reported to promote the activity of c-jun NH_2 terminal kinase (JNK), a member of MAPK family [De Windt et al., 2000b]. JNK activation has also been associated with myocardial infarction and human heart failure [Li et al., 1998; Cook et al., 1999]. Extracellular signal regulated kinase (ERKs) have been involved in hypertrophic responses of cardiomyocytes both in vitro [Glennon et al., 1996] and in vivo [Izumi et al., 1998]. Isoproterenol-induced activation of ERKs was significantly suppressed by CaN inhibitors in cultured cardiomyocytes as well as in the heart of mice [Zou et al., 2001].

Under normal physiological conditions, Ca^{2+} has several important functions in the heart, including driving the contraction of heart muscle. Our understanding of the relationship between changes in intracellular Ca^{2+} levels and its role in heart disease and cell death is still limited. In our present study, we observed conflicting results on CaN activity and its protein expression. In comparison to control, heart CaN activity significantly increased in ischemic-reperfused tissues, whereas the protein expression of CaN A significantly decreased. These contradictory results may be due to the calpain mediated mechanism. It has been observed that in vitro proteolytic cleavage of CaN by *m*-calpain resulted in degradation of CaN A and a simultaneous increase in CaN activity. The weak expression of CaN A in ischemic tissue was due to its proteolysis of

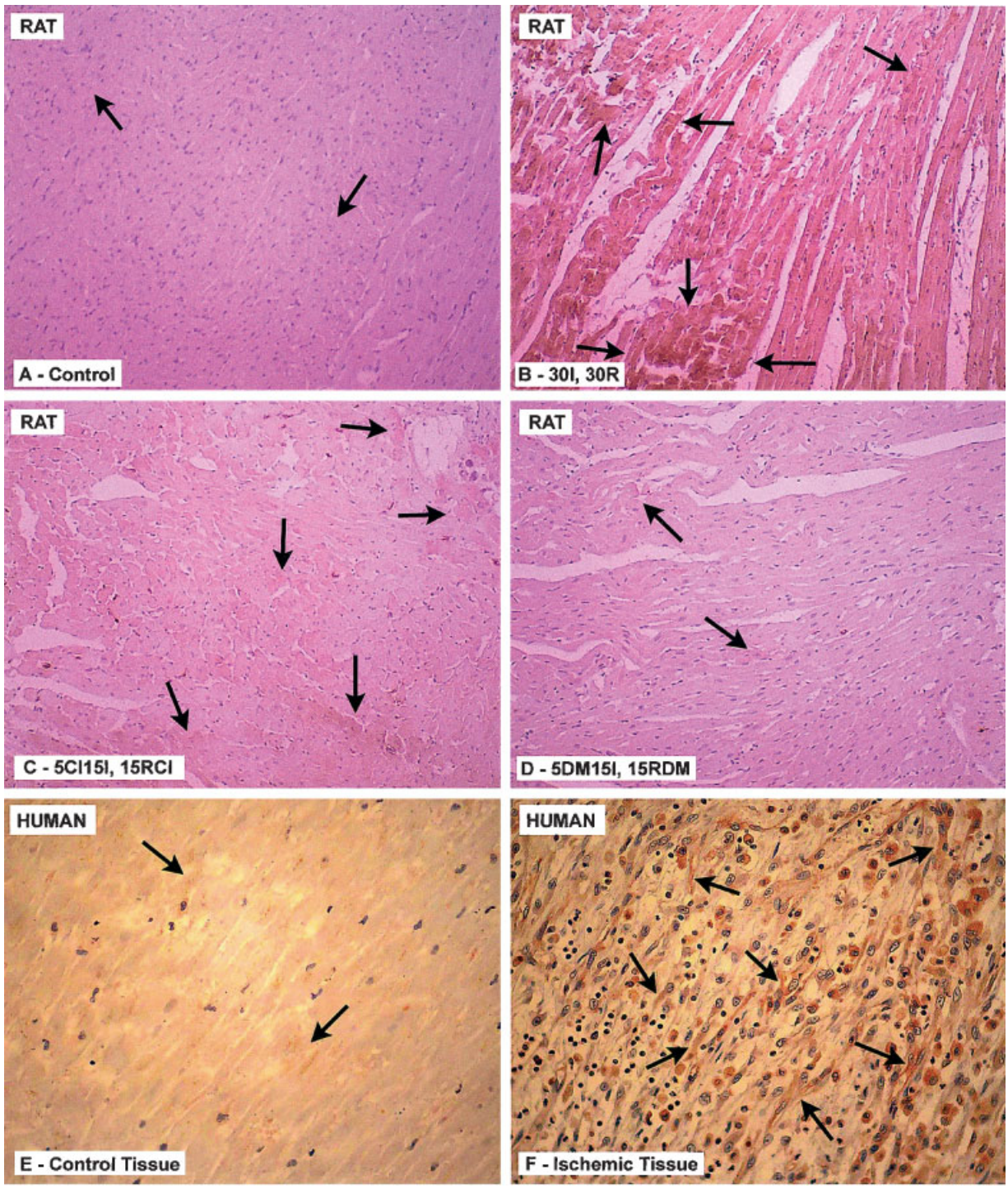

Fig. 3. Immunohistochemical localization of CaN in ischemia-reperfused rat cardiac and human cardiac muscle. Cardiac muscle sections from control rat (**A**) and ischemic/reperfused rat (B-30I, 30R; C-5CI15I, 15RCI; D-5DM15I, 15RDM); human control cardiac tissue (**E**) and human ischemic cardiac tissue (**F**) hearts were probed with CaN polyclonal antibody (magnification ×20). Arrows indicate CaN staining.

m-calpain, which was highly expressed in ischemia-reperfusion. This was confirmed by the presence of immunoreactive proteolytic fragments in experimental tissues.

During ischemia and reperfusion, there is increased influx of Ca^{2+} into the cells, which can activate Ca^{2+} dependent neutral proteases, calpains [Iizuka et al., 1993; Yoshida et al.,

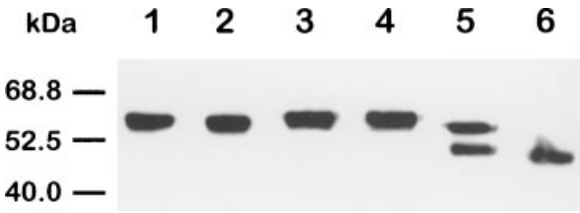

Fig. 4. In vitro proteolysis of purified bovine heart CaN by *m*-calpain. Five micrograms of purified bovine heart CaN was incubated with 3.5 μg of *m*-calpain either in presence of Ca^{2+}/CaM or Ca^{2+} alone. **Lane 1**: CaN and Ca^{2+} alone; **lane 2**, CaN and *m*-calpain alone; **lane 3**, CaN, *m*-calpain and 1 mM EGTA; **lane 4**, CaN, *m*-calpain, 1 mM Ca^{2+} and 1.5 μg of HMWCaMBP (calpain inhibitor); **lane 5**, CaN, *m*-calpain, 1 mM Ca^{2+} and 4 μg CaM; **lane 6**, CaN, *m*-calpain and 1 mM Ca^{2+}. The reaction was stopped with SDS sample buffer and immunoblotting carried out as described under Materials and Methods by using antibody directed against CaN.

1995]. Previous studies from our laboratory suggested that the expressions of μ-calpain and *m*-calpain were increased in ischemia-reperfusion. Simultaneously, the expression of a calpain inhibitor, a high molecular weight CaM binding protein (HMWCaMBP), was significantly decreased [Kakkar et al., 2001]. Calpastatin, another inhibitor of calpain, was also down regulated after ischemia-reperfusion [Sorimachi et al., 1997]. These inhibitors may play important roles in the action of calpain on its substrates in normal myocardium, but are proteolysed during ischemia-reperfusion, leaving calpain free to act on its substrates. The involvement of the caspase family of proteases in apoptosis has been clearly demonstrated in various systems. It has also been reported that calpastatin is fragmented by caspases during apoptosis of neuronal cells, jurkat-T lymphocytes, and monocytic leukemic cells [Wang et al., 1998]. Moreover, calpain has been linked to the activation of caspase-12 in apoptosis [Nakagawa and Yuan, 2000]. Induction of FasL and TNF-α critically depends on caspase activation [Longthorne and Williams, 1997].

Ischemia is associated with multiple alterations in the extracellular and intracellular milieu of cardiomyocytes that may act as inducers of apoptosis [Gill et al., 2002]. In patients with ischemic cardiomyopathy, 5–35% of cardiomyocytes are affected by apoptosis [Haunstetter and Izumo, 1998]. The mechanism by which CaN is involved in apoptosis may be due to the dephosphorylation of Bad, which then translocates to the mitochondria. In mitochondria, it forms a complex with Bcl-2 and induces cytochrome c release [Wang et al., 1999a]. This was supported by the report of Saito et al. [2000] showing that isoproterenol significantly induced the release of cytochrome c from mitochondria to the cytosol, and this isoproterenol induced release was reduced by pretreatment with CaN inhibitors.

Fas, also named Apo-1 or CD 95, a member of the tumor necrosis factor (TNF) receptor family of proteins, is markedly upregulated in cardiomyocytes during ischemia and hypoxia, and cardiomyocytes may thus become susceptible to apoptotic cell death by interaction with FasL [Haunstetter and Izumo, 1998]. The implication of CaN in Fas ligand induction was demonstrated by the partial inhibition of Fas ligand induction with cyclosporin A [Anel et al., 1994]. Another mechanism suggesting the involvement of CaN in FasL expression is through Bcl-2. Bcl-2 binds to CaN and inhibits the dephosphorylation of NFAT. Thus, NFAT is unable to translocate to the nucleus and FasL transcription does not occur [Srivastava et al., 1999]. Earlier reports from our laboratory suggested that Bcl-2 expression did not change in ischemia-reperfusion [Kakkar et al., 2001]. However, Bcl-2 down regulation was observed in the acute ischemia and prolonged reperfusion [Maulik et al., 1999]. More studies will be needed to elucidate the role of CaN and Fas expression in ischemia and apoptosis.

In conclusion, CaN activation plays a supporting role in inducing cardiomyocyte apoptosis in ischemia-reperfusion in rats. The increase in CaN activity and expression observed in ischemic and reperfused rat heart may be due to the calpain mediated proteolysis of CaM-stimulated phosphatase. Our present work in the rat model may shed light on signaling of CaN in cardiomyocyte apoptosis with the involvement of Ca^{2+} regulated protease. Whether similar and relevant results can be observed in vivo in the whole animal heart may prove to be an interesting and challenging issue. Therefore, further studies are needed to confirm our observations in the whole animal heart. This definitely merits further in-depth studies.

ACKNOWLEDGMENTS

Ponniah Selvakumar is a recipient of a Post Doctoral Fellowship from the Dean's Committee on Research, College of Medicine, University

of Saskatchewan, Saskatoon, Saskatchewan, Canada. Dr. R. Kakkar was a recipient of a Research Fellowship from the Health Services Utilization and Research Commission of Saskatchewan. We wish to express our appreciation to Dr. Svein A. Carlsen, Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, for the reading of this manuscript. The authors are thankful to Mr. Todd Reichert for the photographic work.

REFERENCES

Anel A, Buferne M, Boyer C, Schmitt-Verhulst AM, Golstein P. 1994. T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by tyrosine kinase inhibitors and cyclosporine A. Eur J Immunol 24:2469–2476.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 248–254.

Buttini M, Limonta S, Luyten M, Boddeke H. 1995. Distribution of calcineurin A isoenzyme mRNAs in rat thymus and kidney. Histochem J 27:291–299.

Clipstone NA, Crabtree GR. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 357:695–697.

CooK SA, Sugden PH, Clerk A. 1999. Activation of c-Jun N-terminal kinases and p38-mitogen-activated protein kinases in human heart failure secondary to ischemic heart disease. J Mol Cell Cardiol 31:1429–1434.

Crabtree GR, Clipstone NA. 1994. Signal transmission between the plasma membrane and nucleus of lymphocytes. Ann Rev Biochem 63:1045–1083.

Croall DE, DeMartino GN. 1991. Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. Physiol Rev 71:813–847.

De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW II, Kitsis RN, Molkentin JD. 2000a. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure. Circ Res 86:255–263.

De Windt LJ, Lim HW, Haq S, Force T, Molkentin JD. 2000b. Calcineurin promotes protein kinase C and c-jun NH2-terminal kinase activation in the heart: Cross talk between cardiac hypertrophic signaling pathways. J Biol Chem 275:13571–13579.

Ding B, Price RL, Borg TK, Weinberg EO, Halloran PF, Lorell BH. 1999. Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin. Circ Res 84:729–734.

Eto Y, Yonekura K, Sonoda M, Arai N, Sata M, Sugiura S, Takenaka K, Gualberto A, Hixon ML, Wagner MW, Aoyagi T. 2000. Calcineurin is activated in rat hearts with physiological left ventricular hypertrophy induced by voluntary exercise training. Circulation 101:2134–2137.

Gill C, Mestril R, Samali A. 2002. Losing heart: The role of apoptosis in heart disease—a novel therapeutic target? FASEB J 16:135–146.

Glennon PE, Kaddoura S, Sale EM, Sale GJ, Fuller SJ, Sugden PH. 1996. Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. Circ Res 78:954–961.

Hara K, Ichihara Y, Takahashi K. 1983. Purification and characterization of a calcium-activated neutral protease from monkey cardiac muscle. J Biochem 93: 1435–1445.

Haunstetter A, Izumo S. 1998. Apoptosis: Basic mechanisms and implications for cardiovascular disease. Cir Res 82:1111–1129.

Hill JA, Karimi M, Kutschke W, Davisson RL, Zimmerman K, Wang Z, Kerber RE, Weiss RM. 2000. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. Circulation 101:2863–2869.

Hsu SM, Raine L, Fanger H. 1981. Use of avidin–biotin peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29: 577–580.

Iizuka K, Kawaguchi H, Kitabatake A. 1993. Effects of thiol protease inhibitors on fodrin degradation during hypoxia in cultured myocytes. J Mol Cell Cardiol 25:1101–1109.

Ingebritsen TS, Stewart AA, Cohen P. 1983. The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. Eur J Biochem 132:297–307.

Izumi Y, Kim S, Murakami T, Yamanaka S, Iwao H. 1998. Cardiac mitogen-activated protein kinase activities are chronically increased in stroke-prone hypertensive rats. Hypertension 31:50–56.

Jiang H, Xiong F, Kong S, Ogawa T, Kobayashi M, Liu JO. 1997. Distinct tissue and cellular distribution of two major isoforms of calcineurin. Mol Immunol 34:663–669.

Kakita T, Hasegawa K, Iwai-Kanai E, Adachi S, Morimoto T, Wada H, Kawamura T, Yanazume T, Sasayama S. 2001. Calcineurin pathway is required for endothelin-1-mediated protection against oxidant stress-induced apoptosis in cardiac myocytes. Circ Res 88:1239–1246.

Kakkar R, Wang X, Radhi JM, Rajala RVS, Wang R, Sharma RK. 2001. Decreased expression of high molecular weight calmodulin binding protein and its correlation with apoptosis in ischemia reperfused rat heart. Cell Calcium 29:59–71.

Klee CB, Krinks MS. 1978. Purification of cyclic 3′, 5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. Biochemistry 17:120–126.

Klee CB, Ren H, Wang X. 1998. Regulation of the calmodulin stimulated protein phosphatase, calcineurin. J Biol Chem 273:13367–13370.

Li WG, Zaheer A, Coppey L, Oskarsson HJ. 1998. Activation of JNK in the remote myocardium after large myocardial infarction in rats. Biochem Biophys Res Commun 246: 816–820.

Lim HW, De Windt LJ, Steinberg L, Taigen T, Witt SA, Kimball TR, Molkentin JD. 2000a. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. Circulation 101:2431–2437.

Lim HW, De Windt LJ, Mante J, Kimball TR, Witt SA, Sussman MA, Molkentin JD. 2000b. Reversal of cardiac

hypertrophy in transgenic disease models by calcineurin inhibition. J Mol Cell Cardiol 32:697–709.
Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL. 1992. Inhibition of T-cell signaling by immunophilin–ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry 31:3896–3901.
Longthorne VL, Williams GT. 1997. Caspase activity is required for commitment to Fas mediated apoptosis. EMBO J 16:3805–3812.
Luo Z, Shyu KG, Gualberto A, Walsh K. 1998. Calcineurin and cardiac hypertrophy. Nat Med 10:1092–1093.
Maulik N, Sasaki H, Galang N. 1999. Differential regulation of apoptosis by ischemia-reperfusion and ischemic adaptation. Ann N Y Acad Sci 874:401–411.
Meguro T, Hong C, Asai K, Takagi G, McKinsey TA, Olson EN, Vatner SF. 1999. Cyclosporine attenuates pressure overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. Circ Res 84:735–740.
Molkentin JD. 2000. Calcineurin and beyond: Cardiac hypertrophic signaling. Circ Res 87:731–738.
Molkentin J. 2001. Calcineurin, mitochondrial membrane potential, and cardiomyocyte apoptosis. Circ Res 88: 1220–1222.
Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93:215–228.
Morimoto T, Hasegawa K, Wada H, Kakita T, Kaburagi S, Yanazume T, Sansayama S. 2001. Calcineurin-GATA4 pathway is involved in b-adrenergic agonist responsive endothelin-1 transcription in cardiac myocytes. J Biol Chem 276:34983–34989.
Muramatsu T, Giri PR, Higuchi S, Kincaid RL. 1992. Molecular cloning of a calmodulin-dependent phosphatase from murine testis: Identification of a developmentally expressed nonneural isoenzyme. Proc Natl Acad Sci USA 89:529–533.
Müller JG, Nemoto S, Laser M, Carabello BA, Menick DR. 1998. Calcineurin inhibition and cardiac hypertrophy. Science 282:1007.
Nakagawa T, Yuan J. 2000. Cross talk between cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150:887–894.
Pallen CJ, Wang JH. 1983. Calmodulin-stimulated dephosphorylation of *p*-nitrophenyl phosphate and free phosphotyrosine by calcineurin. J Biol Chem 258:8550–8553.
Rusnak F, Mertz P. 2000. Calcineurin: Form and function. Physiol Rev 80:1483–1521.
Saito S, Hiroi Y, Zou Y, Aikawa R, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I. 2000. beta-Adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes. J Biol Chem 275:34528–34533.
Seitz DP, Pasha MK, Singh B, Chu A, Sharma RK. 2002. Localization and characterization of calcineurin in bovine eye. Invest Ophthalmol Vis Sci 43:15–21.
Sharma RK, Desai R, Waisman DM, Wang JH. 1979. Purification and subunits structure of bovine brain modulator binding protein. J Biol Chem 254:4276–4282.
Sharma RK, Taylor WA, Wang JH. 1983. Use of calmodulin affinity for purification of specific calmodulin-dependent enzymes. In:Means AR, O'Malley BW, editors. Methods in enzymol. UK: Academic press. pp 210–219.
Sharma RK, Adachi AM, Adachi K, Wang JH. 1984. Demonstration of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by monoclonal antibodies. J Biol Chem 259:9248–9254.
Shimoyama M, Hayashi D, Takimoto E, Zou Y, Oka T, Uozumi H, Kudoh S, Shibasaki F, Yazaki Y, Nagai R, Komuro I. 1999. Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. Circulation 100:2449–2454.
Sorimachi H, Saido TC, Suzuki K. 1994. New era of calpain research. Discovery of tissue specific calpains. Biochem J 328:721–732.
Sorimachi Y, Harada K, Saido TC, Ono T, Kawashima S, Yoshida K. 1997. Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. J Biochem (Tokyo) 122:743–748.
Srivastava RK, Sasaki CY, Hardwick JM, Longo DL. 1999. Bcl-2-mediated drug resistance: Inhibition of apoptosis by blocking nuclear factor of activated T Lymphocytes (NFAT)-induced Fas ligand transcription. J Exp Med 190:253–265.
Stewart AA, Ingebristen TS, Manalan A, Klee CB, Cohen P. 1982. Discovery of a Ca^{2+}- and calmodulin-dependent protein phosphatase: Probable identity with calcineurin (CaM-BP80). FEBS Lett 137:80–84.
Taigen T, De Windt LJ, Lim HW, Molkentin JD. 2000. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 97:1196–1201.
Takaishi T, Saito N, Kuno T, Tanaka C. 1991. Differential distribution of the mRNA encoding two isoforms of the catalytic subunit of calcineurin in the rat brain. Biochem Biophys Res Commun 174:393–398.
Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. 1979. Proc Natl Acad Sci 76:4350–4359.
Wang JH, Desai R. 1976. A brain protein and its effect on the Ca^{2+} and protein modulator-activated cyclic nucleotide phosphodiesterase. Biochem Biophys Res Commun 72:926–932.
Wang KK, Posmantur R, Nadimpalli R, Nath R, Mohan P, Nixon RA, Talanian RV, Keegan M, Herzog L, Allen H. 1998. Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch Biochem Biophys 356:187–196.
Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, Mckeon F, Bobo T, Franke TF, Reed JC. 1999a. Ca^{2+} induced apoptosis through calcineurin dephosphorylation of BAD. Science 284:339–343.
Wang X, Wu L, Aouffen M, Mateescu MA, Nadeau R, Wang R. 1999b. Novel cardiac protective effects of urea: From shark to rat. Br J Pharmacol 128:1477–1484.
Wilkins BJ, Molkentin JD. 2002. Calcineurin and cardiac hypertrophy: Where have we been? Where are we going? J Physiology 541:1–8.
Yoshida K, Sorimachi Y, Fujiwara M, Hironaka K. 1995. Calpain is implicated in rat myocardial injury after ischemia or reperfusion. Jpn Circ J 59:40–48.
Zhang W, Kowal RC, Rusnak F, Sikkink RA, Olson EN, Victor RG. 1999. Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats. Circ Res 84:722–728.

Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I. 2000. Ca^{2+}/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. J Biol Chem 275: 15239–15245.

Zou Y, Yao A, Zhu W, Kudoh S, Hiroi Y, Shimoyama M, Uozumi H, Kohmoto O, Takahashi T, Shibasaki F, Nagai R, Yazaki Y, Komuro I. 2001. Isoproterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through calcineurin. Circulation 104: 102–108.