Antia apoptotic Activity of Akt Is Down-regulated by Ca²⁺ in Myocardial H9c2 Cells

EVIDENCE OF Ca²⁺-DEPENDENT REGULATION OF PROTEIN PHOSPHATASE 2Ac§

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Chie Yasuoka‡§¶, Yoshihito Ihara‡¶, Satoshi Ikeda‡¶, Yoshiyuki Miyahara‡, Takahito Kondo‡, and Shigeru Kohno‡

From the (Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute and the ‡Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan

Cell survival signaling of the Akt/protein kinase B pathway was influenced by a change in the cytoplasmic free calcium concentration (Ca²⁺) for over 2 h via the regulation of a Ser/Thr phosphatase, protein phosphatase 2Ac (PP2Ac) in rat myocardial H9c2 cells. Akt was down-regulated when [Ca²⁺] was elevated by thapsigargin, an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, but was up-regulated when it was suppressed by 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid tetra(acetoxyethyl)ester (BAPTA-AM), a cell permeable Ca²⁺ chelator. The inactivation of Akt was well correlated with the susceptibility to oxidant-induced apoptosis in H9c2 cells. To investigate the mechanism of the Ca²⁺-dependent regulation of Akt via the regulation of PP2A, we examined the transcriptional regulation of PP2Ac in H9c2 cells with Ca²⁺ modulators. Transcription of the PP2Ac gene was increased by thapsigargin but decreased by BAPTA-AM. The promoter activity was examined and the cAMP response element (CRE) was found responsible for the Ca²⁺-dependent regulation of PP2Ac. Furthermore, phosphorylation of CRE-binding protein increased with thapsigargin but decreased with BAPTA-AM. A long term change of [Ca²⁺] regulates PP2Ac gene transcription via CRE, resulting in a change in the activation status of Akt leading to an altered susceptibility to apoptosis.

Calcium (Ca²⁺) plays a signaling role in many important cellular functions, such as fertilization, embryonic pattern formation, differentiation, proliferation, contraction, secretion, and metabolism (1). The versatility of the Ca²⁺-signaling mechanism in terms of speed, amplitude and spatio-temporal patterning enables elevations of Ca²⁺ to regulate many processes of cell activity. Ca²⁺ exhibits cross-talk with a variety of signaling pathways (1). Ca²⁺ affects the protein kinase A pathway by regulating the metabolism of cAMP. It also activates nitric-oxide (NO) synthase to generate NO, which in turn activates the cGMP pathway through the activation of guanylyl cyclase. The Ras/mitogen-activated protein kinase (MAPK) and Ca²⁺/calmodulin/calmodulin kinase (CaMK) pathways are also controlled by Ca²⁺ (1, 2).

On the other hand, a cellular Ca²⁺ overload or the perturbation of intracellular Ca²⁺ compartmentalization can cause cytotoxicity and trigger apoptosis or necrosis (3, 4). Under such circumstances, various Ca²⁺-dependent signaling cascades with kinases and phosphatases directly or indirectly influence cellular signaling. Protein kinase C family has been proposed to play an important role in the Ca²⁺-mediated signaling of apoptosis (5). Calcineurin/PP2B, a Ca²⁺-dependent Ser/Thr phosphatase (6), also appears to be involved in apoptosis (7). Together, these findings show that Ca²⁺ has a pivotal role in the regulatory mechanism of signaling pathways in cell survival and death, although the precise mechanism of Ca²⁺-dependent cross-talk has not been fully clarified.

Akt/protein kinase B is a pleckstrin homology domain-containing Ser/Thr kinase (8, 9, 10). Akt is presently recognized as a cell survival or an antiapoptotic cellular signaling mediator. Akt is activated through a growth factor receptor-mediated activation of the phosphatidylinositol 3-kinase (PI3K) pathway (10). With growth factor signals, Akt is recruited to the plasma membrane and is activated through phosphorylation at Ser-473 and Thr-308 by phosphatidylinositol 3-phosphate-dependent protein kinase-1 (PDK1) or integrin-linked kinase (9, 10). Akt can phosphorylate Bad, caspase-9, and forkhead-related transcription factors, leading to their inactivation and to enhanced cell survival (8, 9, 10). Inhibitor of nuclear factor κB (IκB) kinase is also phosphorylated by Akt leading to an up-regulation of its activity and resulting in a promotion of the nuclear factor κB (NFκB)-mediated inhibition of apoptosis (8, 11, 12).

Akt has been found to be involved in cell death following the withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation, treatment with drugs and ischemic stress (10). However, in spite that a variety of cellular stressors influence cells through Ca²⁺ signaling, the number of studies

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† Both authors contributed equally to this work.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel.: 81-95-849-7099; Fax: 81-95-849-7100; E-mail: y-ihara@net.nagasaki-u.ac.jp.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid tetra(acetoxyethyl)ester; CRE, cAMP response element; CREB, CRE-binding protein; ER, endoplasmic reticulum; FTF, fluorescein isothiocyanate; PFS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; LDH, lactate dehydrogenase; CaMK, calmodulin kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; EMSA, electrophoretic mobility shift assay.
on Akt signaling and Ca\(^{2+}\) is limited. As for Akt, Ca\(^{2+}\)-dependent activation was reported in several studies (13, 14). On the other hand, there was a report that the activation of Akt is independent of Ca\(^{2+}\) (15). In contrast, we found that Akt was suppressed by an elevation of [Ca\(^{2+}\)], in myocaridic H9c2 cells overexpressing the calreticulin gene (16). In the cells overexpressing calreticulin, protein phosphatase 2A (PP2A) was upregulated by Ca\(^{2+}\) to decrease the phosphorylation level of Akt, and the inactivated status of Akt was well correlated with the susceptibility to apoptosis in H9c2 cells under conditions for differentiation induced by retinoic acid. Collectively, these results suggest that the Ca\(^{2+}\)-dependent regulatory mechanism of Akt signaling may be important to a variety of apoptotic signaling mechanisms, although how has not been fully clarified.

In the present study, to investigate the mechanism of the Ca\(^{2+}\)-dependent regulation of Akt signaling, we examined the influence of a change of [Ca\(^{2+}\)] on susceptibility to oxidative stress-induced cell injury and on the Akt signaling pathway in myocardiad H9c2 cells overexpressing the calreticulin gene (16). In the cells overexpressing calreticulin, protein phosphatase 2A (PP2A) was upregulated by Ca\(^{2+}\) to decrease the phosphorylation level of Akt, and the inactivated status of Akt was well correlated with the susceptibility to apoptosis in H9c2 cells under conditions for differentiation induced by retinoic acid. Collectively, these results suggest that the Ca\(^{2+}\)-dependent regulatory mechanism of Akt signaling may be important to a variety of apoptotic signaling mechanisms, although how has not been fully clarified.

**MATERIALS AND METHODS**

**Antibodies and Reagents**—Antibodies against Akt, phospho-Akt (Ser-473), and phospho-CREB (Ser-133) were purchased from Cell Signaling Technology (Beverly, MA). The antibody against Sp1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The reagents used in the study were all of high grade and from Sigma or Wako Pure Chemicals (Osaka, Japan).

**Cell Culture**—H9c2 cells from embryonic rat heart (16, 17) were obtained from American Type Culture Collection (CRL-1446). H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO\(_2\) at 37 °C. Before reaching confluency, the cells were split, and plated at low density in culture medium containing 10% fetal bovine serum.

**Measurement of Cytoplasmic Free Ca\(^{2+}\)**—The cytoplasmic free Ca\(^{2+}\) concentration, [Ca\(^{2+}\)], was measured using Fura-2-AM essentially as described previously (16). Briefly, cultured cells on glass coverslips were loaded with 5 μM Fura-2-AM (Dojindo, Kumamoto, Japan) for 20 min in Earle’s balanced salt solution (EBSS) in the presence of 0.01% pluronic acid F-127. After four washes with EBSS, the cover glass was positioned in a quartz cuvette containing 3.5 ml of fresh EBSS at a 45° angle to both excitation and emission light paths. The fura-2 fluorescence was determined at 37 °C using a spectrofluorophotometer operating at an emission wavelength of 505 nm with an excitation wavelength of 340 and 380 nm. The maximal signal ([R]\(_{\text{max}}\)) was obtained by adding ionomycin at a final concentration of 4 μM. Then the minimal signal ([R]\(_{\text{min}}\)) was obtained by adding EGTA at a final concentration of 7.5 mM, followed by Tris-free base to a final concentration of 30 mM, to increase the pH to 8.3. R is the ratio (F1/F2) of the fluorescence of Ex 340 nm, Em 505 nm (F1) to that of Ex 380 nm, Em 505 nm (F2). The actual Ca\(^{2+}\) concentration was calculated as K\(_{\text{d}}\) X (R - [R]\(_{\text{min}}\))/([R]\(_{\text{max}}\) - R) with the K\(_{\text{d}}\) equal to 224 nM (18).

**Luciferase Activity Assay**—Luciferase activity was assayed using an Akt activity kit (Cell Signaling Technology) according to the manufacturer’s instructions. 

**Luciferase Activity Assay**—Luciferase activity was assayed photometrically using Ser/Thr phosphatase assay kit 1 (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer’s directions. The activity was assayed in the presence or absence of 10 μM okadaic acid, and the okadaic acid-sensitive activity was determined as PP2A-specific activity. The phosphorylated Ser/Thr phosphatase (KpTPHR) was used as a phosphatase substrate. Protein concentrations were determined using a BCA assay kit (Pierce).

**Generation of Luciferase Reporter Constructs**—A 1.6-kb fragment of rat PP2Ac gene promoter (−1350 to +258) (23) was amplified with rat genomic PCR using Pfu turbo DNA polymerase (Stratagene). The fragments were used as a forward primer (5′-CCTCCTGACCTTCTCTCAGGAACTACTG-3′) and a reverse primer (5′-GTCAGCTCCTTCTGGTGAAACAATCTC-3′). The PCR product was subcloned into pUC18 to obtain pUC18-pro-PP2Ac. The nucleotide sequence was confirmed by sequencing with an ALFexpress II system (Amersham Biosciences). pUC18-pro-PP2Ac was digested with HindIII and the resulting fragment containing the promoter region from −1209 to +258 was inserted into the HindIII site of the reporter vector pGL3-Basic (Stratagene) to give pGL3-pro-PP2Ac. To generate deleted mutants of the luciferase reporter construct, pGL3-pro-PP2Ac was digested with XhoI and KoxI, and deletion mutants were made using a deletion kit for kilo sequence (Takara Biomedicals).

**Site-directed Mutagenesis for Luciferase Vectors**—In vitro mutagenesis was performed with pGL3-pro-PP2Ac-del (-729 to +258) and del (-145 to +258) as templates by using a QuikChange site-directed mutagenesis kit (Stratagene). Oligonucleotides used are as follows: GC box (−155), 5′-CCCTGCCGGGAGGACCAACCAAGCACACAAGGAA-CCACACCTCC-3′; CRE (−26), 5′-GTCGAGCTGCGGTTGCTGCCA-CCGCGGCGGGCGGCGGATTAC-3′. The nucleotide sequences were confirmed by sequencing with an AlExpress II system (Amersham Biosciences).

**Luciferase Activity Assay**—Each vector was transfected into H9c2 cells by using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, cells were treated with thapsigargin (5 μM) or BAPTA-AM (10 μM), or left untreated for the periods indicated in the text. Luciferase activity was assayed with cellular extracts by using a dual-luciferase reporter assay system (Promega).
Elevation of [Ca^{2+}], Gradually Accelerates Cell Damage and Apoptosis in H9c2 Cells—To modify the level of [Ca^{2+}], in H9c2 cells, the cells were treated with 5 μM thapsigargin (25), an inhibitor for sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase to increase [Ca^{2+}], or with 10 μM BAPTA-AM (26), a cell permeable Ca^{2+} chelator to decrease [Ca^{2+}], for 0–4 h. As shown in Fig. 1A, with thapsigargin, [Ca^{2+}] shows a transient increase within the first 10 min. It then decreases to the basal level till 30 min, but again increases and retains elevated until 120 min, before gradually decreasing to the initial level. In contrast, with BAPTA-AM, [Ca^{2+}] shows a continuous lowering during the treatment. To investigate the influence of the long term change of [Ca^{2+}] on susceptibility to oxidative stress-induced cell injury, cells were treated with Ca^{2+} modulators (i.e. thapsigargin and BAPTA-AM) for 4 h then exposed to 75 μM hydrogen peroxide (H_{2}O_{2}) for 0–120 min, and cell damage was examined at predetermined times using the LDH release assay as described in the methods. In Fig. 1B, the release of LDH by H_{2}O_{2} was observed only at 120 min in the medium of untreated cells. However, the release by H_{2}O_{2} was initially observed at 60 min and increased at 120 min in the cells treated with thapsigargin. In contrast, BAPTA-AM treatment completely suppressed the release of LDH by H_{2}O_{2} throughout the 120 min. Next, to investigate whether apoptosis is involved in the mechanism of thapsigargin-induced cell damage, the cells were treated with Ca^{2+} modulators for 4 h then exposed to H_{2}O_{2} (75 μM) for 2 h, and apoptosis was examined. Fig. 1C shows that TUNEL-positive fluorescence intensity was increased slightly by H_{2}O_{2} (upper), but was significantly enhanced after the pretreatment with thapsigargin (middle). In contrast, no change was observed in the fluorescent intensity of the BAPTA-AM-treated cells with or without H_{2}O_{2} (lower). Collectively, these results indicate that the susceptibility to apoptosis was enhanced with a long term elevation of [Ca^{2+}], but was suppressed with the lowering of [Ca^{2+}], in the cells under oxidative stress with H_{2}O_{2}, suggesting that the continuous change of [Ca^{2+}], influences the susceptibility to apoptosis.

The Expression of PP2Ac in H9c2 Cells—To modify the level of [Ca^{2+}], in H9c2 cells, the cells were treated with 5 μM thapsigargin or 10 μM BAPTA-AM for 2 h, cells incubated on Lab-Tek chamber slides (Nunc) were fixed with 3% paraformaldehyde for 20 min at room temperature and washed three times with PBS. Cells were permeabilized in 1% Triton X-100 in PBS for 10 min and washed three times with PBS. They were blocked with 1% bovine serum albumin in PBS for 30 min at room temperature, washed three times with PBS, and then incubated with antiphospho-CREB (Ser-133) overnight at 4 °C. Cells were washed with PBS four times and incubated with FITC-labeled anti-rabbit IgG antibody for 30 min in a dark room. The immunoreactive signals were visualized by indirect immunofluorescence microscopy.

RESULTS

Elevation of [Ca^{2+}] Gradually Accelerates Cell Damage and Apoptosis in H9c2 Cells—To modify the level of [Ca^{2+}], in H9c2 cells, the cells were treated with 5 μM thapsigargin (25), an inhibitor for sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase to increase [Ca^{2+}], or with 10 μM BAPTA-AM (26), a cell permeable Ca^{2+} chelator to decrease [Ca^{2+}], for 0–4 h. As shown in Fig. 1A, with thapsigargin, [Ca^{2+}] shows a transient increase within the first 10 min. It then decreases to the basal level till 30 min, but again increases and retains elevated until 120 min, before gradually decreasing to the initial level. In contrast, with BAPTA-AM, [Ca^{2+}] shows a continuous lowering during the treatment. To investigate the influence of the long term change of [Ca^{2+}] on susceptibility to oxidative stress-induced cell injury, cells were treated with Ca^{2+} modulators (i.e. thapsigargin and BAPTA-AM) for 4 h then exposed to 75 μM hydrogen peroxide (H_{2}O_{2}) for 0–120 min, and cell damage was examined at predetermined times using the LDH release assay as described in the methods. In Fig. 1B, the release of LDH by H_{2}O_{2} was observed only at 120 min in the medium of untreated cells. However, the release by H_{2}O_{2} was initially observed at 60 min and increased at 120 min in the cells treated with thapsigargin. In contrast, BAPTA-AM treatment completely suppressed the release of LDH by H_{2}O_{2} throughout the 120 min. Next, to investigate whether apoptosis is involved in the mechanism of thapsigargin-induced cell damage, the cells were treated with Ca^{2+} modulators for 4 h then exposed to H_{2}O_{2} (75 μM) for 2 h, and apoptosis was examined. Fig. 1C shows that TUNEL-positive fluorescence intensity was increased slightly by H_{2}O_{2} (upper), but was significantly enhanced after the pretreatment with thapsigargin (middle). In contrast, no change was observed in the fluorescent intensity of the BAPTA-AM-treated cells with or without H_{2}O_{2} (lower). Collectively, these results indicate that the susceptibility to apoptosis was enhanced with a long term elevation of [Ca^{2+}], but was suppressed with the lowering of [Ca^{2+}], in the cells under oxidative stress with H_{2}O_{2}, suggesting that the continuous change of [Ca^{2+}], influences the susceptibility to apoptosis.

The Long Term Change of [Ca^{2+}], Influences Akt Signaling in H9c2 Cells—To modify the level of [Ca^{2+}], in cell survival signaling, we focused on the effect of Ca^{2+} modulators on Akt signaling. Previously, we found that the Akt signaling pathway is responsible for the cytoprotective mechanism in H9c2 cells under conditions of stress such as serum starvation with retinoic acid (16), and oxidative stress with hydrogen peroxide (27). Although elevations in Ca^{2+} act as a signal, a prolonged increase in the concentration of Ca^{2+} can be lethal (1). Moreover, cell signaling molecules including transcription factors are activated differentially by the amplitude and duration of the response to Ca^{2+} (28). In the present study, H9c2 cells were treated with Ca^{2+} modulators such as thapsigargin (5 μM) and BAPTA-AM (10 μM) for over 2 h to induce a long term change of [Ca^{2+}]. After the treatments, the phosphorylation levels of Ser-473 and Thr-380 of Akt were examined. The phosphorylation of Thr-380 located in the kinase catalytic domain of Akt is necessary for the activation, and the phosphorylation of Ser-473 located in the regulatory domain of Akt supports the activation. As shown in Fig. 2, A (right) and B, the treatment with BAPTA-AM increased the phosphorylation of Akt both at Ser-473 and Thr-380, and the phosphorylation level increased to a maximum at 2 h, and was sustained thereafter till 4 h. In contrast, the phosphorylation of Akt decreased in a time-dependent manner with thapsigargin (Fig. 2, A (left) and B). Next we examined whether Ca^{2+} modulators also have an effect on the kinase activity of Akt. Fig. 2C shows that Akt activity is suppressed after the treatment with thapsigargin, but increased with BAPTA-AM. These results were consistent with the change in the phosphorylation status of Akt on treatment with Ca^{2+} modulators such as thapsigargin and BAPTA-AM. Together, Akt signaling was suppressed by the long term elevation of [Ca^{2+}], with thapsigargin, but was enhanced by the long term lowering of [Ca^{2+}], with BAPTA-AM. This suggests a Ca^{2+}-dependent regulation of Akt signaling in H9c2 cells. Furthermore, the Ca^{2+}-induced suppression of Akt signaling was compatible with the enhanced susceptibility to apoptosis in H9c2 cells treated with H_{2}O_{2} (Fig. 1, B and C).

The Expression of PP2Ac is Transcriptionally Regulated by Ca^{2+} Modulators—3-Phosphoinositide-dependent protein kinase (PKD1) is known to be responsible for phosphorylating Akt at Thr-380, and is activated by both phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4,)-bisphosphate, products of PI3K (9, 10). To investigate whether the upstream kinases are involved in the regulation of Akt by the change of [Ca^{2+}], the activities for PI3K and PKD1 were measured in the cells treated with Ca^{2+} modulators. However, neither activities showed any significant change even if the cells were treated with thapsigargin or BAPTA-AM for 0–4 h (data not shown). Therefore, we focused on Ser/Thr protein phosphatases that could dephosphorylate and inactivate Akt to regulate the Akt signaling pathway (7). To investigate whether [Ca^{2+}], levels affect the expression of protein Ser/Thr phosphatases, the cells were treated with 5 μM thapsigargin or 10 μM BAPTA-AM for 0–4 h, and transcriptional levels were estimated by Northern blot analysis for protein phosphatase 2A catalytic subunit α (PP2Acα) and protein phosphatase 1α catalytic subunit (PP1αc). In Fig. 3A, the level of PP2Ac mRNA was increased by thapsigargin but decreased by BAPTA-AM. In contrast, the mRNA level of PP1αc was not significantly changed by the Ca^{2+} modulators. In the immunoblot analysis, the protein level of PP2Acα was increased by thapsigargin but decreased by BAPTA-AM (Fig. 3B). However, the protein level of PP1αc was not influenced by thapsigargin or BAPTA-AM either (data not shown). These results were consistent with results of the change of transcriptional levels for the phosphatases in the cells treated with each Ca^{2+} modulator. The enzymatic activity
FIG. 1. The long term elevation of \([Ca^{2+}]_i\) accelerates apoptosis in H9c2 cells under oxidative stress with H2O2. \(A\), H9c2 cells were treated with thapsigargin (5 \(\mu\)M) or BAPTA-AM (10 \(\mu\)M) for the periods indicated, and \([Ca^{2+}]_i\), was measured using Fura-2-AM as described under "Materials and Methods." Each value represents the mean ± S.D. of four independent experiments. \(B\), after 4 h of treatment with thapsigargin (5 \(\mu\)M) or BAPTA-AM (10 \(\mu\)M) or not, the cells were incubated with H2O2 (75 \(\mu\)M) for the periods indicated. Cell injury was estimated by measuring the release of LDH in the culture medium as described under "Materials and Methods." The LDH release is shown as the proportion of total cellular LDH in the medium to total cellular LDH. Each value represents the mean of three experiments, and the S.D. was always within 10% of the mean. \(C\), DNA double-stranded breaks were detected by the TUNEL method as described under "Materials and Methods." Cells were treated with either thapsigargin (5 \(\mu\)M) or BAPTA-AM (10 \(\mu\)M) for 4 h and untreated cells were prepared as a control. Then the cells were treated with (thick lines) or without (thin lines) H2O2 (75 \(\mu\)M) for 2 h. The results were reproducible in three independent experiments.
of PP2A was also assayed in the cells treated with thapsigargin or BAPTA-AM for 0–4 h. As shown in Fig. 3C, the activity of PP2A increased with thapsigargin by 2-fold compared with that of untreated cells. In contrast, the activity was slightly suppressed after 2 h treatment with BAPTA-AM. Collectively, these results indicate that PP2Ac expression is transcriptionally regulated by the long term change of \([\text{Ca}^{2+}]_i\) to control the phosphorylation status of target molecules including Akt.

**Inhibition of PP2A Activity by Okadaic Acid Enhanced the Phosphorylation of Akt**—

Okadaic acid, a polyether toxin from the marine black sponge *Halichondria okadai* is a highly selective inhibitor of PP2A (29). To establish a link between Akt and PP2A, the influence of okadaic acid on Akt signaling was investigated with cells treated with okadaic acid. The cells were treated with okadaic acid (100 nM) for 0–30 min. The phosphorylation level of Akt was estimated by immunoblot analysis using specific antibodies. Akt kinase and PP2A activities were measured as described above. As shown in Fig. 4A, okadaic acid reduced the activity of PP2A by ~30%, and increased the phosphorylation level of Akt (both Thr-308 and Ser-473) and Akt kinase activity. Next, we examined whether the decrease in phosphorylation of Akt caused by thapsigargin could be reversed by okadaic acid. The cells were preincubated with thapsigargin (5 \(\mu\)M) for 4 h, and treated with or without okadaic acid (100 nM) for another 30 min, then the phosphorylation level of Akt was examined as described above. As shown in Fig. 4B, okadaic acid suppressed PP2A activity, and this reversed the decrease in phosphorylation of Akt with thapsigargin. Okadaic acid enhanced the phosphorylation of Akt at concentrations higher than 50 nM (data not shown). These results indicate that the function of PP2A can regulate the phosphorylation status of Akt in H9c2 cells.

**Inhibition of PP2A Activity by Okadaic Acid Suppressed Apoptotic Cell Damage in H9c2 Cells Treated with Thapsigargin and \(\text{H}_2\text{O}_2\)**—

To establish a link between PP2A and \(\text{Ca}^{2+}\)-dependent enhancement of apoptosis, the influence of okadaic acid on apoptosis was investigated using cells treated with okadaic acid. In Fig. 5A, cells were treated with or without

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**FIG. 2.** \(\text{Ca}^{2+}\) modulators influence the phosphorylation and kinase activity of Akt in H9c2 cells. H9c2 cells were cultured in the presence of either thapsigargin (5 \(\mu\)M) or BAPTA-AM (10 \(\mu\)M) for the periods indicated. A, Akt phosphorylation was detected in the cell lysates by immunoblot analysis (IB) with anti-phospho-Akt (Ser-473), anti-phospho-Akt (Thr-308), and anti-Akt antibodies. B, quantitative data for the phosphorylation status of Akt shown in A. The band intensity was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of the phosphorylated Akt (Akt-P)/Akt. Each value represents the mean ± S.D. of four independent experiments. C, Akt kinase activity was assayed as described under “Materials and Methods” using GSK-3β, Ser-21/9, as a substrate. Phosphorylated GSK-3α/β (Ser-21/9), the enzymatic products of Akt, was detected by immunoblot analysis using specific antibody. The data represent three independent experiments.

**FIG. 3.** The expression and phosphatase activity of PP2Ac are regulated by \(\text{Ca}^{2+}\) modulators in H9c2 cells. H9c2 cells were treated with thapsigargin (5 \(\mu\)M) or BAPTA-AM (10 \(\mu\)M) for the periods indicated. A, transcriptional levels of PP2Ac and PP1ac were evaluated by Northern blot analysis as described under "Materials and Methods." An EcoRI-PvuII fragment of 680 bp and a PstI-SmaI fragment of 600 bp were prepared from cDNAs for PP2Ac and PP1ac, respectively, and were used as cDNA probes after labeling with \([\alpha-\text{32P}]\text{dCTP. B, protein levels for PP2Ac and PP1ac were estimated by immunoblot analysis using specific antibodies. The data represent three independent experiments. C, protein phosphatase activity was assayed photometrically as described under "Materials and Methods." Each value represents the mean ± S.D. of four independent experiments."
rather diminished at concentrations higher than 500 nM (data not shown). Fig. 5A shows the effect of okadaic acid on Ca\(^{2+}\)—dependent enhancement of cell damage in cells treated with thapsigargin and H\(_2\)O\(_2\). Fig. 5B shows a dose-dependent effect of okadaic acid on the Ca\(^{2+}\)—dependent enhancement of cell damage. Cells were treated with thapsigargin (5 \(\mu\)M) for 4 h then treated with different concentrations of okadaic acid (0–500 nM) for 30 min. Thereafter, cells were exposed to H\(_2\)O\(_2\) (75 \(\mu\)M) for 2 h, and cell damage was examined using the LDH release assay. Okadaic acid showed maximal cytoprotective effects at a limited concentration range around 100–200 nM. Figure 5C shows the effect of okadaic acid on Ca\(^{2+}\)—dependent enhancement of apoptosis. Cells were treated with thapsigargin (5 \(\mu\)M) for 4 h then treated with or without okadaic acid (100 nM) for 30 min. Then cells were exposed to H\(_2\)O\(_2\) (75 \(\mu\)M) for 2 h, and apoptosis was examined by the TUNEL method. After thapsigargin treatment without okadaic acid, TUNEL-positive fluorescence intensity was significantly increased by H\(_2\)O\(_2\) (Figs. 1C and 5C, Tg(+)/OA(-)/H\(_2\)O\(_2\)(+)). In contrast, okadaic acid reduced the TUNEL-positive fluorescence intensity even in the cells treated with thapsigargin and H\(_2\)O\(_2\) (Fig. 5C, Tg(+)/OA(+)/H\(_2\)O\(_2\)(+)) compared with that of cells treated with thapsigargin and H\(_2\)O\(_2\) without okadaic acid. Okadaic acid did not solely affect the fluorescence intensity in cells with thapsigargin but without H\(_2\)O\(_2\) (Fig. 5C, Tg(+)/OA(-) and Tg(+)/OA(+)). However, it is noteworthy that okadaic acid shows an antia apoptotic effect at a limited concentration range around 100 nM. At concentrations above 500 nM, the antia apoptotic effect of okadaic acid was diminished or rather it showed enhanced enhancement of apoptosis in the cells treated with thapsigargin and H\(_2\)O\(_2\) (data not shown). This may be explained by the dualistic effect of inhibiting the effects of PP1 and PP2A on both apoptosis and cell proliferation in cells exposed to okadaic acid or microcystin-LR (30). Together, these results indicate that okadaic acid, a specific inhibitor of PP2A, inhibits apoptotic cell damage in H9c2 cells treated with thapsigargin and H\(_2\)O\(_2\), and strongly suggests that PP2A up-regulates the Ca\(^{2+}\)—dependent enhancement of apoptosis by dephosphorylating Akt to inhibit cell survival signaling.
Characterization of PP2Ac Gene Promoter—To investigate the mechanism of the transcriptional regulation of PP2Ac expression, we used the 1.6-kb genomic fragment containing the promoter region of PP2Ac inserted into a luciferase vector, pGL3 Basic. The transcriptional initiation site (nucleotide +1) was denoted in accordance with the report of Kitagawa et al. (23), a fragment of 118 bp (−162 to −44) was defined as the essential region for the gene expression of PP2Ac. As shown in Fig. 6, the deletion fragments of the gene promoter were made from the HindIII-digested fragment (−1209 to +258, full-length) (Construct 1), and subcloned into a pGL3 Basic vector. Then, the activity of luciferase was assayed with the cells transfected with each deletion mutant vector. The activity was fully maintained in the 537-bp fragment (−279 to +258) (Construct 2), but it decreased to ∼65% of the full activity in the 403-bp fragment (−145 to +258) (Construct 3) containing the CRE site. In the upstream fragment of 1064-bp (−1209 to −145) (Construct 6) containing the GC box, the activity decreased to ∼40% of the full activity. However, the activity was almost lost in the upstream fragment of 1044 bp (−1209 to −165) (Construct 5) and the downstream fragment of 259 bp (−1 to +258) (Construct 4). The results indicated that the full promoter activity was located in the sequence between −279 and −1. To examine whether the CRE and GC box contribute to the full promoter activity, disabled mutants were generated for the consensus sequences of CRE at −26 and GC box at −155 in the luciferase vector containing the 537-bp Construct 2. In Construct 2-Mt/G containing a CRE but no GC box, the activity decreased to ∼70% of the full activity, and the level was similar to that of Construct 3. In Construct 2-Mt/G&C, the activity was significantly suppressed to less than 5% of full activity by both mutations. Furthermore, by the mutation with CRE, the activity was completely lost in Construct 3-Mt/C. Taken together, these results indicate that both the CRE and GC box contribute to the basal expression of the PP2Ac gene in H9c2 cells.

Thapsigargin Enhanced Protein-CRE Complex Formation—To examine if a DNA-binding protein like Sp1 or CREB could interact with the PP2Ac promoter, EMSA was performed with nuclear extracts from the cells treated with 5 μM thapsigargin or 10 μM BAPTA-AM using 32P-labeled oligonucleotides designed for the GC box at −155 and CRE at −26. In the case of the CRE, a major band appeared but it disappeared in the presence of an excess of unlabeled probe (not shown) or 32P-labeled probe with the disabled-mutant for CRE (Fig. 7, A and C). The intensity of shifted band for CRE significantly increased with the nuclear extracts treated with thapsigargin for 1–2 h (Fig. 7A, left). In contrast, the intensity decreased with the nuclear extracts treated with BAPTA-AM for 1–2 h (Fig. 7A, right). In the case of the GC box, a major band appeared but it disappeared in the presence of an excess of unlabeled probe (not shown) or 32P-labeled probe with the disabled mutant for GC box (Fig. 7, B and C). However, the band intensity was not influenced by the treatment with thapsigargin or BAPTA-AM (Fig. 7). In the case of the GC box-like site at −10, no major band was observed in EMSA, suggesting that the site is nonfunctional (data not shown). In the case of the RORα site at −553, a gel shift was observed but was not changed by the treatment with Ca2+-modulators such as thapsigargin and BAPTA-AM (data not shown). Furthermore, there was no gel-shift observed with the probe for the RORα site at −778 in EMSA, suggesting that the site is nonfunctional (data not shown). Together, these results indicate that the DNA

![Diagram](image-url)
binding activity involves both the CRE at −26 and GC box at −155, but it is specifically influenced by Ca²⁺ modulators, such as thapsigargin and BAPTA-AM, especially in the CRE at −26.

The Gene Promoter Activity of PP2Ac is Regulated by Ca²⁺ Modulators via CRE—To confirm the CRE-dependent regulation of the gene expression of PP2Ac by Ca²⁺ modulators, the gene promoter activity was examined by assaying the luciferase activity as described above. The cells were transfected with luciferase vector construct 3 (−145 to +258) (Fig. 6), which contains a CRE but no GC box. After 24 h of transfection, the cells were treated with 5 μM thapsigargin (A) or 10 μM BAPTA-AM (B) for the periods indicated. Then, luciferase activity was assayed with cell nuclear extracts as described under “Materials and Methods.” Each value represents the mean ± S.D. of at least three experiments. The statistical analysis was performed with a factorial analysis of variance test.
PP2A is a multifunctional protein Ser/Thr phosphatase that regulates a variety of signaling pathways in eukaryotic cells (35, 36, 37). The core structure is a dimer, consisting of a 36-kDa catalytic subunit (PP2Acα, β) and a 65-kDa constant regulatory (structural) subunit (PP2Bα, β). A third, variable regulatory subunit (B, PR55/60, β, γ, δ, ε; B’, PR55/61α, β, γ, δ, ε, B”, PR48/58/72/130; B”’, PR99/110) can associate with this core enzyme. There are various reports of PP2A as a positive regulator of apoptosis (38), although a specific subunit of PP2A containing B’/PR61 is reported to be inhibitory for apoptosis in Drosophila (39, 40). Bad is a pro-apoptotic member of the Bcl-2 family, whose function is highly regulated by reversible phosphorylation (41). PP2A was responsible for the dephosphorylation of Bad (42), and dephosphorylated Bad bound anti-apoptotic Bcl-2 members at the mitochondrial membrane leading to apoptotic cell death (43). PP2A was also found to co-localize at the mitochondrial membrane with Bcl-2, and the pro-apoptotic sphingolipid ceramide has been shown to activate the PP2A involved (44, 45). In anti-Fas-induced apoptosis, activation of caspase-3 caused cleavage of the regulatory Aα subunit of PP2A, and this in turn increased PP2A activity (46). On the other hand, Liu et al. (47) reported that 4-hydroxynonenal induced dephosphorylation of Akt through activation of PP2A in a caspase-dependent apoptosis of Jurkat cells. In the study, the authors described that PP2A was activated by an altered intracellular localization of tyrosine-dephosphorylated PP2A, but not by the caspase-dependent cleavage of the regulatory Aα subunit of PP2A. Furthermore, C2-ceramide induced dephosphorylation of both GSK3β and Akt by activating PP2A, resulting in apoptosis in rat cerebellar granule cells, and the apoptosis was blocked with lithium by inhibiting PP2A activity (48). Together, these findings indicate that PP2A plays a critical role in the positive regulation of apoptosis by dephosphorylating various apoptotic regulators including Akt, but the molecular mechanism for the activation of PP2A in apoptosis is not clearly understood.

PP2A is considered a phosphatase responsible for the dephosphorylation and inactivation of Akt (47, 48, 49, 50, 51, 52, 53). Previously, we also showed that Akt was dephosphorylated by PP2A in H9c2 cells (16), and PP2A interacted transiently with Akt in H9c2 cells under oxidative stress with H2O2 (27). In the present study, the treatment with okadaic acid decreased PP2A activity to ~70% of the untreated control value, and it reversed the thapsigargin-dependent suppression of the phosphorylation of Akt in H9c2 cells (Fig. 4). Furthermore, treatment with okadaic acid could suppress the thapsigargin-induced enhancement of apoptosis in cells exposed to H2O2, although the effective okadaic acid concentration was limited to a range around 100–200 nM (Fig. 5). These findings strongly suggest that PP2A plays an up-regulating role in the thapsigargin-induced enhancement of apoptosis by inhibiting Akt signaling. Collectively, the finding that up-regulation of PP2Aca gene expression led to an increase of PP2A activity is consistent with the enhanced dephosphorylation and inactivation of Akt in H9c2 cells following the long term elevation of [Ca2+]i due to thapsigargin exposure (Fig. 2). Although the expression of PP2Ac is tightly controlled by an autoregulatory translational mechanism (54), there are reports describing changes in PP2Ac levels, for instance, during all-trans retinoic acid-induced differentiation of HL-60 cells (55, 56), during adipocyte differentiation induced by peroxisome proliferator-activated receptor-γ (57), during stimulation by colony-stimulating factor in macrophages (58), and during a response to the disruption of cellular attachment in mouse C3 10T1/2 cells (59).

**Fig. 9.** The phosphorylation of CREB is regulated by Ca2+-modulators in H9c2 cells. A, H9c2 cells were incubated with thapsigargin (5 μM) or BAPTA-AM (10 μM) for the periods indicated, then cell extracts were prepared. The phosphorylation level of CREB was estimated by immunoblot analysis using specific antibodies. B, after the treatment with thapsigargin (5 μM) or BAPTA-AM (10 μM) for 2 h, the intracellular localization of phosphorylated CREB was examined by indirect immunofluorescence microscopy using specific antibodies as described under “Materials and Methods.” The data represent two independent experiments.

**DISCUSSION**

Akt is a Ser/Thr protein kinase with antiapoptotic and oncogenic activities (8–10). With regard to the Ca2+-dependent regulation of Akt, Conus et al. (15) reported that the activation of Akt was independent of Ca2+ in mouse fibroblasts treated with thapsigargin. However, Yano et al. (13) identified a Ca2+-triggered signaling cascade in which CaMK kinase activates Akt in a PI 3-kinase-independent manner. Then Huber et al. (14) found that Akt was rapidly activated by treatment with thapsigargin through the activation of PI 3-kinase. In the present study, we found that Akt was suppressed by a long term elevation of [Ca2+]i, induced by thapsigargin, but was enhanced by a long term lowering of [Ca2+]i, caused by BAPTA-AM in H9c2 cells. The inactivation of Akt is highly correlated with susceptibility to apoptosis. Recently, Luo et al. (34) reported that inactivation of Akt is a causal mediator of cell death, and this is consistent with our present results. Although the underlying mechanism for these differences in the Ca2+-dependent regulation of Akt was not clear, we showed that transcriptional regulation of PP2Acα was important to control the activation status of Akt in H9c2 cells under conditions where there is a long term change in [Ca2+]i, levels.
We also observed transcriptional activation of PP2Acα in H9c2 cells transfected with the expression vector for calreticulin, a molecular chaperone in the endoplasmic reticulum (16). However, the underlying mechanism for these differences in the regulation of PP2A expression was not clarified.

To investigate the molecular mechanism behind the Ca$^{2+}$-dependent transcriptional regulation of PP2Ac in H9c2 cells, the promoter function of the PP2Acα gene was characterized using a luciferase-based reporter assay in cells treated with Ca$^{2+}$-modulators such as thapsigargin and BAPTA-AM. The results showed that the expression of PP2Acα was transcriptionally regulated by the change of [Ca$^{2+}$]i. The PP2Ac gene has been isolated and characterized in humans (60) and rats (23). In both species, the promoter region of PP2Acα has a high GC content and does not contain either a TATA box or a CAAT box, which suggests that the gene is a typical housekeeping gene. Among various transcription factors including CREB, Sp1, and RORα within the promoter region, CREB was revealed to be responsible for Ca$^{2+}$-dependent regulation of the PP2Acα gene in H9c2 cells treated with thapsigargin and BAPTA-AM. CREB is a bZIP transcription factor that forms homo- or heterodimers with itself or other members of the CREB family including ATF1 and CREM, and is a pivotal transcription factor that regulates cell proliferation, differentiation, and survival in a variety of cell types in vertebrates (33). The CREB dimers interact with a specific DNA sequence having the consensus motif TGACGTCA in the regulatory region of CREB target genes. CREB is inactive as a transcription factor until a cell is exposed to any extracellular stimuli that trigger its phosphorylation at a specific site, Ser-133, within its kinase-inducible domain (33). CREB was originally identified as a target of the cAMP signaling pathway, and regulated in response to diverse signals, including peptide hormones, growth factors, and Ca$^{2+}$. CREB is known to be activated at high [Ca$^{2+}$]i, and this is consistent with our findings that the level of CREB phosphorylated at Ser-133 increased with thapsigargin but decreased with BAPTA-AM in the nucleus of H9c2 cells. Though minute changes in [Ca$^{2+}$]i, are quickly transformed into changes in the activity of several kinases including cAMP-dependent kinase, protein kinase C, MAPKs, Ca$^{2+}$/CaMK and CaMK kinase, it is not clear whether these kinases are influenced in the case of long term change of [Ca$^{2+}$]i, in H9c2 cells treated with Ca$^{2+}$-modulators. Among these kinases, CaMKII and CaMKIV were reported to be able to phosphorylate CREB directly (61, 62). In addition, Rsk protein kinase phosphorylates CREB at Ser-133 through activation of the Ras/MAPK signaling pathway by Ca$^{2+}$ (63, 64). Although CaMKIV mediates the early phase in the phosphorylation of Ser-133 in membrane-depolarized neurons, the MAPK pathway is responsible for prolonging the phosphorylation (65). In the present study, an increase in both the phosphorylation of CREB at Ser-133 and activity of CREB to bind the CRE site was observed after 2 h treatment with thapsigargin, suggesting a late activation of CREB caused by the long term elevation of [Ca$^{2+}$]i. The treatment with BAPTA-AM influenced the phosphorylation of CREB and suppressed the binding of CREB to the CRE after 1 h, and this also suggests a late inactivation of CREB on the long term suppression of [Ca$^{2+}$]i. Taken together, the long term change of [Ca$^{2+}$]i may regulate CREB function through the MAPK pathway rather than CaMK pathway in H9c2 cells treated with Ca$^{2+}$-modulators.

Thapsigargin causes an increase of [Ca$^{2+}$]i, and is also known as an inducer of endoplasmic reticulum (ER) stress (unfolding protein stress in the ER) (66). Recently, Song et al. (67) reported that thapsigargin-induced ER stress induced de-phosphorylation of both GSK3β and Akt by activating PP2A, resulting in apoptosis in neuroblastoma cells, and this was consistent with our results. In the study, the authors described that dephosphorylation of GSK3β by activated PP2A was critical for the activation of caspase-3 in ER stress-induced apoptosis, but the mechanism for the PP2A activation by ER stress was not clarified. In ER stress, a transcriptional up-regulation is seen in the ER stress responsive genes that code a variety of ER proteins related to molecular chaperone functions, such as BiP/Grp78, Grp94, Grp58/ERp57, ERp72, and calreticulin (66). In mammalian cells, the 19-nucleotide motif CCAAT-N9-CACG was identified as an ER responsive element (ERSE) of various ER chaperone genes, and was recognized by the human bZIP transcriptional factor ATF6 for ER stress response (68). However, we could not find a consensus sequence within the 1.6-kbp promoter region of the PP2Acα gene, and this suggests that the PP2Acα gene is not a direct target for the ER stress response in thapsigargin-treated cells.

In this study, CREB was linked to the enhanced susceptibility to apoptosis through the induction of the PP2Ac gene in cells exposed to the long term elevation of [Ca$^{2+}$]i. To further investigate whether CREB is specifically responsible for the up-regulation of apoptosis in cells treated with thapsigargin and H$_2$O$_2$, CREB expression was suppressed by the short interfering RNA (siRNA) for the CREB gene. Using mammalian CREB siRNA expression plasmid (pKD-CREB-v2, Upstate Biotechnology), the CREB expression level was suppressed in H9c2 cells to ~30% of non-transfected cells. Using the transfected cells, thapsigargin-dependent enhancement of cell damage and apoptosis were examined in cells treated with thapsigargin (5 μM) and H$_2$O$_2$ (75 μM). However, despite the suppression of CREB protein, cell damage and apoptosis were not inhibited in cells treated with thapsigargin and H$_2$O$_2$, but rather were more enhanced (data not shown). This indicates that CREB is not specifically responsible for the mechanism enhancing apoptosis in cells showing long term elevation of [Ca$^{2+}$]i. However, this may be reasonable because CREB is well known as a transcription factor for cell survival and antiapoptotic genes such as Bcl-2 (69), and the suppression of CREB may firstly decrease the expression of such cell survival genes resulting in enhanced susceptibility to apoptosis. The effect of the suppressed expression of CREB on cell survival was also consistent with previous findings using dominant-negative CREB polypeptides (69). Although the suppressed expression of CREB itself did not specifically reduce the thapsigargin-dependent enhancement of apoptotic cell damage in H9c2 cells, it still may be possible that CREB works for apoptosis on some negative feedback-like loop of cell survival signaling in cells demonstrating long term elevation of [Ca$^{2+}$]i.

In conclusion, we found that the Akt kinase pathway was regulated by a long term change of [Ca$^{2+}$]i, through transcriptional regulation of PP2Ac. With an elevation of [Ca$^{2+}$]i, induced by thapsigargin, PP2Acα gene expression is up-regulated through the activation of CRE at a late phase of the response. As a consequence, Akt is dephosphorylated and inactivated by PP2A, and this leads to an increase in susceptibility to apoptosis under the conditions with thapsigargin. Although the activation of CRE has been considered to function as a cell survival signaling, Ca$^{2+}$-induced late activation of CRE leads to an enhancement of apoptotic signaling, and this suggests some feedback mechanism of CRE-mediating cell survival signaling. Luo et al. (34) reported that NMDA-induced deactivation of Akt was causative of neural cell death, and this suggests some Ca$^{2+}$-dependent mechanism is involved in the inactivation of Akt. Although the Ca$^{2+}$-dependent regulation of cell survival and death has been extensively studied (1, 3, 4), the
Ca$^{2+}$-dependent Regulation of Akt

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