Journal of Cellular Biochemistry 100:653–664 (2007)

18β-Glycyrrhetinic Acid Promotes Src Interaction With Connexin43 in Rat Cardiomyocytes

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Abstract The mechanism by which 18β-glycyrrhetinic acid regulates gap junction intercellular communication (GJIC) remains poorly understood. In this study, treatment of cultured rat neonatal cardiomyocytes with 18β-glycyrrhetinic acid resulted in dose-dependent inhibition of GJIC as assessed by fluorescent dye transfer analysis. 18β-Glycyrrhetinic acid induced time-dependent serine/threonine dephosphorylation and redistribution of connexin43 (Cx43) in cardiomyocytes and the induced Cx43 dephosphorylation was prevented by the protein phosphatase inhibitor, calyculin A. However, functional analyses showed that the inhibitory effect of 18β-glycyrrhetinic acid on dye spreading among cardiomyocytes was not blocked by calyculin A, but was blocked by the Src-selective tyrosine kinase inhibitor, PP2. 18β-Glycyrrhetinic acid also induced an increase in the levels of phosphorylated Src, and this effect was prevented by PP2. Immunoprecipitation using anti-Cx43 and anti-p-Src antibodies showed that 18β-glycyrrhetinic acid increased the association between p-Src and Cx43 and induced tyrosine phosphorylation of Cx43. We conclude that the inhibitory effect of 18β-glycyrrhetinic acid on GJIC in cardiomyocytes involves Src-mediated tyrosine phosphorylation of Cx43. J. Cell. Biochem. 100: 653–664, 2007.

Key words: gap junction; calyculin A; tyrosine phosphorylation of Cx43; Src kinase

Gap junctions, intercellular communicating channels enabling adjacent cells to directly exchange ions, metabolites, and small molecules with molecular weights less than about 1 kDa, play a crucial role in cardiac development and disease [Wei et al., 2004]. A complete gap junction channel is composed of two hexameric hemichannels, each provided by one of the two communicating cells. Each hemichannel consists of six connexin (Cx) molecules, each of which crosses the plasma membrane four times. In cardiac tissue, gap junctions allow the propagation of electrical activation among cardiomyocytes and the synchronous contraction of the myocardium. The major gap junction protein of mammalian cardiomyocytes is Cx43. Suppression of Cx43 expression by Cx43 antisense oligonucleotide reduces gap junction intercellular communication (GJIC) and is associated with an increase in apoptotic death of cultured rat cardiomyocytes [Yasui et al., 2000]. Several isoforms of Cx43, including the non-phosphorylated (Cx43-NP or Cx43-P0) and phosphorylated (Cx43-P1 and Cx43-P2) forms, have been identified [Lampe and Lau, 2004]. The presence of Cx43-P2 has been correlated with the assembly of gap junctional plaques, and dephosphorylation promotes internalization of Cx43 into the cytoplasm [Musil et al., 1990; Guan et al., 1996]. An array of kinases, including protein kinase C (PKC), protein tyrosine kinases, and mitogen activated protein kinases (MAPK), has been shown to phosphorylate various sites in the C-terminal region of Cx43, giving rise to various phosphorylated forms [Lau et al., 1996; Warn-Cramer et al., 1998; Doble et al., 2000; Bowling et al., 2001]. Changes in Cx43 phosphorylation state have dramatic effects on GJIC by affecting electrical and chemical coupling, and are associated with a change in GJIC in cardiomyocytes [Laird et al., 1991; Musil and Goodenough, 1991; Saez

Grant sponsor: National Science Council of the Republic of China; Grant number: NSC-92-2320-B-002-215.

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Received 7 April 2006; Accepted 19 May 2006

DOI 10.1002/jcb.21018

et al., 1997; Lampe and Lau, 2000]. There is ample evidence suggesting that GJIC is regulated by phosphorylation of Cx43 tyrosine residues (Tyr265 and Tyr247 by Src) or serine residues (Ser368 by PKC and Ser255, Ser279, and Ser282 by MAPK) [Lampe and Lau, 2004]. Binding of activated c-Src to the C-terminal domain of Cx43 and the subsequent phosphorylation of Tyr265 causes inhibition of gap junction coupling in transfected Rat-1 fibroblasts and cardiomyocytes [Giepmans et al., 2001; Toyofuku et al., 2001].

Glycyrrhetinic acid (GA), an aglycone saponin extracted from licorice root, which is known for its anti-inflammatory and anti-ulcerous activities, has been widely used as a potent inhibitor of GJIC [Ulmann et al., 1975; Pinder et al., 1976; Armanini et al., 1982]. Of the various GA derivatives, 18α-GA blocks GJIC in human fibroblasts and rat alveolar cells by a mechanism independent of mineralocorticoid or glucocorticoid receptors [Davidson et al., 1984; Regan and Guo, 1997]. In isolated rat hearts, 18α-GA reduces the cell-to-cell diffusion of fluorescent dye and inhibits the propagation of rigor contracture among cardiomyocytes subjected to metabolic inhibition [Ruiz-Meana et al., 2001]. In rat liver epithelial and adrenocortical cells, 18β-GA blocks dye-coupling between cells and disassembles gap junctional plaques [Guan et al., 1996; Huang et al., 2003]. 18β-GA also induces electrical uncoupling between smooth muscle and endothelial cells in guinea pig mesenteric arterioles [Yamamoto et al., 1998]. Although 2 h treatment with GA has been suggested to induce a conformational change in the hemichannel [Davidson and Baumgarten, 1988] and the GA-mediated disassembly of gap junction plaques is attributed to phosphatase-mediated dephosphorylation of Cx43 [Guan et al., 1996; Huang et al., 2003], the mechanisms that underlie the action of GA in regulating gap junction function remain poorly understood. In the present study, we examined the short-term (<1 h) effect of 18β-GA on gap junctions in cultured neonatal rat cardiomyocytes by fluorescent dye scrape loading, microinjection, immunoblotting, and immunoprecipitation using antibodies specific for Tyr416-phosphorylated Src (p-Src), the active form of Src. We present evidence that 18β-GA promotes Src association with Cx43 and induces an increase in p-Src and tyrosine phosphorylation of Cx43. The mechanisms involved in the Src-mediated 18β-GA inhibition of GJIC are discussed.

MATERIALS AND METHODS

Reagents and Antibodies

Protein G-Sepharose bead slurry was purchased from Pharmacia (Uppsala, Sweden). Calyculin A was purchased from Calbiochem (San Diego, CA). 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was purchased from Biomol International L.P. (Plymouth Meeting, PA). Affinity purified, rabbit polyclonal antibodies against both phosphorylated and non-phosphorylated Cx43 (total Cx43, 71-0700) and mouse monoclonal antibody against non-phosphorylated Cx43 (Cx43-NP, 13-8300) were purchased from Zymed (San Francisco, CA). Affinity purified, rabbit polyclonal antibodies against phospho-Src (Tyr416, #2101) or against Src (#2108) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies against phosphotyrosine (4G10), against c-Src (GD11), or against phospho-Src (Tyr416, 2N8) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal antibody against Cx43 (MAB3067) and FITC-conjugated goat anti-rabbit IgG antibody were purchased from Chemicon (Temecula, CA). Mouse monoclonal antibodies against β-actin or against β-tubulin, 18β-glycyrrhetinic acid, Lucifer Yellow CH (MW 457), and 6-carboxyfluorescein (6-CF) were purchased from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Promega (Madison, WI). Texas red-conjugated horse anti-mouse IgG antibodies were purchased from Vector (Burlingame, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG antibodies and Luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Culture of Neonatal Rat Cardiomyocytes

Rat neonatal cardiomyocyte cultures were prepared from 3-day-old Wistar rat pups of both sexes as described previously [Chung et al., 2004]. The heart was dissected out and the ventricles isolated and washed with Ca^{2+}-/Mg^{2+}-free Hank's balanced salt solution (HBSS, Sigma) to remove excess blood, then minced on a watch-glass, and incubated for 8 min at 37°C

in HBSS with 0.125% trypsin/1 mM EDTA (GIBCO, Grand Island, NY) and 0.083% collagenase type II (Sigma). The dissociated cells were collected, mixed well with ice-cold plating medium (10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in minimal essential medium (MEM) containing Earle's salts; all from GIBCO), and collected by centrifugation at 160*g* for 10 min. The cells were resuspended in 10 ml of plating medium, preplated onto a 10 cm culture dish, and maintained in a 5% CO_2 incubator at 37°C for 2 h to remove contaminating fibroblasts. After fibroblast attachment, the suspended cells were collected, diluted with plating medium, and grown at 37°C on rat tail collagen-coated 35 mm culture dishes or glass coverslips at a density of $2.5–4.5 \times 10^4$ cells/cm^2. The plating medium was replaced with growth medium (10% calf serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin in MEM; all from GIBCO) the day after plating.

Drug Treatment of Cardiomyocytes

For time-course studies, cardiomyocytes were washed three times with growth medium without serum, then treated for 15, 30, or 60 min at 37°C with 5 μM 18β-GA. For protein phosphatase and Src tyrosine kinase inhibition studies, cardiomyocytes were treated for 30 min at 37°C with 5 μM 18β-GA alone or together with a protein phosphatase inhibitor (10 nM calyculin A) or a Src family-selective tyrosine kinase inhibitor (30 nM PP2). All compounds were prepared as stock solutions dissolved in dimethyl sulfoxide (DMSO) and were added to growth medium in the absence of serum at less than 0.1% of the final volume.

Immunofluorescence Microscopy

Cardiomyocytes cultured on coverslips were fixed in cold acetone for 5 min at −20°C. After rehydration with a brief wash of phosphate-buffered saline (PBS), pH 7.4, they were incubated for 90 min at 37°C with a 1:100 dilution of rabbit antibodies against total Cx43 (71–0700), then for 90 min at 37°C with a 1:50 dilution of mouse antibodies against Cx43-NP (13–8300). For staining of p-Src, cardiomyocyte cultures were fixed with 10% formalin in PBS for 10 min at room temperature. After washing in PBS, they were permeabilized with 0.2% (v/v) Triton X-100 for 5 min, quenched in PBS containing 0.1% sodium borohydride for another 5 min, and blocked in Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing 1% bovine serum albumin for 1 h at room temperature. Cultures were incubated overnight at 4°C with a 1:50 dilution of rabbit antibodies against phospho-Src (2N8), then for 90 min at 37°C with a 1:100 dilution of mouse antibodies against total Cx43 (MAB3067). Bound primary antibodies were detected by incubation for 90 min at room temperature with a mixture of FITC-conjugated goat anti-rabbit IgG antibodies and Texas red-conjugated goat anti-mouse IgG antibodies (both diluted 1:50). After washing in PBS, the cultures were mounted in a mixture of 2% *n*-propyl gallate and 60% glycerol in 0.1 M PBS, pH 8.0, and sealed with nail polish. The immunofluorescence-labeled cardiomyocytes were examined using a Zeiss Axiophot microscope (Carl Zeiss, Oberkocheu, Germany) equipped with epifluorescence, and the images acquired and digitized using a Nikon D1X digital camera (Nikon, Tokyo, Japan).

Immunoprecipitation

Immunoprecipitation was performed using a modification of previously described method [Toyofuku et al., 1998; Wu et al., 2003]. Cardiomyocytes were washed three times with ice-cold PBS, then extracted for 30 min at 0°C with 100 μl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin) containing phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4), sonicated for 30 s, and insoluble material removed by centrifugation at 13,000*g* for 30 min at 4°C. The cell lysate was pre-cleared by addition of 10 μl of protein G-Sepharose bead slurry, incubation at 4°C for 30 min on a rocker, and removal of the protein G-Sepharose beads by centrifugation at 13,000*g* for 10 min at 4°C. The supernatant was transferred to a microtube and mixed overnight at 4°C with constant rocking with 10 μl of rabbit polyclonal antibodies against total Cx43 (71–0700) or against phospho-Src (#2101), then 50 μl of protein G-Sepharose bead slurry was added and the suspension mixed for 1 h at 4°C. The Sepharose-bound immune complexes were then spun down by centrifugation at 13,000*g* for 1 min at 4°C and washed five times with 50 mM Tris-HCl, pH 7.5,

1% Triton-X 100, 1 mM EDTA, and 1 mM PMSF, then the pellets were heated for 5 min at 95°C in 100 μl of Laemmli sample buffer, and the supernatant collected and used for SDS–PAGE.

Detergent Extraction and Immunoblotting

To obtain Triton-soluble and Triton-insoluble fractions, cardiomyocytes were lysed in RIPA buffer lacking NP-40, but containing 1% Triton-X 100, as described previously [Li et al., 2005]. The cell lysates were sonicated, then centrifuged at 13,000*g* for 30 min at 4°C. The Triton-soluble supernatant was collected and transferred to an Eppendorf microtube and the protein concentration determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). An equal volume of 2× Laemmli sample buffer was added to the cell lysate, then the samples were boiled for 5 min, run on a 10% SDS polyacrylamide gel and electrophoretically transferred to PROTRAN® BA85 nitrocellulose membranes (PerkinElmer Life Science, Boston, MA). Strips of the membranes were blocked for 1 h at room temperature in Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, pH 8.2; TBS) containing 5% skimmed milk and 0.1% Tween-20, then incubated overnight at 4°C with rabbit antibodies against total Cx43 (1:500 dilution in TBS) or with mouse antibodies against Cx43 (1:500), Cx43-NP (1:250), phosphotyrosine (1:1000), or c-Src (1:200), followed by incubation for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies (both 1:7,500 dilution in TBS), as appropriate. Bound antibody was detected by substrate development using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 100 mM Tris-base, 5 mM $MgCl_2$, pH 9.5. In membrane stripping experiments, the blots were stripped using 25 mM glycine-HCl, pH 2.0, 1% (w/v) SDS, and reprobed using a 1:1,000 dilution of mouse antibody against β-actin, a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibody, and Western Blotting Luminol Reagent (Santa Cruz) and Hyperfilm ECL (Amershampharmacia Biotech, Buckinghamshire, England, UK) before X-ray film exposure and development. Bands on nitrocellulose membranes and films were scanned and quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD).

Microinjection and Scrape-Loading Dye Transfer Analyses

Microinjection was performed using a micromanipulator with a glass capillary micropipette connected to a Picospritzer II (Parker Hannifin Co., Fairfield, NJ) as described previously [Chung et al., 2004]. Cardiomyocytes cultured on glass coverslips in culture dishes were transferred to the stage of an inverted Nikon Diaphot microscope equipped with epifluorescence and a Nikon Coolpix 995 digital camera (Nikon). Clusters of synchronously beating cardiomyocytes were selected and a single cell within each cluster microinjected using a micropipette loading with 2.5% Lucifer Yellow CH in PBS, then the cells were examined for dye coupling 10 s later. The images were captured using the digital camera and the percentage of coupling (percentage of injections resulting in dye transfer to other cells in the cluster) assessed using Adobe Photoshop imaging software.

Scrape-loading was performed and dye transfer assessed as described previously with minor modifications [Li et al., 2005]. Briefly, cardiomyocytes were grown to confluence in 35 mm culture dishes, scrape-loaded for 3 min with 0.05% 6-CF in HBSS, and fixed with 1% formalin. After washing in PBS, the culture dishes were transferred to a Nikon Diaphot microscope and the images captured using a Nikon D70 digital camera (Nikon). The spread of 6-CF was measured and analyzed in several fields at the center of the scrape line using Scion Image software (Frederick, MD).

Statistical Analysis

All experimental data are the mean ± SD of at least three independent experiments from three different preparations. The difference between the means was considered statistically significant when the P-value was < 0.05 by Student's t test.

RESULTS

18β-GA Induces Dose-Dependent Inhibition of Dye Coupling Between Cardiomyocytes

The extent of dye coupling between cultured rat neonatal cardiomyocytes was evaluated by intracellular microinjection of Lucifer yellow CH. In DMSO-treated control cells, considerable dye transfer between the microinjected

cardiomyocytes and adjacent cells was seen (Fig. 1A,C). The effect of 18β-GA on dye coupling was assessed by pretreating the cells with 18β-GA for 15 min before Lucifer yellow CH microinjection. As shown in Figure 1E, dye coupling was inhibited in a dose-dependent manner ($IC_{50} = 1.78$ μM). The concentration of 5 μM 18β-GA was chosen for use throughout the rest of this study; Figure 1B,D shows the effect of 15 min treatment with 5 μM 18β-GA on dye coupling between cardiomyocytes.

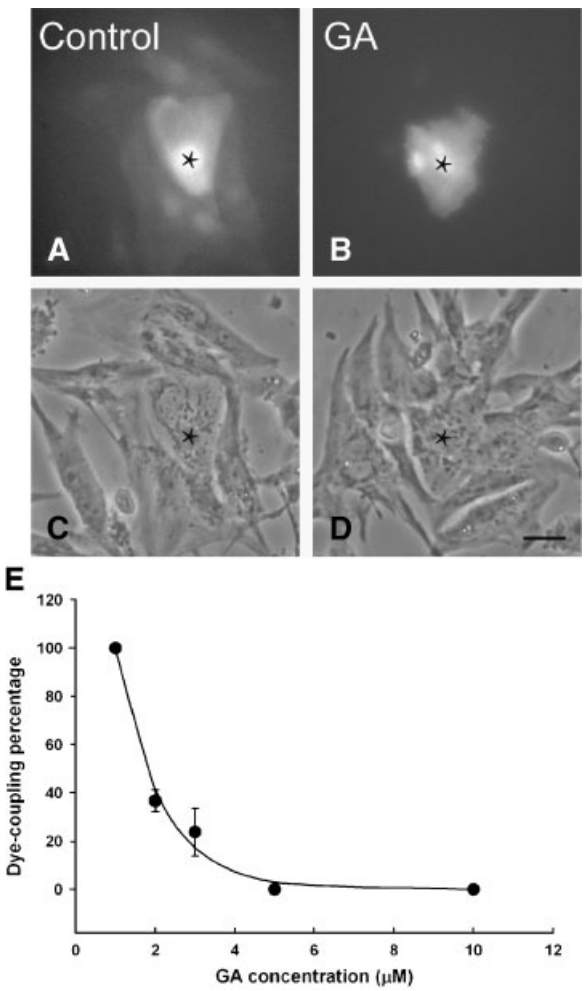

Fig. 1. Dose-dependent effect of 18β-GA on dye coupling in cultured cardiomyocytes. Cardiomyocytes treated for 15 min with 0.1% DMSO (Control) (**A, C**) or 5 μM 18β-GA (GA) (**B, D**) were microinjected with Lucifer yellow CH (*) and examined for dye coupling 10 s later. C and D are phase-contrast micrographs of the fluorescent field shown in A and B, respectively. Bar = 20 μm. **E**: Cardiomyocytes treated for 15 min with 1, 2, 3, 5, or 10 μM 18β-GA were assayed for dye coupling by microinjection of Lucifer yellow CH. The percentage of dye-coupling is the percentage of injections resulted in dye transfer to neighboring cardiomyocytes. Fitting of the data to a non-linear regression curve gave an IC_{50} of 1.78 μM. Each point represents the mean ± SD for the percentage of dye-coupling for three cultures.

Effects of 18β-GA on Cx43 Immunoreactivity of Cardiomyocytes

In a time-course study, double immunofluorescence staining with rabbit antibody against total Cx43 and mouse antibody against Cx43-NP showed changes in Cx43 immunoreactivity after treatment for various times with 18β-GA. In control cardiomyocytes, aggregates of total Cx43 staining were seen at cell–cell contacts corresponding to gap junction plaques (Fig. 2A), while little or no Cx43-NP labeling was detected (Fig. 2B). Although no obvious change in total Cx43 staining was seen at gap junction plaques after 15 min of 18β-GA treatment (Fig. 2D), Cx43-NP staining was clearly detected (Fig. 2E). Treatment with 18β-GA for 30–60 min resulted in a decrease in staining for total Cx43 (Fig. 2G,J) and Cx43-NP (Fig. 2H,K) at gap junctions and a corresponding increase in cytoplasmic staining. Cx43-NP staining colocalized with total Cx43 staining at both gap junctional plaques (Fig. 2A–L, arrows) and in the cytoplasm (Fig. 2G,H,J,K, arrowheads).

18β-GA Induces Global Cx43 Dephosphorylation in Cardiomyocytes

In order to investigate the correlation between phosphorylation status and Cx43 distribution in cardiomyocytes after 18β-GA treatment, whole cell lysates were analyzed by immunoblotting. In control lysates, three major bands, a fast-migrating band (Cx43-P0) and a slow-migrating doublet (Cx43-P1 and Cx43-P2), were detected using antibody against total Cx43 (Fig. 3A, top panel), and a single band was detected using antibody against Cx43-NP (Fig. 3B, top panel) as reported previously [Chung et al., 2004]. Treatment with 18β-GA for 30 min induced dephosphorylation of Cx43, as shown by a decrease in the levels of phosphorylated Cx43 (Cx43-P2 plus Cx43-P1) and a concurrent increase in the levels of Cx43-P0 (Fig. 3A) and Cx43-NP (Fig. 3B) compared to controls. When control cells were treated for 30 min with calyculin A, an inhibitor of protein phosphatases type 1 and type 2A, background Cx43 dephosphorylation was inhibited and phosphorylated Cx43 levels increased, with a concurrent decrease in Cx43-P0 and Cx43-NP (Fig. 3A,B). In addition, co-treatment with

calyculin A prevented 18β-GA-induced Cx43 dephosphorylation (Fig. 3A,B). Densitometric analysis showed that calyculin A significantly prevented the 18β-GA-induced dephosphorylation of Cx43 and the increase in Cx43-P0 and Cx43-NP levels ($P < 0.05$) (Fig. 3A,B, bottom panels). Since it is well established that cytoplasmic Cx43 is extractable by the non-ionic detergent, Triton X-100 [Musil and Goodenough, 1991], we examined changes in Cx43-NP levels in the Triton X-100-soluble fraction after different lengths of treatment with 18β-GA, and found a marked time-dependent increase (Fig. 4A,B).

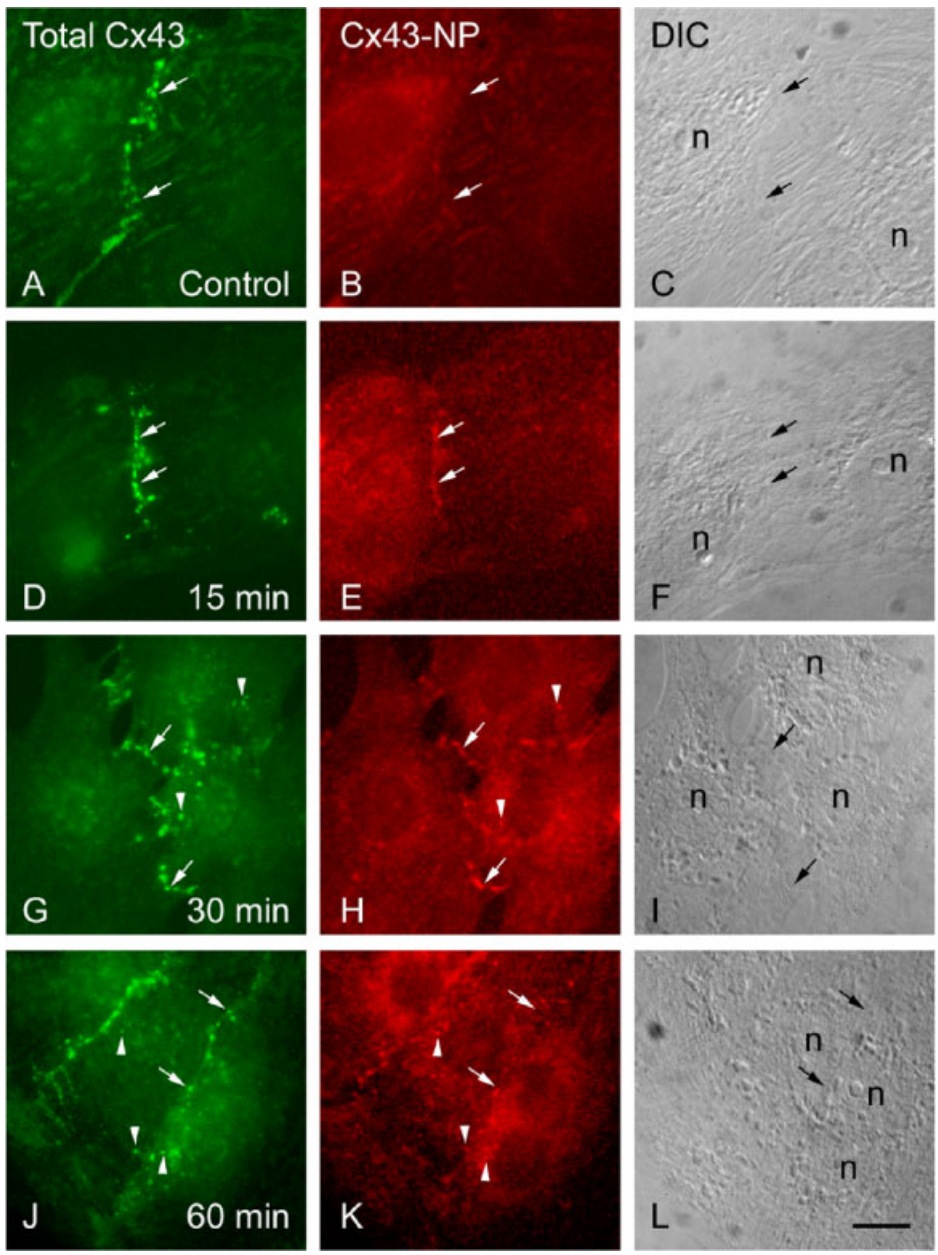

Fig. 2. Double immunofluorescence staining of cardiomyocytes showing that 18β-GA treatment results in dephosphorylation of Cx43. Cardiomyocytes treated with 0.1% DMSO for 60 min (Control) (**A**–**C**) or with 5 μM 18β-GA for 15 min (**D**–**F**), 30 min (**G**–**I**), or 60 min (**J**–**L**) were double-labeled with antibody recognizing both phosphorylated and non-phosphorylated Cx43 (**left panels**) (total Cx43) and antibody recognizing only non-phosphorylated Cx43 (**center panels**) (Cx43-NP). The arrows in the left panels indicate the junctional distribution of total Cx43 and those in the center panels the corresponding position on the Cx43-NP-stained samples. The arrowheads indicate the cytoplasmic distribution of total Cx43 (G, J) and Cx43-NP (H, K), respectively. C, F, I, and L are DIC images of (A, B), (D, E), (G, H), and (J, K), respectively. n, nucleus of cardiomyocytes (Bar = 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

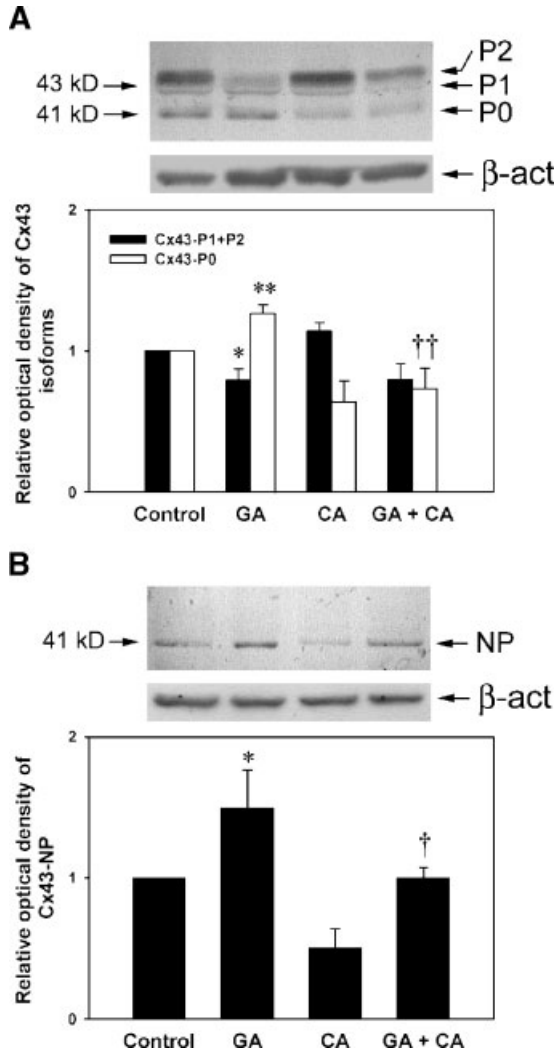

Fig. 3. Western blots of total cell lysates showing that calyculin A prevents 18β-GA-induced Cx43 dephosphorylation. Cardiomyocytes treated for 30 min with 0.1% DMSO (Control), 5 μM 18β-GA (GA), 10 nM calyculin A (CA), or 5 μM 18β-GA plus 10 nM calyculin A (GA + CA) were harvested and the cell lysates analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. Blots were probed with antibody against total Cx43 (**A**, **top panel**) or antibody specific for Cx43-NP (**B**, **top panel**). The blots were then stripped at a low pH and re-probed with β-actin antibody (β-act) (**center panels**) as an internal loading control. The **bottom panels** in A and B are, respectively, densitometric analyses of total Cx43 and Cx43-NP blots from three experiments. Staining is expressed relative to that of the untreated control. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control sample, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to the GA sample (unpaired Student's *t* test).

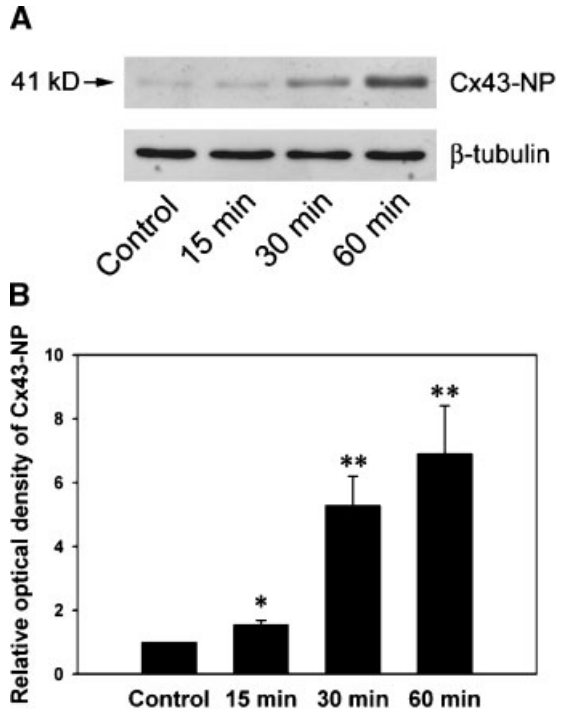

Fig. 4. Western blots of the Triton X-100-soluble fraction of cardiomyocytes showing time-dependent increase of Cx43-NP after treatment with 18β-GA. Cardiomyocytes treated with 0.1% DMSO for 60 min (Control) or with 5 μM 18β-GA for 15 min (15 min), 30 min (30 min), or 60 min (60 min) were lyzed in RIPA buffer containing 1% Triton X-100, and the Triton-soluble fractions were collected by centrifugation and analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting using antibody specific for Cx43-NP. **A**: Typical blots (**upper panel**); the blots were then stripped at a low pH and re-probed with β-tubulin antibody (β-tubulin) as an internal loading control. **B**: Densitometric analyses of the Cx43-NP blots from three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control sample (unpaired Student's *t* test).

PP2 Blocks 18β-GA-Induced Inhibition of Dye-Spreading in Cardiomyocytes

Functional analyses assessed by scrape-loading with 6-CF were performed to evaluate the effects of 18β-GA on dye spreading in cardiomyocytes. In accordance with the micro-injection results (Fig. 1), considerable dye transfer occurred in control cardiomyocytes (Fig. 5A). Treatment with 18β-GA for 30 min inhibited dye-spreading (Fig. 5B), but co-treatment with calyculin A failed to prevent this effect (Fig. 5C). Constitutively active c-Src has been shown to disrupt GJIC in transfected cells [Giepmans et al., 2001]. Although treatment with the Src-specific tyrosine kinase inhibitor, PP2, alone had no significant effect on dye transfer, the inhibitory effect of 18β-GA on GJIC was blocked by co-treatment with PP2 (Fig. 5D). The effects of various drug treatments on dye transfer distance were quantified and are summarized in Figure 5E, confirming the above results.

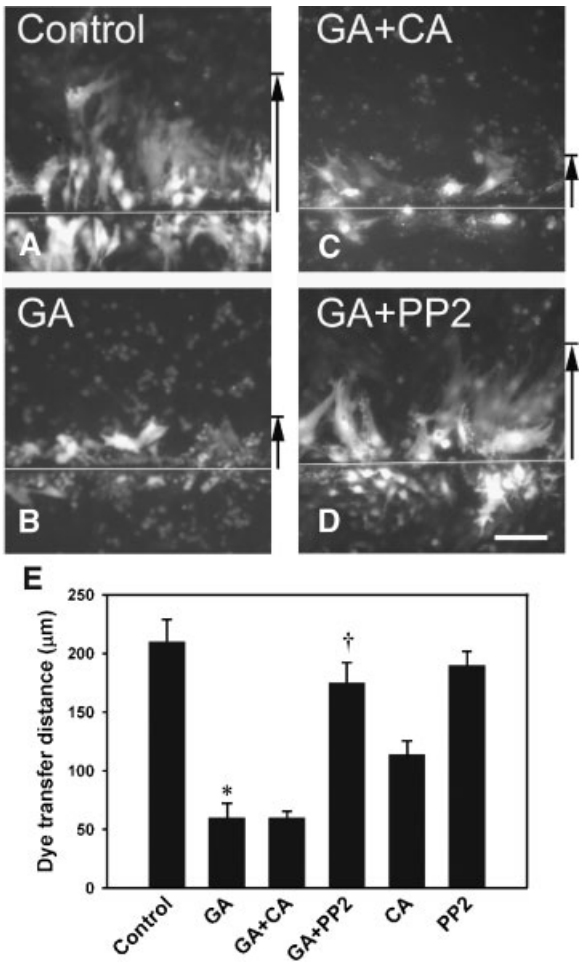

Fig. 5. PP2, but not calyculin A, blocks the inhibitory effect of 18β-GA on dye-spreading. Cardiomyocytes treated for 30 min with 0.1% DMSO (Control), 5 μM 18β-GA (GA), 10 nM calyculin A (CA), or 30 nM PP2 (PP2), or co-treated with 18β-GA and calyculin A (GA + CA), or with 18β-GA and PP2 (GA + PP2) were subjected to 6-CF scrape-loading as described in the Materials and Methods, then dye migration, which is defined as the distance of dye spreading from the scrape line and equals to the length of the indicated arrow, was measured (**A–D**). Bar = 100 μm. **E**: Each bar represents the mean ± SD for the dye transfer distance for three cultures from three experiments. $^{*}P < 0.01$, compared to the control sample, $^{\dagger}P < 0.01$, compared to the GA sample (unpaired Student's *t* test).

18β-GA Induces Src Phosphorylation

Immunoblot analyses and densitometry were performed to test whether 18β-GA induced Src phosphorylation and whether PP2 prevented this effect. In a time-course study, 18β-GA induced a progressive increase in p-Src levels, which peaked at 30 min of 18β-GA treatment (data not shown). The increase in p-Src levels caused by 30 min treatment with 18β-GA was prevented by co-treatment with PP2 (Fig. 6). Double immunofluorescence staining with rabbit antibody against p-Src and mouse antibody against total Cx43 showed changes in p-Src immunoreactivity after 18β-GA treatment. In control cardiomyocytes, only weak p-Src staining was seen at cell–cell contact sites (Fig. 7A). In contrast, a dramatic increase in p-Src at gap junction was detected after 18β-GA treatment (Fig. 7C), which was attenuated by co-treatment with PP2 (Fig. 7E).

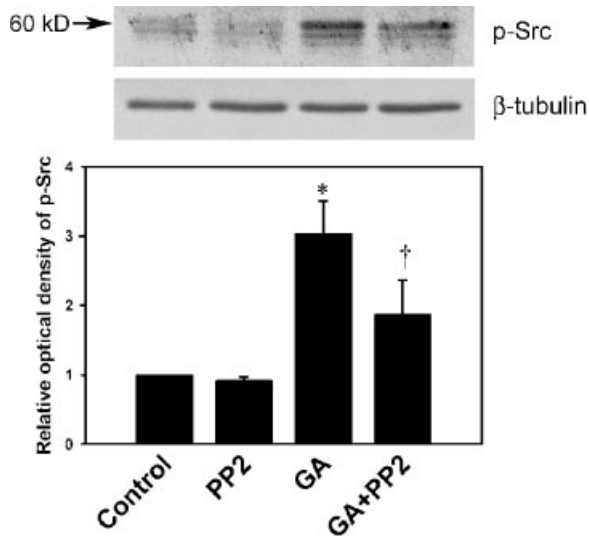

Fig. 6. 18β-GA treatment increases p-Src levels and PP2 prevents this effect. Control cardiomyocytes treated for 30 min with 0.1% DMSO (Control), 30 nM PP2 (PP2), or 5 μM 18β-GA (GA) or co-treated for 30 min with 5 μM 18β-GA and 30 nM PP2 (GA + PP2) were harvested, and the cell lysates analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting. Blots were probed with antibody against p-Src (**top panel**), then stripped and re-probed with β-tubulin antibody (**center panel**). The p-Src blots from three experiments were subjected to densitometric analysis (**bottom panel**). $^{*}P < 0.01$, compared to the control sample, $^{\dagger}P < 0.05$, compared to the GA sample (unpaired Student's *t* test).

18β-GA Enhances Association of p-Src With Cx43

To test whether 18β-GA promoted the association between Cx43 and c-Src and induced tyrosine phosphorylation of Cx43, we immunoprecipitated cell extracts using a polyclonal antibody against Cx43 and probed the membrane either with a monoclonal anti-c-Src antibody or with a monoclonal anti-phosphotyrosine antibody. Our results showed that treatment with 18β-GA for 30 min resulted in an increase in Cx43-associated c-Src (Fig. 8A) and slightly increased tyrosine phosphorylation of Cx43 (Fig. 8B). In addition, when the proteins were immunoprecipitated with a polyclonal antibody against p-Src and the membrane probed with a

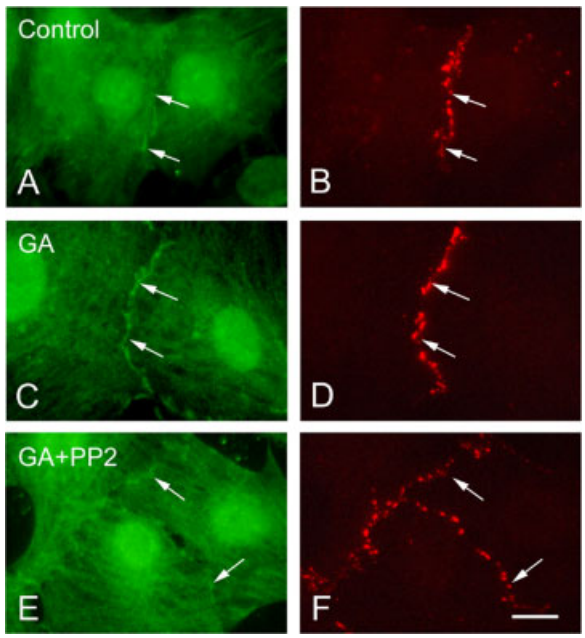

Fig. 7. Effects of PP2 on 18β-GA-induced changes in p-Src immunoraectivity in rat cardiomyocytes. Control cardiomyocytes treated for 30 min with 0.1% DMSO (Control), 5 μM 18β-GA (GA), or co-treated for 30 min with 5 μM 18β-GA and 30 nM PP2 (GA + PP2) double-labeled for p-Src (**A**, **C**, **E**) and total Cx43 (**B**, **D**, **F**). The arrows in the **left panels** indicate the junctional distribution of p-Src and those in the **right panels** the corresponding position on the total Cx43-stained samples. (Bar = 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

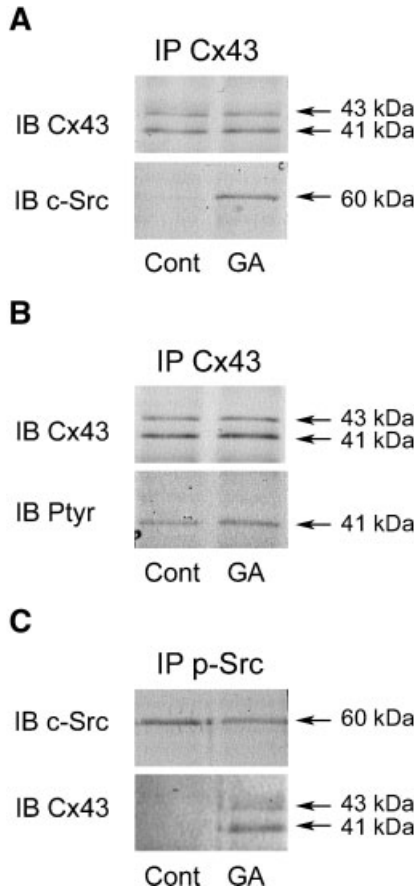

Fig. 8. 18β-GA increases p-Src binding to Cx43. Cardiomyocytes treated for 30 min with 0.1% DMSO (Cont) or 5 μM 18β-GA (GA) were harvested, and the cell lysates immunoprecipitated using rabbit polyclonal antibodies against total Cx43 (**A** and **B**) or p-Src (**C**). The immune complexes were then analyzed for total Cx43 (IB Cx43), phosphotyrosine (IB Ptyr), or c-Src (IB c-Src) by immunoblotting. Arrows on the right indicate the positions of c-Src (60 kDa), Cx43-P1 (43 kDa), and Cx43-P0 (41 kDa), respectively.

monoclonal anti-total Cx43 antibody, p-Src-associated Cx43 levels were increased after 18β-GA treatment (Fig. 8C).

DISCUSSION

In the present study, short-term treatment with 18β-GA induced inhibition of GJIC in cardiomyocytes in a dose-dependent manner ($IC_{50} = 1.78$ μM). Although 18β-GA induced serine/threonine dephosphorylation of Cx43, tyrosine phosphorylation of Cx43 was slightly increased. 18β-GA enhanced the association between Src and Cx43 and induced an increase in p-Src levels, which was inhibited by co-treatment with the Src kinase inhibitor, PP2, suggesting that tyrosine phosphorylation of Cx43 in cardiomyocytes is mediated by activation of Src. In a recent study, Giepmans et al. [2001] showed that constitutively active c-Src phosphorylates Tyr265 in the C-terminal tail of Cx43 and disrupts GJIC in the transfected cells, and that removal of Tyr265 from the C-terminal tail restores GJIC. In the same study, a direct association between Cx43 and c-Src was demonstrated by immunoprecipitation, and it was concluded that a stable interaction between Cx43 and c-Src leads to inhibition of GJIC [Giepmans et al., 2001]. Moreover, in a related study, Lin et al. [2001] demonstrated that activated v-Src phosphorylates both Tyr247 and Tyr265 in Cx43, and that, while Tyr247 phosphorylation is responsible for the closure of Cx43 gap junction channels, Tyr265 phosphorylation by v-Src seems to play a critical role in the subsequent Tyr247 phosphorylation. In a modified "ball-and-chain" model of the gap junction gating mechanism, Cx43 channel closure is proposed to be mediated through Src phosphorylation, and the negatively charged phosphoryl group on Tyr247 is suggested to interact with positively charged amino acids in

the cytoplasmic loop of Cx43, leading to channel blockade [Warn-Cramer and Lau, 2004]. Whether 18β-GA induces phosphorylation of Cx43 at Tyr247 and/or Tyr265 in cardiomyocytes requires further investigation.

In the present study, 18β-GA induced redistribution of Cx43 and cytoplasmic accumulation of Cx43-NP. In a freeze fracture analysis, GA treatment was shown to induce the disassembly of gap junctions by changing the arrangement and increasing the particle space of gap junctional plaques [Goldberg et al., 1996]. In a transmission electron microscopic study, Goldberg et al. [1996] showed relocation of nonannular gap junctions from the plasma membrane to the cytoplasm in 18α-GA-treated rat C6 glioma cells. 18β-GA was also shown to reversibly dephosphorylate Cx43 in rat liver epithelial cells, and this dephosphorylation clearly initiated Cx43 redistribution from gap junctional plaques to the cytoplasm; this Cx43 relocation was not due to Cx43 degradation, as it was not prevented by treatment of the cells with a microtubule inhibitor or a lysosomal enzyme inhibitor [Guan et al., 1996]. Consistent with the results of previous studies, our study showed that 18β-GA-induced Cx43 dephosphorylation in cardiomyocytes was prevented by co-treatment with calyculin A, indicating that it was mediated by a Type 1 or Type 2A protein phosphatase.

The rapid inhibition of GJIC by GA suggests that it acts on existing gap junctions and that the rapid reversal is due to the re-opening of existing gap junctions, rather than the formation of new junctions [Davidson et al., 1986]. In our present study, the short-term inhibitory effect of 18β-GA on GJIC was blocked by co-treatment with PP2, but not by co-treatment with calyculin A, showing that the effect is not mediated by protein phosphatase, but by an Src tyrosine kinase. Although co-treatment with PP2 greatly increased the dye transfer distance in 18β-GA-treated cardiomyocytes, it was unable to completely overcome the effect of 18β-GA (87% of dye transfer distance compared to control cells); this partial recovery may be due to the inability of PP2 to prevent the internalization and disassembly of a proportion of Cx43 gap junctions caused by protein phosphatase. Alternatively, high concentrations of 18α-GA have been shown to downregulate Cx43 mRNA expression in type II alveolar cells [Guo et al., 1999]. Our results showed that total amount of Cx43 was slightly reduced in the samples treated with 18β-GA (Fig. 3A); this may also explain the lack of a complete recovery in GJIC by PP2 in 18β-GA-treated cardiomyocytes. Moreover, retention of gap junctions on the plasma membrane is also regulated by the binding of Cx43 to the membrane-associated guanylate kinase protein, zonula occludens-1 (ZO-1) [Toyofuku et al., 1998; Wu et al., 2003]. The binding of Cx43 to ZO-1 is regulated by c-Src, as constitutively active c-Src inhibits this interaction in cardiomyocytes, while a Cx43 mutant lacking Tyr265 is able to bind to ZO-1 in HEK 293 cells cotransfected with active c-Src [Toyofuku et al., 2001]. Thus, 18β-GA may also exert its inhibitory effect on GJIC by interfering with the binding of Cx43 to ZO-1 via c-Src activation.

In a recent study on cultured astrocytes, Li et al. [2005] showed that chemical ischemia/hypoxia-induced serine/threonine dephosphorylation of Cx43 is accompanied by increased binding of dephosphorylated Cx43 to c-Src and that blockade of Cx43 dephosphorylation by treatment with phosphatase inhibitors prevents this effect. It was suggested that changes in the phosphorylation state of Cx43 regulate binding. In contrast, our results showed that blockade of serine/threonine dephosphorylation of Cx43 by co-treatment with calyculin A did not significantly alter the 18β-GA-induced increase in p-Src levels (data not shown), and that 18β-GA treatment resulted in enhanced binding of p-Src to both phosphorylated and non-phosphorylated Cx43 (Fig. 7C), suggesting that binding of p-Src to Cx43 is independent of the phosphorylation state of Cx43. Moreover, an increase in intracellular H^+ levels by chemically induced acidosis enhances the binding of Cx43 to c-Src [Duffy et al., 2004]. Whether 18β-GA induces an increase in intracellular H^+ levels and whether the effect of 18β-GA on Src activation is mediated via an increase in intracellular H^+ levels in cardiomyocytes requires further investigation.

In conclusion, we have demonstrated that the inhibitory effect of 18β-GA on GJIC in cardiomyocytes is associated with serine/threonine dephosphorylation of Cx43 and concomitant tyrosine phosphorylation of Cx43. The 18β-GA-induced increase in tyrosine phosphorylation of Cx43 as a result of Src activation is probably a cause of gap junction gating, while 18β-GA-induced serine/threonine

dephosphorylation of Cx43 appears to facilitate the internalization of junctional Cx43 into the cytoplasm. Further studies on the relationships between Cx43 and its associated proteins, and the characterization of the phosphatases and kinases regulating Cx43 phosphorylation will help to elucidate the mechanisms by which 18β-GA inhibits GJIC.

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