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Effects of 18-glycyrrhetinic acid on serine 368 phosphorylation of connexin43 in rat neonatal cardiomyocytes

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Abstract

18β-Glycyrrhetinic acid (18β-GA) regulates serine/threonine dephosphorylation of connexin43 (Cx43). Phospho-specific antibodies were used here to determine the effect of 18β-GA on serine 368-phosphorylated Cx43 (pSer368Cx43) in cultured rat neonatal cardiomyocytes by immunofluorescence microscopy and immunoblot analyses. 18β-GA caused a time-dependent increase in pSer368Cx43 levels and induced gap junction disassembly, shown by a change in pSer368Cx43 immunostaining from large aggregates to dispersed punctates at cell–cell contact areas. 18β-GA also induced a time-dependent increase in the levels of serine 729-phosphorylated PKCε, the active form of PKCε. The 18β-GA-induced increase in pSer368Cx43 levels and changes in pSer368Cx43 staining pattern were abolished by the PKC inhibitor, chelerythrine. Furthermore, 18β-GA increased the co-immunoprecipitation of Cx43 with PKCε. However, the 18β-GA-induced increase in pSer368Cx43 levels and increased association of Cx43 with PKCε were inhibited by co-treatment with the protein phosphatase type 1 and type 2A inhibitor, calyculin A. We conclude that 18β-GA induces Ser368 phosphorylation of Cx43 via PKCε.
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Keywords: 18β-Glycyrrhetinic acid; Gap junction; Connexin43; Protein kinase C; Cardiomyocyte

1. Introduction

Gap junctions are aggregates of aqueous channels between contiguous cells that provide a direct route for cytoplasmic diffusion of ions and small molecules (Söhl and Willecke, 2004). Each gap junction channel is formed by 2 connexons (or hemichannels), one from each of the contiguous cells, which dock with the apposing plasma membrane. Each connexon oligomer is composed of 6 connexin (Cx) subunits. The predominant gap junction protein expressed in ventricular cardiomyocytes is Cx43 (Söhl and Willecke, 2004). Multiple phosphorylation sites in the C-terminus of Cx43 have been identified, and phosphorylation of Cx43 has been implicated in regulation of its trafficking, the assembly/disassembly of gap junctions, and channel gating (for review, see Solan and Lampe, 2005). It is well established that serine 368 in the C-terminus of Cx43 is phosphorylated by protein kinase C (PKC) (Sáez et al., 1997; Lampe et al., 2000). Phosphorylation of serine 368 results in a change in function by increasing channel permeability and decreasing channel conductance (Lampe et al., 2000; Ek-Vitorin et al., 2006). PKC-mediated serine 368 phosphorylation of recombinant Cx43 reconstituted into liposomes has been demonstrated to lead to hemichannel gating (Bao et al., 2004). In cultured cardiomyocytes, serine 368 phosphorylation is implicated in the 17β-estradiol-mediated attenuation of chemical ischemia-induced dye uncoupling and oleic acid-induced gap junction disassembly (Chung et al., 2004; Huang et al., 2004).

18β-Glycyrrhetinic acid (18β-GA), a hydrolysis product of the triterpene, saponin, isolated from licorice root, has been used as a gap junction uncoupler, which inhibits gap junction intercellular communication between liver epithelial cells and induces electrical uncoupling between endothelial and smooth

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muscle cells in arterioles (Davidson et al., 1986; Yamamoto et al., 1998). 18β-GA also disassembles gap junctional plaques in liver epithelial and adrenocortical cells, an effect ascribed to phosphatase-mediated dephosphorylation of Cx43 (Guan et al., 1996; Huang et al., 2003). Furthermore, 18β-GA causes dose-dependent inhibition of dye coupling in cardiomyocytes by a mechanism involving tyrosine phosphorylation of Cx43 by Src kinase (Chung et al., 2007). Although 18β-GA has been considered as a PKC inhibitor, O'Brian et al. (1990) have shown that low concentrations of 18β-GA enhance PKC activity. The aims of the present study were therefore to determine whether 18β-GA induces phosphorylation of Cx43 at serine residues, and whether this phosphorylation involves PKC. Our results show that, in cardiomyocytes, 18β-GA induced a selective dephosphorylation of Cx43 concomitant with an increase in the levels of serine 368-phosphorylated Cx43 (pSer368Cx43) and serine 729-phosphorylated PKCε (pPKCε, the active form of PKCε). 18β-GA also induces a time-dependent change of pSer368Cx43-immunoreactive gap junctional plaques from large aggregates to dispersed punctates. The 18β-GA-induced changes in Ser368Cx43 levels and staining pattern of gap junctions were both attenuated by co-treatment with a PKC inhibitor, chelerythrine, indicating a mechanism involving PKC activation.

2. Materials and methods

2.1. Reagents

18β-Glycyrrhetinic acid, mouse anti-β-actin or anti-β-tubulin antibodies, rabbit anti-β-tubulin antibody, nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma–Aldrich (St. Louis, MO). Calyculin A and chelerythrine chloride were purchased from Calbiochem (San Diego, CA). Affinity purified, mouse monoclonal antibody against non-phosphorylated Ser368 of Cx43 (Cx43-NP, 13-8300) and rabbit polyclonal antibody against total Cx43 (71-0700) were purchased from Zymed (San Francisco, CA). Affinity purified goat polyclonal antibody against serine 729-phosphorylated PKCε (pPKCε, sc-12355), affinity purified rabbit polyclonal antibody against PKCε (sc-214), horseradish peroxidase-conjugated goat anti-mouse IgG antibody, and Luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against pPKCε (#06-821) and mouse monoclonal antibody against N-cadherin (#05-915) were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against total Cx43 (MAB3067), rabbit polyclonal antibody against pSer368Cx43 (AB3841), and FITC-conjugated goat anti-rabbit IgG antibody were purchased from Chemicon (Temecula, CA). 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). Protein G-Sepharose bead slurry was purchased from Pharmacia (Uppsala, Sweden).

2.2. Primary culture of rat neonatal cardiomyocytes and drug treatment

Primary neonatal cardiomyocytes were prepared from postnatal day 3 Wistar rat pups of both sexes as described previously with minor modification (Chung et al., 2004). The hearts were isolated, the atria removed, and the ventricles washed with Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Sigma–Aldrich) to remove excess blood, minced on a watch-glass, and incubated for 5 min at 37 °C in HBSS containing 0.125% trypsin/1 mM EDTA (GIBCO, Grand Island, NY) and 0.083% collagenase type II (Sigma–Aldrich). The supernatants containing cell debris and connective tissues were discarded and the residual ventricle fragments subjected to 4 more digestion steps as above; in each of these, the dissociated cells in the supernatants were collected and mixed with an equal volume of ice-cold plating medium (10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in Dulbecco's modified Eagle's medium (DMEM); Sigma–Aldrich). The suspended cells were then pooled, collected by centrifugation at 160*g* for 10 min, resuspended in 10 ml of plating medium, preplated on a 10 cm culture dish, and placed 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 plated at 37 °C at $3–4 \times 10^4$ cells/cm^2 on rat tail collagen-coated 35 mm culture dishes or glass coverslips. On the day after plating, the plating medium was replaced with growth medium (10% calf serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin in DMEM) and was then changed every 2 days. The percentage of cardiomyocytes was greater than 90% as determined by the proportion of cells showing spontaneous contraction. The cultures were used 4 days after plating.

For the time-course study, cardiomyocytes were washed 3 times in growth medium without serum, incubated for 15, 30, or 60 min at 37 °C with 5 μM 18β-GA (Chung et al., 2007). For PKC and protein phosphatase inhibition studies, cardiomyocytes were treated for 30 min at 37 °C with 5 μM 18β-GA alone or combined with a PKC inhibitor (0.5 or 5 μM chelerythrine) or a protein phosphatase inhibitor (10 nM calyculin A). All compounds were stock solutions in dimethyl sulfoxide (DMSO) in the absence or presence of alkaline phosphatase (8 unit/10 μg) and were added to growth medium in the absence of serum at less than 0.1% of the final volume; 0.1% DMSO was added to the controls. The cardiomyocytes were processed for immunofluorescence microscopy, immunoprecipitation, or immunoblot analysis.

2.3. Immunofluorescence microscopy

Cardiomyocyte cultures were fixed in cold acetone for 5 min at −20 °C as described previously (Chung et al., 2004). After a brief wash with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4; PBS), they were incubated for 90 min at 37 °C with a 1:100 dilution of rabbit antisera against pSer368Cx43, then

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