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Effects of 18β-Glycyrrhetinic Acid on the Junctional Complex and Steroidogenesis in Rat Adrenocortical Cells

Shih-Horng Huang,[1] Jiahn-Chun Wu,[2] Ra-Der Hwang,[2] Hui-Lin Yeo,[2] and Seu-Mei Wang[2]*

[1]Far Eastern Memorial Hospital, Department of Surgery and Division of General Surgery, Taipei, Taiwan 220
[2]Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan 100

Abstract Cellular junctions play important roles in cell differentiation, signal transduction, and cell function. This study investigated their function in steroid secretion by adrenal cells. Immunofluorescence staining revealed the presence of gap junctions and adherens junctions between adrenal cells. The major gap junction protein, connexin43, was seen as a linear dotted pattern of the typical gap junction plaques, in contrast to α-, β-, and γ-catenin, which were seen as continuous, linear staining of cell–cell adherens junction. Treatment with 18β-glycyrrhetinic acid, a gap junction inhibitor, reduced the immunoreactivity of these proteins in a time- and dose-dependent manner, and caused the gap junction and adherens junction to separate longitudinally from the cell–cell contact sites, indicating the structural interdependency of these two junctions. Interestingly, 18β-glycyrrhetinic acid stimulated a two- to three–fold increase in steroid production in these adrenal cells lacking intact cell junctions. These data raise the question of the necessity for cell communication for the endocrine function of adrenal cells. Pharmacological analyses indicated that the steroidogenic effect of 18β-glycyrrhetinic acid was partially mediated by extracellular signal-related kinase and calcium/calmodulin-dependent kinase, a pathway distinct from the protein kinase A signaling pathway already known to mediate steroidogenesis in adrenal cells. J. Cell. Biochem. 90: 33–41, 2003.

Key words: 18β-glycyrrhetinic acid; gap junction; adherens junction; steroidogenesis; adrenocortical cells; signaling pathway

Gap junctions are intercellular protein channels involved in the exchange of ions and small molecules. In addition to providing the structural basis for electrical coupling, they also permit metabolic coupling between neighboring cells. Most studies on the junctional complex in adrenocortical cells have focused on the gap junction [Meda et al., 1993; Murray et al., 1995; Munari-Silem et al., 1995; Murray and Pharrams, 1997]. The main gap junction protein, connexin (Cx), consists of several isoforms with different molecular weights [Goodenough et al., 1996]. Among them, Cx43 molecules are named nonphosphorylated form (NP) and phosphorylated forms, P′, P1, P2, and P3, on the basis of different phosphorylation levels on Cx43 [Musil et al., 1990; Budunova et al., 1993]. Cx43 is the isoform predominantly expressed in adrenocortical cells in several species [Murray and Pharrams, 1997]. The development of gap junctions in the different zones of the adrenal cortex of postnatal rats is hormonally- and temporally-regulated [Palacios, 1979]. The importance of gap junctions and cell communication in steroid secretion is well established [Munari-Silem et al., 1995; Oyoyo et al., 1997]. The density of gap junctions varies

Abbreviations used: 18βGA, 18β-glycyrrhetinic acid; PKA, protein kinase A; ERK, extracellular signal-related kinase; CaMK, calcium/calmodulin-dependent kinase; MAPK, mitogen-associated protein kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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*Correspondence to: Seu-Mei Wang, Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, 1-1 Jen-Ai Road, Taipei, Taiwan 100.
E-mail: smwang@ha.mc.ntu.edu.tw

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in different adrenal cortex zones and is high in the zona fasciculata and reticularis [Murray et al., 1995; Murray and Pharrams, 1997; Murray et al., 2000], reflecting the connection between morphological and functional differences. Gap junctions mediate communication between hormone-responsive and nonresponsive adrenal cells, since the gap junction blocker, 18β-glycyrrhetinic acid (18βGA), markedly decreases the steroidogenic response to a sub-maximal dose of adrenocorticotrophic hormone (ACTH) [Munari-Silem et al., 1995]. Furthermore, transfection with Cx43 antisense DNA greatly reduces the steroidogenic response to ACTH [Shah and Murray, 2001]. ACTH increases Cx43 expression via an increase in cAMP levels and stimulates the secretory activity of adrenal cells [Murray and Taylor, 1988; Murray et al., 1995]. In contrast, human chorionic gonadotropin increases rat Leydig cell steroidogenesis, but reduces the expression of Cx43 mRNA and protein [You et al., 2000]. Thus, the relationship between Cx expression and sterodiogenesis varies in different steroidogenic cells.

The adherens junction is a complex of proteins, including transmembrane cadherin and its associated proteins, α-, β-, and γ-catenin [Ozawa et al., 1989; Takeichi, 1990], and is involved in the formation and function of gap junctions. During the assembly of intercalated discs in rat adult cultured cardiomyocytes, the adherens junction appears prior to the assembly of Cx43 into gap junction plaques [Hertig et al., 1996; Kostin et al., 1999]. Treatment of teratocarcinoma PCC cells with anti-E cadherin antibody results in the failure of dye coupling via gap junctions [Kanno et al., 1984], incorporation of anti-N-cadherin antibodies into hepatoma cells inhibits dye transfer and gap junction assembly [Meyer et al., 1992], and the expression of an N-cadherin mutant in rat cardiomyocytes results in disassembly of gap junctions [Hertig et al., 1996]. Although both cortical and medullary adrenal tumors express N-cadherin [Khorram-Manesh et al., 2002], the expression pattern of the cadherin/catenin complex in the adherens junction of adrenal cells has not yet been established. Furthermore, the interrelation between the gap junction and adherens junction and whether the gap junction is important for the endocrine function of adrenal cells remain to be elucidated. In this study, we investigated the effects of 18βGA on the gap junction and adherens junction and on sterodiogenesis in cultured rat adrenal cells.

MATERIALS AND METHODS

Cell Culture

Female Wistar rats, aged 8–10 weeks, were purchased from the facility for Research Animal of the National Taiwan University. The maintenance and use of the animals were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). Rats were anesthetized with 7% chloral hydrate by intraperitoneal injection and the adrenal glands were removed, cut into small pieces, and incubated for 30 min at 37°C with gentle shaking with collagenase (0.5 mg/ml; Sigma) in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), pH 7.2, as described previously [Wang et al., 2000]. After mechanical dispersion by repeated passage through a Pasteur pipette, the cell suspension was diluted with cold growth medium [DMEM/F-12 (1:1), containing 25 mM HEPES, 5% fetal calf serum, 2.5% horse serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin] and the cells were collected by centrifugation at 1,000*g* for 5 min at room temperature. They were then plated on coverslips in 35-mm culture dishes (for immunofluorescence) or 24-well plates (for radioimmunoassay) in growth medium, maintained for 3 days in a 5% CO_2 incubator at 37°C, and used on the 4th day.

Immunofluorescence microscopy. Adrenal cells were incubated for 6 h at 37°C with 30 μM 18βGA (Aldrich, Milwaukee, WI), a gap junction blocker, then, after fixation in cold acetone for 5 min and a brief wash in phosphate-buffered saline (PBS), were incubated for 2 h at 37°C with a 1:50–100 dilution of primary antibody [rabbit antibody against nonphosphorylated and phosphorylated Cx43 (Zymed, South San Franscisco, CA), mouse monoclonal anti-nonphosphorylated Cx43 (Zymed), rabbit anti-α-catenin (Sigma, St. Louis, MO), mouse anti-β-catenin (Transduction Laboratory, Lexington, KY), or mouse anti-γ-catenin (Transduction Laboratory)]. After PBS washes, they were then incubated for 1 h at 37°C with FITC-conjugated, goat anti-rabbit or anti-mouse IgG (Sigma), as appropriate, then washed with PBS, mounted using 3% n-propyl gallate

and 50% glycerol in PBS, and examined with a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkocheu, Germany) equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

Western Blotting

After various treatments, the cells were collected from the culture dishes and sonicated in lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, pH 6.9), and the protein concentration of the homogenate determined using a Biorad protein assay kit. An equal volume of gel sample buffer was added and the mixture heated at 90°C for 3 min, then the proteins (40 μg per lane) were electrophoresed on 10% SDS polyacrylamide gels [Fritz et al., 1989] and transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). Strips from the membrane were blocked for 1 h at room temperature with 5% nonfat milk in PBS, then incubated overnight at 4°C with rabbit anti-nonphosphorylated and phosphorylated Cx43 (Zymed; 1:500), mouse anti-nonphosphorylated Cx43 (Zymed; 1:250), rabbit anti-phosphorylated Cx43 (Chemicon, Temecula, CA), or mouse anti-cytochrome C (BioVision Research Products, Mountain View, CA; 1: 250) antibodies. After washes with PBS containing 0.1% Tween-20, the strips were incubated with alkaline phosphatase-conjugated secondary antibodies (Promega, Madison, WI) and positive bands visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogen.

Drug Treatment

In one series of experiments, the cells were treated for 6 h at 37°C with different concentrations (5–75 μM) of 18βGA, then the culture supernatants were collected and assayed for corticosterone. In a second series, the cells were treated for 6 h with 30 μM 18βGA alone or combined with an MAPK (mitogen-associated protein kinase) kinase inhibitor (50 μM PD98059; purchased from Calbiochem, La Jolla, CA) or a protein kinase A (PKA) inhibitor (300 nM KT5720) or a calcium/calmodulin-dependent kinase II (CaMKII) inhibitor (10 μM KN-93) (both from Biolmol Research Laboratories, Inc., Plymouth Meeting, PA), then the culture supernatants were collected for corticosterone measurement.

Corticosterone Radioimmunoassay

Five microliters of the culture medium was diluted 20-fold with assay buffer (0.05 M Tris-HCl, pH 8.0, containing 0.1 M NaCl, 0.1% NaN_3, and 0.1% BSA), then added to the assay tube containing 500 μl of 1:10 diluted anti-corticosterone antiserum (Sigma). After 20 min at 37°C, 100 μl of ^{3}H-labeled corticosterone (10,000 cpm) in assay buffer was added and incubation continued for 1 h at 37°C and 1 h at 4°C. Free hormone was then adsorbed by addition of 300 μl of dextran-coated charcoal (0.5% dextran and 1.25% charcoal in assay buffer) for 10 min at room temperature, then bound hormone was removed by centrifugation at 12,000*g* for 10 min at 4°C. A sample (0.7 ml) of the supernatant was transferred to a counting vial containing 3 ml of counting solution (Ecoscient H) and counted in a β-counter (Beckman, LS600IC) for 1 min. A standard curve was established using a corticosterone standard (Sigma) and corticosterone production was expressed as nanogram per milligram (ng/mg) total protein. Three independent experiments were performed, each in triplicate, and the data represent the mean ± SD. A P-value of < 0.05 using Student's t-test was considered significant.

RESULTS

In DMSO (vehicle)-treated control adrenal cells, adherens junctions, identified by positive immunostaining for α-, β-, or γ-catenin, were seen as continuous linear structures between cells (Fig. 1B, arrows). In contrast, gap junctions, identified by positive immunostaining for Cx43 (phosphorylated and nonphosphorylated Cx43) appeared as discontinuous, punctate structures in regions of cell–cell contact (Fig. 1A, arrowheads). The parts of the adrenal cell membrane not involved in cell–cell contact were devoid of any staining for these junctional proteins. The immunofluorescence using anti-nonphosphorylated Cx43 antibodies was weaker than that using antibodies against phosphorylated and nonphosphorylated Cx43 (data not shown). Western blotting using anti-phosphorylated and nonphosphorylated Cx43 and anti-phosphorylated Cx43 antibodies showed that the three phosphorylated forms of Cx43, P2, P1, P′, and the nonphosphorylated form, NP, were expressed in rat adrenal cells (Fig. 2A,B).

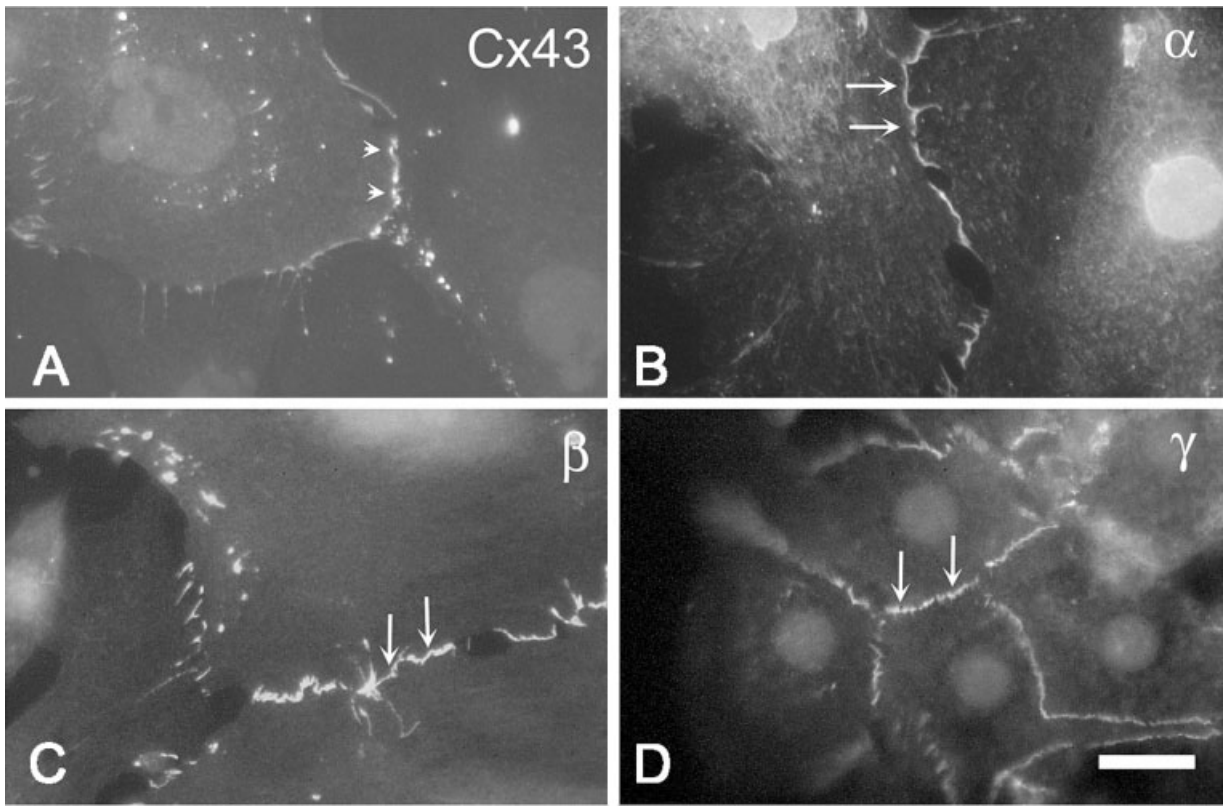

Fig. 1. Distribution of Cx43 and α-, β-, and γ-catenin in DMSO-treated control adrenal cells. The panels show staining with antibodies against phosphorylated and nonphosphorylated Cx43 (**A**, arrowheads), α-catenin (**B**), β-catenin (**C**), or γ-catenin (**D**). The adherens junctions are seen as linear staining between cells (arrows). Bar = 10 μm.

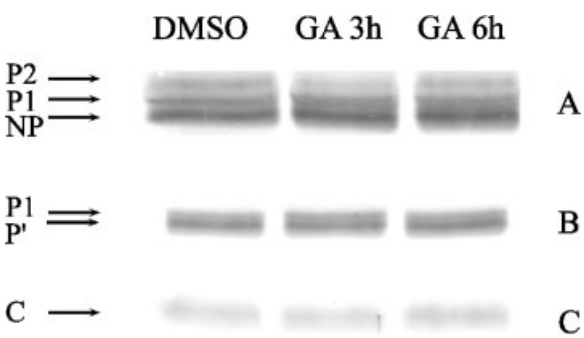

Fig. 2. Effect of 18βGA on the expression of phosphorylated and nonphosphorylated Cx43. Adrenal cells were treated with DMSO or 30 μM 18βGA (3 or 6 h), then harvested for Western blot analysis using rabbit antibodies against nonphosphorylated and phosphorylated Cx43 (**A**) or phosphorylated Cx43 (**B**) or mouse antibody against cytochrome C (**C**), used as an internal standard. P2, P1, P′ are phosphorylated Cx43. NP is nonphosphorylated Cx43. Anti-phosphorylated Cx reacts with P′ and P1. Anti-phosphorylated and nonphosphorylated Cx43 reacts with P2, P1, P′, and NP. Note the amount of P2 decrease with time.

When a time-course study was carried out to study Cx43 distribution after treatment for various times with 18βGA, Cx43 expression at cell–cell junctions decreased from 1 to 6 h (Fig. 3B–D, arrows; Fig. 4A), as compared with the control cells (Fig. 3A, arrows). 18βGA treatment also resulted in a dose- and time-dependent decrease in α-catenin and γ-catenin immunoreactivity at adherens junctions. Figure 4 shows structural changes at adherens junctions after 6 h of treatment. 18βGA induced the longitudinal splitting of the adherens junction, as shown by the two half-junctions, which only stained weakly for α-catenin (Fig. 4B, arrows) and γ-catenin (Fig. 4D, arrows). Although β-catenin staining intensity was not significantly affected, β-catenin were often located in two half junctions (Fig. 4C, arrows). Western blotting showed that 18βGA treatment resulted in a gradual decrease in the amount of P2 Cx43 with time (Fig. 2A), while it did not affect the protein levels of P′ and P1

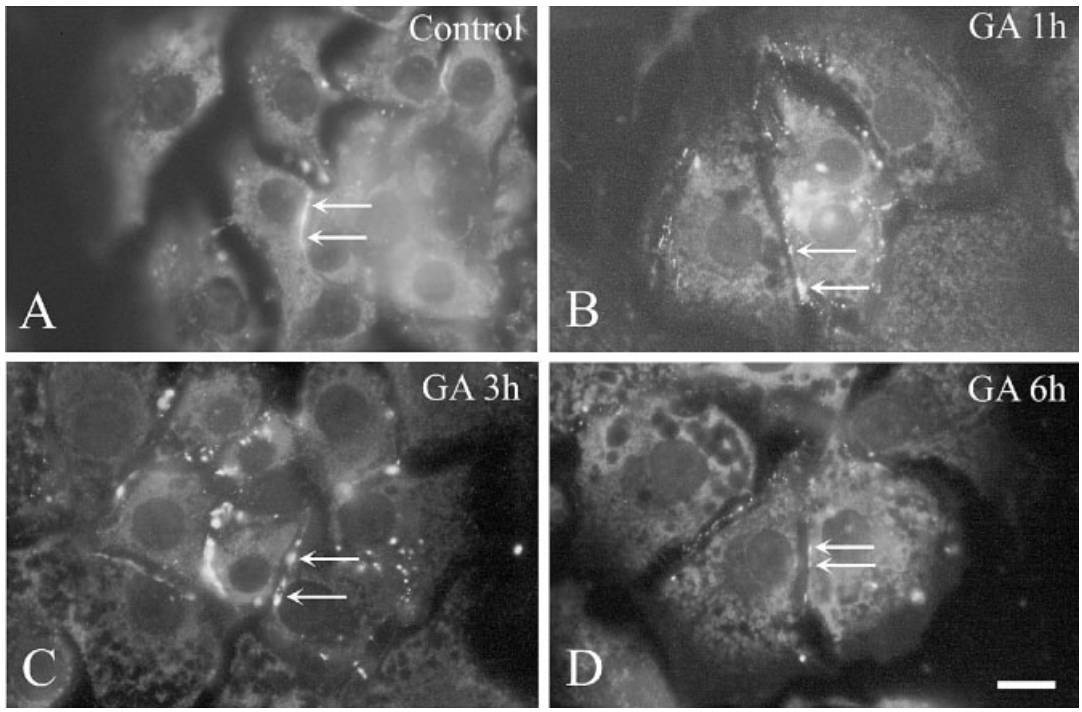

Fig. 3. Time-course study of the effect of 18βGA on the distribution of Cx43. Cells were treated with DMSO (**A**) or 30 μM 18βGA for 1 h (**B**), 3 h (**C**), or 6 h (**D**), then immunostained with antibodies against phosphorylated and nonphosphorylated Cx43. Arrows indicate Cx43 staining. Bar = 10 μm.

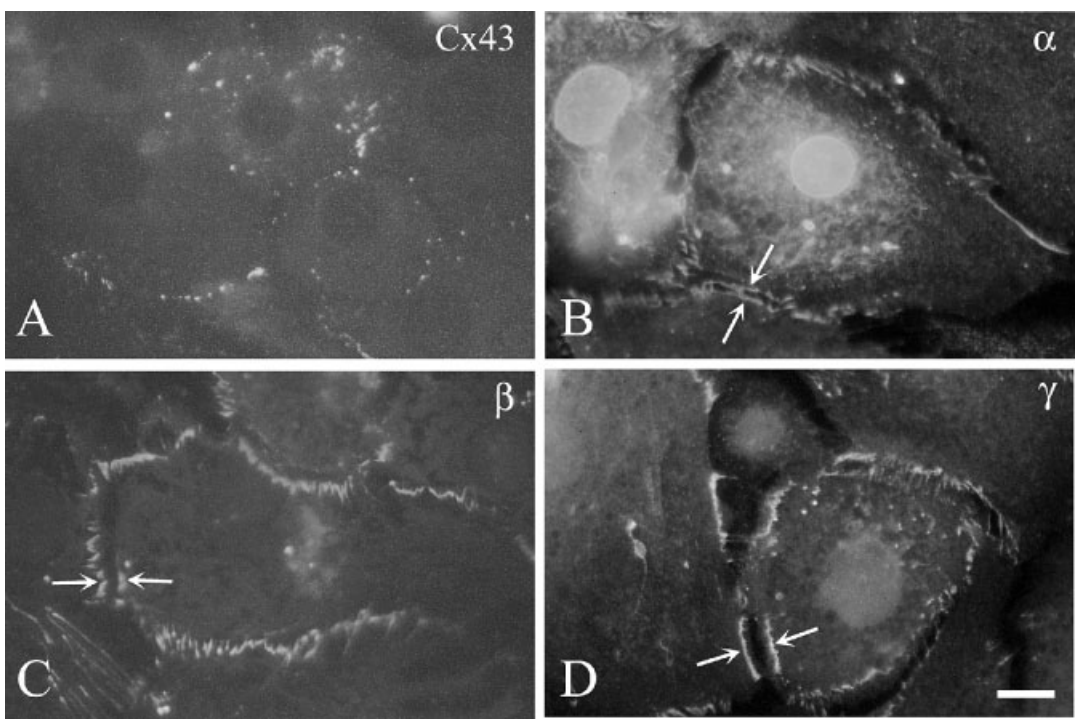

Fig. 4. Effect of 18βGA on the distribution of Cx43 and α-, β-, and γ-catenin in adrenal cells. Adrenal cells were treated with 30 μM 18βGA for 6 h, then immunostained for Cx43 (**A**), α-catenin (**B**), β-catenin (**C**), or γ-catenin (**D**). Arrows indicate the half-junction staining of catenins. Bar = 10 μm.

(Fig. 2B) or cytochrome C (Fig. 2C, an internal standard).

To examine whether intact gap junctions were required for the endocrine function of adrenal cells, we studied the effect of 18β-GA on corticosterone production. Figure 5 shows that 18βGA increased corticosterone production in a dose-dependent manner over the range of 5–75 μM. Since concentrations higher than 45 μM caused detachment of about 10% of the cells, a concentration of 30 μM was used in subsequent experiments. In order to elucidate the signaling pathway involved in the effect of 18βGA on steroidogenesis, we examined possible involvement of the PKA pathway. The PKA inhibitor, KT5720, had no effect on either the basal level of sterodiogenesis or on 18βGA-induced steroidogenesis (Fig. 6). Since ERK regulates hormone-sensitive lipase (HSL) activity in adipocytes [Greenberg et al., 2001; Zhang et al., 2002] and, in other cell types, such as smooth muscle cells and neuronal cell lines, CaMK regulates ERK activity [Abraham et al., 1997], HSL has been recently identified to be responsible for the major cholesterol ester hydrolase activity in adrenal cells [Kraemer and Shen, 2002]. Therefore, we investigated the roles of these two kinases in 18βGA-induced sterodiogenesis by combined application with specific inhibitors, which prevent ERK phosphorylation or CaMKII phosphorylation. As shown in Figure 7, treatment alone with either the MAPK kinase inhibitor, PD98059, or the CaMK II inhibitor, KN-93, resulted in a significant decrease ($P < 0.05$) in basal corticosterone production, suggesting the involvement of ERK and CaMKII in basal steroidogenesis. Cotreatment of 18βGA with either PD98059 or KN-93 significantly inhibited ($P < 0.01$) of 18βGA-induced steroid production. These data provide strong support for the involvement of ERK and CaMK in 18βGA-induced steroidogenesis.

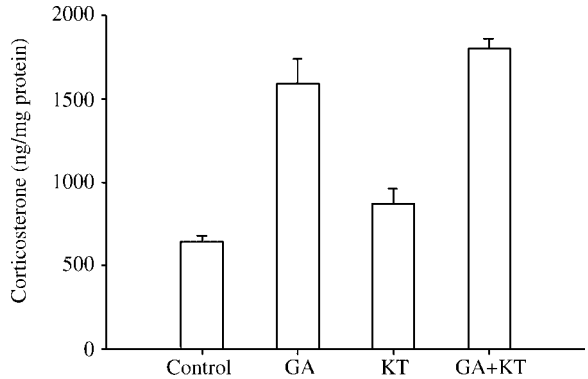

Fig. 6. Effect of the PKA inhibitor, KT5720, on 18βGA-induced steroidogenesis. Adrenal cells were treated for 6 h with DMSO (DMSO), 30 μM KT5720 (KT), 30 μM 18βGA(GA), or 30 μM 18βGA plus 30 μM KT5720 (GA + KT), then the medium was assayed for corticosterones (n = 3).

DISCUSSION

Several lines of evidence show that the formation and stability of the adherens junction indirectly affects the assembly and function of the gap junction. Treatment of MDCK epithelial

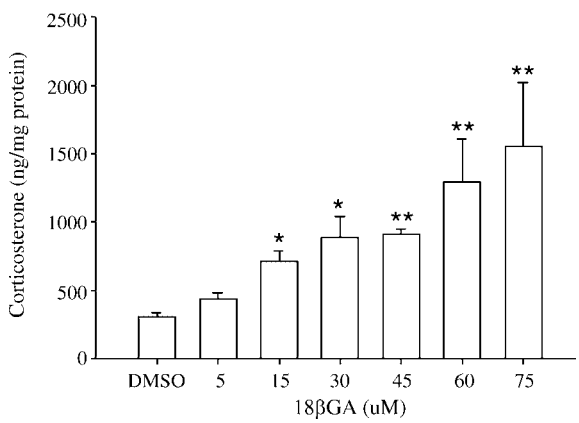

Fig. 5. Dose-dependent effect of 18βGA on steroidogenesis. Different final concentrations (5–75 μM) of 18βGA were added to adrenal cultures for 6 h, then the medium was assayed for corticosterone. *$P < 0.05$, **$P < 0.01$. DMSO, DMSO control (n = 3).

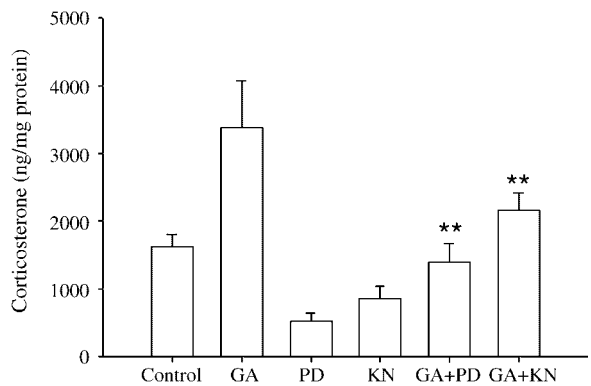

Fig. 7. Effect of a CaMK inhibitor or an ERK inhibitor on 18βGA-induced steroidogenesis. Adrenal cells were treated for 6 h with DMSO (DMSO), 30 μM 18βGA (GA), 50 μM PD98059 (PD), 10 μM KN-93 (KN), 30 μM 18βGA plus 50 μM PD (GA + PD), or 30 μM 18βGA plus 10 μM KN-93 (GA + KN), then the medium was assayed for corticosterone. **$P < 0.01$ compared to the 18βGA group (n = 3).

cells with anti-E-cadherin antibodies inhibits the formation of the junctional complex, which consists of tight junctions, gap junctions, adherens junctions, and desmosomes [Gumbiner et al., 1988]. Incubation with anti-cadherin antibodies abolishes dye transfer among PCC3 teratocarcinoma stem cells and among hepatoma cells [Kanno et al., 1984; Kostin et al., 1999]. Moreover, treatment of hepatoma cells with antibodies against the extracellular domain of Cx32 prevents the formation of both gap junctions and adherens junctions [Meyer et al., 1992]. The present finding that 18βGA disrupted both gap junctions and adherens junctions confirms their structural dependency. In addition to the known function of the ZO-1-β-catenin complex in mediating Cx43 targeting to the gap junction [Itoh et al., 1997; Toyofuku et al., 1998; Wu et al., 2003], a recent report suggested that ZO-1 is also involved in the remodeling of the gap junction during enzymatic dissociation of adult ventricular cardiomyocytes [Barker et al., 2002]. Thus, the underlying mechanism of gap junction-dependent disassembly of the adherens junction may involve the 18βGA-induced dephosphorylation of Cx43, thus affecting the interaction between Cx43, ZO-1, and β-catenin, major components of the adherens junction.

The disruption of gap junction plaques by 18βGA in rat liver epithelial cells is mediated by the activation of type 1 or 2A phosphatase, which then dephosphorylates Cx43 [Goldberg et al., 1996]. In addition, the study by Goldberg et al. [1996] suggests that GA may alter connexon particle packing in gap junction plaques. Furthermore, in alveolar epithelial cells, 18αGA causes downregulation of Cx protein and mRNA expression, which results in disassembly of gap junction plaques and blocking of gap junction intercellular communication [Guo et al., 1999]. In the present study, P2 expression was decreased, suggesting that dephosphorylation of Cx induced by 18βGA may contribute to the disassembly of gap junctions, as reported in other cell types [Guan et al., 1996].

When we examined the signaling pathway involved in 18βGA-induced steroidogenesis, we found that either PD98059 (an ERK inhibitor) or KN-93 (a CaMK inhibitor) significantly inhibited the stimulatory effect of 18β-GA on steroidogenesis, thus indicating the involvement of these two kinases. Gap junction channels are inhibited by several growth factors, including epidermal growth factor and platelet-derived growth factor [Warn-Cramer et al., 1996, 1998; Vikhamar et al., 1998; Rivedal and Opsahl, 2001]. In rat liver epithelial cells, MAP kinase (ERK) can directly phosphorylate Cx43 on Ser255, Ser279, and/or Ser 282, leading to disassembly of gap junction plaques [Kanemitsu and Lau, 1993; Warn-Cramer et al., 1996, 1998; Hossain et al., 1999; Vikhamar et al., 1998; Rivedal and Opsahl, 2001]. However, conflicting results were obtained using the human kidney epithelial cell line, K7, in which MAP kinase, activated by epidermal growth factor, increases both gap junction intercellular communication (by phosphorylating Cx43) and Cx43 synthesis and transport to gap junction plaques [Vikhamar et al., 1998]. Thus, MAP kinase-induced Cx43 phosphorylation may result in either a reduction or an increase in gap junction intercellular communication in a cell type-dependent manner. It remains to be elucidated whether 18βGA-induced ERK activation is related to the disassembly of gap junction plaques in rat adrenal cells. In hepatocytes, CaMKII can phosphorylate Cx32 at threonine residues [Saez et al., 1990]. Although the current study suggested that CaMKII was activated by 18βGA, there is, as yet, no evidence that CaMKII can phosphorylate Cx43, the major isoform of Cx in rat adrenal cells.

Our study provided evidence that intact gap junctions and adherens junctions were not essential for the secretory function of rat adrenal cells, since 18βGA stimulated corticosterone production in adrenal csells in the absence of gap junctions or of complete adherens junctions. In contrast, in bovine and human adrenal fasciculata cells, 18βGA was found to inhibit gap junction intercellular communication and decreases steroidogenic responsiveness to ACTH [Munari-Silem et al., 1995]. The gap junction is thought to mediate cAMP signaling among blastomeres, as shown by the observation that 18βGA blocks both gap junctions and cAMP transmission among cells through gap junctions, thus indirectly causing decreased expression of the cAMP-dependent transcription factor, Oct-3/4 [Burnside and Collas, 2002]. The present study showed that inhibition of PKA by KT5720 did not abrogate 18βGA-induced steroidogenesis, suggesting a cAMP-independent mechanism in rat adrenal cells. Since the action of 18βGA on

steroidogenesis was not mediated by cAMP, this may explain why 18βGA could exert its steroidogenic effect in the absence of intact gap junctions and therefore in the absence of any cAMP-mediated pathway. GA has corticoid-like functions, including anti-inflammation and anti-allergy effects, and can delay the development of certain autoimmune diseases in the spontaneously autoimmune mouse strain, MRL lpr/lpr [Horigome et al., 2001], although the precise mechanism is unknown. On the basis of this research, 18βGA-induced adrenal steroidogenesis may partially account for these anti-inflammation and anti-allergy actions of this drug.

In addition to translocating to the nucleus and activating transcription factors, activated ERK can phosphorylate HSL and accelerate lipolysis in 3T3-L1 adipocytes [Greenberg et al., 2001; Zhang et al., 2002]. It is now clear that neutral cholesterol hydrolase, the major enzyme responsible for hydrolysis of cholesterol esters, is the HSL in adrenal cells [Cook and Yeaman, 1982; Kraemer and Shen, 2002]. It is therefore possible that activation of ERK may stimulate HSL activity in rat adrenal cells. This hypothesis is supported by the finding that in mouse Y-1 cells, phosphorylation of p42/44 ERK greatly promotes steroidogenesis [Gyles et al., 2000]. Taken together, our results demonstrate that in rat adrenal cells, in addition to disrupting gap junctions and adherens junctions, 18βGA can stimulate steroidogenesis by a pathway involving ERK and CaMK.

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