Bimodal inhibition of connexin 43 gap junctions decreases ACTH-induced steroidogenesis and increases bovine adrenal cell population growth

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Abstract
In order to elucidate the role of gap junctions in adrenal cell responses, we measured the effect of inhibiting gap junctions with 18-α-glycerrhetinic acid (GA; a potent inhibitor of cell–cell communication) and connexin antisense transfection on cell proliferation and adrenocorticotropin (ACTH)-stimulated steroidogenesis. In these experiments we utilized a bovine adrenocortical cell (SBAC) population, which responds to ACTH treatment with a dose-dependent increase in steroid production, an increase in connexin 43 (α1-Cx43) gap junction protein concentrations, and a decrease in cell population growth. SBAC cell populations treated with GA had increased growth rates, decreased ACTH-stimulated steroidogenesis, but no reduction in α1-Cx43 gap junction protein contents. In contrast, when SBAC cells were transfected with α1-Cx43 antisense cDNA, gap junction protein concentration was dramatically reduced as expected, unlike the GA-treated cell populations. Cell populations transfected with α1-Cx43-antisense also exhibited increased growth rates and a decreased steroidogenic response to ACTH treatment as compared with control or vector-only transfected cell populations. The decreased responsiveness and increased number of cells in the population after gap junction function was decreased by either GA treatment or antisense transfection, suggests that gap junctions may be necessary factors in ACTH-stimulated responsiveness and growth control in the adrenal gland.

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Introduction
It has been suggested that endocrine cells require signals from their extracellular environment and from one another to coordinate normal physiological responses (Meda et al. 1982, Meda 1996, Morand et al. 1996, Oyoyo et al. 1997). Furthermore, cell–cell communication via gap junctions is hypothesized to have a significant role in endocrine cell growth, differentiation and hormone response (Murray & Fletcher 1984, Loewenstein & Rose 1992, Meda 1996, Munari-Silem 1996, Oyoyo et al. 1997, Yamasaki et al. 1999). Gap junctions have been shown to provide channels between adjoining cells (Loewenstein 1981). Each gap junction channel is composed of a family of membrane proteins called connexins (Kumar & Gilula 1996, Nicholson & Bruzzone 1997). To date, at least 13 connexin species have been isolated (White et al. 1995, Kumar & Gilula 1996), one of which, connexin 43 (α1-Cx43), has been demonstrated to be abundant in the adrenal gland and in adrenal cell cultures (Murray et al. 1995, 1997, Murray & Pharrams 1997, Oyoyo et al. 1997, Murray & Shah 1998). Adrenocorticotropin (ACTH) treatment of adrenal cells maintained in culture increases the number of gap junction protein plaques as revealed by immunocytochemistry (Oyoyo et al. 1997, Murray & Shah 1998) or freeze-fracture techniques (Decker et al. 1978). The abundance of α1-Cx43 gap junctions in the adrenal gland and the increased presence of gap junctions after ACTH treatment suggest that gap junctions have the potential to play a pivotal role in adrenal gland function.

In these studies, we have utilized two separate techniques (18-α-glycerrhetinic acid (GA) treatment and antisense transfection) to assess the role of gap junctions in endocrine processes. In previous studies it has been shown that GA treatment disrupts gap junction functions, as measured by cell-to-cell transfer of dye (Hirata et al. 1998, Bani-Yaghoub et al. 1999) and electrical coupling (Yamamoto et al. 1998, Santicioli & Maggi 2000), while simultaneously decreasing steroid production in adrenal cell cultures (Munari-Silem et al. 1995). In this study we have tested the hypothesis that the inability to form gap junctions after antisense transfection would also have disruptive effects on steroid production and cell population growth, and that such effects would be similar to those produced by GA treatment. The data support the conclusion that adrenal
cell population growth, like steroidogenesis, is regulated by gap junctions in the adrenal cell.

Materials and Methods

Bovine adrenal cortical cells (SBAC) purchased from American Type Culture Collection (Bethesda, MD, USA) were grown in Ham’s F-12 media containing 0.86 mg/l ZnSO4, 10% fetal calf serum, 50 ng/ml basic fibroblast growth factor (Becton-Dickinson, Franklin Lakes, NJ, USA), 200 U/ml penicillin, 200 µg/ml streptomycin and 5 µg/ml fungizone (Amphotericin B) (all purchased from Gibco BRL, Grand Island, NY, USA). This medium will be referred to subsequently as ‘complete F-12’ medium. Cells were fed every 2 or 3 days and incubated at 37 °C with 5% CO2. Treatments included 20, 40 or 80 mU/ml ACTH (Sigma, St Louis, MO, USA), GA (1 mM) and subcloned into the SmaI site of the pNUT vector containing antisense sequences to the cytoplasmic domains of four different gap junction types. The following antibodies were used: α1-Cx43 gap junction cDNA coding for 43,000 molecular weight protein (Beyer et al. 1987), β1-Cx32 gap junction cDNA coding for 32,000 molecular weight protein (Paul et al. 1986, Zhang & Nicholson 1989), β2-Cx26 gap junction cDNA coding for 26,000 molecular weight protein (Paul et al. 1986, Hoh et al. 1991), and β3-Cx31 gap junction cDNA coding for 31,000 molecular weight protein (Risek et al. 1990).

cDNA vectors

Purified rat α1-Cx43 antisense cDNA (residues 180–1864, 1.68 kb) and pNUT vectors were obtained as gifts from Dr Nalin Kumar as part of a current collaboration. A pNUT vector containing the rat α1-Cx43 antisense cDNA and encoding neomycin resistance was then constructed (Kumar et al. 1995).

Stable transfection

SBAC populations were co-transfected with vectors containing antisense sequences to α1-Cx43 gap junction cDNA and vectors containing G418 resistance genes. An EcoRI–SmaI fragment containing nucleotides 1–1061 of the human α1-Cx43 gap junction cDNA was blunted and subcloned into the SmaI site of the pNUT vector in an antisense orientation with respect to the mouse metallothionein promoter (Durnam & Palmiter 1987). A lipofectamine transfection procedure was used (LipofectAMINE, Gibco, Grand Island, NY, USA) as previously described (Oyoyo et al. 1997).

Cells transfected with pNUT Neo vectors only (transfected control cells) and cells transfected with pNUT Neo vectors and α1-Cx43 antisense were cultured for 2 weeks in F-12 complete medium containing 400 µg/ml G418. The surviving cells were fed F-12 complete medium alone or medium supplemented with 400 µg/ml G418 every other feeding time, to select and maintain a transfected population for 2 months. Cells were fed every 2–3 days and incubated at 37 °C with 5% CO2. Transfected populations were pooled and used as one population (Cx43 AS Neo). Changes in adrenal cell function and gap junction protein expression measured in α1-Cx43 antisense transfected cells were compared with cells transfected with the pNUT neo vector only, thus allowing for a control of variables related to the transfection procedure alone.

Immunocytochemistry

SBAC cells were seeded at 1 × 105 cells/cm2 onto sterile coverslips and treated with ACTH (20, 40 or 80 mU/ml) or diluent and were fixed in 3% formaldehyde for 20 min at room temperature and permeabilized in anhydrous acetone for 7 min at −20 °C. Cells were then incubated at 37 °C for 60 min in primary antibody (rabbit IgG diluted 1:100 in PBS) or pre-immune serum and prepared for immunocytochemical analysis with previously described methods (Oyoyo et al. 1997). The antibody α1-Cx43 gap junction cDNA coding for 43,000 molecular weight protein was used. After being rinsed with PBS, cells were incubated in secondary antibody (Cy3-conjugated goat anti-rabbit IgG; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 45 min at 37 °C. Coverslips were washed thoroughly in PBS and mounted onto glass slides with a drop of Fluoromount-G anti-quench mounting medium (Southern Biotechnical Lab, Birmingham, AL, USA). Immunolabeling was viewed and photographed with a Nikon Microphot FXA fluorescence phase microscope (Oyoyo et al. 1997).

Image analysis of gap junctions

Gap junction number and distribution in adrenal cell populations were characterized with a Nikon Microphot FXA fluorescence phase microscope interfaced to an Optimas Image Analysis Computer program (Media Cybernetics, Silver Springs, MD, USA) as previously described (Oyoyo et al. 1997). The experiments were run in triplicate with two plates per treatment group and 10 areas from each slide sample were randomly selected for data analysis. Each data point represents the pooled data from six samples. The number of cells in an area was determined by counting the number of nuclei stained by Hoechst dye (Molecular Probes, Eugene, OR, USA).

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Fluorescent data were analyzed with one-way analysis of variance and the Student’s t-test and expressed as means ± s.e.m. A value of $P \leq 0.05$ was considered significant.

**Population growth analysis**

Cell population numbers were determined by Trypan Blue dye exclusion cell-counting techniques as previously described (Freeman 1976). A period of 24 h was allowed for control, transfected and GA-treated cells seeded at $1 \times 10^5$ cells/cm$^2$ to attach before treatment with ACTH (20, 40 or 80 mU/ml) or diluent. Cells were maintained for various times and then lifted in 0.25% trypsin–Earle’s balanced salt solution (EBSS) and counted in 0.4% trypan blue dye with a hemocytometer. A total of six 35-mm plates per treatment group were used. Eight data points were gathered per experimental plate. Population number data were initially analyzed by one-way analysis of variance and further analyzed with the Student’s t-test and expressed as number of cells/ml $\times 10^4$ ± s.e.m. A value of $P \leq 0.05$ was considered significant.

**Steroid production analysis**

Culture medium from SBAC populations seeded at $1 \times 10^5$ cells/cm$^2$ and treated with ACTH (40 mU/ml) or diluent alone was collected and analyzed for $\Delta^4$, 3-ketosteroid production with a modification of the procedure of Vernikos-Danellis et al. (1966). Media were collected from the samples and stored at $-20^\circ$C. The medium was diluted to 5 ml with 0.9% saline and 6 ml methylene chloride were added. The mixture was shaken, centrifuged at 10,000 r.p.m., and filtered through a 0.2-µm phase filter paper. The resulting methylene chloride extract was reacted with 3 ml 35:65 ethanol:sulfuric acid phase and read on a Perkin-Elmer Spectrophotometer. Fluorescence was measured at 470 nm as the primary wavelength and 536 nm as the secondary wavelength. The mixture was reacted with 3 ml 35:65 ethanol:sulfuric acid phase and then pipetted out and read on a Perkin-Elmer Spectrophotometer. Data were initially analyzed by one-way analysis of variance and the Student $t$-test and expressed as number of cells/ml $\times 10^4$ ± s.e.m. A value of $P \leq 0.05$ was considered significant.

**Western blot analysis of gap junction expression**

Gap junction proteins were isolated with Laemmli lysing buffer (Bio-Rad, Hercules, CA, USA). Adrenal cell monolayers were lysed by addition of the solution to the culture plate and swirling. The cellular extracts were then boiled and centrifuged and the supernatants collected and assayed for protein quantity with the Pierce BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Protein samples (15 µg/lane) were separated on 10% polyacrylamide gels and transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA). The membrane was probed with monoclonal mouse $\alpha_1$-Cx43 antibody (Transduction Laboratories, Lexington, KY, USA) diluted in PBS-minus milk. After a washing in PBS, the membrane was incubated in goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma). The membrane was then stained with chemiluminescence (Amersham, Arlington Heights, IL, USA) and developed through a mini-med X-ray film processor (AFP imaging, Elmsforth, NY, USA). Protein bands were further analyzed with a Phosphor Imager (Bio-Rad) by exposing the membrane to a chemiluminescence–image screen for 2 h and then evaluating the volume per mm$^2$ per band.

**Results**

**Connexin 43 concentrations**

The presence of abundant $\alpha_1$-Cx43 gap junction protein at areas of cell-to-cell contact was demonstrated with immunocytological analysis in SBAC populations (Fig. 1A–D, F). Gap junction, $\alpha_1$-Cx43, labeling appeared mainly as bright puncta at regions of cell–cell contacts. The level of labeling remained consistent in control cultures for 60 passages (data not shown). A protein with a molecular mass of 43 kDa, consistent with $\alpha_1$-Cx43, was revealed with western blot analysis (Fig. 1E); however $\beta_1$-Cx32, $\beta_2$-Cx26, and $\beta_3$-Cx37 proteins were not found in SBAC cell populations (data not shown). To measure the effects of hormonal stimulation on the amount of $\alpha_1$-Cx43 gap junction protein, SBAC cell populations were treated for 24 h with ACTH (20, 40 or 80 mU/ml). ACTH treatment resulted in a dose-dependent increase in the amount of $\alpha_1$-Cx43 protein above that measured in non-ACTH-treated control cultures (Fig. 1). From these findings, it is suggested that gap junctions may be regulated by ACTH and therefore may also have a role in ACTH responsiveness.

To elucidate the relationship between gap junctions and ACTH responsiveness, the effects of treatments known to inhibit either gap junctional communication or gap junction protein expression were studied. Furthermore, as it had been reported that gap junction communication could be blocked by GA treatment in a number of different cell types (Davidson & Baumgarten 1988, Hirata et al. 1998, Bani–Yaghoub et al. 1999), including adrenal cortical cells (Munari-Silem et al. 1999), we examined the effect of GA on gap junction protein concentrations and on the ACTH-induced changes in the amounts of gap junction proteins.

GA (1 mM) treatment for 24 h at a concentration used in other studies to eliminate communication did not
change the level of α₁-Cx43 protein as measured by either immunocytochemistry (data not shown) or western blot analysis (Fig. 2, lanes 1 and 5). Neither the ACTH-induced increase in the number of α₁-Cx43 gap junctions per cell determined by immunocytochemical analysis (Fig. 1F) nor the amounts of α₁-Cx43 gap junction protein were altered by GA (1 mM) treatment (Fig. 2, lanes 1 and 5). Thus gap junctions still formed and persisted in the GA-treated SBAC population.

When cells were transfected with antisense α₁-Cx43 cDNA, however, as expected the number of α₁-Cx43 gap junctions per cell was decreased considerably as compared

Figure 1 Amounts of α₁-Cx43 protein in SBAC cell populations. SBAC cells were treated for 24 h with diluent (A), or with 20 (B), 40 (C) or 80 (D) mU/ml ACTH and prepared for immunocytochemistry (A–D), or western blot analysis (E). A–D, arrows point to gap junction plaques. D, bar = 10 µm. (F) Gap junction plaque number counts from immunocytochemical preparations treated with ACTH (0–80 mU/ml) with or without GA (1 mM). Data are expressed as mean number of gap junctions/cell ± s.e.m. *P ≤ 0.05 compared with untreated populations.
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ACTH stimulation resulted in a decrease in cell population growth compared with non-ACTH treated control populations (Fig. 5). The decreased population growth in response to ACTH was dose dependent such that the high (80 mU/ml) dose caused a more significant decrease in growth rates than did treatment with the lower ACTH dose (40 mU/ml) (Fig. 5A). Treatment with 20 mU/ml ACTH did not significantly alter population growth rates. GA reduced ACTH-induced inhibition of population growth at the lower ACTH (40 mU/ml) treatment dose, but had no effect on ACTH inhibition of population growth at the higher dose (80 mU/ml) (Fig. 5B and C).

Treatment with ACTH (40 mU/ml) significantly decreased the population growth rates of both control vector (pNUT)-transfected and Cx43-antisense-transfected populations (Fig. 4C). However, ACTH had less effect on the Cx43-antisense-transfected cells (21% change compared with non-ACTH treated populations) than on the pNUT-transfected populations (40% change compared with non-ACTH treated populations) (Fig. 4C). The greatest effect of ACTH on cell number was observed between days 7 and 14 of treatment, which is concurrent with the cell populations being in greater contact.

Steroid production
ACTH treatment (20, 40 or 80 mU/ml) significantly increased the amount of steroid produced per cell compared with that produced by cells in non-ACTH-stimulated populations (Fig. 6A). Cell populations treated with 40 mU/ml ACTH produced at least twice as much steroids as those treated with 20 mU/ml ACTH (Fig. 6A). Increasing the ACTH concentration from 40 to 80 mU/ml did not, however, further enhance steroid production. Although GA had no effect on the basal level of steroids being produced, GA decreased the ability of the cell population to produce steroids in response to ACTH (20 and 40 mU/ml) (Fig. 6A). GA treatment however did not alter the steroidogenic response produced after treatment with the higher ACTH dose (80 mU/ml).

Similar to the effects seen with GA treatment, the steroidogenic capacity of SBAC cells to respond to ACTH was also decreased in populations in which α1-Cx43 gap junction expression had been reduced by antisense transfection (Fig. 6B). The basal amounts of steroids per cell in non-hormone-stimulated control, pNUT-transfected and antisense-transfected populations did not significantly differ from one another. However, control vector-transfected populations produced more steroids per cell in response to ACTH (40 mU/ml) than did ACTH-treated cells in which gap junctions had been reduced by antisense transfection (Fig. 6B).

Discussion
To test the hypothesis that gap junctions play a significant role in adrenocortical function, we measured adrenal

Population growth
SBAC cell population growth was measured in populations after either GA treatment or transfection procedures (Fig. 4). The number of SBAC cells in control or control vector (pNUT) transfected cell populations increased steadily from day 3 to day 14 (Fig. 4). GA (1 mM) treatment resulted in an increase in cell population growth in comparison with the diluent-treated control populations (Fig. 4A). Similarly, SBAC populations in which gap junction protein expression was suppressed by transfection with α1-Cx43 antisense grew faster than control vector-transfected populations (Fig. 4B).

with that in control populations (Fig. 3). As seen in Fig. 3, typical gap junction plaques were present in pNUT control transfected cell populations, whereas the α1-Cx43 antisense-transfected populations had few or no gap junction plaques. Consistent with the morphological finding of a decreased number of gap junction puncta, it was demonstrated that the amounts of α1-Cx43 protein measured by western blot were greatly diminished in α1-Cx43 antisense-transfected populations (Fig. 2, lanes 3 and 4). As in non-transfected control populations, ACTH (40 mU/ml) treatment of the control vector (pNUT) transfected populations resulted in an increase in α1-Cx43 protein expression compared with non-ACTH treated pNUT controls (Fig. 2, lanes 2, 6 and 7). ACTH treatment did not increase the amount of α1-Cx43 protein (Fig. 2, lanes 3 and 4) or change the size or number of α1-Cx43 gap junction plaques in cell populations transfected with α1-Cx43 antisense cDNA (data not shown).

Figure 2 Western blot analysis of α1-Cx43 gap junction protein in SBAC cell populations. Control (lane 1), pNUT-transfected (lane 2), α1-Cx43 antisense-transfected (lane 3), ACTH- (40 mU/ml for 24 h) α1-Cx43 antisense-transfected (lane 4), GA- (1 mM) treated (lane 5), ACTH- (40 mU/ml for 24 h) (lane 6), and pNUT-transfected cells treated with 40 mU/ml ACTH for 24 h (lane 7).

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cell population growth and steroidogenesis in an adrenal cortical cell line (SBAC) in which gap junction function was disrupted by two distinct methods: chemical treatment with GA and antisense transfection. Increased cell population growth and decreased ACTH-induced steroidogenesis occurred in adrenal cell populations treated with either method.

A considerable body of evidence has accumulated to suggest that a number of chemicals, including GA, act by blocking or otherwise disrupting the gap junction pore (Davidson et al. 1986, Davidson & Baumgarten 1988). We found that, in the presence of GA, adrenal cells retained the ability to respond to ACTH with an increase in gap junction number and conclude that GA treatment does not negatively affect ACTH-induced gap junction protein insertion or aggregation in the plasma membrane. The finding of a decreased ACTH-stimulated steroidogenic responsiveness in GA-treated adrenal cultures similar to that seen in antisense-treated cultures is consistent with GA acting to block the gap junction pore and further suggests that GA is capable of blocking both established and newly formed gap junctions.

Unlike GA treatment, which did not, however, affect gap junction number, antisense transfection resulted in a dramatic decrease in gap junction (α1-Cx43) protein expression and number. Although GA and antisense transfection treatments differed in amounts of α1-Cx43 protein expression as measured by western blot analysis and immunocytochemistry, their effects on cell population growth and ACTH-induced steroidogenesis were similar.

Figure 3  Immunohistochemical localization of α1-Cx43 gap junction protein in SBAC cell populations. Note the punctate fluorescence (arrows) indicative of α1-Cx43 gap junction antigen in the pNUT control (A), whereas little or no α1-Cx43 is seen in antisense-transfected cultures (B). Gap junction plaque number was counted from immunocytochemical preparations and presented as a histogram (C) in which data are expressed as mean number of gap junctions/cell ± S.E.M. The cell nuclei (n) in (A) and (B) were colocalized with DAPI staining. Bar represents 20 μm. *P ≤ 0.05 compared with control populations. Cx43 AS, antisense α1-Cx43.
The differences in connexin concentrations measured after the differing methods of treatment almost certainly reflect differences in mechanism of gap junction channel inhibition. With GA treatment, the channel is formed but is believed to be non-functional (Davidson et al. 1986, Davidson & Baumgarten 1988), whereas with antisense transfection few gap junction channels can be formed. Any difference seen between the two methods might be attributable to the fact that GA treatment is less specific and is affecting processes in addition to gap

**Figure 4** Cell population growth determined by trypan blue dye exclusion counting techniques. SBAC cells were either treated with GA (1 mM) or diluent (control) (A) or transfected with α1-Cx43 antisense or control vector pNUT (B). Data are expressed as number of cells × 10⁷ ± S.E.M. (C) Data from transfected cells treated with diluent or ACTH (40 mU/ml) are presented as doubling time in hours ± S.E.M. *P ≤ 0.05 compared with timed controls (A, B) or non-ACTH-treated controls (C).
junction pore blockage (Bicikova et al. 1997). These studies have however eliminated the possibility that GA alters cell function by reducing the number of gap junction plaques on the cell surface, and therefore supports the central dogma that GA acts by reducing pore permeability. This suggestion that the effects of GA are restricted to the gap junctional complex is further supported by the fact that reduced steroidogenesis and increased cell number were similarly observed in this cell line when it was genetically altered to prevent the formation of gap junctions.

It is believed that SBAC cells represent a heterologous population in terms of their ability to respond to ACTH. In such populations, gap junctional communication may provide a mechanism for amplifying hormone response by providing second messengers to cells, which are not as capable of responding directly (Murray & Fletcher 1984, Munari-Silem & Rouset 1996). Decreasing the number of gap junctions with antisense transfection or GA treatment would eliminate the capacity for communication and thus result in the inability of cells to transfer factors needed for steroidogenic or growth control. We demonstrate that at low doses (20 and 40 mU/ml), GA treatment reduces steroid production, but at the higher dose (80 mU/ml) of ACTH, steroid production is not significantly altered. This result suggests that functional gap junctions may be a necessary component in the steroidogenic pathway, to communicate steroid-producing signals in order to amplify response at lower doses of hormone stimulant but not at higher doses. It is possible that gap junctional communication of signals facilitates hormone responsiveness when stimulation is submaximal. At the higher ACTH dose, all the cells may be maximally stimulated and thus intercellular communication does not further enhance hormone responsiveness in these populations.

Our steroid data are consistent with those of Munari-Silem et al. (1995), who found that primary cultures of cortical cells exhibit a dose-dependent inhibition of ACTH-induced steroidogenesis after GA treatment. In their studies the relationship between population growth rates and gap junction inhibition was not investigated. We found that transfection of α1-Cx43 antisense cDNA into SBAC cells and GA treatment both resulted in increased population growth and a decrease in ACTH-induced steroidogenesis. The increased cell number within the population may reflect a change in proliferation, increased cell survival, or both.

Our results are consistent with the theory that gap junctions are required for the passage of molecules between cells and serve to facilitate adrenal responses to physiological stimuli. This study is novel in altering gap junctions by two different mechanisms and subsequently evaluating changes in both cell population growth and steroidogenesis. The results strengthen the hypothesis that gap junctions in the adrenal gland facilitate steroid production and control of population growth control via communication of signal molecules.
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