Expression and regulation of connexin43 in rat Leydig cells

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Abstract

Gap junctions are intercellular protein channels which provide a pathway for the exchange of ions and small molecules. This exchange of materials allows metabolic coupling of cells. Gap junction channels are made up of connexins, integral membrane proteins encoded by a multigene family. Rat testes contain mRNAs for at least five different connexins: Cx26, Cx32, Cx33, Cx37 and Cx43. Immunocytochemical studies have shown that Cx43 assembles gap junctions between Leydig cells. The present study investigated the expression and regulation of the Cx43 gene in rat Leydig cells. Purified Leydig cells were obtained from 40- to 80-day-old Sprague–Dawley rats using a combination of arterial perfusion, collagenase digestion, centrifugal elutriation and Percoll gradient centrifugation. Leydig cells from 20- and 30-day-old rats were isolated without arterial perfusion or centrifugal elutriation. Cx43 mRNA was present in 20-day-old rat Leydig cells, reached a plateau at day 40, and remained at high levels in 65- and 80-day-old rat Leydig cells. To evaluate the regulation of Cx43 gene expression, Leydig cells were cultured overnight and then treated with human chorionic gonadotropin (hCG) for variable periods of time. Addition of hCG (10 ng/ml) increased cytochrome P450 side-chain cleavage and steroidogenic acute regulatory protein mRNA levels and testosterone formation. However, Cx43 mRNA levels were inhibited by hCG in a time- and dose-dependent manner. Cx43 mRNA levels decreased 27% as early as 2 h after the addition of hCG and decreased 60% by 24 h. Treatment of Leydig cells with 8-bromo-cAMP (0·1 mM) for 6 and 24 h also reduced Cx43 mRNA levels by 36 and 56% respectively. Primary cultured Leydig cells stained strongly positive with anti-Cx43 monoclonal antibody. Treatment with hCG for 24 h reduced Cx43 signals and caused Cx43 to redistribute to the periphery of the cells. To evaluate the regulation of Cx43 *in vivo*, rats were treated with hCG (300 ng i.p.) and testes were removed 24 h later. Frozen section of testes revealed that these interstitial cells stained positive for 3β-hydroxysteroid dehydrogenase (3β-HSD) by histochemical staining and were positive for Cx43 by immunofluorescence staining. The adjacent seminiferous tubules stained only weakly positive for Cx43. Twenty-four hours after hCG treatment, 3β-HSD activity increased while Cx43 immunostaining of Leydig cells was reduced. In conclusion, gap junction channels of Leydig cells are regulated by hCG both *in vivo* and *in vitro*. hCG increased Leydig cell steroidogenesis and steroidogenic enzyme mRNA levels but caused a redistribution of Cx43.

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Introduction

Gap junctions are intercellular protein channels that provide a pathway for the exchange of ions and small molecules. This exchange of materials allows electrical and metabolic coupling of cells. Metabolic coupling via gap junctions involving the exchange of metabolites and second messengers has been implicated in embryonic development and control of cell growth (Loewenstein 1979, 1981, Caveney 1985, Goodenough *et al.* 1996, Kumar & Gilula 1996).

Connexins are the principal protein components of gap junctions. The connexin is organized into a basic unit of structure, the connexon, which is a hexameric structure with a toroid appearance. The family of connexin proteins includes at least 13 members in rodents and 5 of them (Cx26, Cx32, Cx33, Cx37 and Cx43) have been identified in various types of testicular cells using specific antibodies and/or cDNAs. An individual connexon from one cell docks or associates with a corresponding connexon on a neighboring cell to form a gap junction channel, and multiple channels, in turn, cluster or aggregate in the plane of the membrane to form gap junction plaques (Goodenough *et al.* 1996, Kumar & Gilula 1996).

The extensive intercellular coupling between Leydig cells has been demonstrated both *in vitro* and *in situ* (Kawa 1987, Risley *et al.* 1992, Varanda & Campos de Carvalho 1994). Immunocytochemical studies have shown that Cx43 assembles gap junctions between Leydig cells (Risley *et al.* 1992, Pellitier 1995, Tan *et al.* 1996).

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Junctional properties of Leydig cells mechanically dissociated from mouse testes have also been characterized by dye injection and electrophysiological measurements. These cells were found to be extensively coupled (Varanda & Campos de Carvalho 1994). Western blot analysis using purified Leydig cells confirmed the presence of Cx43 (Varanda & Campos de Carvalho 1994). The present study investigated the expression and regulation of the Cx43 gene in rat Leydig cells.

Materials and Methods

Isolation and purification of rat Leydig cells

Male Sprague–Dawley rats were obtained from Charles Rivers (Raleigh, NC, USA). The animals were freely fed rat chow and maintained on a 12 h light:12 h darkness cycle. Animals were killed by CO_2 asphyxiation prior to dissection. Highly purified Leydig cells were isolated from 40-, 55-, 65- and 80-day-old rat testes using a combination of arterial perfusion, collagenase digestion, centrifugal elutriation and Percoll gradient centrifugation as described by Klinefelter *et al.* (1987) with minor modification (Lin *et al.* 1992). The protocol was approved by the local Animal Study Committee. Leydig cells from 20- and 30-day-old rats were isolated without arterial perfusion and centrifugal elutriation. Freshly isolated Leydig cells were plated in 100 mm culture dishes and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2 overnight. The medium was removed and replaced with fresh medium containing human chorionic gonadotropin (hCG) (10 ng/ml) or 8-bromo-cAMP (Sigma, Chemical Co., St Louis, MO, USA) (0·1 mM). Cultures were continued for an additional 2–24 h and total RNA was extracted. Supernatants were centrifuged and stored at − 20 °C for testosterone RIA as described previously (Lin 1985). The highly specific anti-testosterone antibody cross-reacted only 7% with 5α-dihydrotestosterone and less than 1% with other steroids (progesterone, 17α-hydroxyprogesterone, pregnenolone, androstenedione, estrone and estradiol). All samples from a single study were analyzed in a single assay.

Northern blot hybridization

Total RNA of Leydig cells was isolated with TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD, USA). Twenty micrograms RNA per well were loaded in a 2% agarose denatured gel. After 5 h electrophoresis at 55 V, the RNA was transferred to a positively charged nylon membrane (Ambion, Austin, TX, USA). The hybridization was carried out at 65 °C overnight. The antisense RNA probes for Cx43 gene (the template plasmid cDNA from Dr David Kiang, University of Minnesota, Minneapolis, MN, USA) and β-actin gene (pTRI-beta-actin mouse control template cDNA, from MAXIscript kit, Ambion), were generated with a MAXIscript *in vitro* transcription kit (Ambion) and labeled with a BrightStar Psoralen-Biotin Nonisotopic labeling kit (Ambion) and detected with the BrightStar BioDetect Nonisotopic detection kit (Ambion). The cytochrome P450 side-chain cleavage (P450 scc) cDNA (from Dr JoAnne Richards, Houston, TX, USA) or steroidogenic acute regulatory protein (StAR) cDNA (from Dr Douglas M Stocco, Lubbock, TX, USA) probes were labeled with [α-^{32}P]dCTP with a Random Primers DNA Labeling system (Gibco-BRL, Grand Island, NY, USA) and hybridization was performed at 42 °C overnight (Lin *et al.* 1998). The membranes were exposed to Kodak XAR5 film with an intensifier screen at − 80 °C for 2 days.

The photographs were scanned with the Scanmaker III scanner (Microtek, Redondo, CA, USA) and quantified with the Sigmascan Pro 4 program (Sigma). Our previous study has shown that both P450 scc and StAR mRNA levels were up-regulated by hCG in a time- and dose-dependent manner (Lin *et al.* 1998). P450 scc or StAR mRNA levels were used in the present study to monitor the response of Leydig cells to hCG treatment. Transcription of the β-actin gene, which was not affected by any of these treatments, was used as the internal control for each specimen.

Immunofluorescence staining

Leydig cells were isolated and cultured in two-well chamber slides (Lab-Tek Chamber Slide; Nalge Nunc International, Naperville, IL, USA) overnight. The cells were treated with hCG (10 ng/ml) for 24 h.

To evaluate *in vivo* effects of hCG on Cx43 gene expression, 65-day-old rats were treated with hCG (300 ng i.p.) and testes removed 24 h later. Freshly isolated rat testes were immediately frozen in liquid nitrogen and kept at − 80 °C for storage. Five-micron sections were cut and mounted on 3-aminopropyltriethoxy-silane (Sigma)-coated slides. The tissue and cell slides were both fixed in 10% buffered formalin for 15 min and washed with PBS, then incubated in avidin, biotin blocking solution (Avidin/Biotin Blocking kit; Vector, Burlingame, CA, USA) and 5% BSA /PBS buffer (containing 0·02% Triton X-100) for 15 min at room temperature respectively. The monoclonal anti-Cx43 antibody (Chemicon MAB 3068, Temecula, CA, USA), diluted 1:50 in the above BSA/PBS buffer, was applied to the sections and they were incubated at 4 °C overnight. The biotinylated second antibody from the Vectastain ABC kit (Universal kit, Vector) was used as conjugator. After washing with PBS, the sections were labeled with streptavidin fluorescein for 75 min (at 3·6 μg/ml). The sections were covered with cover-slides with Vectashield mounting medium (Vector), examined microscopically and photographed

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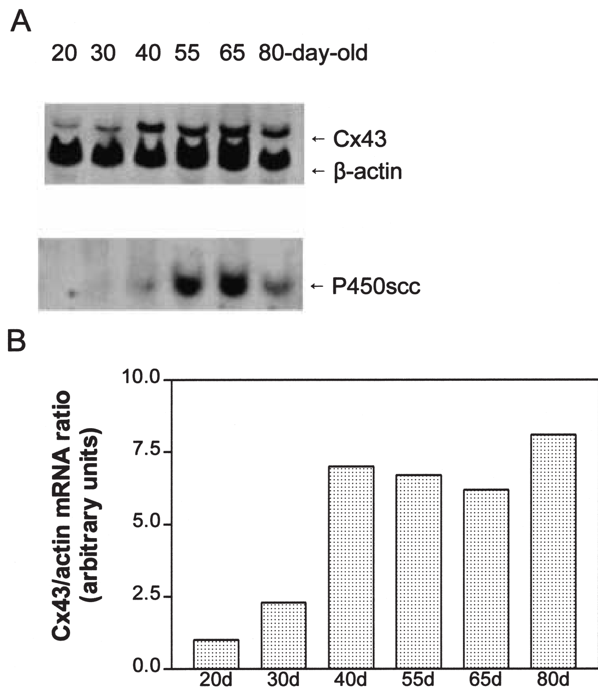

Figure 1 Effects of age on Cx43 gene expression. Leydig cells were isolated from 20-, 30-, 40-, 55-, 65- and 80-day-old rats as described in Materials and Methods. Total RNAs were extracted for Northern blot analyses. Each lane contained 20 µg total RNA. The blot was first hybridized with antisense Cx43 and β-actin RNA probes generated with a MAXIscript *in vitro* transcription kit and labeled with a BrightStar Psoralen-Biotin Nonisotope labeling kit. P450 scc cDNA probe was labeled with [α-^{32}P]dCTP. (A) A representative Northern blot; similar results were observed in two other separate experiments. (B) Cx43/actin mRNA ratios.

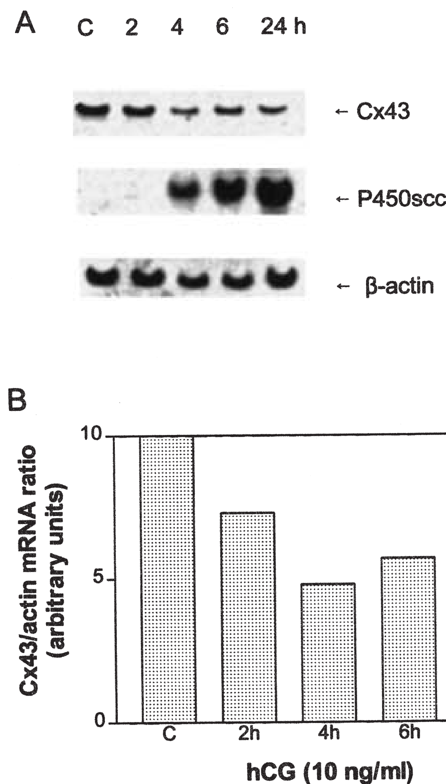

Figure 2 Effects of hCG on Cx43 gene expression. Purified Leydig cells were obtained from 55- to 65-day-old rats. Cells were cultured overnight and then treated with hCG (10 ng/ml) for 2, 4, 6 or 24 h. Total RNAs were extracted for Northern blot analyses. (A) A representative Northern blot; similar results were obtained with two separate experiments. (B) Cx43/actin mRNA ratios.

with a confocal microscope (BioRad MRC 1000; Bio-Rad, Richmond, CA, USA). Quantification of Cx43 immunofluorescence content was performed with the Sigmascan Pro 4 program (Sigma).

Histochemical staining

Histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme activity was carried out as reported by Klinefelter *et al.* (1987). Frozen section testis slides or air-dried purified Leydig cells were covered with staining solution prepared by mixing solution A (1 mg nitroblue tetrazolium dissolved in 0·6 ml 1 mg/ml etiocholanolone in dimethylsulfoxide) with solution B (10 mg β-NAD+ in 9·5 ml warm PBS). Tissue slides or cells were allowed to stain for 90 min, rinsed in distilled water, and fixed in 10% formalin in PBS with 5% sucrose, pH 7·4 (Klinefelter *et al.* 1987). As assessed by 3β-HSD staining, more than 95% of the cells were stained positive for Leydig cells.

Statistical analyses

All experiments were repeated at least three times. One-way ANOVA followed by Newman–Keuls multiple comparison tests were used for statistical analyses (GraphPad Prism, Version 2·01, GraphPad Software Inc., San Diego, CA, USA). $P \leq 0{\cdot}05$ was considered significant.

Results

Ontogeny of Cx43 gene expression in developing rat Leydig cells

We first evaluated the transcription of the Cx43 gene in freshly isolated Leydig cells from different age rats, 20–80 days old. As shown in Fig. 1, P450 scc mRNA levels progressively increased as Leydig cells matured. Only a very faint band was observed with Cx43 antisense probe hybridization in the RNA from day 20 rat Leydig cells. By day 30, Cx43 mRNA levels increased about 2-fold. The

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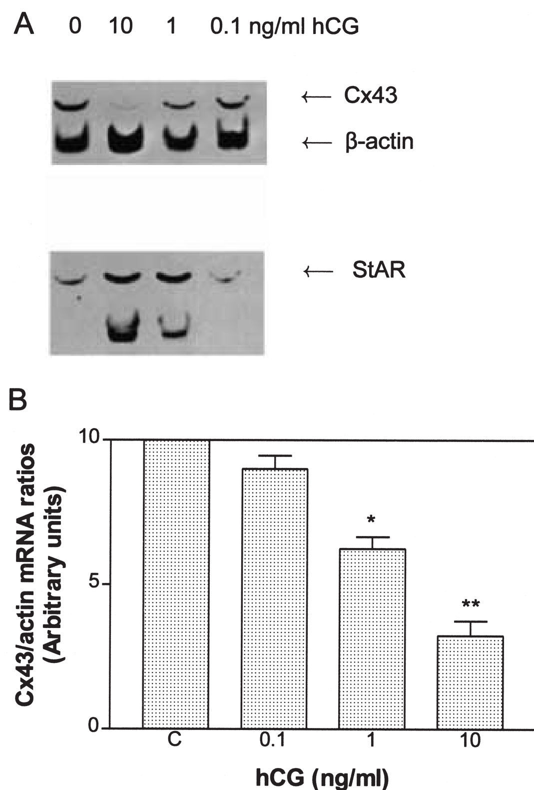

Figure 3 Dose–response curve of the effect of hCG on Cx43 gene expression. Purified Leydig cells were obtained from 55- to 65-day-old rats. Cells were cultured overnight and then treated with hCG (0·1, 1 or 10 ng/ml) for 6 h. Total RNAs were extracted for Northern blot analyses. (A) A representative blot. (B) Cx43/actin mRNA ratios. Results are the mean ± S.E. of three separate experiments. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ compared with controls without hCG treatment.

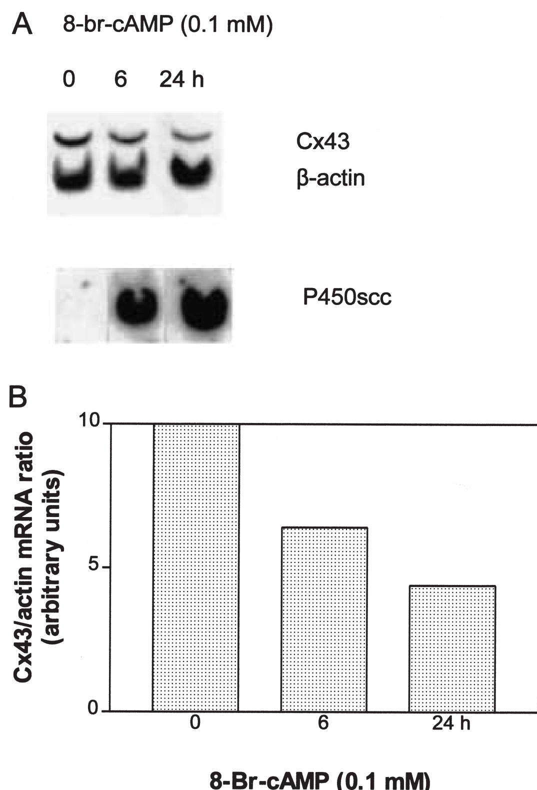

Figure 4 Effects of 8-bromo-cAMP on Cx43 gene expression. Purified adult Leydig cells were cultured overnight. Cells were then treated with 8-bromo-cAMP (0·1 mM) for 6 or 24 h. Total RNAs were extracted for Northern blot analyses. (A) A representative blot; similar results were obtained with two other separate experiments. (B) Cx43/actin mRNA ratios

Cx43 transcript increased dramatically by day 40, with a level about 7-fold higher than that of day 20 rat Leydig cells. Cx43 mRNA levels remained in high levels in 65- and 80-day-old rat Leydig cells. Increased Cx43 mRNA expression correlated with increased P450 scc mRNA levels.

Effects of hCG on Cx43 mRNA levels

Since Cx43 gene expression correlated with male rat sexual maturation, we evaluated the effects of hCG on Cx43 mRNA levels. Purified Leydig cells (60-day-old rats) were cultured overnight and hCG (10 ng/ml) was then added for various periods of time. hCG inhibited Cx43 mRNA expression as early as 2 h and reached a nadir by 24 h (Fig. 2). In contrast, P450 scc mRNA levels were increased dramatically by hCG treatment. hCG also increased testosterone formation from basal 3·2 ± 0·4 (mean ± S.E.; n=3) ng/ml to 95·2 ± 5·1 ng/ml ($P<0{\cdot}001$) by 24 h. Figure 3 shows the dose–response curve of Cx43 in response to hCG. Purified Leydig cells were cultured overnight. After medium change, hCG (0·1, 1 or 10 ng/ml) was added and cultures were continued for an additional 6 h. hCG caused a dose-dependent inhibition of Cx43 mRNA expression; in contrast, StAR mRNA levels were increased markedly.

Effects of cAMP on Cx43 mRNA expression

The effect of hCG is mediated by the adenylate cyclase–cAMP pathway (Dufau 1988). We next evaluated the effect of 8-bromo-cAMP on Cx43 gene expression. Purified Leydig cells were cultured overnight. After medium change, the cells were treated with 8-bromo-cAMP (0·1 mM). As shown in Fig. 4, treatment of cells with 8-bromo-cAMP for 6 and 24 h reduced Cx43 mRNA levels by 36 and 56% respectively, comparable to that observed with hCG treatment. In contrast, P450 scc mRNA levels increased markedly in response to 8-bromo-cAMP. Testosterone levels in the supernatant

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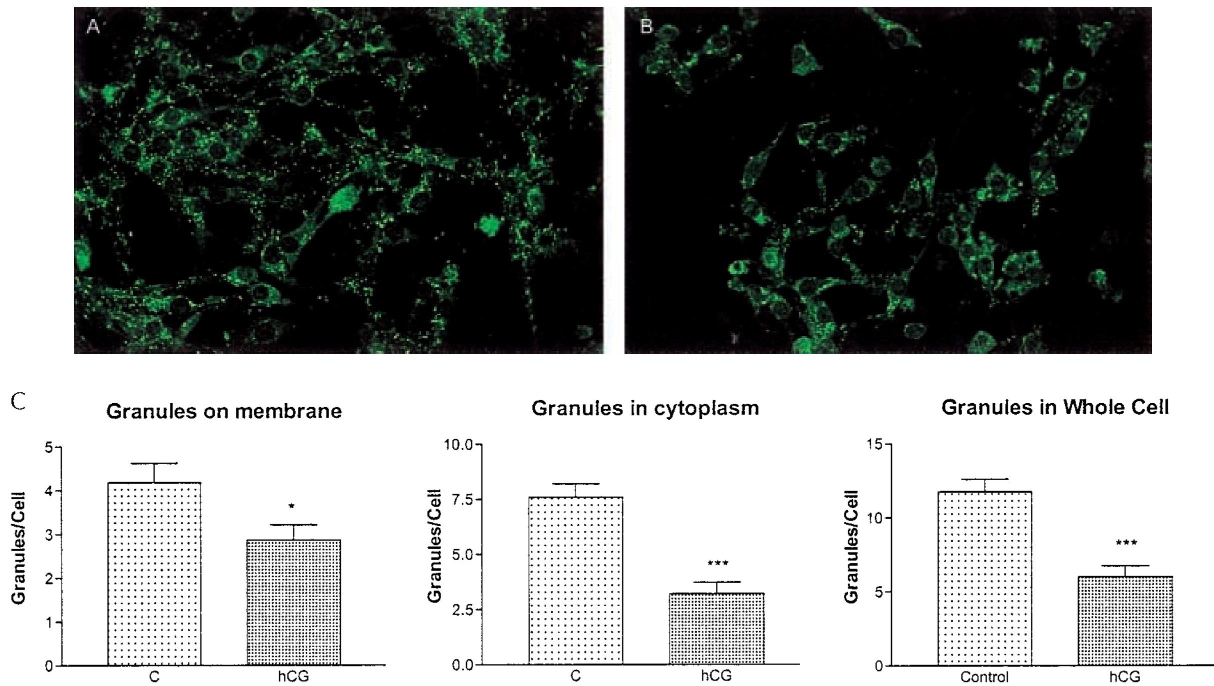

Figure 5 Immunofluorescence staining of Leydig cells. Purified adult Leydig cells were cultured overnight. After medium change, cells were treated with hCG (10 ng/ml) for 24 h. Leydig cells were stained with anti-Cx43 monoclonal antibody. (A) Control, (B) hCG treated. (C) Quantitative measurement with the Sigmascan Pro 4 program of immunostaining. **P*<0·02; ****P*<0·001 compared with controls without hCG treatment.

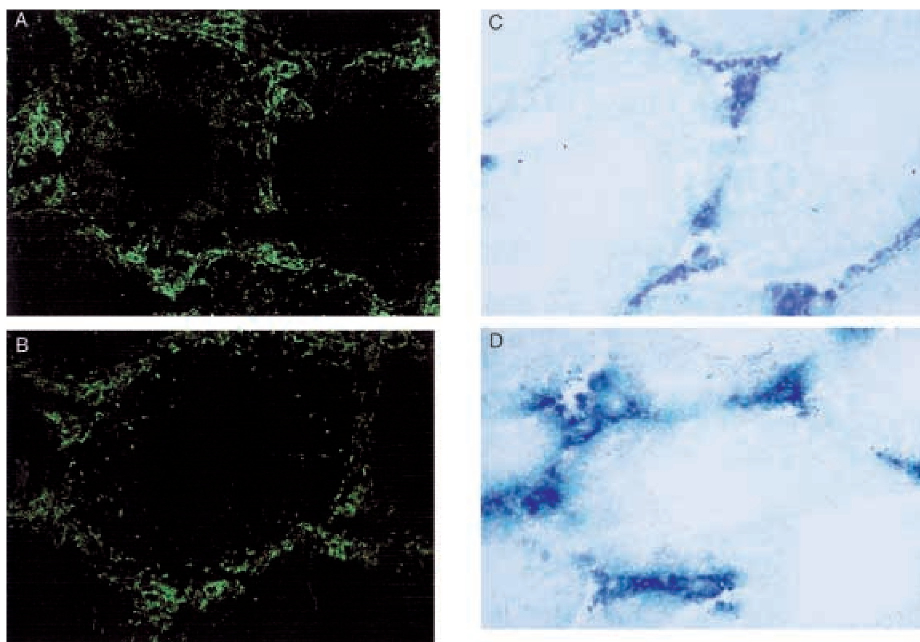

Figure 6 *In vivo* effect of hCG on Cx43 gene expression. Sixty-five-day-old rats were treated with hCG (300 ng i.p.) and testes were removed 24 h later. Section was stained with anti-Cx43 monoclonal antibody and immunofluorescence; (A) control, (B) hCG treatment. Frozen section of testes revealed that these interstitial cells were stained positive for 3β-HSD; (C) control, (D) hCG treatment.

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were also increased markedly from basal levels of 4·2 ± 0·3 to 102 ± 5·6 ng/ml (P<0·001) by 8-bromo-cAMP treatment.

Effects of hCG on Cx43 protein levels in vivo *and* in vitro

In order to confirm that hCG indeed down-regulated Cx43 gene expression, primary cultured Leydig cells were stained with anti-Cx43 monoclonal antibody and immunofluorescence staining. Control Leydig cells without hCG treatment stained strongly positive with multiple punctate signals (Fig. 5A). hCG treatment for 24 h reduced the Cx43 signal; in addition, the punctate signals were redistributed to the cell membrane (Fig. 5B). Figure 5C shows quantitative measurement of Cx43 immunofluorescence. hCG treatment decreased Cx43 signals by more than 49% in the whole cells. We also treated 65-day-old rats with hCG (5 units i.p.) and testes were removed 24 h later. Frozen section of testes revealed that these interstitial cells stained positive for 3β-HSD (Fig. 6). 3β-HSD activity increased following hCG treatment while Cx43 staining of Leydig cells was reduced and strong punctate immunostaining of Cx43 was observed on the surface of Leydig cells. The adjacent seminiferous tubules were only weakly stained 24 h after hCG treatment (Fig. 6).

Discussion

In the present study, we demonstrated that Cx43 is expressed in rat Leydig cells and that its expression increases with age, reaching a plateau by day 40. hCG and 8-bromo-cAMP increase Leydig cell steroidogenesis and steroidogenic enzyme mRNA levels but down-regulate Cx43 and cause a redistribution of Cx43 to the periphery of the cells.

Leydig cells occur as aggregates of cells interconnected by gap junctions that coordinate their secretory activity. A total of 13 mammalian gap junction proteins, known as connexins, have been cloned (Goodenough *et al.* 1996, Kumar & Gilula 1996). Five of these have been identified in the rat testis (Kadle *et al.* 1991, Risley *et al.* 1992, Tan *et al.* 1996). Cx33 and Cx43 are expressed in Sertoli cells in a stage- and age-dependent manner (Risley *et al.* 1992). Cx26 and Cx32 were localized faintly in the apical region of the seminiferous epithelium (Risley *et al.* 1992). Cx37 is localized in the endothelia of blood vessels, whereas only Cx43 is localized in the membrane appositions between Leydig cells and in some Sertoli–Sertoli gap junctions (Tan *et al.* 1996). Expression of Cx43 in Leydig cells increases with age and reaches maximum levels of expression in the adult (Risley *et al.* 1992), which is consistent with the idea that gap junctional communication is involved in the control of hormone secretion. Using purified Leydig cell preparations, we found in the present study that Cx43 is expressed in 20-day-old rat Leydig cells and reaches a plateau by day 40. Tan *et al.* (1996) reported that Cx43 in the intersitium was localized predominantly to Leydig cell gap junctions. In the tubules, Cx43 was distributed in linear arrays at the periphery of tubules, between the basal and adluminal compartments, and in the region containing the Sertoli occluding junctions. Pellitier (1995) showed that Cx43 is present between Leydig cells in the fetal testis. The appearance of Cx43 concurred with the onset of spermatogenesis. In the seminiferous epithelium, the distribution of Cx43 coincided with the gap junctions of the Sertoli cell junctional blood barrier in guinea pigs. The distribution of Cx43 correlated with germ cell differentiation in a stage-dependent manner (Pellitier 1995). Gap junctions in pairs of Leydig cells mechanically dissociated from mouse testes have been studied with the double whole cell patchclamp technique (Varanda & Campos de Carvalho 1994). Leydig cells were found to be extensively coupled by dye injection and electrophysiological measurements.

The effects of cAMP on Cx43 gene expression have been reported previously in various cell systems. Mehta *et al.* (1992) and Schiller *et al.* (1992) reported that forskolin, an activator of adenylate cyclase, increased Cx43 mRNA 6-fold in a hepatoma cell line, with the increase preceding any detectable increase in dye coupling. Atkinson *et al.* (1995) investigated the exposure of a mouse mammary tumor cell line, MMT22, to 8-bromo-cAMP. An increase of permeance was noted within 30 min of treatment and increased 4-fold by 24 h. The permeability change was accompanied by an increase in gap junction. However, Cx43 mRNA and total cellular content of Cx43 did not change. In our present study, we found that exposure of Leydig cells to 8-bromo-cAMP or hCG decreased Cx43 mRNA levels. However, Cx43 was redistributed to the cell membrane, suggesting that a greater proportion of Cx43 was utilized for channel formation.

In conclusion, gap junction channels of rat Leydig cells are regulated by hCG both *in vivo* and *in vitro*. hCG increased StAR and P450 scc mRNA expression in Leydig cells, but reduced Cx43 expression. Most importantly, hCG caused a redistribution of Cx43.

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