Modulation of gap junction mediated intercellular communication in TM3 Leydig cells

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Abstract

Long-term modulation of intercellular communication via gap junctions was investigated in TM3 Leydig cells, under low and high confluence states, and upon treatment of the cells for different times with activators of protein kinase A (PKA) and protein kinase C (PKC). Cells in low confluence were readily coupled, as determined by transfer of the dye Lucifer Yellow; on reaching confluence, the cells uncoupled. Western blots and RT-PCR revealed that connexin 43 (Cx43) was abundantly expressed in TM3 Leydig cells and its expression was decreased after the cells achieved confluence. Stimulation of PKA or PKC induced a decrease in cell-cell communication. Staurosporin, an inhibitor of protein kinases, increased coupling and was able to prevent and reverse the uncoupling actions of dibutyryl cAMP and 12-O-tetradecanoyl-phorbol-13-

acetate (TPA). Under modulation by confluence, Cx43 was localized to the appositional membranes when cells were coupled and was mainly in the cytoplasm when they were uncoupled. In addition, cAMP and TPA reduced the surface membrane labeling for Cx43, whereas staurosporin increased it. These data show a strong correlation between functional coupling and the membrane distribution of Cx43, implying that this connexin has an important role in intercellular communication between TM3 cells. Furthermore, increased testosterone secretion in response to luteinizing hormone was accompanied by a decrease in intercellular communication, suggesting that gap junction mediated coupling may be a modulator of hormone secretion in TM3 cells.

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Introduction

Gap junctions are plasma membrane specializations responsible for the transfer of small molecules (up to ~1 kDa) and ions between adjoining cells (Bennett *et al.* 1991). Gap junction mediated intercellular communication has been implicated in fundamental cellular processes such as embryonic development, cell differentiation, proliferation and growth control. The proteins forming gap junctions, named connexins, are phosphoproteins (with the exception of connexin 26 (Cx26)) that can be phosphorylated by protein kinases activated by neurotransmitters, growth factors, hormones, oncogenes and exogenous chemicals. In particular, activation of protein kinases A and C is known to modulate junctional communication between cells. PKC has been shown to phosphorylate Cx43 in serine and threonine residues of the carboxyl tail of the protein (Moreno *et al.* 1994, Oh *et al.* 1991), but to date the phosphorylation of Cx43 by PKA has not been

unequivocally shown. Nevertheless, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and cAMP have been found to induce a variety of effects in cells expressing Cx43, leading both to inhibition and to enhancement of gap junction communication, depending on cell type and developmental stage (Oh *et al.* 1991, Risley *et al.* 1992, Xie & Hu 1992, Hünster & Weingant 1993, Lampe 1994).

In the testis, cellular interactions are essential for adequate functioning of many different cell types (Skinner 1982) and, to date, 11 types of connexin have been identified in its constituent cells (Kadle *et al.* 1991, Haefliger *et al.* 1992, Risley *et al.* 1992, Risley 2000). Freshly dissociated Leydig cells show both electrical and metabolic coupling, and Cx43 has been reported as the main functional component of their gap junctions (Pérez-Armendariz *et al.* 1994, Varanda & Campos de Carvalho 1994). Although electrical characteristics of the gap junction in these cells have been well characterized, modulation processes have not yet been extensively

studied. Given that gap junction communication has been shown to modulate secretion in endocrine and exocrine organs (Rüegg & Burgess 1989, Granot & Dekel 1994, You *et al.* 2000), it is important to study the regulation of junctional communication in TM3 Leydig cells. The main purpose of the present study was to examine the extent to which coupling between these cells can be influenced by PKA and PKC, and to correlate this modulation of coupling to the expression of Cx43 and the secretion of testosterone.

Materials and Methods

Cell culture

TM3 Leydig cells, a non-tumorigenic cell line derived from mouse testis, were obtained from the American Type Culture Collection. We decided to use this cell line in our studies because they respond to luteinizing hormone (LH) by increasing testosterone production and secretion, through mechanisms similar to those encountered in freshly isolated cells. The cells were grown in a mixture of Ham's F-12 medium plus Dulbecco's Modified Eagle Medium (1:1 v:v), supplemented with 5 mM NaHCO₃, 15 mM *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethane sulfonic acid], 7.5–10% fetal bovine serum and 1% penicillin/streptomycin, pH 7.4. Serum concentration (7.5 or 10%) did not affect cell properties. Cells were plated at either low or high density, depending on the type of experiment intended, for at least 12 h before the experiment, and then treated with a specific drug for times ranging from 15 min to 12 h. For subconfluent cultures, the cells were plated at approximately 200 cells/mm². For confluent cultures, the cells were plated at 400 cells/mm². At the time of the experiments, the low-density cultures had 320 cells/mm² and high-density cultures had 650 cells/mm². The cells were always cultured in the presence of fetal bovine serum and kept in an atmosphere of 5% CO₂ and 95% O₂ at 37 °C in an incubator (Forma Scientific).

Dye transfer

TM3 cells in low- or high-confluence cultures were plated in plastic dishes and injected with Lucifer Yellow CH (5% in 150 mM LiCl without pH adjustment), with the aid of glass microelectrodes (resistance between 40 and 70 MΩ) by application of short hyperpolarizing current pulses (0.1 nA, 100 ms), using a WPI amplifier model 7060. Dye transfer was observed in a Nikon Diaphot inverted microscope, equipped with xenon arc illumination, and photographs were taken using Kodak TMAX400 film, 2 min after injection of dye into one cell (Srivinas *et al.* 1999). For measurement of modulation of coupling by pharmacological agents, a minimum of 90 cells were injected for

every time point in each of at least three independent experiments; values were plotted as a function of time after drug application. The percent of coupled cells was evaluated as the number of injections that resulted in the transfer of Lucifer Yellow to neighboring cell(s), in relation to the total number of injections in a given assay. Results are presented as means ± S.E.M.

Immunofluorescence and Western blots

Cultured TM3 cells were plated in high or low confluence on 25 cm² plastic culture flasks or glass coverslips, previously treated with poly-L-lysine, for Western blot and immunofluorescence respectively. Cells were kept in culture for at least 12 h before experimental maneuvers. For western blots, the TM3 cells were treated with dibutyryl (db)-cAMP or TPA for 12 h. Staurosporin was used in TM3 cells after the 12 h treatment with PKA and PKC activators, and cells were incubated for 1 h with staurosporin. Homogenates from sister cultures (control and treated) were obtained by scraping 25 cm² flasks in 1 mM NaHCO₃ (pH 8.3) containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and were centrifuged at 10 000 r.p.m. for 10 min. Total protein content was determined by the Bradford method (Bradford 1976) and 100 µg samples were loaded in each lane of a 10% SDS-PAGE system. Protein was then transferred to nitrocellulose and the membrane was incubated with polyclonal antibody to residues 346–360 of the Cx43 sequence (kindly provided by Dr E L Hertzberg, Albert Einstein College of Medicine, New York, USA), as previously described (Varanda & Campos de Carvalho 1994). Heart homogenate was used as positive control and densitometry was performed using the gel analysis software Sigma Gel (v1.1, Jandel Scientific, Chicago, IL, USA). Results are presented as means ± S.E.M.

For immunofluorescence, the cells were treated with db-cAMP, TPA or staurosporin for the same time intervals described above and then fixed in ethanol (70%) at –20 °C for 20 min. Cx43 was detected as described previously for other cell types (Dermietzel *et al.* 1991). In short, cells were incubated, for 30 min at room temperature, with 0.1% IgG-free bovine serum albumin (BSA) to reduce non-specific binding. This was followed by incubation with the polyclonal antibody for Cx43, described above, diluted 1/500, for 1 h at room temperature. Then the cells were washed four times, for 10 min each time, with phosphate-buffered saline (PBS) and incubated with the secondary antibody (goat anti-rabbit IgG conjugated with fluorescein isothiocyanate diluted 1/150), for 1 h at room temperature. The coverslips were then washed with PBS four times, for 10 min each time, and mounted with 10 µl of a solution containing *P*-phenylenediamine + glycerol + PBS in the proportions 29:9:1. Fluorescence was observed on a Zeiss Axiovert 100 microscope. Because of the repeated washes and poor adherence of TM3

cells to the coverslips, it was common to lose cells during processing for immunofluorescence.

Radioimmunoassay

Leydig cells were cultured in 75 cm² flasks and subjected to treatment with LH (LH-RP3 No. AFP7187B; National Institute of Diabetes, Digestive and Kidney Diseases, USA) for the indicated times. Testosterone concentration was assayed in the incubation media by radioimmunoassay. Cells in five different incubation bottles were treated with either 1 or 10 ng/ml LH. The results are normalized for 10⁶ cells (number of cells determined by counting in Neubauer chamber) and the initial value (zero time) was taken as the concentration of testosterone detected in the incubation medium (DMEM+10% FCS) without cells. ³H-Testosterone was obtained from NEN Life Science Products and the specific antibody was a generous gift from Dr José Antunes Rodrigues (Department of Physiology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil). The lower limit of detection of the assay was 0.3 ng/ml sample and the intra-assay coefficient of variation was 5.4%.

Isolation of total RNA

Total RNA from all subconfluent or confluent cells and rat heart tissue was extracted by Trizol reagent (Gibco BRL, Grand Island, NY, USA). RNase-free DNase I (1 U/μl) was used to treat the isolated RNA for 1 h, to eliminate contamination with genomic DNA. The treatment was terminated by extraction with phenol–chloroform–isoamyl alcohol (PCI) in the proportions 25:24:1 (vol/vol) and precipitation with ethanol.

Reverse transcriptase-polymerase chain reaction

In order to prepare first-strand DNA, total RNA isolated from TM3 Leydig cells ($n = 3-4$) and rat heart cells was reverse-transcribed with SuperScript (Gibco BRL) at 37 °C for 60 min, extracted with PCI and precipitated with ethanol. The cDNA synthesis was primed with oligo-dT primer. A PCR technique was used to amplify the synthesized cDNA. The following solution was used: 0.2 μmol/l primers, 0.2 mmol/l each dNTP, 50 mmol/l KCl, 10 mmol/l Tris HCl (pH 8.3) and 1.5 mmol/l MgCl₂ plus 2.5 U AmpliTaq (Perkin Elmer, New Jersey, NJ, USA). The primers synthesized for Cx43 (sense: 5'-ATGAGCAGTCTGCCTTTCGT-3'; antisense: 5'-CACCACCAGCATGAAGATGA-3') correspond to nucleotides 346–366 and 2076–2093 respectively of the rat Cx43 sequence (GeneBank accession number: XM 027460) and are predicted to amplify a 459 bp PCR product. PCR was performed with 34 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min). Rat glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) primers predicted to amplify a 221 bp PCR product (sense: 5'-GTCTTCACCACCAT GGAG-3'; antisense: 5'-CGATGCCAAAGTTGTCA TG-3') corresponding respectively to nucleotides 326–343 and 518–536 of the rat GAPDH gene were added into the same RT-PCR reaction tubes and their products were used as internal controls (GeneBank accession number: M17701). PCR products were size-fractionated by agarose gel electrophoresis. The expected Cx43 and GAPDH bands from the same sample were submitted to a densitometry analysis using the gel analysis software Sigma Gel (v1.1, Jandel Scientific) and normalized by dividing the Cx43 values by the corresponding GAPDH values.

The semi-quantitative method of RT-PCR used for studies of expression of Cx43 mRNA in TM3 Leydig cells was validated in preliminary experiments. Firstly, the optimal PCR conditions that yielded a single band on agarose gel electrophoresis were determined for Cx43 and GAPDH genes in the same reaction tube. Secondly, to determine whether the method was semi-quantitative, serial quantities of total RNA (62.5, 125, 250, 500, 1000 and 2000 ng) extracted from TM3 cells were used for RT-PCR amplification for Cx43 and GAPDH genes in the same reaction tube. Thirdly, experiments were performed to determine the optimal number of PCR cycles that yielded PCR products in the linear phase of amplification of both genes in the same reaction. Finally, to ensure that the reactions were consistent, PCR reactions were performed at least twice. Only one of these reactions was included for final densitometry analysis, and the selection was arbitrary. For the semi-quantitative PCR comparing the confluent and subconfluent cells and the drug treatments, the optimal total amount of RNA was 500 ng and the optimal number of PCR cycles was 34. All reactions included a negative control RT(–). The identity of the amplification was confirmed by determination of the molecular size on agarose gel electrophoresis with a 100 bp DNA molecular marker (Gibco BRL). Results are presented as means ± S.E.M.

Statistical analysis

One-way analysis of variance followed by the Newman–Keuls multiple comparison test was used for data presented in Figs 2, 3 and 6. For data given in Fig. 1 we used the χ^2 test, and for those in Figs 4, 5, 8 and 9 we performed Student's non-paired *t*-test. In all cases, statistical significance was considered to be present at $P < 0.05$.

Results

When examined at high density in confluent cultures, TM3 Leydig cells showed a poor degree of dye coupling (Fig. 1A, B) demonstrated by the low proportion of injections that resulted in the spread of Lucifer Yellow

Figure 1 Gap junction coupling between TM3 Leydig cells. (A) Phase and (B) fluorescence pictures of cells cultured under high confluence; (C) phase and (D) fluorescence pictures of cells forming pairs in a low-confluence culture. In (B) and (D) Lucifer Yellow was injected in one cell and pictures taken 2 min later. (E) Histogram of the percentage of coupled cells in each condition. Low, low-confluence cultures; High, high-confluence cultures; Pairs, cells in low confluence forming pairs; n, number of cells that were injected with Lucifer Yellow in each condition. Calibration bar in (D) represents 20 μ m.

between the cells (3% ($n=108$), histogram in Fig. 1). In contrast, when cultured at low density as subconfluent cultures, the cells showed a high degree of coupling, with 80% of the injected cells ($n=20$) showing transfer of the dye. This number is very similar to that observed in cell pairs (Fig. 1C, D) in which 87% of the pairs were coupled ($n=131$, histogram in Fig. 1), and is in agreement with previous reports based on electrophysiological measurements (Pérez-Armendariz *et al.* 1994, Varanda & Campos de Carvalho 1994, Cristancho *et al.* 2000).

Western blots revealed the presence of Cx43 in TM3 cells. Figure 2 illustrates an experiment in which Cx43 was detected in subconfluent and confluent sister cultures. Densitometry shows that the labeling was more intense for subconfluent (lane 1) than for confluent (lane 2) cultures, indicating that the expression of this protein is modulated by contact between the cells.

The results of semi-quantitative RT-PCR using Cx43-specific oligonucleotides and RNA extracted from Leydig cells in confluent and subconfluent cultures are shown in Fig. 3. Expression of Cx43 mRNA was reduced in confluent cultures (to 47.5% of subconfluent culture values, $P<0.05$), in agreement with the diminished expression of protein.

It is widely accepted that db-cAMP and TPA activate PKA and PKC respectively (Berridge 1984). PKA activation generally leads to increased intercellular communication, but PKC activation usually has the opposite effect. Figure 4A shows the effect of PKA activation on gap junction intercellular communication 1, 6 and 12 h after a single addition of db-cAMP (0.5 mM) to the culture medium. Figure 4B illustrates similar experiments using TPA (50 nM) to activate PKC. Both cAMP and TPA greatly reduced gap junction intercellular communication in subconfluent TM3 cell cultures. A significant reduction was already apparent in the first 1 h after addition of the

drugs to the medium (>80% of the cells uncoupled), and this was maintained after 12 h of a single drug application.

Staurosporin, an inhibitor of protein kinases, is able to couple TM3 cells in confluent cultures and to reverse the uncoupling effects of both TPA and c-AMP in subconfluent cultures. Figure 5 illustrates a typical experiment in which cells in a subconfluent culture were injected with

Figure 2 Western blot for Cx43. Lanes 1 and 2: homogenates of TM3 Leydig cells cultured under low and high confluence, respectively. Lane C: a rat heart homogenate used as positive control for Cx43. Each lane of the gel was loaded with 100 μ g total protein before transfer to nitrocellulose. The band for cells in low confluence was more intensely marked than that for high-confluence cells. As the amount of protein loaded in the gel was the same, this difference can be used as a semi-quantitative estimate of Cx43 expression. The histogram represents the mean $\pm$ S.E.M. ($n=3$) of the relative densitometry values for Cx43 labeling, taking the rat heart homogenate as control. *Protein expression in confluent cultures was significantly different from that in subconfluent ones ($P<0.05$).

Figure 3 Relative expression of Cx43 in total RNA isolated from subconfluent and confluent TM3 Leydig cells: results obtained by amplifying Cx43 and GAPDH RT-PCR products. The histogram represents the mean $\pm$ S.E.M. for the ratio between the Cx43 and GAPDH bands ($n=4$). The insert shows the agarose gel of a semi-quantitative RT-PCR experiment. RT(-), negative control, performed with 500 ng total RNA derived from subconfluent cells in the absence of reverse transcriptase. *Expression of mRNA in confluent cultures was significantly different from that in subconfluent cultures ($P<0.05$).

Lucifer Yellow, db-cAMP (500 μ M) was added for 1 h and dye coupling was again measured, and then 1 mM staurosporin was added, in the presence of db-cAMP, for 30 min and coupling was assayed once more. The histogram shows that staurosporin restored gap junction intercellular communication to the control condition even in the presence of db-cAMP. Similar results were obtained when cells were treated first with TPA and then with staurosporin (not shown).

Interestingly, total expression of the Cx43 protein in TM3 Leydig cells treated with cAMP, TPA and staurosporin was not altered in relation to control (Fig. 6A). Accordingly, semi-quantitative RT-PCR using total RNA from the same treated cells showed that Cx43 mRNA was not altered in relation to that in control cultures (Fig. 6B).

Indirect immunofluorescence was used to evaluate whether the cellular localization of Cx43 was directly

Figure 4 Junctional coupling in TM3 Leydig cells was decreased by cAMP (A) or TPA (B). The drugs were added to subconfluent cultures at a concentration of 500 μ M for cAMP or 50 nM for TPA, for 1, 6 and 12 h. Control, no drug added. The degree of coupling was assessed by injecting Lucifer Yellow in one cell of a group and counting the number of cells labeled after 2 min. A total of 720 cells was injected in three different culture dishes for each group (control, 1 h, 6 h and 12 h). For each experiment, the percentage of coupled cells (number of injected cells that passed dye to neighbors/total number of injected cells) was determined and means $\pm$ S.D. were plotted for each experimental condition. *Coupling in the control was significantly greater than that in any of the treated cultures ($P<0.05$).

Figure 5 Staurosporin reversed the uncoupling effect of cAMP. At least 30 cells were injected with Lucifer Yellow in each of these subconfluent-culture dishes. The percentage of coupled cells in each dish was determined and means $\pm$ s.d. were plotted (number of cells injected in each condition was 90). Control coupling was determined 12 h after plating (black bar); cAMP (500 μ M) was applied to the same cultures for 1 h and coupling was again determined (cross-hatched bar). Staurosporin (1 mM) was then added in the presence of cAMP, restoring the degree of coupling to control values (open bar). *Coupling during cAMP treatment was significantly lower than that in the two other groups ($P < 0.05$).

related to coupling in cells at low and high confluence. In low-confluence cultures and in pairs of TM3 Leydig cells, immunoreactivity for Cx43 was detected at appositional membranes, in addition to diffusely in the cytoplasm (Fig. 7A). In high-confluence cultures, TM3 Leydig cell immunoreactivity was detected mainly as points dispersed in the cytosol, with no detectable labeling in appositional membrane regions (not shown). In agreement with the findings of the dye-coupling experiments, when subconfluent cultures or cell pairs were treated with 500 μ M db-cAMP (Fig. 7B) or 50 nM TPA (Fig. 7C) for 1 h, they showed labeling over the cytosol and the perinuclear region, with disappearance of the appositional labeling (compare Figs 7A, 7B and 7C). In contrast, confluent cells treated with 1 mM staurosporin for 1 h showed intense reactivity at the appositional membranes (Fig. 7D). Incubation with secondary antibody alone did not label the cells (not shown).

To investigate the effects of a physiological modulator of Leydig cells, we exposed TM3 subconfluent cultures to LH and measured hormone secretion and gap junction intercellular communication in the cultures. When testosterone secretion by the TM3 cells in subconfluent cultures was determined during a 12 h period, control values were within the ranges reported in the literature (Pérez-Armendariz *et al.* 1996). Upon exposure of the cells to LH (1 or 10 ng/ml), testosterone secretion increased during the first hour and then was maintained at high levels

during the 12 h period (Fig. 8). Figure 9 shows that, 15 min and 1 h after application to TM3 Leydig cells, LH in concentrations of 1 and 10 ng/ml significantly reduced gap junction intercellular communication and that, 3 h after exposure of the cells to LH, coupling was totally abolished. These results were to be expected, because LH acts by increasing adenyl cyclase activity in these cells, resulting in activation of PKA.

Discussion

In this study we have shown that TM3 Leydig cells are coupled through intercellular gap junctions in a manner similar to that described for freshly dissociated Leydig cells from mouse testis (Pérez-Armendariz *et al.* 1994, 1996, Varanda & Campos de Carvalho 1994). We have also shown that the junctional communication between TM3 cells is modulated by cell density, as coupling in cells under high confluence was lower than that observed under low confluence or in cell pairs.

Cx43 has an important role in gap junction communication in TM3 Leydig cells, based on the correlation between modulation of cell-cell coupling and the expression of Cx43 at appositional membranes shown in this study. Previous experiments in which we showed that junctional conductance properties in TM3 cells were similar to those obtained in communication-deficient cell lines transfected with Cx43 cDNA reinforce this idea. To date, 11 different connexins have been reported to be present in the testis, communicating between the different cell types that constitute this organ (Kadle *et al.* 1991, Haefliger *et al.* 1992, Risley *et al.* 1992, Risley 2000). Preliminary experiments using RT-PCR in TM3 Leydig cells have identified six connexin transcripts, of which connexins 31, 37, 40, 43, 45 and 50 have been described in testis (Risley 2000). Whether or not all these connexins contribute to functional coupling between TM3 cells remains to be investigated.

Our experiments show that, in TM3 Leydig cells, db-cAMP and TPA drastically reduce junctional coupling 1 h after application. A rapid decrease (in minutes) in electrical coupling has been reported (Cristancho *et al.* 2000) after activation of PKA and PKC, indicating that these protein kinases act to reduce intercellular communication by both short- and long-term mechanisms in TM3 cells. These findings are consistent with the hypothesis that their activation may induce effects directly on the channel – with respect to the fast reduction in junctional conductance – and may also interfere with the assembly, degradation, or both, of channel protein. In agreement with that, a 12 h exposure to cAMP or TPA induced disappearance of Cx43 membrane reactivity, restricting it to the cytoplasm of cells in subconfluent cultures, whereas total Cx43 protein concentrations and message levels were unaltered by these treatments.

Figure 6 cAMP, TPA and staurosporin did not affect the expression of Cx43 protein (A) or mRNA (B). (A and B) Lanes 1–4: homogenates of TM3 subconfluent Leydig cell cultures treated with TPA+staurosporin, cAMP+staurosporin, TPA, and cAMP respectively; lane 5: control TM3 subconfluent culture (not treated); lane 6: a rat heart homogenate used as a positive control for Cx43. (A) Each lane of the gel was loaded with 100 µg total protein before transfer to nitrocellulose. The histogram represents relative densitometry values for Cx43 labeling, taking the rat heart homogenate as control ($n=3$). Expression of Cx43 was not altered by any of the treatments. (B) Relative expression of Cx43 mRNA in subconfluent TM3 Leydig cells, obtained by amplified Cx43 and GAPDH RT-PCR products. The histogram represents the mean $\pm$ S.E.M. values for the ratio between the Cx43 and GAPDH bands ($n=3$). The insert shows the agarose gel of a semi-quantitative RT-PCR experiment. RT(–), negative control, performed with 500 µg total RNA derived from subconfluent cells in the absence of reverse transcriptase. *Expression of mRNA in rat heart tissue was significantly different from treated and un-treated subconfluent TM3 cells ($P<0.05$). There were no significant differences between all groups of TM3 cells.

The results reported here for db-cAMP are the opposite of what has been found in the majority of other cell types, in which cAMP enhances coupling in spite of the fact that it decreases single channel conductance (Kwak *et al.* 1995, Moreno *et al.* 1994). Nonetheless, Sacai *et al.* (1992) and Cole & Garfield (1986) reported cAMP-dependent reduction of coupling in myometrial cells and argued that their results were dependent on the stage of maturation of the cell. In addition, in immature rat prefrontal cortex, both cAMP and β -adrenoceptor activation reduced gap junctional coupling, as demonstrated by a decline in transfer of dye between cells (Rorig *et al.* 1995).

In contrast, our results with TPA are in agreement with most of those reported in the literature, in which a reduction in junctional conductance has been clearly seen in preparations in which Cx43 seems to be the main gap junction forming protein (Yada *et al.* 1985, De Mello 1991, Giaume *et al.* 1991, Spray *et al.* 1991). Nonetheless, exceptions have been observed: an increase in coupling after treatment with TPA has been reported in Sertoli cells (Grassi *et al.* 1986) and in corpus cavernosum smooth muscle cells (Moreno *et al.* 1993).

It should be pointed out that the data obtained with dye coupling, western blot, immunofluorescence and semi-quantitative RT-PCR in the TM3 cells were consistent. Immunofluorescence showed that the TM3 cells displayed Cx43 reactivity localized to the appositional membranes when in pairs or in low confluence, compatible with the high degree of dye coupling measured under these

Figure 7 Indirect immunofluorescence for Cx43. (A) Cells forming pairs showed immunoreactivity mainly localized to the appositional membranes, where the gap junction plaques are localized. (B) Treatment of cells in low confluence with 500 µM db-cAMP displaced the immunoreactivity from the appositional membranes and dispersed it in the cytoplasm. (C) Immunoreactivity of cells in low confluence treated with 50 nM TPA. Labeling was diffuse in the cytoplasm, but there was no appositional membrane labeling. (D) Intense membrane labeling in high-confluence cultures treated with 1 mM staurosporin for 1 h. Calibration bar represents 5 µm for (A), (B) and (C), and 10 µm for (D).

Figure 8 LH-induced testosterone secretion in TM3 Leydig cells. Cells were incubated in the absence of LH (control) and in the presence of 1 ng/ml or 10 ng/ml of the hormone. Values are means $\pm$ S.E.M. ($n=5$). *Statistically different from control (Student's non-paired *t*-test, $P<0.05$).

conditions. In contrast, confluent cultures not only showed poor dye coupling, but also displayed immunoreactivity to Cx43 localized in the cytoplasm, and not in appositional membranes. Accordingly, western blots and the semi-quantitative RT-PCR of confluent cultures showed a lower expression of Cx43 protein and mRNA respectively, when compared with subconfluent cultures. However, Cx43 mRNA and Cx43 protein concentrations differed by approximately twofold, whereas the percentage of coupling varied by almost 30-fold when we compared subconfluent and confluent cultures, suggesting that other connexins may form gap junction channels between TM3 cells, or that the localization of Cx43 at membrane appositional areas is the relevant variable.

Granot & Dekkel (1994) and You *et al.* (2000) have reported that the expression of gap junctions between rat ovarian cells and between rat Leydig cells respectively can be modulated by PKA and LH – known to induce an increase in the intracellular concentration of cAMP (Duffau *et al.* 1980, Rüegg & Burgess 1989). Both groups showed that LH induced a significant decrease in Cx43 mRNA levels after incubation for 24 h, and in the ovarian cells staurosporin blocked these effects.

Many authors have shown that secretion is accompanied by uncoupling in endocrine and exocrine organs. You *et al.* (2000) have directly measured the production of testosterone and levels of Cx43 mRNA during stimulation of rat Leydig cells by human chorionic gonadotrophin (hCG), both in culture and *in vivo*. As expected, hCG treatment led to an increase in testosterone formation and a decrease in Cx43 mRNA levels – an effect that was also mimicked by cAMP. These results are consistent with

Figure 9 Junctional coupling in TM3 Leydig cells was decreased by LH. Treatment of the cells with 1 ng/ml and 10 ng/ml LH decreased the degree of coupling in subconfluent cultures exposed to the hormone for 15 min, 1 h and 3 h compared with control (no drug added). The number of cells injected in each condition was 90. *The degree of coupling in the non-treated cells was significantly different from that in treated cells ($P<0.05$).

experiments in which we found that LH at concentrations ranging from 1 to 10 ng/ml induced a marked uncoupling of the TM3 cells and a sustained increase in production of testosterone by the TM3 cells. Taken together, these data imply that gap junction mediated intercellular communication may have an important role in modulating hormone production in Leydig cells.

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