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Follicle-Stimulating Hormone Increases Gap Junction Communication in Sertoli Cells from Immature Rat Testis in Primary Culture

F. Pluciennik, M. Joffre, J. Délèze

Unité de Recherche Associée au CNRS 1869 "Biomembranes," Faculté des Sciences, 40 Avenue du Recteur Pineau, 86022-Poitiers, France

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Abstract. The gap junction communication in Sertoli cells from immature rat testes, cultured either in absence or in presence of follicle-stimulating hormone (FSH), was studied by microinjection of a fluorescent dye and by Fluorescence Recovery After Photobleaching (gapFRAP).

The cells cultured for 2–4 days in the absence of FSH showed a flattened "epithelial-like" appearance. They were poorly coupled, as judged by the low frequency of cell-to-cell spread of microinjected Lucifer Yellow, and by the value of the rate constant of dye transfer (k) estimated in gapFRAP experiments. However, when two different subpopulations of cells were separately analyzed, namely the cells forming small groups contacting over part of their circumference ("adjoining cells"), and the cells arranged in tight clusters, we found that the value of k in the latter group was much higher, reaching about 75% of that obtained in the presence of FSH.

The cells cultured for two days in a medium containing ovine FSH underwent striking morphological changes and presented a rounded, "fibroblast-like" appearance. They were arranged in networks or in clusters. The frequency of cell-to-cell dye diffusion after microinjection and the rate constant of dye transfer were rapidly increased to the same final level by FSH, although they were initially different in these two groups. A concentration dependence of k, in the range 0.05 to 3 ng/ml, was observed in the cells in networks, contrasting with an all-or-none increase in the cells in clusters.

Two days after FSH withdrawal, the dye transfer constant returned to prestimulation control values in the cells in clusters, but not in the cells in networks, which maintained a stable degree of coupling comparable to that of the unstimulated cells in clusters. This observation suggests (i) that an initial promoting effect of FSH already exists in the immature rat testis, which is preserved after enzymatic treatment in the cell clusters, but not in the more dispersed cells, and (ii) that the decreased junctional coupling is re-established in the dispersed cells by FSH, through a synthesis or a membrane insertion of connexin.

The effects of FSH were mimicked by a brief exposure to 1 mM dibutyryl-cyclicAMP, but not to 10 nM human chorionic gonadotropin (hCG), indicating that the gap junction communication in Sertoli cells is upregulated by FSH through a specific membrane receptor, with cyclicAMP acting as a second messenger.

Key words: Sertoli cells — Gap junction — GapFRAP method — Dye diffusion — Follicle-stimulating hormone — CyclicAMP

Introduction

Evidence has been rising in support of the hypothesis that the cell-to-cell exchange of small ions and molecules through gap junctions is an important determinant of tissue growth, differentiation and metabolic activity (*reviewed in* Loewenstein, 1981, 1986; Warner, 1988; Loewenstein & Rose, 1992).

Changes in cell-to-cell communication through gap junctions seem to take part in the control of secretory function. This is indicated by the uncoupling effect of acetylcholine on the salivary gland (Iwatsuki & Petersen, 1978) and on the lacrimal gland (Neyton & Trautman, 1986), or by the changes of insulin-secretion following the development of gap junctions in pancre-

Correspondence to: M. Joffre

atic beta cells (Meda et al., 1983), and their uncoupling by heptanol (Meda et al., 1990).

The Sertoli cells are part of the epithelial wall of the seminiferous tubules of the mammalian testis. The synthesis and transformation of several sexual steroids, together with the expression and release of a number of proteins (among them an androgen binding protein), point to an important contribution of these cells to the development and maintenance of spermatogenesis. Another well-known function of the Sertoli cells is secretion of a potassium-rich testicular fluid into the lumen of the seminiferous tubule (*for review:* Jégou, 1992).

A junctional complex, comprising tight and gap junctions, is present in the seminiferous tubules of mature rat testis (Dym & Fawcett, 1970; Gilula, Fawcett & Aoki, 1976). An analogous structure is also observed in cultures of whole seminiferous tubules (Meyer, Posalaky & McGinley, 1977) and in monolayer cultures of Sertoli cells, isolated from rat testes and grown in a FSH-containing medium (Solari & Fritz, 1978; Posalaky et al., 1981; Hadley et al., 1985).

Formation of the junctional complex coincides with the establishment of the blood-testis barrier (Johnson, 1970) and is a prerequisite for the onset of fluid secretion, which in the rat starts between 15 and 19 days after birth (Vitale, Fawcett & Dym, 1973). The frequency of gap junctions is greater in immature rat testes and decreases when spermatogenesis is initiated. At that time, the gap junction particles have been seen to become closely associated with tight junctions (Gilula et al., 1976). In keeping with these morphological observations, cell-to-cell electrical coupling is known to be present in cultured Sertoli cells (Eusebi et al., 1985; Grassi et al., 1986).

The follicle-stimulating hormone (FSH) elicits a number of morphological and biochemical events in the Sertoli cells of immature testes (*reviewed by* Jégou, 1992). For instance, in Sertoli cells isolated from 18-day-old rats and cultured in a two-compartment chamber, FSH promotes the growth of tight junctions and increases the electrical resistance measured across the cell monolayer (Janecki, Jakubowiak & Steinberger, 1991*a, b*).

It has been shown that FSH substantially increases the membrane potential of cultured Sertoli cells from immature rat testis by cyclic AMP-dependent processes, which simultaneously stimulate active ion transport by a Na/K pump and induce a mixed Na/Ca influx. Membrane hyperpolarization then results from outward K^+ currents activated by the rise of intracellular calcium concentration (Joffre & Roche, 1988). The hyperpolarization promoted by FSH is maintained in a hormone-free medium, but is rapidly and reversibly reduced by four different treatments known to induce electrical uncoupling (Roche & Joffre, 1989). To account for these data, the hypothesis that FSH modulates the membrane potential and cell-to-cell communication through gap junctions in Sertoli cells was proposed.

In the present study, we examine the effects of FSH on dye transfer through gap junctions in Sertoli cells by microinjection of Lucifer Yellow (LY) and by Fluorescent Recovery After Photobleaching (gapFRAP). We show that the gap junctional communication is increased by FSH. This effect is mimicked by dibutyryl-cyclicAMP, and is partly reversed when withdrawing the hormone-containing medium. It cannot be reproduced by human chorionic gonadotrophin (hCG), another glycoprotein hormone with a heterodimeric structure similar to that of FSH. We conclude that gap junction communication in cultured Sertoli cells is upregulated by FSH through a specific membrane receptor, which triggers the cyclicAMP enzymatic cascade. Preliminary accounts of the main results have been published in abstract forms (Pluciennik, Joffre & Délèze, 1991, 1992).

Materials and Methods

Sertoli Cell Preparation

The experiments were performed on cultured Sertoli cells isolated in sterile conditions from 15–17-day-old Wistar rats. The animals, raised at constant temperature (20°C) under a controlled light-dark cycle (12 hr/12 hr), were killed by decapitation and the testes removed aseptically. After decapsulation, the parenchyma of six to ten testes were submitted to three consecutive enzymatic treatments at 37°C, by collagenase, pancreatin and trypsin, according to a procedure modified from Verhoeven, Dierickx and de Moor (1979). The first dissociation step was performed in 25 ml of a Ca^{2+} - and Mg^{2+} - free solution (medium A) containing 12.5 mg collagenase (Serva, 0.6–0.8 U/mg). The interstitial tissue was separated from the tubules by incubation with continuous shaking (110 cycles/min, 60 min). The tissue was then allowed to sediment for 5 min and the supernatant was removed. The remaining tissue was resuspended in 10 ml of medium A and shaken by hand for 10 sec. After repeating this washing procedure, the tissue was minced with scissors and washed five times with 10 ml of medium A, then allowed to sediment for 5 min.

A second incubation was carried out to detach the peritubular cells, comprising myoid cells and fibroblasts embedded in a collagen network, from the seminiferous tubules. This was performed under continuous shaking (110 cycles/min, 20 min) in 25 ml of medium A containing 12.5 mg of pancreatin (Sigma, St. Louis, MO, grade VI). The pancreatin solution was then discarded, and the fragments of seminiferous tubules were separated from the peritubular cells by gentle shaking in the presence of 10 ml of medium A (five times).

The seminiferous tubules were finally dissociated into aggregates of Sertoli and germinal cells by a third enzymatic incubation in 5 ml of a trypsin-EDTA mixture (GIBCO, 0.5 g/100 ml), under continuous shaking at 110 cycles/min for 5 min. The cells were resuspended in 5 ml of medium A containing 0.5 mg of a trypsin inhibitor (Sigma, soybean-type 1S), transferred to a 50 ml polycarbonate tube and allowed to sediment for 15 min. The supernatant was discarded, the sedimented fragments washed twice with 10 ml of medium A and passed about ten times through a syringe needle (No. 17 gauge) at a slow rate. The cell suspension was adjusted to a volume of 5–7 ml with medium RPMI 1640 (GIBCO, UK) and the cell density was measured in a haematocytometer.

Medium A was a Ca^{2+} and Mg^{2+}-free modified Earle's solution containing (in mM): 116.3 Na^+, 5.4 K^+, 121.7 Cl^-, 0.9 H_2 Po_4^-, 5.5 glucose, supplemented with 53.5 mannitol and 20 HEPES and with streptomycin sulfate (100 mM) and penicillin G (100 IU/ml) (Serva). The pH was adjusted to 7.4 and the osmolarity to 300 mOsmol by mannitol addition. The medium was sterilized by filtration through a 0.22 μm pore filter (Millipore).

Sertoli Cell Culture

The final cell preparations were plated at low density in 35-mm plastic petri dishes (Nunclon, Nunc, UK). On the first day, 5×10^5 cells were suspended in 2 ml RPMI 1640 supplemented with 2 mM L-glutamine (Sigma), transferrin (Sigma, 0.05 mg/ml), insulin (Sigma, 0.1 mg/ml), bovine serum albumin (Sigma, 1 mg/ml), HEPES (Sigma, 10 mM), sodium bicarbonate (20 mM) and with streptomycin sulfate (100 mM) and penicillin G (100 IU/ml). The culture dishes were incubated at 34°C for two to five days in a humidified CO_2 incubator (5% CO_2/95% ambient air). From day two on, the medium was renewed at two-day intervals.

Cell-to-cell dye diffusion was assayed from day two to day five on dispersed groups of Sertoli cells attached to the plastic (*see* Fig. 1). All observations were performed at room temperature (20–23°C) and in ambient air, after replacing the culture medium with a Tyrode saline containing (in mM): 147 Na^+, 5.4 K^+, 1.8 Ca^{2+}, 1 Mg^{2+}, 148 Cl^-, 5.5 glucose, and 10 HEPES to maintain the pH at 7.4 in the absence of CO_2.

Dye-Diffusion Experiments

Cell-to-Cell Dye Transfer after Microinjection

In a first series of experiments, the extent of cell-to-cell communication has been estimated simply by counting the cells which became fluorescent after a microinjection of Lucifer Yellow (LY) into one Sertoli cell. This highly fluorescent and membrane impermeant molecule (MW 457) is known to pass through the junctions (Stewart, 1978).

The experiments were carried out on the stage of a Zeiss Standard microscope equipped for Nomarsky interference contrast and epifluorescence observations. Micropipette tips were filled by capillarity with a solution containing 65 mM Lucifer Yellow-CH (dilithium salt, Sigma) and 140 mM LiCl. The micropipettes were inserted, under interferential contrast optics at a magnification of 400×, into one cell situated inside randomly selected groups of Sertoli cells. After switching to epifluorescence illumination, the microinjection was preformed by means of an apparatus (Socolar & Loewenstein, 1979) that allowed us to apply gas pressures of slowly increasing strength to the micropipettes, until a weak fluorescence began to appear in the injected cell. Injection proceeded for a few seconds and was interrupted when the cell became uniformly fluorescent. Care was taken to avoid cell swelling, and all experiments showing signs of cell injury under interference contrast were rejected. Two minutes after completing the injection, the cells that had become fluorescent inside the group containing the injected cell were counted.

Cell-to-Cell Dye Transfer Estimated by Fluorescence Recovery after Photobleaching

Principle of Method. The FRAP method has been applied to the study of cell-to-cell communication through gap junctions (gapFRAP) by Wade, Trosko and Schindler (1986). Cells in a thin monolayer are uniformly loaded with a fluorescent molecule that easily passes through gap junctions, and the fluorescent emission of one cell is suppressed or substantially reduced by a light pulse of sufficient intensity (a photobleaching process). If the bleached cell is connected to its neighbors by open gap junctions, a fluorescence recovery takes place by diffusion of dye molecules from adjacent cells.

Dye Loading of the Cells for GapFRAP. For gap-FRAP measurements, the Sertoli cells were loaded for 10 min at room temperature with a fluorogenic substrate (Rotman & Papermaster, 1966), the diacetate ester of 6-carboxyfluorescein (6-CF; Sigma) dissolved in DMSO (0.25% of the volume of Tyrode solution) to give a final concentration of 7 μg/ml. This nonpolar compound penetrates readily into the cells, where it is hydrolyzed by cytoplasmic esterases, releasing the highly fluorescent and polar molecule 6-carboxyfluorescein. The latter accumulates inside the cells because of its very low membrane permeability.

When dye loading was completed, the extracellular fluorogenic ester was washed off carefully several times with Tyrode. Labeling the cells with 6-CF did not affect their viability, and the same dishes could be restained on successive days.

Data Acquisition. Fluorescence intensities of the 6-CF-loaded cells were observed and recorded, and photobleaching was performed by means of the cytofluorimetric system ACAS 570 (Anchored Cell Analysis and Sorting, Meridian Instrument, Okemos, MI), which allows convenient digital video imaging and analyzing. The ACAS 570 workstation basically consists of an inverted phase contrast microscope (Olympus IMT-2) equipped with a computer-controlled (PC-AT 286) source of laser light (5 W argon ion laser), a high speed step-by-step scanning stage, and with devices for light detection and measurement, data storage, display and processing. Records of the fluorescent emission of the 6-CF-loaded cells, excited by weak pulses of the argon laser tuned at 488 nm, are performed by moving a selected field of the tissue culture dish in front of the microscope objective (40×, numerical aperture 0.55, from which a theoretical depth of field of 1.5 μm can be calculated), which focuses the light pulses down to about 1 μm. The fluorescent emission, collected by the microscope objective and separated from the excitation wavelength by appropriate dichroic (510 nm) and barrier (530 ± 30 nm) filters, is measured at each step and recorded together with its x-y coordinates. A computer-reconstructed color-coded digital image of the fluorescence intensities simultaneously appears on a video screen, and can be subsequently analyzed and printed either in a false color or in a gray density scale (*see* Fig. 3).

A number of Sertoli cells in a 180 × 180 μm field were selected for measurement and delineated by drawing a polygon (*see* Fig. 3), into which the emission intensity can be automatically integrated. A control image of the initial distribution of fluorescence was obtained under an intensity of the excitation light chosen to avoid cell damage and to minimize photobleaching of the fluorescent molecules during the subsequent scans. Photobleaching of several cells (e.g., cells labeled *1,2,3,4* in Fig. 3*B* and *E*) was then performed by flashing a preset number of laser pulses of higher intensity (8 mW), which had been chosen by trial and error in preliminary assays to induce a sufficient bleaching (remaining fluorescent intensity 10 to 20% of initial values) without causing cell damage. The fluorescence levels of the cells in the selected field were then recorded immediately after photobleaching and in a series of successive scans (usually six) taken at intervals of 2 min.

Image Analysis and Estimate of the Dye-Transfer Constant. The software provided with the ACAS Meridian system allows for convenient off-line time-resolved integration of fluorescence intensities in any selected area of the recorded fields (e.g., Fig. 4). A spontaneous fluorescence decay, of less than 10% of the initial intensity level, occurred

in unbleached cells during the 12 min of an experiment, due to the outflux of 6-carboxyfluorescein through nonjunctional cell membranes and to photobleaching in the successive scans. In contrast, the fluorescence intensities integrated in the bleached cells increased with time, provided they were in contact with unbleached neighbors. The fluorescence recovery curves from the bleached cells were automatically corrected for the spontaneous fluorescence decay measured in unbleached cells from the same records.

As expected from diffusion kinetics across a concentration boundary, the initial rise of concentration of fluorescent molecules in bleached cells closely follows an exponential time course. If the gap junctional membrane is the rate-limiting step in this cell-to-cell diffusion process, the rate constant (k) of the exponential fluorescence recovery in the bleached cells provides a quantitative measure of the dye transfer through the junctional membrane. In thin layers (up to 100 μm) and at low dye concentrations (range 10^{-8} to 10^{-3} M), the integrated fluorescence intensities vary in proportion to the dye concentration (Barrows et al., 1984). Therefore, the rate constant of the fluorescence recovery (k) can be obtained from the equation:

$$(F_i - F_t)/(F_i - F_o) = e^{-kt} \quad (1)$$

(Peters, 1983), where F_i, F_o and F_t are fluorescence intensities integrated to the whole cells before, immediately after, and at time t after photobleaching, respectively. The rate constant (k) of cell-to-cell dye transfer (the inverse value of the time constant in $10^{-2} \times \text{min}^{-1}$) is related to the permeability coefficient P of the junctional membrane by:

$$P = (V/A)k \quad (2)$$

where V is the volume of the bleached cell and A the area of the gap junctions.

Statistical Analysis

Ideally, measurements of fluorescence recovery should be performed on the same set of cells submitted to different experimental conditions. As this was not possible in a study of delayed hormonal effects, the statistical significance of the difference of the means was established using Student's t-test for unpaired data, with the n numbers sufficiently large. Results are expressed as means ± SEM.

Hormones and Drugs

Ovine FSH (NIAMDD ovineFSH-S16) was provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Hormone Distribution Program. It was added to the culture medium on day one.

Human chorionic gonadotropin (hCG; Organon) and dibutyryl-cyclicAMP (Sigma) were dissolved either in the culture medium during the incubation, or in the Tyrode solution before the measurements of dye transfer.

Results

Sertoli Cells in Primary Cultures

Freshly dispersed Sertoli cells, obtained from testes of 15–17-day-old rats by the three-step enzymatic procedure described in Materials and Methods, are mainly isolated, or associated in small aggregates of two to ten cells. When plated at low density on 35-mm plastic petri dishes in RPMI medium, they progressively attach themselves to the plastic and flatten out. Two days later, the cells cultured in the absence of FSH form nonconfluent cultures that, under phase contrast microscopy, present an "epithelial-like" appearance (Hutson, 1978) (Fig. 1*A*). They either remain isolated, or they contact side-by-side in small dispersed groups of two to four adjoining cells, or they form small clusters of more tightly aggregated cells (Fig. 1*A*).

When cultured for 2–5 days with ovine FSH, the Sertoli cells take on a more rounded and thicker appearance, displaying fine spine-like projections, which make contact with other isolated cells and organize them into networks or clusters (Fig. 1*B*). This conversion to a "fibroblast-like" morphology (Posalaki et al., 1981) is also observed after dibutyryl-cyclicAMP stimulation but not after hCG treatment. Addition of FSH to unstimulated cells at any time during the 1–5 days of culture promotes the changes of morphology depicted in Fig. 1*B*.

Dye Transfer through Gap Junctions in Unstimulated Sertoli Cells

Dye Diffusion after Microinjections

The numbers of cells that became fluorescent, counted after LY-injections into one cell situated inside 112 randomly chosen groups, are represented in the frequency histogram of Fig. 2 (open bars). In 88% of the assays, the diffusion tracer remained confined to the injected cell, or propagated to one adjacent cell. Dye spread to two cells or more was observed only in 12% of the cases.

Dye Diffusion Measured by GapFRAP

After a 15 min incubation with 6-carboxyfluorescein-diacetate, the intracellular concentration of the fluorescent moiety 6-CF reaches a level sufficient for photobleaching assays. An example of dye transfer in unstimulated Sertoli cells cultured for four days is illustrated in Fig. 3*A–C*. Figure 3*A* is a computer-generated gray density image of the distribution of fluorescence intensities obtained immediately before photobleaching.

The unequal fluorescence levels inside one cell and between different cells reflect the variations in cell thickness. The cells marked by polygons *1-2-3-4* are selected for subsequent photobleaching. Figure 3*B* shows that, immediately after applying 10–12 strong pulses of

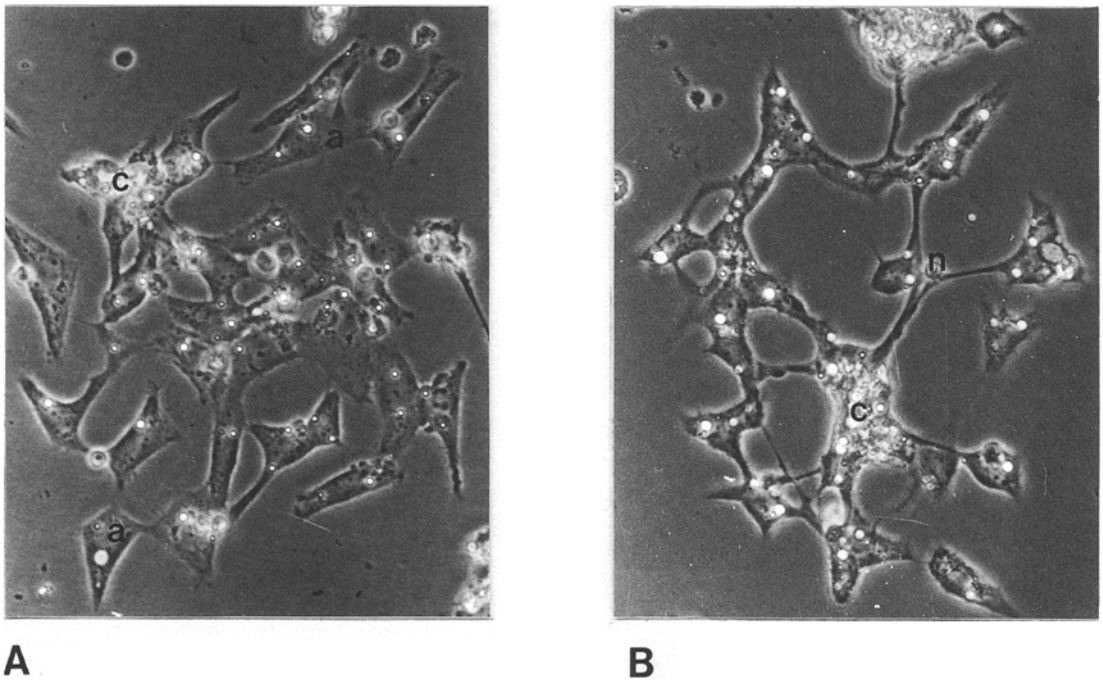


Fig. 1. Phase contrast micrographs of enzymatically dissociated Sertoli cells from 15–17-day-old rats cultured for three days in RPMI alone (*A*), or after addition of ovine FSH (0.6 μg/ml) (*B*) (350×). (*A*) Control cells. In the absence of hormone, the flat, epithelial-like cells appear either isolated, or in small groups of 2–4 cells with few and small contact areas ("adjoining cells," *a*) or they are arranged more tightly "in clusters" (*c*). (*B*) FSH-stimulated cells. With the hormone, the Sertoli cells round up and become thicker, taking on a fibroblast-like morphology. They are grouped either in tight "clusters" (*c*) or in "networks" by extended processes (*n*).

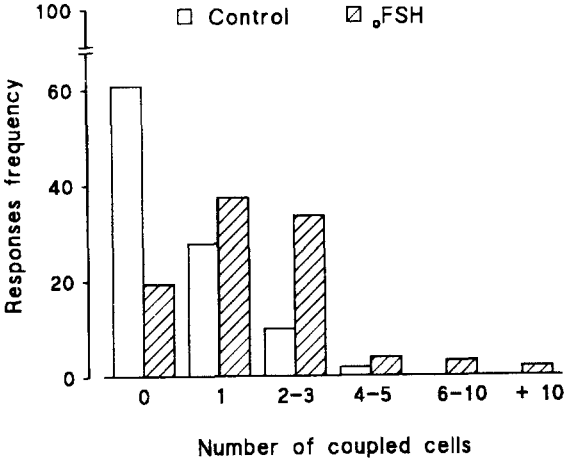
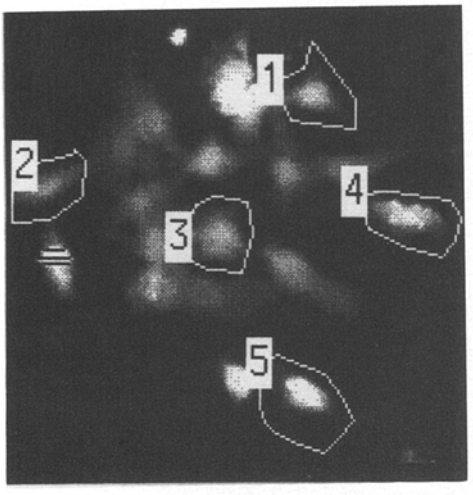


Fig. 2. Frequency histograms showing the numbers of cells that became fluorescent 2 min after microinjecting Lucifer Yellow into Sertoli cells. *Open bars:* cells grown without FSH. *Hatched bars:* FSH-stimulated cells.

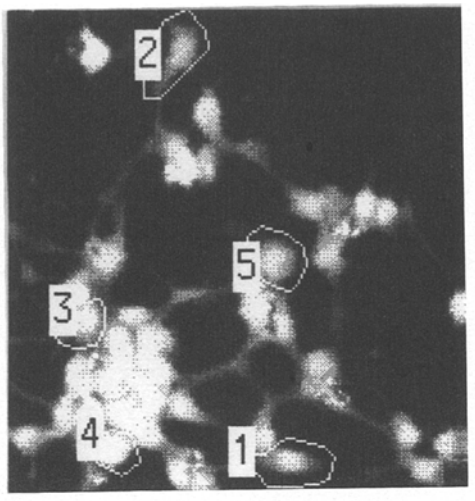
laser light, the level of fluorescence emission in these cells is markedly decreased. Fluorescence intensity subsequently increases, as shown by the same field recorded 12 min later (Fig. 3*C*, the cell number *5* is not bleached and will be used as a control).

The fluorescence levels stored for each data point during the successive scans are then integrated in the polygons which define the selected cells. Measurements corresponding to the experiment of Fig. 3, expressed as percentages of the prebleach intensity, are plotted in Fig. 4*A* and *B*. A 5 to 10% decrease of fluorescence intensity is observed in unbleached cells in the course of an experiment. As pointed out in Materials and Methods, all fluorescence intensities are corrected for this decrease, including those of the unbleached cells, which thus remain at the 100% level (Fig. 4*A* and *B*).

The fluorescence intensities measured in the scan recorded immediately after photobleaching (time 0 in Fig. 4) are decreased to about 10 to 20% of the initial levels. The subsequent records show that fluorescence recovers in the Sertoli cells that contact other cells (Fig. 4*A* and *B*). Twelve minutes later, the fluorescence intensity of unstimulated Sertoli cells reaches about 50% of the initial level. In contrast, fluorescence intensity does not increase in isolated cells (*not illustrated*), indicating that fluorescence recovery occurs by dye diffusion through gap junctions.



A



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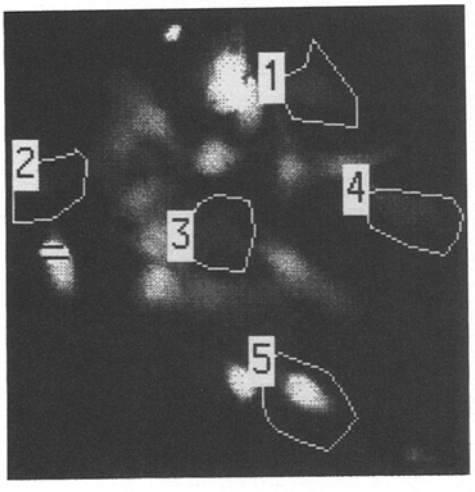
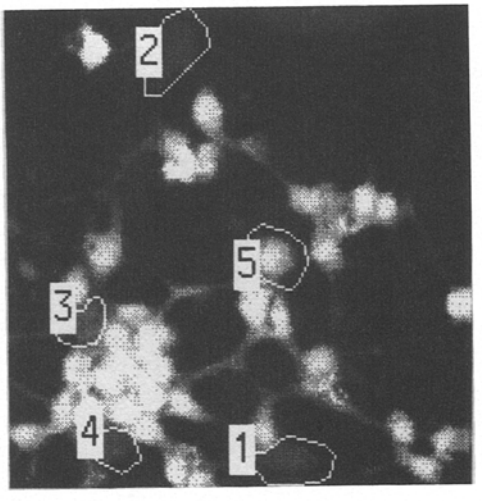
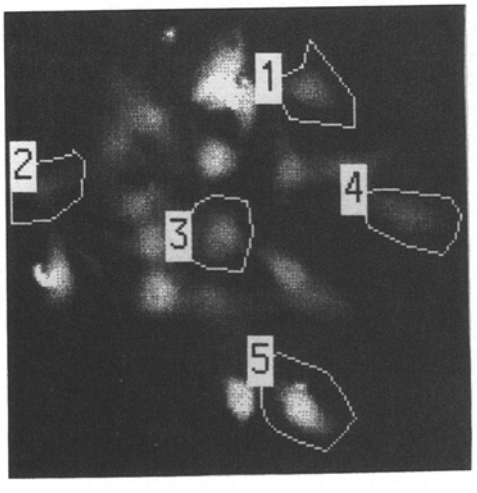


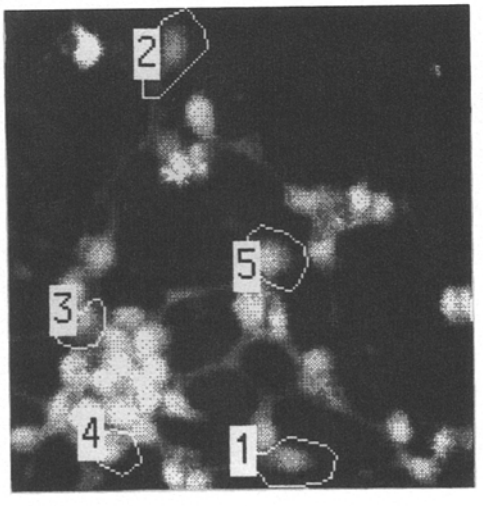
B



E



C



F

Fluorescence recovery is slow in photobleached cells grown in control conditions (RPMI medium without oFSH) (Fig. 4*A* and *B*). In these conditions, the relative permeability constant k, determined from a lot of 87 cells, varies from 0 to 20.7×10^{-2} min^{-1}.

It can be inferred from the two different cell topologies illustrated in Fig. 1*A* that the rate constant of fluorescence recovery could differ in the "adjoining cells," which show but a few contact areas with each other, and in the much more tightly packed cells "in clusters." The rate of fluorescence recovery was therefore measured in these two groups (*see* Fig. 6*A* and *B*). The k values of these two subpopulations are not modified in the course of a culture period of two to five days (n = 158) (*not illustrated*). The rate constant of adjoining cells varies from 0 to 5.5×10^{-2} min^{-1} (n = 58), and as many as 36% of them are uncapable of dye transfer (k = 0). In the coupled cells, the mean value of k is $2.7 \pm 0.3 \times 10^{-2}$ min^{-1} (n = 37). A much smaller proportion of cells in clusters are uncoupled (2 out of 29 cells). In the coupled cells, the k value averaged $7.6 \pm 0.8 \times 10^{-2}$ min^{-1}, which is significantly higher than the k of adjoining cells ($P < 0.01$).

The k values of unstimulated Sertoli cells taken in clusters has also been compared to the number of closely related cells. No significant relationship was observed in this case.

Dye Transfer through Gap Junctions in Sertoli Cells Stimulated by Ovine FSH

Sertoli cells were exposed for three to five days to 0.6 μg/ml of ovine FSH, a concentration which induces a 50% increase in the membrane potential of similarly cultured cells (Joffre & Roche, 1988). Exposure to oFSH induces morphological modifications as described above (Fig. 1*B*). After microinjections of LY or loading with 6-CF, the fluorescence intensity from FSH-stimulated Sertoli cells is generally higher than that of control cells, which is related to the increase in thickness of the cells under FSH.

Dye Diffusion after Microinjections

Figure 5*A* shows a typical Sertoli cell network selected for a microinjection experiment, and Fig. 5*B* depicts the cell-to-cell spread of fluorescence in the same field 2 min after LY injection. The numbers of fluorescent cells counted in 161 successful injections are represented in the frequency histogram of Fig. 2 (hatched bars). The dye was *restricted* to the injected cell in 20% of the trials, and propagated to two cells or more (up to 20) in 43%.

Dye Diffusion Measured by GapFRAP

Figures 3(*D–F*) and 4(*C–D*) illustrate a representative experiment performed on Sertoli cells cultured with ovine FSH. Following the photobleaching of selected cells, the fluorescence intensity decreases to about 20% of the initial level, then returns with time more rapidly than in the unstimulated cells (Fig. 4*C*). Twelve minutes later, the fluorescence intensity from stimulated Sertoli cells generally reaches 50 to 80% of the initial intensity.

FSH-stimulated cells are always capable of dye transfer ($k > 0$) (Fig. 6*C* and *D*). The relative permeability constant k, determined from 40 Sertoli cells cultured for 3–4 days, varies from 0.5 to about 23×10^{-2} min^{-1}. The values of k were measured separately for the two cell topologies (in clusters and in networks) that could be distinguished in the FSH-stimulated cultures. As shown by the histograms of Fig. 6*C* and *D*, no significant difference in the k values of these two subpopulations can be detected ($k = 9.0 \pm 0.5 \times 10^{-2}$ min^{-1}, n = 191 for the cells in networks and $10.8 \pm 0.5 \times 10^{-2}$ min^{-1}, n = 213 for the cells in clusters). In these two samples, a correlation analysis demonstrates that the rate constant of dye diffusion into the photobleached cells is independent of the number of closely related unbleached cells (Fig. 7*A* and *B*).

In the course of this study, we have observed that, in contrast to the unstimulated cells, the k values obtained from ovineFSH-stimulated cells in clusters and in networks varies within the culture period. This parameter is lower in two-day-stimulated cells ($8.0 \pm 0.7 \times 10^{-2}$ min^{-1}, n = 61, $P < 0.01$) and higher in five-day-stimulated cells ($12.7 \pm 1.1 \times 10^{-2}$ min^{-1}, n = 36, $P < 0.01$) than in 3–4 day cultures of Sertoli cells. For practical reasons, the subsequent experiments have been performed on 3–4-day cultures of Sertoli cells and the results have been compared to control values obtained from cells of the same bath cultured without hormone for the same duration.

Fig. 3. Typical computer-generated grey density images of fluorescence distribution in fourth day primary cultures of Sertoli cells grown in the absence of FSH (*A–C*), and with 0.6 μg/ml of ovine FSH added to the culture medium (*D–F*) (500×). These black and white transcripts from color-coded data were printed after subtraction of a background fluorescence. Sertoli cells were loaded with 6-carboxyfluorescein diacetate for 10 min before proceeding to the gapFRAP experiment. These fluorescence pictures represent the Sertoli cells immediately before photobleaching (*A, D*), immediately after photobleaching the cells delineated by polygons *1* to *4* (*B, E*), and 12 min later (*C, F*). Cells in polygons *5* (left and right panels) are left unbleached as controls.

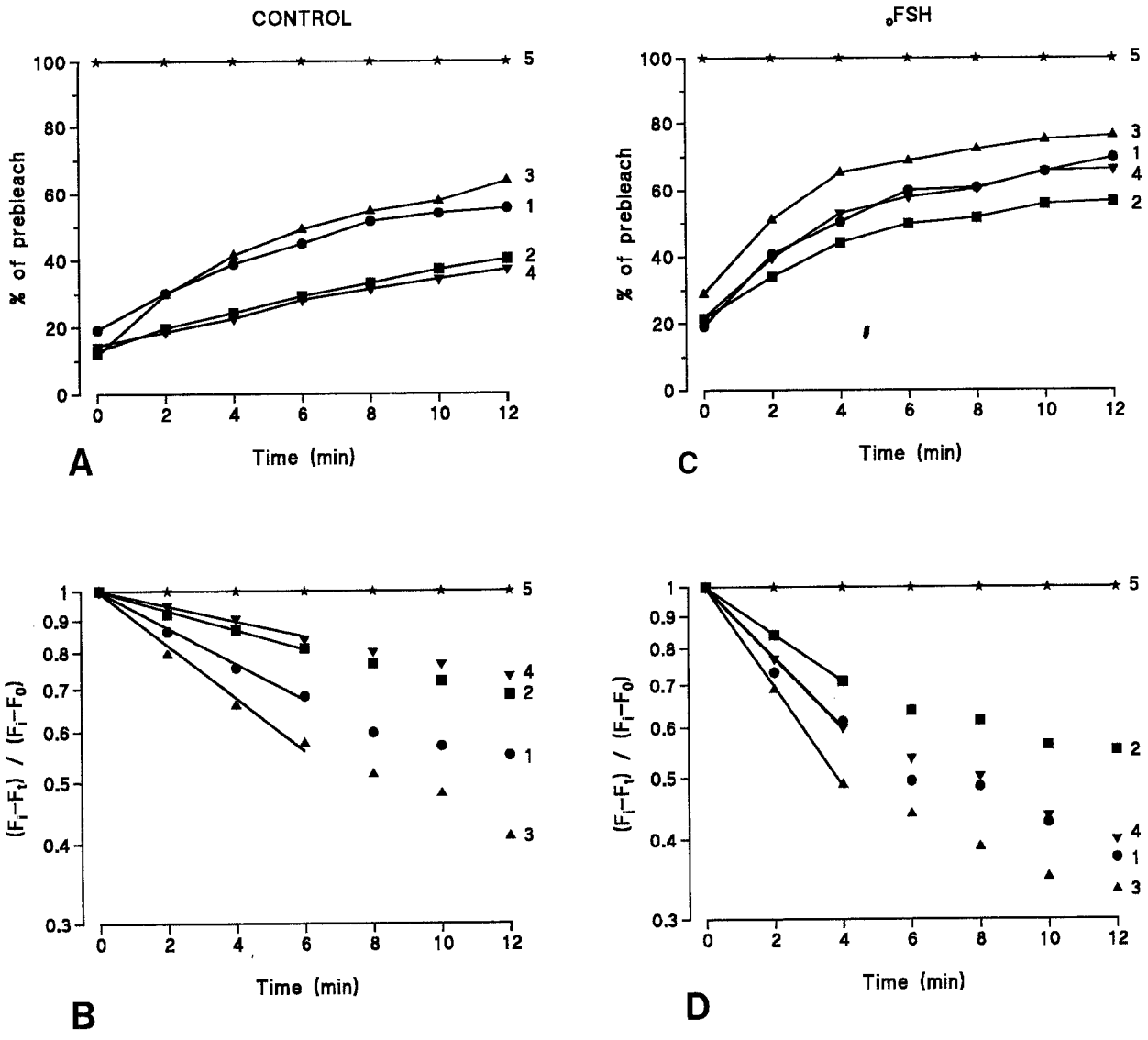


Fig. 4. Fluorescence recovery curves for Sertoli cells in the absence of FSH (*A–B*), and with 0.6 μg/ml of ovine FSH (*C–D*). These curves are constructed from the experiments illustrated in Fig. 3. The values of fluorescence recovery in photobleached polygons are corrected for the leak and photobleaching of fluorescent molecules that may have occurred in successive scans, by using data collected from polygons *5* (unbleached cells in left and right panels). Corrected values for unbleached cells are represented by straight lines. In *B* and *D*, the rate constants k are respectively determined from the fluorescence recovery curves in *A* and *C*, as $F_i - F_t/F_i - F_o = e^{-kt}$, where F_i, F_o and F_t are fluorescence intensities, integrated to the whole polygons, before photobleaching, and at 0 and t minutes after photobleaching. The rate constant k is related to the permeability coefficient of the junctional membrane.

Partial Reversal of FSH Effects

Cell coupling was determined after the hormone was removed by washing the petri dishes three times with RPMI medium, or with a low-pH glycine buffer (50 mM glycine, 150 mM NaCl; pH 3; 4°C) for 4 min to remove the remaining membrane-bound FSH (Bernier & Saez, 1985). The k values of cells (adjoining cells, cells in clusters or in network), incubated in a hormone-free medium for a new period of two days, were always lower than in the presence of FSH (Fig. 8). Although these values return to the initial prestimulation level in the cells in clusters, by contrast, in adjoining cells or in cells in networks, they remain higher than in the controls.

Absence of Effect of Human Chorionic Gonadotropin on Dye Diffusion in Sertoli Cells

The effect of human chorionic gonadotropin (hCG), another glycoprotein hormone which possesses a similar (α, β) subunit structure as FSH, with an identical α component (Segaloff et al., 1990), was assayed on Sertoli cells, using the same experimental approach. Even when added to the culture medium at a high concentra-

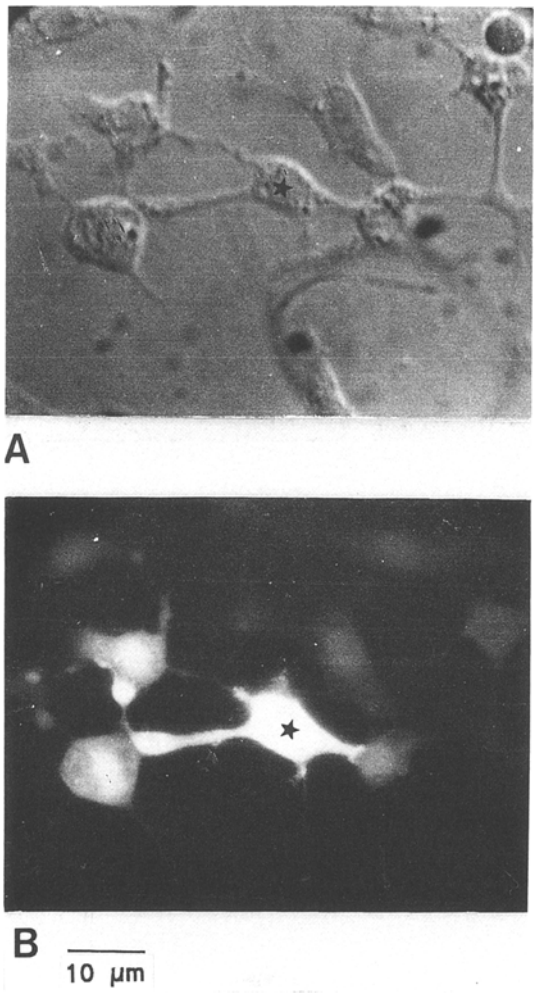


Fig. 5. Microphotographs of FSH-stimulated cells. (*A*) A network of cell viewed with Nomarsky interference optics prior to a microinjection of Lucifer Yellow. (*B*) Fluorescent emission of the same network photographed two minutes after microinjecting LY into the marked cell (★).

tion (10 ng/ml) for four days, hCG does not induce the morphological changes typically observed after FSH. Similarly, this hormone has no effect on the relative permeability constant of junctional membranes ($k = 2.3 \pm 0.5 \times 10^{-2}$ min^{-1}, $n = 22$, in the presence of hCG, comparing with $2.2 \pm 0.5 \times 10^{-2}$ min^{-1}, $n = 18$, observed in the controls.

Concentration Dependence of FSH Effects

The concentration dependence of the ovine FSH effects, in the range of 0.005 to 600 ng/ml, is represented in Fig. 9 for the groups of cells in networks and in clusters.

Concentrations below 0.05 ng/ml do not induce any morphological modifications of isolated Sertoli cells in 3–4-day cultures. These cells remain flattened and epithelial-like as the control cells. Higher concentrations induce network formation and increase the k values simultaneously (Fig. 9A). As the FSH concentration rises from 0.05 to 3 ng/ml, the relative permeability constant increases linearly with the logarithm of concentration (regression line: $Y = 7.0 \log x + 13.3$; $r^2 = 0.95$; $n = 57$, $P < 0.01$). In the highest concentration range (5 to 600 ng/ml), the k values decrease to a lower constant value of $9.0 \pm 0.7 \times 10^{-2}$ min^{-1} ($n = 28$, $P < 0.01$).

Whatever the FSH concentration, in the range of 0.005 to 600 ng/ml, the k values obtained from cells in clusters are significantly increased ($P < 0.01$, Fig. 9*B*). But in these cases, no correlation can be calculated, which suggests an all-or-none effect, whose amplitude appears to be dependent on the initial value of k.

Effects of Dibutyryl-CyclicAMP

At a concentration of 1 mM, dibutyryl-cyclicAMP (dB-cAMP) produces a maximal stimulation of testosterone metabolism by the Sertoli cells in culture (Verhoeven et al., 1979). A two-day exposure to 0.5 and 1 mM induces fine spine-like lateral projections, which make contact with other isolated cells and organize them into networks. This conversion to the fibroblast-like morphology is similar to that observed with ovine FSH (Fig. 1*B*). At these concentrations, dB-cAMP also increases the k values significantly ($P < 0.01$, Fig. 10) in the same way as done by 0.6 μg/ml ovine FSH.

Early Effects of Ovine FSH and Dibutyryl-CyclicAMP

FSH (0.6 μg/ml) or dB-cAMP (1 mM) were dissolved in the control medium (RPMI) of two-day-old primary cultures of Sertoli cells, and dye diffusion in cell clusters was analyzed. Ovine FSH increases k values significantly after a 120 min latency period ($P < 0.01$, Fig. 11*A*). These values are similar to those recorded after a two-day period of FSH stimulation ($13.2 \pm 1.7 \times 10^{-2}$ min^{-1}, $n = 3$, against $12.7 \pm 1.1 \times 10^{-2}$ min^{-1}, $n = 36$). In contrast, 1 mM dB-cAMP does not significantly increase the relative permeability constant of unstimulated cells at times shorter than 300 min (Fig. 11*B*). With this concentration, the k values nevertheless tend to increase at times greater than 6 hr ($11.5 \pm 0.5 \times 10^{-2}$ min^{-1}, $n = 4$).

Discussion

SERTOLI CELL CULTURES

Conventional cultures of Sertoli cells on plastic petri dishes have been extensively developed for studying cell

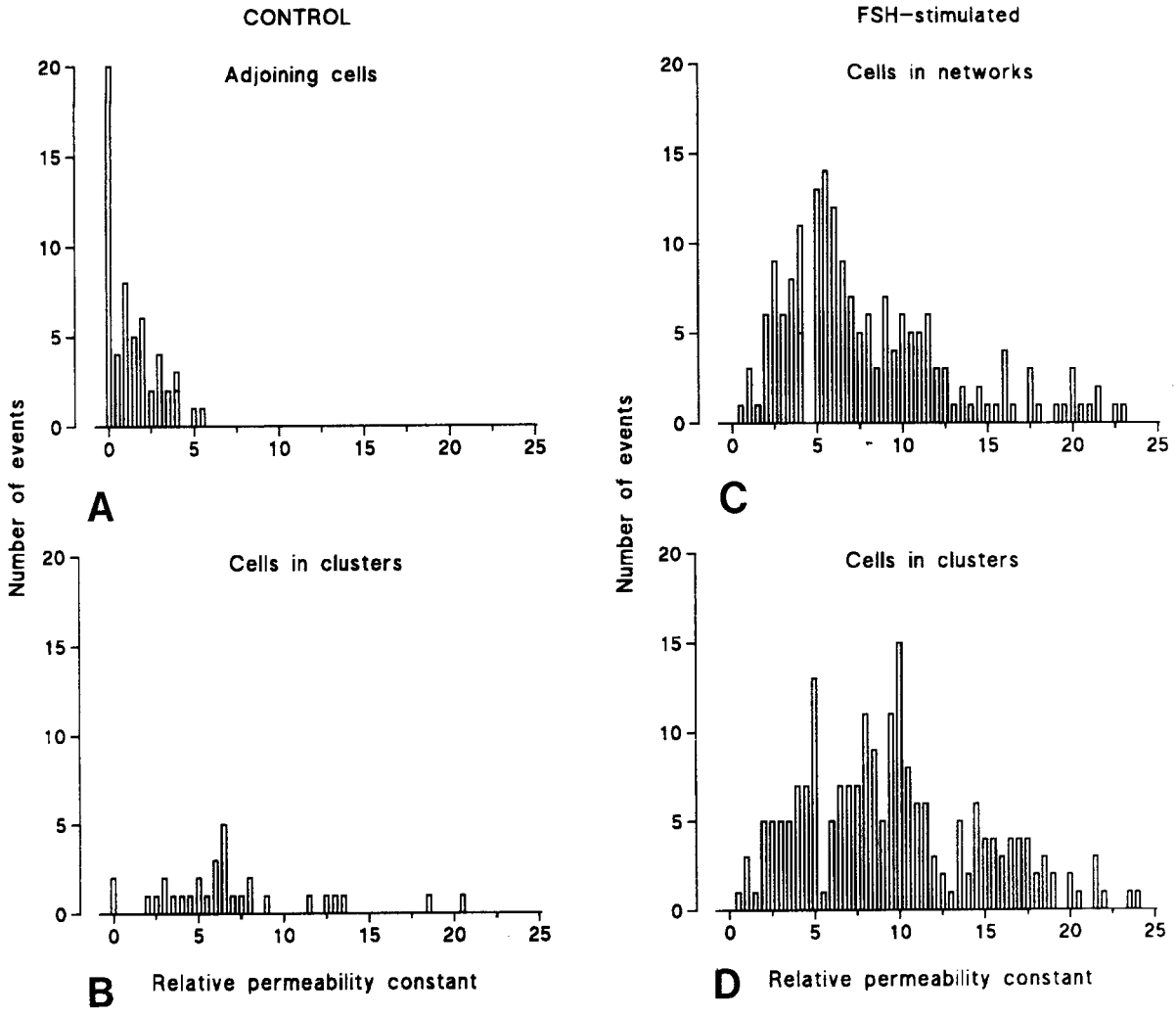


Fig. 6. Frequency distributions of the relative permeability constants k obtained from Sertoli cells cultured for 3–4 days. The cells were either unstimulated (*A–B*), taken in adjoining configuration (*A*) and in clusters (*B*) or stimulated by FSH (0.6 μg/ml ovine FSH) (*C–D*) taken in networks (*C*) and in clusters (*D*). *Abscissa:* k ($min^{-1} \times 100$).

structure and function and FSH regulation of cellular metabolism and secretion (Dorrington, Roller & Fritz, 1975; Steinberger et al., 1975; Welsh & Wiebe, 1975; Verhoeven, et al., 1979; Rich et al., 1983), although these cells present little resemblance to the highly polarized cells in vivo and fewer cell-cell junctions (Solari & Fritz, 1978). Thus, attempts have been made to improve the morphology of Sertoli cells and to prolong their secretory activity in culture. That includes the use of various types of reconstituted basal membranes and cell culture chambers with two compartments (Hadley et al., 1985; Djakiew et al., 1986; Janecki & Steinberger, 1987). In these systems, Sertoli cells are polarized with characteristic basally located tight and gap junctions. But they are frequently organized in thick confluent layers and always have a dense structure unsuitable for analysis of cell-to-cell dye diffusion. For these reasons, conventional primary cultures of Sertoli cells on plastic were used to investigate cell-to-cell dye diffusion by microinjection and by the gapFRAP methods, as previously done on normal human fibroblasts and human teratocarcinoma cells (Wade et al., 1986), on astrocytes (Anders & Salopek, 1989) or on bovine luteal cells (Redmer, Grazul-Bilska & Reynolds, 1991).

Very few fibroblasts are present in the culture dishes, their number being limited to less than 2% by the cell isolation procedure. Moreover, since the cultures are performed in a serum-free RPMI medium, the division and growth of the remaining fibroblasts are greatly curtailed. Finally, these fibroblasts can easily be recognized by morphological criteria under phase contrast microscopy, and by their low level of fluorescence in gapFRAP experiments.

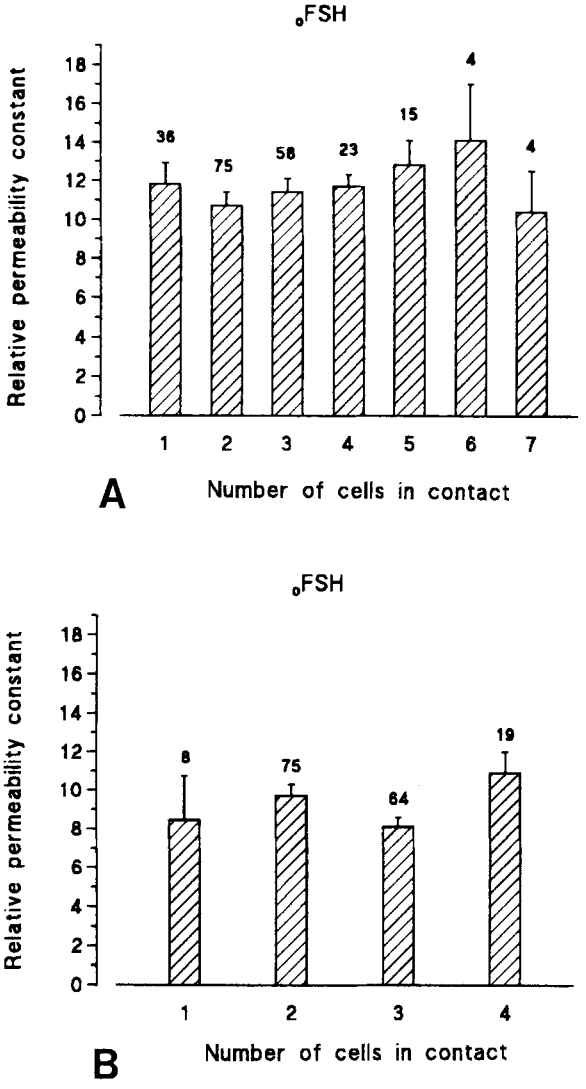


Fig. 7. Correlations between k values and numbers of closely related cells in FSH-stimulated cells either in clusters (*A*) or in networks (*B*). *Bars* indicate k values ($min^{-1} \times 100$) measured in 3–4 different cultures, and expressed as means ± SEM, with (n) numbers of measurements.

Effects of FSH on Sertoli Cell Cultures

Low-density cultures of Sertoli cells from immature rat testes have been previously used to determine the morphological changes induced by FSH and by cyclicAMP derivatives (Tung, Dorrington & Fritz, 1975; Hutson, 1978; Hutson, Garner & Stocco, 1980; Spruill et al., 1981). The present study largely confirms all the FSH-induced morphological changes described by these authors. Control cells in cultures are thin, flat and epithelium-like with few cytoplasmic extensions, whereas cells that have been exposed to either FSH or dB-cAMP develop extensive outgrowths of cytoplasmic processes making contacts with adjacent cells.

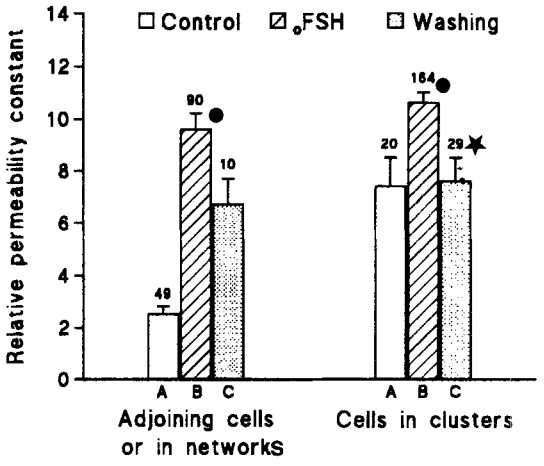


Fig. 8. Effect of FSH-withdrawal on the FSH-induced increase of the relative permeability constant of the junctional membrane. After 3–4 days exposure to 0.6 μg/ml of ovine FSH followed by a 2-day culture in RMPI alone, histograms of k values were obtained from FSH-stimulated cells taken either in networks (left panel) or in clusters (right panel). *Bars* indicate k ($min^{-1} \times 100$) values expressed as means ± SEM for (n) cells taken in 3–4 different cultures. (*A*) Control cells. (*B*) FSH-stimulated cells. (*C*) FSH-stimulated cells after a reversal period in the control medium without FSH for two days. ●$P < 0.01$ by unpaired Student's t-test against control cells. ★$P < 0.05$ by unpaired t-test against FSH-stimulated cells maintained in the FSH-containing medium.

Analysis of Dye Transfer in Dispersed Sertoli Cell Cultures by Means of the GapFRAP Method

The experimental records of fluorescence recovery in photobleached Sertoli cells always comprised an initial exponential part (Fig. 4*B,D*). In a first approximation, the bleached cell with its gap junctional membranes and the set of immediately adjacent (first order) cells were therefore treated as a two-compartment aqueous system separated by a diffusion barrier. As stated in Materials and Methods, with this simple assumption the inverse value (k) of the time constant for the initial first order part of the fluorescence recovery curve provides a quantitative estimate of dye transfer through the junctional membranes. The rate of fluorescence recovery tends to slow down at later times (at $t > 4$ min with FSH and at $t > 6$ min without FSH, Fig. 4*B,D*), as expected in a multicompartment system (in this case a set of second order cells indirectly connected to the bleached cell). In practice, the variable topology of the cell groups in these dispersed cultures precluded a more accurate quantitative analysis of our data by means of a multicompartment model, and values of the transfer constant k were obtained from the initial part of the recovery curves, at a time when the effects of more remote compartments on the net flux of dye molecules into the bleached cells can be neglected. To obtain comparable

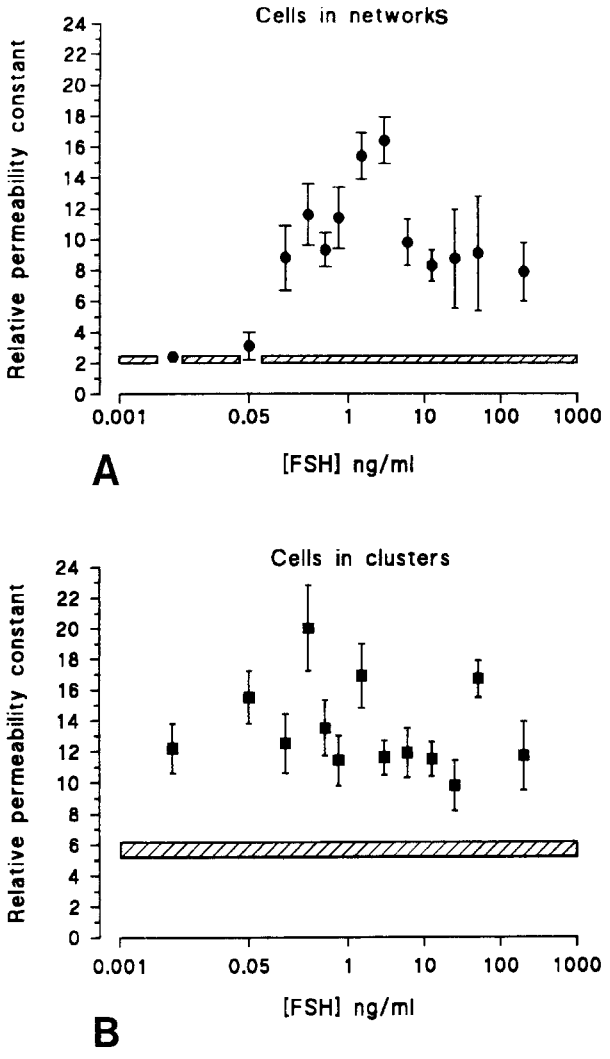


Fig. 9. Concentration dependence of the FSH-induced increase of the relative permeability constant. The cultures were exposed to concentrations of ovine FSH-S_{16} in the range 0.005 to 600 ng/ml for three to four days. Values were obtained from FSH-stimulated cells grouped either in networks (n = 85) (*A*), or in clusters (n = 117) (*B*). k ($min^{-1} \times 100$) values are expressed as means ± SEM for n (4 to 20) cells taken in 3–4 different cultures. *Hatched horizontal bars* indicate k values in the control unstimulated cells.

data, the cells that became fluorescent after microinjections of Lucifer Yellow were counted at 2 min.

Although the theoretical depth of field on our microscope (1.5 μm) seems adequate relatively to the largest cell thickness (about 2 μm), some contributions from out-of-focus object planes add up to the recorded fluorescence intensities. This factor is not considered an important cause of error, because measurements were integrated over the whole cell area, which could be manually redrawn during the measuring process if the cell shape appeared to have changed, a rare event within the twelve minutes of experiments.

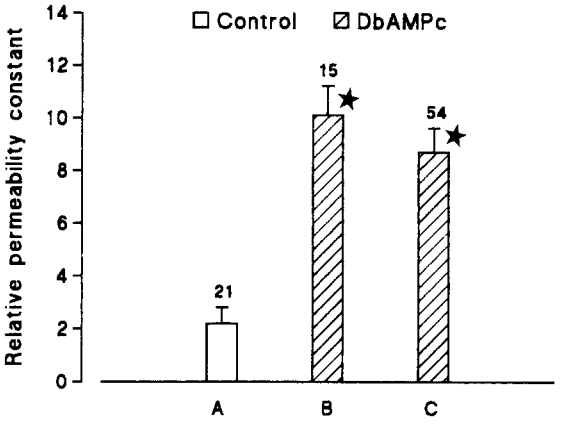


Fig. 10. Effects of dibutyryl-cyclicAMP (dB-cAMP) on the relative permeability constant k. Cultures were exposed to constant concentrations of dB-cAMP for three to four days. Measurements were obtained from dB-cAMP-stimulated cells taken in networks. The k values ($min^{-1} \times 100$) are expressed as means ± SEM for (n) cells taken in 3–4 different cultures. (*A*) Control cells (n = 21); (*B*) Cells cultured with 0.5 mM (n = 15), and (*C*) with 1 mM dB-cAMP (n = 54); ★$P < 0.01$ by unpaired Student's t-test against control cells.

Cell-to-Cell Communication in Sertoli Cells

Dye-injection experiments showed that Sertoli cells cultured in control conditions (RPMI without FSH) are much less frequently coupled than those cultured with FSH (Fig. 2). This effect was confirmed with the gapFRAP method, which allows us to obtain a satisfactory estimate of the relative permeability constant of the junctional membrane.

Dye Transfer through Gap Junctions in Control Sertoli Cells

Morphological features of Sertoli cells grown in RPMI alone for three to four days led us to analyze diffusional coupling in two different cell populations: adjoining cells and cells in clusters. On the basis of their relative position, a group of cells was classified as "adjoining cells" when they contact only over part of their circumference. The relative permeability constant between adjoining cells was either frequently equal to zero or to a low value of about $2–3 \times 10^{-2}$ min^{-1}. The k values from cells "in clusters" were significantly higher and appeared to be independent of culture duration and, rather surprisingly, of the number of neighboring cells. The high k values from cells in clusters, close to those obtained in the presence of FSH, suggest that a pre-existing coupling between cells in clusters was not suppressed by the three-step enzymatic treatment. This confirms by a dye-diffusion measurement the results ob-

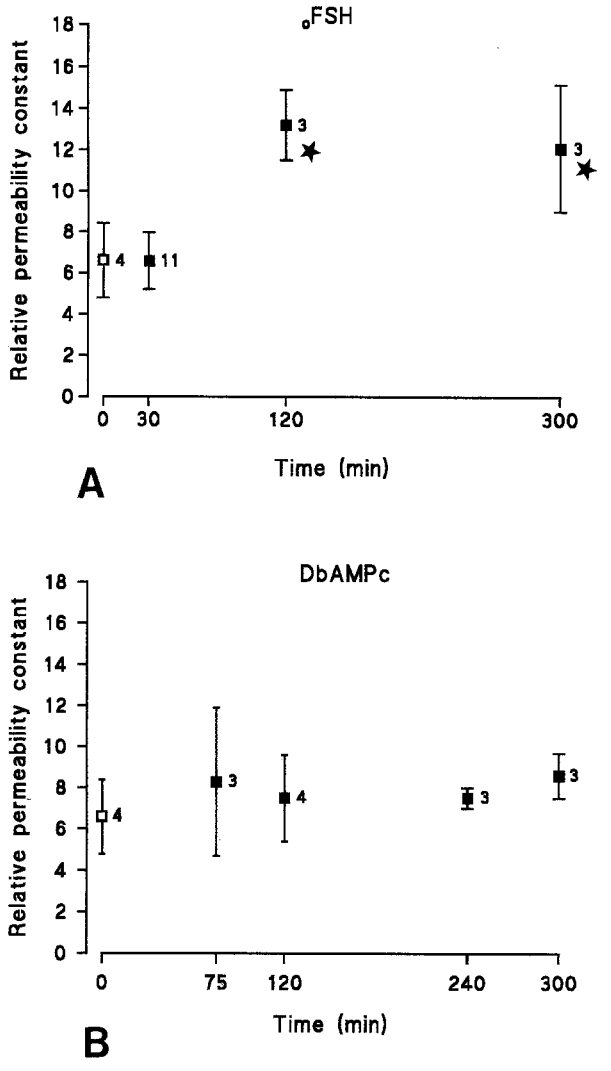


Fig. 11. Early effects of ovine FSH (*A*) and dB-cAMP (*B*) on the relative permeability constant (k). Two-day cultures of control cells in clusters were exposed to 0.6 μg/ml ovine FSH (*A*) or 1 mM dB-cAMP (*B*). The k values ($min^{-1} \times 100$) are expressed as means ± SEM for (n) cells taken in two different cultures. (□) Control values; (■) FSH or dB-cAMP values.

tained by Eusebi et al. (1985) in primary cultures of Sertoli cells by an electrophysiological technique.

No dye diffusion was seen in about 36% of the adjoining cells. A decrease of a pre-existing coupling as a consequence of the disappearance of internal cyclicAMP stores, as in hepatocytes (Saez et al., 1989), may be excluded since such a decrease would also be expected in the cells in clusters. This indicates that, in more dispersed cells a recoupling did not spontaneously occur in the absence of FSH. But, since a weak coupling was also encountered in about 64% of the adjoining cells and in all cells in cluster, we may not exclude that, in the absence of stimulating messenger, there was either a spontaneous recoupling, or a pre-existing coupling between cells, which was maintained independently of a decreasing cell metabolism.

Dye Transfer through Gap Junctions in FSH-Stimulated Sertoli Cells

The diffusional coupling between Sertoli cells in primary culture was rapidly increased by FSH, while it was unchanged in the presence of high concentrations of hCG. Since hCG receptors are lacking in the membrane of Sertoli cells (*reviewed by* Jégou, 1992), this indicates that FSH in Sertoli cells, as in granulosa cells (Amsterdam, Knecht & Catt, 1981), promotes the gap junction communication of cultured cells through a receptor-dependent mechanism. Comparable conclusions can be reached for thyroid cells (Munari-Silem, Audebert & Rousset, 1991) and luteal cells (Redmer et al., 1991), following their stimulation by TSH and LH, respectively.

Considering the previously observed effects of ovine FSH-S_{16} on membrane potential (Joffre & Roche, 1988), we performed our first assays with a FSH concentration of 0.6 μg/ml, the EC_{50} value for the electrophysiological effect, then with decreasing values. In the range of 0.05 to 3 ng/ml FSH, the relative permeability constant k increases linearly (EC_{50} of about 0.5 ng/ml) with the FSH concentration in the Sertoli cells in networks. In the same range, FSH significantly promoted the coupling between cells in clusters, and similar maximal values of coupling were observed between cells in clusters and in networks. Higher concentration (5 ng/ml and more), however, induced lower values of coupling, indicating a possible receptor desensitization.

FSH acting upon plasma membrane receptors located on the basolateral surface of Sertoli cells triggers an increase of the cytosolic cyclicAMP level (Dorrington et al., 1975; Means et al., 1980). FSH as well as dB-cAMP promote morphological changes and both increase the gap junctional communication, substantiating the hypothesis of a cyclicAMP-dependent phosphorylation process responsible for this FSH effect. These results confirm the cyclicAMP-dependent mechanism of the potentiation of gap junctional communication observed in a number of other cell systems (Loewenstein, 1981; Hertzberg, Lawrence & Gilula, 1981; Spray & Bennett, 1985), but do not confirm the cyclicAMP-dependent decrease of the gap junction communication between Sertoli cells described by Grassi et al. (1986).

In the course of this study, we observed that micromolar concentrations of FSH are more potent to increase the gap junctional communication than millimolar concentrations of dB-cAMP. It is known that the

transduction of the FSH signal in Sertoli cells is a complex process involving intracellular free calcium (Means et al., 1980; Grasso & Reichert, 1989, 1990; Gorczynska & Handelsman, 1991). Grasso, Joseph and Reichert (1991) suggest that FSH-induced changes in the intracellular calcium levels result in an influx of extracellular calcium, possibly through voltage-activated calcium channels (Grasso & Reichert, 1989; Gorczynska & Handelsman, 1991; D'Agostino, Menè & Stefanini, 1992). This might occur either through a cyclicAMP-dependent phosphorylation secondary to receptor binding (Gorczynska & Handelsman, 1991), or by a direct interaction of FSH with its receptor (Grasso, Santa-Coloma & Reichert, 1991). An increase of cytosolic calcium as a result of an inhibition of a Na/Ca exchanger by FSH has also been suggested (Grasso, et al., 1991). For these reasons, a possible amplifying role of calcium current or translocation in the control of gap junction communication triggered by FSH might be hypothesized.

The promoting effects of FSH were not totally reversed upon returning to a control medium without hormone and were only decreased to levels similar to those of the cells in clusters cultured in the absence of stimulating hormone. It is now well established that gap junctional communication in other cells is generally modulated either by controlling the number of gap junctions through synthesis or cell membrane insertion, and/or by controlling the gating of gap junctional channels (*see review:* Loewenstein, 1986). In this context, our results highly suggest that the initial promoting effect of FSH in primary cultures of Sertoli cells depends on a synthesis or on a cell membrane insertion of connexin, a mechanism that is not reversed by the withdrawal of hormone and that is preserved by a gentle enzymatic treatment since, in the absence of FSH, the coupling remains high in isolated cell clusters. Thereafter, this initial gap junctional re-coupling is secondarily controlled by a cyclicAMP-dependent mechanism which is reversed in the absence of FSH.

At the present time, caution is appropriate in proposing an *in situ* physiological role for these effects of FSH on diffusional coupling between Sertoli cells in vitro. An FSH effect on tight junctions has been observed in an ultrastructural study of monolayers of Sertoli cells cultured in the same conditions as ours (Solari & Fritz, 1978). It was shown that the Sertoli cells isolated from testes of 10-day-old rats and cultured in a medium without FSH did not develop complete junctional complexes. These appeared only when the cells were stimulated by FSH, and did regress when returning to a hormone-free medium, but an FSH effect on gap junctions was not reported. Direct evaluation of the electrotonic coupling between Sertoli cells in culture has provided information on the gap junction status. Using two microelectrodes applied to Sertoli cells from 15-day-old rats, Eusebi et al. (1985) demonstrate that unstimulated cells are electrically coupled, which is in conformity with the present dye-diffusion data obtained on unstimulated cell clusters from immature rat testis.

CyclicAMP and its analogues have diverse effects on cell-to-cell communication in different systems, ranging from a delayed upregulation, blocked by inhibitors of protein synthesis, in cultured mammalian cells (Flagg-Newton, Dahl & Loewenstein, 1981), to a rapid down regulation of dye transfer and electrical coupling in horizontal cells from the turtle and fish retina (Piccolino, Neyton & Gerschenfeld, 1984; Lasater, 1987). The delayed effect seems to depend on protein synthesis — either connexins or phosphorylating enzymes — and the short-term regulations are possibly effected by changes in the phosphorylation state of membrane-inserted connexons. Divergent effects of cyclicAMP have been reported even in cell systems expressing the same connexins. A rapid upregulation of junctional conduction has been seen by Burt & Spray (1988) and De Mello (1988) in cardiac myocytes, which express connexin43 (Beyer, Paul & Goodenough, 1987) (this effect of cAMP seems difficult to confirm: *see* Sugiura et al., 1990), but cyclicAMP promotes a decreased coupling in uterine muscle (Cole & Garfield, 1986), which expresses the same connexin (Risek et al., 1990).

DB-cAMP rapidly inhibits electrical coupling in confluent cultures of Sertoli cells and in isolated seminiferous tubules (Grassi et al., 1986), in which the only connexin so far detected is connexin43 (Risley et al., 1992). At first sight, this result contradicts the present report on the promoting effect of dB-cAMP on dye transfer in the same cell system. However, looking into the data of Grassi et al. (1986, Table 1) dB-cAMP certainly has a large inhibitory effect when the experiments were performed in a calcium-free, EGTA-containing medium, but this effect is very much smaller in a calcium-containing medium. The significance of the latter result is difficult to appreciate, since no statistical tests are given. In this context, it may be recalled that Sertoli cells have an abnormally low membrane potential in calcium-free, EGTA-containing medium (Roche & Joffre, 1989). Thus, we may provisionally suggest that the absence of extracellular calcium and the concomitant depolarization may suffice to promote a different effect of dB-cAMP.

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