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Contraceptive gossypol blocks cell-to-cell communication in human and rat cells

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Abstract

Gossypol (a polycyclic lipophilic agent naturally present in cottonseed, known as a potent non-steroid antifertility agent and a non-specific enzyme inhibitor) irreversibly impaired the intercellular communication between homologous pairs of various cultured cells, from man or rat, involved (Sertoli or trophoblastic cells) or not involved (ventricular myocytes) in steroidogenesis, in a dose-dependent manner. In serum-free assays, a rapid junctional uncoupling occurred in non-cytotoxic conditions. At 5 μM (approximately twice the peak plasma concentration measured in human patients during chronic administration), gap junctional communication was interrupted within 4 to 10 min, without concomitant rise in the intracellular Ca^{2+} concentration. The latter importantly increased when gossypol treatment was prolonged (cytotoxic effect). The short term uncoupling effect of gossypol was prevented by serum proteins, but long-lasting treatments (48 h) with moderate concentrations (3 μM) elicited junctional uncoupling and impeded the in vitro differentiation of human trophoblasts.

Keywords: Gap junction: Cell-to-cell conductance; Dye diffusion; Trophoblast differentiation, human; Ca^{2+}, intracellular; Patch clamp, dual

1. Introduction

Cell-to-cell communication by the exchange of small ions and molecules (up to about 1 kDa in size) between adjacent cells takes place through gap junctions, which are clusters of transmembranous channels that provide a pathway for the diffusion of amino acids, small peptides, sugars, vitamins, second messengers (cyclic nucleotides, inositol triphosphate), etc. Each channel is formed by two connexons (one per cell) tightly associated in the intercellular space. Proposed physiological functions for gap junctions include transmission of intracellular signals, contribution to cellular homeostasis, regulation of growth and tissue development. The permeability of junctional channels is finely regulated at both transcriptional and post-transcriptional levels, e.g. via cycles of phosphorylation and dephosphorylation of junctional proteins (connexins), fluctuations of intracellular Ca^{2+}, H^+ or cAMP concentrations, oncogene activations, growth factors, hormones, etc. (for review, see Bennett et al., 1991; Loewenstein and Rose, 1992).

Junctional communication can be interrupted by a variety of lipophilic substances, including aliphatic alcohols (Johnston et al., 1980), aldehydes (De Haan et al., 1994) and fatty acids (Aylworth et al., 1986), and some cyclic compounds, such as phorbol esters (Murray and Fitzgerald, 1979), retinoic acid (Mercier et al., 1993), glycyrrhetinic acid derivatives (Davidson et al., 1986), steroids, particularly certain bile acids (Noda et al., 1981), sterols (Zwijsen et al., 1992) or sex steroid hormones (Kihara et al., 1990; Pluciennik et al., 1991a,1996; Hervé et al., 1996).

In some mammalian cell lines, but not in all of them, gossypol, a polyphenolic dialdehyde (see Fig. 1) naturally present in cottonseed (*Gossypium* sp., Malvacae), interrupted gap junctional intercellular communication at concentrations that do not cause cell killing or mutations (Ye et al., 1990). Gossypol is a potent male non-steroid contra-

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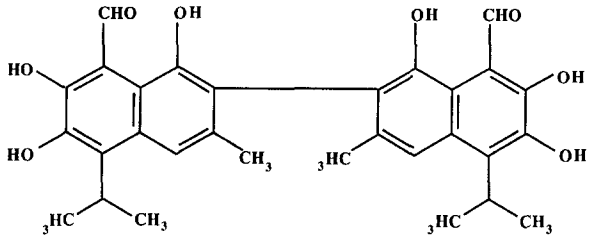

Fig. 1. Chemical structure of gossypol, a symmetrical molecule with several functional groups including two identical aldehyde groups.

ceptive (National Coordinating Group on Male Antifertility Agents, 1978; Quian and Wang, 1984; Segal, 1985) as well as a female antifertility agent (Gu and Anderson, 1985; Lin et al., 1985). This highly reactive agent has been shown to interfere with numerous physiological processes, such as metabolism and growth, by acting as a non specific enzyme inhibitor in vitro, affecting various types of reactions essential for cellular signalling, but the exact mechanism for its effects remains unknown. This drug can inhibit a variety of enzymes, including adenylate cyclase, protein kinase C, DNA polymerase, topoisomerase, malate dehydrogenase, lactate dehydrogenase isozymes, gluthathione-*S*-transferase, enzymes involved in ion transport, in metabolism of nucleotides, in steroidogenesis, etc. (for review, see Quian and Wang, 1984).

In vivo, the tolerance to gossypol was said to vary according to species, and it was for example reported that rats were sensitive to lower doses than men (Bertrand and Belleville, 1991). In vitro, the membranes of steroidogenic subcellular organelles (e.g. adrenal cortex mitochondria) were less affected by gossypol than membranes from non steroidogenic tissues (as heart or kidney), and the difference was attributed to their different lipidic composition (Cuéllar and Ramírez, 1993).

In the present study, the effects of gossypol on the functional state of gap junction channels, quantified by measuring either the junctional permeability for fluorescent dyes or the junctional electrical conductance, have been investigated in three cell types, namely Sertoli cells and ventricular myocytes of young rats, and human trophoblastic cells. Both the testicular Sertoli cells, which belong to the somatic components of seminiferous tubules, and the trophoblast of the placenta villi, express important exocrine functions, including secretion of proteins, steroids, growth factors and hormones. In contrast, cardiac myocytes have a contractile function and are not involved in these secretory activities. Some characteristics of the gossypol effects were examined such as the uncoupling concentration range, the kinetics of channel closure, the protective action of serum proteins and the possible involvement of the cytosolic Ca^{2+} concentration. Preliminary reports of this study have been published in abstract form (Pluciennik et al., 1991b; Bastide and Délèze, 1991; Cronier et al., 1996).

2. Materials and methods

2.1. Cell cultures and solutions

2.1.1. Sertoli cells

Sertoli cells were isolated as previously described (Pluciennik et al., 1994). The testes of anaesthetised immature (15–17 days old) rats were removed and exposed to three successive enzymatic digestions by respectively collagenase, pancreatin and trypsin, in Ca^{2+}- and Mg^{2+}-free Earle's solutions. Cells were plated at a density of 55 000 cells/cm^2 in 35 mm dishes, in a culture medium (RPMI 1640, Gibco, Gaithersburg, MD, USA) supplemented with sodium bicarbonate (20 mM) and Hepes (10 mM, both from Gibco). L-Glutamine (2 mM), bovine serum albumin (1 mg/ml), transferrin (5 μg/ml), insulin (10 μg/ml), streptomycin sulfate (68 μM) and penicillin G (100 IU/ml) (all from Sigma, St Louis, MO, USA) were also added to the medium, as well as 0.6 μg/ml ovine follicle stimulating hormone (oFSH), provided by the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD, USA. The culture dishes were maintained in a humidified atmosphere of 95% air and 5% CO_2, at 34°C, for 2 to 5 days. After the second day, the media were changed every 2 days.

2.1.2. Rat cardiac myocytes

Rat cardiac myocytes were prepared from about ten 1–2 day old animals. Briefly, ventricles were minced and serially digested in 0.15% crude trypsin (1 mg/ml; Boehringer, Mannheim, Germany) in a Ca^{2+}- and Mg^{2+}-free culture medium (Spinner) at 37°C, under gentle stirring for 50 to 75 min. Enzymatically released cells from serial digestions 2–5 were centrifuged (500 × *g* for 5 min), re-suspended in Ham's F10 culture medium (Gibco) and preplated in polystyrene flasks for 90 min to allow attachment of non-muscle cells. Cardiac myocytes, seeded in 35 mm dishes (about 55 000 cells/cm^2) were cultured at 37°C in a Ham's F10 medium supplemented with 10% foetal calf serum (Boehringer), 10% heat inactivated horse serum (Gibco), streptomycin sulfate (17 μM) and penicillin G (40 IU/ml; both from Serva), in a humidified atmosphere of 95% air and 5% CO_2. After 24 h, the culture medium was replaced with Ham's F10 supplemented with the same amounts of heat inactivated horse serum and antibiotics.

2.1.3. Human cytotrophoblastic cells

Human cytotrophoblastic cells were processed from normal term placentas, obtained after uncomplicated caesarean sections, as recently described (Cronier et al., 1994b). Briefly, after serial trypsin and DNase digestions, the cell suspension was purified first on a discontinuous Percoll gradient, then by means of a negative selection procedure with a monoclonal antihuman leucocytic antigen-A, -B, and -C antibody (W_6-32HL, Sera Lab, Crawley Down, UK). Cytotrophoblastic cells were plated at a density of 110 000 cells/cm^2 in 35 mm dishes in Minimal

Essential Medium (MEM, Gibco) supplemented with 10% foetal calf serum, streptomycin (68 μM) and penicillin G (100 IU/ml) (both from Sigma). Purified human Chorionic Gonadotropin (3000 IU/mg; Organon, Séri-Fontaine, France) was also added. The culture dishes were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and the media were renewed every day.

In the three cell types, measurements of intercellular coupling were made (except when different procedures are mentioned in the Results section) after replacement of the culture medium by a Tyrode's solution containing (in mM): NaCl 144, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 2.5, NaH_2PO_4 0.3, glucose 5.6, Hepes 5 (buffered to pH 7.4 with NaOH). The dishes were then transferred onto the stage of an inverted microscope and the cells were visualized by phase-contrast microscopy. All measurements of cell-to-cell communication were performed at room temperature (22–24°C).

Gossypol (2,2′-bi[8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphtalene], see Fig. 1), purchased from Sigma, was dissolved in dimethyl sulfoxide (DMSO, stock solution 5 mM, daily prepared) and added to the Tyrode's solution at the desired concentration. The maximal amount of DMSO in the medium (0.1% v/v) did not affect the junctional coupling of these cells in control experiments. The test solutions were either added to the culture dishes for the times specified in the Results section, or applied very rapidly by directing a streamline flow from the opening of a stainless steel capillary (internal diameter 30 μm), positioned in the bath near the investigated cell pair. Washing of the test fluids was then similarly performed by switching to a Tyrode's solution flowing out of a second capillary juxtaposed to the first at the opening.

2.2. Cytotoxicity assays

Possible cytotoxic effects were examined by means of the tetrazolium salt colorimetric assay for cell growth and survival (Mosmann, 1983). Tetrazolium (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma) was dissolved in RPMI-1640 (5 mg/ml), filtered (0.2 μm). 100 μl of this stock solution were added per ml of culture medium after the cells had been exposed to gossypol-containing solutions at uncoupling concentrations, and the dishes were incubated at 37°C for 4 h. The reaction mixture was then removed and 1 ml of DMSO was added in order to dissolve the dark blue crystal of formazan formed in the mitochondria of living cells. After 10 min of incubation at room temperature, solutions from 3 culture dishes were pooled and the optical density was measured at a reference wavelength of 630 nm and a test wavelength of 570 nm.

2.3. Quantitative measurement of cell-to-cell dye transfer

The permeability of gap junction channels for the fluorescent diffusion tracer 6-carboxyfluorescein was measured by analysing the Fluorescence Recovery After Photobleaching (gap FRAP, Wade et al., 1986) of one cell in a pair or in a small group of cells. Cells were loaded for 10 min at room temperature in Tyrode's solution containing 6-carboxyfluorescein diacetate (Sigma). This compound easily penetrates into the cells, then intracellular esterases hydrolyse it (Rotman and Papermaster, 1966) releasing the highly fluorescent and membrane impermeant moiety, which accumulates inside the cells. After washing off carefully the excess extracellular fluorogenic ester to prevent further loading, FRAP analysis was performed using a cytofluorimetric system (Anchored Cell Analysis and Sorting, ACAS 570, Meridian Instruments, Okemos, MI, USA). In selected cells, the fluorescence was photobleached by strong light pulses from an argon laser, then the redistribution of unbleached dye molecules was monitored as a function of time, as previously described (Pluciennik et al., 1994).

When the bleached cell was connected by open junctional channels to unbleached adjacent cells, its fluorescence level gradually increased. The initial recovery of fluorescence in the bleached cells followed an exponential time course. Therefore, the rate constant k of the exponential fluorescence recovery (the inverse value of the time constant) provided a quantitative measure of dye transfer through junctional channels. It is known that in a thin layer of solution (up to 100 μm) and at low dye concentrations (range for fluorescein 10^{-8} to 10^{-3} M), the integrated fluorescent intensities vary linearly with the dye concentrations. Assuming that these conditions are satisfied in dispersed cultured cells, the initial rate constant k can be obtained from the equation:

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

where F_i, F_o and F_t are the fluorescence intensities in the bleached cells, respectively before photobleaching, immediately after and at time t after photobleaching.

The validity of Eq. (1) was checked by plotting the pattern of fluorescence recovery following photobleaching in a logarithmic scale vs. time in linear scale (see Fig. 4a,b). The increase in fluorescent emission remained linear for at least 4 min for rat cardiac cells, 6 min for rat Sertoli and human trophoblastic cells, showing that fluorescence recovery obeyed Eq. (1) during this time interval.

After evaluation of the cell-to-cell transfer of dye in control conditions, culture dishes were exposed to gossypol for 15 min, then a second photobleach allowed to compare the dye diffusion in the same cell population after treatment. In both cases, for each culture dish, 1 to 3 cells in contact with others were photobleached and the redistribution of fluorescence was measured from sequential scans at 1-min intervals. A single non-photobleached cell served as positive control to monitor background photobleaching due to image scans and to detect any increase in fluorescence that would indicate the presence of uncleaved dye.

Results were expressed as means ± S.E.M. and the

statistical significance of the difference of the means was established using Student's t-test for unpaired data, with a sufficiently large n number.

2.4. Ca^{2+} measurements

A possible involvement of a rise in the intracellular Ca^{2+} concentration during gossypol action was examined by photometry of the light emission of the fluorescent Ca^{2+} indicator Fluo-3 (Kao et al., 1989). Briefly, the fluorescent dye was loaded in the form of acetoxymethyl ester (Fluo-3AM, 5 μM) for 1 h at room temperature in the dark, with addition of pluronic acid to facilitate dispersion. Both Fluo-3AM and pluronic acid F-127 (purchased from Molecular Probes, Eugene, OR, USA) were stored in stock solutions in anhydrous DMSO at −20°C. At low concentrations, Fluo-3 fluoresces in direct proportion to its degree of Ca^{2+} saturation, with an adequate resolution in an extended range of concentrations, and can be conveniently excited with the 488 nm emission line of the argon laser of the ACAS workstation, like the 6-carboxyfluorescein used for dye-coupling measurements.

2.5. Measurement of the junctional conductance

Junctional conductance was determined using a dual voltage clamp technique in whole-cell configuration according to the method of Weingart (1986). Low resistance (1.5 to 5 MΩ) patch pipettes obtained using an horizontal puller (Mecanex BB-CH-PC) were backfilled with a filtered solution containing (in mM): KCl 140, Mg-ATP 5, Hepes 10, EGTA 5, glucose 10, GTP 0.1 (buffered to pH 7.2 with KOH) and connected to their respective feed back amplifiers (Biologic RK300, Grenoble, France).

Both cells of the pair were first clamped to a common holding potential (V_h, close to −80 mV), then a voltage difference was established by pulsing one cell to a membrane potential different from the common holding potential. If permeable junctional channels connected the cytoplasms of adjacent cells, a current flowed through them, and the junctional conductance was estimated as the current supplied by the feed-back amplifier connected to the cell held at V_h divided by the difference in membrane potentials between the cells. Changes in its intensity reflected variations of the functional state and of the proportion of open junctional channels. Currents and potentials records were digitized, stored and analyzed using a personal computer by means of a software package (Pclamp, Axon Instruments, Burlingname, CA, USA).

3. Results

3.1. Characteristics of the cell-to-cell dye transfers in control conditions

Enzymatically dispersed cells from the three used tissues, when seeded in Petri dishes, progressively attach themselves to the plastic bottom, flatten and emit protrusions and pseudopodia towards neighbouring cells. After cells enter in contact, a cell-to-cell diffusion of the fluorescent dye can be observed. This step is interpreted as corresponding to the establishment of gap junctions and to the opening of channels connecting directly the cytoplasms of cells in contact. An important intercellular coupling spontaneously occurs in cardiac cells but, in the two other cell types, it is largely enhanced by the presence of specific hormones in the culture medium, as recently demonstrated. In Sertoli cells (Pluciennik et al., 1994), the Follicle Stimulating Hormone (FSH, a glycoprotein secreted by the adenohypophysis, essential for normal sexual development and reproductive function, 0.6 μg/ml), as in human trophoblastic cells (Cronier et al., 1994b) the human chorionic gonadotropin (a glycoprotein hormone produced by the trophoblast, essential for the maintenance of pregnancy through its luteotrophic effect, 500 mIU/ml), greatly enhance gap junctional communication.

Dye transfer occurring through junctional channels is illustrated in Fig. 2, upper panel (a–c). Sertoli cells were cultured for 4 days in presence of FSH. After staining with 6-carboxyfluorescein, the computer-generated images of the fluorescent dye distribution were obtained before (Fig. 2a), then respectively just after photobleaching (Fig. 2b) and 10 min (Fig. 2c) later, in Tyrode's solution. Immediately after the photobleach, the light emission of the three selected cells was reduced to about 20 to 40% of its initial level. Then, in control conditions, the intensity of the fluorescent emission of the cells in contact with others progressively increased, reflecting the arrival of unbleached molecules issuing from adjacent cells. This recovery followed, at least during the first min, a monoexponential time course (see Fig. 3, obtained from human trophoblasts cultured for 2 days in presence of human chorionic gonadotropin, continuous line, □). By fitting Eq. (1) to the experimental data, kt was plotted versus time (Fig. 4) and, from the slope of this relationship, it was possible to determine the rate constant, k, that provided a measure of the relative junctional permeability. k values were found equal to 0.07 ± 0.01 min^{-1} (mean ± S.E.M.; $n = 27$) in neonatal rat Sertoli cells, to 0.11 ± 0.01 min^{-1} ($n = 20$) in human trophoblasts (both after hormone stimulation), and to 0.26 ± 0.06 min^{-1} ($n = 40$) in ventricular myocytes. In other words, in normal culture conditions, the intensity of fluorescent emission increased after photobleaching, following an exponential time course with mean time constants of 3.8 min in cardiac cells, 9.1 min in human trophoblasts and 14.3 min in Sertoli cells.

However, in human trophoblastic cells, this step of intercellular communication was ephemeral and rapidly led to a membrane fusion and to the formation of a syncytium, whatever the elements that were in contact (contiguous cytotrophoblastic cells, adjacent syncytiotrophoblasts or contacting cytotrophoblastic cell and syncytiotrophoblast). Intercellular communications via gap junction channels or through cytoplasmic bridges (while cell fusion is occur-

ring) are then easily distinguished by using junctional blockers such as heptanol, and by analyzing the time course of fluorescence recovery (Cronier et al., 1994b). Only cell elements communicating through junctional channels were taken in account to examine the effects of gossypol.

3.2. Cytotoxicity of gossypol

On human peripheral blood mononuclear cells, the cytotoxicity of the (+)-enantiomer of gossypol, as determined by the IC_{50}, was estimated at 52 μM whereas the (−)-enantiomer had an $IC_{50} > 100$ μM (Lin et al., 1993). Gossypol isolated from cottonseed is a racemic mixture and was used at a maximal concentration of 5 μM in the present study. However, at this concentration, cytotoxic effects were manifest in cells exposed for periods exceeding 2 or 3 h in serum-free conditions, as shown by progressive alterations of their morphology (shrinkage of the cells, development of vacuoles in the cytoplasm). At these concentrations, the presence of foetal calf serum in the culture medium did not prevent these effects but when lower, moderate concentrations of gossypol (up to 3 μM) were used, the cell membrane integrity was preserved, even after two days in culture, and cell aggregation occurred.

However, even during pulse exposures to gossypol,

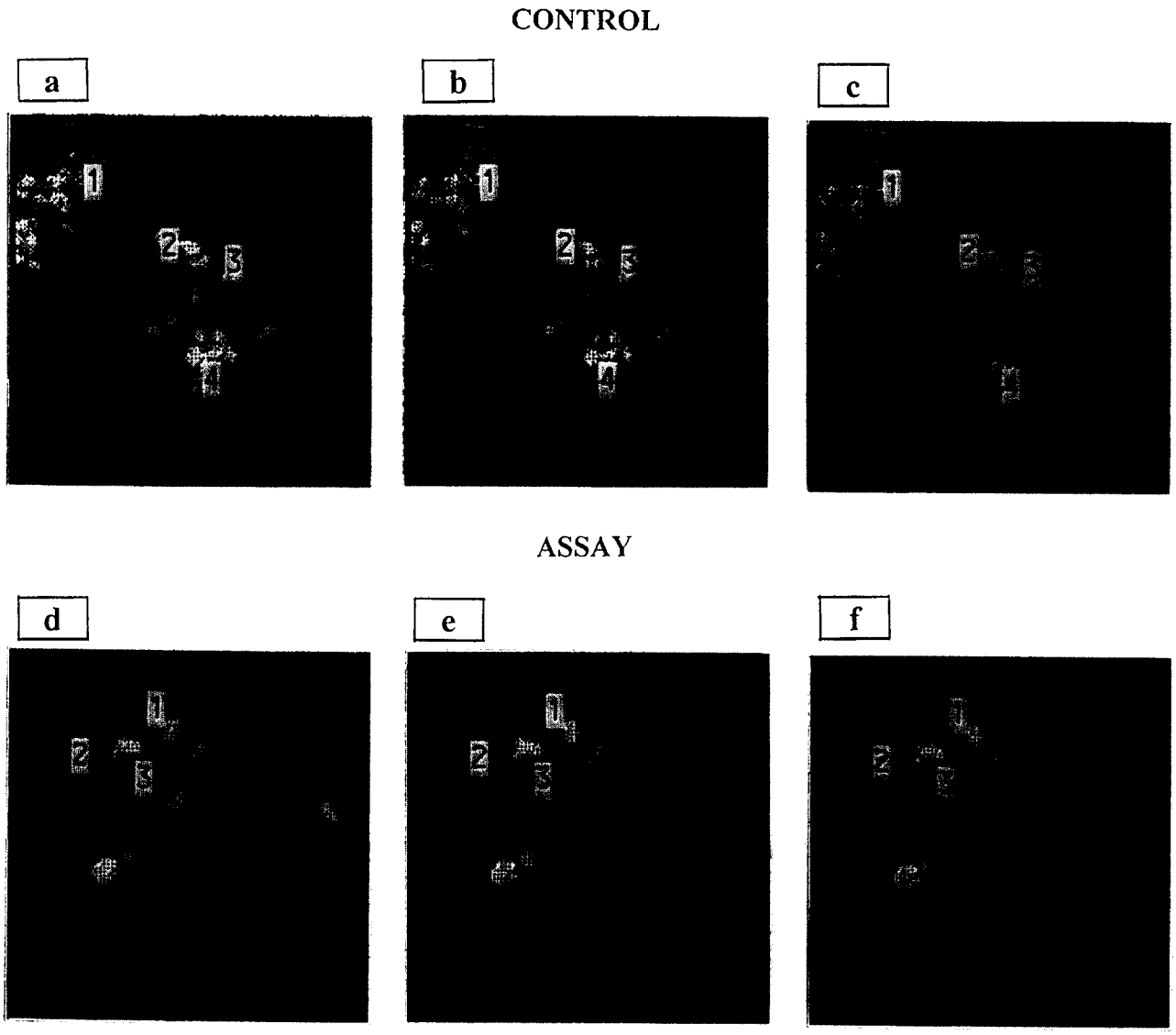

Fig. 2. Examples of quantification of the fluorescent dye distribution in Sertoli cells of immature rat testes, cultured for four days in a FSH-containing medium. The grey density images of fluorescence intensities were obtained by scanning a field of 270 × 270 μm with low intensity light pulses. After a pre-bleach scan (left panel), 6-carboxyfluorescein was photobleached in some selected areas (polygons 2 to 4, upper row; 2 and 3, lower row) by means of a strong illumination. The evolutions of the fluorescence levels in the selected cells was investigated in control conditions (upper panel) and after a 15 min exposure to gossypol (3 μM) in serum-free conditions (lower panel), just after photobleaching (middle column), then 12 min later (right column). In the photobleached cells, dye reappearance was observed in control conditions (2 to 4) whereas recovery was lacking in gossypol-treated cells (2 and 3). Cells in polygons 1 (both panels) were kept unbleached as controls for the spontaneous fading of fluorescent emission.

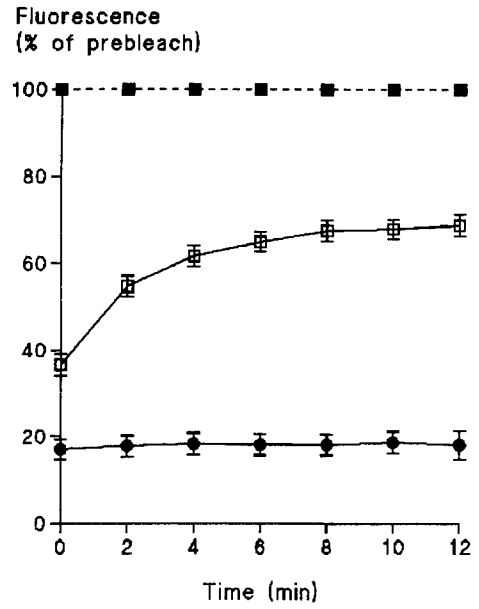

Fig. 3. Time courses of the fluorescent emission of human trophoblastic cells stimulated with human chorionic gonadotropin, drawn from the experimental data in control conditions (□, $n = 34$) or in presence (●, $n = 13$) of gossypol (3 μM). The upper stippled horizontal line (■) represents the light emission of unbleached isolated cells. The fluorescence intensity of the selected cells is represented in percentage (mean ± S.E.M.) of the prebleach emission versus the time after photobleaching. In control conditions, the fluorescent emission of the bleached cells increased progressively. In contrast, in presence of gossypol, the fluorescence levels did not significantly increase in the bleached cells. In the isolated unbleached cells, the light emission remained unchanged.

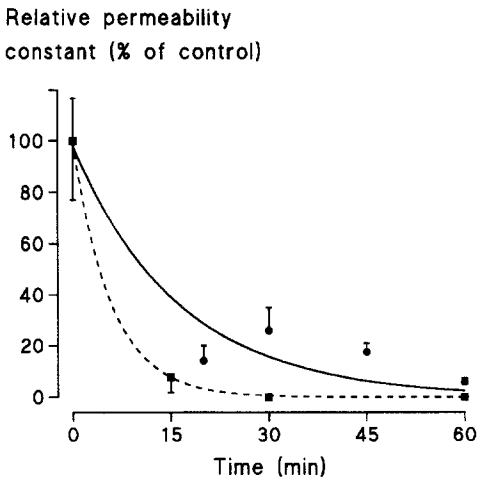

Fig. 5. Time-dependent decrease of diffusional coupling after application of gossypol to Sertoli cells (●, continuous line) and ventricular myocytes (■, dotted line) of young rat, at concentrations of 3 and 5 μM, respectively. Data were obtained from (from left to right) 21, 5, 7, 12 and 8 measurements in Sertoli cells and from 9, 6, 5, 4 and 6 measurements in cardiac myocytes, respectively. Results are expressed in percent of the relative permeability constant (k) measured in the same culture dishes in control conditions; bars indicate S.E.M.

cytotoxic effects could influence the cellular metabolism. This eventuality was examined by performing cytotoxicity assays to measure the mitochondrial activity by means of the tetrazolium salt colorimetric assay (MTT test), after gossypol treatments causing a complete interruption of the

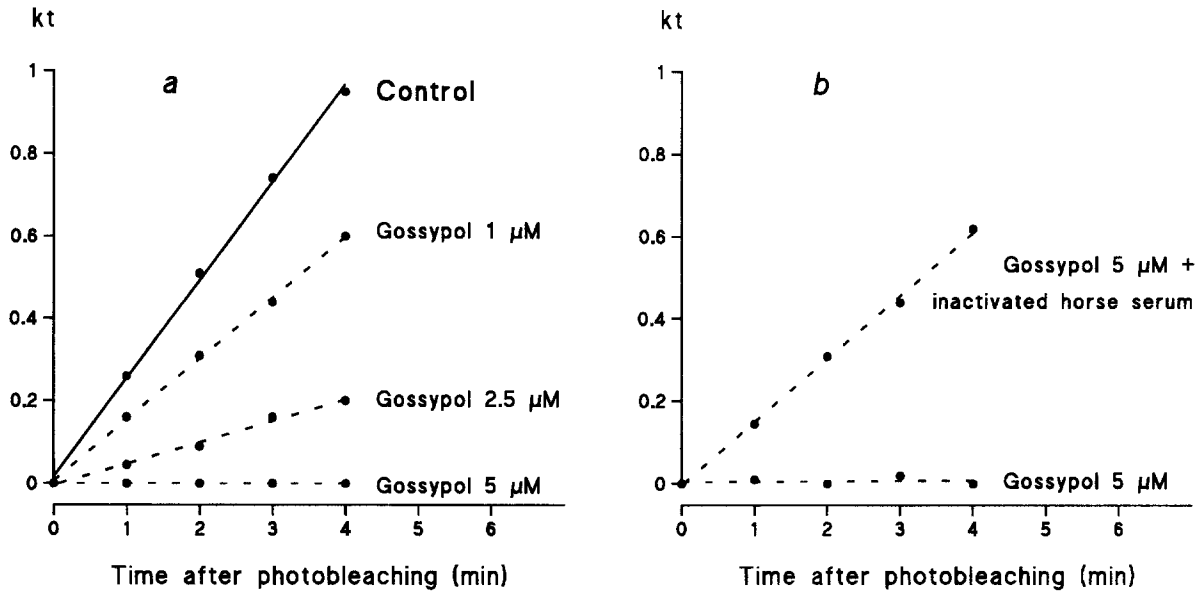

Fig. 4. Illustrations of the graphic determination of the relative permeability constant k in neonatal rat myocytes, in control conditions (a, continuous lines) and in cells exposed to different concentrations of gossypol for 15 min (a and b, dotted lines). From the fluorescence intensities integrated in the polygons circumscribing the cells, measured every min, kt was determined using Eq. (1): $kt = -\ln (F_i - F_t)/(F_i - F_0)$. When plotted vs. time, its evolution was linear, at least during the first 4 min after photobleaching. The slope of the fluorescent emission recovery was progressively lowered when increasing gossypol concentrations were used, and dye transfer was abolished at 5 μM. In contrast, in presence of serum, an important intercellular coupling was still observed (b). Data were obtained from respectively 9 measurements in control conditions, 25 in presence of gossypol 1 μM, 25 in presence of gossypol 2.5 μM and 10 in presence of gossypol 5 μM (6 in serum-free conditions, 4 when horse serum was present).

Table 1
Colorimetric tetrazolium assay for gossypol cytotoxicity

Cell type	Control conditions	After gossypol treatment	Air-dried cells
Human trophoblasts	0.44 ± 0.04	0.38 ± 0.06	0.11 ± 0.05
Rat cardiac myocytes	0.54 ± 0.05	0.53 ± 0.04	0.07 ± 0.01
Rat Sertoli cells	0.40 ± 0.03	0.41 ± 0.08	0.07 ± 0.04

After extraction of the formazan formed in the mitochondria of living cells, the optical density (OD) was measured at a wavelength of 570 nm with background subtraction at 630 nm. Numbers ± S.D. represent the mean values of the OD-(570–630) of 3 measurements, each sample being composed by pooling the solutions obtained from 3 culture dishes. Air-dried cells were used as negative controls; cultures were washed in Tyrode's solution and air-dried for 10 min before the colorimetric assay.

cell-to-cell communication, and the results of optical density measurements are summarised in Table 1. This parameter is proportional to the level of formazan reaction product formed in active mitochondria of living cells. In the three cell types, optical densities were similar in the control and gossypol-treated cells, showing that the short-term exposure to gossypol had no cytotoxic consequences.

3.3. In serum-free solutions, gossypol rapidly interrupts the cell-to-cell diffusion of a fluorescent dye

After 15 min of exposure to gossypol (3 μM), the spontaneous decay of the fluorescent emission of isolated cells, resulting from a background photobleach by the successive scans and from a dye outflux through non-junctional membranes, was very similar in control and in gossypol-treated cultures (e.g. Fig. 2, polygons 1 in both rows), showing that the integrity of the plasma membrane was preserved.

In photobleached cells in contact with unbleached cells, the level of fluorescent emission did not rise (Fig. 2, bottom panel, and Fig. 3, ●), revealing an obstruction to the dye transfer, due to the closure of the intercellular channels triggered by gossypol. The cells replaced in culture for up to 18 h after gossypol removal did not recover the ability of cell-to-cell exchange.

3.4. Concentration dependence of the gossypol effect on junctional coupling

The effects of increasing concentrations of gossypol (1 to 5 μM) on the cell-to-cell transfer of fluorescent dye were quantified in rat Sertoli cells and in cardiac myocytes after brief (15-min) or sustained (several hours) exposures. After a 15-min treatment, intercellular dye diffusion was not affected by gossypol at concentrations up to 1 μM. With higher gossypol concentrations (2.5 to 5 μM), the slope of the fluorescence recovery decreased to very low

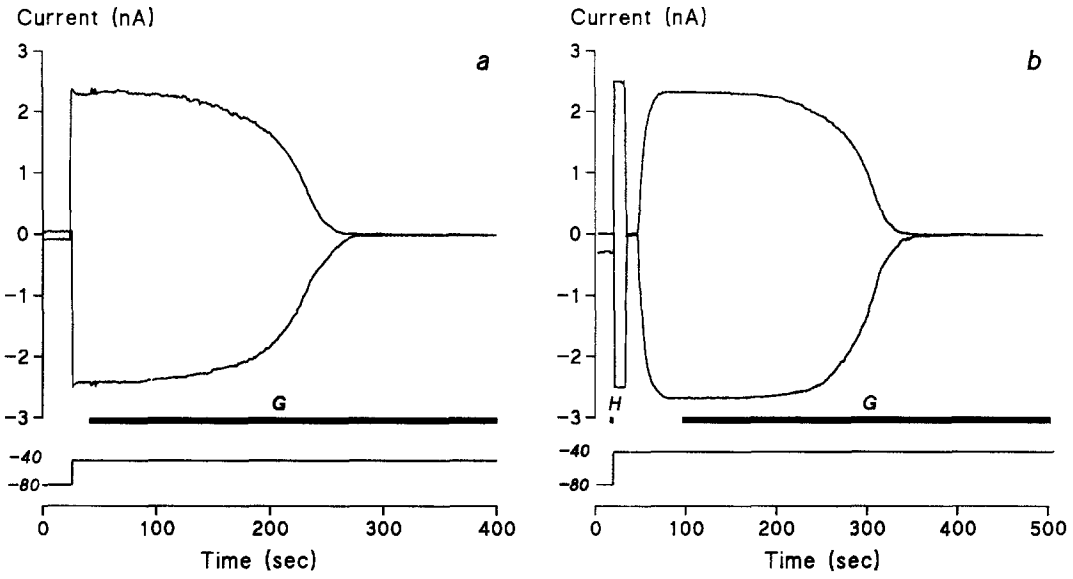

Fig. 6. Typical examples of the time course of the decrease in current responses recorded under voltage clamp conditions, in double whole-cell configuration, in pairs of ventricular myocytes, when gossypol (5 μM) was applied in the vicinity of the cell pairs. A continuous voltage step (to −40 mV, bottom trace) was applied to one cell from the common holding potential (−80 mV), while the potential of the second cell remained unchanged. Due to the transjunctional voltage difference, a large amplitude current crossed the cell-to-cell junction, compensated by an opposite current supplied by the feed-back amplifier connected to the cell maintained at −80 mV. Gossypol applications (a and b, G) triggered a progressive decrease of the current intensity, reflecting the gradual closure of gap junctional channels. (b) Comparison of the time courses of electrical uncouplings elicited by heptanol (3 mM) and gossypol. When a brief pulse of heptanol-containing solution was applied (H), the junctional current was rapidly but reversibly abolished. The subsequent total electrical uncoupling caused by gossypol application took place within 4 to 6 min.

values (Fig. 4a), reflecting the impairment of cell-to-cell communication, except when serum proteins (5% w/v) were present in the test medium (Fig. 4b). The known great affinity of gossypol for serum albumin (Royer and Vander Jagt, 1983) could explain this lower uncoupling efficiency by the formation of biologically inactive protein-gossypol adducts.

The effects of high (5 μM) and moderate (3 μM) concentrations of gossypol on the relative permeability constant k are plotted versus time in Fig. 5. After 1-h treatment, a complete interruption of gap junctional communication was observed in both cell types. With lower gossypol concentrations, a sizeable intercellular coupling persisted for a long period. In ventricular myocytes for example, k slowly decreased and, from 0.24 ± 0.03 ($n = 9$) in control conditions, was lowered to 0.11 ± 0.02 after 1 h, 0.08 ± 0.03 after 2 h and 0.03 ± 0.01 after 6 h in presence of gossypol 2.5 μM, and to 0.21 ± 0.02, 0.18 ± 0.04 and 0.14 ± 0.03 respectively after the same duration in presence of gossypol 1 μM ($4 < n < 6$). In Sertoli cells, at the latter concentration, a measurable cell-to-cell dye transfer was still present after a 32 h exposure, with $k = 0.065 \pm$

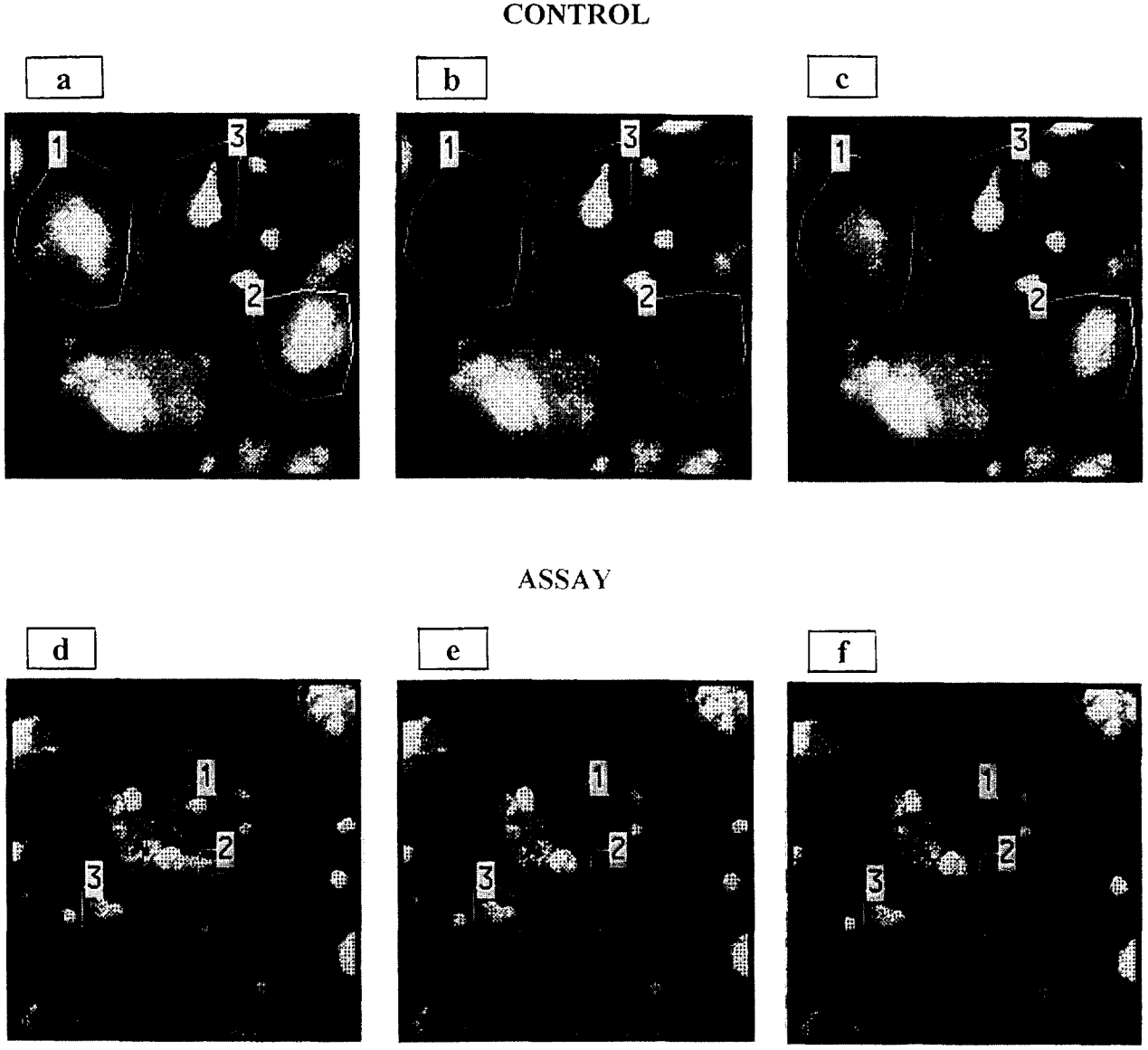

Fig. 7. Comparison of computer-generated images of fluorescence distribution in human trophoblasts cultured for 2 days in a foetal calf serum (10%) and human chorionic gonadotropin-containing medium, in absence (upper row) or in presence of gossypol (3 μM, lower row). After a pre-bleach scan (a), the light emission of the photobleached cells in contact with other cellular structures increased in the cells grown in control conditions (c, polygons 1 and 2) whereas fluorescence recovery was lacking in gossypol-treated cells (f, polygons 1 and 2). The isolated cell elements in polygons 3 were used as controls of fluorescence fading. The large syncytial structures observed in control conditions contrast with the aggregated cytotrophoblastic cells persisting in the gossypol assay.

0.01 ($n = 3$) vs. 0.07 ± 0.01 ($n = 27$) in control conditions.

3.5. Kinetics of the interruption of cell-to-cell communication

An estimation of the kinetics of the uncoupling process was made possible by continuously monitoring the junctional conductance in double voltage clamp conditions (Fig. 6). A gossypol-containing solution was administered by means of a continuous flow directed to the cell pair under investigation. In experiments performed on neonatal rat myocytes cultured for 2–3 days and exposed to gossypol 5 μM, the junctional currents, elicited in dual voltage clamp conditions by application of a continuous transjunctional voltage difference, decreased progressively to immeasurably low values, reflecting the gradual closing of junctional channels, and a total electrical uncoupling was obtained after 4 to 10 min (Fig. 6a,b). The junctional conductances (mean ± S.E.M.: 41.2 ± 2.3 nS, $n = 249$ in control conditions) had become nil at this time, reflecting a complete closure of all junctional channels.

This relatively slow time course is in sharp contrast with the fast junctional closure observed when short (1 s) pulses of a heptanol-containing solution were applied, triggering an abrupt channel closure, complete in less than 500 ms, which was rapidly and totally reversible, as illustrated in Fig. 6b where the cell pair was consecutively uncoupled by heptanol (3 mM) then gossypol (5 μM) applications.

3.6. Effects of a prolonged exposure of the cells to gossypol in serum-containing media

Cytotrophoblastic cells are able to differentiate to form syncytiotrophoblasts within 1 to 2 days. During this in vitro differentiation, the cell fusion occurs after a transient step during which cells in contact establish a direct communication via gap junctional channels (Cronier et al., 1994b). These characteristic properties were employed to investigate the consequences of prolonged exposures (2 days) of these cells to a moderate concentration of gossypol (3 μM), in a serum-containing medium.

In presence of the drug, the aggregation of cytotrophoblastic cells occurred and, as shown in Fig. 7, the spontaneous dye-fading in isolated cells was not accelerated, showing that the permeability of the non-junctional plasma membrane for organic molecules was not altered, but no cell-to-cell dye transfer took place, reflecting the closing of intercellular channels. Moreover, the fusion of the cytotrophoblastic cells into syncytiotrophoblasts was then impeded. Indeed, after 2 days in culture in presence of gossypol (Fig. 7, lower panel), cytotrophoblastic forms persisted, in contrast with the large syncytial elements already formed in control conditions after the same delay (Fig. 7, upper panel).

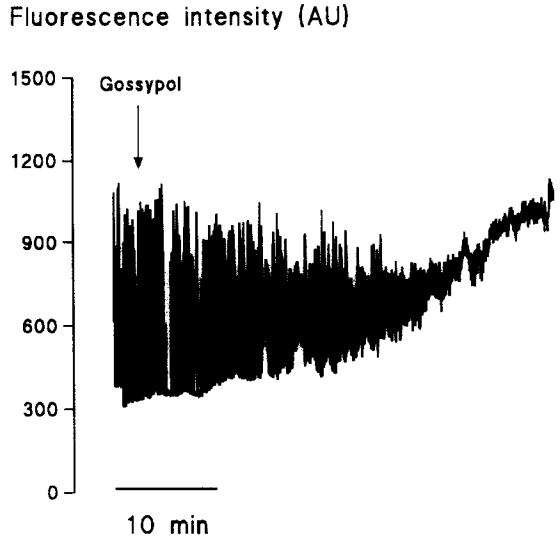

Fig. 8. Record of the Fluo-3 fluorescence level, reflecting changes of the intracellular calcium concentration in rat ventricular myocytes during gossypol application (5 μM). The ample oscillations of the calcium concentration that occurred before the introduction of the drug in the medium reflected a spontaneous activity of the myocytes.

Thus, the presence of serum did not durably prevent the long-term effects of gossypol on cell-to-cell communication. And a consequence of the intercellular uncoupling was the inhibition of the tissue differentiation. Indeed, it was recently established that the development of a cell-to-cell communication is a requisite step in the in vitro trophoblast differentiation, that a physiological syncytium precedes the formation of a morphological syncytium (Cronier et al., 1994b). The latter step remained inhibited as long as a junctional uncoupler (heptanol) was present in the culture medium and only took place after its washing (Cronier et al., 1994a). The irreversibility of the action of gossypol did not allow to examine the effects of its removal on cellular differentiation.

3.7. This intercellular uncoupling is not mediated by an increase in cytosolic Ca^{2+} concentration

Gossypol was seen to induce a release of Ca^{2+} from rat liver mitochondria (Martinez et al., 1993). To examine the eventuality that interruption of the cell-to-cell communication by gossypol might be due to an increase in cytosolic Ca^{2+} concentration sufficient to gate the junctional channels, a fluorescent indicator (Fluo-3) was used to investigate the effect of gossypol on the intracellular Ca^{2+} concentration of ventricular myocytes. As shown in Fig. 8, the bulk cytosolic Ca^{2+} concentration was only modestly increased during the initial 10 to 15 min of action of gossypol (5 μM), when junctional uncoupling developed. Subsequently, an important increase in Ca^{2+} concentration took place, that might result from other non-specific effects of gossypol (e.g. inhibition of several membrane

transport mechanisms) leading to a delayed cytotoxic effect by overloading the Ca^{2+} buffering capacities of the cells.

4. Discussion

Gossypol, a yellow polyphenolic aldehyde contained in cottonseed products consumed in human and animal diets, found to have a reversible infertility effect on adult man (see Moh et al., 1993), was seen in the present study to impair the functional state of the channels connecting adjacent cells, from man or rat, in tissues involved in steroidogenesis (trophoblastic or Sertoli cells) or not involved (ventricular myocytes). When high concentrations of gossypol (3 to 5 μM) were applied in serum-free conditions, a gap junctional uncoupling rapidly occurred in the three cell types in a similar concentration range, approximately twice the maximal plasma concentration reported in man. Indeed, the peak concentration of gossypol in the plasma of the human male following a single 20 mg oral dose is approximately 2 μM, with a half life of 10 days (Wang et al., 1985). At these concentrations (3 to 5 μM), gossypol was seen to cause marked negative inotropic and arrhythmogenic effects in Langendorff preparations of guinea pig and rat hearts (Ye et al., 1987). In contrast to other lipophilic uncouplers (aliphatic alcohols or fatty acids, sex steroid esters), reversibility was never observed after gossypol-triggered uncoupling.

When present, serum proteins prevented the short-term uncoupling effect of gossypol. These results are consistent with the previously observed long delays necessary to achieve junctional uncoupling when foetal calf serum was present, such as 5 h in rat liver WB-344 cells exposed to 3–5 μM gossypol or several hours in LC540 rat Leydig cells treated with 15 μM gossypol (Ye et al., 1990). Other protective effects of serum proteins against gossypol action have also been reported for BCL-D1 human diploid fibroblasts (Joseph et al., 1986), murine erythroleukemia cells (Haspel et al., 1984) or bovine luteal cells (Gu et al., 1990), suggesting that protein-gossypol adducts would be less active biologically. Trophoblastic cells were able to survive to treatments with moderate concentration (3 μM) of gossypol, but their in vitro differentiation was stopped. This absence of trophoblastic syncytialisation when intercellular coupling is impeded confirms that gap junctional communication is a requisite for trophoblastic differentiation (Cronier et al., 1994b), as similarly observed for myoblast fusion (Mège et al., 1994).

In conditions sufficient to induce a complete intercellular uncoupling, the slow spontaneous decay of the fluorescent emission was not accelerated, showing that the permeability of the non-junctional plasma membrane for organic molecules was not altered. Gossypol showed no evidence of cytotoxicity in these conditions, and the closure of the gap junctional channels occurred during the first minutes of action and preceded the disorder in Ca^{2+} homeostasis. It can be concluded that the uncoupling effects of gossypol are temporally dissociated from subsequent cytotoxic effects observed after exposures of the cells to high concentrations (5 μM) in serum-deprived conditions.

The possibility of a causal involvement of Ca^{2+} in the gossypol-induced junctional uncoupling has been directly examined by measuring the fluorescent emission of Fluo-3, an intracellular Ca^{2+} indicator. In the time interval requested for complete uncoupling, the rise in Ca^{2+} concentration was very modest. In double patch clamp conditions, intercellular uncoupling occurred while an increase in the intracellular Ca^{2+} level was made unlikely by the presence of EGTA (5 mM) in the pipette filling solution. Similarly, a possible decrease of the internal pH in cells exposed to gossypol would then have been prevented by the presence of Hepes (10 mM). But, during prolonged exposures to gossypol, a delayed and important rise in Ca^{2+} concentration was recorded. This observation is consistent with the reported inhibition of the ATP-dependent uptake of Ca^{2+} by gossypol in preparations from dog heart (Ye et al., 1987). Gossypol is considered to directly inhibit the electron transport chain of mitochondria (Abou-Donia and Dieckert, 1974), causing a drop in the intracellular ATP level that, by reducing the active transport of Ca^{2+}, might induce an increase in cytosolic Ca^{2+} concentration. Ye et al. (1990) examined the possible involvements of protein kinase C (that gossypol was reported to inhibit) and of free radicals release in the abolition of cell-to-cell communication, and discarded these eventualities.

Gossypol, a very reactive reagent, can easily bind either to proteins (such as the junctional proteins) or to the phospholipid bilayers of biological membranes (e.g. the boundary lipids surrounding the membrane proteins) and then alter their structure.

Gossypol can readily interact with proteins (Lyman et al., 1959), through either non covalent interaction or covalent binding between aldehyde groups of gossypol and the amine groups of cellular proteins, by Schiff's base formation (Strøm-Hansen et al., 1989). The cytotoxicity of gossypol was lowered by modifying the aldehyde functional groups of the molecule (Radloff et al., 1986). The junctional channels are formed by units (connexons) made of related proteins (connexins), considered to span the membrane four times, with both ends located on the cytoplasmic side of the membrane (Laird and Revel, 1990). Connexin-43 is the major gap junction protein present in rat ventricular myocytes (Beyer et al., 1987) and Sertoli cells (Risley et al., 1992), and the only protein identified in human trophoblastic cells (Cronier et al., 1994c). It appears from its amino acid sequence (Beyer et al., 1987) that residues with an extra basic group (lysine or arginine) capable to interact with aldehyde radicals are particularly abundant in domains considered important for responses to gating stimuli and long term regulation (e.g. the extracellular loops and cytoplasmic domains, *see* Sáez et al., 1993).

On the other hand, gossypol, in micromolar concentrations in the aqueous phase, is also able to strongly bind to lipid monolayers or bilayers (Reyes et al., 1984). The different sensitivities of membranes to gossypol have been attributed to their different lipidic composition, expressed as their different phosphatidylcholine/phosphatidylserine (De Peyster et al., 1986) or phospholipid/cholesterol (Cuéllar and Ramírez, 1993) ratios. The membrane lipidic composition could, according to Wu et al. (1991) undergo adaptive changes during chronic gossypol treatment, comparable to the adaptive changes in lipidic content caused by chronic ethanol exposures (Waring et al., 1981). Specific lipids appear to be required for the assembly of gap junction particles in functional arrays, particularly a high cholesterol-to-phospholipid molar ratio (Malewicz et al., 1990). The comparable sensitivity of the three investigated cell types to gossypol exposures may be the consequence of similar lipidic environments of the junctional proteins.

The gossypol action has often been considered to result from the observed decrease of membrane fluidity (Reyes et al., 1984; De Peyster et al., 1986; Radloff et al., 1986; Wu et al., 1989, 1991; Cuéllar and Ramírez, 1993). This hypothesis would account for the large variety of its inhibitory effects on membrane-associated proteins. A generalized increase in the microviscosity of the cell membranes, by reducing the freedom of movement of membrane-associated proteins or glycoproteins within the lipid bilayer, would hinder their optimal activity.

A decrease in the fluidity of this microenvironment was also seen to occur when the gap junctional communication was interrupted by heptanol (Bastiaanse et al., 1993). Another lipophilic uncoupler, 17β-estradiol (Hervé et al., 1996) shares this property (Clarke et al., 1987). The uncoupling delay after gossypol treatment is comparable with that of 17β-estradiol (4 to 10 min) and considerably longer than after heptanol applications (< 0.5 s; Bastide et al., 1995). This slower time course might reflect a protracted interaction of the active molecule with the membrane, may be due to a different partitioning coefficient of the two drugs in the cell membranes. It was however observed that the incorporation of lipophilic substances into myocytes membranes was not well predicted by their partition coefficient between lipids and water (Massey et al., 1992).

The present results are consistent with mechanisms involving a direct action of this highly reactive redox reagent on the channel proteins and/or with localized disordering effects on the membrane-channel structure caused by gossypol, similar to those produced by other lipophilic molecules, which are thought to affect the junctional channels by interfering with the arrangement of the membrane lipid bilayer, and thereby altering the conformation of the gap junction channels. Serum proteins prevented the acute effects of high concentrations of gossypol but failed to protect the cells from long-lasting exposures to moderate, non cytotoxic, concentrations of the drug, that can be encountered in therapeutic conditions.

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