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Gap Junction Coupling and Apoptosis in GFSHR-17 Granulosa Cells

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Abstract. Recently, we found that intracellular washout of cGMP induces gap junction uncoupling and proposed a link between gap junction uncoupling and stimulation of apoptotic reactions in GFSHR-17 granulosa cells. In the present report we show that an inhibitor of guanylyl cyclase, ODQ, reduces gap junction coupling and promotes apoptotic reactions such as chromatin condensation and DNA strand breaks. To analyze whether gap junction uncoupling and induction of apoptotic reactions are related, the cells were treated with heptanol and 18β-GA, two known gap junction uncouplers. Gap junction coupling of GFSHR-17 cells could be restored if the incubation time with the gap junction uncouplers was less than 10 min. A prolonged incubation time irreversibly suppressed gap junction coupling and caused chromatin condensation as well as DNA degradation. The promotion of apoptotic reactions by heptanol or 18β-GA was not observed in cells with low gap junction coupling like HeLa cells, indicating that the observed genotoxic reactions are not caused by unspecific effects of gap junction uncouplers. Additionally, it was observed that heptanol or 18β-GA did not induce a sustained rise of $[Ca^{2+}]_i$. The effects of gap junction uncouplers could not be suppressed by the presence of 8-Br-cGMP. It is discussed that irreversible gap junction uncoupling can be mediated by cGMP-dependent as well as cGMP-independent pathways and in turn could lead to stimulation of apoptotic reactions in granulosa cells.

Key words: Granulosa cells — cGMP — Gap junction — Apoptosis — Chromatin condensation — DNA strand breaks

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Introduction

Gap junctions are aggregates of cell-to-cell channels that enable neighboring cells to exchange small molecules (≤ 1 kDa) like Ca^{2+}, cAMP, IP_3, and to synchronize electrical activities (Bruzzone, White & Paul, 1996; Goodenough, Goliger & Paul, 1996; White & Paul, 1999; Harris, 2001). Gap junction channels are composed of connexins (Cx), which are products of 19 and 20 genes by mouse and human, respectively, and are specifically expressed and regulated in the different tissues (Willecke et al., 2002). Six connexins oligomerize and form a connexon that is inserted into the cellular membrane. Two connexons of adjacent cells associate and form a cell-to-cell channel. Since gap junction channels allow a direct intercellular exchange of metabolites and second messengers, a specific role in diseases and regulation of cellular reactions like proliferation, transformation, differentiation and apoptosis has been hypothesized (Bruzzone et al., 1996; Goodenough et al., 1996; White & Paul, 1999; Nakase et al., 2003).

Granulosa cells in the ovular follicles are eliminated by apoptosis during atresia or the demise of the corpus luteum (Quirk, Harman & Cowan, 2001; Sasson & Amsterdam, 2003). During atresia excess follicles are removed and dominant follicles, which undergo maturation, are selected. With the demise of corpus luteum, the ovary gets rid of superfluous cells, which otherwise represent a risk of tumor formation. Death-receptor as well as mitochondria-dependent apoptotic pathways have been characterized in different cell systems. Although the start of both pathways differs, they converge in activation of apoptosis executioner caspases: caspase-3, -6 and -7, which induce chromatin condensation and DNA degradation, the hallmarks of apoptosis (Gottlieb, 2000; Quirk et al., 2001; Johnson, 2003; Peter & Krammer, 2003; Sasson & Amsterdam, 2003). Additionally, a caspase-independent mitochondrial pathway for apoptosis has been described. This pathway is activated in

response to depletion of cellular NAD^+. Mitochondria are then disrupted and cytochrome c and AIF (apoptotis-inducing factor) are liberated. By association with Apf-1 (apoptosis-promoting factor-1) and procaspase 9 in the cytosol, cytochrome c stimulates apoptosis by a caspase-dependent pathway, whereas AIF diffuses into the nucleus, cleaves DNA and thereby stimulates apoptosis by a caspase-independent pathway (Chiarugi & Moskowitz, 2002; Lipton & Bossy-Wetzel, 2002).

Granulosa cells express different connexins during their hormone-regulated proliferation and differentiation (Okuma et al., 1996; Sommersberg et al., 2000; Wright et al., 2001). An involvement of gap junction coupling in follicular development and atresia has been proposed (Wiesen & Midgley, 1996; Ackert et al., 2001; Wright et al., 2001). It has been found that follicular growth is suppressed in Cx43-deficient mice (Ackert et al., 2001) and that induction of atresia is correlated with reduced Cx43 expression (Wiesen & Midgley, 1996). Additionally, we could recently demonstrate that at double whole-cell patch-clamp configuration, the gap junction conductance (G_j) of GFSHR-17 granulosa cells, an in vitro model of granulosa cells (Keren et al., 1993), was significantly reduced in the absence of cGMP in the intracellular solution (Ngezahayo, Altmann & Kolb, 2003). The decrease of G_j was related to a sustained $[Ca^{2+}]_i$ rise and shrinkage of cell volume and followed by formation of blebs. Since these cellular changes were not observed in presence of intracellular cGMP, it was proposed that cGMP-depletion inhibited gap junction communication and contributed to stimulation of apoptotic processes like formation of blebs in granulosa cells (Ngezahayo et al., 2003).

In this report, data are presented, which correlate inhibition of gap junction coupling and stimulation of apoptotic pathways in GFSHR-17 granulosa cells. Using the double whole-cell patch-camp technique, DAPI-staining and comet assay, it was investigated whether the inhibitor of guanylyl cyclase ODQ affects G_j and causes genotoxic effects like chromatin condensation and DNA strand breaks. Furthermore, two chemically non-related gap junction uncouplers, heptanol and 18β-GA, were applied to elucidate the proposed correlation between gap junction uncoupling and stimulation of apoptotic reactions in GFSHR-17 granulosa cells. To ensure that the stimulation of apoptotic reactions was not caused by unspecific effects of the gap junction uncouplers on cells, like change in membrane fluidity (Enders et al., 2004), HeLa cells, which exhibit low coupling by gap junctions (Elfgang et al., 1995; Breidert et al., 2005), were used. We found that neither heptanol nor 18β-GA altered the structure of the chromatin of HeLa cells. The involvement of gap junction uncoupling in stimulation of apoptotic reactions in granulosa cells is discussed.

Materials and Methods

Cell Culture

GFSHR-17 granulosa and HeLa cells were seeded ($2–5\times10^5$ cells/ml) on cover slips in petri dishes containing Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 5% fetal calf serum (Sigma), penicillin and streptomycin (Sigma). Cells were cultivated at 37°C under an atmosphere of 5% CO_2 – 95% air. The culture medium was replaced every 2–3 days. Cells were used for experiments 3–6 days after plating. GFSHR-17 cells were utilized up to a total of 25 passages.

Cell Treatment

To study the effects of 1H-[1, 2, 4] oxadiazolo [4, 3-a]quinoxalin-1-one (ODQ) on GFSH-R17 cells, cover slips with adherent cells were distributed in three petri dishes containing fresh culture medium. ODQ/DMSO solution was added to one petri dish at a final concentration of 20 μM ODQ/0.2% DMSO. Two control dishes were prepared: one dish contained culture medium and the second 0.2% DMSO. The three dishes were kept for further 2–4 h in the incubator.

For analysis of effects of 18β-glycyrrhetinic acid (18β-GA) or heptanol, the same protocol was used. 18β-GA was diluted in the culture medium to 100 μM (or 25 μM), heptanol to 2 mM (or 0.5 mM). The cells were incubated for 20–40 min in the incubator. In further experiments, cells were pre-incubated in culture medium containing 200 μM 8-Br-cGMP (Sigma) 1 h before treatment with either heptanol or 18β-GA.

Measurement of Gap Junction Coupling in the Double Whole-Cell Patch Configuration

To study the effect of ODQ on gap junction coupling, gap junction conductance (G_j) was determined using the double whole-cell patch-clamp technique as previously described (Ngezahayo et al., 2003). A cover slip was transferred into a patch-clamp chamber containing 300–500 μl bath solution (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 Glucose, 10 HEPES, pH 7.4. Depending on the pre-treatment, 20 μM ODQ/0.2% DMSO or 0.2% DMSO was added to the bath solution. Double whole-cell configuration was established using a pipette-filling solution that does not induce gap junction uncoupling (Ngezahayo et al., 2003). G_j was measured within 5 min after establishment of the double whole-cell configuration.

To explore the effects of heptanol or 18β-GA on G_j, the cells were superfused with a solution containing either heptanol or 18β-GA during the double whole-cell patch-clamp recording.

Analysis of Chromatin Condensation by DAPI Staining

Cover slips with adherent cells were washed twice with PBS (phosphate buffered solution) and fixed with 4% formaldehyde for 20 min. Thereafter, the cells were permeabilized by incubation in 0.3% Triton X-100 for 5–10 min and washed twice with PBS. Staining was performed by incubation in 1 μM DAPI (Molecular Probes/Invitrogen, Karlsruhe, Germany) for 10 min. The cells were washed with PBS and placed in PBS for further analysis.

Observation of the chromatin structure was performed using an inverted fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a monochomator polychrome II (Hamamatsu, Herrsching, Germany). The excitation light (348 nm) was produced

by a 75 W XBO xenon lamp. Fluorescence images were acquired using a CCD camera (Hamamatsu) connected to a computer. The monochromator as well as the camera were controlled by the software Aquacosmos (Hamamatsu). The quantitative evaluation of the results was performed by counting the total number of cells as well as the cells exhibiting chromatin condensation in four different areas of a cover slip. The percentage of cells with condensed chromatin was calculated for each cover slip. The results are given as mean ± standard deviation (SD); *n* denotes the number of cover slips used for the corresponding treatment. At least 1,200 cells were analyzed per treatment.

Analysis of DNA Strand Breaks By Comet Assay

Comet assay was performed according to Ivancsits et al. (2002). After incubation of the cells in culture medium containing either DMSO, ODQ, heptanol or 18β-GA, the cells were trypsinized, collected and centrifuged for 10 min at 1000 × g. The pellets were resuspended in PBS to 2×10^6 cells/ml. The following procedure was performed in the dark. 20 µl of cell suspension was mixed with 100 µl of low melting agarose (0.6%) at 37°C. 100 µl of the cell-agarose mixture was placed on agarose-coated glass slides and covered with a cover slip. The cell-agarose mixtures were allowed to solidify for 10 min at 4°C. The cover slip was removed and further 100 µl of low-melting agarose was added. After solidification at 4°C, the samples were incubated for 90 min in a lysis buffer containing 2.5 M NaCl, 100 mM NA_2EDTA, 10 mM Tris, 1% lauryl sarcosin, 1% Triton X-100, 10% DMSO, pH 10. Subsequently, the samples were drained and placed in a horizontal gel electrophoresis chamber near to the anode. The chamber was filled with electrophoresis buffer for alkaline comet assay (1 mM Na_2EDTA, 300 mM NaOH, pH > 13) and electrophoresis was performed (25 V, 300 mA, 4°C, 20 min). After electrophoresis the slides were washed three times with Tris-buffer (400 mM Tris, pH 7.5), dried at room temperature and conserved for further analysis. Comets were visualized by ethidium bromide staining (20 µg/ml) and examined at 200-fold magnification with a fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a xenon lamp and ethidium bromide filter set (excitation, 520 nm; fluorescence, 605 nm). The fluorescence images were recorded with a CCD color camera connected to a computer. The quantitative analysis was performed with a comet-scoring software (http://www.autocomet.com/home.php). The tail moment, as marker of DNA strand breaks, was calculated according to the software. To obtain the relative tail moment for each treatment, the tail moment of pre-treated cells (at least 1000 cells/treatment) was normalized to the tail moment of untreated cells after each experiment. The data are given as mean ± SEM for $n = 5$ experiments, respectively.

Statistical Analysis

Statistical analysis was performed using the software Origin (Microcal Software, Inc., Northampton, MA). The differences between control/DMSO and ODQ, heptanol or 18β-GA exposed cells were tested for significance by independent Student's *t*-test. A difference at $P < 0.05$ was considered statistically significant.

Measurement of Intracellular Concentration of Free Ca^{2+}

The intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was measured as described previously (Ngezahayo et al., 2003). Cells were loaded with Fura 2-AM (Molecular Probes/Invitrogen, Karlsruhe, Germany) for 30 min at room temperature. Fura 2-AM-loaded cells were than transferred into a superfusion chamber and mounted onto an inverted microscope. The cells were superfused with a bath solution (2 ml/min) for at least 5 min to remove the extracellular Fura 2-AM and DMSO. Fura-2 within the cells was excited at 340 nm and 380 nm using the monochromator polychrome II. The fluorescence was registered with the digital CCD camera. The ratio of the fluorescence images to an excitation at 340 nm and 380 nm (F_{340}/F_{380}) was calculated using the program Aquacosmos.

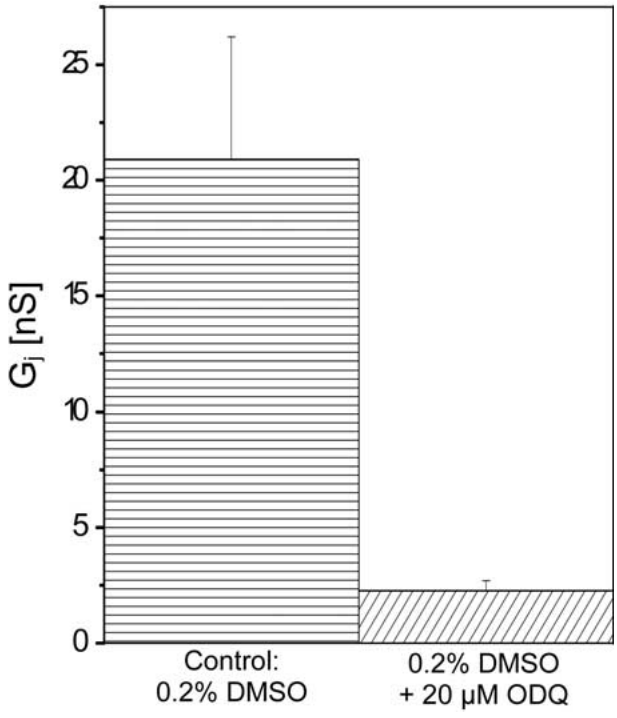

Fig. 1. Effect of ODQ on gap junction conductance (G_j) of GFSHR-17 cells. Cells were pre-incubated for 2–4 h in cell culture solution containing 20 µM ODQ and 0.2% DMSO. Double whole-cell patch-clamp configuration was established and G_j was measured within the first 5 min of recording. Control cells were incubated in culture medium containing 0.2% DMSO. G_j was significantly reduced by ODQ-treatment ($P < 0.01$). The mean ± SD of $n = 8$ independent experiments for each treatment is given.

Results

ODQ Uncouples Gap Junctions and Stimulates Chromatin Condensation and DNA Degradation

We proposed that cGMP depletion leads to gap junction uncoupling and stimulates apoptotic processes in granulosa cells (Ngezahayo et al., 2003). To obtain experimental evidence for this hypothesis, GFSHR-17 cells were pre-incubated in 20 µM ODQ for at least two hours to block the activity of guanylyl cyclase. As shown in Fig. 1, pre-treatment with ODQ significantly reduced gap junction conductance (G_j) compared to DMSO-treated (control) cells.

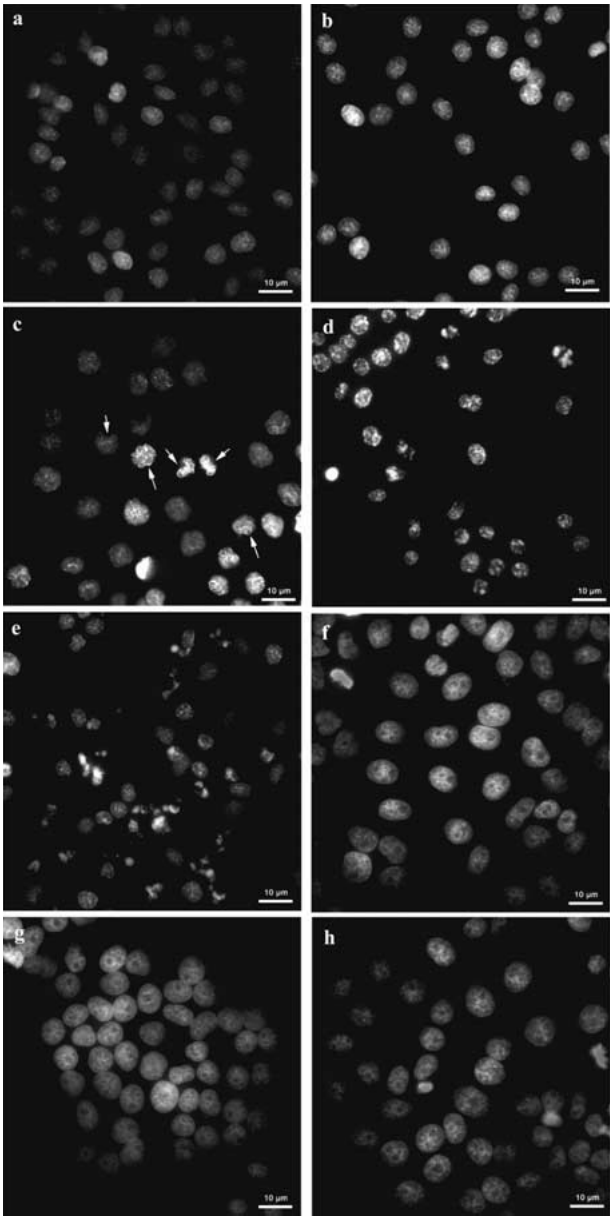

Fig. 2. Chromatin condensation of GFSHR-17 cells under various incubation conditions: (*a*) untreated cells, (*b*) 0.2% DMSO-treated cells, (*c*) cells treated with 20 µM ODQ for 4 h, (*d*) cells incubated with 2 mM heptanol for 20 min and (*e*) cells incubated with 100 µM 18β-GA for 20 min. Characteristic chromatin condensation is indicated by arrows (*c*). (*f*) Untreated HeLa cells, (*g*) HeLa cells incubated with 2 mM heptanol and (*h*) HeLa cells incubated with 100 µM 8β-GA for 30 min (for more detail *see* text).

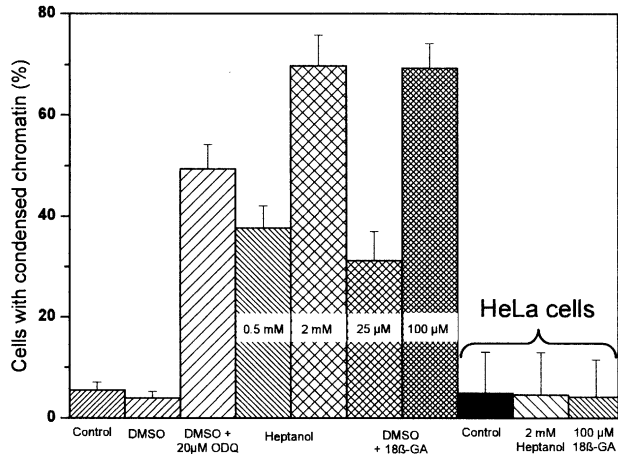

Fig. 3. Percentage of GFSHR-17 and HeLa cells exhibiting condensed chromatin for the corresponding treatment as used for Fig. 2. Treatment of GFSHR-17 cells with ODQ, heptanol as well as 8β-GA significantly increased the portion of cells with condensed chromatin ($P < 0.01$). The mean ± SD for $n = 10$ independent experiments for each treatment is given.

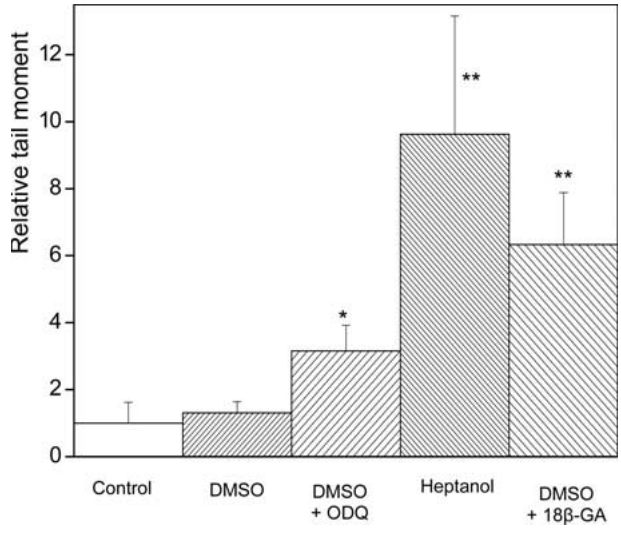

Fig. 4. Relative tail moment of cells exposed to different incubation solutions. Addition of 20 µM ODQ, 2 mM heptanol and 100 µM 18β-GA significantly (*: $P < 0.05$; **: $P < 0.01$) increased the tail moment of the cells compared to 0.2% DMSO or cell culture solution (*Control*). Mean ± SEM for $n = 5$ experiments for each treatment is given.

DMSO-exposed cells did not show a significant reduction of G_j compared to DMSO-untreated cells. This result confirms the previous finding that absence of cGMP inhibits gap junction communication in GFSHR-17 cells (Ngezahayo et al., 2003). In addition, it was found that ODQ induced apoptotic reactions such as chromatin condensation (Fig. 2*c*, Fig. 3) and DNA-strand breaks (Fig. 4).

Gap Junction Uncouplers Heptanol and 18β-GA Stimulate Chromatin Condensation, DNA Degradation but no Sustained $[Ca^{2+}]_i$ Increase

To analyze whether gap junction uncoupling is involved in stimulation of apoptotic reactions, heptanol and 18β-GA, two chemically non-related and well-known gap junction uncouplers, were applied. In contrast to DMSO-treated or untreated cells (Fig. 2*a*, *b*; Fig. 3 and Fig. 4), incubation with 2 mM heptanol

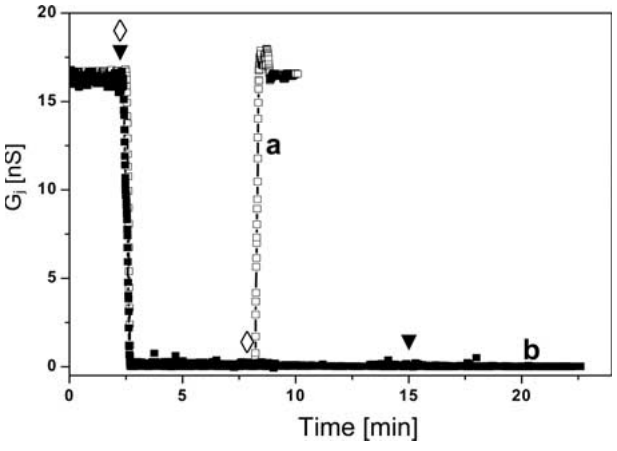

Fig. 5. Effect of heptanol on the time course of G_j. A rapid decrease of G_j was induced by superfusion of the cells with 2 mM heptanol (representatives for 5 experiments for each treatment, respectively). If heptanol was removed within 10 min, a recovery of the G_j was observed (curve *a*). However, incubation of GFSHR-17 cells with heptanol for more than 10 min irreversibly uncouples the gap junctions (curve *b*). The symbols ◇ and ▼ represent the time points of application and washout of heptanol for curve *a* and curve *b*, respectively. A comparable result was obtained for 100 μM 18β-GA.

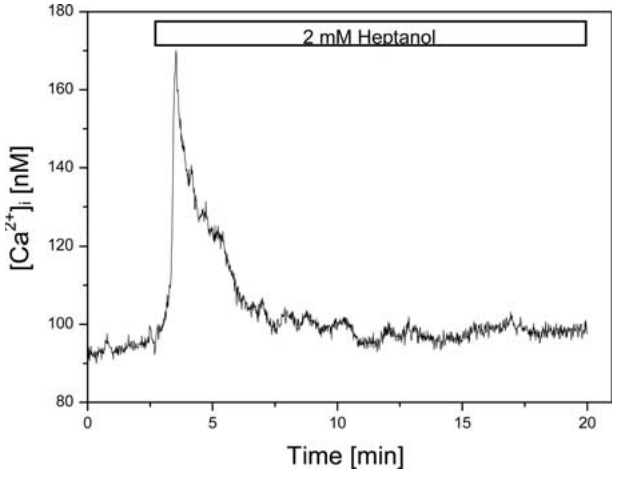

Fig. 6. Effect of heptanol on the time course of $[Ca^{2+}]_i$. Addition of 2 mM heptanol to the bath induced a $[Ca^{2+}]_i$ spike. Comparable results were obtained after application of 100 μM 18β-GA.

or 100 μM 18β-GA induced chromatin condensation (Fig. 2*d*, *e*, Fig 3) and DNA-strand breaks (Fig. 4). It is interesting to note that in comparable experiments, neither heptanol nor 18β-GA affected the chromatin structure of HeLa cells (Fig. 2 *f*, *g*, *h*; Fig. 3). Furthermore, it was observed that heptanol as well as 18β-GA induce chromatin condensation in GFSHR-17 cells in a time-dependent manner. Incubation with heptanol or 18β-GA for less than 10 min did not significantly alter the chromatin structure. In line with this finding, gap junction uncoupling by 2 mM heptanol or 100 μM 18β-GA was reversible if the incubation time did not exceed about 10 min (Fig. 5). Furthermore, the rate of gap junction uncoupling appeared to be concentration-dependent. At 2 mM heptanol or 100 μM 18β-GA, the amplitude of gap junction coupling decreased by about 50% within 10–15 s. This lag time increased to more than 30 s by lowering the concentration of heptanol or 18β-GA to 0.5 mM or 25 μM, respectively. Since lower concentration of the used uncouplers suppressed gap junction coupling more slowly, it can be expected that reduction of the concentration of the uncouplers delays the onset of apoptotic reactions. Regarding the stimulation of apoptotic reactions it was found that reduction of the concentration of heptanol or 18β-GA to 0.5 mM or 25 μM, respectively, significantly reduced the percentage of the cells with condensed chromatin compared to 2 mM heptanol or 100 μM 18β-GA (Fig. 3). Additionally, we analyzed the effect of gap junction uncouplers on G_j in the presence of 8-Br-cGMP. We observed that simultaneous presence of 200 μM 8-Br-cGMP in pipette filling solution and in the bath did not inhibit the decrease of G_j by heptanol or 18β-GA. In parallel, the stimulation of chromatin condensation by heptanol or 18β-GA was also not suppressed by 8-Br-cGMP (*results not shown*). Finally, we examined the effects of gap junction uncouplers on $[Ca^{2+}]_i$. As shown in Fig. 6, 2 mM heptanol induced a rapid $[Ca^{2+}]_i$-increase from about 100 nM to about 200 nM. The $[Ca^{2+}]_i$-increase was maximal within 10–30 s and was followed by a decline to the original $[Ca^{2+}]_i$ value within further 3–5 min. In all performed experiments using either 2 mM heptanol or 100 μM 18β-GA (5 experiments respectively) a similar $[Ca^{2+}]_i$-spike was detected, but a sustained $[Ca^{2+}]_i$-increase was not observed.

Discussion

Previously, we presented evidence that absence of intracellular cGMP significantly reduced gap junction coupling and caused apoptosis-related cellular changes, such as cell-volume shrinkage and formation of blebs in GFSHR-17 cells (Ngezahayo et al., 2003). In this report we show that ODQ-dependent inhibition of guanylyl cyclase, which causes cGMP depletion, induced gap junction uncoupling (Fig. 1) and stimulated typical apoptotic reactions like chromatin condensation (Fig. 2*c*, Fig. 3) and DNA degradation (Fig. 4) in GFSHR-17 granulosa cells. Thus, this is an evidence that in addition to modulation of gap junction coupling, cGMP-depletion is involved in regulation of apoptosis. It is known that cGMP controls cellular reactions by activation of protein kinase G (PKG). However, there are controversies about the sensitivity of gap junction channels composed by different connexins towards PKG-dependent

phosphorylation: PKG activation does not affect the conductance of gap junctions formed by Cx45 (van Veen, van Rijen & Jongsma, 2000) and PKG-dependent phosphorylation reduces the conductance of gap junction formed by rat Cx43 (Kwak et al., 1995). According to these observations, cGMP depletion should not affect G_j of GFSHR-17 granulosa cells, which are cells of rat and express mainly Cx43 and Cx45 (Okuma et al., 1996).

It has been observed in granulosa cells and other cell systems that cGMP is a blocker of apoptosis (Perez et al., 2000; Barsacchi et al., 2002; Fiscus, 2002; Peluso, 2003). The underlying mechanisms involve activation of PKG, blockage of sphingomyelinase and K^+ efflux. In monocytic cells, cGMP-dependent activation of PKG blocked apoptosis by inhibition of acid sphingomyelinase (Barsacchi et al., 2002). In granulosa cells, it was demonstrated that progesterone-dependent promotion of cell survival could be mimicked by cGMP and attenuated by PKG antagonists (Schreiber et al., 1993). Furthermore, activation of K^+-efflux was shown to be critical in induction of apoptosis in granulosa cells (Perez et al., 2000; Ngezahayo et al., 2003). Accordingly, it can be speculated that cGMP depletion reduces PKG activity, stimulates sphingomyelinase as well as K^+ efflux and thereby promotes apoptotic reactions in granulosa cells.

The finding that ODQ reduced gap junction coupling and stimulated apoptotic reactions raises the question whether gap junction uncoupling is involved in stimulation of apoptotic reactions. To elucidate this question, two chemically non-related gap junction uncouplers, heptanol and 18β-GA, were used and a possible activation of apoptotic processes was analyzed at the level of chromatin and DNA. Increased chromatin condensation (Fig. 2*d*, *e*) as well as DNA strand breaks (Fig. 4) were induced by an incubation of the cells with heptanol or 18β-GA for 20–30 min. It is known that heptanol as well as 18β-GA rapidly and reversibly inhibit gap junction coupling (Harris, 2001). In GFSHR-17 granulosa cells however, we observed that the gap junction coupling could only be restored if the incubation with either heptanol or 18β-GA-containing solution did not exceed 10 min (Fig. 5). A prolonged incubation with gap junction uncouplers induced an irreversible gap junction uncoupling (Fig. 5). In parallel, incubation with heptanol or 18β-GA-containing solution for less than 10 min did not significantly alter chromatin structure. It is therefore proposed that sustained gap junction uncoupling is a prerequisite for significant changes in chromatin structure. This hypothesis is also in line with the following observation: after addition of 2 mM heptanol or 100 μM 18β-GA, G_j was reduced by about 50% within 10 to 15 s. At 0.5 mM heptanol or 25 μM 18β-GA, the corresponding lag time increased to more than 30 s, which indicates that reduction of the concentration of uncouplers lowers the rate of gap junction uncoupling. This effect might in turn delay the onset of apoptotic reactions. As presented in Fig. 3, at 0.5 mM heptanol or 25 μM 18β-GA, the percentage of the cells with condensed chromatin is reduced compared to that found for 2 mM heptanol or 100 μM 18β-GA. It can therefore be suggested that apoptotic reactions in granulosa cells are related to a significant gap junction uncoupling. It can be argued that gap junction uncouplers stimulated the apoptotic reactions by unspecific effects like alteration of the membrane fluidity (Enders et al., 2004). This assumption is, however, challenged by the result obtained on HeLa cells (Fig. 2*f*, *g*, *h*; Fig. 3). In HeLa cells, which are poorly coupled by gap junctions (Elfgang et al., 1995; Breidert et al., 2005), it is observed that the gap junction uncouplers do not promote apoptotic reactions. We therefore propose that a long-lasting blockade of gap junction coupling in granulosa cells is involved in stimulation of apoptotic reactions. In line with this assumption, it was found that brain infarct induced an increased apoptosis in heterozygote Cx43 null mice (Cx43 +/−) compared to wild-type animals (Nakase et al., 2003). Furthermore, it was shown that reduction of gap junction coupling in cell lines of glial origin is related to induction of apoptosis (Robe et al., 2000; 2005).

With respect to the experiments with ODQ and gap junction uncouplers, it can be speculated that cGMP is involved in the effects of heptanol and 18β-GA on gap junction coupling and apoptotic reactions. To test this hypothesis, gap junction uncouplers and 8-Br-cGMP were simultaneously applied. We found that 8-Br-cGMP did not suppress the heptanol or 18β-GA-induced chromatin condensation. Also addition of 0.2 mM 8-Br-cGMP to bath and pipette-filling solution did not block the heptanol- or 18β-GA-induced G_j-reduction (*data not shown*). We therefore postulate that cGMP is not involved in the effects of gap junction uncouplers on the magnitude of gap junction conductance, structure of chromatin, or on DNA.

In the following it will be discussed whether gap junction uncouplers affect gap junction coupling and stimulate apoptotic reactions by increase of $[Ca^{2+}]_i$. Heptanol as well as 18β-GA induced a Ca^{2+} spike of less than 200 nM in GFSHR-17 granulosa cells (Fig. 6). In different cell systems, rise in $[Ca^{2+}]_i$ induces gap junction uncoupling. This is particularly true if $[Ca^{2+}]_i$ adopts constant values in the 10 μM range (Harris, 2001). In our experiments, heptanol as well as 18β-GA caused a transient $[Ca^{2+}]_i$ spike, which did not exceed 200 nM. Previously, we have shown that the occurrence of a Ca^{2+} spike comparable to the Ca^{2+} signal presented in Fig. 6 does not stimulate gap junction uncoupling or apoptotic reactions (Ngezahayo et al., 2003). It is therefore proposed that both heptanol and 18β-GA inhibit gap

junction coupling and stimulate apoptotic reactions in a Ca^{2+}-independent manner. This conclusion is in agreement with the assumption that heptanol or glycerrhetinic acid uncouple gap junction by mechanisms independent of intracellular signals (Harris et al., 2001).

Taken together our results give evidence that long-lasting gap junction uncoupling is involved in promotion of apoptotic processes in GFSHR-17 granulosa cells. It is well known that gap junction coupling allows exchange of metabolites between adjacent cells. It can therefore be postulated that gap junction uncoupling in GFSHR-17 granulosa cells inhibits exchange of metabolites. NAD^+ is a known metabolite that can permeate through connexons (Bruzzone et al., 2001). Furthermore, depletion of intracellular NAD^+ stimulates apoptotic reactions in different cell systems by disrupting the mitochondria (Chiarugi & Moskowitz, 2002; Lipton & Bossy-Wetzel, 2002). It can be speculated that gap junction uncoupling in GFSHR-17 granulosa cells hinders NAD^+ exchange between neighboring cells, which causes an NAD^+ depletion in some cells and thereby stimulates caspase-dependent as well as caspase-independent pathways, leading to apoptosis. Further experiments are necessary to identify the molecular mechanisms involved in stimulation of apoptosis in response to gap junction uncoupling in granulosa cells.

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