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J Clin Invest. 1995;**95**(4):1429-1439. <https://doi.org/10.1172/JCI117813>.

Research Article

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Gap Junction-mediated Cell-to-Cell Communication in Bovine and Human Adrenal Cells. A Process whereby Cells Increase Their Responsiveness to Physiological Corticotropin Concentrations

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Abstract

We have studied the role of gap junction-mediated intercellular communication on the steroidogenic response of bovine (BAC) and human (HAC) adrenal fasciculo-reticularis cells in culture to corticotropin (ACTH). Indirect immunofluorescence analyses showed that intact human and bovine adrenocortical tissue as well as HAC and BAC in culture expressed the gap junction protein connexin43 (also termed $\alpha 1$ connexin). Both HAC and BAC were functionally coupled through gap junctions as demonstrated by microinjection of a low molecular mass fluorescent probe, Lucifer yellow. The cell-to-cell transfer of the probe was blocked by 18 α -glycyrrhetic acid (GA), an inhibitor of gap junction-mediated intercellular communication. GA markedly decreased the steroidogenic response (cortisol production) of both HAC and BAC to low (10 pM) but not to high (5 nM) concentrations of ACTH. GA had no inhibitory effect on the steroidogenic response to 8 Br-cAMP (at either low or high concentrations) and did neither modify the binding of [¹²⁵I]-ACTH to its receptor nor the ACTH-induced cAMP production. BAC cultured at high or low cell densities (2.4×10^5 vs. 0.24×10^5 cells/cm²) exhibited distinct levels of intercellular communication and were differently responsive to sub-maximal ACTH concentrations. The ACTH ED₅₀ values for cortisol production were 8.5 ± 1.3 and 45 ± 14 pM ($P < 0.02$) for BAC cultured at high and low density, respectively. In the presence of GA, there was a shift of the ACTH concentration-response curves in the two culture conditions. The ACTH ED₅₀ of high density and low density cultured BAC increased 25- and 5-fold, respectively, and became similar (220 ± 90 and 250 ± 120 pM). These results demonstrate that gap junction-mediated communication between hormone-responsive and nonresponsive cells is one mechanism by which adrenal cells increase their responsiveness to low ACTH concentrations. (*J. Clin. Invest.* 1995; 95:1429–1439.) Key words: gap junctions • adrenal cells • cell-to-cell communication • corticotropin • steroidogenesis

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Received for publication 30 March 1994 and in revised form 7 November 1994.

J. Clin. Invest.

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0021-9738/95/04/1429/11 \$2.00

Volume 95, April 1995, 1429–1439

Introduction

The biological response of an organ or a tissue to a stimulus is, at any time, the result of the activity of numerous cells that adjust their functioning to provide an integrated response adapted to the needs of the organism. Cells are therefore expected to adapt their individual responsiveness to physiological modifications of their environment in a coordinated way. Coordination is primarily achieved by extracellular signals which act on individual cells expressing the same specific receptors. Another mechanism by which cells could coordinate and sometimes synchronize their responses is the direct cell-to-cell communication through specialized intercellular junctions named gap junctions (GJ).¹ In most organs and tissues, cells (of the same type or more exceptionally of distinct types) can exchange molecules of low molecular mass ($< 1,000$ D), naturally dissolved in their cytoplasm, through GJ (1, 2). By allowing the cell-to-cell transfer of informative molecules, GJ are involved in the control of cell growth, differentiation and metabolic cooperation (for reviews see references 3–8). GJ channels between adjacent cells can be open or closed; the transition from one state to the other is a regulated process (7, 8). GJ are known to be permeable to inositol 1, 4, 5 triphosphate and calcium ions (9–14) and to cyclic AMP (15–18). It has been elegantly demonstrated that cells that do not respond to a given external signal could be committed to respond by receiving the adequate second messenger from neighboring responsive cells with which they are coupled through GJ (16–18). These data show how gap junctional communication can participate in the coordination of cell responses. There is now need to define physiological situations or functional circumstances in which GJ-mediated cell-to-cell transfer of second messengers actually participates in the determination of the overall response of a cell population to an external signal.

We have used two well-characterized cell culture systems, adrenocortical cells of bovine or human origin in primary culture that produce cortisol in response to corticotropin (ACTH) via the cAMP pathway (19, 20) and a method of analysis of GJ-mediated cell-to-cell communication by microinjection of a fluorescent probe (Lucifer yellow) to examine the functional impact of gap junctional communication. First, we examined whether compounds known to cause GJ closure such as 18 α -glycyrrhetic acid (GA) (21, 22) or octanol (23, 24), could

1. *Abbreviations used in this paper:* BAC, bovine adrenal fasciculata-reticularis cells; connexin43: $\alpha 1$ connexin, 43-kD cardiac gap junction protein; connexin32: $\beta 1$ connexin, 32-kD liver gap junction protein; F₁₂/DME, Ham's F₁₂/Dulbecco Modified Eagle's Medium; GA, 18 α -glycyrrhetic acid; GJ, gap junction; HAC, human adrenal fasciculata-reticularis cells

alter the secretory response of adrenal cell populations to ACTH. We then analyzed the extent that the hormone concentration-response relationship could depend on the level of GJ coupling within a cell population. Variations of GJ coupling were obtained by culturing cells at different densities and by applying a GJ inhibitor. Our results suggest that cell-to-cell communication determines the responsiveness of adrenocortical cells to low ACTH concentrations. We have found that the concentration of ACTH required to obtain the half-maximum cortisol production could vary up to 25-fold depending on the level of cell-to-cell coupling within the cell population. These results provide at least one explanation for the known discrepancy between the K_d value for the interaction of ACTH with its receptor and the ACTH concentrations active on steroidogenesis both in physiological conditions (25, 26) and in Cushing's disease (27).

Methods

Cell culture. Bovine adrenal cells (BAC) were isolated and cultured as previously described (28). Briefly, bovine adrenal glands were collected from a local slaughterhouse, dissected, freed of fat, and sliced with a Stadie-Riggs microtome delivering slices of 0.5 mm. The first slice containing zona glomerulosa was discarded, whereas the second and third slices were used for preparation of fasciculata cells. Dispersed cells were obtained by four successive 20-min treatments with trypsin (0.19%) in Ham's F-12/Dulbecco's modified Eagle's medium (F12/DME) (1:1) containing gentamycin (20 μ g/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml) nystatin (100 U/ml), 14 mM NaHCO_3 , and 15 mM Hepes, pH 7.4. The cells were harvested, washed and placed in a growth medium composed of F12/DME containing insulin (10 μ g/ml), transferrin (10 μ g/ml), vitamin C (0.1 mM) and fetal calf serum (1%). The next day, the medium was removed and replaced by the same medium without serum. Adult human adrenals were obtained from patients in cerebral death at the same time that the kidneys were removed for transplantation. Although it is likely that patients with cerebral death have marked changes in endocrine function, the basal cortisol secretion of both HAC and BAC was low after 2 to 3 d in culture, suggesting a "normalization" of adrenal cell activities. This protocol was approved by the ethical committee of the Hospices Civils de Lyon (France). After removal of the major part of the medulla, the zona fasciculata-reticularis was separated from the capsula and used for cell preparation. In preliminary experiments, attempts were made to isolate human adrenal cells (HAC) with the trypsin digestion method used for BAC dispersion. The yield was very low ($2-4 \times 10^6$ cells/adrenal), and more than 50% of cells were not viable. HAC were dispersed by two successive 20 min-treatments with collagenase (1 mg/ml) and DNase (0.1 mg/ml) in F12/DME medium, supplemented with NaHCO_3 (14 mM) and Hepes (10 mM) and antibiotics (the same mixture as that used for BAC dispersion). The yield was greatly improved ($30-35 \times 10^6$ cells/adrenal), and only 10% of cells were not viable. Isolated cells were then seeded in Petri dishes in F12/DME containing 10% fetal serum plus insulin (10 μ g/ml), transferrin (10 μ g/ml) and vitamin C (0.1 mM). After 24 h, the medium was changed and replaced by the same medium without serum. For both BAC and HAC, plating efficiency varied between 70 and 80% and, in our culture conditions, cell multiplication was negligible. The precise number of cells used in each experimental protocol was determined at the end of the experiments using a Coulter counter.

Measurements of cell activities. For both cell culture types, the medium was changed every two days. The inhibitors of GJ were added 10 min before the effectors. cAMP was measured in the medium after 1 h of incubation, at which time > 80% of the total cAMP was extracellular. cAMP accumulation in the medium is related to the concentration of ACTH used (29-31). Medium cortisol was measured after a 2-h incubation period because a linear increase has been observed during this period after ACTH stimulation (30, 32). Cortisol and cAMP were

measured by specific radioimmunoassays as previously described (28). The binding of ^{125}I -labeled human (Tyr 23) ACTH (1-39) (Amersham, Les Ulis, France; specific activity: 74 TBq/nmol) to BAC was performed as described (31).

Assessment of direct cell-to-cell communication. Gap junction-mediated intercellular communication was assayed by direct cell-to-cell transfer of a low Mr fluorescent dye: Lucifer yellow, a probe originally used by Stewart (33). Lucifer yellow (Sigma Chemical Co., St. Louis), 5% (wt/vol) in 0.3 M LiCl, was microinjected into the cytoplasm, under nitrogen pressure applied for 0.1-0.3 s using a micromanipulator 5170 and a microinjector 5242 from Eppendorf (Hamburg, Germany), installed on an Axiovert 35 M inverted microscope from Zeiss (Oberkochen, Germany). Microinjections were performed using glass micropipettes (Femtotips from Eppendorf) in cultured cells maintained at 37°C using a control heating unit (TRZ 3700 from Zeiss). Lucifer yellow distribution was visualized using the filter combination BP 395-440/LP 470 from Zeiss. Fluorescence images of probe distribution were taken within 5 min after microinjection using either 400 ASA T-MAX Kodak films (Eastman Kodak Co., Rochester, NY) or a SIT camera (Lhesa electronique, Cergy-Pontoise, France) coupled to an image processing system (Sapphire from Quantel, Montigny-le Bretonneux, France) and stored on a Bernoulli box.

Immunofluorescence labeling. Cultured cells were washed with the culture medium without serum and fixed with 4% paraformaldehyde in PBS for 30 min in Petri dishes. The affinity-purified anti-connexin antibodies used at the concentration of 3 μ g/ml in PBS containing 1mg/ml BSA were applied onto fixed cells overnight at 4°C. Hen anti-connexin32 antibodies and rabbit anti-connexin43 antibodies were produced against synthetic peptides corresponding to residues 266-282 of rat connexin32 and to residues 314-322 of rat connexin43 (34, 35), respectively. Immunodetection of hen IgGs was achieved using affinity-purified goat anti-hen IgG (H+L) coupled to fluorescein from KPL Laboratories (Gaithersburg, MD). Rabbit IgGs were detected using F(ab) $_2$ fragments of goat anti-rabbit affinity-purified antibody coupled to fluorescein from Sigma Chemical Co. Fluorescein-conjugated secondary antibodies were applied for 30 min at room temperature. Cells were mounted in Fluoprep mounting preparation (Biomérieux, France) and examined on an Axiophot microscope from Zeiss. Fluorescence images were recorded using a SIT camera coupled to the Sapphire image processing system, and photomicrographs were made using a video printer UP 5000 (Sony, Japan).

Results

Inhibition of GJ-mediated intercellular communication in cultured bovine adrenocortical cells (BAC) by 18 α -glycyrrhetic acid (GA). BAC cultured at a high concentration of 2.4×10^5 cells/cm 2 formed subconfluent monolayers. Cells had a typical epithelial morphology (Fig. 1, a and d). Monolayer cells were tested for their ability to communicate via GJ. Lucifer yellow microinjected into the cytoplasm of a cell was detected within seconds in those cells surrounding the injected one (Fig. 1, b and c). By microinjecting a large number of BAC, we observed that the level of intercellular communication was rather heterogeneous within the cell population. The number of dye-coupled cells varied from 2 to about 20, and the average number of communicating cells per injection was 8.5 ± 1 (mean \pm SEM). Furthermore, ~ 20% of cells which were microinjected did not communicate with any neighboring cells. The effects of 18 α -glycyrrhetic acid (GA) on GJ-mediated intercellular communication in BAC are illustrated in Fig. 1, d-f and quantified in Fig. 2. Treatment of BAC with 10 μ M GA caused a time-dependent inhibition of cell-to-cell transfer of Lucifer yellow. A marked reduction in cell coupling was already evident after 5 min (Fig. 2). A complete inhibition of cell-to-cell communi-

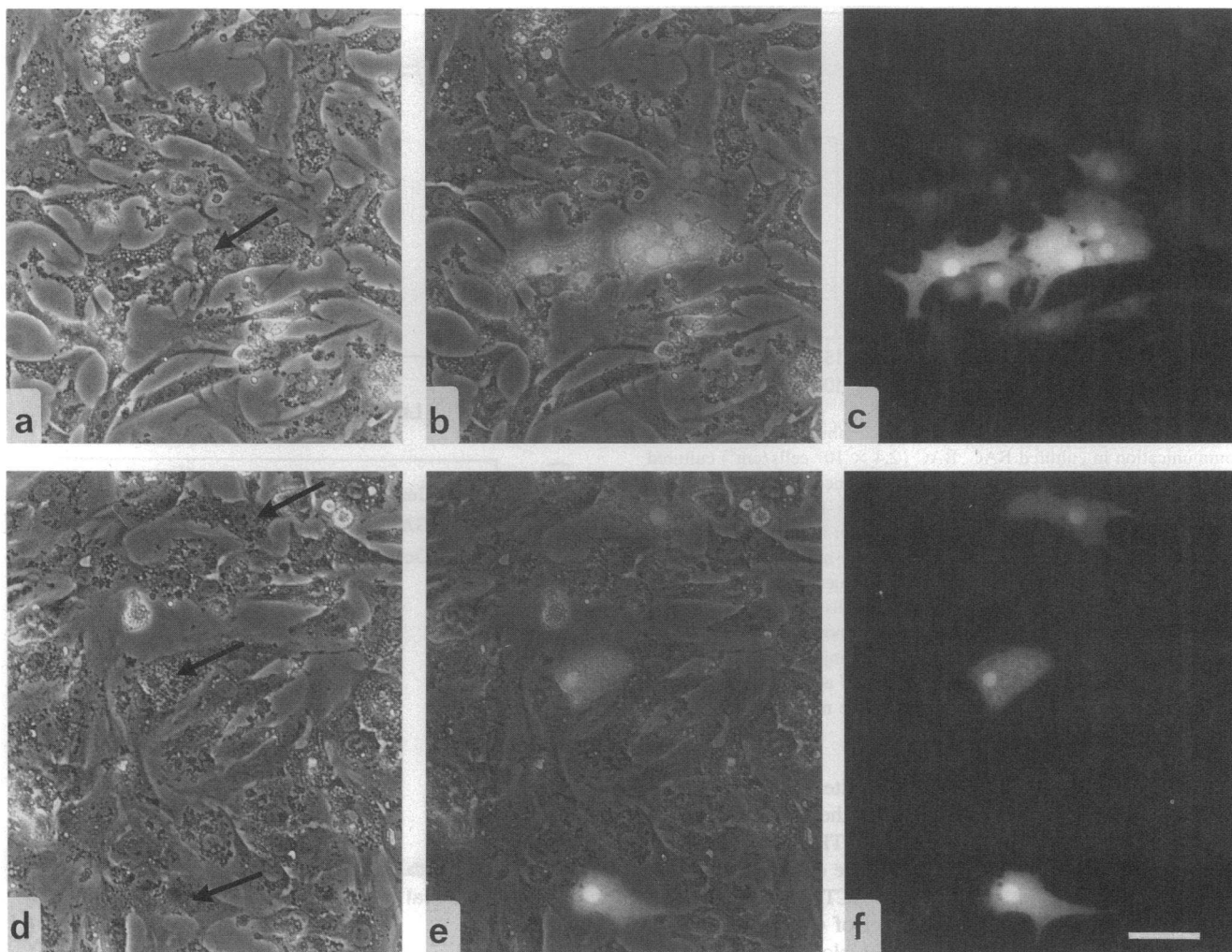


Figure 1. Intercellular communication between BAC in primary culture. Inhibition by GA of GJ-mediated intercellular communication visualized by the cell-to-cell diffusion of Lucifer yellow. BAC (2.4×10^5 cells/cm²) were cultured for 5 d and incubated without (*a-c*) or with (*d-f*) 10 μ M GA for 30 min at 37°C. Lucifer yellow was then microinjected into the cytoplasm of one, two, or three distant cells (identified by arrows on the phase contrast micrographs). The distribution of the fluorescent dye was examined 5 min after microinjection. The panels from the left to the right correspond to the phase contrast (*a* and *d*), phase contrast plus fluorescence (*b* and *e*) and fluorescence (*c* and *f*) images of the same field. Lucifer yellow introduced in one cell of the untreated BAC monolayer (*a*) diffused in numerous adjacent cells (*b* and *c*). In contrast, none of the microinjected cells (*d*) of the GA-treated BAC monolayer transferred the dye to neighboring cells (*e* and *f*). Bar, 50 μ m.

cation was obtained after 30 min of incubation; Lucifer yellow remained located in the microinjected cells in 100% of the cases (Fig. 1*f*). When GA was used at lower concentrations (between 1 and 10 μ M), longer incubation times were required to abolish GJ-mediated intercellular communication. A complete inhibition was obtained after a 1 h-incubation of BAC with 5 μ M GA.

The GA-induced inhibition of GJ-mediated cell-to-cell communication influences the steroidogenic response of BAC to ACTH. At concentrations ≤ 10 μ M, GA did not affect the basal cortisol production by BAC. GA exerted a concentration-dependent inhibitory action on the steroidogenic effects of submaximal concentrations of ACTH, whereas those of 8 Br-cAMP were only slightly reduced at 10 μ M (Fig. 3*A*). For all subsequent experiments, a GA concentration of 5 μ M was selected since GJ-mediated cell-to-cell communication was completely inhibited (Fig. 2) and the ACTH-response was reduced $\sim 70\%$, but the response to 8Br-cAMP was not affected (Fig. 3*A*).

BAC in culture exhibited a low basal cortisol production which was increased up to 70-fold in response to maximal concentrations of ACTH or 8 Br-cAMP. Results of Fig. 3*B* shows that GA differently affected the secretory response to low (10 pM) and high (5 nM) ACTH concentrations. GA decreased the cortisol production in response to a submaximal concentration of ACTH (10 pM) but was without effect on the response to a maximal or saturating concentration of ACTH (5 nM). Interestingly, GA treatment did not affect the response of 0.1 mM 8Br-cAMP in eliciting a submaximal cortisol production or the response of 1 mM 8 Br-cAMP in yielding the maximal cortisol production. Similar results were obtained with another GJ blocker, octanol.

We examined whether GA inhibition of the steroidogenic response to submaximal concentrations of ACTH could be due to drug effects other than that on GJ closure. For instance, we tested whether GA affected the binding of ACTH to its membrane receptors or the ACTH-induced activation of adenyllyl

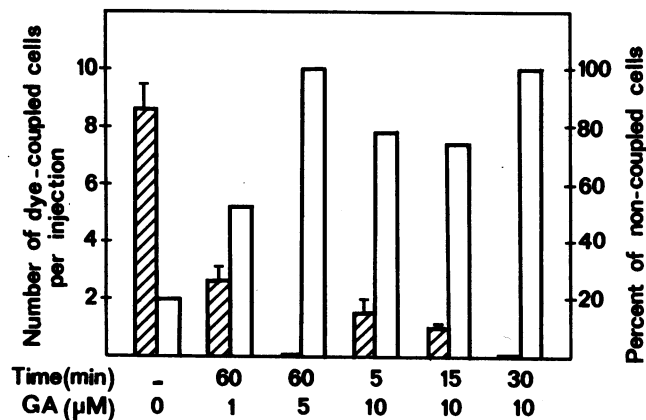


Figure 2. Quantitative analysis of GA-induced inhibition of intercellular communication in cultured BAC. BAC (2.4×10^5 cells/cm²) cultured for 5 d were incubated with GA at different concentrations for 5–60 min at 37°C. In each experimental condition, 10–60 randomly selected cells in the Petri dishes were microinjected with Lucifer yellow. The number of fluorescently labeled cells around the microinjected one was determined within 5–15 min after a series of injections; it varied from 0 (uncoupled cell) to up to 20. The histogram displays two parameters of cell-to-cell communication: the percentage of non-coupled cells (*open columns*) and the number of coupled cells per injection when Lucifer yellow was transferred from the injected cell to at least one other cell (*hatched columns*); results are expressed as the mean \pm SEM.

cyclase (Table I). GA (5 μ M) neither altered the binding of labeled ¹²⁵I-ACTH to BAC, nor modified the cAMP generation in response to either 10 pM or 5 nM ACTH. Under the same experimental conditions, GA decreased the cortisol production in response to low but not to maximal ACTH concentrations.

To further document that the effect of GA on the ACTH response was actually related to its GJ blocking activity, we compared the action of the drug on monolayer cells in Petri dishes having established GJ as shown in Fig. 1 and on corresponding cells in suspension that were collected from Petri dishes and dispersed by a mild trypsin treatment to disrupt cell interactions via GJ. As shown in Fig. 4, the steroidogenic response to 10 pM ACTH was decreased by GA when cells were kept as monolayers but was not affected by GA when cells were dispersed and maintained in suspension. The response to a maximal ACTH concentration (10 nM) was unaffected by the GA treatment in both cell systems. It is noteworthy that a concentration of ACTH of 10 pM which produced about half-maximum response on monolayer cells only gave a response equal to 30% of the maximum response on dispersed cells. The dispersed cells did have a lower density of ACTH receptors compared with cultured cells (31) which may have been a consequence of trypsin treatment.

GA also inhibits GJ-mediated cell-to-cell communication and the steroidogenic response to ACTH on human adrenocortical cells (HAC). To further document the general relevance of our findings, the same experiments were performed using HAC in culture. HAC cultured at the same concentration as BAC (2.4×10^5 cells/cm²) did not spread on the Petri dishes but formed cell clusters of various size (Fig. 5, *a* and *d*). After microinjection of Lucifer yellow in one cell or possibly a few cells in a three-dimensional aggregate, there was rapid diffusion of the fluorescent dye to a large number of cells and often into almost all the cells of the cluster (Fig. 5, *B* and *C*). A 30 min

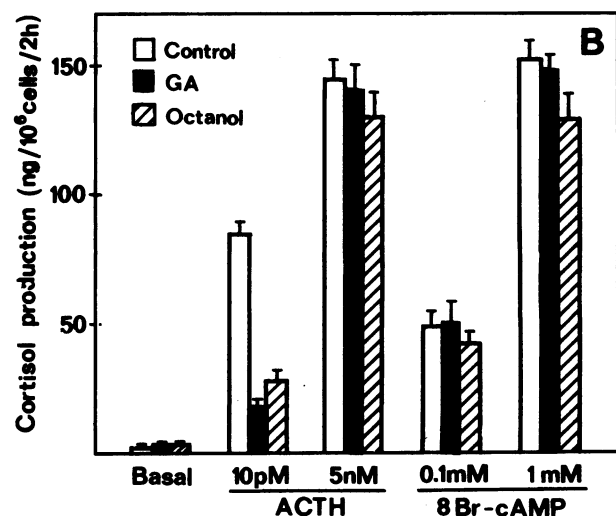
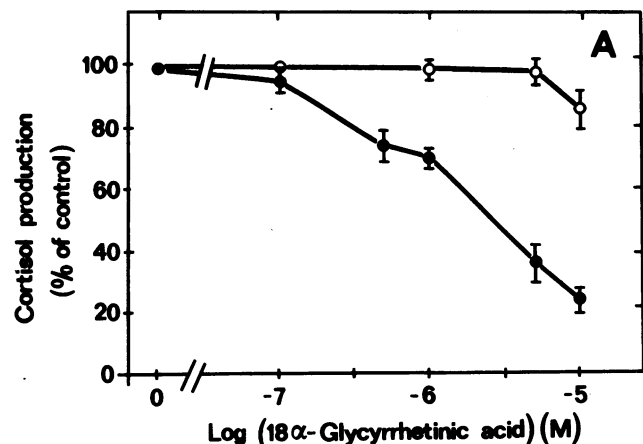


Figure 3. Incidence of the inhibition of GJ-mediated intercellular communication on the ACTH- or 8 Br-cAMP-stimulated cortisol production by BAC. (A) Effects of increasing concentrations of GA on ACTH and 8Br-cAMP-induced cortisol production. BAC (2.4×10^5 cells/cm²) cultured for 5 d were incubated without or with 10 pM ACTH (●) or 0.1 mM 8Br-cAMP (○) in the presence of various concentrations of GA from 0.1 to 10 μ M for 2 h at 37°C. Cortisol was assayed in the medium. GA at all concentrations tested, did not affect the basal cortisol production. Thus, results of the effects of GA on ACTH- or 8Br-cAMP-stimulated cortisol production have been expressed as percent of control. Each point and vertical bar represent the mean \pm SEM of triplicate. (B) Effects of GA and octanol on the response of BAC to ACTH or 8 Br-cAMP. BAC (2.4×10^5 cells/cm²) cultured for 5 d were incubated without or with ACTH (10 pM or 5 nM) or Br-cAMP (0.1 or 1 mM) in the absence or the presence of 5 μ M GA or 1mM octanol. Cortisol was assayed in the incubation medium. Results represent the mean \pm SEM of the values obtained in two separate experiments; each experimental condition was done in quadruplicate. GA and octanol had the same effects; the GJ inhibitors only decreased the response to the submaximal concentration of ACTH.

incubation of HAC with 5 μ M GA led to a marked inhibition of the cell-to-cell transfer of Lucifer yellow (Fig. 5, *E* and *F*). In almost all cases, few cells displayed fluorescence around the site of microinjection even after a longer period of exposure to GA. This may have been due to the injection of Lucifer yellow into several cells of the clusters and not into a single cell as was possible with the BAC monolayers. As compared with

Table 1. Effect of GA on ACTH Binding and ACTH-induced cAMP and Cortisol Productions

Effector	¹²⁵ I-ACTH bound (cpm/10 ⁶ cells)		cAMP pmoles/1 h per 10 ⁶ cells		Cortisol production ng/1 h per 10 ⁶ cells	
	Control	GA	Control	GA	Control	GA
None	2200±220	2100±150	0.6±0.1	0.8±0.2	0.9±0.2	1.4±0.4
ACTH (10 pM)	—	—	4±0	5±3	43±3	8±2
ACTH (5 nM)	—	—	23±3	21±4	78±5	73±4

BAC (2.7×10^5 cells/cm²) were cultured for 3 d. For the measurement of ACTH binding, cells were incubated with 80 pM ¹²⁵I-ACTH for 1 h at 37°C in the presence or the absence of 5 μM GA. Specific binding was measured as previously described (31). Cells were incubated with the indicated concentration of ACTH in the presence or absence of 5 μM GA, and the cAMP and cortisol productions were measured after 1 h of incubation at 37°C (28). Results are the mean±SEM of two experiments.

BAC, HAC produced about 10 times more cortisol in their basal state: 20–30 versus 2–3 ng/10⁶ cells/2 h for BAC. A 2-h treatment of HAC with 5 μM GA did not alter the basal cortisol release but reduced by 60–70% the cortisol production in response to 10 pM ACTH, a hormone concentration eliciting the half-maximum steroidogenic response (Fig. 6). The response of HAC to a maximal ACTH concentration (5 nM) as well as the responses to low or maximal 8 Br-cAMP concentrations were not affected by the GA treatment. Octanol, 1 mM, produced the same effects as GA.

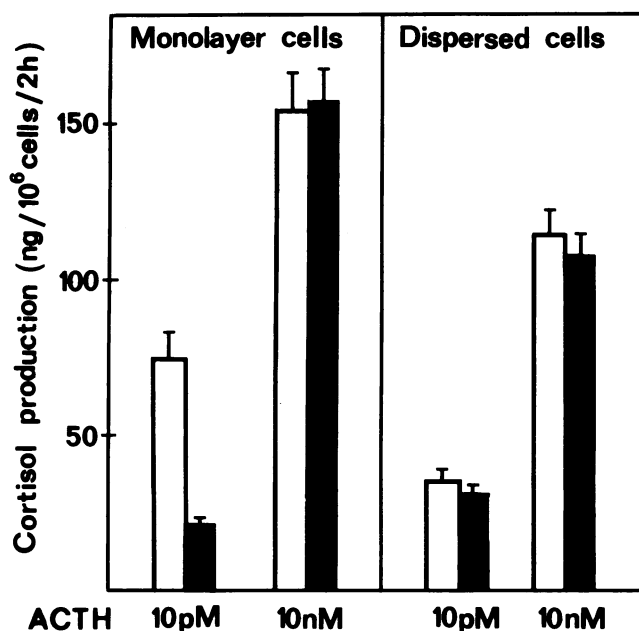


Figure 4. Evidence that the inhibitory effect of GA on the response of BAC to ACTH is related to the GA-induced inhibition of intercellular communication. BAC (3×10^5 cells/cm²) were cultured for 3 d. Some of the dishes were treated with 0.25% (wt/vol) trypsin, 1 mM EDTA for 10 min to detach the cells. For obtaining completely isolated cells, the cell suspension was submitted to repeated pipetting. Cells were then washed several times and suspended in the culture medium. Intact monolayers and corresponding isolated cells were incubated without or with ACTH (10 pM or 10 nM) in the absence (open columns) or in the presence (closed columns) of 5 μM GA for 2 h at 37°C. Cortisol was assayed in the medium. Columns and vertical bars represent the mean±SEM of triplicate.

Immunodetection of connexin43 in cultured BAC and HAC. GJ were visualized by indirect immunofluorescence in BAC and HAC using antibodies directed against two different GJ proteins, connexin43 and connexin32. Purified anti-connexin43 antibodies labeled rather large spots distributed along regions of cell-cell contacts in BAC monolayers (Fig. 7, a). HAC were also positive for connexin 43. The anti-connexin43 antibodies labeled a rather large number of small spots grouped in clusters (Fig. 7 c). Due to the superimposition of HAC, the labeled structures could not be clearly identified. Nevertheless, fluorescence labeling was presumably located between the cells but some labeling of connexin43 in the cytoplasm cannot be ruled out. Both BAC and HAC were not labeled by connexin32 antibodies. Similar results were obtained on tissue sections of bovine and human adrenal glands (data not shown).

The responsiveness of BAC to ACTH depends on the level of GJ-mediated intercellular communication. We performed full ACTH concentration-response studies on monolayer cell preparations that display distinct levels of GJ coupling. Experiments were designed to obtain three different levels of GJ coupling: 1. cells were cultured at high density (2.4×10^5 cells/cm²) to promote cell-cell interactions and favor GJ coupling, 2. cells were cultured at low density (0.24×10^5 cells/cm²) to make cell contacts scarce and limit GJ coupling and 3. cells were cultured at high or low density and acutely treated with GA to close all the existing GJ.

The morphological features and the intercellular communication capabilities of BAC cultured at high or low density are illustrated in Fig. 8. Seeded at high density, cells formed almost confluent monolayers (e and f). When seeded at low density, cells spread rather homogeneously over the entire surface of the Petri dish (a and b) and appeared as single cells or groups of few cells (generally 3 or 4). Lucifer yellow microinjected into the cytoplasm of one of these cells diffused to the other cells of the group (Fig. 8, c and d). Cells cultured at high density were highly coupled through GJ as shown in g and h. Cortisol production by BAC cultured under these two conditions was measured in response to increasing concentrations of ACTH, from 0.1 pM to 10 nM (Fig. 9). The concentration-response curve obtained with cells cultured at low density was shifted towards the right as compared to that obtained with confluent monolayers. The maximum cortisol production as well as the ACTH concentration required to reach it were similar for BAC cultured at high and low density. It is noteworthy that increasing concentrations of ACTH produced the same gradual

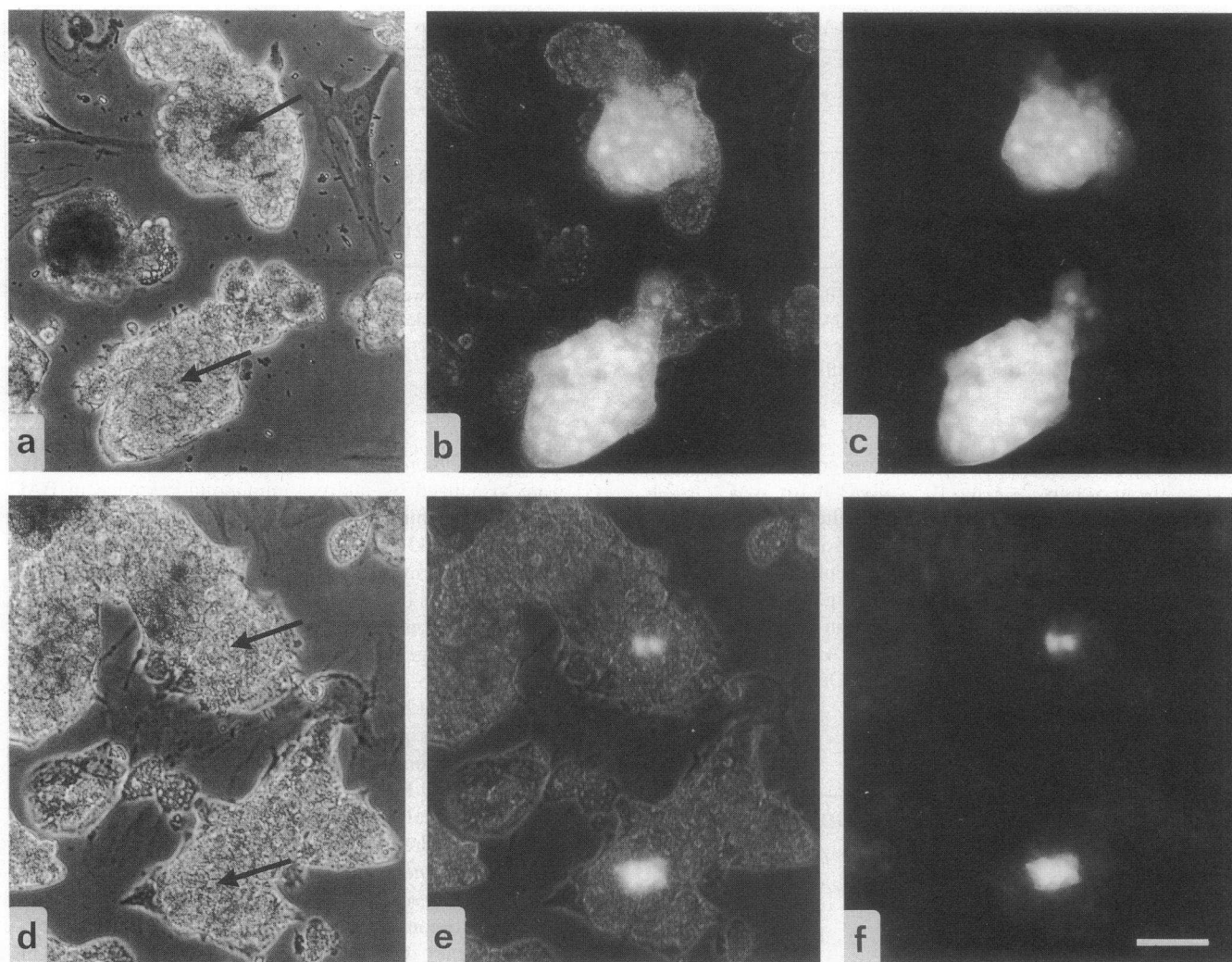


Figure 5. Intercellular communication between HAC in culture. Inhibition of the cell-to-cell diffusion of Lucifer yellow by GA. HAC (2.1×10^5 cells/cm²) were cultured for 5 d and incubated without (*a-c*) or with (*d-f*) $10 \mu\text{M}$ GA for 30 min at 37°C . Lucifer yellow was microinjected at the surface of cell clusters. Since the cells were round and closely packed, possibly two or three received the fluorescent dye. The sites of injection are indicated by arrows on the phase contrast micrographs. The distribution of the fluorescent probe was examined within 5 min after microinjection. The panels from the left to the right correspond to phase contrast (*a* and *d*), phase contrast plus fluorescence (*b* and *e*) and fluorescence (*c* and *f*) images of the same field. HAC were highly coupled through GJ (*a-c*), and GA strongly inhibited cell-to-cell communication; the few fluorescent cells found in GA-treated HAC probably correspond to cells which received Lucifer yellow by microinjection. Bar, $50 \mu\text{m}$.

cAMP increase in the two conditions of culture. Results of Fig. 9 also illustrate the well-established discrepancy (29, 36) between the ACTH concentrations required for stimulation of cortisol production and ACTH concentrations active on cAMP generation.

If the difference in the responsiveness of BAC cultured at high and low density to ACTH is directly linked to the difference in GJ coupling and therefore to a difference in the ability of cells to exchange informative molecules (here cAMP), one would expect a complete disappearance of this difference by blocking cell-to-cell communication by GA treatment. Results reported in Fig. 10 are in complete agreement with this prediction. The ACTH concentration-response curves obtained with BAC cultured at high or low density and exposed to a 2-h incubation period with $5 \mu\text{M}$ GA (Fig. 10 B) were superimposed and shifted towards the right as compared with the dose-response curves obtained with untreated cells (Fig. 10 A). As

observed in all the above-mentioned experiments, the maximum responses to ACTH were not altered by the GA treatment. The changes in the responsiveness of BAC to ACTH were quantified by comparing the ACTH ED_{50} . Average values obtained on three different cell preparations are reported on Table II. Preventing highly coupled BAC (that is, BAC cultured at high density) from communicating by GA treatment resulted in a 25-fold increase in the ACTH ED_{50} .

Discussion

In this study we have used adrenal cortical cells, which remain fully differentiated in primary culture, as a model system to demonstrate that the responsiveness of a cell population to an external signal, such as a polypeptide hormone, is dependent on the intercellular communication determined by the level of cell-to-cell coupling mediated by GJ. Adrenal cortical cells of

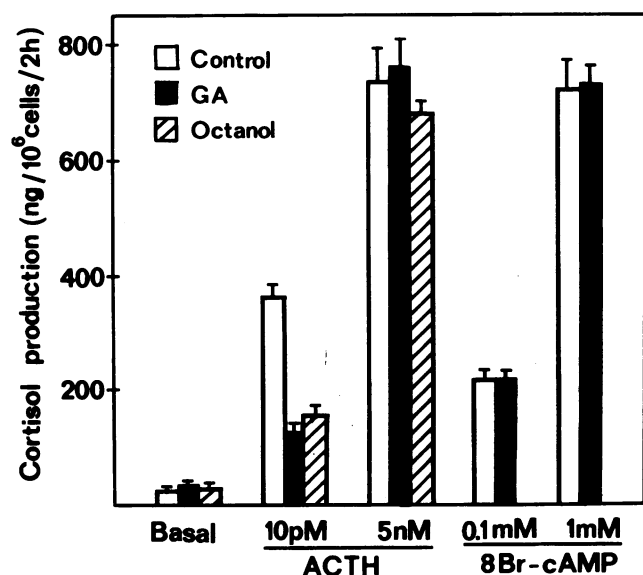


Figure 6. Incidence of the inhibition of GJ-mediated intercellular communication on ACTH-stimulated cortisol production by HAC. HAC (2.1×10^5 cells/cm²) cultured for 5 d were incubated without or with ACTH (10 pM or 5 nM) or 8Br-cAMP (0.1 or 1 mM) in the absence or the presence of 5 μ M GA or 1 mM octanol for 2 h at 37°C. Cortisol was assayed in the medium. Results are the mean \pm SEM of the values obtained in two separate experiments; each experimental condition was done in quadruplicate. Both GA and octanol decreased the response to the submaximal concentration of ACTH.

bovine or human origin in primary culture possess GJ, the functional state of which was measured by analysis of the cell-to-cell transfer of the fluorescent dye: Lucifer yellow. Most of the cells were capable of communicating with many of their neighbors. However, $\sim 20\%$ of cells, especially in cultured BAC, were uncoupled as measured by the microinjection assay. A similar observation has been made on another type of endocrine cells in primary culture: thyroid epithelial cells (37). The cells which do not exchange low molecular mass substances with their neighbors could either be devoid of GJ or could have their GJ channels in a closed conformation. The latter hypothesis is more likely since it has been possible to convert such a sub-population of uncoupled thyroid cells into communicating cells by a short-term hormone treatment (37). BAC and HAC in primary culture possess GJ composed of connexin43, alternatively referred to as α_1 connexin (38). This is in agreement with a recent report (39) showing that rat adrenal glands express connexin43 but no connexin32. Since many cell types express more than one connexin (40–43), one cannot exclude the presence of GJ formed by other connexins. The GJ inhibitor, 18 α -glycyrrhetic acid or GA, originally described by Davidson and his colleagues (21) was found remarkably potent on adrenal cortical cells. GA has been preferred to the more common GJ inhibitor, octanol, since GA completely inhibits GJ-mediated intercellular communication in the micromolar concentration range whereas millimolar concentrations of octanol are required. Furthermore, GA at a concentration of 5 μ M had no detectable effect on cell morphology and did not modify any of the metabolic parameters tested: hormone binding, hormone-activation of adenylyl cyclase and maximum cortisol production. Due to its lipophilic properties, GA may insert into the

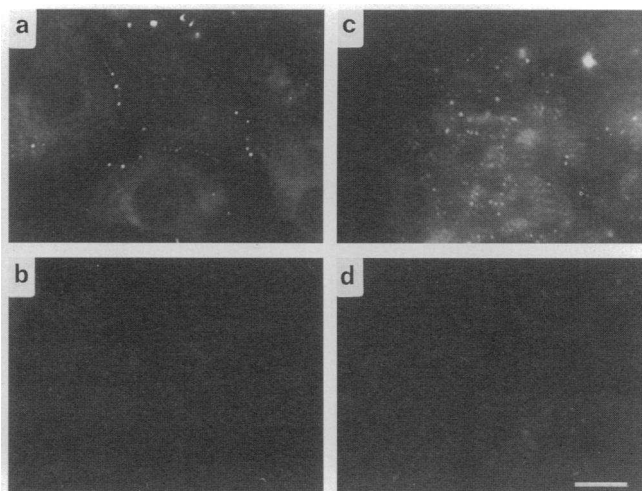


Figure 7. Immunodetection of connexin43 in BAC and HAC in primary culture. BAC and HAC (2×10^5 cells/cm²) cultured for 5 d were fixed and permeabilized as indicated in "Experimental procedures." Fixed BAC (a and b) and HAC (c and d) were incubated with either the affinity-purified anti-connexin43 antibodies (a and c) or the corresponding fraction of the preimmune serum (b and d). Immune complexes were detected using anti-rabbit IgG antibody conjugated to fluorescein. Bar, 50 μ m.

plasma membrane and interact with GJ protein oligomers, the connexons, and thereby induce a conformational change causing closure of GJ channels (22).

When the concentration of ACTH is sub-maximal, GA-induced GJ closure produces a concentration-dependent inhibition of ACTH-stimulated cortisol production. This finding indicates that the action of ACTH at low concentration necessitates a GJ-mediated cell-to-cell transfer of cytoplasmic molecule(s). ACTH is known to regulate adrenocortical cell activities such as cortisol production by the activation of adenylyl cyclase and the generation of cAMP as the intracellular messenger (19, 20). Hence, it is reasonable to postulate that the signaling molecule in question is cAMP. The cell-to-cell transfer of cAMP appears to be operative in other cell types (16–18) and could represent the limiting step in the action of low ACTH concentrations on adrenal cells. At low hormone concentrations, only a fraction of the cell population would respond by an increase of intracellular cAMP. If GJ are closed, only these cells would be engaged in steroid production. If cell-to-cell communication is possible, cAMP would be transferred to neighboring cells and therefore more cells would be activated and participate in hormone production. The number of recruited cells is expected to depend not only on the number of cells which communicate with the responder cells, but also on the amounts of cAMP produced by these cells and the diffusion and dilution of the nucleotide. After ACTH stimulation of adrenal cells, maximal intracellular cAMP content has been observed within 15–20 min (29, 36). Thereafter, it remains stable or slightly decreases, whereas the extracellular cAMP progressively increases for at least 60 min, indicating continuous cAMP production. At 15 min, the cAMP content of cells treated with 10 pM or 1 nM ACTH was ~ 3 pmoles/ 10^6 cells and 20 pmoles/ 10^6 cells, respectively (29, 36, and our own unpublished results). Considering that the diameter of BAC is ~ 16 μ m, one can calculate that cAMP would reach, if uniformly distributed in the total cell volume, a concentration

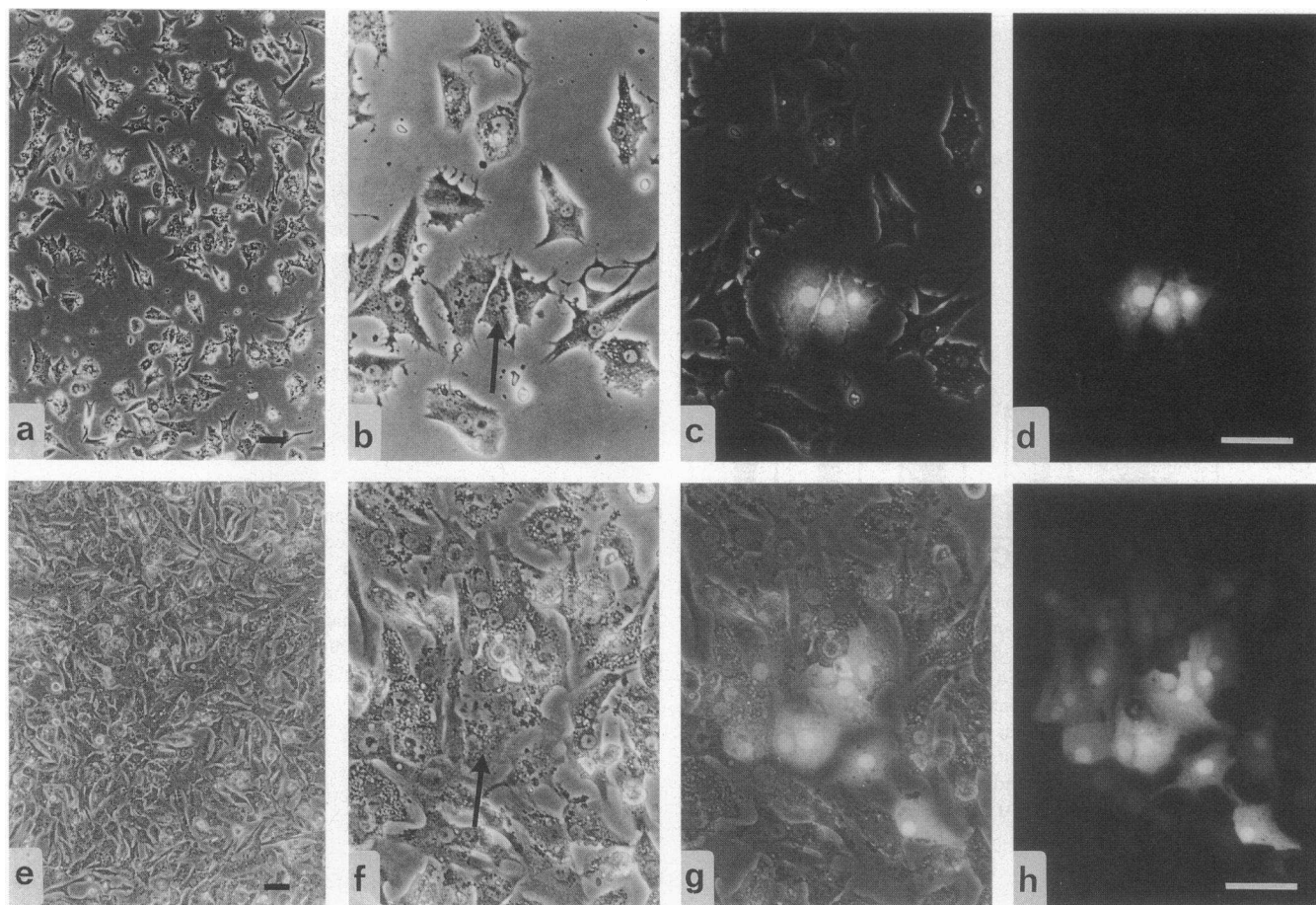


Figure 8. Level of GJ-mediated intercellular communication in BAC cultured at high or low density. BAC were cultured for 5 days at a density of 0.24×10^5 (a-d) or 2.4×10^5 (e-h) cells/cm². The difference in the cell organization is illustrated in the low magnification phase contrast micrographs of a and e. Cells identified by arrows in b and f were microinjected with Lucifer yellow. The fluorescent probe largely diffused in a large number of adjacent cells in high density cultured BAC (g and h) whereas its diffusion among cells cultured at low density was clearly limited by the number of cells in contact with the injected one (c and d). The phase contrast image (b) and the fluorescence image (d) are superimposed in c. The same is true for f and h which give g. Bar, 50 μ m.

of 1 and 10 μ M, respectively. These concentrations appear to be one or two orders of magnitude higher than the cAMP concentration required for a half-maximal activation of cAMP-dependent protein kinase in cytosolic extracts from adrenal cells (44, 45). Thus, diffusion of the nucleotide to 10 to 20 neighboring cells through GJ could theoretically activate the protein kinase A of these cells. These calculations are rough estimates since they do not take into consideration the true intracellular distribution of the nucleotide and the amounts of cAMP that are degraded or leave the cells. However, they are consistent with the observations of Fletcher and Greenan (18) showing that the increased cAMP production by a single cell was able to activate protein kinase A of several neighboring cells which communicated through GJ. The interpretation of the effects of GA on the response of either BAC or HAC to sub-maximal concentrations of ACTH, based on an inhibition of the diffusion of cAMP between responder and non-responder cells coupled via GJ, is strengthened by two distinct experimental observations. First, the cell responses to maximal ACTH concentrations were never affected by blocking GJ-mediated intercellular communication. This indicates that each cell within the cell population, whatever its intrinsic sensitivity to ACTH, is capable of

responding to elevated concentrations of the hormone by increasing its intracellular cAMP concentration and therefore the cortisol production. Under these conditions, cyclic AMP diffusion between coupled cells is not necessary and GJ communication can be inhibited without influencing the overall hormone response of the cell population. Second, the steroidogenic response of isolated BAC in suspension (dispersed by mild trypsin treatment) to a submaximal ACTH concentration was not altered by GA. As a consequence of the disruption of GJ, cells behave as separate units with their own responsiveness to the hormone.

It is generally believed that the dose-response curves obtained with a hormone acting through membrane receptors is due either to the activation of an increasing number of cells when the concentration of the agonist increases (threshold response model) or to a progressive activation of each cell in response to increasing concentrations of the agonist (graded response model) (46). In the first model, the threshold concentration of agonist necessary to initiate the response differs among individual cells. This all-or-none response suggests a quantal process of response. A threshold concentration to induce an increase of intracellular Ca^{2+} has been observed in many

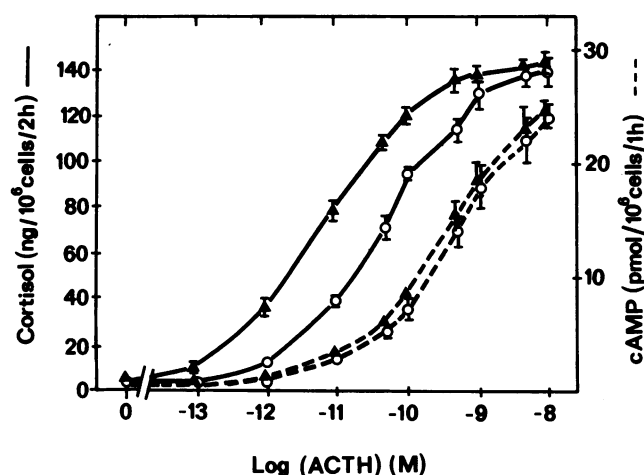


Figure 9. ACTH concentration-response curves for cortisol and cAMP production by BAC cultured at high or low density. BAC at a density of 2.4×10^5 (\blacktriangle) or 0.23×10^5 (\circ) cells/cm² were incubated with increasing concentrations of ACTH. Cortisol and cAMP were measured after 2 and 1 h of incubation, respectively. Results are expressed as the mean \pm SEM of quadruplicate of a representative experiment. BAC cultured at high or low density exhibited the same maximum capacity to produce cortisol in response to elevated ACTH concentrations but gave very different cortisol production in response to sub-maximal ACTH concentrations; a fact illustrated by the distance between the ACTH dose-response curves (continuous lines). Increasing concentrations of ACTH induced the same gradual increase in cAMP production on BAC cultured at high or low density (dashed lines).

cell types (46–50). A threshold concentration has also been observed in the secretory response of pituitary cells to several peptides (46, 50, 51). At present, no information exists concerning the type of response of adrenal fasciculata cells to corticotropin, but the response of intercellular Ca^{2+} in adrenal glomerulosa cells to angiotensin-II follows the threshold model (48, 49). Recent results dealing with another steroidogenic cell type, rat Leydig cells, indicate that the response induced by human chorionic gonadotropin fits with the threshold model (52). Our data give clear indications on the type of response (threshold versus graded) of adrenocortical cells to ACTH. If the response of adrenocortical cells to ACTH was of the graded type, one would not expect an inhibition of the response by blocking GJ-mediated intercellular communication. In contrast, a response of the threshold type in association with the intercellular communication fits well with the observed results.

To our knowledge, we demonstrate for the first time that GJ-mediated intercellular communication has a major functional impact on the response of a cell population to non-saturating concentrations of an external signal. We show that, depending on the level of cell-to-cell communication, the hormone concentration required for half-maximal response of the cell population varied up to 25-fold. The ACTH ED_{50} , which was equal to 8 pM on BAC cultured at high density, increased to 220 pM when the same cells were prevented from communicating. The ED_{50} values obtained with GA-treated cells are comparable with those reported in the literature for isolated adrenal cells from various species (29, 53–55).

Our findings may provide an explanation for certain pathophysiological observations. The K_d of ACTH for its adrenal receptor in several species (31, 56–58) including human (59)

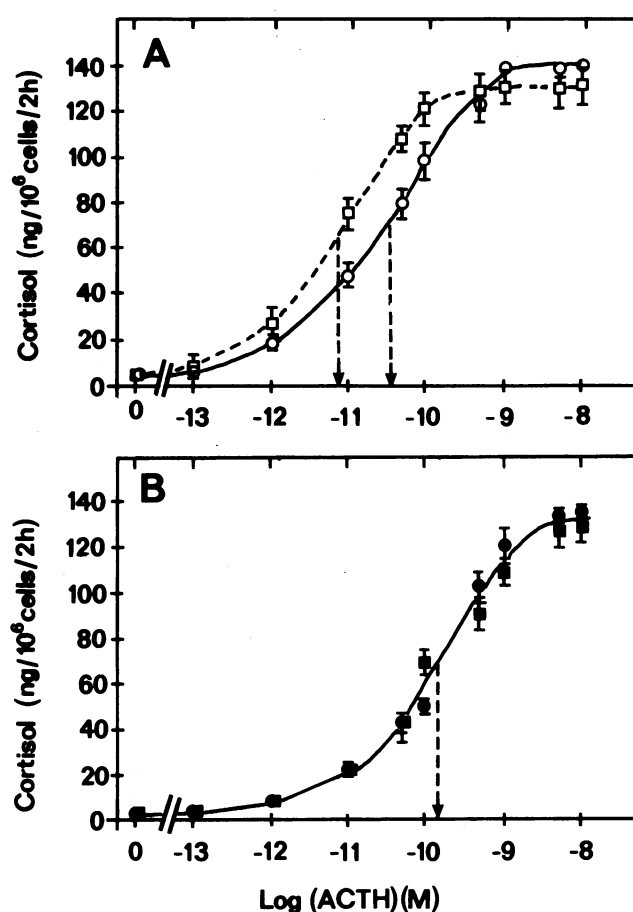


Figure 10. Evidence that GJ-mediated intercellular communication regulates the responsiveness of BAC to submaximal ACTH concentrations. BAC cultured at high (2.4×10^5 cells/cm²) (\square , \blacksquare) or low (0.24×10^5 cells/cm²) (\circ , \bullet) density for 5 d were incubated with increasing concentrations of ACTH in the absence (\square , \circ) or in the presence (\blacksquare , \bullet) of 5 μM GA. Cortisol production was measured after 2 h of incubation. For the clarity of the figure, dose-response curves obtained with untreated BAC are presented in A and those obtained with GA-treated BAC are presented in B. Results are expressed as the mean \pm SEM of the values obtained on three distinct cell preparations. The vertical dashed lines allow appreciation of the difference in the ACTH concentration that caused the half-maximal cortisol production in each experimental condition. When treated by GA, BAC cultured at either high or low density exhibited the same sensitivity to ACTH.

is about 0.5–1 nM. However, in humans, under physiological conditions, plasma ACTH concentrations are very low, less than 5 pM, and an 8–10-fold increase (to 40–50 pM) is known to induce almost maximum acute cortisol secretion (25, 26). Similarly, plasma cortisol levels are very high in many patients with Cushing's disease, despite the fact that plasma ACTH concentrations are between 50 and 100 pM (27). Thus, in this range of ACTH concentration, the functional state of GJ and therefore the level of cell-to-cell communication could be of major importance in determining the amplitude of the overall response of the gland. Finally, the present work establishes that human and bovine adrenal cells are coupled via GJ and that GJ-mediated cell-to-cell communication enhances the responsiveness to low ACTH concentrations. These data might be of general significance and apply to many cell types, because GJ

Table II. The Responsiveness of BAC to ACTH Depends on the Level of GJ-mediated Intercellular Communication

Culture conditions	ACTH ED ₅₀ (×10 ¹⁰ M)		GA-induced ACTH ED ₅₀ increase
	Control	GA (5 μM)	
Low cell density	0.45±0.14	2.5±1.2 [‡]	×5.5
High cell density	0.085±0.013*	2.2±0.9 [‡]	×25.8

Effects of GA on ACTH ED₅₀ for cortisol production by BAC cultured at low or high density. Experimental conditions are the same as those described in Fig. 10. Results are expressed as the mean±SEM of the values obtained on three separate cell preparations. Statistical analysis was performed by the Student paired *t* test.

* Significant difference (*P* < 0.05) with the value of the same column.

‡ Significant difference (*P* < 0.05) with the value of the same line.

are widespread structures and most cells are under control of regulatory signals acting through plasma membrane receptors and intracellular messengers which can diffuse from cell-to-cell via GJ.

Acknowledgments

The authors thank Professor D. Gros (UA CNRS 179, Marseille) for the supply of anti-connexin antibodies and to D. Langlois, A. Penhoat and C. Jaillard for their help in the preparation of bovine adrenal cells. M.C. Lebrethon was supported by a fellowship from "La Fondation pour la Recherche Médicale" (France).

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