Hormone-induced Intercellular Signal Transfer Dissociates Cyclic AMP-dependent Protein Kinase

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ABSTRACT   We used co-cultures of porcine ovarian granulosa cells and mouse adrenocortical tumor cells (Y-1) to examine the kinetics of contact-dependent intercellular signal transfer and to assess the molecular mechanisms employed by this process. Exposure to follicle-stimulating hormone (FSH) caused cAMP-dependent protein kinase dissociation in granulosa cells and, with time, in Y-1 cells if, and only if, they contacted a responding granulosa cell. Y-1 cells close to a granulosa cell but not touching it failed to respond similarly. In reciprocal experiments, co-cultures were stimulated with adrenocorticotropic hormone (ACTH). Y-1 cells dissociated protein kinase as did granulosa cells in contact with Y-1 cells; however, granulosa cells that were not in contact with Y-1 cells failed to respond to the hormone. Fluorogenic steroids were secreted by Y-1 cells cultured alone and stimulated with ACTH, but were not secreted by cultures exposed to FSH. Neither hormone caused fluorogenic steroid production by granulosa cells. On the other hand these steroids were secreted in co-cultures stimulated with ACTH and to a lesser degree in co-cultures exposed to FSH. Autoradiography revealed that I^{125}-FSH bound only to granulosa cells, never to Y-1 cells, even if they were in contact with an ovarian cell. The possibility of cell fusion was tested by experiments in which Y-1 cell membranes were labeled with cationized ferritin. These cells were then placed in co-culture with ovarian granulosa cells that had previously been allowed to ingest latex spheres. At regions of gap junctions between Y-1 and granulosa cells ferritin remained attached to the adrenal cell membrane and was never observed to migrate to the granulosa cell membrane. From these data, we conclude that hormone specific stimulation of one cell type leads to protein kinase dissociation in heterotypic partners only if they contact a hormone responsive cell. This signal transfer is bidirectional, exhibits temporal kinetics and occurs in the absence of apparent cell fusion. The only structural feature connecting Y-1 and granulosa cells were gap junctions implying they provided the communication channels; however, alternative mechanisms cannot be excluded. We have not established the identity of the signal being transferred although cAMP is a logical candidate.

Intercellular communication is a generic term referring to the ability to exchange molecules as large as 1,000 mol wt directly between the cytoplasm of adjacent cells (1). The evidence is convincing that extracellular routes are not involved and that communication is coincident with the presence of gap junctions (1–7). Of necessity, intercellular communication is probed by indirect approaches such as metabolic cooperation (1, 2, 4) ionic coupling or the transfer of fluorescent dyes of various sizes (3, 7). From these studies we have learned that

small molecules when introduced into the cytoplasm of a donor cell can migrate to the cytoplasms of adjacent recipient cells (1–7) providing they contact the donor cell. This has led to the hypothesis that a similar path would be taken by regulatory signals such as cyclic nucleotides, calcium, or prostaglandins (1, 2, 7, 8). If so, it is postulated that these signals could regulate growth and/or synchronize various other cellular activities (2, 7, 8). Indirect evidence supporting this view has been reported by Yotti et al. (9) and others (10, 11) who

find that chemical tumor promoters (growth stimulators) can prevent the establishment of intercellular communication (metabolic cooperation) and eliminate established channels of communication. Using a different approach, Lawrence et al. (12) observed that ovarian granulosa cells and myocardial cells when contacting in co-culture were coupled ionically and metabolically. Stimulation by a hormone specific for one cell type initiated biologic activity in a heterotypic partner that did not respond similarly when cultured alone. They speculated that the intercellular transfer of cyclic AMP probably by gap junctions could account for these observations, but no tests were made of alternatives, such as anomalous hormone binding or cell fusion, other than by morphologic inspection. This is a problem in view of the fact receptors for peptide hormones can be isolated from one cell type reinstated into recipient membranes from a different cell type and express normal function (13). Thus, the postulate that the intercellular transfer of biologic signals may be influential in regulating cell growth or function remains unproven. Perhaps most importantly nothing is known of the molecular mechanisms that are involved in this process.

Protein phosphorylation-dephosphorylation reactions are the means by which many, perhaps all, aspects of cell growth and function are regulated (14). The most widely studied mediator of this regulation is cyclic $3'-5'$-adenosine monophosphate (cAMP),[1] which seems to act exclusively through its dependent protein kinases (14, 15). Binding of cAMP to the regulatory subunit of protein kinase holoenzymes causes them to dissociate free catalytic subunits which, with their phosphotransferase activities, are the effectors of cAMP actions (14, 15). Given this, we reasoned that if there exists a biologically valid intercellular communication of regulatory molecules, then those signals, when transmitted to a recipient cell, should initiate the dissociation of cAMP-dependent protein kinases.

To test this hypothesis, we developed a method that allows the direct cytochemical localization of free catalytic subunits of cAMP-dependent protein kinase at single cell resolution (16). The validation and use of this procedure has been previously described (16, 17). Using this method, we have been able to follow the kinetics of intercellular communication between two hormone responsive, phenotypically distinct, cell types.

MATERIALS AND METHODS

Cultures: Porcine ovarian granulosa cells were aseptically harvested from preovulatory (day 18-20) follicles of cycling sows. Debris was removed by centrifugation of cells through a 1:1 mixture of DME:F-12 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS [Gibco Laboratories]). No antibiotics were used. Granulosa cells cultured alone (solo culture) were seeded into 10 well chamber slides (Bellco Glass, Inc., Vineland, NJ) at a density of 1×10^5 cells/cm^2 in 200 μl of complete medium.

Murine adrenocortical tumor cells clone Y-1 (Y-1 cells) were originally obtained from Dr. Bernard Schimmer (Toronto, Canada) and continuously cultured by previously described methods (18). For these studies, cultures were resuspended using 4 mM EDTA for 3-4 min at 37°C then transferred to the DME:F-12 medium through which they were centrifuged twice to remove debris. Cells were plated into 10 well chamber slides at 1×10^5 cells/cm^2.

Co-cultures were established by mixing freshly harvested granulosa cells with resuspended Y-1 cells, usually in a 2:1 or 5:1 ratio of granulosa to Y-1, then seeding each chamber slide well with a total of 1×10^5 cells/cm^2. Alternatively,

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[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropin; cAMP, $3'-5'$-cyclic-adenosine monophosphate; FSH, follicle-stimulating hormone; PKI, protein kinase inhibitor; F:PKI, fluorescent PKI; 8BrcAMP; Y-1, murine adrenocortical tumor cells clone Y-1.

one cell type (e.g., granulosa) was solo cultured at low density for 24 h then the other cell type (e.g., Y-1) was added, again keeping the ratio of granulosa:Y-1 cells at 2:1 or 5:1 in the final mixture. This type of preparation was especially useful for those experiments in which one of the cell components was prelabeled with rhodamine conjugated latex spheres or cationized ferritin (below). In all cases, co-cultures or solo cultures were maintained at 37°C in an atmosphere of 5% CO$_2$:95% air and 100% humidity.

Cell Prelabeling: Although Y-1 and ovarian granulosa cells are easily distinguished using phase-contrast optics they are less readily differentiated in the electron microscope where, in any given section, only small portions of one or the other cell may be viewed. To overcome this granulosa cells, after one day of culture, were challenged with rhodamine-conjugated latex spheres prepared as described below, for 30 min to 2 h at 37°C (2.4 × 10^9 spheres/ml culture medium). Cultures were then washed three times with complete medium to remove excess spheres. With this procedure >90% of granulosa cells ingested 30 or more spheres that were predominantly confined to cortical cytoplasm. 2-4 h after labeling, cells were suspended with 4 mM EDTA, centrifuged twice through complete medium, resuspended, and mixed with an appropriate number of Y-1 cells. In separate experiments, attempts were made to similarly label Y-1 cells. However, in no case did they ingest the spheres. This fact made latex sphere labeling of granulosa cells useful for distinguishing them from Y-1 cells and in addition for determining whether or not fusion between the two cell types occurred. Although this was adequate for assessing the occurrence of large sites of fusion (0.48 μM, the diameter of the spheres) it would be inadequate to test for microfocal fusion that may be a problem in co-cultures of interspecific cells (2, 5-7). To control for this possibility, we washed cultures of Y-1 cells three times with 37°C serum-free culture medium. Fresh serum free medium containing cationized ferritin (Miles-Yeda, Rehovoth, Israel) amounting to 0.5 mg protein/ml was added to cultures and cell surfaces labeled by the method of Danon et al. (19). Cultures were then washed twice with serum free medium after which complete medium was readded. 2-4 h later granulosa cells labeled as described above with latex spheres were added in a three-fold excess to these cultures of ferritin-labeled Y-1 cells. Cells were allowed to intermingle for 12 h, after which they were prepared as a monolayer for electron microscopy.

Latex beads (~0.48 μM average diameter) were purchased in a 1% solution from Pelco Inc. (Tustin, CA). The beads were removed from the shipping solution by centrifuging at 20,000 rpm in a Beckman a21c centrifuge (Beckman Instruments, Inc., Palo Alto, CA) then suspended in 1 ml of a pH-9 carbonate buffer. Tetramethyl rhodamine isothiocyanate (Research Organics, Inc., Cleveland, OH) was added (2 mg/ml buffer) and the suspension was vortexed for 2 min at room temperature, cooled on ice for 2 min, and centrifuged at 19,000 rpm for 15 min in a Beckman a21c centrifuge. The pellet was resuspended in carbonate buffer and dialysed against three daily changes of triple distilled water (3.5 liters) for 3 d. The rhodamine particles were added to the granulosa culture in a concentration of 2.4 × 10^9 particles/ml of incubation media after they had been sterilized by three centrifugations through 70% ethanol.

Steroid Production: The production and secretion of fluorogenic steroids (Δ^4, three ketosteroids) was tested using a modification of the procedure of Vernikos-Danellis et al. (20). ACTH was purchased from Armour Pharmaceutical Co. (Chicago, IL) or Sigma Chemical Co. (St. Louis, MO) and diluted for use in Earle's balanced salt solution. The recovered medium was diluted to 5 ml with 0.9% saline, 6 ml of methylene chloride was added and the mixture was shaken. The mixtures were centrifuged at 10,000 rpm and filtered through Whatman IPS filter paper (Whatman Laboratory Products, Inc., Clifton, NJ). The resulting methylene chloride extract was reacted with 3 ml of a 35:65 ethanol:sulfuric acid solution for 70 min. The acid layer was read on a Mark IV spectrofluorometer with 415 and 505 nM as the primary and secondary wavelengths. Corticosterone dissolved in ethanol was used as a standard.

Morphologic Procedures: All preparations for electron microscopy were those described previously for use on cultured cells (21).

Cytochemical localization of free catalytic units from cAMP-dependent protein kinase was carried out as described previously (16, 17). Briefly, the procedure relies on a fluorescent conjugate (F:PKI) of homogenously pure protein kinase inhibitor (PKI). The F:PKI binds specifically to the substrate site of free catalytic units from protein kinase for which it has an affinity orders of magnitude ($K_a < 2 \times 10^{-9}$ M) greater than any known biologic substrate (22). The F:PKI serves as a reliable and sensitive probe for detecting free catalytic units at subcellular resolution limited only by the fluorescence optics used (16, 17). After stimulation with nucleotide analogue, hormone or the appropriate control solution for 0-120 min cultures were washed in room temperature phosphate-buffered saline, pH 7.4, then fixed for 15 min in −30°C anhydrous acetone, washed with 4°C phosphate-buffered saline, pH 7.4, for 3 min and covered with F:PKI (250 ng/10^5 cells). Preparations were then placed in a humidity chamber and incubated for 48 h at 4°C. After washing, cultures were coverslipped and examined with the fluorescence microscope. As in earlier

descriptions (16), incubation times of 6, 12, 24, 48, 72, and 96 h with various amounts of F:PKI (1.25 ng-1.25 μg/10^5 cells) were tried. The method described above gave the most reproducible results in terms of fluorescence intensity in all subcellular compartments. Controls for specificity of F:PKI binding were those used previously (16) namely substrate competition; competitive inhibition of F:PKI by a 10-20 molar ratio excess of native PKI and reduction in F:PKI binding by affinity column purified antibody to catalytic units (23).

Fluorescence quantitation with a Leitz MPV microspectrophotometer was done by one of us (S. A. Murray) or by students who were unaware of the nature of the protocols. The procedure used is described elsewhere (16). For the data presented here, the photometer's aperture was set at 3×3 μm using a stage micrometer. No fewer than 25 readings were made of each possible situation (Y-1 noncontacting or contacting and granulosa noncontacting or contacting) in duplicate wells of each preparation. Each preparation was repeated at least three times giving a minimum of 100 readings for every dose and time of stimulation for each agent (8BrcAMP, FSH, ACTH). Data were then analyzed by Duncans one way analysis of variance and are presented as percentage increases (average $\pm$ SD) in fluorescence (i.e., amount of F:PKI bound) relative to that of noncontacting cells in unstimulated cultures that were stained with F:PKI probe. Unlike previous studies it was not possible to assess the amount of F:PKI bound (i.e., fluorescence intensity) by cells randomly encountered during microscopy. This was due to the complexity of the co-cultures and the need to evaluate the amount of F:PKI bound by stimulated cells and their immediately nearby neighbors, whether contacting or noncontacting. In addition, the co-cultures, themselves, appeared to be nonrandom in that granulosa and Y-1 cells often segregated from each other forming clusters of varying size that consisted almost exclusively of one cell type. Therefore, photography and microspectrophotometry were confined to those areas of the co-cultures possessing an apparently random admixture of granulosa and Y-1 cells. This tendency to segregate out was not due to the mode used to plate cultures as it occurred with similar frequency whether cultures were initiated with both cell types simultaneously or with one type added 24 h before addition of the second cell component, as described above.

The average fluorescence intensity, indicative of free catalytic unit content, of unstimulated, noncontacting Y-1 or granulosa cells was subtracted from that of all other preparations. Thus, the data is presented as percentage increase in protein kinase dissociation relative to control. The actual values from the microspectrophotometer for Y-1 or granulosa cells were respectively 1.7 ± 0.4 mV and 1.1 ± 0.2 mV for the experiment documented in Table I. The interassay variation for these studies ($n = 7$) was $\pm 30\%$.

Fluorescence photomicrography was done using a varioorthomat camera (Leitz) with optics specific for either fluorescein isothiocyanate (Leitz K2) or tetramethyl rhodamine isothiocyanate (Leitz N2). The exposure time for each protocol was based on the average of ten or more automatic exposures (Leitz varioorthomat) of the most fluorescent cell component. All images were obtained using TR1-X pan film (Kodak) which was developed with D-76 (Kodak).

For autoradiography, co-cultures of Y-1 and ovarian granulosa cells were exposed to I^{125}-FSH (New England Nuclear, Boston, MA; 65.2 μCi/μg) at 1.0-0.1 μCi/10^5 cells for 0, 30, 60, or 120 min. Cultures were then washed exhaustively in sterile 4°C phosphate-buffered saline pH 7.4, fixed with anhydrous acetone and allowed to dry at room temperature. They were then coated with NTB-3 emulsion (Kodak) diluted 1:1.5 with sterile water, placed in a dark box with a dessicant, sealed and stored at 4°C for 2-8 wk. After exposure autoradiographs were developed with D19 (Kodak), fixed, and slides stained with hematoxylin and eosin. Nonspecific binding was controlled by treating cultures with I^{125}-FSH in the presence of a 20 molar ratio excess of unlabeled FSH (NIH-FSH-S13).

Reagents: Affinity purification of the inhibitor protein and preparation of F:PKI were done precisely as described previously (16, 22). Hormones were a gift of the National Pituitary Agency (National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health). 8BrcAMP and cAMP were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The iodinated hormones used were generously supplied by New England Nuclear.

RESULTS

Morphology of Co-cultures

The light and electron microscopic appearance of Y-1 or ovarian granulosa cell cultures has been documented elsewhere (18, 24).

When placed in co-culture the two cell types adhered to substrate quickly and within 6-8 h they had flattened appreciably. During the first 12 h Y-1 and granulosa cells intermin-

gled randomly and clusters appeared to consist of each cell type in proportion to its density at seeding. After the first day there was noticeable segregation of cells forming islands wherein one or the other cell type predominated. By the end of the second day about one-third of each co-culture was composed of a random mixture within which heterotypic cell contacts were common. Because the Y-1 cells divide much more rapidly than do the highly differentiated granulosa cells, within 48 h they predominated in those cultures seeded with a Y-1:granulosa ratio of 1:2 but remained a slight minority in co-cultures where the starting ratio was 1:5. In the first case (a 1:2 starting ratio) there tended to be a greater incidence of multiple Y-1 cells contacting a single granulosa cell, whereas in the latter case (1:5 starting ratio) there were more instances of one-one heterotypic content. The photographic and microfluorometry data from both of the preparations were, however, similar as long as they were obtained solely from areas of the culture where Y-1 and granulosa cells freely intermingled as shown in Fig. 1. All of the data to be presented is from such regions. The segregated islands of cells were avoided as they presented few opportunities for heterotypic contacts. It is noteworthy that within these large clusters, of Y-1 or granulosa cells only, the kinetics of protein kinase dissociation in response to 8BrcAMP or specific hormone stimulation were not significantly different from those when these cell types were cultured alone. The details of these observations will be reported separately (Murray, S. A., C. V. Byus, and W. H. Fletcher, manuscript submitted for publication; Fletcher, W. H., J. F. O'Neal, and P. J. Tuso, manuscript submitted for publication), but for the purpose of this study they indicate that the co-culture conditions did not interfere with the normal subcellular mechanisms of cAMP-dependent protein kinase dissociation. Upon exposure to 8BrcAMP for up to 60 min cAMP-dependent protein kinase dissociation was equally evident in granulosa and Y-1 cells whether or not they formed heterotypic contact (Fig. 2). Within 1 min barely detectable amounts of free catalytic units were localized in
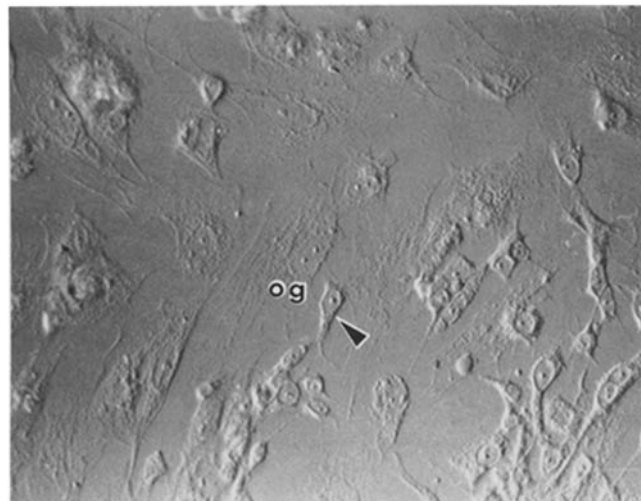

FIGURE 1 Overview of a viable coculture showing a region of random cell mixture. These areas, amounting to about one-third or less, of each co-culture were used for this study. Areas such as the upper left quadrant, comprised of granulosa cells alone, or the lower right quadrant, composed mainly of Y-1 cells, were avoided. Note the distinctive appearance of the small, slightly rounded Y-1 cell (arrowhead) relative to the much larger, flattened ovarian granulosa cell (og). × 750.
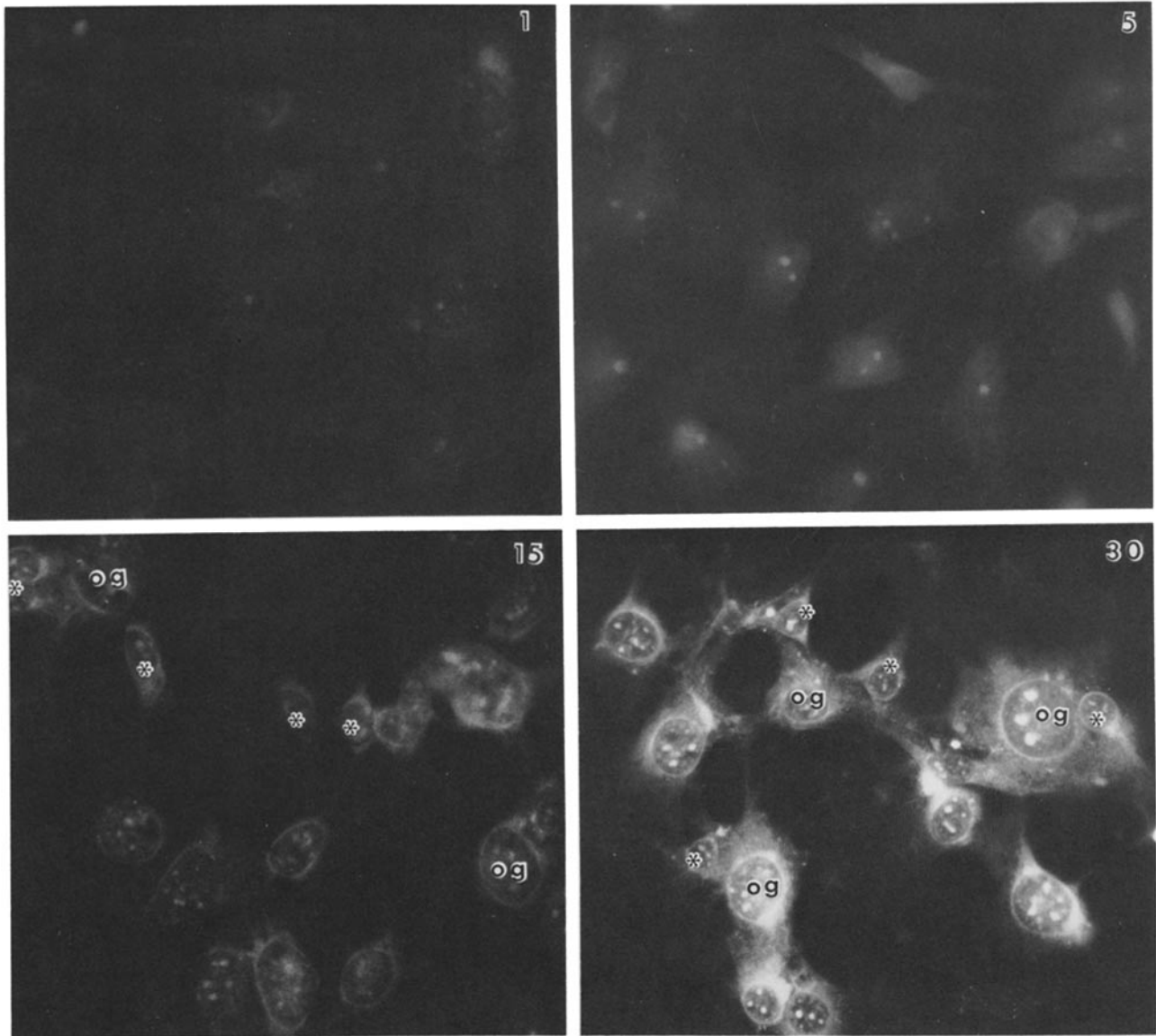
FIGURE 2 Co-cultures were stimulated for 1, 5, 15, or 30 min (times indicated on the image) with 0.1 mM 8BrcAMP then prepared for the localization of free catalytic units dissociated from cAMP-dependent protein kinase. Increasing time of exposure caused a progressive rise in the amount of free catalytic units and in the percentage of both cell types responding. After 15 min or more of stimulation Y-1 cells (*) and granulosa cells (og) are easily differentiated. There is a heterogeneity of response (i.e., fluorescence intensity) although at all times Y-1 cells appear to contain less free catalytic units than granulosa cells. All images × 1,200.

the nucleolus and cytoplasm of both cell types. At subsequent time intervals free catalytic unit continued to increase in these subcellular regions with an apparent maximum net dissociation occurring by 30 min, at which time nucleoplasm also possessed significant amounts of free catalytic units. As shown in Fig. 2 between the 1 min and 5 min intervals of exposure to 8BrcAMP there is a perceptible rise in nucleolar and cytoplasmic content of free catalytic unit and an increase in the percentage of cells responding to the nucleotide. However, it required 15 min to 30 min (D) of stimulation to initiate protein kinase dissociation in most, perhaps all, cells of these co-cultures. Even then, there were consistent differences among cells in their content of free catalytic units (i.e., fluorescence intensity). This heterogenous response in protein kinase dissociation is commonly encountered (17) especially in cultures treated for a brief time or with low doses of stimulating agent. 30 min exposure to 0.1 mM 8BrcAMP

yielded an optimal response in that most cells, Y-1 and granulosa, contained peak amounts of free catalytic units. Thus, these preparations were used to obtain the microspectrophotometric data that allowed a semiquantitative comparison of F:PKI binding by each cellular component of stimulated and control co-culture preparations (Table I). In a separate experimental series using Y-1 (Murray, S. A., C. V. Byus, and W. H. Fletcher, manuscript submitted for publication) or granulosa cells (Fletcher, W. H., J. F. O'Neal, and P. J. Tuso, manuscript submitted for publication) cultured alone 30 min proved to be a good exposure time for observing the effects of cyclic nucleotides analogues or peptide hormones upon protein kinase dissociation.

As shown in Table I, 8BrcAMP led to a doubling of protein kinase dissociation in Y-1 tumor cells and a trebling of dissociation in ovarian granulosa cells, relative to unstimulated noncontacting controls (see Materials and Methods). In

TABLE I

Treatment	Noncontacting		Contacting	
	Y-1	Granulosa	Y-1	Granulosa
Unstimulated	0	0	24.67 ± 12.68	12.01 ± 6.3
8BrcAMP	113.37* ± 22.3	160.06* ± 4.85	138.78* ± 3.87	244.56* ± 76.35
FSH	24.63 ± 24.63	188.65* ± 13.82	93.49*[l] ± 19.10	237.78* ± 60.00
ACTH	199.8* ± 79.6	22.97 ± 18.2	231.5* ± 46.4	559.4*[s] ± 31.8

* $P < 0.01$ compared to unstimulated noncontacting control.
[l] $P < 0.05$ compared to stimulated noncontacting homologous cells in same culture.
[s] $P < 0.01$ compared to stimulated noncontacting homologous cells in same culture.
This table presents the microspectrophotometric quantitation of F:PKI binding to each component of the co-cultures using procedures described in Materials and Methods. Each data point is the average ± SD of no few than 100 cells in duplicate or triplicate cocultures. Noncontacting means cells not connected to a heterotypic partner but which may have been in contact with a like cell type. Contacting refers to those instances wherein a cell contacted one or more heterotypic cells, as in Fig. 3 and 4. In all experiments, unstimulated cells in such contact had a slightly, but never significantly, greater amount of endogenously dissociated kinase than noncontacting cells of the same culture. Dose and time dependence studies (text) revealed that kinase dissociation was maximal following 30 min of exposure to 0.1 mM 8BrcAMP, 10^{-9} M NIH-FSH-S13 or ACTH 6 × 10^{-10} M, which were the parameters used to obtain the data shown here. 8BrcAMP caused a highly significant elevation in free catalytic units in Y-1 and granulosa cells whether or not they were in heterotypic contact. FSH caused significant kinase dissociation in contacting and noncontacting granulosa cells, but had no effect on Y-1 cells that were not contacting a granulosa cell partner. Y-1 cells contacting FSH responsive granulosa cells also dissociated kinase in amounts comparable to that initiated by 8BrcAMP. The status of contact had no significant effect on ACTH-induced kinase dissociation in Y-1 cells, although in all experiments they contained slightly more free catalytic units when connected to a granulosa cell partner.

all but one of the seven experiments in this series cells contacting heterotypic partners dissociated more protein kinase than cells of the same culture that were not in such contact (Table I). While this was a persistent observation the difference never reached levels of statistical significance. Y-1 and granulosa cells in contact with one another in unstimulated cultures had slightly elevated amounts of free catalytic units relative to unstimulated, noncontacting controls (Table I). This may not be attributed solely to intercellular contact as Y-1 cells not contacting granulosa cells in FSH-stimulated cultures and granulosa cells not touching Y-1 cells in ACTH-stimulated cultures also had slightly, but never significantly, elevated contents of dissociated protein kinase when compared to controls. It seems therefore, that the co-culture condition itself may induce slight elevations in basal dissociation of protein-kinase, although some contribution of intercellular contact also seems likely (compare noncontacting vs. contacting cells in unstimulated cultures). It should be emphasized that the absolute amplitude of fluorescence increase, indicative of basal free catalytic unit content, in contacting cells relative to noncontacting controls, was less than that of co-cultures stimulated for 1 min with 8BrcAMP (Fig. 2).

A strikingly different picture emerged when co-cultures were stimulated with a hormone specific for only one cell type. Fig. 3 displays results from co-cultures challenged for 1–60 min with 6 × 10^{-10} M ACTH. After 5-min exposure to hormone (Fig. 3, top left) the Y-1 cells possessed abundant free catalytic units. Two of these adrenocortical cells contacted an ovarian granulosa cell which also has dissociated cAMP-dependent protein kinase in the nucleolus and in cytoplasmic regions adjacent to the areas of heterologous intercellular contact. F:PKI has also bound to cytoplasmic free catalytic units distal to the area of contact but to a much lesser degree. Comparing this with the top right panel of Fig. 3, which is a phase-contrast image of this same cluster verifies that two granulosa cells in contact with each other, but not with the nearby Y-1 cells, fail to dissociate protein kinase. It should be noted that the intense fluorescence of Y-1 cells is somewhat artifical in that it is due to intentional overexposure of the negative needed to demonstrate the F:PKI bound to the granulosa cell. Nevertheless, the top panels of Fig. 3 show unequivocally that only those granulosa cells contacting

ACTH responsive Y-1 cells dissociate cAMP-dependent protein kinase. After 30-min exposure to ACTH (Fig. 3, bottom) ovarian granulosa cells in contact with Y-1 tumor cells contain abundant free catalytic units. In fact, as the image and Table I indicate, the granulosa cells have at this time dissociated more than twice the amount of catalytic units as have contacting Y-1 partners. Equally close but noncontacting granulosa cells do not dissociate protein kinase above basal levels. In the 22 co-culture experiments performed we have only rarely observed the asymmetric subcellular distribution of protein kinase dissociation shown in the top left panel of Fig. 3 and in those cases this occurred only in co-cultures stimulated for 10 min or less. At longer times of exposure to ACTH responsive heterotypic cell pairs had a more uniform distribution of cytoplasmic catalytic units. On the other hand while the rise in nucleolar protein kinase activity in recipient cells was at least as rapid as that of cytoplasm, nucleoplasmic free catalytic units were consistently evident only in cultures stimulated for 30 min or more as shown in (Fig. 3, bottom).

Exposure of co-cultures to FSH (NIH-FSH-S 13) for 1–60 min revealed that contact dependent intercellular signal transfer was reciprocal. The hormone induced protein kinase dissociation in ovarian granulosa cells and in contacting but not in noncontacting Y-1 cells (Fig. 4). FSH caused free catalytic unit accumulation first in responsive ovarian granulosa cells that with time (5–15 min) initiated protein kinase dissociation in connected Y-1 cells. Although dissociation was significantly greater than that of noncontacting Y-1 cells in FSH stimulated or in unstimulated co-cultures (Table I) it was significantly less than that of Y-1 cells stimulated with ACTH ($P < 0.01$) or with 8BrcAMP ($P < 0.05$). This is in marked contrast to co-cultures wherein granulosa cells contacting ACTH responsive Y-1 cells dissociated significantly more protein kinase than they did upon exposure to FSH or 8BrcAMP ($P < 0.01$ for both cases). There are a number of feasible causes of this phenomenon (see Discussion). The relevant fact is that with ACTH or FSH stimulation only those cells that formed heterotypic intercellular contact with a responsive, hormone receptor bearing partner dissociated cAMP-dependent protein kinase. We never observed dissociation in noncontacting Y-1 or granulosa cells of co-cultures stimulated, respectively, with FSH or ACTH. However, we did not always observe
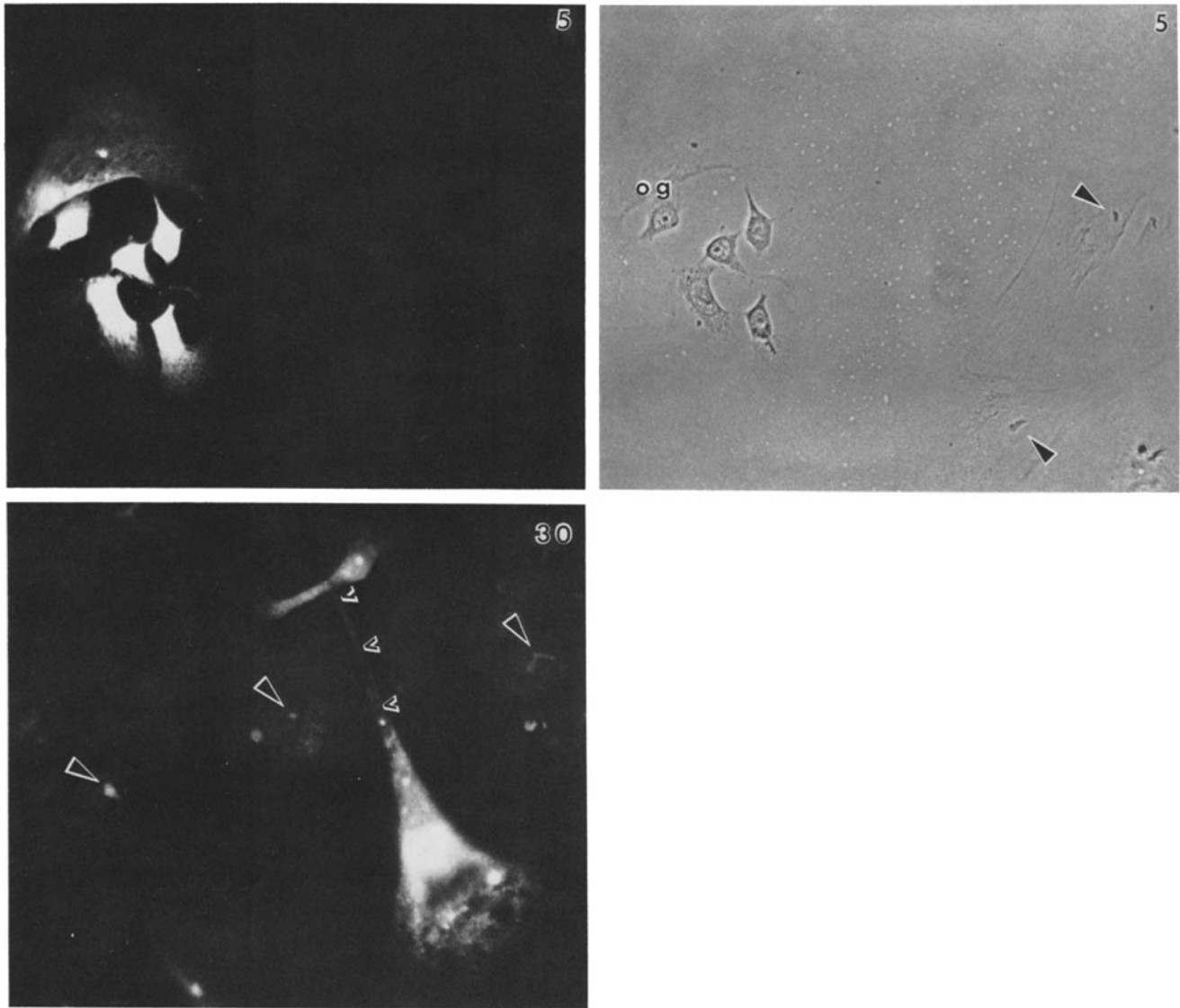
Figure 3 Co-cultures were exposed for 0–30 min to 6×10^{-10} M ACTH. While not shown unstimulated, 0 time, F:PKI stained controls bound negligible amounts of the probe. After 5 min of stimulation (*upper* panels) Y-1 cells (five are shown) possessed abundant free catalytic units. A granulosa cell that is contacted by two Y-1 cells also has dissociated protein kinase in the nucleolus and in cytoplasm adjacent to the points where it is in contact with Y-1 cells. As the right hand panel reveals nearby the responding granulosa cell (og) are two granulosa cells which do not contact Y-1 cells and have failed to dissociate protein kinase. (Black triangles point to the nucleus with its prominent phase-dense nucleolus in each granulosa cell.) The *lower* panel depicts a mixed cell aggregate after 30-min exposure to ACTH. The small Y-1 cell contains free catalytic units in cytoplasm, nucleoplasm, and nucleolus. It is connected to an ovarian granulosa cell by a slender process (pointed to by the chevrons) from the ovarian cell, which itself contains active cAMP-dependent protein kinase in cytoplasm, nucleoplasm, and nucleolus. In contrast three adjacent but noncontacting granulosa cells (arrowheads indicte the nucleus of each) have not dissociated protein kinase. Note that the nucleoli (just beyond the tip of the arrowheads) of the nonresponding granulosa cells contain a small amount of free catalytic unit. All images $\times$ 1,200.

positive effects of intercellular signal transfer. In fact, in FSH-stimulated co-cultures, less than a third of the observed instances of Y-1 cells contacting granulosa cells led to protein kinase dissociation in both the primary responding granulosa cell and signal recipient Y-1 cell. The predominate basis of this was revealed by additional experiments (below). Importantly, Y-1 cells did not dissociate protein kinase when contacting nonresponsive granulosa cells of FSH-stimulated co-cultures.

Similar observations were made using ACTH-stimulated co-cultures; although there were fewer cases (10% or less) of

Y-1 cells not responding to some degree to ACTH stimulation. Each of these instances of negative data will be considered (Discussion). The only salient observation is that following ACTH or FSH stimulation cAMP-dependent protein kinase dissociation occurred in receptor competent cells and in heterotypic partners if, and only if, they were in direct contact.

Collectively, these data (Figs. 1–4, Table I) allow the conclusion that hormone-specific stimulation of one member of a heterotypic cell pair leads to cAMP-dependent protein kinase dissociation in the contacting partner that is otherwise
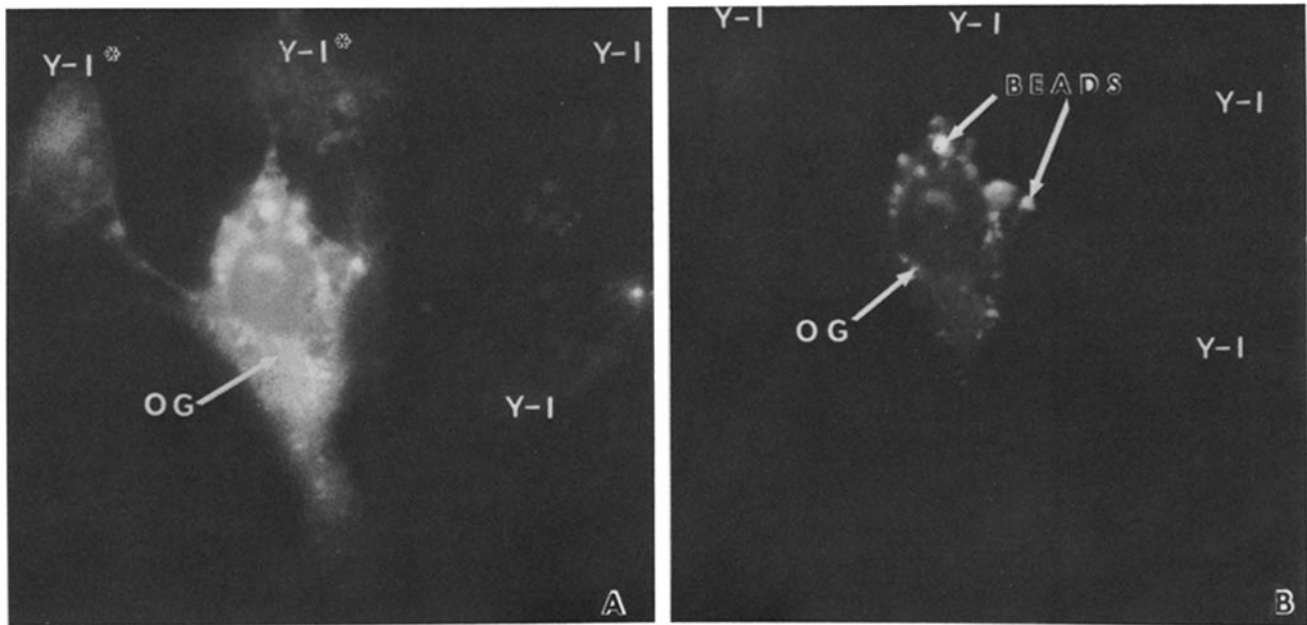
FIGURE 4   Both panels are images of the same cell cluster viewed with optics specific for fluorescein isothiocyanate (A) or rhodamine isothiocyanate (B). The co-culture was exposed to FSH for 30 min. The granulosa cell (og) has dissociated abundant free catalytic unit. Two contacting Y-1 cells (Y-1*) also possess appreciable protein kinase, whereas two equally near, but noncontacting Y-1 cells (Y-1) fail to dissociate enzyme B verifies the identity of the granulosa cell that was prelabeled (Materials and Methods) with rhodamine derivatized latex spheres (beads).

unresponsive to the stimulus and that this response is bidirectional. They do not, however, speak to the route of this contact dependent cell-cell signal transfer. In this regard a number of possibilities were considered. A medium-borne effect is excluded by the fact that heterotypic cells failed to dissociate kinase even though they were equally near, but not in contact with, the hormone responsive cell. Furthermore, addition of 1 mM cAMP a potential extracellular signal (1, 2, 7) had no effect on the hormone specific response observed; nor, over a 30-min period, did it cause detectable protein kinase dissociation in either Y-1 or granulosa cells cultured alone (data not shown). This does not indicate that the nucleotide had no effect on intercellular communication or on gap junction assembly as reported by others (25). Although this aspect deserves further consideration, it is beyond the scope of the present study.

In some cases co-culture of interspecific cell types appears to lead to cell fusion (2). If this occurred between Y-1 and granulosa cells, then receptors could migrate into the membrane of a heterotypic cell thereby giving rise to anomalous hormone binding (13). To test this possibility co-cultures were exposed to I^{125}-FSH and autoradiographs were prepared by standard procedures (Materials and Methods). As can be seen in Fig. 5 only the granulosa component of the co-cultures bound hormone. Y-1 cells whether or not in contact with granulosa cells failed to bind I^{125}-FSH above background levels. Clearly, adventitious hormone binding due to receptors moving from granulosa to Y-1 cells through points of cell fusion does not seem likely. While this strengthens the case for the hormone specificity of intercellular signal transfer observed between granulosa and Y-1 cells it does not eliminate intercellular fusion as the basis for the contact dependency of this process. This is because membrane macromolecules like receptors for peptide hormones might not move to an adjacent cell through sites of fusion if they were linked to submembranous cytoskeletal elements (26). As the results of Frye and
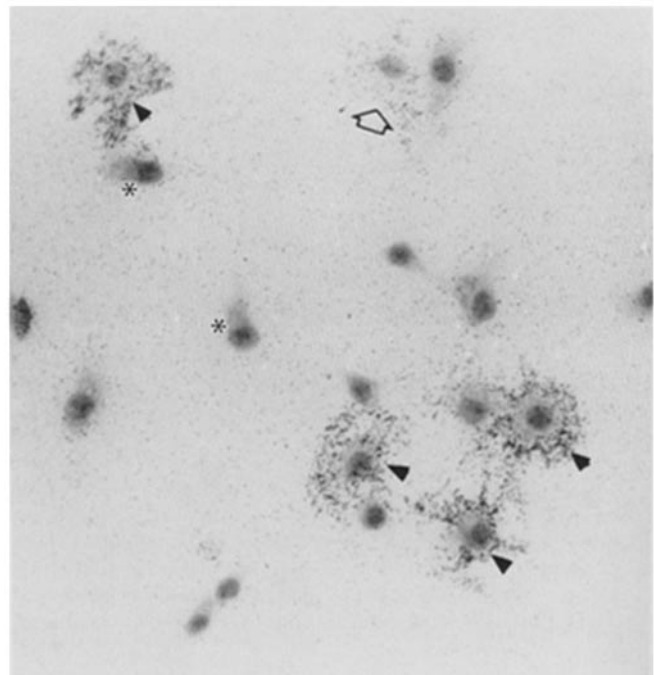

FIGURE 5   Co-cultures were treated for 1 h with 0.5 μCi I^{125}-FSH/ 10^5 cells. Four granulosa cells (black triangles) have bound appreciable hormone while one (open arrow) has bound very little. The region depicted had the greatest density of FSH receptor bearing granulosa cells. Even so, none of the small Y-1 cells (examples at asterisk) bind hormone even when they contact heavily labeled granulosa cells (upper left and lower right quadrants). × 1,200.

Edidins' experiments demonstrated (27) many cell specific surface determinants freely intermingle following fusion of two cell types, forming heterokaryons. Thus, to test the possibility that microfocal fusion provided the pathway for signal transfer, the surface membrane of Y-1 cell was labeled using

TABLE II

Addition (M)

	None	FSH (10⁻⁹)	ACTH (10⁻⁹)	8BrcAMP (10⁻⁴)
GRAN	0.08 ± 0.02	0.09 ± 0.03	0.12 ± 0.02	0.11 ± 0.02
Y-1	0.09 ± 0.02	0.10 ± 0.02	1.16 ± 0.04*	1.01 ± 0.08*
GRAN + Y-1	0.12 ± 0.03	0.42 ± 0.13*	1.08 ± 0.08*	0.89 ± 0.10*

Data presented as micrograms of corticosterone/250 μl/24 h ± SD of triplicate assays.
* $P \leq 0.001$ compared to unstimulated controls.

low density (10-20% confluent) cultures exposed to cationized ferritin (Materials and Methods). A threefold excess of granulosa cells that had been previously labeled with latex spheres was then added to the Y-1 culture and 12 h later cultures were prepared for electron microscopy. In each of the three protocols, residual surface bound ferritin remained attached to Y-1 cells and did not migrate to the connected granulosa cell, even at regions morphologically specialized as gap junctions (data not shown).

Biologic Effects of Signal Transfer

It was important to test whether or not the protein kinase dissociation due to intercellular signal transfer led to a normal cellular response. To this end, ovarian granulosa and Y-1 cells were separately cultured on 12-×-12-mm glass coverslips one each per well of a 12 well plate (Flow Laboratories, Inc., Inglewood, CA) at a density of 1.0-1.5 × 10⁵ cells/cm² (~1.5 × 10⁵ cells). Co-cultures were established using the full surface of the well itself (4.5 cm²) and consisted of a twofold excess of granulosa cells relative to Y-1 cells, however, they contained the same number of Y-1 cells as did the solo culture control. Thus, both culture conditions were at the same density (1.5 × 10⁵/cm²) and had the same number of adrenal tumor cells. All cultures were bathed in 1.0 ml of complete medium, which was replaced after 20-24 h with fresh medium. 3-4 h later cultures were challenged with FSH, ACTH, or 8BrcAMP or with diluent only. A repeat dose of each agent was added at 12 and 24 h after the initial stimulus, medium was recovered, centrifuged to remove debris and frozen to be assayed (Materials and Methods) within 7 d. As shown in Table II ovarian granulosa cells do not secrete fluorogenic steroids upon exposure to hormone or nucleotide analogue. Y-1 cells, secreted a 10-fold or greater amount of steroids when stimulated with ACTH or 8BrcAMP, but failed to respond to FSH which was no more effective than the diluent control. In the co-cultures, however, FSH led to a four- to fivefold rise in steroid secretion relative to controls while ACTH and 8BrcAMP were even more effective in this regard. FSH stimulation of co-cultures never led to steroid production equivalent to that caused by ACTH, presumably because not all granulosa cells were FSH responsive and of those only a subpopulation was in communication with Y-1 cells. This would be fully in accord with the autoradiographic results described above.

DISCUSSION

We have questioned whether or not intercellular communication involves transmission of biologically valid signals directly among cells in contact with one another and, if so, what molecular mechanisms are thereby set in motion? This has been a vexing problem for many years because only indirect approaches such as ionic or metabolic coupling or dye transfer were available for assessing potential intercellular signal exchange (reviewed in references 1, 2). While useful, these studies have not tested the subcellular actions, if any, of intercellular communication (1, 7, 28, 29), which requires following the kinetics of these events at single cell resolution. The data from this study allow us to conclude that hormone-induced intercellular communication does, in fact, initiate a defined biologic response, the dissociation of cAMP-dependent protein kinase and consequent steroid secretion. This event is hormone dependent, bidirectional, and shows temporal kinetics. It requires direct intercellular contact but does not involve cell fusion, and the possiblity of an extracellular route seems extremely remote. This is particularly so in view of the consistent observation that recipient cells, those lacking the appropriate hormone receptor, dissociated cAMP-dependent protein kinase only if they contacted a hormone competent partner. Equally nearby, occasionally closer (Fig. 3, bottom), but noncontacting potential recipients failed to respond to the stimulus. Also, attempts to mimic putative extracellular signals such as cAMP by adding them to the medium gave only negative results.

Identity of the Communicating Channel

The most extensively implicated structural basis for intercellular communication is the gap junction (1-12). In this study, morphological typical gap junctions were observed uniting Y-1 cells and latex bead labeled granulosa cells. Freeze-fracture also revealed intramembrane particle aggregates characteristic of gap junctions (8, 18). However, we were not able to convincingly identify these as occurring between heterotypic partners, as the nonjunctional membrane of Y-1 and granulosa cells are quite similar. Even so, there were adequate numbers of bona fide gap junctions observed between Y-1 cells and labeled ovarian granulosa cells in thin section preparations to assure they were a feature of the co-cultures. This does not indicate that structurally identifiable gap junctions were the only basis for the intercellular signal transfer documented above; although the brunt of current literature suggest this to be the case (1, 4, 5, 7). Other forms of contact-dependent cell interaction were considered. Fusion between Y-1 and granulosa cells seems unlikely for a number of reasons. In experiments in which granulosa cells were prelabeled with rhodamine-derivatized latex spheres, the spheres were never observed in Y-1 cells in contact with granulosa cells. Similarly, the results from protocols using Y-1 cells whose membrane has been labeled with cationized ferritin revealed that even at sites of junction with granulosa cells ferritin had not migrated to the granulosa membrane. These findings indicate an absence of widespread fusion between Y-1 and granulosa cells. They do not, however, eliminate the possibility of other forms of fusion, such as restricted changes in membrane bilayer permeability at points of heterotypic contact that could go undetected in our studies.

Throughout these studies, cultures were monitored with electron microscopy. In no case were viruses or other similar contaminants, which might cause cell fusion, observed.

Overall, it seems that the most logical candidate for a communication channel is the gap junctions connecting Y-1 and granulosa cells. We cannot, however, exclude a contribution by any of the other mechanisms discussed above, excepting organismal contamination. We can, on the other hand, say unequivocally there are *functional* communicating channels between Y-1 and granulosa cells. The *morphologic* basis of these channels can be debated.

There are many interesting features of these observations that may speak to the physiological relevance of intercellular signal transfer as a means of modulating cAMP-dependent protein kinase dissociation. The finding that one-third or fewer of connected granulosa and Y-1 cells dissociated protein kinase in response to FSH stimulation was initially troubling because Lawrence et al. (12) found a high incidence (small standard of the mean with $n = 4$) of FSH stimulated intercellular communication (assessed by increased beat frequency) between ovarian granulosa cells and mouse myocardial cells. The problem was resolved by the autoradiographic protocols done for our study that revealed that about one-third to one-half of preovulatory phase granulosa cells possess FSH receptors. This agrees well with the percentage of granulosa cells that upon exposure to FSH dissociate protein kinase themselves and with time in connected Y-1 tumor cells. It also is consistent with the pattern of I^{125} luteinizing hormone binding by porcine granulosa cells in vitro (32) and rat granulosa cells in vivo (see references 32 and 33) and in vitro (33). Perhaps the difference between the results of Lawrence et al. (12) and those detailed here are due to their using rat granulosa cells whereas we used porcine granulosa cells.

The hormone-induced intercellular signal transfer documented above while it is clearly bidirectional, based on the specific hormone used, may be unequal. This is indicated by the finding that Y-1 cells contacting granulosa cells in FSH-stimulated cultures dissociated significantly less protein kinase than they did in response to ACTH or 8BrcAMP, whereas granulosa cells dissociated significantly more protein kinase when contacting an ACTH responsive Y-1 cell than they did in response to FSH or 8BrcAMP (Table I). A number of interpretations for this effect were considered.

The signal could be attentuated in one direction (towards Y-1 cells) more so than in the other. Two independent lines of evidence suggest this possiblity, presuming signal transfer is mediated by gap junctions. cAMP accumulation has a positive effect on the porosity of gap junctions and on the kinetics of intercellular signal transfer (34). The opposed connexons of gap junctions may not be in perfect register but instead may occasionally be skewed (33). While this conclusion is controversial (34) it does in the context of cAMP actions on gap junctions (32) suggest a means for altering, by phosphorylation-dephosphorylation reaction, the permeability of a communication channel more so on one side than on the other.

It could be that this apparent asymmetry of signal transfer is due to the use of phenotypically distinctive cell types. Conceivably, Y-1 and granulosa cells use different signals with that of the Y-1 cell being a more potent inducer of protein kinase dissociation by contacting granulosa cells than is the signal of granulosa cells when transmitted to Y-1 cells.

While the above possibilities cannot be discarded, the inter-

pretation we most favor is that the signal exchanged between granulosa and Y-1 cells is metabolized. This notion is consistent with the observation that it required 5 min or more for signal transfer to cause detectable protein kinase dissociation (Figs. 3 and 4) in recipient Y-1 or granulosa cells. Yet, when cells are injected with fluorescent dyes abundant probe is transmitted to contacting cells in seconds to a minute (6, 32). Clearly, if in this study the signal were metabolized its intracellular level would represent the difference in the amount generated versus the amount that is degraded, complexed with binding proteins or otherwise sequestered. In this regard metabolism of the signal transmitted would also explain the possible asymmetry of signal transfer. Thus, the small Y-1 cells could generate a greater concentration of signal than the more voluminous granulosa cells and thereby provide a proportionally greater amount of it for intercellular transfer. This would be in accord with the "signal dilution" hypothesis proposed by Lowenstein (2) and with the "sink and source" concept suggested by Sheridan (7).

Contact But No Signal Transfer

One feature of the co-cultures that was quite surprising were the numerous instances in which heterotypic cells were in intimate contact but upon stimulation with hormone signal transfer did not occur. These negative results in no way detract from the clear evidence of protein kinase dissociation as a result of signal transfer. They are, however, of interest. The brunt of these observations were made in co-cultures stimulated with FSH and were of two types. In the first a granulosa and a Y-1 cell contacted by abutment or through slender processes yet after 30 min exposure to FSH neither cell dissociated protein kinase. We feel these cases represented contact of Y-1 cells with granulosa cells lacking hormone receptors as discussed above (Fig. 5). The second category of observation were those in which the granulosa cell dissociated protein kinase to apparently normal levels, whereas the contacting Y-1 cell failed to do so. A reasonable interpretation for these events would be that no communicating channels formed at the point of contact or, if they did exist, they were not patent.

Similar observations were made of co-cultures stimulated with ACTH through to a far lesser degree. This is probably due to the fact that Y-1 is a clonal cell line selected for its sensitivity to ACTH. Thus, most if not all cells would be expected to have available hormone receptors appropriately coupled to adenylate cyclase.

Identity of the Intercellular Signal

The data presented and discussed above make it amply clear that intercellular signal transfer, probably via gap junctions, does have a biologic role the basis of which involves the dissociation of cAMP-dependent protein kinase. This does not imply that the protein kinase mechanism is the only one modulated by direct signal transfer. It is, however, the only one for which there is now evidence.

On the other hand we have not established the identity of the signal being exchanged between Y-1 and granulosa cells, if in fact there is only one such moiety. Others (2, 7, 12), including one of us (8, 21), have previously suggested that cAMP is a likely candidate because of its known regulatory functions and because its size and charge would allow it to permeate junctional channels (2, 3). These channels with an

apparent molecular cut off of ~1,000 mol wt (3), makes it unlikely that free catalytic units or protein kinase holoenzymes could act as signals (40,000 mol wt or more). The only data from this study that hints at the identity of the intercellular signal is that 8BrcAMP and hormone, induced signal transfer with similar temporal kinetics of protein kinase dissociation.

There are other molecules that could function as a signal such as prostaglandins, calcium, polyamines, or a host of alternatives. In fact, it seems likely that more than one signal, working through cAMP-dependent protein kinase, could exist. This might allow a greater diversity of responses. At the moment we are attempting to test some of the alternatives using the contact dependent intercellular signal transfer protocols and cytochemical monitoring of protein-kinase dissociation documentated here.

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