# INTERCELLULAR COMMUNICATION IN THE RAT ANTERIOR PITUITARY GLAND

## An In Vivo and In Vitro Study

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The concept of "stimulus-secretion coupling" suggested by Douglas and co-workers to explain the events related to monamine discharge by the adrenal medulla (5, 7) may be applied to other endocrine tissues, such as adrenal cortex (36), pancreatic islets (4), and magnocellular hypothalamic neurons (6), which exhibit a similar ion-dependent process of hormone elaboration. In addition, they share another feature, that of joining neighbor cells via membrane junctions (12, 26, and Fletcher, unpublished observation). Given this, and the reports that hormone secretion by the pars distalis also involves a secretagogue-induced decrease in membrane bioelectric potential accompanied by a rise in cellular [$Ca^{++}$] (27, 34, 41), it was appropriate to test the possibility that cells of the anterior pituitary gland are united by junctions.

## MATERIALS AND METHODS

### *Electron Microscopy*

Pieces of rat anterior pituitary were processed for electron microscopy using isosmotic solutions (cf. reference 12). Tissues were immersed for 4–6 h in Karnovsky's dialdehyde fixative (19) buffered to pH 7.4 with 0.1 M sodium cacodylate containing 0.05% calcium chloride, then rinsed in buffer containing 4% sucrose. After this, they were postfixed in 2% osmium tetroxide containing 3% sucrose and either dehydrated in a graded series of ethanol or placed for 1–4 h in a solution of 0.5% uranyl acetate and 5% sucrose, after which they were dehydrated. Cultured cells were fixed *in situ* using the dialdehyde described at half strength. They were then briefly rinsed in buffer as described above, postfixed in similarly buffered 1% osmium tetroxide and dehydrated. Tissues and cultures of cells both were imbedded in Epon 812 (Shell Chemical Co., New York).

For some protocols, tissues were exposed to electron-dense extracellular tracers either before or during fixation. Lanthanum was used as described by Revel and Karnovsky (31), potassium pyroantimonate (20) as a 1–2% solution, and ruthenium red according to Luft's method (23). In each case, $Ca^{++}$ was eliminated from buffer. Treatment of cultures with any of these tracers, especially lanthanum, before fixation caused cells to lift free of the substrate; thus, tracers were used only after dialdehyde fixation was begun.

Tissues to be used for freeze-fracture were fixed in dialdehyde for 4–6 h, then passed through progressively increasing concentrations of glycerol buffered to pH 7.4 with 0.1 M sodium cacodylate containing 0.1% calcium chloride, and allowed to stay in the final concentration (20%) of glycerol for 4–6 h. Fracturing at −115°C was done in a Balzers 360-m device (Balzers High Vacuum Corp., Santa Ana, Calif.) at less than $6 \times 10^{-7}$ torr. Replicas were cast with platinum-carbon and stabilized with carbon.

### *Preparation of Cell Cultures*

Anterior pituitary glands were removed from decapitated rats, rinsed in complete culture medium (Dulbecco's) plus 10% fetal-calf serum and 100 μg/ml Gentamicin (Schering Diagnostics, Port Reading, N.J.), and diced into small pieces. The "clean" bits of gland were placed in a sterile centrifuge tube (15 ml) to which 3 ml of enzyme solution (0.1% trypsin-EDTA in balanced salt solution) were added. The contents of the tube were continually stirred. Every 15 min, agitation was stopped and the dispersed cells and enzyme solution were removed, and fresh enzyme solution was added and stirring restarted. The suspension of freshly disaggregated cells was centrifuged for 5 min at 500 *g* and the cell pellet resuspended with 1 ml of complete culture medium. An

aliquot of this pellet was used to obtain an estimate of cell number and viability (trypan blue exclusion). Cells thus disaggregated were seeded into sterile plastic petri dishes and placed in a 37°C chamber, gassed with 95% $O_2$:5% $CO_2$. The cells were allowed to incubate for 4 days, by which time they had firmly attached to the substratum and flattened slightly.

### *Assessment of Electrotonic Coupling*

Intercellular electrotonic coupling measurements were carried out on an inverted phase microscope with Leitz micromanipulators (E. Leitz, Inc., Rockleigh, N. J.) The cultures were maintained at 35–37°C on the microscope stage, and a thin layer of mineral oil spread over the medium prevented desiccation. A soft, continuous flow of 95% $O_2$:5% $CO_2$ was maintained over the mineral oil. Pyrex microelectrodes were filled with 3 M KCl, and only those with tip resistances of 30–50 MΩ were used. One electrode was attached to a modified Wheatstone bridge circuit, and the second electrode was used for monitoring electrotonic potentials in the adjacent cell. Microelectrode resistance was checked before and after recording from cells by monitoring balance for the bridge electrode and by a brief voltage ramp to the voltage-sensing electrode.

## RESULTS

Extensive examination of anterior pituitary tissue revealed that some endocrine cells were joined by gap junctions. In thin sections the contact regions usually extended for less than 0.2 μM (range ca. 0.1–0.25 μM) and consisted of apposed plasma membranes separated by a 3–5-nm intercellular cleft (as in Fig. 1). When filled with electron-opaque tracers, this "gap" appeared wider due to staining of the outer aspects of subjacent membranes. In some instances the intercellular cleft broadened to ca. 6 nm, but reclosed quickly. However, in other cases the entire intermembrane distance was this dimension. Occasionally, functional characterization of both partners contributing to the junction could be made using established ultrastructural criteria (8, 15, 16, 25). In these cases, mammotropes (cells producing prolactin) were the prevalent cell type joined by gap junctions while such contacts were less frequent between somatotropes (producing growth hormone), gonadotropes [producing either luteinizing hormone (LH) or follicle-stimulating hormone (FSH)] or thyrotropes (producing thyroid-stimulating hormone). More often than not, only one cell of a conjoined pair could be characterized, usually because the junction occurred between the body of one cell and a sometimes deeply invaginating process of a distant, unidentifiable, partner. In other instances, two cell processes, neither of which contained secretion granules, were joined by a gap junction. Whether these events represented contact between folliculo-stellate cells, corticotropes (producing ACTH) or chromophobes (resting endocrine cells? [15, 16]) is uncertain, as each of these pituitary constituents can send out undifferentiated processes. As described by various authors (8, 15, 16), secretory cells often were united homologously[1] and heterologously by desmosomes and intermediate junctions (9).

Freeze-fracture of the gland efficiently revealed the diminutive gap junctions (Fig. 2). When replicated, they appeared as macular, sometimes oblate, regions of apposed cell membranes specialized by polygonally arrayed 8–9-nm particles associated with the "*A*" fracture face[2] and similarly arranged depressions in the "*B*" face. While this procedure unveiled a number of gap junctions, it was seldom possible to identify both contributors because one of them was usually removed by the fracturing process. Mammotropes and gonadotropes could be readily distinguished and differentiated from nonendocrine follicle cells whose surfaces were specialized by microvilli. However, somatotropes and thyrotropes, while easily found under certain experimental conditions (Fletcher and Everett, unpublished observations) were less conspicuous in the basal-state preparations used for this study. Thus, the junctions possibly uniting these latter cell types could not be verified by freeze-fracture.

Besides these authentic gap junctions, numerous loose aggregates of 11–11.5-nm particles were routinely seen on replicas of the *A* fracture face relatively devoid of the ubiquitous, scattered 6–8-nm intramembrane particles (inset, Fig. 2). Except for the absence of a filamentous component, these clusters of large particles are reminiscent of the freeze-fractured desmosomes found in cervical epithelium (cf. reference 24). Also, the frequency with which they were encountered here was comparable to that of the desmosomes seen in

[1] The terms homologous and heterologous are used to describe the secretion product, not the embryonic origin of the endocrine cells.

[2] The complementary intramembrane faces exposed by splitting within the hydrophobic bilayer (3, 29) are conventionally labeled "*A*" face, the leaflet associated with cell interior, and "*B*" face, the leaflet adjacent to extracellular space.

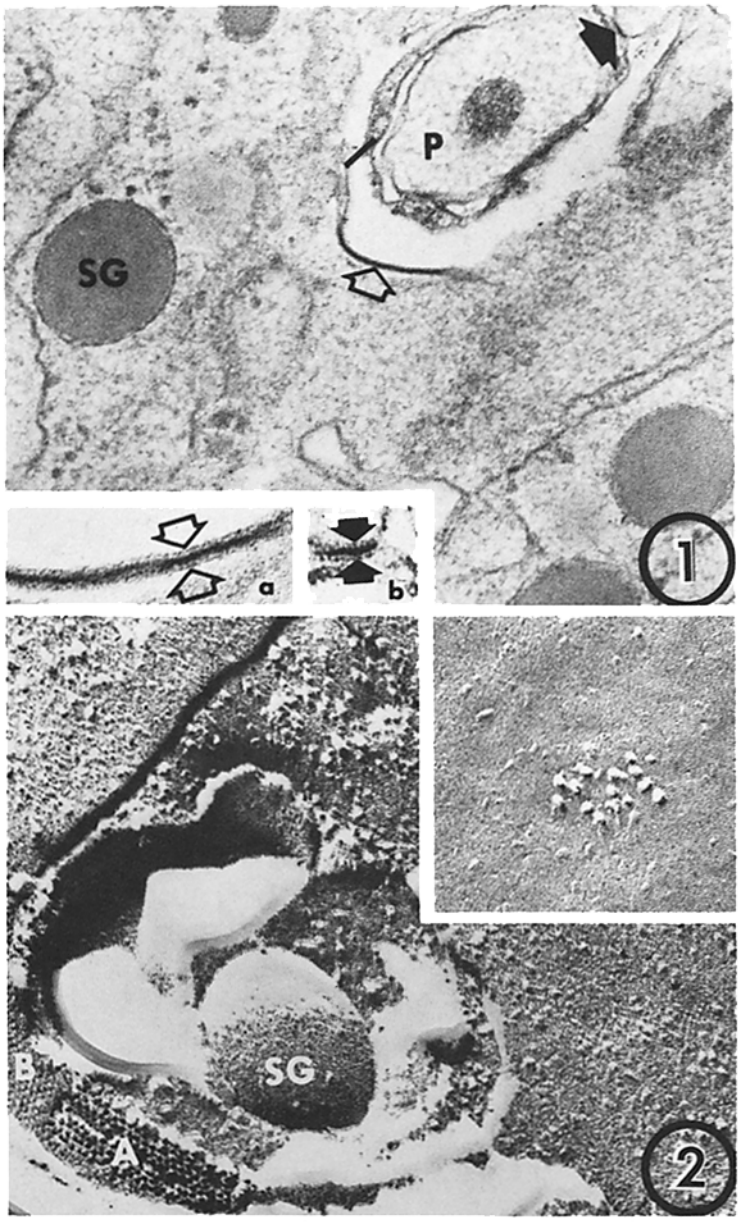


FIGURE 1 Principal figure, × 45,000. 4-day culture. A gonadotrope containing secretion granules (*SG*) varying from 200 to 400 nm in size has formed a gap junction (*open arrow*) with a process (*P*) of a neighboring cell which in turn joins another cell by a similar though smaller junction (*solid arrow*). This is the only observation of three cells bridged by gap junctions. The central process has withdrawn slightly (*black line*), and the junction has split asymmetrically, remaining with one partner. Inset (*a*), × 140,000. Higher magnification of the larger junction in the principal figure. The specialized region (*open arrows*) consists of apposed membranes separated by a 4-nm cleft, here filled with potassium pyroantimonate. This tracer does not seem to stain nonjunctional membrane. Inset (*b*), × 140,000. At the smaller of the two junctions, the contact region has not divided; however, the intercellular space has widened to ca. 6 nm at the solid arrows, but remains at 4 nm to the left of that.

FIGURE 2 Principal figure, × 100,000. Intact anterior pituitary. A fortuitous fracture has revealed the *A* and *B* faces of a gap junction, then dipped to the cell interior and exposed a nearby secretion granule (*SG*). Judged by granule size (300 nm), this appears to be a gonadotrope joined to an unidentified neighbor. The center of each *B*-face depression has a 2-nm diam elevated stub. Inset, × 100,000. Intact anterior pituitary. A commonly seen region of fracture face A relatively devoid of 6–8-nm particles but containing a loose cluster of 11–11.5-nm particles.

thin sections of the pituitary. Even so, since desmosomes do not seem to always involve modification of the membrane interior (13) and do not occupy obligatory positions between secretory cells of the anterior pituitary, this interpretation must remain provisional.

After 4 days in culture, the pituitary cells uniformly appeared healthy, and occasional mitoses were seen. Prolactin cells were typically (8, 15, 16) hyperactive, yet the relative numbers of this and other cell types was not remarkably changed from those found 90 min after their dispersion. These observations are consistent with the report of Hopkins and Farquhar (17), except that by using trypsin and neuraminidase to disaggregate the gland, they reduced cell losses during the 1st day to "less than 10%" whereas our losses appeared to be slightly higher. Fibroblast contamination was not a problem in these short-term cultures, but in the few preparations maintained for up to 3 wks increasingly greater numbers of agranular, fibroblast-like cells were found. As shown in Fig. 1, gap junctions, presumably destroyed during cell disaggregation, had reformed such that 4 days later they could be readily identified and were indistinguishable from the junctions found in intact glands. Commonly, cells retracted from one another during preparation of the cultures for electron microscopy, particularly in those cases where extracellular tracers were used. When this occurred, the junction did not divide in the intercellular space but withdrew intact and remained with one partner. Similar asymmetric splitting of gap junctions has been observed after enzyme dispersion of liver cells (2) and may well be a common feature of in vitro preparations (19), at least in those cases where the methods of tissue disaggregation and cell maintenance described here are used.

Electrophysiological studies of similar cultures revealed that endocrine cells (judged by their containing numerous refractile granules when viewed with phase optics) are ionically coupled (Fig. 3). In 6 of 14 cases, current pulsed into one cell (V1) resulted in an electrotonic potential in the adjacent cell (V2) which was only slightly attenuated (V2/V1) ca. 0.90–0.94). In five cases the cells were not coupled, and in three instances the bridge balance after otherwise successful impalements was low. No systematic analysis of resting membrane potentials was made, although they were generally low (ca. −10 mV) and negative relative to the external bath potential. These potentials are consistent with those reported by York et al. (41), except that they found numerous instances of positive inside potentials.

## DISCUSSION

We have shown that endocrine cells of the intact and disaggregated rat anterior pituitary gland are united by gap junctions. Both in vivo and in vitro, the thin section appearance of these membrane specializations conformed to the structural criteria established by Revel and co-workers (31; also see 32) and which have been repeatedly confirmed

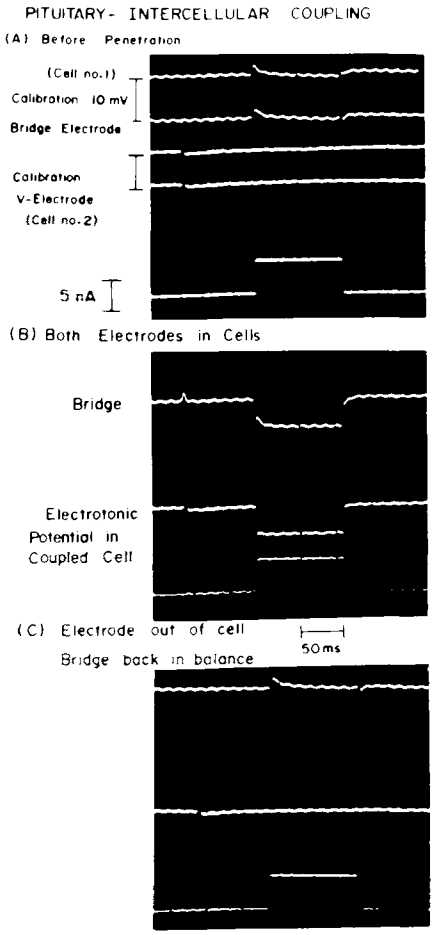


FIGURE 3 Representative record of electrical coupling in a 4-day-old culture of pituitary cells. (A) Current and voltage calibrations with both electrodes positioned in the bath near cells to be impaled. (B) Electrodes in adjacent cells demonstrating that current injected into cell 1 produces an electrotonic potential change in the adjacent cell 2. (C) Bridge electrode withdrawn from the cell to a position just outside. Under these conditions the current pulse produces no potential change in cell 2.

since then (cf. references 13, 24, 35). At the pituitary junctions, potassium pyroantimonate and lanthanum are sequestered in the intercellular gap and stain the outer leaflets of adjacent membranes. This may indicate, as it does in other tissues (12, 20), that polyvalent cation-binding sites face the extracellular space differentiated by these intimate appositions. Freeze-fracture of anterior pituitary tissue has established these structures as true gap junctions in that they involve extensive modification of the membrane interior. In replicas the junctions appeared as packed aggregates of intramembrane particles on the *A* face and similarly arranged depressions into the apposing *B* membrane leaflet. In the pituitary, the size range of particles (8–9 nm) is similar to that found in other tissues (cf. references 12–14, 38) although it is slightly larger than the 6–7-nm dimensions described for the gap junctions (nexus) of cardiac muscle (cf. reference 24). This variation is most likely due to differences in the thickness of replicas used, a problem which has been considered in detail by McNutt and Weinstein (24).

The combined morphological evidence retrieved by these two preparative methods clearly demonstrates that cells producing the same tropic-hormone (homologous cells) can contact one another via gap junctions. The data from sectioned material indicate that the mammotropes, gonadotropes, somatotropes, or thyrotropes, and possibly other types of secretory cells, are united by this means. For reasons discussed in Results, only the junctions between mammotropes or gonadotropes could be verified with the freeze-fracture method, although it is likely that the junctions between other cell types are equally authentic. Recently, Hopkins and Farquhar (17) mentioned that "shallow gap junctions" are found between secretory cells of the rat adenohypophysis; however, their observations were confined to thinly sectioned material, and the authors did not indicate the functional categories of cells united by these specializations. Even so, when considered in the light of the detailed descriptions given here, the findings of Hopkins and Farquhar essentially agree with ours.

In an effort to establish whether or not cells of the adenohypophysis could communicate ionically pooled anterior pituitaries were enzymatically disaggregated and the harvested cells maintained in culture. While the preparative methods employed here differed slightly from those used by Hymer et al. (18) and Hopkins and Farquhar (17), the viability and percentage of cells retrieved from glands were similar in all three cases. In addition, the morphology of cells 90 min after dissociation compared favorably with that described by Hymer et al. and Hopkins and Farquhar, indicating that the functional competence of preparations used for this study was also comparable. Because the cells were small (ca. 8–10 $\mu$M) and often lysed after introduction of a microelectrode, many attempts to impale adjacent cells were abortive. In those cases where valid, repetitive measurements of direct intercellular current passage were made, about 40% of the cell pairs tested were electrotonically coupled. This frequency of ionic communication in these heterotypic cultures is consistent with the finding of gap junctions only between homologous cells. If heterologous pairing was widespread, more, if not most, cells should have been coupled. This is clearly not the case. It is conceivable that junctions form only during certain functional states. This might also explain the existence of coupling in less than half the cell pairs; however, we have no evidence to support this notion. The possibility that coupling involved nonsecretory cells is considered unlikely for two reasons: (*a*) only those cells with numerous, refractile granules were impaled with microelectrodes, which could be expected to eliminate the agranular follicle cells and (*b*) a contribution by fibroblasts, which do couple ionically and metabolically via gap junctions (14) can be partially discounted for reasons given in (*a*). Also, fibroblasts were infrequently seen and could not, on a basis of random distribution, account for the high rate of microelectrode impalements resulting in positive coupling. Therefore, when this is taken together with the morphological evidence already considered, it seems likely that the direct intercellular coupling is between tropic-hormone-producing cells and, in view of comparable data from other systems (1, 13, 14, 22, 24, 35, 37, 38), this communication is probably mediated by the gap junctions described here.

Before we arrived at this conclusion, the possibility was considered that the route of intercellular communication was established by other forms of cell-to-cell contact. No gross continuities such as cytoplasmic bridges (10) united secretory cells in vivo or in vitro, and the number of such structures that might exist between early postmitotic cells would be very small, given the low incidence of cytokinesis observed. The desmosomes and intermediate junctions which, like the gap junctions, reform after pituitary disaggregation have long

been known to unite adenohypophysial cells (8, 15, 16). However, neither of these types of contact has been shown to play a role in the cell-to-cell exchange of molecules (1, 13, 14, 22, 24, 35). Finally, the loose aggregates of 11–11.5-nm *A*-face particles unveiled by freeze-fracture could represent a novel type of junction, rather than desmosomes as suggested in the Results section. This is especially so since such modification of the membrane interior could correspond to those "gap junctions" which had an excessively wide intercellular cleft (ca. 6 nm). Similarly unusual gap junctions have been reported in intestinal epithelium (38), vertebrate retina (30), and crayfish axon (28) and have been recently reviewed by McNutt and Weinstein (24) who categorize them as "large subunit gap junctions." In contrast to each of the occurrences indicated above, the clusters of large intramembrane particles in the anterior pituitary gland lack a complementary *B*-face image. As the *B*-face impressions revealed by freeze-fracture seem to be an invariable component of junctions thought to mediate intercellular communication (13, 24, 35), the absence of this feature suggests that the aggregated large particles are not a true junction and most likely do not subserve electrotonic coupling.

## FUNCTIONAL IMPLICATIONS

It seems likely from the data already discussed that homologous endocrine cells of the anterior pituitary gland when joined by gap junctions can act as a functional syncytium. Even so, in view of the instances where these membrane specializations united gonadotropes which could not be characterized as exclusively producing LH or FSH, the possibility that heterologous intercellular junctions occur cannot be discounted. This inability to determine the mode of intercellular communication in the gland, however, does not obscure the findings of gap junctions and electrotonic coupling documented here. These observations, together with the wealth of literature devoted to anterior pituitary function, allow us to discuss the potential importance of intercellular communication to the gland as an endocrine organ.

The demonstration that disaggregated anterior pituitary cells are coupled electrically strongly supports the proposition that they share a common intracellular space, at least in terms of inorganic ions. In view of this, it is possible that activated cells united homologously or heterologously with neighbors via gap junctions may act, respectively, as sinks or sources for the ionic requirements of the secretory process. Thus, secretion by a cell responding to its specific stimulus could be facilitated by $Ca^{++}$ (18, 27, 33, 34, 41) or other ions from contiguous neighbors.[3] While we have not shown in this study that pituitary cells exchange larger molecules, this would be consonant with the findings of others that cells coupling ionically and joined by gap junctions also cooperate metabolically (1, 13, 14, 22, 24, 39). Given this, it is conceivable that when a releasing factor binds to a competent tropic-hormone cell it initiates the production or activation of some "secretion signal" that is capable of passing to adjacent, homologous cells. This would have the advantage of retaining the apparent specificity of releasing factors and allow a way for their action to be amplified and evenly distributed throughout the gland. Supporting this contention is the report by Rubin et al. (33) that, in adrenal cortical cells which are extensively united by junctions (12), ACTH initiates the production (unmasking?) of a labile, intracellular factor responsible for stimulating steroid synthesis, even after ACTH has been withdrawn. They speculate that this factor could be $Ca^{++}$, prostaglandin, messenger RNA, or cyclic 3′-5′ adenosine monophosphate (CAMP). In the anterior pituitary gland, intercellular transfer of such a factor generated by action of secretagogue may well be mediated by the specialized junctional membrane areas described here. Sheridan has proposed a similar scheme for the brown fat cells that he demonstrated were electrotonically coupled (37). To the extent that his model applies to the pituitary, CAMP passed between cells united by gap junctions may assume a similar role in the normalization of releasing factor information among homologous cell types.

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[3] Perhaps related to this is the finding that, in the central nervous system, glial cells act as ion sources for neurons (40), to which they can join via gap junctions (11) and certain, more elaborate, intermembrane specializations (21).

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