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HETEROGENEOUS DISTRIBUTION OF ECTO-Ca-ATPases IN PRIMARY CULTURES OF HUMAN ADENOHYPOPHYSEAL CELLS

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Abstract

The aim of this study was to investigate the localization of ecto-Ca-adenosine-triphosphatase (ecto-Ca-ATPase) in different parenchymal cells of the human pituitary in tissue culture. The distribution of ecto-ATPases on the surface membrane of a particular parenchymal cell varied with the type of cells in contact with this parenchymal cell; the membrane portions immediately exposed to the medium showed low if any ecto-ATPase activity. These results suggest that ecto-Ca-ATPases of the parenchymal cells may be involved in cell adhesion processes and may be of crucial importance in the organization (*in vivo*) and reorganization (*in vitro*) of human adenohypophyseal tissue.

Key Words: Ecto-Ca-adenosine-triphosphatase (ecto-Ca-ATPase), cell-cell adhesion molecule (CCAM), growth hormone cell (GH cell), prolactin cell (PRL), human adenohypophysis, tissue culture.

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Introduction

Heterogeneous distribution of surface domains is a well-known characteristic feature of several normal cell types (e.g., Somosy *et al.*, 1989; Wild and Schraner, 1990; Scheetz and Dubin, 1994). We have previously shown that there are differences in the distribution of Ca-dependent ATPase in the plasma membrane of the adenohypophyseal parenchymal cells in rat (El-sherif and Bácsy, 1989) and this was confirmed by Soji *et al.* (1991). According to several authors, there are some cell adhesion molecules [cell-cell adhesion molecules (CCAMs), and neural adhesion molecules (NCAMs), which show ecto-ATPase activity (Lin *et al.*, 1991; Cunningham *et al.*, 1993; Dzhandzhugazyan and Bock, 1993; Murphy *et al.*, 1994; Sawa *et al.*, 1994). We decided to examine whether any correlation can be found between the localization pattern of ecto-ATPase activity and the association of the different cell types of the adenohypophysis. In order to eliminate technical problems arising from the original compact tissue structure, we used a primary culture of dispersed human adenohypophyseal cells.

Materials and Methods

Cell cultures from an adult human adenohypophysis were prepared essentially as described by Bácsy *et al.* (1992). After pre-embedding localization of the Ca-ATPase activity, combined immunocytochemical demonstration of growth hormone (GH) and prolactin (PRL) was carried out on ultrathin Araldite sections.

Tissue culture

Human hypophyses were dissected out within 2 hours post mortem under sterile conditions, with permission of the Ethics Committee of the Hungarian Council of Medical Sciences. The organ was transported to the tissue culture laboratory in cooled (4°C) Medium 199 (about 15 minutes after tissue removal). After rinsing in a new volume of Medium 199 with about 1% (v/v) Neomycin (Sigma, St. Louis, MO, USA) solution (10 mg/ml in 0.9% NaCl) added, the adenohypophysis was

dissected free of the capsule and extraneous tissues, cut in small pieces, washed in Ca,Mg-free Tyrode's solution (Reanal, Budapest, Hungary), and incubated overnight in 0.25% trypsin (Difco, Detroit, Michigan, USA) in the refrigerator. (Difco Trypsin was dissolved in Ca,Mg-free Tyrode's solution and Millipore-filtered at 0.22 μm.) The next day, the pieces, in the enzyme solution, were placed on a magnetic stirrer at 37°C. Every 15 minutes, the cell suspension was collected, and an equal volume of Medium 199 supplemented with 20% fetal calf serum, 20% of 1.2% D-glucose (Reanal, Hungary) solution, and 1.5% of 0.1 M HEPES (Gibco, Paisley, Scotland) was added to stop digestion. The remaining tissue pieces were digested further with new trypsin solution. Each fraction of the cell suspension was centrifuged (800 rpm, 10 minutes), and the pelleted cells were resuspended in the supplemented Medium 199. Five-six fractions of 10 ml each could be collected. The cell suspension was explanted in 8-well plastic chamber slides (Nunc, Naperville, IL, USA) with 70,000 cells in 0.1 ml medium. The medium was changed every second day. The cultures were kept in an ASSAB incubator in an atmosphere of 5% CO_2 and 95% air for 7-8 days. Cell viability was checked by the Trypan-blue exclusion test.

Ca-ATPase enzyme cytochemistry

For Ca-ATPase cytochemistry we used a cerium-precipitation method as described by Kittel (1994). The cells were fixed in the plastic chamber with 3% paraformaldehyde (Merck, Darmstedt, Germany), 0.5% glutaraldehyde (Merck) and 0.25 M sucrose in 0.05 M cacodylate buffer (pH 7.4) for 30 minutes at 4°C in the chamber slides. After washing in 0.05 M cacodylate buffer with 0.25 M sucrose (pH 7.4) for 3 x 10 minutes, the cells were incubated in a medium containing Tris-maleate buffer (70 mM, pH 7.2), ATP (1 mM), $CaCl_2$ (3 mM), $CeCl_3$ (2 mM), $MnCl_2$ (5 mM) and levamisole (Janssen, Beerse, Belgium) (5 mM) for 30 minutes at room temperature. Following rinses (3 x 10 minutes) in the buffer, pre-embedding counterstaining with 2% uranyl acetate in 70% ethanol for 20 minutes, and finally, dehydration in graded ethanol and embedding in Araldite 6005 were carried out in the original wells. The resin blocks were detached from the plastic slides and cut with a diamond knife in an ultramicrotome parallel with the surface. The following controls were applied to check the specificity of the reaction product: (a) $CaCl_2$ was deleted and 10 mM ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N-tetraacetic acid (EGTA) added to the reaction mixture; (b) ATP was omitted from the incubation medium; and (c) In order to confirm that the reaction product did not represent Na,K-ATPase or the Ca pump, parallel experiments were carried out in the presence of 1 mM ouabain (inhibitor of Na,K-ATPase) or 300 μm suramin (Ca pump inhibitor).

Immunocytochemistry

The ultrathin sections mounted on nickel grids were floated on a drop of water for 5 minutes and for another 5 minutes on 0.02 M phosphate buffer saline (PBS), 2 x 5 minutes and PBS with 0.2% gelatin to block non-specific protein-binding sites. The incubation for the first hormone, human GH or PRL (one hour), was carried out carefully on one side of the grid with a rabbit antibody [supplied by Bio Genex Laboratories (Dublin, California) in the final recommended dilution), this side of the sections rinsed in PBS for 3 x 5 minutes, followed by the incubation with 15 nm gold particles coated with goat anti-rabbit IgG (Auro Probe TM EM GAR IgG 15, Amersham, U.K.) for 90 minutes, rinsed with PBS, then with bidistilled water, and air dried. The same procedure was repeated on the other side of the grid with the other primary antibody and the 30 nm gold probe (Auro Probe TM EM GAR IgG 30). Finally, the sections were briefly counterstained with uranyl acetate (12 minutes) and lead citrate (1 minute) before examination in the transmission electron microscope (Hitachi 2001) operated at an accelerating voltage of 80 kV. In some instances, monolayer cell islets, detached from their plastic substrates, were re-embedded in Epon so that sections perpendicular to the surface can be made.

Results

Ca-ATPase reaction product was seen between the membranes of numerous parenchymal cells and folliculi-stellate cell processes. Large free cell surfaces were either free of reaction product, or the reaction intensity was appreciably lower than between the contact surfaces. The overview of cell groups suggests that the intensity and pattern of enzyme activity depends on the adjacent cell types, and that they may vary along the membrane of the same cell (Figures 1, 2 and 3). If calcium ions were eliminated from the cells and from the ATPase medium the amount of reaction product was reduced to traces. In the absence of ATP, no cerium precipitate was formed. Neither suramin nor ouabain modified the intensity or pattern of the reaction. Folliculi-stellate cell processes usually display moderate enzyme activity at their free surfaces, fibroblasts have low if any activity at their surface bordering on the culture medium or other cells and were free of reaction product towards the bottom of the plastic dish (Figure 1). At least two morphologically different types of GH cells were observed in this culture: one polygonal with secretory granules filling most of the cytoplasm (Figures 3, 4 and 5) and

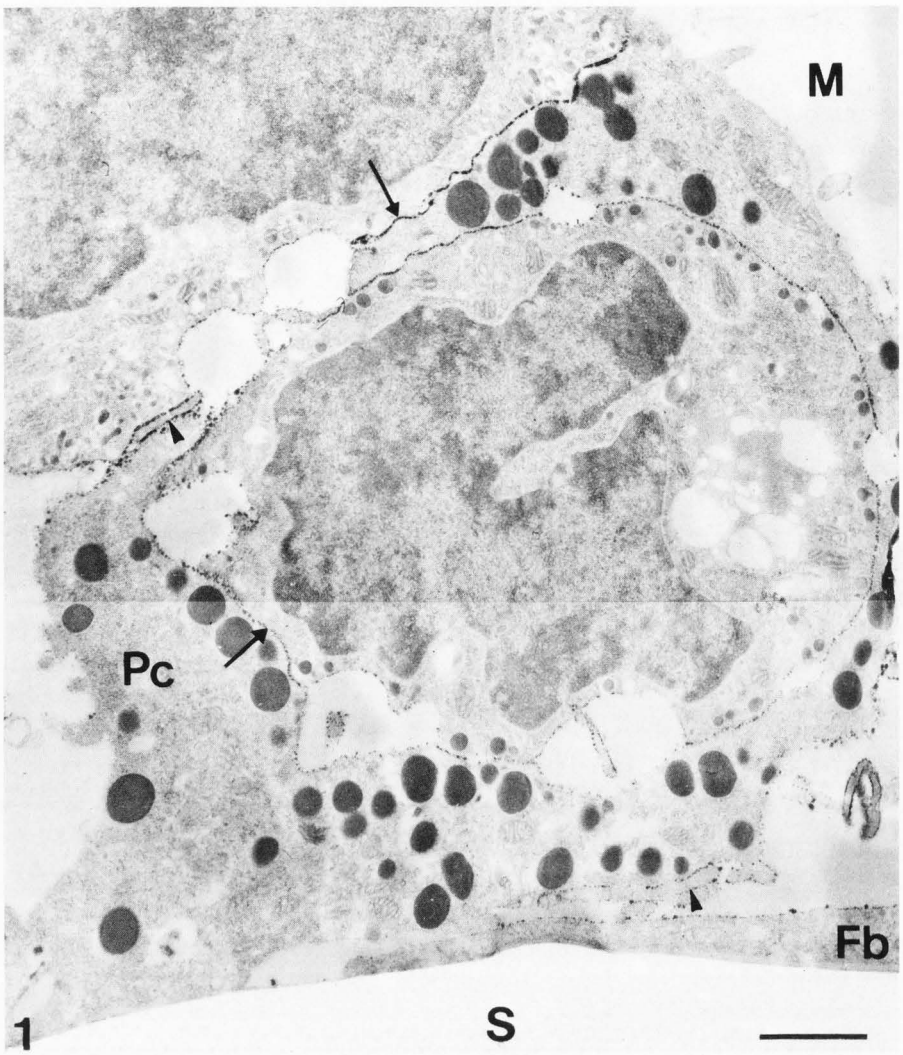
another type with a columnar shape with smaller secretory granules lining the surface membrane (Figure 4). Very often, the continuously free surfaces of the polygonal type were free of ATPase related reaction product while the intercellular cleft adjoining contacting cells could contain a heavy reaction deposit (Figures 1 and 3). If the two different GH subtypes are in contact with the precipitate, or if other cell types, e.g., prolactin containing cells, adjoin the precipitate, it is usually abundant (Figure 6). In the space between two polygonal GH cells, the reaction may be absent (Figure 5). The columnar GH cell may have a high ATPase activity even on its free surface (Figure 4). In the short unattached intervals of the cell membrane between two contact parts, the pattern of the deposition is not changed (Figures 1 and 3). Typical prolactin cells with very regular round secretory granules were rich in ecto-ATPase activity, and some of this activity was maintained even at surfaces not touching any other cells (Figure 6). In this particular culture co-localization of GH and PRL was not frequently observed; the occurring mammosomatotrophs bore the morphological and enzyme-cytochemical characteristics of the polygonal GH cells.

Discussion

Ecto-ATPases are glycoproteins that hydrolyze nucleotide triphosphates, and their active site is located on the extracellular side of the cell membrane. The presence of these enzymes has been shown in a variety of animal and human tissues (Banerjee, 1981; Pearson, 1985). The physiological roles of these enzymes are not completely understood but it is very likely that they are particularly important in cells that secrete ATP or cells that are activated by ATP; that means ecto-ATPases may be involved in the regulation of intercellular processes controlled or modulated by extracellular ATP. Recently, numerous enzymes which previously were suggested to be Ca- or Ca,Mg-ATPases involved in the regulation of the intracellular calcium level, have been identified as ecto-ATPases. It has also been shown that some ecto-ATPases belong to the immunoglobulin superfamily (e.g., Aurivillius *et al.*, 1990; Lin *et al.*, 1991). Some authors claim some NCAM and CCAM molecules to be similar or identical to ecto-ATPases (Lin *et al.*, 1991; Cunningham *et al.*, 1993; Dzhandzhugazyan and Bock, 1993; Murphy *et al.*, 1994). Kirley and coworkers, on the other hand, deny that either T-cadherin, an adhesion molecule, or NCAM would be identical with the suggested ecto-ATPase (Stout *et al.*, 1994; Stout and Kirley, 1994). The situation is complicated because of the lack of specific and selective inhibitors of ecto-ATPases. Although ecto-ATPase molecules have been purified from only a few tissues, it seems quite likely that different forms exist in different tissues. Thus, it is also likely that a universal specific ecto-ATPase inhibitor will never be found (Ziganshin *et al.*, 1994). In order to assess details of the cytochemical distribution of ecto-ATPase activity on human adenohypophyseal cells in culture with the hope that some conclusion can be drawn concerning the function of this enzyme in some pituitary cells, we identified, immunocytochemically, growth hormone and prolactin secreting cell types simultaneously with enzyme localization. We found two morphologically different types of GH cells (Figures 3-5, and Figure 4) and PRL cells (Figures 2 and 6). Our findings revealed marked correlation between ecto-ATPase activity and the type of parenchymal cells with which a given cell type is connected. For instance, the polygonal type GH cell showed strong ecto-ATPase activity in contacts with prolactin cells and other kinds of parenchymal cells (Figures 1, 2, 3 and 6) but the deposit could be absent between two cells of the same type. On the other hand, a heavy reaction product was visible on the membrane part of both GH cells in contact with another, non GH positive cell (Figure 5). Free surfaces of parenchymal cells were usually weakly or not labelled (e.g., many PRL cells, Figures 1, 2, 6). The only elements that displayed uniform enzyme activity on their surface, were the folliculi-stellate cells.

Our findings strongly suggest that ecto-ATPases can be involved in cell-cell contacts between parenchymal cells of the human adenohypophysis. The ecto-ATPase of folliculi-stellate cells seems to have a different character. According to some authors, the surface ATPase activity of these cells in several species may be of importance in the regulation of extracellular calcium concentration (Bambauer *et al.*, 1985; Perryman, 1989; Peute *et al.*, 1990). The heterogeneous distribution of several enzymes in membrane domains of some cell types may be connected to cell polarity (e.g., Wild and Schraner, 1990; Rodriquez-Boulan and Powell, 1992; Murphy *et al.*, 1994). We think, however, that in the case of the pituitary parenchymal cells, the differential distribution of ecto-ATPase activity does not depend on the inherent property of single cells but is a result of the interaction between cells. The special role of this type of ecto-ATPase may thus be connected with the association of cells. A variety of cell associations is a characteristic feature of the intact adenohypophysis, enabling an intricate mutual short-distance modulation of the secretory activity. Cell contacts may also have significance in the reassociation of the dispersed cells *in vitro*.

The present study strongly supports the possibility that these ecto-ATPases may play a specific role in the cellular organization of the anterior pituitary tissue, and may settle the dispute as to whether ecto-ATPases in the human adenohypophysis are cell adhesion molecules.



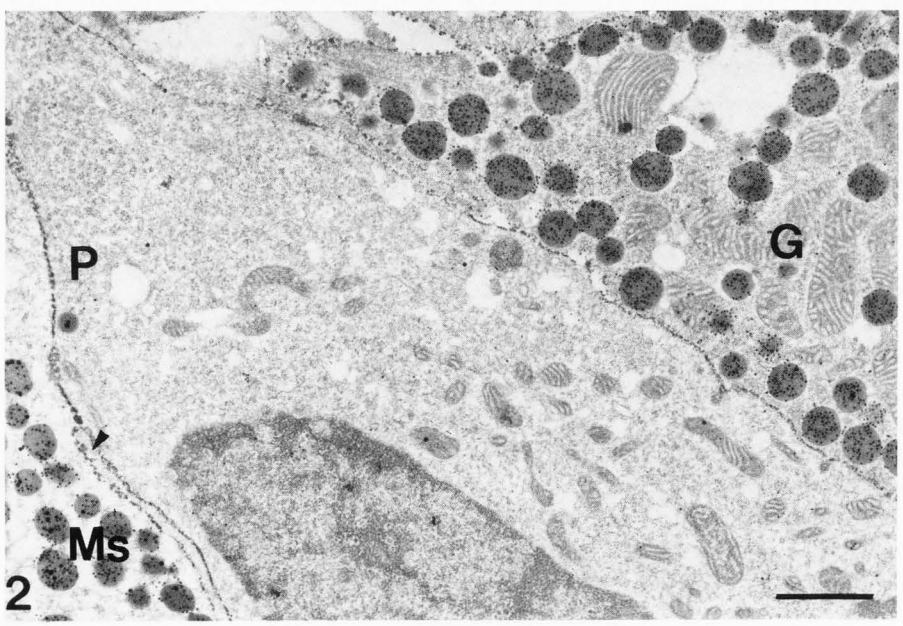
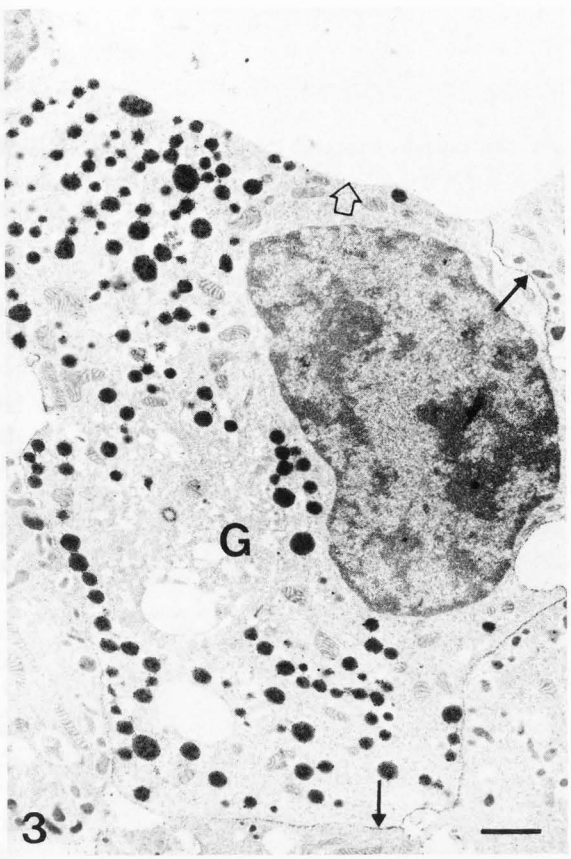


Figure 1. Ultrathin section perpendicular to the culture plane. Incubation for ecto-ATPase activity, uranyl acetate and lead citrate counterstaining. Fb = fibroblast, Pc = parenchymal cell, arrowhead: folliculi-stellate cell process, arrows = cerium precipitate on and between contact surfaces, S = where the plastic support used to be located, M = culture medium. Bar = 1 μm.

Figures 2-6. Combined immuno- and enzyme-cytochemical electron micrographs. Large (30 nm) gold particles label PRL, small gold particles (15 nm) GH, amorphous cerium precipitate indicates ecto-ATPase activity. The sections were lightly contrasted with uranyl acetate and lead citrate, except for Figure 4. G = GH cell, P = PRL cell, MS = mammosomatotrop, arrowhead = folliculi-stellate cell process. Bars = 1 μm. **Figure 2.** A scarcely granulated PRL cell between a GH and a mammosomatotropic cell. **Figure 3.** Polygonal GH cell with ATPase positive contact surfaces (arrows). The free surface (empty arrow) is free of reaction product. **Figure 4.** Two subtypes of GH cell. **Figure 5.** Two GH cells and a non-identified cell. Cerium deposit between the heterogeneous cell types. Arrow = ATPase-negative border between the two GH cells. (No contrast staining in section). **Figure 6.** Regular PRL cell contacting a GH and a non-identified parenchymal cell. Arrows indicate different localization patterns on the three cell types.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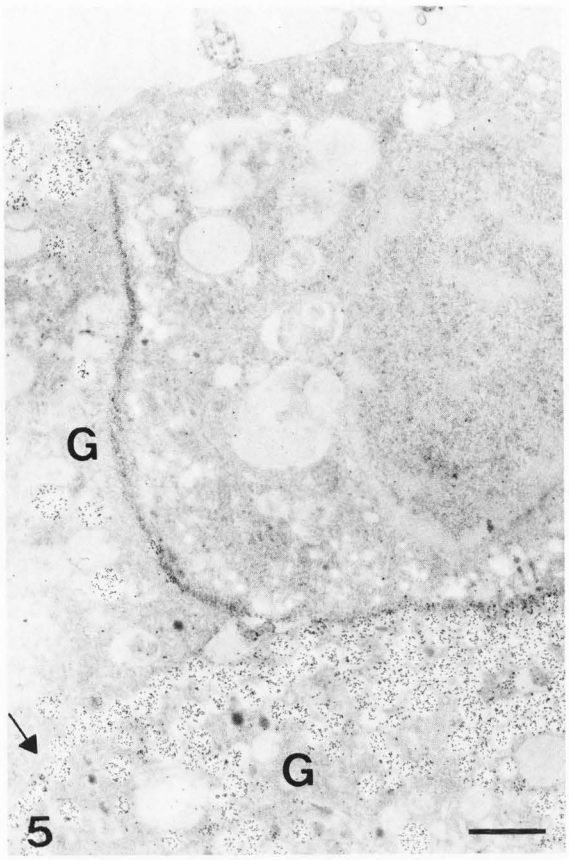
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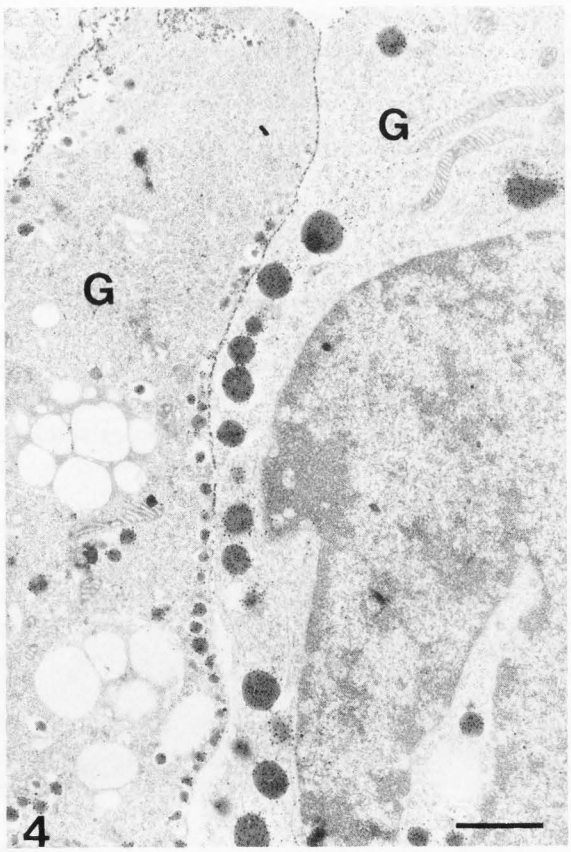
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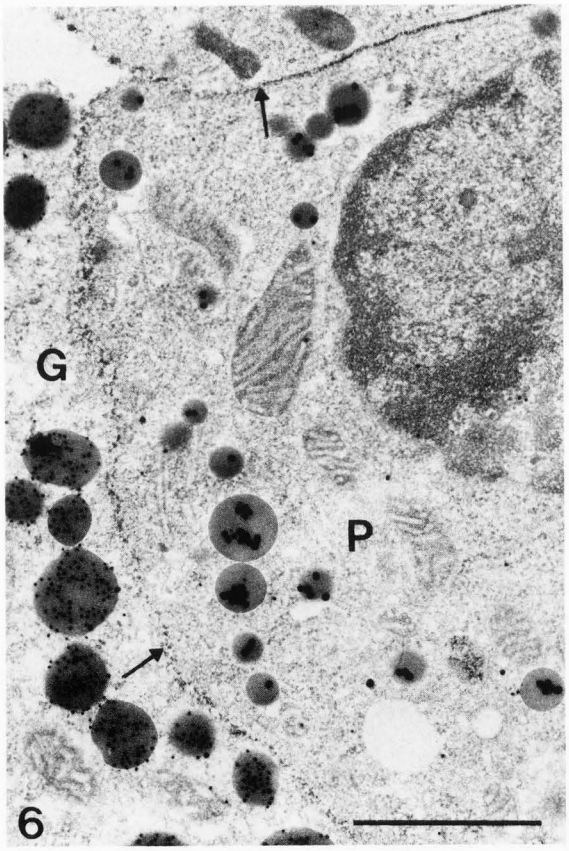
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Discussion with Reviewers

W.C. de Bruijn: How sure are the authors that the relative heterogeneity of the distribution can be objectively determined? In a situation as described, can this relative difference be clearly discriminated? When we assume that each single membrane is ideally contributing with a same number of reaction sites (e.g., 50 sites/nm), can the authors be sure to observe the difference between (100 sites/nm) positions where two membranes are opposed to each other and those where that distance has increased (to larger than, e.g., 25 nm) and the relative number of sites has fallen below the expected 50 sites/nm? This is especially important, because, in cultured cells, the chance to have truly cross-sectioned membranes is relatively low as compared to the situation in tissue.

Authors: This question concerns the philosophy of the

quantitative character of the reaction product under different experimental conditions. To answer the first part of the question, it is clear that a relative quantitative statement based on a qualitative evaluation can only be made if the differences are of order(s) of magnitude and are very reproducible. We feel that this is the case between some contact and free surfaces. We agree that in surface-labeling methods, an underestimation, and in catalytic reactions, a partial overestimation of the intensity may occur. The latter distorts the statement of intensity differences. However, if a quantitative study is undertaken, one is able to determine the range of experimental parameters where the quantity of the end product is proportional to activity. In order to be able to state marked intensity differences in a qualitative study, the incubation time should be shorter than needed for the maximal reaction product at the highest activity sites. The effect of oblique sections of the membranes on reaction intensity is practically eliminated in the case of surface labeling because the real apposition of the membrane to the cut surface is unidimensional. For catalytic reactions, the area of the membrane contained in the section is increased as compared to the perpendicular section. This results in an increased overall amount of reaction product issued from the same active site density in the membrane. This is accompanied by decreased density and increased enlarged virtual membrane thickness. In the case of monolayers, this effect can more easily be eliminated because sections perpendicular to the monolayer meet two membrane surfaces of each cell perpendicularly with little error. You are correct in assuming that narrow membrane contacts may influence the quantity of reaction product in several ways. One is diffusion hindrance; another is steric hindrance; a third one is the promotion of early precipitation by preventing dilution of the primary reaction product. It is practical to look for free surfaces of the same cells, or surfaces contacting other cell types, to assess this problem.

S. Enerström: What is the reason for leaving small tissue pieces for such a long time in the enzyme?
Authors: The long trypsin digestion time at low temperature is a usual practice in dissociation of tissues containing massive connective tissue septa. In our experience, this schedule was usually more effective in collecting a sufficient number of viable cells than a short trypsinization at 37°C.

S. Enerström: The double immunostaining technique used by the authors is quite unsatisfactory and does not allow for safe, restricted localization of the two hormones. How was the immunostaining controlled for specificity?
Authors: We could exactly reproduce the method described by Bendayan (1982) which is supported by the fact that single and double localization of the two hormones was regularly found in different cells of the same section. In addition to compulsory tests, e.g., deletion of the primary antibody and using a dilution series of the primary antibody, specificity was supported by the repetition of the standard procedure on parallel grids with the reverse combination of gold size and primary antibody.

S. Enerström: The authors have not identified the folliculi-stellate (FS) cells by other means than by electron microscopy. Which criteria were used for recognition of this cell and stroma cell processes without immunocytochemistry?
Authors: We really have not identified FS cells biochemically. Our interpretation of some very thin processes between parenchymal cells, that never contained any granules and always were thinner than the smallest secretory granules, depends on our experience from the intact human pituitary tissue. Stroma cell processes are expected to contain some collagen fibres and their contact with an epithelial cell involves some intercellular substance.

S. Enerström: The cell size or shape does not tell us anything about metabolic activity, and could be an effect of events during culture. The authors do not give any details about sizes or labeling density of "somatotrophic granules" in Figure 4.
Authors: Immunological identification of a cell is a direct method, in contrast to quantitative morphological identification using sizes and proportions of cytoplasmic structures, which is indirect. The indirect method may be reliable under normal conditions (e.g., *in situ*, without any physiological alteration), but is less certain when the cells are cultured or experimentally treated, or when neoplastic transformation takes place. On the other hand, discrete cell populations may show a uniform set of morphological criteria, even under artificial conditions.

S. Enerström: How do the authors explain the "hollow" secretory granules in Figure 5?
Authors: Figure 5 is not post-stained with lead citrate. The density of the secretory granule matrix may vary extensively depending on small details of fixation and embedding conditions if no osmium postfixation or lead staining is applied. The well-localized, dense gold labelling unequivocally differentiates between hormone storage granules and other "hollow" cytoplasmic structures. There is a marked advantage in evaluating the specific label avoiding the background created by staining.

S. Enerström: The search for functional relations be-

tween reassociated adenohypophyseal cells in tissue culture is difficult due to the artificial environment with loss of hypothalamic stimulation/inhibition. One could thus argue that usual activity parameters are not relevant for this system and that only simple morphological criteria are applicable. A simplistic interpretation of the authors data would be that the distribution of cell adhesion molecules has impact on cell shape and polarity.

Authors: We agree that no conventional parameter (neither activity nor morphological) can, in principle, be expected from *in vitro* systems. However, we argue in favor of a close correspondence between the influence of bioactive components of the medium as well as activity and morphological features of the cells. The main virtue of *in vitro* models in cell biology is the recognition of individual regulatory elements not accessible in the extremely complex *in vivo* situation. Among decisive organizational properties of the tissue may be cell polarity and the inclination of cells to reassociate.

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