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Regulation of Steroid Secretion by the Testis

THE ACTION OF LUTEINIZING HORMONE ON THE TESTIS

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Summary—Luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) receptors are coupled to intracellular effector systems, most notably adenylate cyclase, through guanyl nucleotide-binding proteins or G-proteins. The molecular mechanism involved in the dynamic coupling of the LH/hCG receptor however, are not known. It has been postulated that receptor aggregation at the molecular level plays a critical role in this process. There have been attempts to understand the receptor association and dissociation phenomena at the molecular level. One of them involves the participation of the major histocompatibility complex (MHC) class I antigen in the mechanism of receptor activation and/or expression. One molecular basis for these mechanisms consists of a physical interaction between MHC proteins and receptors to form "compound receptors" able to transfer a hormonal signal to the cell. Using a photo-reactive probe we demonstrated that the LH/hCG receptors and the class I antigens are closely associated in the membrane. Thus, it is possible to form covalent complexes of hCG and class I antigens through the binding of the hormone to specific receptors. These findings imply that LH/hCG receptors and the MHC class I antigens may interact at the level of the plasma membrane in the mechanism of LH action. We also performed experiments using a single cell and limiting stimulation to a patch of membrane. The results stimulating the cell in a localized area suggested that even if all components are entirely free to float there is a constraint in the localization of the receptor, G-protein, and/or the effector, supporting the constraint dissociation model. Within a limited area subunits could dissociate, but they would not be free to diffuse throughout the membrane. Moreover the concept of compartmentalization that has been utilized to explain some inconsistencies in second-messenger action now can be proved by experimental design.

LUTEINIZING HORMONE (LH) AND HUMAN CHORIONIC GONADOTROPIN (hCG)

The main physiological functions of the testis are hormonal secretion and gametogenesis. The first includes the synthesis and secretion of testosterone, which is conducted by the interstitial Leydig cells located in groups disseminated between the seminiferous tubules. The function of the Leydig cells is mainly under the control of LH. LH is a glycoprotein belonging to a family of heterodimeric proteins such as hCG, follitropin and thyrotropin which share a common α subunit but differ in their hormone-specific β subunit [1].

The targets for LH and hCG are the gonadal tissues and the most commonly accepted function of both hormones is the regulation of steroidogenesis. A great deal has been learned during the past ten years about the mechanism whereby LH and hCG exert their endocrine control upon the gonadal target cells. The effects of LH and hCG on gonadal tissue are initiated by the binding of circulating hormone to specific high affinity receptors in the plasma membrane [2]. The molecular characterization of the LH/hCG receptors has turned out to be a difficult task. Attempts to identify the LH/hCG receptors using cross-linking techniques has produced contradictory results. However, it is now very well demonstrated that the LH/hCG receptor is a single 90 kDa glycoprotein. Using affinity cross-linking techniques, several research groups have reported findings that support the existence of only one hormone binding unit of molecular size 90 kDa [3–8]. This concept is also supported by a recent study in which 5 different monoclonal antibodies that inhibit hCG binding to the membrane receptor recognize only a 90 kDa polypeptide in a deter-

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gent extract of ovarian cell membranes [9]. Moreover, very recently, the LH/hCG receptor has been cloned [10, 11]; it is postulated that the LH/hCG receptor consists of a large extracellular N-terminal region (containing 6 potential sites for N-linked glycosylation) attached to a region that spanned the membrane 7 times, terminating with a small C-terminal cytoplasmic domain. The deduced amino acid sequence of the receptor shows that it is 700 amino acids long, the first 26 being the signal sequence.

DYNAMIC COUPLING OF THE LH/hCG RECEPTOR

LH/hCG receptors are coupled to intracellular effector systems, most notably adenylate cyclase, through guanyl nucleotide-binding proteins or G-proteins [2, 12–14]. The molecular mechanisms involved in the dynamic coupling of the LH/hCG receptors however, are not known. It has been postulated that receptor aggregation at the molecular level appears to play a critical role in this process [15]. Studies using epidermal growth factor (EGF) [16], insulin [17] and LH [18], suggest that hormone binding alone is a biologically unproductive process without a subsequent step of dimerization, micro-redistribution, aggregation or cross-linking that seems to be a limiting determining process, although the mechanism by which the bound hormone triggers this process is unkown. There have been attempts to understand the receptor association and dissociation phenomena at the molecular level. Hakimuddin and Bahl [19] suggested that the receptor occurs primarily as a monomer and not a dimer and that the association of the receptor into an oligomer is probably caused by intermolecular oxidation of free thiol groups present in the receptor to form disulfide bonds. Covalent cross-linking of hCG, with isotope in either the α or β subunit, to membrane bound receptors, produced complexes that contain a single receptor component of approx. 90 kDA. The cross-linking studies indicated that both subunits interact with receptors. and also suggested receptor dimerization [20]. Kusuda and Dufau [21] suggested that native receptors are dimers of identical subunits and even after dissociation into the subunits the receptors are still able to bind hormones. Although the individual subunits can bind hormones, it is conceivable that the dimeric form is necessary for signal transduction [22]. It is possible that the association of receptors may be triggered by the formation of the hormone–receptor complex in the membrane. In this regard it has been demonstrated that EGF induces rapid dimerization of EGF receptors in living cells, suggesting that this process may play a role in transmembrane signaling mediated by EGF [23]. Two separate models have been proposed to explain how ligand binding to the extracellular domain can activate the intracellular receptor domain. In one model, it is proposed that the ligand binding alters the interaction of the extracellular domain within the plasma membrane and that this alters the position of the transmembrane region. This, in turn, causes a conformational change in the catalytic domain leading to enhanced enzymatic activity [24, 25]. An alternative model is an allosteric oligomerization model [26, 27], in which ligand-induced receptor oligomerization leads to the activation of the catalytic properties of the intracellular domain by subunit interaction between neighboring cytoplasmic domains. Using mutants in the transmembrane region results have been obtained that are consistent with the intramolecular allosteric oligomerization model for receptor activation rather than with a model that assumes intramolecular receptor activation.

CLASS I ANTIGENS AND LH/hCG RECEPTORS

An alternative hypothesis involves the participation of the major histocompatibility complex (MHC) class I antigens in the mechanism of receptor activation and/or expression. MHC molecules have been suggested as anchorage proteins in the membrane for proteins that direct organogenesis [28]. One molecular basis for this mechanism consists of a physical interaction between MHC proteins and antigens occurring at the surface of the target cells [29–31]. MHC class I antigens consist of a family of polymorphic glycoproteins expressed in most cells of the organism. They are composed of a transmembrane 45 kDa heavy chain non-covalently associated on its extracellular domain with a smaller subunit, B2 microglobulin of 12 kDa. The best documented function of class I antigens is to allow the recognition of foreign antigens by the T-cell receptor on cytotoxic T-cells.

It is now generally accepted that, on a target cell, foreign antigens and self class I molecules are co-recognized by a single receptor of

the T-lymphocyte. This implies that foreign antigens and MHC class I antigens interact with each other during the recognition of the target cell by the T-lymphocytes. Moreover, the class I antigens interact during the expression of the foreign antigen to form a selective molecular complex to serve as a receptor during the recognition by T-lymphocyte. Recently, a broader role of the MHC antigens has been envisioned, mainly owing to growing evidence that class I antigens may be involved in hormone signaling, indeed monoclonal antibodies to MHC class I antigens inhibit insulin [32] and EGF binding to human cultured cells [33]. Moreover, insulin receptors and class I antigens have been shown to form selective molecular complexes in mouse liver [34, 35]. It can thus be assumed that, besides their immunological function as restriction elements for the recognition of foreign antigens in the context of cells, MHC antigens also play a role in the recognition of hormones or growth factors by their respective receptors, possibly by interacting with the latter to form "compound receptors" able to transfer a hormonal signal to the cell. Recently, a physical interaction between MHC class I antigens and LH receptors has been reported [36]. Moreover, monoclonal antibodies against class I antigen stimulate testosterone production in mouse Leydig cells [37]; these findings may imply that LH/hCG receptors and MHC class I antigens may interact at the level of the plasma membrane in the mechanism of LH action.

Although the mechanism by which antibodies against class I antigens are able to mimic the stimulation of receptors is difficult to explain, our data demonstrate that a specific preimmunization is required to bring out the steroidogenic responses. The possibility that any antibodies recognizing membrane antigens would trigger cellular response is ruled out since some antibodies recognizing other membrane antigens, such as the LH/hCG receptors with antagonist properties, do not activate the cellular response [18]. Sera against class II antigens, even if they showed cytotoxic titres compatible with Ia region haplotypes from tested strains, had no pharmacological effect. In addition, our data indicate that the antibodies need to interact with the receptors; this notion is supported by the following results: (a) after blockade of LH/hCG receptors with an antagonist, the stimulatory action of alloimmune IgG was blunted, (b) alloimmune IgG inhibited in dose-dependent manner the binding of ^{125}I-hCG to LH/hCG receptors. These data also ruled out that the binding of the antibody to class I antigens *per se* may induce the activation of the membrane adenylate cyclase system.

The pharmacological mechanism by which the antibodies against class I antigens activate the Leydig cells, may involve aggregation of receptors. Whichever is the mechanism of this phenomenon, it should be noted that other investigators have reported the existence of comparable effects of anti-MHC antibodies on different target organs [32, 38–40] and it has been suggested that cross-linking of HLA antigens appears to be required for the antibody–receptors interaction [33].

The finding that an antagonist of LH/hCG receptors inhibited the action of alloimmune IgG on testosterone production in Leydig cells, would suggest that the receptors cannot be aggregated. This is in line with the failure of the antagonist to produce aggregation of receptors [18].

The pharmacological effect of the antibodies suggested that the physiological effect of the hormone may require the association of the receptor with the MHC class I antigens. To study this hypothesis, we carried out experiments to investigate the possible structural relationship between LH/hCG and MHC class I molecules on the surface of membranes carrying the LH/hCG receptors. Monoclonal antibodies against MHC class I antigens are able to precipitate the soluble LH/hCG receptors, both, as a hormone–receptor complex or as a free receptor [36–37]. Precipitation was observed in LH/hCG receptors from C3H and BALB/c mouse cells. The mAbs were active on receptors from the strains carrying the H-2 haplotype corresponding the haplotypes to whom the mAbs are reactive. These observations correlate with the ability of the same mAbs to stimulate testosterone synthesis. In all cases the efficiency of mAbs to precipitate LH/hCG receptors, was higher when the solubilization of the receptors was done as a hormone–receptor complex. The data described above suggest that the physiological effect of the hormone may require the association of the LH/hCG receptor with the MHC class I antigen. This hypothesis is supported by the finding that antibodies reacting with distinct epitopes on H-2 class I antigens were able to precipitate the soluble LH/hCG receptors, with more efficiency as hormone–receptor complex. This precipitation of LH/hCG receptors by H-2 antibodies could

be due either to the presence of common topes on LH/hCG receptors and H-2 antigens (cross-reactivity), or to the formation of a molecular complex between the two molecules (co-precipitation). The demonstration that different mAbs, known to react with distinct antigen determinants [41] are able to precipitate the LH/hCG receptors, strongly supports the co-precipitation possibility. Moreover, in the case of cross-reaction, the precipitation of LH/hCG receptors should not depend on the presence of the appropriate antigens. In fact, LH/hCG receptors were only precipitated by anti-H-2 antibodies corresponding to the appropriate haplotypes. This is in agreement with reports on other membrane receptors [35, 42] and with a recent paper showing very strong evidence of interaction between insulin receptors and class I antigens [43]. Precipitation of LH/hCG receptors was obtained and it was invariably associated with the same antibodies that produce testosterone synthesis. This was also shown by [43] where haplotype-specific precipitation of insulin receptors restricted to class I, but not class II, antigen was shown, but without the unique property of a particular haplotype. The precipitation was significantly higher when the solubilization of LH/hCG receptors was performed after the formation of the hormone–receptor complex. The precipitation of detergent soluble LH/hCG receptors was not total, as was shown for the insulin receptor [43]. It was suggested that there may be a subpopulation of receptors, some of which are associated with different class I antigens. In this context, the highest precipitation in our experimental conditions was obtained with the monoclonal antibodies recognizing the H-2K and H-2D haplotypes. It is worthwhile to point out that the amount of receptor precipitated when solubilization was done without preincubation with the hormone, referred to as free receptor, correlates with the amount of receptor which was demonstrated to be aggregated in basal state, not only for LH [44–46], but also for other systems [47]. These results were observed with all the monoclonal antibodies used and suggested that the hormone triggers the association of receptors with MHC antigens on the plasma membrane, and also the higher efficiency of the mAbs to precipitate the receptor as a homone–receptor complex. Recently, very elegant studies indicate that MHC class I heavy chain and the tetrameric insulin receptor are structurally associated in the cell membrane and suggest that this association may occur by displacement of β_2 microglobulin by the insulin receptor [42].

The co-precipitation experiment suggested that the receptors and the class I antigens may be closely related in the membrane, however there is still the possibility that some proteins in between are solubilized together with the receptors and the MHC molecules. Affinity cross-linking techniques have proved to be highly useful for the identification and structural characterization of membrane receptors. Several research groups, using affinity cross-linking techniques, have reported findings that support the existence of only one hormone binding unit of molecular size 90 kDa. However, other research groups, using the same techniques, have identified the 90 kDa band and several radiolabeled complexes including a 144 and 75 kDa band [48].

Since it is now known that the receptor is a 90 kDa protein, the radiolabeled bands shown by Zhang and Menon [48], probably represent proteins closely associated with the receptors.

PHOTO-AFFINITY DERIVATIVE OF hCG

In order to study whether the class I antigens are closely associated to the LH/hCG receptors the physical interaction was studied by the development of a photo-affinity derivative of hCG to covalently bind the hormone to the receptor and proteins associated with it. Since the derivative possess an arm of 10 Å, it is the only molecular entity able to produce covalent cross-linking and since it is the specific ligand it will be possible to bind the hCG derivative to the receptor and the derivative may covalently bind a protein in very close association with the receptor. The photo-reactive probe was prepared by introducing *p*-azidophenacyl groups [49] into a reduced disulfide bridge of hCG. The native hormone and the derivative (AP–hCG) were radiolabeled using limiting amounts of chloramine-T. The binding properties of the derivative were similar to the native hormone ($Ka = 0.9 \pm 0.02$ nM). Moreover, when Leydig cells from Balb/c mice were stimulated with the AP–hCG the testosterone dose–response curve was similar to the native hormone (Table 1).

After radiodination, knowing that the derivative showed biological properties indistinguishable from the native hormone, the ^{125}I-AP–hCG

Table 1. AP–hCG stimulation of testosterone production by isolated mouse Leydig cells

hCG or AP–hCG (ng)	Testosterone (ng)	
	hCG	AP–hCG
0	4.65 ± 0.22	4.8 ± 0.33
1.0	6.66 ± 0.43	6.5 ± 0.59
2.5	11.2 ± 0.99	11.7 ± 1.05
5.0	16.5 ± 1.30	16.0 ± 1.22
10.0	18.4 ± 1.39	18.0 ± 1.45

Cells were incubated with the hormones for 2 h at 37°C in the presence of methyl-isobutyl-xanthine (10 μM) under CO_2–O_2 (5:95). Testosterone was determined by RIA.

was used to form covalent complexes with the receptor and proteins associated with it. Mouse Leydig cells were incubated with the derivative at 15°C and irradiated; the membranes were isolated and analyzed by SDS–PAGE and autoradiography (Fig. 1). Besides the α subunit (16–20 kDa), β subunit (25–35 kDa) and the α–β dimer (40–45 kDa) two other complexes were also found: the already described 105–115 kDa complex corresponding to the α subunit covalently bound to the receptor and the second one, a molecular species of 70–75 kDa. Subtracting the molecular weight of the α subunit the latter corresponds to a protein of 40–45 kDa. This molecular size is similar to the class I antigens. The 70–75 kDa band can be transferred to a nitrocellulose sheet stained with monoclonal antibodies to class I antigens. In order to determine the specificity of the labeling, we carried out two sets of experiments: (1) the cells were incubated with the derivative in the presence of unlabeled hormone and (2) the incubation was performed with cells from down regulated testes. In both experiments the 70–75 kDa band was not present. However, there was still the possibility that in the down regulated testes the 70–75 kDa protein was also down regulated. Therefore, we studied the possibility that the binding of hCG to this polypeptide exhibited the saturability of the LH/hCG receptors. The appearance of the 70 kDa band parallels the affinity binding constant of the hCG for the LH/hCG receptors (0.7 nM). On the basis of these results, we have proposed that the 70–75 kDa polypeptide represents the covalently bound α subunit of hCG to the class I antigen molecules.

Experiments were also done to study the influence of the incubation temperature in the formation of the 70–75 kDa covalent complex. Mouse Leydig cells were incubated at 4 or 15°C for 12 h and at 37°C for 1 h. These incubation times were chosen in order to saturate the receptor with the ligand. The densitometry of the autoradiography of the SDS–PAGE [Fig. 2(A–C)] showed that the ratio of the receptor/class I antigens complexes increased with the rise of the incubation temperature. As can be seen in Fig. 2(A), peak 2, corresponding to the AP–hCG–receptor, shows an area of 14.5% and peak 3, corresponding to the AP–hCG–class I antigen, an area of 3.4%. The areas of peaks 2 and 3 when the incubation was performed at 15°C were 15.2 and 12.4%, respectively and 8.6 vs 7.5% when the incubation was performed at 34°C. These results may indicate that the movements of proteins in the membrane as a consequence of the increase in the temperature facilitate the association of the class I antigens with the LH/hCG receptors, thus the increase in the formation of the covalent complex AP–hCG–class I antigens.

The above experiments using the photo-reactive probe argue that, the LH/hCG receptors and the class I antigens are closely associated in

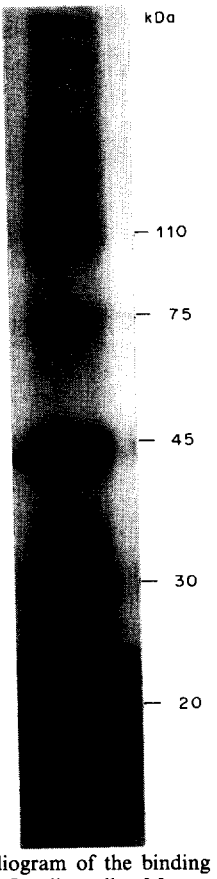

Fig. 1. Autoradiogram of the binding of ^{125}I-AP–hCG to isolated mouse Leydig cells. Mouse Leydig cells, were incubated with the ^{125}I-AP–hCG derivative at saturating concentration (500,000 dpm), at 15°C for 12 h. The membranes were isolated, analyzed by SDS–PAGE and autoradiography. The numbers on the right represent the molecular weight of the standard markers. The lines indicate the covalent complex.

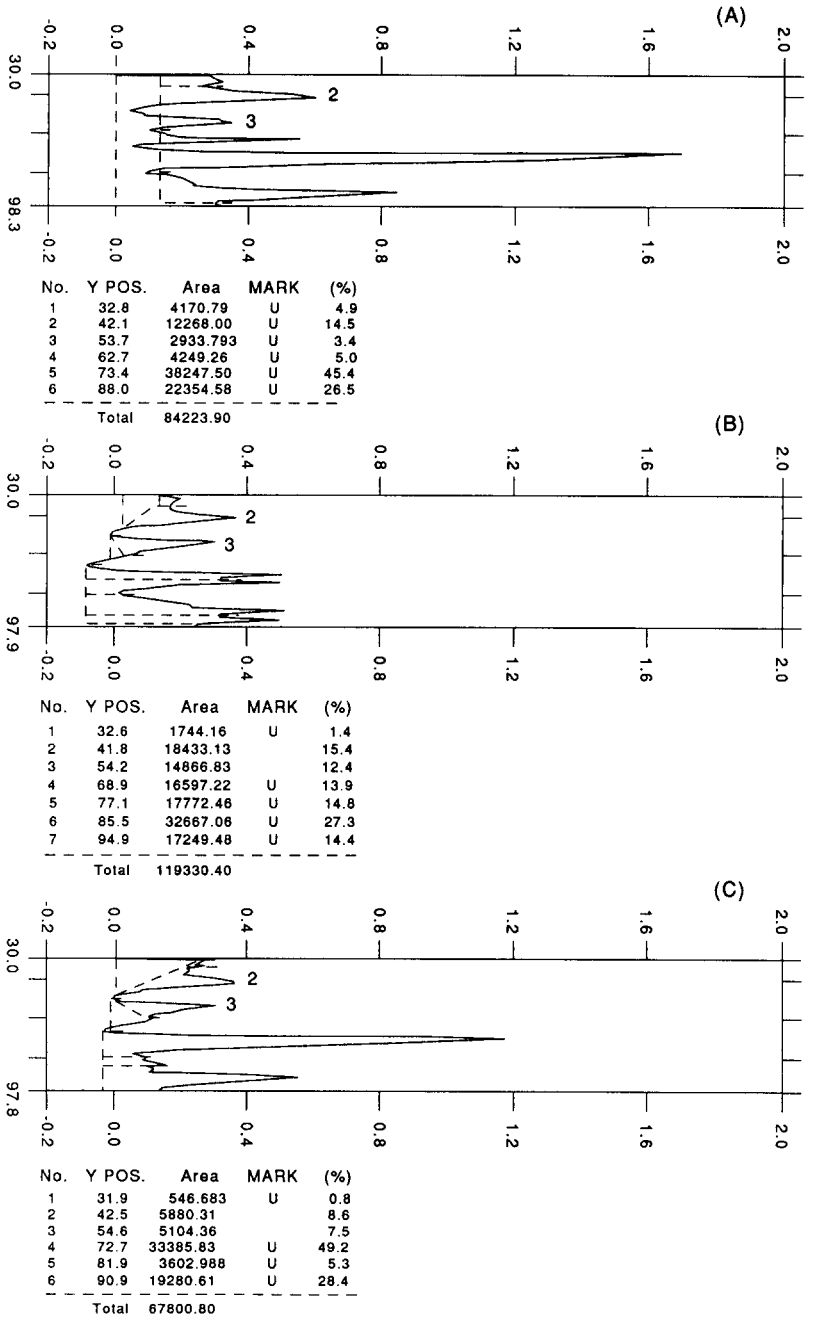

Fig. 2. Densitograms of autoradiograms. Mouse Leydig cells were incubated as described in the legend to Fig. 1 at (A) 0, (B) 15 and (C) 37°C. The membranes were isolated, analyzed by SDS–PAGE, autoradiography and densitometry. Peak 2 represents the 105–115 kDa and peak 3 the 65–70 kDa complexes, respectively.

the membrane. Thus, it is possible to form covalent complexes of hCG and the class I antigens through the binding of the hormone to the specific receptors. This finding may imply that LH/hCG receptors and the MHC class I antigens may interact at the level of the plasma membrane in the mechanism of LH action. In this regard a recent paper suggested the participation of MHC class I antigens in the expression of the insulin receptor [50].

INTRACELLULAR EFFECTOR SYSTEMS

LH/hCG receptors are coupled to the adenylate cyclase system, through guanyl nucleotide-binding proteins or G-proteins. The G-proteins

are composed of three subunits, α, β and γ and are associated with the plasma membrane. In the current view of the mechanism of activation of G-proteins, after the formation of the hormone–receptor complex the activated α-GTP subunit and the β-, γ subunit dissociate and one of both interacts with effectors. Alternatively a free α subunit may bind other subunits [51]. The overall structural similarity within the α and the β, γ subunit families, the *in vitro* ability of some subunits to exchange for others, and the multiplicity of cellular functions carried out by each class of subunits raise questions about the mechanism insulating hormonal signals from excessive crosstalk.

There is good evidence that some components of the lipid bilayer can move in the plane of the lipid bilayer. Hormone-sensitive adenylyl cyclase can be reconstituted by fusing cells which contain either receptor or adenylyl cyclase catalytic units indicating that components from two types of cells must have diffused in the plane of the plasma membrane [52]. In this regard we have demonstrated that electrofusion of Leydig cells and adrenal cells generated hybrids capable of simultaneously synthesizing testosterone and corticosterone [53]. Evidence was also obtained that under such circumstances, heterologous lutropin receptor–adrenal cyclase complexes were formed. However, if all components are entirely free to float and if there is potential crosstalk at the receptor–G-protein or G-protein–effector interfaces, then one way to ensure specificity of G-protein action is to constrain the localization of the receptor, G-protein and/or the effector. Neer and Clapham [51] named this the constraint dissociation model. Within a limited area subunits could dissociate, but they would not be free to diffuse throughout the membrane.

The concept of compartmentalization has been utilized to explain some inconsistencies in second-messenger action. One example is the fact that resting intracellular cyclic AMP concentrations are three orders of magnitude higher than the measurable Ka of cyclic AMP-dependent protein kinase, which is, nevertheless, not fully activated under these conditions. Is the cyclic AMP-dependent protein kinase compartmentalized or protected from resting cyclic AMP levels and thus only responds to *de novo* synthesis of cyclic AMP localized to the vicinity of the receptor-activated cyclase? We have demonstrated that certain responses generated by cyclic AMP are much more efficient when the messenger is produced through receptor activation [9]. Cyclic AMP generated by adenylyl cyclase molecules coupled to a gonadotropin receptor is directed to a small pool of protein kinase [54, 55].

Using a single cell and limiting stimulation to a "patch" of membrane has made it possible to understand how one form of signal transduction proceeds, i.e. the mechanism by which neurotransmitter occupied-receptors lead to the gating of individual ion channels [56]. A similar approach can be used to try to understand another form of membane signal transduction at a single cell level: the mechanism of LH action upon the spatial characteristics of a second-messenger signal propagation.

We have utilized the MA-10 Leydig tumor cell line in culture [57], generously provided by Dr Mario Ascoli, activated by either oLH or 8-bromo-3′-5′-adenosinemonophosphate (8-Br-cAMP), to try to address some of the above questions. The MA-10 is a clonal strain of Leydig tumor cells, adapted to culture by Ascoli [57], and named MA-10 cell line. This cell line originated from a transplantable tumor designed M5480P that was originated spontaneously in a C57BL-6 mouse. The presence of LH/hCG receptors that are coupled to the steroidogenic pathway has been retained by the MA-10 cells; the affinity of the LH/hCG receptors and the number of similar in a normal Leydig cell or in a MA-10 cell. The major steroid produced by MA-10 cells is progesterone rather than testosterone, because of the decrease in the activity of one of the enzymes, the 17-α hydroxylase. In spite of these differences, the main feature of the differentiated function of normal Leydig cells, the mechanisms of regulation of steroid synthesis by LH/hCG has been retained.

Two approaches were taken: the first consists of stimulation of a limited area of the monolayer of cells by puffing, and the second of patching an individual cell's membrane. When MA-10 Leydig tumor cells were grown in the absence of oLH, most of the cells grew in a monolayer of flattened-out cells [Fig. 3(a)]. When the cells are grown in the presence of hormone, dramatic changes occurred in their morphological appearance. Most of the cell became irregular in shape [Fig. 3(b)] with numerous partial rounding up and several secretory bubbles. The change in cell shape is due to a centripetal retraction of the cytoplasm towards the cell body. The mitochondria were

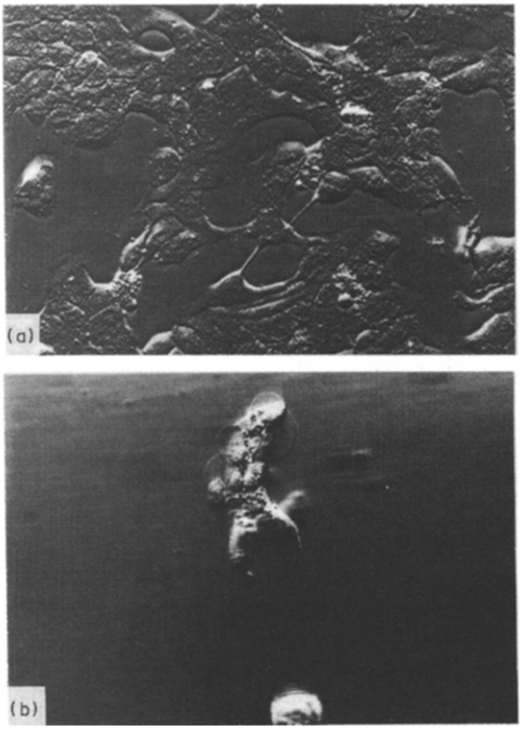

Fig. 3. Cellular "rounding-up". Hoffman-modulated contrast micrographs of MA-10 cells in culture. (a) Leydig tumor cells in only Locke's saline. Note that cells are generally flattened-out and (b) cells after perfusion with 2 ng/ml of oLH in the same saline.

enlarged, while the endoplasmic reticulum and Golgi complex were more developed [58].

When the cells are stimulated for 13 min or more via a section of membrane tightly sealed to an electrode containing LH (20 ng/ml), in every case ($n = 5$) a very localized area, corresponding to the patch of membrane which was in the electrode, exhibited a morphological change. Figure 4 shows a cell prior to patching the Fig. 4(b) the same cell 15 min later. This compartmentalized response was clearly a partial rounding up, visible as such when focusing up and down. Under Hoffmann-modulated contrast this zone appears as a lighter zone against the darker, flat background. The membrane of a different MA-10 cell was patched using an electrode (R = 10M) containing 10 μM 8-Br-cAMP. Figure 5(a) shows the cell 5 min after application of the electrode containing 8-BR-cAMP. After 20 min a total rounding-up of this cell occurs [Fig. 5(b)] as a result of this cell-attached patch. Surprisingly some of the connecting cells (in particular to the left of patched cells) were also stimulated. Unlike patches formed with LH, those formed with the cAMP derivative in the electrode do not lead to a localized response ($n = 3$). Scanning electromicrographs of control, total rounding-up and partial rounding-up are shown in Fig. 6(a–c). When a single cell shows a rounded-up profile the plasma membrane is essentially covered with numerous long microville and are irregular in shape. The rounding-up changes in cell shape are directly proportional to the amount of steroid secreted in hormone treated cells [58]. Rounding-up, even if it is a crude parameter, is one of the best ways to observe the kinetics of localized stimulation at the single cell level.

The present work described for the first time, the stimulation by a peptide hormone of an individual cell in a prescribed region of its plasma membrane. The area of rounding-up is dose-dependent in terms of the magnitude of the stimulation, i.e. the area of the application. The specificity of this morphological response for the hormone and second messenger was shown by the lack of response to patch formation without these agents. This also ruled out the possibility of mechanical contact artefact during the patch formation. It appears

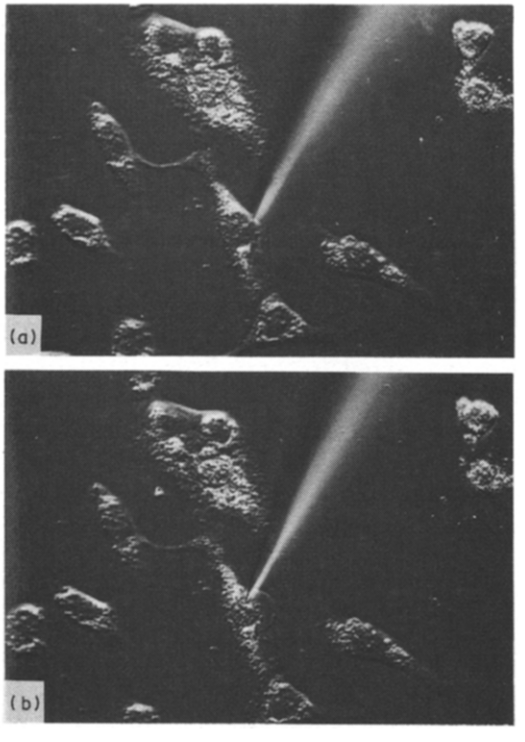

Fig. 4. Hoffman-modulated contrast micrographs of MA-10 cells in culture. (a) Patch electrode (R = 20 M) filled with 20 ng/ml oLH was positioned (out of focus, in the middle) above the central cell and (b) 15 min after patch (R = 10 M) formation but shortly after the electrode became cell-detached (i.e. lost seal). Note localized "rounding-up" around tip and large semi-circle of secretion appearing to the right of the cell.

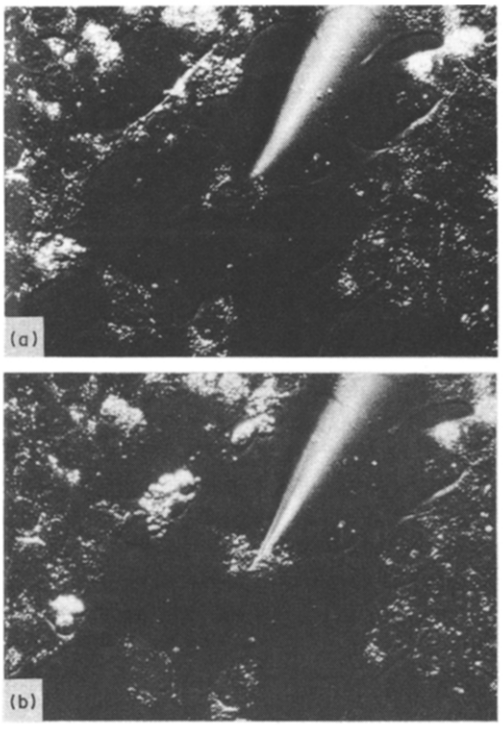

Fig. 5. Hoffman modulated contrast micrographs of MA-10 cells. (a) Electrode (R = 10 M) filled with 10 μM 8-Br-cAMP positioned above central Leydig tumor cell in field. Note intercellular connection between the central cell and surrounding cells, and the cells above and to lower left appear to be already partially "rounded-up" and (b) 5 min after patch (R = 40 M) formation with electrode still cell-attached.

that each individual cell responds to a peptide stimulation gradually in a dose-dependent manner.

The morphological changes "rounding-up" is a phenomenon already described for LH and other hormones acting via stimulation of adenylate cyclase and cAMP-dependent protein kinase [59]. We have shown that stimulated receptors, which are coupled to adenylate cyclase, lead to an internal response which remains localized to the site of stimulation. Thus, the hormone–receptor complex does not transmit an intramembranous or an intracellular signal to activate receptors and enzymes locally. Thus, locally stimulated receptors are not free to move through the membrane, in the area of the patch, the hormone–receptor complexes, the adenylate cyclase, and the cAMP-dependent protein kinase must be closely associated so that their interactions are localized. It appears that each individual cell responds to peptide hormones in a compartmentalized manner localized to the site of receptor occupancy, but also gradually in a dose- and time-dependent manner. Thus, when sufficient occupancy of receptors has occurred it will produce a generalized response, this response can be transmitted to neighboring cells. These results suggest that in a certain tissue the presence of receptors in the entire population of cells is not necessary. The response could be initiated in only a few cells possessing the receptors and generalized to the rest of the tissue through intercellular connections. This could thus be a mechanism for cellular synchronization and, for cellular differentiation, even in the absence of a receptor in many of the cells comprising a tissue organ.

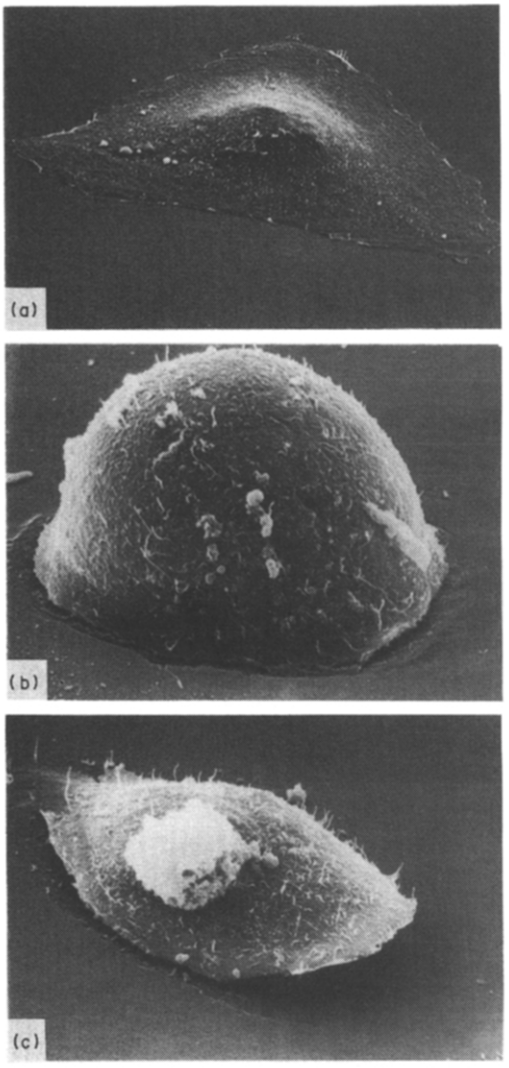

Fig. 6. SEM micrographs of MA-10 cells in culture. (a) Leydig tumor cell in only saline. Note that the cell is generally flattened-out, except for the nuclear region, and mostly free of microvilli, (b) similar cell after puffing with broken (R < 1 M) electrode containing 20 ng/ml oLH. Note total "rounding-up" and the appearance of microvilli and (c) similar cell after puffing with unbroken electrode (R = 10 M) containing 20 ng/ml oLH in saline. Note large ($\sim$13 x 10 nM) localized "rounding-up" where numerous microvilli are concentrated and that the rest of the cell is flattened.

The same idea has also been drawn utilizing a different approach [60].

The results, i.e. stimulating the cell in a localized area, may lead us to suggest that even if all components are entirely free to float there is a constraint in the localization of the receptor, G-protein, and/or the effector, supporting the model of Neer and Clapham [51] i.e. the constraint dissociation model. Within a limited area subunits could dissociate, but they would not be free to diffuse throughout the membrane. Thus, if hormone receptors, G-proteins and effectors indeed exist in specialized membrane domains then it will be important to define the structures that target the molecules to their common meeting ground and keep them there.

The concept of compartmentalization that has been utilized to explain some inconsistencies in second-messenger action can now be proved by utilizing this experimental design at a single cell subcellular level.

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