Stimulation of an individual cell with peptide hormone in a prescribed region of its plasma membrane results in a compartmentalized cyclic AMP-dependent protein kinase response

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ABSTRACT

This work describes the stimulation by a peptide hormone of an individual cell in a prescribed region of its plasma membrane. When Leydig cells were stimulated via a section of membrane tightly sealed to an electrode containing LH, a very localized area exhibited the morphological change known as 'rounding up', which is a cyclic AMP-dependent protein kinase-mediated response. This localized stimulation did not produce a wider response through intracellular, intermembranous or extracellular signals. Each individual cell responded to peptide stimulation gradually, with an increase over time and with dose. In contrast, when the stimulation was accomplished using a non-hydrolysable cyclic AMP analogue in the patch electrode, a general response throughout an individual cell was produced. Locally stimulated peptide hormone receptors, adenylate cyclases and cyclic AMP-dependent protein kinases appear to be closely associated so that second messenger production and the effects it mediates are compartmentalized.

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INTRODUCTION

It is well recognized that the interaction of a peptide hormone with its receptors on the surface of a cell and the initiation of signal transduction is one of the most important steps in its mechanism of action. There has been a great amount of data describing the kinetics of the hormone–receptor interaction as well as the activation of second messenger formation (Cuatrecasas & Hollenberg, 1976; Minton, 1981; Cuatrecasas, 1982; Sibley, Benovic, Caron & Leffkowitz, 1988). There remain, however, several important questions regarding the mechanism of peptide hormone action.

The concept of 'compartmentalization' has been utilized to explain some inconsistencies in second messenger action (Greengard, 1978). One example is the fact that resting intracellular cyclic AMP (cAMP) concentrations are higher than the measurable association constant of cAMP-dependent protein kinase, which is, nevertheless, not fully activated under these conditions (Rubin & Rosen, 1975). Is the cAMP-dependent protein kinase compartmentalized or 'protected' from resting cAMP levels and thus responds only to de-novo synthesis of cAMP localized to the vicinity of the receptor-activated cyclase? Another example is the observation that certain responses generated by cAMP are much more efficient when the messenger is produced through receptor activation, suggesting a compartmentalization between the site of cAMP production and the cAMP-dependent kinase system (Richards & Midgley, 1976; Podesta, Milan, Stephan & Neher, 1979; Podesta, Milani, Stephen & Neher, 1980; Podesta, Solano & Sanchez, 1986). Unfortunately, direct evidence for such limitations has been scarce. It is thus important to determine whether specific hormone receptors, adenylate cyclases, phosphodiesterases and cAMP-dependent protein kinases are closely associated so that their interactions are limited or compartmentalized (Podesta, Solano & Lemos, 1989).

In this paper we have utilized the MA-10 Leydig tumour cell line in culture (Ascoli, 1985), which can
be activated by either ovine luteinizing hormone (oLH) or 8-bromo-cAMP (8-Br-cAMP). Using single cells 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 leads to the gating of individual ion channels (for review see Siegelbaum & Tsien, 1983). A similar approach is used here to try to understand another form of membrane signal transduction at the level of the single cell: the mechanism of peptide hormone action. We have taken advantage of the patch-clamp technique to stimulate a Leydig cell in a defined portion of its plasma membrane and to determine whether its response is likewise localized (Podesta et al. 1989). LH can interact only with a limited number of its receptors, since the tight seal prevents leakage of the peptide in or out of the patch (Neher, Sakmann & Steinbach, 1978), and different recording configurations (Hamill, Marty, Neher et al. 1981) were utilized to analyse the steps involved in hormone action. The response of the Leydig cells was observed by a phenomenon already described for LH, human chorionic gonadotrophin (hCG) and other hormones: the alteration in cell shape called ‘rounding up’. Changes in cell shape and size due to gonadotrophin stimulation have been described both in vivo and in vitro (Amsterdam, Knecht & Catt, 1981). After treatment with gonadotrophin or cyclic nucleotide the cells became highly aggregated and ‘rounded up’ with increased numbers of microvilli on the cell surface (Amsterdam & Rotmensch, 1987). Microvilli are rich in thin filaments (actin), and accumulation of microfilaments and microtubules beneath aggregates of receptor-bound gonadotrophin has been demonstrated (Amsterdam & Rotmensch, 1987). Clustering of organelles in the perinuclear region, concurrent with hormone-induced shape change, might facilitate movements of substrate among organelles and thus enhance steroid synthesis. It has also been established that this phenomenon of rounding up is mediated by cAMP stimulating a cAMP-dependent protein kinase (Rae, Gutman, Tsao & Schimmer, 1979; Amsterdam et al. 1981).

MATERIALS AND METHODS

MA-10 cells

MA-10 cells are a clonal strain of Leydig tumour cells adapted to culture conditions from a transplantable tumour originated spontaneously in a C57B1/6 mouse (Ascoli, 1985). They have the capacity to convert cholesterol into steroid hormones and to respond to LH and/or hCG with an increase in steroid biosynthesis and subsequent release. The major steroid produced and subsequently secreted is progesterone (Ascoli, 1985). LH and/or hCG also have the capacity to produce the series of morphological changes leading to the so-called rounding up of the cells.

The observations of cellular rounding up were performed by four different people. In all instances the observer did not know what the electrode was filled with, in order to avoid any bias.

Ultrastructural analysis

After experimental manipulations the MA-10 cell cultures were immediately fixed with 2% (v/v) glutaraldehyde in 100 mM phosphate-buffered saline (pH 7-2) for 30–60 min. All preparative steps for scanning electron microscopy (SEM) were performed at room temperature. Cultures were washed for 3 × 10 min in the same buffer and post-fixed in buffered 1% (w/v) osmium tetroxide for 30–60 min. After two washes for 10 min in distilled water, the cultures were dehydrated in ethanol, rinsed in acetone, critical-point dried in CO₂ and sputter coated with 25 nm gold. The cultures were then examined using an AMR 1000A SEM at 10–20 kV.

Patch-clamp technique

Fire-polished hard-glass electrodes (Jencon Scientific Ltd, Leighton Buzzard, Beds, U.K.) with resistances (R) of 10–20 megaohms (MΩ) were used to achieve seal R values of >10 gigaohms (GΩ). Seals were most easily achieved during the first hour after removal of culture medium. The size of the pipette tip openings were calculated to be between 0.5 and 1 µm², giving the minimum size of the patches of plasma membrane sealed off. In fact, the areas of patches are considerably (approximately ten times) larger (Sakmann & Neher, 1982) and most probably correspond in size to the localized responses observed with oLH in the pipette (see e.g. Fig. 5b). Standard patch-clamp techniques (Hamill et al. 1981; Lemos, Nordmann, Cooke & Stuenkel, 1986) were used to achieve cell-attached patches. Sometimes the GΩ seal itself was ‘broken’ but the patch was left intact on the cell.

‘Puff’ application of agents to single cells was achieved either by using the above pipettes or by breaking off a normal electrode, filled with either 2–20 ng oLH/ml or 120 µM 8-Br-cAMP (Sigma, St Louis, MO, U.S.A.) dissolved in saline (145 mM NaCl, 1 mM MgCl₂, 10 mM glucose, 2.2 mM CaCl₂ and 10 mM Na-Hepes; pH 7.2), to a resistance of <1 MΩ and applying positive pressure by mouth. Negative pressure was applied at all other times in

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order to ensure that there was no leakage of pipette contents. During patch formation the cells were perfused with saline. This solution was also used to fill the patch microelectrodes, with or without the appropriate concentrations of oLH or 8-Br-cAMP.

RESULTS

Patch clamping and puffing

Control dishes, with only saline inside the electrode, are shown in Fig. 1. The layer of MA-10 cells and

**Figure 1.** Hoffman-modulated contrast micrographs of MA-10 Leydig cells in culture under control conditions. (a) Patch electrode of low resistance ($R < 1 \, \text{M} \Omega$, note large tip), filled only with saline, was placed just to the left of a process connecting two Leydig tumour cells. (b) Application of maximum positive pressure, by mouth, to the electrode produced buckling of this process (arrow). (c) The same field as in $a$ and $b$ is shown 60 min after pressure application of control saline; the electrode had been previously removed. (d) Different field with a new patch electrode of higher resistance ($R = 10 \, \text{M} \Omega$), also filled only with saline, positioned (out of focus, in middle) just above the central cell. (e) The same field as in $d$ is shown 20 min after patch ($R = 10 \, \text{M} \Omega$) formation with the same electrode, as in $d$, on this cell. Scale bar = 30 \mu m for all panels.
the broken-off ($R<1$ MΩ) electrode, before application of positive pressure, are shown in Fig. 1a. The effect of a brief (1–2 s) positive pressure (puff) applied to the electrode is illustrated in Fig. 1b, where the movement of an intercellular process (arrowed) during the application of the puff is visible, corroborating that the electrode contents were indeed expelled. Figure 1c shows the same layer of cells 60 min after the puff. Note that there was no change in their morphology. Figure 1d shows another monolayer of cells before patch formation, and Fig. 1e shows a cell 20 min after patching with an electrode ($R=10$ MΩ) which contained only normal saline. There were no morphological changes in any ($n=6$) patched cell (Table 1) or in the area surrounding any ($n=4$) cells puffed with only saline.

**Morphological response to oLH**

When MA-10 Leydig tumour cells were grown in the presence of a threshold concentration (2 ng/ml) of oLH for 60 min, the culture consisted mostly of rounded-up cells (Fig. 2a and Table 1). However, when maximal doses of oLH (200–2000 ng/ml) were used and the culture cells observed after less than 5 min, before the total rounding-up phenomenon was complete, it was possible to observe several localized responses (Fig. 2d). Furthermore, associated with these limited morphological responses were localized ‘extracellular bubbles’ (Fig. 2b and c).

**Puffing**

Localized oLH stimulation accomplished without patch formation is shown in Fig. 3. Within 1–2 µm of the cell at the center of the dish, puffing was carried out using an unbroken ($R=15$ MΩ) electrode containing 20 ng oLH/ml. Figure 3a shows this cell, with the electrode positioned above it, and its surroundings before stimulation. After 3 min the same electrode was broken ($R<1$ MΩ) and moved to the upper left portion of the field and the same positive pressure (puff) was applied. This resulted in the expulsion of greater amounts of hormone but over a much larger area. Figure 3b shows a limited rounding up, clearly identifiable as such when focusing up and down, in the central cell 30 min after puffing with the unbroken electrode. At the upper left, however, it is possible to see multiple rounding up and the finger-like ‘footprints’ remaining from the flattened position that the two cells had before (Fig. 3a) the larger puff with oLH. This response is similar to that seen with bath applications of high concentrations of oLH (compare with Fig. 2b and c). There was, however, a multiple or total response in all cells ($n=9$) stimulated by puffing with a larger or broken electrode (Table 1). Figure 3c shows the cells 4 h after puff stimulation with oLH and indicates that the morphological changes were maintained.

**Ultrastructure**

In order to determine whether the observed localized response was comparable to the total rounding-up phenomenon, changes in the ultrastructural surface morphology of cultured MA-10 Leydig cells were examined in cells stimulated in either a general or in a limited area of their plasma membrane. Figure 4a shows a single cell under control conditions, exhibiting a flattened appearance and being almost devoid of microvilli on its surface. In contrast, after perfusion with a low concentration of oLH (2 ng/ml), a completely round shape with numerous microvilli on the cell surface was observed (Fig. 4b). Limited stimulation of a cell by puffing with oLH (20 ng/ml) produced numerous microvilli in the area of stimulation, associated with partial rounding up in which the flattened appearance was less pronounced (Fig. 4c). This is in agreement with the observation in cultured granulosa cells that treatment with gonadotrophin or cAMP produced rounded-up cells with numerous microvilli on their surface (Amsterdam & Rotmensch, 1987).

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**Table 1. Morphological responses by MA-10 Leydig cells to general applications of peptide hormone or second messenger**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Application</th>
<th>Response</th>
<th>Response time</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Br-cAMP</td>
<td>Bath</td>
<td>None</td>
<td>&gt; 1 h</td>
</tr>
<tr>
<td>0.05 µM</td>
<td>Bath</td>
<td>None</td>
<td>&gt; 1 h</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>Bath</td>
<td>None</td>
<td>&gt; 1 h</td>
</tr>
<tr>
<td>1 µM</td>
<td>Bath</td>
<td>General</td>
<td>20 min</td>
</tr>
<tr>
<td>32.5 µM</td>
<td>Bath</td>
<td>General</td>
<td>20 min</td>
</tr>
<tr>
<td>120 µM</td>
<td>Puff*</td>
<td>General</td>
<td>20–40 min</td>
</tr>
<tr>
<td>oLH</td>
<td>2 ng/ml</td>
<td>Puff*</td>
<td>None</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>Bath</td>
<td>General</td>
<td>60 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>Puff†</td>
<td>Localized</td>
<td>30 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>Puff*</td>
<td>Localized, multiple</td>
<td>&lt; 30 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>Bath</td>
<td>General</td>
<td>30–40 min</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>Bath</td>
<td>Localized, multiple</td>
<td>&lt; 30 min</td>
</tr>
</tbody>
</table>

The cyclic AMP derivative 8-bromo-cyclic AMP (8-Br-cAMP) or the peptide hormone ovine LH (oLH) were applied at increasing concentrations either by perfusion to the bath or puffed via an electrode onto individual Leydig cells in culture. *The third column notes the extent of the morphological change termed ‘rounding up’ (see Introduction), while the fourth column refers to the time when such a response was first observed. A general response indicates that entire, individual cells became rounded, while a localized response was a single (or multiple) ‘rounding up’ which was restricted to a small region(s) of a cell.

*Broken (< 1 MΩ) electrode was used; †unbroken (15 MΩ) electrode was used.

**Figure 2.** Hoffman-modulated contrast (except d) micrographs of MA-10 Leydig cells in culture illustrating cellular 'rounding up'. (a) Leydig tumour cells were exposed to 20 ng ovine LH (oLH)/ml, and exhibited general rounding-up. (b) A single cell from a different plate is shown after addition of 200 ng oLH/ml. Note the multiple rounding up (small arrows) and 'extracellular bubbles' (large arrows). (c) Different cells from the same plate are shown, also exhibiting multiple rounding up (small arrows) and extracellular bubbles (large arrows); note totally rounded up cells at bottom. (d) Phase micrograph, at lower magnification, of b and c together. Scale bars = 30 μm for all micrographs.

**Patching**

When the cells were stimulated for 13 min or more via a section of membrane tightly sealed to an electrode containing oLH, in every case (n = 5) a very localized area, corresponding to the patch of membrane which was in the electrode (see Materials and Methods), exhibited a morphological change (see Fig. 5 and Table 2). Figure 5a shows a cell prior to patching, and Fig. 5b and c the same cell 15 min and 3 h later respectively. This compartmentalized response was clearly a partial rounding-up (small arrow), visible as such when focusing up and down. Furthermore, associated with this limited morphological response there was a localized extracellular bubble (large arrow), observable due to the difference in viscosity of the cellular secretion and the incubation buffer (Fig. 5b and c). The morphological changes were maintained after the loss of the seal and removal of the stimulating electrode (Table 2).

**Morphological response to second messenger**

The membrane of a different MA-10 cell was patched using an electrode (R = 10 MΩ) containing 10 μM 8-Br-cAMP. Figure 6a shows the central cell prior to the formation of a patch, and Fig. 6b is the same cell 5 min after application of the electrode containing 8-Br-cAMP. After 20 min a total rounding-up of this cell occurred (Fig. 6c) as a result of this cell-attached patch. Surprisingly, some of the connected cells (particularly to the left of the patched cell) were also stimulated. Unlike patches formed with oLH (see Table 2), those formed with the cAMP derivative in the electrode did not lead to localized responses (n = 3). In fact, the generalized response could be observed in other cells (see Fig. 6c), suggesting that this non-compartmentalized response may be intercellularly transmitted. Furthermore, puffing the cell with a
broken electrode, containing either oLH (Fig. 3) or the cAMP analogue, gave a general response similar to that observed with bath application (see Table 1).

**Kinetics of the morphological response**

To determine whether the tight GΩ seal physically limited the response, the seal was broken at various times after cell-attached patch formation (Table 2 and Fig. 5b). The morphological responses were dependent upon concentration (Table 1) and on the time of exposure (Table 2). Surprisingly, the responses remained localized for oLH (Fig. 5c) and general for cAMP stimulation (Table 2). Therefore, the tight seal between the pipette and the cell membrane would not appear to be "causing" the localized rounding up. This was further verified by the fact that puffing, which does not require seal formation, could also stimulate a local response (Fig. 3). Thus locally stimulated LH receptors do not appear to migrate in the plasma membrane to cause a wider response.

**DISCUSSION**

The present work describes, for the first time, the stimulation by a peptide hormone of an individual steroidogenic cell in a prescribed region of its plasma membrane. The parameter utilized to monitor responses was the morphological change, cellular rounding up. This rounding-up phenomenon has been observed at the ultrastructural level using electron microscopy on groups of cells in culture (Soto, Kliman, Strauss & Paavola, 1986). In that study the time-course for the steroidogenic response of cells to the hormones LH and hCG and the morphological rounding up of cells paralleled each other. The extent of these changes in cell morphology was also directly proportional to the amount of steroid secreted (Zion, Benzdina, Mastroianni et al. 1988). The steroidogenic response of the MA-10 Leydig tumour cells to gonadotrophin or 8-Br-cAMP took 4 h at 37°C to reach its maximum level. The shortest time to evoke a significant response was around 35 min (Ascoli, 1985). This is not unique to MA-10 cells; isolated rat interstitial

the multiple rounding up of the two cells at the upper left corner of the field (compare with a). The same electrode was broken to <1 MΩ resistance and maximal pressure was applied for 3 s, just after the micrograph in a was taken, to only these corner cells. (c) The same field is shown 4 h after the two applications of the peptide hormone; note that the morphological changes were maintained. Scale bar = 30 μm.
cells also produced maximal steroidogenesis only after 3–4 h, and the shortest time to evoke steroidogenesis with a maximal dose of gonadotrophin was around 20–30 min (Catt, Tsuruhara, Mendelson et al. 1974). The most rapid increase in steroid neosynthesis after hormonal stimulation is observed using superfusion of adrenocortical cell suspensions (Schulster & Jenner, 1975). The discrepancy between these two techniques is probably due to the different sensitivities in measuring the response. In our experimental design, the response can be observed within 15 min. These experiments were performed under microscope illumination and thus the temperature of the incubation medium was between 25 and 30 °C. Nevertheless, the time-course for the steroid response paralleled the hormone-induced shape change. This is also true at 37 °C (Soto et al. 1986). In the present study, the area of rounding up in individual cells was dose dependent in terms of the magnitude of the stimulation, i.e. the area of application (compare Figs 3 and 5). The specificity of this morphological response for the hormone and second messenger was shown by the lack of response to puffing or patch formation without these agents (Fig. 1). However, there existed the possibility that the mechanical contact artifacts which are associated with pipette application could be the cause of the effect observed on cell shape. This possibility is ruled out by the observations that in none of the controls using saline within the electrode was rounding up observed (Table 2), and that when the LH stimulation was applied by the puff technique there was no contact with the electrode, yet localized rounding up occurred (Fig. 3). In contrast, this kind of stimulation did not elicit rounding up in any of the controls studied (Fig. 1). Moreover, in some cases the same cell when used as a control did not produce any morphological change if there was saline in the electrode, but did when there was hormone or second messenger in the electrode. In addition, formation of the seal with the electrode filled with peptide hormone vs cAMP produced different time lags in the morphological response (Table 2). If contact were the cause of the response the lag period should be the same with either agent. Moreover, if the seal was broken the effect was also time dependent (Table 2) and could be observed only

'rounding up' and the appearance of microvilli. (c) A similar cell is shown after puffing with an unbroken electrode \((R = 10 \, \text{M} \Omega)\) containing 20 ng oLH/ml in saline; note the large \((> 13 \times 10 \, \mu m)\) localized rounding up (arrow) where numerous microvilli are concentrated (compare with Fig. 3c) and that the rest of the cell is less flattened. Scale bar = 10 \, \mu m for each micrograph.

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TABLE 2. Morphological responses by MA-10 Leydig cells to applications via a pipette of peptide hormone or second messenger to a small patch of membrane

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Seal duration</th>
<th>Response</th>
<th>Response time</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>oLH</td>
<td>36 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>5 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>7 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>9 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>15 min</td>
<td>Localized</td>
<td>30 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>3 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>7 min</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>13 min</td>
<td>Localized</td>
<td>20 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>15 min</td>
<td>Localized</td>
<td>20 min</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>30 min</td>
<td>Localized</td>
<td>20–30 min</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>30 min</td>
<td>Localized</td>
<td>15–20 min</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>30 s</td>
<td>None</td>
<td>&gt; 3 h</td>
</tr>
<tr>
<td>10 µM</td>
<td>5 min</td>
<td>General</td>
<td>20 min</td>
</tr>
<tr>
<td>10 µM</td>
<td>20 min</td>
<td>General</td>
<td>20 min</td>
</tr>
</tbody>
</table>

In this series of experiments, cell-attached patches were formed on Leydig cells in culture using electrodes filled with saline only (none) or with different concentrations of ovine LH (oLH) or 8-bromo-cAMP (8-Br-cAMP). The second column indicates how long the tight seal between the electrode and cell membrane endured before it was lost. The patch of membrane which was inside the pipette remained with the cell under these circumstances. Saline in the pipette was used as a control for the effect of losing the seal on cell morphology. The third column notes the extent of the morphological change termed 'rounding up' (see Introduction), while the fourth column refers to the time when such a response was first observed. A general response indicates that entire, individual cells became round, while a localized response was a 'rounding up' which was restricted to a small region of a cell.

when the electrode was filled with either LH or 8-Br-cAMP. The time and hormone dependence of the response strongly argues against it being solely the result of mechanical contact artifacts. Examination by SEM (Fig. 4) demonstrated that the localized response was similar to total rounding up. Moreover, the fact that the different responses fall on a morphological continuum (compare Figs 2c, 2b, 3b and 5b) also suggests that they are equivalent.

In contrast, when the stimulation was accomplished using 8-Br-cAMP in the patch electrode, a generalized response was produced in the entire cell (Fig. 6c). Furthermore, this response appeared to be transmitted to neighbouring cells (Fig. 6c), probably via intercellular connections or gap junctions (Gilula, Reeves & Steinbach, 1972). Leakage of the cAMP derivative through the cell membrane could not account for the response in other cells, since substantial dilution (at least 1:10) would have occurred. The concentration in the pipette (e.g. in Fig. 6) was 10 µM, and much higher rounding up (small arrow) remains in the same location on the cell, but that the extracellular bubble is larger (large arrow). Scale bar = 30 µM.
Concentrations (＞30 μM) were necessary to elicit rounding up when it was applied generally to the bathing medium or puffed onto the cells (Table 1). It must be pointed out that we used 8-Br-cAMP in the patch electrodes, which has a greater capacity to pass through membranes than does cAMP. Nevertheless, Fletcher & Greenan (1985) have demonstrated a similar effect via the production of endogenous cAMP.

The kinetics of the cellular response to hormone was observable in terms of the time (Table 2), dose (Table 2) and extent of the area of stimulation (Figs 3 vs 5). Breaking the seal after different times showed that there was a lag in the response which was dependent upon the concentration or agent (LH vs cAMP) used for stimulation. The kinetics of the response also depended upon the size of the pipette tip, the agent used within the electrode and on the application technique (patch vs puff). Cyclic AMP had a shorter lag period for the response than did LH, and this was true even after breaking the seal (Table 2). This is in accordance with the fact that the peptide hormone must first interact with its membrane-bound receptor in order to elicit the response.

The differences between application of hormone and second messenger were not due simply to the utilization of sub-threshold concentrations of hormone. We were careful to use saturating concentrations (≥20 ng/ml) of oLH when stimulating via a patch. Such concentrations (20−100 ng/ml) of oLH maximally stimulate adenylate cyclase, and therefore the production of cAMP, in these cells (Ascoli, 1985). In fact, up to 200 ng oLH/ml still gave localized responses when applied to a patch on these Leydig cells (Table 2). Furthermore, the converse situation also indicates that the compartmentalized effect was not due simply to sub-threshold production of the second messenger, since lower concentrations (<1 μM) of 8-Br-cAMP did not produce a local (or, indeed, any) response, as would be expected if the area of the response was simply dependent upon concentration (Table 1).

Localized stimulation of the plasma membrane may also produce localized secretion. An extracellular bubble could be visualized because of the differential density between the secreted products and the incubation medium (see Figs 3 and 5). The possibility that this could be simply a result of mechanical artifact due to touching the cell can be ruled out since bath application (see Fig. 2) could also produce extracellular bubbles. The extracellular bubble is suggestive of localized secretion, because its production was always correlated with stimulation (Figs 2, 3 and 5). That is, we have never observed an extracellular bubble in the absence of

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FIGURE 6. Hoffman-modulated contrast micrographs of MA-10 Leydig cells in normal medium showing the effects of localized application of 8-bromo-cyclic AMP (8-Br-cAMP). (a) An electrode (R=10 MΩ) filled with 10 μM 8-Br-cAMP is positioned above the central Leydig tumour cell. Note the intercellular connections between the central and surrounding cells, and that the cells above and to lower left appear to be already partially ‘rounded up’. (b) The same cell is shown 5 min after patch (R=40 GΩ) formation with the electrode still attached to the cell. (c) The same field is shown 20 min after patch formation. There is a large general effect in the central cell, and the connected cells above and to the left have also rounded up. Scale bar = 30 μm.
either localized or individual cellular rounding up (\( n = 7 \)) and it does not develop until 30 min after touching the cell. This observation again argues against the possibility that the bubble simply reflects a localized loss of membrane due to mechanical contact artifacts. In addition, individual cell secretion can be observed after LH puff stimulation (authors’ unpublished observations) by the use of the reverse haemolytic plaque assay (Neill & Frawley, 1983). In this case it is possible to observe the end point of hormone secretion, at the single cell level, in parallel with the effect of LH on the morphology of the cell.

It appears that each individual cell responds to peptide stimulation gradually in a dose- and time-dependent manner (Tables 1 and 2) and, perhaps, at multiple local areas (Figs 2 and 3). As a result of localized hormonal stimulation, we have observed a compartmentalized cAMP response, within an intact cell, which is limited to the area where the receptors were occupied – the patch. The cAMP effect appears to be localized to the area of adenylate cyclase activation, which suggests either that second messenger concentrations are limited by these sites of synthesis or that endogenous cAMP cannot diffuse freely throughout the cell cytoplasm. This compartmentalization also implies, since rounding up is a cAMP-dependent protein kinase-mediated response, that the activated kinase is restricted to sites near the adenylate cyclase. Furthermore, such compartmentalization appears to occur under ‘normal stimulation’ (see Fig. 2b, c and d). LH receptors desensitize rapidly and local application might, therefore, lead to a local response due to turn off of the signalling mechanism. In the MA-10 tumour Leydig cells, however, desensitization as a result of exposure to hCG (40 ng/ml) is maximal after 24 h at 37 °C, with a half-maximal decrease observed at 2 h (Freeman & Ascoli, 1981). Since desensitization will turn off the signal only after 24 h, it is unlikely that the observed localized response was due simply to the turn off of the signalling mechanism by desensitization.

In conclusion, stimulation of LH receptors, which are coupled to adenylate cyclase, leads 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 unoccupied receptors, cyclases or kinases. The hormone only binds to and activates receptors and enzymes locally. Thus locally stimulated receptors are not free to move through the membrane, the hormone is not able to stimulate other receptors, and the newly formed second messenger is not able to diffuse and activate kinases outside the patch of stimulation. In the area of such a patch, the hormone–receptor complexes, the adenylate cyclases and the stimulated cAMP-dependent protein kinases must be closely associated so that their interactions are localized. Thus second messenger production and the effects it mediates appear to be compartmentalized.

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