Hormones and their Actions
Part I

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CHAPTER 9

Internalization of peptide hormones and hormone receptors

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1. Introduction

Peptide hormones are one class of many agents present in the bloodstream that affect the multiplication and differentiated functions of mammalian cells. The ability of a particular peptide hormone to elicit an effect in the appropriate target cell is dictated by the presence of receptors on the surface of the target cell which specifically bind that hormone. Although the cellular responses to the different peptide hormones vary, as do many of the mechanisms of signal transduction that translate the binding of the hormone to the cellular response, there is one salient feature that all peptide hormones studied to date share. This is the receptor-mediated endocytosis (RME) of the hormone.

The idea that proteins could be internalized by a receptor-mediated mechanism by their target cells was sparked by the pioneering studies of Goldstein and co-workers [1] and by Cohen and co-workers [2,3], who obtained evidence for the receptor-mediated internalization and degradation of low-density lipoprotein (LDL) and epidermal growth factor (EGF), respectively, in the mid 1970s. Although endocytosis of a non-specific nature had been described by then, the concept of endocytosis of a specific ligand being mediated by the binding of that ligand to a cell surface receptor was unprecedented.

These investigators were one of the first to study the binding of ^{125}I-labelled ligands to intact cells (as opposed to studying the binding of the ligand to membranes, which was the prevailing approach at the time). Interestingly, their studies showed that when the binding studies on the cultured cells were performed at 37°C, but not at 4°C, there was a time-dependent accumulation of degradation products

Abbreviations and trivial names used are: RME, receptor-mediated endocytosis; LDL, low density lipoprotein; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; LH, luteinizing hormone; hCG, human chorionic gonadotropin; and G protein, guanine nucleotide binding protein.

of the ligand in the culture medium. That the degradation of these ligands was occurring as a result of internalization of the ligand into the cell was suggested by observations that the accumulation of degradation products in the medium was both energy- and temperature-dependent and that it could be inhibited by agents known to inhibit lysosomal function. By using specific treatments to release the surface-bound [^{125}I]LDL or [^{125}I]EGF, it was possible to document the appearance of intracellular radioactivity (representing intact or partially degraded ligand) prior to the release of degradation products into the medium. Furthermore, it was found that some compounds (such as metabolic inhibitors) prevented the accumulation of intracellular ligand (presumably by inhibiting internalization); whereas other compounds known to inhibit lysosomal function (such as NH_4Cl or chloroquine) allowed internalization, but prevented degradation of the ligand [3–7].

Concomitant morphological studies by electron microscopy on the fates of receptor-bound LDL and EGF (using ligands covalently attached to electron-dense ferritin) elegantly confirmed the inferences from the biochemical data that these ligands were internalized and degraded in the lysosomes [8–11]. Since the internalization and degradation of ligand was strictly dependent upon binding of the ligand to the cell surface receptor, this process was called receptor-mediated endocytosis (RME).

RME has since been shown to occur with other transport proteins, other growth factors, and with peptide hormones (for reviews see Refs. 12–16). The general features of RME as they are understood today from biochemical and morphological studies on a variety of ligands are discussed below as they pertain to peptide hormones.

2. *General features of receptor-mediated endocytosis*

A schematic overview of RME is shown in Fig. 1. The cell surface receptors for a particular hormone are either located in areas of the plasma membrane referred to as coated pits or they are randomly distributed throughout the cell surface and migrate to the coated pits upon binding of the hormone. Coated pits are indented areas of the plasma membrane where there is an intracellular 'lining' of the membrane with the protein clathrin and they constitute a small percentage (<5%) of the total area of the plasma membrane [8,17,18]. In the cases where the hormone-receptor complexes migrate to coated pits, there often is a microaggregation of the complexes (two to four per group) during this redistribution [19]. Following this microaggregation there is a more masssive clustering of hormone-receptor complexes in the coated pits.

Coated pits containing receptor-bound hormones become invaginated and pinch off intracellularly to form what are called coated vesicles. The coated vesicles still have clathrin associated with them, forming basket-like structures around the ves-

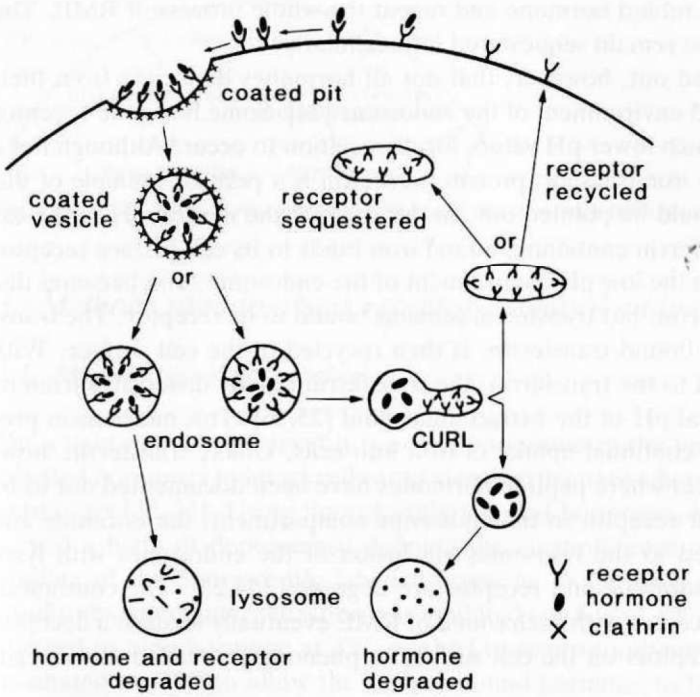

Fig. 1. Schematic representation of the possible routes of receptor and hormone during RME.

icles [20]. The lumen (fluid-filled interior) of the coated vesicles does not have any free hormone. At this stage, the hormone is still bound to the receptor, facing the lumen [10]. With time, the coated vesicles shed their clathrin coats and fuse with other similar vesicles; all this time these vesicles are moving further into the interior of the cell [15]. The prelysosomal vesicles resulting from these fusions are called endosomes or endocytic vesicles and have a critical role in RME due to the acidic environment of their lumen.

Although not as acidic as lysosomes (with an intra-compartmental pH of 4.5, see Ref. 21), the pH 5.5 environment of the endosome [22] is sufficiently low to cause the dissociation of some hormones from their receptors. When this occurs, there is a subsequent sequestering of the free hormone from the receptor in a related vesicle and tubule compartment called CURL (compartment for uncoupling of receptor from ligand, see Ref. 23), where the free hormone is sequestered into the vesicular structure while the receptor accumulates in the membrane of the tubule structure. A subsequent physical separation of these compartments allows for the differential processing of the hormone versus the receptor. Thus, while the free hormone is ultimately delivered (via vesicle fusion) to the lysosome where it is degraded, the free receptor may be recycled (via the Golgi compartment) to the cell

surface, where it can rebind hormone and repeat the whole process of RME. The free receptor may also remain sequestered intracellularly.

It should be pointed out, however, that not all hormones dissociate from their receptor in the pH 5.5 environment of the endosome [24]. Some hormone-receptor complexes require much lower pH values for dissociation to occur. Although not a peptide hormone, the iron-transport protein transferrin is a peculiar example of this phenomenon and should be pointed out. In this case, at the neutral pH of the extracellular fluid transferrin containing bound iron binds to its cell surface receptor and is internalized. In the low pH environment of the endosome, iron becomes dissociated from transferrin, but transferrin remains bound to its receptor. The transferrin receptor, with bound transferrin, is then recycled to the cell surface. With iron no longer bound to the transferrin, the transferrin readily dissociates from its receptor at the neutral pH of the extracellular fluid [25,26]. This mechanism provides for an efficient continual uptake of iron into cells. Unlike transferrin, however, in those instances where peptide hormones have been documented not to be dissociated from their receptor in the endosome compartment, the hormone and receptor are delivered to the lysosomes via fusion of the endosomes with lysosomes, where both hormone and receptor are degraded [24,27]. The continuous degradation of the receptor with each round of RME eventually leads to a decrease in the number of receptors on the cell surface, a phenomenon called down-regulation.

The distinction between a given receptor being recycled versus degraded is not always an all-or-none phenomenon. In fact, in many cases both processes occur to different degrees. Thus, even though the majority of receptors may be recycled, each round of endocytosis can result in the degradation of a small percentage of receptors. If the rate of synthesis of new receptors plus the rate of recycling of internalized receptors is slower than the rate at which receptors are degraded with each round of RME, there will eventually be a down-regulation of the cell surface receptors. Another factor to be taken into account is that some receptors may be spared degradation, but they may not be immediately recycled back to the cell surface (i.e., they may be sequestered intracellularly). These possible routes of receptor disappearance and appearance on the cell surface are summarized schematically in Fig. 2.

Whether a given hormone receptor is recycled or not during RME depends not only upon which hormone the receptor binds, but also upon the cell type and stage of differentiation of a given cell. Thus, the insulin receptor has been shown to be recycled during RME in rat adipocytes [28,29], but not in lymphocytes [30]; and it is down-regulated in the adult rat liver [31], but not in the fetal rat liver [31].

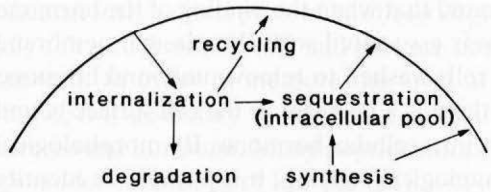

Fig. 2. Possible routes of receptor appearance and disappearance from the cell surface.

3. *Methods used to assess receptor-mediated endocytosis*

3.1. Morphological approaches

On a light microscopic level it is possible to visualize the binding of fluorescently labelled hormones to intact cells or to visualize the native hormone with fluorescent antibodies [32–35]. Using fluorescently labelled hormones, investigators have observed a band of fluorescence defining the circumference of each cell when the binding of the fluorescently labelled hormone to the cells was performed under conditions where internalization was inhibited (such as at 4°C). When the cells were allowed to bind hormone at 4°C, washed to remove unbound hormone, and then incubated at 37°C to allow the surface-bound hormone to be internalized, it was possible to observe a concentration of the fluorescence into small patches on the cell surface and a subsequent increase in diffuse fluorescence located inside the cell. This experimental approach is powerful in that it allows one to visually determine whether under different conditions a hormone is bound to the cell surface or is internalized, and therefore it has been widely used. In order to identify the particular organelles with which the internalized hormone becomes associated, however, it is necessary to examine the cells using an electron microscope.

Using electron microscopy, one can 'follow' the fate of a given peptide hormone in its target cell by using preparations of hormone that have been coupled to electron dense particles, such as ferritin or colloidal gold; or by using hormone preparations that have been radiolabelled to a high specific activity (typically with ^{125}I) and performing autoradiography [8,9,11]. Alternatively, one can bind the unaltered hormone to the cell, prepare the sample for electron microscopy and then bind an electron-dense anti-hormone antibody to the sample to visualize the hormone [23]. The latter approach is generally preferable in that one need not be concerned that the electron dense or radiolabelled hormone is handled by the cell differently than the native hormone. Since colloidal gold is available in a range of sizes, if an antibody to the receptor is available (that can recognize the receptor even when hormone is bound to it), then by using an anti-receptor antibody coupled to colloidal gold of one diameter and an anti-hormone antibody couple to colloidal gold of a different diameter, one can simultaneously follow the fate of both the hormone and the receptor during RME [23].

Using these approaches, it has been found that when the binding of the hormone to the cells is done at 4°C the hormone is associated with the plasma membrane only. If the binding is done at 4°C, the cells washed to remove unbound hormone and then subsequently warmed to 37°C, there is a decrease in the cell surface-bound hormone and a concomitant increase in intracellular hormone. By morphological appearances and by enzymatic or immunological staining, it is possible to identify the intracellular compartments with which the hormone is associated. Furthermore, if one uses a hormone (or antibody to the hormone) made electron dense, the resolution is usually fine enough that one can assess whether the hormone is associated with the organelle membrane (and thus probably receptor-bound) or is free in the lumen [10].

Typically, a morphometric analysis is performed where a large number of micrographs taken at each time point are examined and the number of grains of ferritin or gold particles associated with a given cellular organelle (plasma membrane, coated vesicle, endosome, lysosome, etc.) are tabulated. As such, one can calculate the percentage of grains or particles associated with a given organelle at each time point and arrive at a statistically valid conclusion as to the route of the hormone (and/or receptor) during RME [9,11,36].

3.2. Biochemical approaches

In order to study the RME of a peptide hormone biochemically, it is necessary to be able to radiolabel the hormone to a high specific activity with ^{125}I, while retaining the normal binding and biological properties of the hormone. As discussed in the introduction, if one binds the iodinated hormone to intact cells at 37°C and detects ligand degradation products in the medium, that is an indication that RME of the hormone may be occurring. Ligand degradation can be ascertained by analyzing the molecular size of the radioactive products by gel filtration or by testing the precipitability of the radioactivity by trichloroacetic acid [37]. Since single amino acids and small peptides are not precipitable by trichloroacetic acid, the percentage of acid-soluble radioactivity in the medium represents the percentage of degraded ligand. It is then necessary to document that the accumulation of acid-soluble radioactivity in the medium is dependent upon the extent of hormone binding, the length of the incubation, and the temperature (such that degradation of the hormone should not be apparent at 4°C). Furthermore, one should be able to inhibit the appearance of acid-soluble radioactivity with metabolic inhibitors (such as NaN_3) or with compounds that inhibit the delivery of the hormone to the lysosomes or inhibit lysosomal function (such as leupeptin, NH_4Cl, chloroquine, or monensin; see Refs. 38,39).

When one measures the amount of hormone bound to an intact cell at 37°C, this represents a sum of surface-bound hormone plus hormone that has since been internalized (and is in an intact or partially degraded form). It should be noted that

once an internalized protein has been degraded to free amino acids, these are rapidly released from the cell, and thus are not detected to an appreciable extent within the cell. In order to measure the level of surface-bound versus internalized hormone, it is necessary to develop a method that will quantitatively release the surface-bound hormone. Many peptide hormones can be dissociated from their receptor under conditions of low pH (pH 3–4) and thus this has been a commonly used method [6,36]. An advantage of this method is that it is a mild treatment and thus in some cases one can treat the cells with acid to remove the surface-bound hormone and then rebind fresh hormone and observe a cellular response [36]. Another method that is generally applicable is to degrade the surface-bound hormone by adding proteases using conditions that do not lyse the cells or allow penetration of the added enzyme [3]. It should be noted, however, that this treatment may also damage the receptor and thus cannot be used if one wishes to subsequently rebind fresh hormone to the cells. Lastly, a variety of other methods tailored to the binding characteristics of a given ligand have also been used [5,40,41]. With any given treatment, however, it is necessary to document that one is indeed releasing most (or all) of the surface-bound hormone. This can be done by saturating the binding sites of the cell with radiolabelled hormone under conditions where no internalization should occur (such as at 4°C) and then testing if the treatment releases all the cell-associated radioactivity. Thus, by measuring total cell-associated radioactivity in one set of cells and releasable radioactivity in another set of cells, one can calculate the amount of internalized hormone by subtraction. Therefore, one can in fact measure hormone binding to an intact cell at 37°C and construct a time course of cell surface-bound hormone, internalized hormone and degraded hormone. Under these conditions, cells are continuously exposed to hormone in the medium and thus are undergoing many rounds of RME. If the internalized receptor is not recycled back to the cell surface, the cell surface receptor will become down-regulated. A schematic example of a time course of hormone binding and internalization to intact cells where the receptor is down-regulated is shown in Fig. 3A. In contrast, Fig. 3B depicts a representation of such a time course when the internalized receptor is not down-regulated. It should be pointed out that the maximal amount of hormone internalized and/or degraded will vary depending upon the extent of receptor recycling. Thus, if the receptors do not recycle, the maximal amount of hormone internalized and/or degraded should be less than or equal to the number of cell surface receptors. Therefore, the amount of hormone that is processed in this case is dictated by the number of hormone receptors. If the receptors do recycle, then the maximal amount of hormone internalized and/or degraded should exceed the number of cell surface receptors. In this case the cells can theoretically degrade all the added hormone, regardless of the number of hormone receptors.

From the biochemical approaches discussed thus far, one can conclude that a given hormone may be internalized by RME. Conclusive evidence for such internalization, however, can only be obtained by concurrent morphological data as described

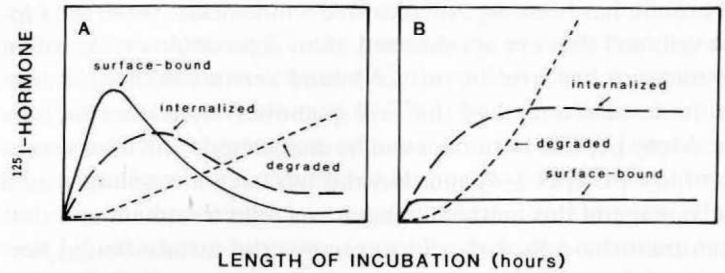

Fig. 3. Distribution of hormone bound to cells during many rounds of endocytosis. Cells are incubated with radiolabelled hormone for increasing lengths of time at 37°C. At various time points, hormone that is surface-bound, internalized or degraded and released into the medium is determined as described in the text. Panel A represents a case where the cell surface receptor becomes down-regulated; Panel B represents a case where the cell surface receptor is not down-regulated, but instead is recycled.

above. For data on the rate of internalization of the hormone and possible down-regulation and/or recycling of the receptor one must again use biochemical approaches.

A commonly used method to calculate the rate of internalization of a hormone is to bind the hormone to intact cells at 4°C (where no internalization should occur), wash the cells to remove unbound hormone and then measure the amount of surface-bound radioactivity remaining as a function of time after warming the cells. Unlike the experimental approach described above where the cells are allowed to continuously bind and internalize hormone at 37°C and thus undergo many rounds of RME (see Fig. 3), under these conditions the cells are internalizing only the pre-bound hormone and thus are undergoing only one round of RME. A schematic example of results of this kind of experiment is shown in Fig. 4. Typically, one observes a loss of surface-bound radioactivity with a concomitant increase in the levels of internalized radioactivity. Since the internalized hormone is degraded, the levels of internalized radioactivity subsequently decline and there is an increase in the levels of degradation products in the medium. It should be noted that when these experiments are done it is difficult to detect a lag in the appearance of the internalized radioactivity; however, there is a lag in the appearance of degradation products in the medium [36]. This lag is a composite of the rate of accumulation of hormone in the lysosomes, the rate of hormone degradation and the rate of release of degradation products. Among these processes, the rate of hormone degradation appears to be limiting [36]. The use of the loss of cell surface-bound radioactivity as a measure of the rate of hormone internalization is valid, though, only when there is little or no dissociation of the hormone from the receptor during the 37°C incubation (which can be assessed by the appearance of trichloroacetic acid-insoluble radioactivity in the medium). Otherwise, the rate of loss of surface-bound hormone would reflect both the rate of internalization of receptor-bound hormone and the rate of dissociation of the hormone from the cell surface receptor [42].

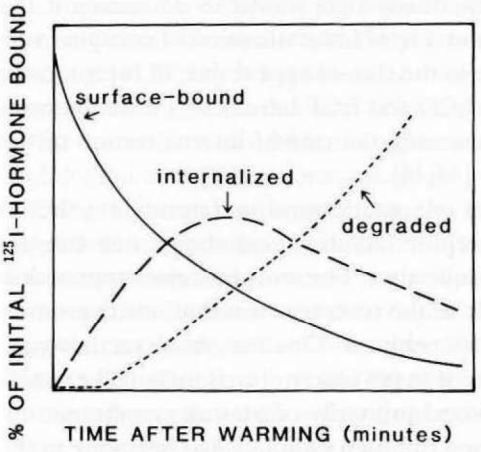

Fig. 4. Distribution of hormone bound to cells during one round of endocytosis. Cells are incubated with hormone at 4°C to saturate the cell surface receptor. At $t=0$, the cells are washed to remove unbound hormone and warmed to 37°C. At various time points after warming, the hormone that is surface-bound, internalized, or degraded and released into the medium is determined as described in the text.

A more valid approach to calculating the rate of internalization of a hormone is to use a steady-state approach as originally described by Wiley and Cunningham [43–45]. In this approach, the cells are incubated with the hormone at 37°C under conditions where the cells undergo many rounds of RME as they continuously bind and internalize hormone (c.f., Fig. 3). Under these experimental conditions, the rate of internalization can be calculated from the ratio of internalized to surface-bound hormone provided that **(i)** the time course chosen is shorter than the observed lag of appearance of degradation products in the medium (see above); and **(ii)** the level of surface-bound radioactivity is at a steady state [43]. Although the first of these two criteria must always be met, one can also perform this experiment while the surface-bound radioactivity is approaching a steady state. If this is done, however, the rate of internalization is calculated from the ratio of the internalized radioactivity (which is, by definition, an integral since the experiment is done before any degradation products are released into the medium) versus the integral of the surface bound radioactivity [42,44,45]. In addition to the rate of internalization, the steady-state analysis described by Wiley and Cunningham allows one to calculate many other parameters pertaining to the hormone-receptor interaction during RME. These include the steady-state association constant for the hormone-receptor complex (a steady state equivalent of the K_a calculated by Scatchard analysis), the number of cell surface receptors, the rate of receptor appearance at the cell surface, the rate constant for the internalization of occupied receptors, the rate constant for the internalization of unoccupied receptors and the rate constant for the degradation of the internalized hormone. Furthermore, if the receptor for the hor-

mone is down-regulated, one can use this steady-state model to determine if the down-regulation is due to an increase in the rate of internalization of occupied versus unoccupied receptors or to a decrease in the rate of appearance of receptors on the cell surface. Using this approach, both hCG and EGF have been shown to down-regulate their respective receptors by increasing the rate of internalization of the occupied versus the unoccupied receptor [44,46].

Another important aspect of RME that one would want to determine is the intracellular route of the hormone and receptor. As discussed above, one can use morphological approaches to address this question. The morphological approach is particularly elegant if one has an antibody to the receptor such that one can simultaneously detect both the hormone and its receptor. One can, however, also address this question biochemically. Indeed, it is possible to fractionate cell extracts on Percoll gradients into fractions composed primarily of plasma membrane, endosomes or lysosomes [24,47,48]. Thus, one can bind radiolabelled hormone to the cells, allow the cells to internalize the hormone for a given length of time, and then fractionate the cells to determine in which intracellular compartment the hormone (i.e., radioactivity) is located. One can determine whether the internalized hormone is free or receptor-bound by precipitation of the internalized radioactivity by polyethylene glycol or ammonium sulfate [24,49]. Furthermore, by analyzing the internalized radioactivity in the different compartments on SDS-polyacrylamide gels, one can assess whether the hormone is intact or partially degraded [24,48]. Thus, one can determine in which compartment the hormone dissociates from its receptor and in which compartment degradation of the hormone occurs. Using these tools, it has been possible to document that unlike many other hormones which dissociate from their receptor in the endosome, hCG remains receptor bound. Thus, the hCG-receptor complex is delivered to the lysosome intact, whereupon the complex is dissociated [24]. Although it can only be directly ascertained that the hormone is then degraded (since it is the hormone which is radiolabelled), it is assumed that delivery of the hCG receptor to the lysosome also results in its degradation (which is consistent with the down-regulation of the hCG receptor in these cells).

Another frequently used tool to assess the intracellular route of internalized hormones and their receptors is the use of compounds or conditions that allow hormone binding and internalization to occur, but impede the intracellular route of the internalized hormone receptor. By using an inhibitor of lysosomal enzymes, such as leupeptin, one can 'trap' undegraded hormone (and possibly receptor) in the lysosome [24]. By performing the experiment at 16–20°C, it is possible to internalize receptor-bound hormone, but 'trap' it in the endosome compartment [50]. Other compounds such as monensin and NH_4Cl can be used to raise the pH in intracellular organelles [39]. Unfortunately, since pH gradients exist in both endosomes and lysosomes (and other intracellular organelles), these compounds may impede any one (or many) of the steps in the transit of the hormone and receptor, and thus one must use additional approaches (as outlined above) to determine in which organelle

the hormone (or receptor) has been trapped. Thus, although it has been shown that in many cases monensin and NH_4Cl trap the ligand-receptor complex in the endosomes [47,48,51], it has been documented that in murine Leydig tumor cells these compounds allow the delivery of hCG (bound to its receptor) from the endosome to the lysosome but inhibit the subsequent dissociation of hCG from its receptor and degradation of the hormone [24].

Once the hormone-receptor complex has been internalized, the receptor may be degraded, sequestered intracellularly, and/or recycled back to the cell surface. If the receptor were sequestered intracellularly, then one should be able to allow cells to internalize hormone and then detect a pool of intracellular receptors in a detergent extract of the cells. To do this, one would allow the cells to bind and internalize unlabelled hormone and then measure the binding of radiolabelled hormone to the intact cells versus a detergent extract of the cells (where both the cells and extract have been treated with acid to remove the unlabelled hormone prior to adding the radiolabelled hormone). Since the detergent extract would be composed of both cell surface and intracellular receptors, an increase in binding activity of the detergent extract and a decrease in binding to the intact cells would be indicative that the receptors internalized during RME of the unlabelled hormone were being sequestered intracellularly. Alternatively, if one detected a decrease in the binding activity in the intact cell and in the detergent extract, this would indicate that the internalized receptor was being degraded (or inactivated).

Indications that receptor recycling may be occurring are **(i)** if the level of cell surface binding and internalized hormone attain a steady state instead of decreasing with increasing time of exposure to the hormone; **(ii)** if the amount of hormone degraded over a period of time far exceeds the steady-state level of surface-bound and internalized hormone (c.f., Fig. 3B). To document receptor recycling, one typically allows the cells to bind and internalize unlabelled hormone, washes the cells free of unbound hormone, and then proteolyzes the cells to destroy the cell surface receptors. The rapid reappearance of cell surface receptors (as measured by binding of radiolabelled hormone), especially if observed under conditions where de novo synthesis of new receptors has been inhibited, is suggestive of receptor recycling [29]. One possible explanation for these data other than receptor recycling, however, is that there may exist a preformed intracellular pool of receptors which can be rapidly mobilized to the cell surface. Whether such an intracellular pool exists can be determined by comparing the binding activity of intact cells that had not been incubated with hormone versus a detergent extract of similarly unexposed cells [52].

It must be stressed again that, even if receptor recycling is documented, a hormone receptor may still become down-regulated if with each round of RME there is some receptor degradation also (the rate of which exceeds the rate of de novo synthesis). The relative degrees of recycling versus degradation of the receptor will determine the rapidity with which down-regulation occurs.

4. *Biological consequences of receptor-mediated endocytosis*

It has become clear in recent years that different peptide hormones may use different signal transduction systems (a few examples being the activation of adenylate cyclase, the stimulation of protein kinase C, the stimulation of the breakdown of polyphosphoinositides or, as in the case of insulin and EGF, the stimulation of the receptor tyrosine kinase) to translate the binding of the hormone into a cellular response. Furthermore, a given hormone may activate more than one transducing system. It is becoming increasingly apparent, however, that many of these transducing systems are stimulated upon hormone binding to its receptor via the intermediary action of GTP-binding regulatory proteins (called G proteins), which are associated with the plasma membrane. Thus, in most (or all) cases, the process of signal transduction appears to occur at the cell surface. What then is the role of RME in the stimulation of a cellular pathway in response to hormone binding?

4.1. *Microaggregation*

One of the first consequences of hormone binding to receptors that are not already located in coated pits is a microaggregation of the hormone-receptor complexes (two to four complexes per group) [19]. In a classic study by Kahn and co-workers [53] it was found that certain antibodies to the insulin receptor were able to mimic the actions of insulin in that they stimulated glucose oxidation when added to adipocytes. Interestingly, although bivalent $F(ab)_2$ antibody fragments retained this insulin-like action, monovalent F(ab) antibody fragments were not stimulatory. However, when secondary bivalent IgGs directed towards the monovalent F(ab) fragments were subsequently added, a cellular response was observed. These results suggested that sheer occupancy of the insulin receptor was not sufficient to elicit a response, and that a microaggregation of the receptors (caused in this instance either by cross-linking receptors with bivalent anti-receptor antibodies or by cross-linking the monovalent of the F(ab)-receptor complexes amongst each other by the bivalent anti-F(ab) antibodies) was necessary to provoke the cellular response. These studies were also significant in that they suggested that the 'information' for evoking a cellular response lies not in the hormone, but in the receptor; and that the role of the hormone is to confer a given configuration to the receptor that allows it to transduce the signal. Similar kinds of studies with EGF [34,35], GnRH [54], prolactin [55] and LH [32,33] have also suggested an important role for microaggregation in signal transduction. This is intriguing in light of the fact that the structures and functions of some of these receptors are quite different. Thus, although the EGF and insulin receptors are known to contain intrinsic tyrosine protein kinase activity, no such activity has been described for the LH, prolactin or GnRH receptors. Furthermore, although both the LH and the GnRH receptor interact with G proteins, it is not yet clear if either the EGF or insulin receptors also do so. Clearly more will

have to be learned about the precise mechanisms by which microaggregation 'stimulates' these different peptide hormone receptors in order to better understand its role in hormonal stimulation.

4.2. Internalized and degraded hormone

As discussed above, once the hormone-receptor complex has been sequestered into the coated pits of the plasma membrane, it is rapidly internalized, and the hormone is ultimately degraded in the lysosome. It has long been debated (and still is) whether the internalized and/or degraded hormone has a role in provoking a cellular response. The question as to the role of the degraded hormone has been easier to address in that it has been possible in some cases to inhibit the degradation of the hormone (using inhibitors such as NH_4Cl and chloroquine) without inhibiting the hormone-provoked biological response [56]. Thus, it appears that degradation of the hormone per se is probably not necessary to provoke a cellular response.

The question as to whether the internalized hormone has a biological role has not been as easy to answer, however, for technical reasons. Thus, the compounds typically used to block internalization (such as metabolic inhibitors) have general toxic effects on the cells; and although 4°C conditions inhibit internalization, these conditions also 'slow' the biological responses one is measuring. Another means of addressing this question has been to immobilize the hormone onto beads that cannot be internalized and to ask whether the hormone is able to provoke a response. A drawback to this approach, however, is that given positive results, it is difficult to rule out the possibility that free hormone has 'leaked' off the resin and that it is the free hormone (able to be internalized) which is eliciting the response. Given this caveat, however, this approach has been used by some investigators and their results suggest that immobilized hormone is indeed capable of provoking the appropriate response (see Ref. 57 and references therein).

Other indirect methods have also been used to investigate the role of the internalized hormone. One approach has been to ask what happens to the cellular response when the surface-bound hormone is removed or neutralized. Thus, it was found that if one removes surface-bound LH or hCG from Leydig cells, even after a significant amount of hormone has been internalized, there is a rapid cessation of stimulated cAMP and steroid production [58,59]. Similarly, it was found that the addition of neutralizing antibodies to EGF up to 8 h after the addition of EGF (at which time a considerable amount of EGF has been internalized and degraded) prevented the EGF-induced increase in DNA synthesis in human fibroblasts [60,61]. Thus, these kinds of studies have also suggested that it is the hormone-receptor complex on the cell surface that provokes the cellular response, and that the internalization of the complex (with the eventual degradation of the hormone) serves as a means of terminating the response.

For those receptors that do interact with G proteins (such as those that stimulate

adenylate cyclase), internalization of the hormone-receptor complex may be a means of segregating the hormone-receptor from the G proteins (if the G proteins are not co-internalized), and thus terminating the response. Furthermore, even if the G proteins were co-internalized, they would now be facing the lumen of the endocytic vesicle and may not have access to the appropriate substrates. Thus, although earlier events may functionally 'uncouple' the receptor-G protein interactions, internalization of the hormone-receptor may provide a 'failsafe' mechanism for terminating the hormonal response arising from interactions with G proteins.

There is recent data to suggest that there may in fact be a biological role for the internalized insulin and EGF receptors (both of which are themselves tyrosine kinases). Thus, microinjection of insulin-occupied insulin receptors into *Xenopus* oocytes causes the increased phosphorylation of ribosomal protein S6 (a known substrate for the insulin receptor/kinase) [62]; and the EGF receptor in endocytic vesicles has been shown to retain its kinase activity [63]. Whether the internalized insulin receptor/kinase or EGF receptor/kinase has a physiological role or not is as yet unknown. Clearly, though, these data suggest that there is much more to be learned about the role of internalized hormone-receptor complexes, especially those where the receptor possesses intrinsic enzymatic activity.

4.3. Receptor down-regulation

Although the hormone internalized by RME is ultimately degraded in the lysosomes, the internalized receptor may be sequestered intracellularly, recycled back to the cell surface or delivered to the lysosomes and degraded. A consequence of the sequestration of the receptor or the degradation of the internalized receptor is the 'down-regulation' of the cell surface receptor. As discussed above, even if most of the receptor is recycled, if some is degraded with each round of RME, there will still eventually be a down-regulation of the cell surface receptor. There are two possible consequences of receptor down-regulation. If there are 'spare' receptors (i.e., if only a small percentage of the cell surface receptors need to be occupied in order to provoke a response), then a decrease in the number of cell surface receptors will not decrease the maximal possible response, but it will decrease the sensitivity of the cell such that a higher concentration of hormone will be needed to evoke a half-maximal response. If there are few or no spare receptors, then down-regulation of the cell surface receptors will lead to both a decreased sensitivity to hormone and to a decreased maximal response by the cell [64].

The down-regulation of the hormone receptor may therefore constitute one mechanism (usually of many) by which the cell becomes refractory (i.e., less responsive) to a continuous hormonal challenge or to a re-challenge of hormone.

5. Conclusion

In summary, once peptide hormones are bound to their receptors on the appropriate target cells, they are internalized and degraded by the process of RME. It is hoped that this review has provided a general overview of the salient features of this pathway and how it is related to the biological actions of peptide hormones. It cannot be overstressed, however, that within this general pathway there are many variations on the possible routes and fates of the internalized hormone and receptor. Thus, whether a given hormone receptor is down-regulated or not depends not only upon the hormone, but upon the particular cell types that have receptors for this hormone. Furthermore, a given cell may both degrade and recycle internalized receptors. Whether the cell surface receptor is down-regulated in this case would depend upon the quantitative contributions of each of these routes.

Although there is as yet no compelling evidence to suggest that internalized peptide hormones and/or their receptors have a role in the signal transduction processes involved in the hormonal cellular responses, it cannot categorically be ruled out that such a possibility exists. To date, however, most of the studies done on the RME of peptide hormones suggest that the internalization of the hormone serves as a mechanism for terminating the actions of the hormone. Ongoing studies in many laboratories are still aimed at gathering more information on the role of RME in hormone action.

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