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Review

Receptor-Mediated Transport of Peptide Hormones and Its Importance in the Overall Hormone Disposition in the Body

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A remarkable feature of the pharmacokinetics of polypeptide hormones is the contribution of specific binding sites (receptors) to the polypeptide hormone distribution and clearance in the body. The concept of "transport receptor" is now well established, and receptor-mediated endocytosis (RME) is recognized as a general mechanism in the uptake of biologically important peptide hormones. This article focuses on the kinetic analysis of the RME of polypeptides, based mainly upon the observations of the kinetics of epidermal growth factor in the liver. The following points are emphasized: (1) How can we determine the existence and the kinetic constants of polypeptide RME *in vivo* and in the perfused liver system? A liver perfusion method, the single-pass multiple-indicator dilution technique, has been shown to be suitable for analyzing the dynamics of interaction of peptide hormones with their cell surface receptors. (2) What is the importance of down-regulation of transport receptors to the overall kinetics of polypeptides *in vivo?* Time profiles of polypeptide plasma concentrations and their surface receptors in the liver after iv administration of epidermal growth factor were simulated with a physiologic pharmacokinetic model that includes kinetic constants representing the interaction of polypeptides and their receptors.

KEY WORDS: receptor-mediated endocytosis; peptide hormones; epidermal growth factor; down-regulation; silent receptor; internalization; liver perfusion method; multiple indicator dilution method; physiological pharmacokinetic model.

INTRODUCTION

The diversity of biologically active peptides has focused interest on peptides and their analogues as drugs. To aid in the design of effective polypeptide drugs, knowledge of their metabolism, disposition, and activity *in vivo* is essential, but the current knowledge remains fragmentary (1,2).

One of the most remarkable features of the pharmacokinetics of polypeptides, compared to that of small molecules, is the contribution of specific binding sites to peptide distribution and elimination in the body (1,3). Receptor-mediated endocytosis (RME) is well recognized as a general mechanism employed by many cells in the uptake of biologically important polypeptides (4–12). These include peptide hormones [e.g., epidermal growth factor (EGF), insulin, glucagon, vasopressin, and atrial natriuretic polypeptide (ANP)], carrier proteins for nutritional and regulatory substances [e.g., low-density lipoprotein (LDL), transcobalamin, and transferrin], lysosomal enzymes, asialoglycoproteins (ASG), and immunoglobulins. The concept of "transport receptor" is now well established. However, it remains unclear whether these receptors are only transporters or have other still unrecognized functions. Recently Maack *et al.* (3) suggested the presence of biologically silent receptors (C receptors) for ANP in the kidney using a ring-deleted analogue of ANP. This analogue is devoid of detectable renal effects and does not antagonize any of the known effects induced by native ANP which binds to the biologically active receptors (B receptors). Although the issue of whether the C receptor has nothing to do with biological effects has not been concluded (14), C receptors may be considered to serve as specific peripheral storage-clearance binding sites for ANP, controlling the duration of hormonal action.

Whether specific binding sites play a major role in the distribution of certain polypeptides can be determined with the pulse-chase method (15), among other methods. Here, the increase in the serum concentrations of preadministered labeled peptides after an additional intravenous injection of unlabeled peptide is examined. This method indeed revealed specific binding sites for β-endorphin in the peripheral tissues such as liver and lung (16). Such displacements from tissue binding sites usually are not observed for low molecular weight drugs, probably because nonspecific tissue binding is much greater than specific binding. On the other hand, for polypeptides, the nonspecific binding is usually low, causing a detectable increase in the serum concentration of tracer after the chase dose.

The liver and the kidney have been widely accepted as the most important organs in clearing polypeptides from the circulation (1,2,4,11,17). Clearance by the liver is often highly selective compared to that by the kidney and has been studied extensively for several polypeptides, such as ASG,

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insulin, EGF, and transferrin (for review, see Refs. 5, 8, 10, 11, 18, and 19). For polypeptides taken up via the RME mechanism, the liver plays a major role as a "homeostatic regulator" in controlling the concentration of peptide hormones in the circulating blood. The initial step in the RME is the binding of polypeptides to specific plasma membrane receptors. The fate of ligands (polypeptides), once internalized, varies from degradation to transport across the cell and subsequent release. If the internalized ligands are subsequently excreted into the lumen (e.g., into the bile or urine), this process is called receptor-mediated transcytosis (17,20–24). The fates of the surface receptors interacting with specific ligands also vary from reutilization (recycling) to degradation and subsequent down-regulation. The receptor recycling process is rapid; the entire circuit from plasma membrane to intracellular vesicular compartment and back to the membrane is presumably traversed in 8 to 12 min (25). However, even this rate is much slower than the recycling rate of the carriers for small molecules in the membrane. Transport receptors for polypeptides are thus different from the membrane carriers for small molecules in that the receptor density in the membrane can be affected by the plasma concentration–time profiles of the polypeptides. Therefore, polypeptide transport into the tissues cannot be expressed by conventional Michaelis–Menten kinetics, contrary to the case for small molecules.

We have been studying the kinetics of binding and internalization of EGF by hepatocytes using freshly isolated hepatocytes or liver homogenates (26,27), isolated basolateral plasma membranes (28), isolated perfused rat liver (31), and *in vivo* systems (29,30). This article centers on the kinetic analysis of the RME of polypeptides. We emphasize the following questions: (a) How does one experimentally determine whether RME is involved *in vitro* and *in vivo?* (b) How can the kinetic constants of the RME process be determined in these systems? and (c) What is the importance of the down-regulation of transport receptors for the analysis of polypeptide disposition *in vivo?*

WHAT IS KNOWN ABOUT RECEPTOR-MEDIATED ENDOCYTOSIS (RME)?

Figure 1 illustrates the general pathways of cellular uptake, intracellular transport, processing, and metabolism of polypeptides in the liver. Kinetic aspects of each process are summarized as follows.

Binding and the Internalization Process (4–6,10,11,32–35)

The binding properties of polypeptides to hepatocyte plasma membrane receptors have been reviewed (11,32). The primary amino acid sequence is known for several receptors involved in RME, namely, the receptors for LDL, EGF, ASG, insulin, immunoglobulin A, and transferrin. These receptors possess a transmembrane structure consisting of an external hydrophilic ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tail. The cytoplasmic portion of EGF and insulin receptors is known to have intrinsic protein kinase activity (4). After ligand–receptor binding, coated pits form at the cell surface and the pits pinch off from the plasma membrane and become coated vesicles. Coated vesicles contain an abundance of both receptors and structural proteins such as clathrin. The process of coated vesicle formation occurs simultaneously with the loss of the ligand–receptor complex from the cell surface, and this process is called internalization.

Intracellular Processing and the Fate of Receptors (5–7,11,36–38)

Two major intracellular pathways of polypeptide inter-

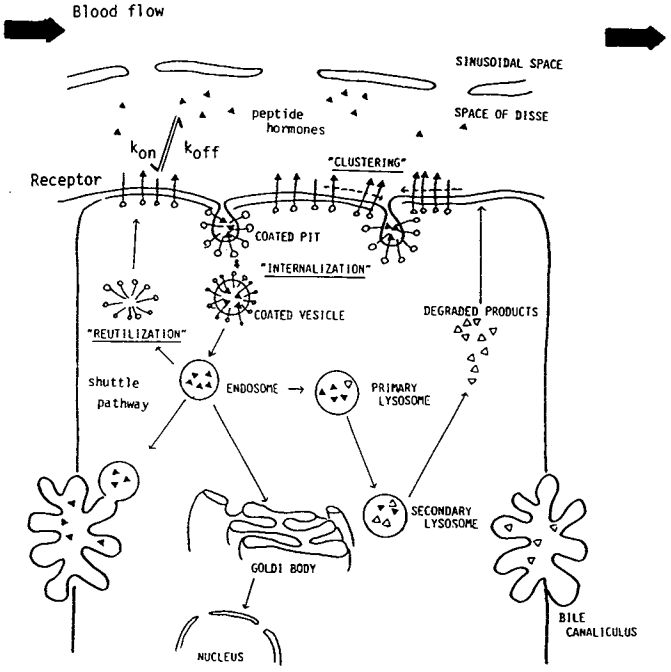

Fig. 1. Schematic diagrams for the pathway of receptor-mediated endocytosis in hepatocytes. See details in the text. (The figures in Refs. 11 and 37 are modified.)

nalization by hepatocytes have been identified (11,39). In one pathway, the endocytic vesicle serves only as a direct shuttle vesicle, forming at the sinusoidal surface, traversing the cell to the peribiliary space, and fusing with the bile canalicular membrane, where the contents are released into the bile by exocytosis. This pathway is known as the direct shuttle pathway, and polymeric immunoglobulin A is effectively secreted by this pathway (17,40).

The second "indirect" pathway involves the interaction of coated vesicles with other cellular compartments. The ligand–receptor complexes proceed through coated vesicles to another intracellular compartment, in which many ligands dissociate from their receptors, and many receptors "sort" from one another (5,6,10). These structures have been termed endocytotic vesicles, endosomes, receptosomes, and the "compartment of uncoupling receptor and ligand (CURL)" (5). Both the endocytotic compartment, like the CURL, and the coated vesicles, which deliver the ligand–receptor complex, are acidified by proton pumps that exist within the membranes of these compartments. The acidic nature of the CURL is critical to the appropriate destinations of the ligand and/or the receptors. That is, the pH value in the compartment could regulate the receptor–ligand binding affinity and consequently the ability of receptor proteins to recycle. It is now widely appreciated that the receptor molecules for certain polypeptides recycle back to the cell surface from the acidic compartment (Type 1; e.g., LDL, ASG, and transferrin receptors). Some receptors undergo degradation after being internalized and subsequent down-regulation (Type 2; e.g., interferon receptor). Receptors for some peptides may show a mixed behavior of Type 1 and Type 2 (Type 3; e.g., EGF and insulin receptors). That is, some fractions of the receptors are recycled and some are degraded. Thus, the efficiency of the receptor recycling depends on the combination of the peptides and cell lines. Receptors can internalize and recycle even in the absence of their ligands ("constitutive recycling") (41,42), although the rate is much slower. Ligand binding to receptors may trigger one of the regulatory events.

In general, the RME process is both of a high capacity because of receptor recycling and of a high affinity due to the highly specific recognition. Initial attempts to use this system for targeted drug (or peptide) delivery are promising (4,43).

IN VIVO ANALYSIS

Dynamic Analysis

A requisite for the demonstration of RME *in vivo* is a delay of the plasma disappearance rate of labeled polypeptides by coadministration with excess unlabeled ligand (isotope dilution method). The authors analyzed the early-phase (up to 3 min) plasma disappearance rate of labeled EGF (^{125}I-EGF) after its iv administration with varying amounts of unlabeled EGF (29). The half-life was prolonged with an increase in the EGF dose. As the distribution volume of the central compartment (V_1) showed no change, the increase in half-life means a decrease in the early-phase clearance, suggesting the saturation of RME. However, in similar experiments with ANP by Ohnuma *et al.* (14), unlabeled ANP decreased the distribution volume (V_1), thereby shortening the plasma half-life of ^{125}I-ANP, while the effects on the early-phase (~4 min) clearance were minimal. These discrepant results might be due to the difference of *in vivo* binding rates of EGF and ANP, which depend on both the on-rate constant (k_{on}) and the density of receptors (P_T).

The amount of labeled EGF taken up by each tissue was determined at 3 min after the coadministration of labeled EGF and unlabeled EGF (29). The distributions of labeled EGF to the liver, kidney, small intestine, stomach, and spleen were greater than expected from the distributions only to the extracellular space of each tissue. The *in vivo* tissue uptake and plasma disappearance of EGF were analyzed according to Eadie–Hofstee and with a kinetic perfusion model under the following assumptions: (i) the tissue compartment (containing cell surface receptors) is well stirred, and the venous (outlet) concentration of labeled EGF in the tissues is the same as that in the capillary; (ii) degradation of labeled EGF during the 3 min period after iv administraiton is low; and (iii) EGF rapidly equilibrates between the capillary bed and the interstitial fluid. Thus, the mass-balance equations of EGF in the extracellular space and the cell compartment of a tissue are described as follows:

$$V_T\, dC_E/dt = Q_T C_P - Q_T C_E - V_{max}\, C_E/(K_m + C_E) - P_{dif} C_E \quad (1)$$

$$dX_T/dt = V_{max}\, C_E/(K_m + C_E) + P_{dif}\, C_E \quad (2)$$

where V_T and C_E are the extracellular volume and the peptide concentration in the extracellular space, respectively, Q_T is the plasma flow rate in the tissue, X_T is the amount of the peptide associated with the tissue, C_P is the plasma concentration of the peptide and varies with time, K_m and V_{max} represent the Michaelis constant and the maximum uptake rate for the receptor-mediated uptake process, respectively, and P_{dif} is the proportionality constant for the receptor-independent uptake process. The calculated apparent Michaelis constants are $\leq$20 n*M*, while the maximum uptake rate shows great intertissue differences (29). The calculated saturable uptake clearance (V_{max}/K_m) in each tissue against the specific binding activity determined *in vitro* using tissue homogenates correlated well, except for lung and spleen (26) (Fig. 2). The receptor-mediated process was estimated to have a clearance of approximately 11 ml/min, 70% of which was contributed by the liver. The hepatic uptake of EGF at the low dose is so large that it is limited by the hepatic plasma flow (29). However, the assumption of a rapid equilibrium of EGF (MW ca. 6000) between the capillary bed and the interstitial fluid may not hold true for tissues other than the liver. The authors successfully simulated the time profiles of tissue distribution of biologically inactive ^{125}I-β-endorphin, by incorporating diffusional transport across the capillary membrane into a physiologically based pharmacokinetic model (44). Experimental evidence from solute permeability studies and interstitial fluid volume measurements predict that the equilibration times of peptide hormones (MW <10,000) are usually less than 3 min for visceral organs but 30 to 80 min for muscle (45). Therefore, saturable uptake by muscle would not be detectable by short-time *in vivo* experiments. In most tissues, however, only a few minutes

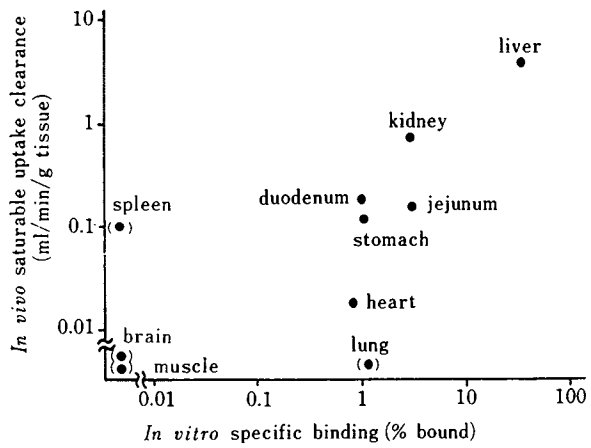

Fig. 2. Correlation between *in vitro* specific binding activity to tissue homogenates and *in vivo* receptor-dependent uptake clearance among various tissues in rats. [With permission from Ref. 26.]

are necessary for peptide hormones to reach a pseudo-steady state between plasma and interstitial fluid.

Static (Equilibrium) Analysis

Whitcomb *et al.* (46,47) have developed a theoretical basis for an *in vivo* radioreceptor assay for polypeptide hormones assuming that the steady-state distribution of labeled peptides in the presence of excess unlabeled peptide represents distribution only to the extracellular space (plasma plus interstitial space). In this method, ^{125}I-insulin and ^{131}I-albumin were infused into rats with increasing amounts of unlabeled insulin. Plasma and tissue samples were taken at 5 min and the radioactivities of each isotope were measured. They further assumed that peptides move freely between the vascular and the interstitial space, whereas albumin molecules are acutely confined to the vascular compartment, except in the liver. Under these assumptions, the amount of hormone specifically bound to receptors in a given tissue (B_c) can be given by

$$B_e = (T_e/P_e - T_c/P_c) \times P_e \quad (3)$$

where T and P are the amount of peptides in a tissue and the plasma, respectively, and the subscripts c and e represent the experimental conditions corresponding to the presence and absence of excess unlabeled peptides, respectively. Using this methodology, they demonstrated specific, displaceable binding of insulin to the receptors in the liver, muscle, fat, adrenal glands, pancreas, small intestines, and spleen (47).

Let us compare the *in vivo* dynamic analysis with the static analysis. The static analysis adopted by Whitcomb *et al.* (46,47) uses an equilibrium condition, and therefore, it is relatively simple and requires minimal tissue preparation and no complex calculation. This method allows calculation of the equilibrium binding activity, but it fails to assess the uptake rate. On the other hand, the dynamic method (29) can yield the values of K_m and V_{max}, individually, and the uptake rate for each tissue can be obtained. These parameters may be "hybrid" parameters, representing the processes of binding to the cell surface receptors and the internalization to the cell interior (48–50). These two subcompartments may be distinguished only with the use of pulse-chase or washout experiments (50).

Other Methods

Spady *et al.* (51,52) have also characterized the *in vivo* receptor-dependent LDL transport process in various tissues in terms of its maximum uptake rate (V_{max}) and Michaelis constant (K_m). The rates of LDL uptake *in vivo* by the different tissues of the rat and hamster were determined using the primed-continuous infusion technique, which allows one to elevate abruptly the plasma concentration of LDL to any desired value. Since the specific activity of the plasma LDL was constant over the 4-hr infusion period and since the tissue accumulation of LDL was linear with respect to the time of infusion, the tissue uptake clearance can be obtained by dividing the rate of uptake into each tissue by the plasma concentration of LDL. Kinetic analysis yielded values for K_m and V_{max} and the proportionality constant representing the receptor-independent transport. Such analysis showed that the receptor-dependent uptake exists not only in the liver and endocrine glands but also in the spleen, kidney, and intestine and that the K_m values (approx. 90 mg/dl) were essentially the same in all of the tissues. Such an experimental design may be suitable for polypeptides with slow tissue uptake.

Pardridge *et al.* (53) have analyzed the *in vivo* kinetics of hepatic clearance of ^{125}I-ASG with a portal vein double-bolus injection technique in rats. A 200-μl bolus of buffered solution was rapidly injected immediately after the hepatic artery was ligated. The injection solution contained ^{125}I-ASG, ^{3}H-water, and various concentrations of competing unlabeled ASG. The ^{3}H-water is used as a highly diffusible internal reference of the uptake process. At 18 sec after the portal injection, the right major lobe was rapidly excised and the animal killed. By 18 sec, the injection bolus completely passes through the liver, yet no measurable recirculation of labels occurs. The unidirectional hepatic extraction ratio of ASG was thus calculated from the obtained data. Assuming that the *in vivo* hepatic receptor concentration is much greater than the receptor K_d (dissociation constant), the maximal binding capacity of the external surface of liver cells *in vivo* was calculated to be 1.2 nmol/g of liver. This value approximates *in vitro* estimates of the total hepatic binding capacity, but it is 10-fold greater than *in vitro* estimates of binding capacity on the external surface of liver cells. These results suggest that the large majority of ASG receptors is located on the liver cell surface in the *in vivo* condition. It may be interesting to analyze the obtained dose-dependent changes in the extraction ratios based on a physiological pharmacokinetic model that includes a receptor–polypeptide interaction as discussed below.

Pardridge *et al.* (53) have also examined the recovery of cell surface receptors using the same portal vein double-bolus technique, wherein a 400-μg dose of unlabeled ASG was followed 30 sec–30 min later by portal vein injection of a tracer amount of ^{125}I-ASG. The specific extraction of the ^{125}I-ASG, which was negligible shortly after the 400-μg loading dose, gradually increased toward normal levels, with a half-time of 21 min (Fig. 3), which may represent the *in vivo*

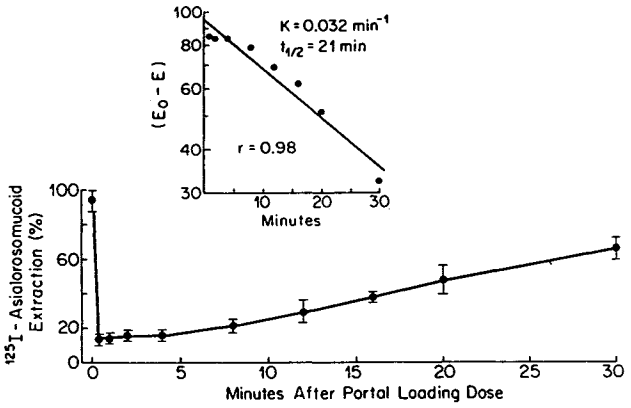

Fig. 3. Recovery of the extraction ratio of ^{125}I-ASG after the first loading dose of unlabeled ASG (400 μg) in rats. The extraction ratio was measured with the double-bolus technique, in which the liver was excised at 18 sec following the second bolus containing ^{125}I-ASG and ^{3}H-water (as a highly diffusible internal reference). The upper graph is a logarithmic plot, where E_0 and E represent the extraction ratio of ^{125}I-ASG after the injection of 0 or 400 μg of ASG, respectively. [From Pardridge *et al.* (53) with permission.]

rate of receptor recycling. Indeed, the time for receptor recycling in rat hepatoma cells was reported to be 15 min (54). As they discussed (53), however, the gradual reappearance of the unoccupied binding sites can also occur by simple dissociation of bound ligand from the cell surface.

KINETIC ANALYSIS OF RME IN THE PERFUSED LIVER SYSTEM

The interaction between peptide hormones and their specific receptors has been analyzed mainly using isolated or cultured cells and plasma membranes (55–57). These systems, however, neither maintain cell polarity nor include delivery of a ligand by blood flow. The use of the tissue perfusion method may be effective in resolving these problems, since the spatial architecture between cells and capillary bed is maintained. Here, examples analyzing the RME of EGF in the liver are given. Two liver perfusion methods have been used, i.e., a single-pass multiple indicator dilution (MID) method and a recirculation method.

MID Analysis

The MID method has been developed by Goresky (58) for analyzing the hepatic transport of small molecules. This method has some advantages: first, various kinetic parameters of uptake, efflux, and sequestration can be determined separately (considering the case for small molecules); and second, relatively rapid kinetic processes can be analyzed (59–61). The injection mixture consisting of ^{14}C-inulin as an extracellular reference substance and a test peptide is given as a rapid single injection into the portal vein. Subsequently, total hepatic vein effluent is collected in about 0.5 to 1-sec aliquots for 1–2 min. Figure 4 shows the time courses of the concentration of labeled EGF and inulin in the outflow after the instantaneous injection together with varying amounts of unlabeled EGF (31). With increasing EGF doses, the recovery of labeled EGF in the outflow is increased, until the dilution curve of labeled EGF is nearly superimposed on that of inulin. The extraction ratio (E_H) and distribution volume (V_d) of EGF are calculated from the dilution curves by a model-independent moment analysis (62). The extraction ratio of EGF is approximately 0.8 at the tracer dose, while it decreased toward zero with excess EGF, together with a decrease in the distribution volume. While EGF has specific binding sites on the liver cell surface, somatostatin tracer, which has no specific binding to liver cells, does not show a

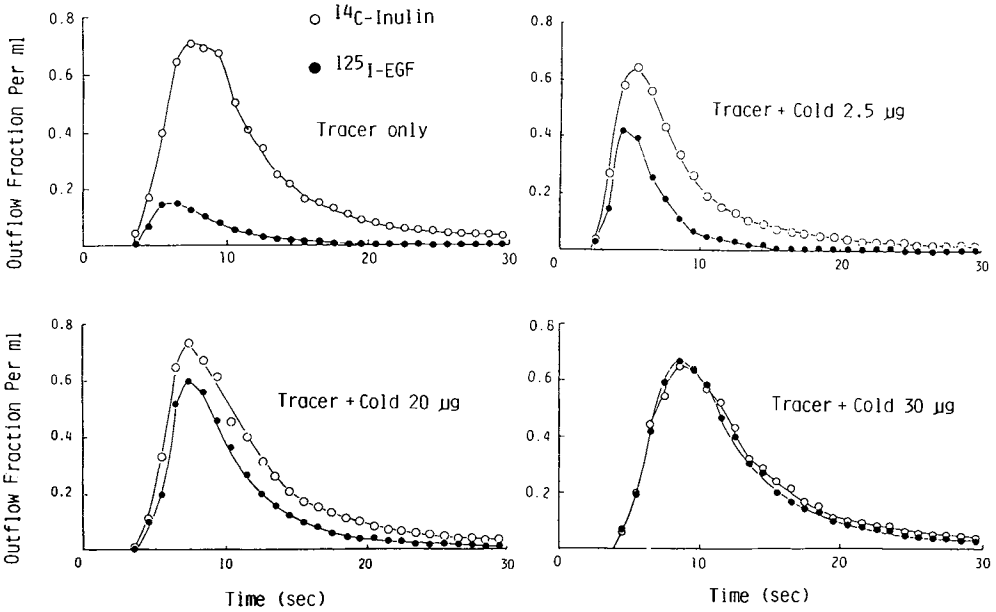

Fig. 4. Indicator dilution curves from rat liver for ^{125}I-EGF and ^{14}C-inulin (as an extracellular reference) in the presence of varied amounts of unlabeled EGF in the injectate. Abscissas, time in seconds (actual time including large vessel and catheter transit time); ordinates, outflow fraction per milliliter (concentration/dose). (Part of the figure is from Ref. 31 with permission.)

changed dilution curve with excess somatostatin. Further, the initial up-slope of the ratio plot of EGF [a plot of ln(inulin/EGF) vs time], representing the relatively slow binding to cell surface receptors, cannot be observed for somatostatin (31). The MID method was also applied to insulin and glucagon (31), both of which are known to have receptors on hepatocytes, and to analyze the receptor-mediated hepatic uptake of ASG (63).

The time course of the recovery of available EGF receptors on the cell surface was also analyzed by MID (31). After a MID control experiment with EGF tracer, excess (20 μg) unlabeled EGF was injected instantaneously, followed again by MID experiments of EGF tracer doses to measure receptor recovery on the cell surface. As shown in the ratio plots (Fig. 5), the initial slopes, which correspond to the product of k_{on} and P_T (the density of available receptors on the cell surface), greatly decreased at 1 min after the injection of excess unlabeled EGF and then gradually recovered to the control value, with a 5~10 min half-life. Whether the mechanism is receptor internalization or ligand–receptor dissociation, the recovery MID experiment assesses available receptors on the liver cell surface.

Assuming first-order kinetics for EGF tracer doses, the dilution curve can be analyzed according to the flow-limited distributed model (58,64,65), with three kinetic constants, k_1 (apparent uptake rate constant), k_2 (efflux rate constant), and k_3 (sequestration rate constant). These three parameters may represent the corresponding kinetic steps in the RME system as shown in Fig. 6 (31). Here, k_{off} and k_{seq} are the off-rate constant of EGF and the sequestration rate constant of the complex from the cell surface receptor compartment, and Vd_{ref} is the volume of extracellular space obtained by the analysis of the dilution curve of ^{14}C-inulin. These considerations suggest that the apparent removal rate constant, k_1, which can be obtained from the initial slope of the ratio plot, includes both k_{on} and the available receptor density. Thus, analysis of the dilution curve at a tracer dose of peptides gives the parameter values of $k_{on}P_T$, k_{off}, and k_{seq}. Furthermore, by simultaneously analyzing the ratio plots at various doses of EGF, the k_{on} and P_T values can be determined separately (31). The parameters thus obtained by the MID analysis are listed in Table I. On the other hand, the K_d and P_T values determined from *in vitro* equilibrium binding studies using rat liver homogenates, isolated hepatocytes, or isolated plasma membranes are 1–15 n*M* and 17–65 pmol/g liver, respectively (26–28,66–68,72). The binding parameters (K_d and P_T) obtained from the MID analysis thus fall within the value range obtained from the direct binding studies. It remains unresolved which kinetic process the parameter k_{seq} represents. This parameter may be related either to the internalization process of the EGF–receptor complex or to its clustering process. However, the calculated k_{seq} value is two orders of magnitude larger than the internalization rate constant (0.001–0.002 sec^{-1}) directly determined in isolated hepatocytes by using an acid-washing technique (27). Therefore, the k_{seq} value may represent more rapid kinetic processes, such as the clustering and the conformational change of the EGF–receptor complex. For analyzing the slower kinetic processes including the internalization and recycling of the receptors, the simultaneous use of a conventional recirculatory method (67,69) would be necessary.

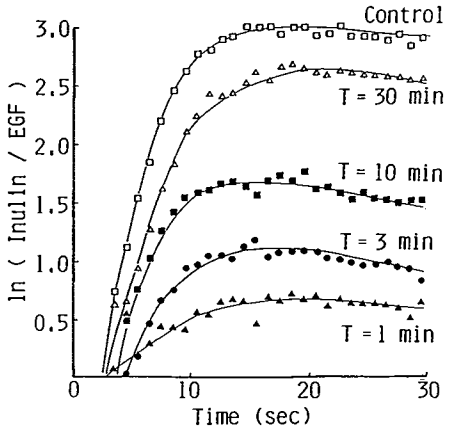

Fig. 5. Recovery of the available EGF receptors on the rat liver cell surface after injection of an excess amount of unlabeled EGF. Following the 15-min perfusion with buffer, 20 μg of EGF dissolved in 200 μl of the perfusate buffer was injected as a bolus into the portal vein. The MID experiment at a tracer dose of ^{125}I-EGF was then performed at 1, 3, 10, and 30 min after the loading dose of unlabeled EGF. The logarithmic ratio (^{14}-inulin/^{125}I-EGF) was calculated from the dilution curve obtained at each time (expressed as *T*) after the loading dose. The initial slope of the plot corresponds to the product of k_{on} and the density of available receptors on the liver cell surface. (With permission from Ref. 31.)

Recirculation Method

Dunn *et al.* (56,67) have used a recirculatory perfusion system to analyze the kinetics of the RME of EGF. Advantages of the recirculatory perfused liver system over an *in vivo* system include the following (56): (a) reagents such as enzyme-impermeable chemical labels or metabolic inhibitors can be introduced into the liver circulation without dilution by total blood volume; (b) these reagents can be recirculated and then removed from the perfusion medium under defined conditions (to establish a "pulse") and replaced with fresh medium lacking the reagent; (c) the temperature of the perfusion medium can be varied over a wide range (4–37°C) to assess the effect on various parameters; (d) the temperature can be rapidly switched from one (4°C) to another (37°C), allowing synchronization of the internalization of receptors; and (e) mild acid treatment (pH 5.0) can remove EGF bound to cell surface receptors without affecting the subsequent EGF binding or uptake, allowing measurement of the internalized EGF. Such an analysis led to the following findings. The ligand pathway was also visualized cytochemically, and rapid transport from endosomes to the Golgi apparatus–lysosome region was observed ($t_{1/2}$, 7 min) (67). At selected times after the addition of saturating doses of EGF, livers were homogenized and homogenates were assayed for accessible (minus Brij 35) and latent receptors (plus Brij 35). These results are shown in Fig. 7. Before the liver is exposed to EGF, the number of accessible and total receptors is the same, suggesting the absence of an intracellular receptor pool. Upon the addition of EGF to a perfused liver, a rapid disappearance of cell surface receptors that paralleled the internalization of EGF in both time and extent was observed. The number of surface receptors decreased by 80%

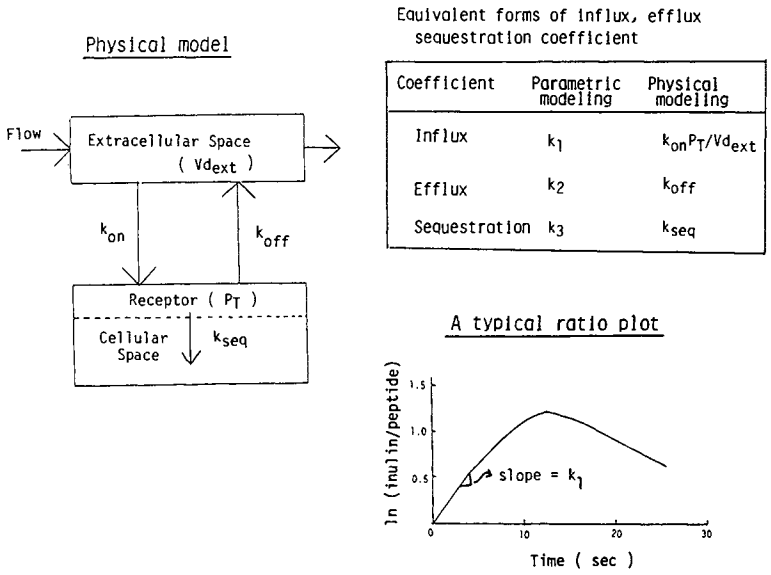

Fig. 6. Equivalent forms of influx, efflux, and sequestration rate coefficient between parametric modeling and physical modeling.

within 20 min and remained at this level for 4 hr. Furthermore, the observed down-regulation of EGF receptors at the surface agreed with the appearance of an internal pool (in endosomes) of receptors. The amount of EGF internalized at 4 hr was three times the total number of EGF binding sites in liver homogenates (67).

In a later paper, Dunn *et al.* (69) measured the EGF-receptor movement in the perfused rat liver system with polyclonal antibodies against the receptor. These results suggested rapid internalization of EGF receptors in response to EGF and degradation within lysosomes (69). However, four times more EGF was degraded at 8 hr than the high-affinity (K_d, 8–15 n*M*) receptors lost, indicating that (a) high-affinity receptors were recycled and/or (b) more than 300,000 receptors were available. To examine the latter possibility, the Dunn group (69) actually identified a latent pool of approximately 300,000 low-affinity receptors (K_d, ~200 n*M*).

The mechanisms of down-regulation (recycling and/or degradation) have not yet been resolved. Receptor degradation was shown to account for down-regulation in 3T3 and A431 cells (70,71). In contrast, Wiley and Cunningham (34) used a mathematical modeling to analyze EGF endocytosis in 3T3 cells and concluded that down-regulation is explained by an increased rate of receptor internalization. The results of Dunn *et al.* (69), as described above, demonstrate the degradation of EGF receptors in the hepatocytes, but the possibility of receptor recycling still remains. In fact, Gladhaug and Christoffersen (72) recently supported the idea of recycling of EGF receptors using results from work done with freshly isolated hepatocytes. These results have provided evidence for receptor externalization, which might be accomplished by either recycling of internalized receptors or recruitment of preexisting receptors (72). Interestingly, Mcclain and Olefsky (73) very recently presented evidence for two independent pathways of insulin-receptor internalization in hepatocytes and hepatoma cells. They proposed that the low-dose pathway is through conventional coated pits and endosomal acidification but that the high-dose pathway may proceed independent of the conventional pathway and is more rapid in both internalization and recycling.

Thus, there have been many controversies with regard to the itinerary of peptide receptors. Issues which we particularly hope to see resolved in the near-future are the control over recycling versus degradation of receptors and the precise mechanism of receptor–ligand dissociation, segregation, and sorting.

Table I. Parameters for the Interaction of EGF with the Receptors on the Rat Liver Cell Surface[a]

k_{on} (on-rate constant)	2.0 μM^{-1} sec^{-1}
k_{off} (off-rate constant)	0.035 sec^{-1}
k_{seq} (sequestration rate constant)	0.069 sec^{-1}
P_T (cell surface receptor density)	36 pmol/g liver
K_d (equilibrium dissociation constant)	k_{off}/k_{on} = 17.5 n*M*

[a] These parameters obtained by fitting the data from MID experiments (37°C) to a kinetic model (31).

MODELING OF *IN VIVO* PHARMACOKINETICS OF POLYPEPTIDE HORMONES INCORPORATING THE DOWN-REGULATION OF RECEPTORS

We attempted to simulate the *in vivo* plasma concentration–time profiles of EGF to understand how the time profiles of the plasma concentration of polypeptides affect the time profiles of cell surface receptors, and vice versa. For this purpose, a simple physiological pharmacokinetic model (74,75) as shown in Fig. 8 was used, in which the liver is considered to be a major organ responsible for the receptor-mediated clearance and distribution of EGF (29,30). The kidney also clears EGF by glomelular filtration (30). The kinetic model (Fig. 8) in the liver compartment with respect to the interaction of ligand with cell surface receptors, the internalization of ligand–receptor complex, and receptor insertion and turnover is specified by three linked differential equations as follows:

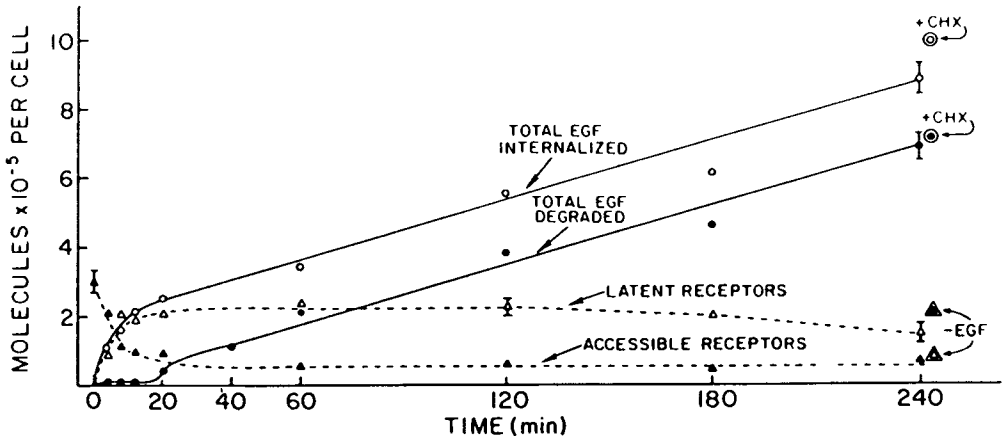

Fig. 7. The uptake and degradation of EGF and effects on receptor number determined in the isolated perfused rat liver (recirculatory system). At selected times after the addition of saturating amounts of ^{125}I-EGF to the perfusate, whole livers and/or biopsies were homogenized. The total EGF internalized (○) equaled the TCA-soluble radioactivity in the perfusate plus the total radioactivity in the liver. The combined TCA-soluble radioactivity in the liver and perfusate yielded the total EGF degraded (●). Cycloheximede (CHX; 0.2–1 m*M*) was perfused through a liver for 30 min prior to ligand addition. EGF binding capacity was measured in the absence (accessible; ▲) or presence (total) of Brij-35 detergent. Latent receptors (△) were calculated from the difference in total and accessible receptors. The effects of long-term perfusion on receptor number were determined in the absence of EGF (△, △). [From Dunn and Hubbard (67) with permission.]

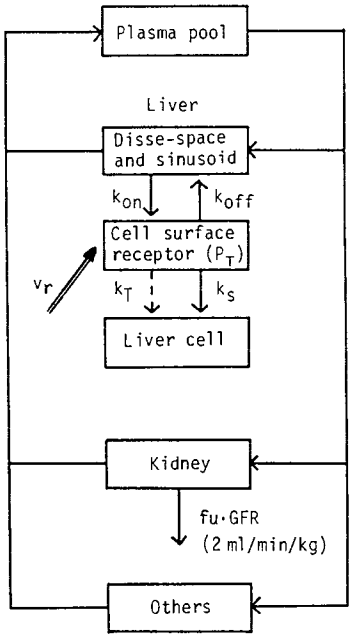

Fig. 8. Physiological pharmacokinetic model for EGF disposition in the rat. Physiological and biochemical parameters used for the simulation are listed in Table II. The bulk of the EGF clearance is assigned to the liver, and some to the glomerular filtration in the kidney according to our experimental results.

$$V_E\, dC_E/dt = Q_H\,(C_P - C_E) - k_{on}(P_T - LR_s)C_E - k_{off}\, LR_s \quad (4)$$

$$dLR_s/dt = k_{on}\,(P_T - LR_s)C_E - (k_{off} + k_s)LR_s \quad (5)$$

$$dP_T/dt = v_r - k_s LR_s - k_t(P_T - LR_s) \quad (6)$$

where Q_H is the hepatic plasma flow rate and V_E is the volume of the hepatic extracellular space; C_P and C_E are the peptide concentrations in the plasma pool and in the extracellular space of the liver, respectively; P_T is the density of cell surface total receptors; LR_s is the concentration of the cell surface ligand–receptor complex; k_s and k_t are the internalization rate constant of occupied and unoccupied receptors, respectively; and v_r is the constant rate of insertion of new receptors and/or the recycled receptors into the membrane. One may notice that this model considers the internalization of free (unoccupied) receptors as well as ligand–receptor complexes, although the internalization rate of free receptors is set to be much slower than that of the complex. This model for the liver compartment is essentially the same as that introduced by Wiley and Cunningham (34). Before administering exogenous EGF, a given number of liver cell-surface receptors should be at a steady state; then

$$v_r = k_t\, P_{T(t=0)} \quad (7)$$

where $P_{T(t=0)}$ is the density of EGF receptors on the liver cell surface before administering exogenous EGF. Also, for extrahepatic compartments (plasma pool, kidney, and others), the mass-balance differential equations can be written, and these simultaneous differential equations were solved numerically using a computer to yield the values of C_P, LR_s, and P_T as a function of time. The physiological and biochemical parameters used for the simulations are listed in Table II. Time profiles of plasma EGF concentrations and the corresponding densities of cell surface EGF receptors are simulated at various iv doses of EGF (Fig. 9). Receptor densities

Table II. Physiological and Biochemical Parameters Used for the Simulation of EGF Disposition in the Rat[a]

Tissue	Physiological parameter: Volume (ml/kg BW)	Physiological parameter: Blood flow (ml/min/kg BW)	Biochemical parameters
Plasma pool	120 (distribution volume)	81	
Kidney	1.6 (extracellular space)	31	2 ml/min/kg BW (renal clearance = plasma unbound fraction × GFR)
Others	80	10	
Liver	11.6 (extracellular space)	40	k_{on} = 0.12 (pmol/ml)$^{-1}$ min^{-1} k_{off} = 1.1 min^{-1} k_s = 0.55 min^{-1} k_T = 0.036 min^{-1} $P_{T(t=0)}$ = 1000 pmol/kg BW = 25 pmol/g · liver v_r = 36 pmol/min/kg BW = 0.9 pmol/min/g · liver

[a] These parameters are taken basically from our data (27,29–31). However, some parameters are not definitive and should be examined more in the future.

on the liver cell surface are shown to be altered with time (down-regulation) depending on the dose of the peptide hormone (Fig. 9b). In addition, the nonlinear kinetics have been reproduced by simulation studies, showing that not only the saturation of EGF binding to the receptors, but also the down-regulation of the cell surface receptors could be causes for the nonlinear kinetics. Figure 10 shows the simulations when the k_t values are varied from 0.0036 to 0.36 min^{-1} with a constant k_s value. If the k_t value is small, the plasma disappearance of EGF is much delayed due to an extensive and prolonged down-regulation of the surface receptors. As the k_t value becomes larger, the turnover of the surface receptors is accelerated [as shown by Eq. (7)] and both the extent and the duration of down-regulation of receptors are minimal, leading to the rapid plasma disappearance of EGF. When the k_t value becomes very large, the density of cell surface receptors is kept constant, and the kinetics can be expressed by a conventional Michaelis–Menten equation which has been widely used for the analysis of carrier-mediated transport of small molecules. In this way, this sim-

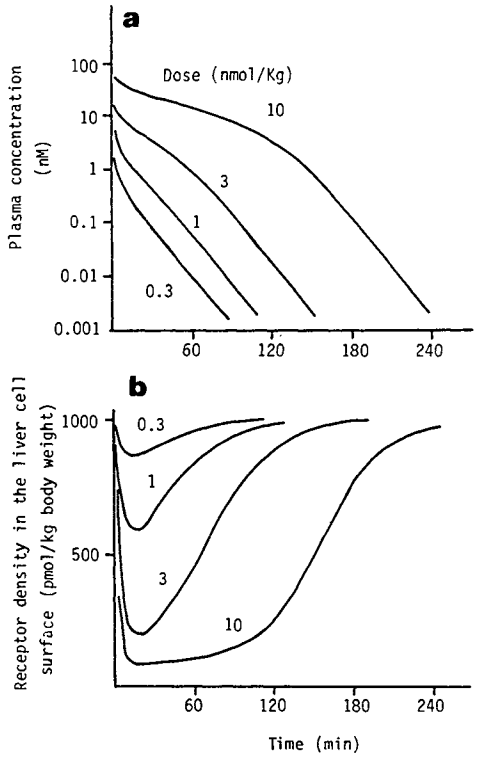

Fig. 9. Simulation of the time profiles of plasma EGF concentrations (a) and the corresponding densities of liver cell surface EGF receptors (b) at various iv doses of EGF.

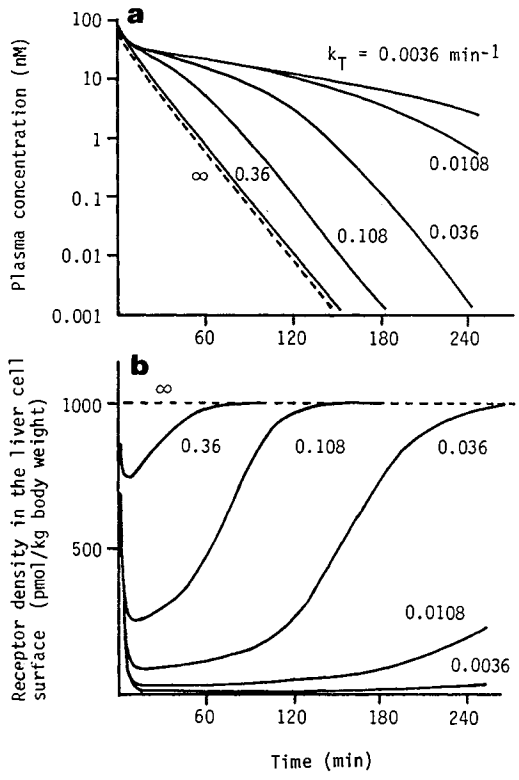

Fig. 10. Simulation of the effect of the internalization rate constant (k_t) for free receptors on the time profiles of plasma EGF concentrations (a) and the corresponding densities of liver cell surface EGF receptors (b) after iv injection of 10 nmol/kg of EGF. The internalization rate constant (K_s) for occupied receptor was kept constant (0.55 min^{-1}).

ulation study suggested that the down-regulation of cell surface receptors can be kinetically explained by a more rapid surface clearance of occupied versus unoccupied receptors. Considering such a down-regulation phenomenon, a conventional dosage regimen such as a constant infusion method to maintain an effective plasma concentration of drugs may not necessarily be appropriate for peptide hormones.

The present kinetic model may still be oversimplified because of the complexity of RME. The following feasible mechanisms could be incorporated into the model, once their mechanisms have been clarified. (i) Can the synthesis rate of receptors and/or the insertion rate of receptors into the cell surface be regulated either by the occupancy of cell surface receptors or by the induced rate of internalization? In the present analysis, the v_r value is assumed constant irrespective of the time profiles of the polypeptide concentrations. However, this may not be true for all cases. It is possible that the v_r value could be affected by a wave of EGF in the cases that the receptors are fractionally degraded after the internalization (69) or that a feedback regulation of receptor synthesis occurs (76). In such cases, the down-regulation could occur through a reduced rate of insertion of receptors into cell surface membranes. (ii) What is the fraction of internalized receptors to be recycled or to be degraded? It is crucial to know this fraction for each peptide hormone to quantify the down-regulation. (iii) Does receptor heterogeneity exist (69,72)? and Does a hepatic lobular gradient (from periportal to centrilobular) of EGF and/or the receptors exist (77)? (iv) Is the internalization rate constant (k_s) affected by the occupancy of cell surface receptors? Although some studies showed that the k_s value is independent of the receptor occupancy (33), this may not hold true for all cases and should be considered. Most recently, Gex-Fabry and Delisi (37,38) presented a more elaborate kinetic model for RME, having incorporated some of the above-mentioned mechanisms. The basic processes considered in this model (37,38) are ligand–receptor binding, diffusion of receptors and ligand–receptor complexes in the plane of the membrane toward and away from coated pits, binding of the complexes to coated pit proteins, endocytosis of coated pit contents, degradation of ligand, and recycling of undegraded receptors.

Finally, the down-regulation mechanism for receptors may exist not only in the clearance organ such as the liver but also in the effect-target organ, which may have different kinetics. These factors must be known to understand the pharmacodynamics of biologically active polypeptides after their administration into the body.

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