Rate Constants for Binding, Dissociation, and Internalization of EGF: Effect of Receptor Occupancy and Ligand Concentration†

Christopher M. Waters, Kerby C. Oberg, Graham Carpenter, and Knowles A. Overholser*
Departments of Biomedical Engineering and Biochemistry, Vanderbilt University, Nashville, Tennessee 37235
Received July 19, 1989; Revised Manuscript Received December 4, 1989

ABSTRACT: We measured the kinetic parameters for interaction of epidermal growth factor (EGF) with fetal rat lung (FRL) cells under two sets of experimental conditions and applied sensitivity analysis to see which parameters were well-defined. In the first set of experiments (method 1), the kinetics of internalization and dissociation of radiolabeled EGF were measured with a temperature-shift protocol in medium initially devoid of free ligand. The initial concentration of radiolabeled EGF bound to the cell surface corresponded to levels of receptor occupancy ranging from approximately 200 receptors per cell to approximately 18 000 receptors per cell, a level at which EGF binding approaches saturation. In the second set of experiments (method 2), carried out at a constant temperature, we began with no surface-bound or internalized ligand. The initial free ligand concentration was varied from 0.2 to 50 ng/mL. In both sets of experiments, we measured surface-bound, internalized, and free ^{125}I-EGF as functions of time and evaluated the parameters of a mathematical model of endocytosis. Sensitivity analysis showed that three rate constants were well-defined in this combination of two experimental approaches: k_e, the endocytic rate constant; k_a, the association rate constant; and k_d, the dissociation rate constant. The endocytic parameter k_e was found to be independent of initial surface receptor occupancy (method 1); there was some indication that it increased with initial free ligand concentration in method 2. Neither k_d nor k_a was found to change with extent of initial surface receptor occupancy or initial free ligand concentration, respectively, a finding of significance, since diffusion theory predicts these parameters will vary with surface receptor occupancy. In addition, the measurement of k_d and k_a enabled us to calculate EGF receptor affinity at 37 °C over a range of ligand concentrations.

At 37 °C, the interaction of epidermal growth factor (EGF)[1] with its receptor on the surface of cells is rapidly followed by clustering of EGF–receptor complexes in coated pits, internalization of the complexes with the formation of endocytic vesicles, and intracellular processing of the endocytic vesicles, ultimately resulting in fusion with lysosomes, where both the ligand and receptor are degraded (Carpenter, 1987). This pathway for the metabolism of EGF–receptor complexes, as well as other growth factors, has been defined with morphologic techniques as well as more indirect biochemical studies. The available data demonstrate that this is a very dynamic pathway and that variations exist for other types of ligand–receptor complexes. For example, LDL and transferrin receptors are not routed to the lysosome after internalization, but are rapidly recycled to the cell surface (Brown et al., 1983; Hanover & Dickson, 1985). Reports exist to indicate that a low level of recycling may occur for EGF–receptor complexes in some cell types (Dunn et al., 1986; Murthy et al., 1986; Teslenko et al., 1987; Gladhaug & Christoffersen, 1988). From these studies, representations of the dynamic pathway have been devised. However, these descriptions do not address the kinetic parameters of individual steps in the pathway.

To better understand and appreciate these parameters, computer-aided mathematical modeling of the endocytosis pathway has been utilized. This approach, it is hoped, may yield meaningful information concerning reaction rates and constants, reaction sequence and molecularity, rate-limiting steps, and the influence of reactant concentrations. For the endocytosis of EGF–receptor complexes, Wiley and his collaborators (Wiley & Cunningham, 1981, 1982; Knauer et al., 1984) have utilized modeling to define and measure a rate constant for endocytosis (k_e) and to determine the influence of culture conditions on this kinetic parameter (Wiley & Cunningham, 1982). Their original studies assumed steady-state conditions, while recent publications (Opresko & Wiley, 1987a,b) have amended the analysis to include non-steady-state conditions. However, other assumptions are inherent in the analysis, e.g., a negligible effect of ligand degradation. Also, values for the parameters have been derived from only one type of experimental protocol, and their utility for predicting results of different experiments has not been tested. The modeling studies described in this paper extend the analysis of endocytosis to include the measurement of several rate constants for endocytosis under a variety of reaction conditions and the utilization of two distinct experimental protocols chosen for their capacity to permit separate determination of binding and dissociation parameters as well as internalization constants. In particular, we have determined the manner in which differing levels of receptor occupancy and free ligand concentration do or do not influence rate constants of the endocytic pathway. Sensitivity analysis of these values is included to indicate the level of confidence one can have in these values. This has not been done in any of the previously reported modeling studies for ligand–receptor complexes.

Materials and Methods

Cell Culture. Fetal rat lung (FRL) epithelial cells were plated into six-well dishes (Costar, Cambridge, MA) at a density of 7800 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum (Gibco, Long Island, NY) as previously described (Oberg et al., 1988). The cells

† This work was supported by Grant CA24701 from the National Institutes of Health. C.M.W. is supported by Training Grant HL07411.
* To whom correspondence should be addressed at Box 6173, Station B, Vanderbilt University, Nashville, TN 37235.

[1] Abbreviations: EGF, epidermal growth factor; FRL, fetal rat lung; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; BSS, balanced salt solution.

0006-2960/90/0429-3563$02.50/0

were grown at 37 °C in a humidified incubator with 5% CO_2. The cells were used in experiments when they were 95–100% confluent (approximately 100000 cells/cm^2). Trypsinized cells from selected wells were counted electronically (Coulter Instruments, Inc., Hialeah, FL).

Radioiodination of EGF. EGF was isolated from the mouse submaxillary gland according to the methods of Savage and Cohen (1972) and iodinated as described by Carpenter and Cohen (1976). The specific activity of the ^{125}I–EGF used in each experiment was determined and is presented in the figure legends.

Binding, Dissociation, and Internalization Kinetics. Two different methods were applied to measure the kinetics of the interactions of ^{125}I–EGF with FRL cells. While both methods are appropriate to the study of the endocytic step itself, method 1 was designed to provide additional information on the kinetics of the dissociation of ligand–receptor complexes. In this approach, all labeled EGF molecules were initially surface-bound at 4 °C; the concentration of free ligand was initially at zero before the experiment began with a rapid temperature shift to 37 °C. Method 2, on the other hand, emphasizes the kinetics of the association (surface binding) of ligand and receptor. In method 2, all of the labeled molecules were initially present as free ligand. Later we will show by means of sensitivity analysis how the combination of both methods maximizes identifiability of the rate constants.

Method 1. Confluent cells (100000 cells/cm^2) were washed twice at room temperature with binding medium: DMEM supplemented with 20 mM HEPES (Calbiochem, La Jolla, CA) and 0.2% bovine serum albumin (BSA) (Miles Scientific, Naperville, IL). After the second wash, 1 mL of binding medium was added to each well, and the plate was chilled on ice for 15 min. An excess of unlabeled EGF ranging from 40- to 100-fold was added to selected wells in order to determine the level of nonspecific binding (Carpenter, 1985). Radiolabeled EGF was then added to all wells at concentrations ranging from 0.2 to 50 ng/mL, and the cells were incubated for 2.5–3.5 h at 4 °C. The low temperature prevents internalization of ^{125}I-EGF–receptor complexes (Carpenter, 1985), while the long incubation time ensures equilibrium binding of the growth factor [as determined by separate time course studies at 4 °C (not shown)]. At the end of the incubation period, the cells were quickly washed twice with ice-cold Hanks' balanced salt solution (BSS) supplemented with 0.2% BSA to remove unbound ^{125}I-EGF. One milliliter of prewarmed (37 °C) binding medium was then added to each well, and the plates were quickly transferred to a 37 °C water bath in which they were incubated for various times (0–15 min). At the end of the timed incubations, the plates were removed from the water bath, and the medium containing dissociated ^{125}I–EGF was quickly pipetted from the wells into sample tubes. The dissociated free ligand concentration was obtained by measuring radioactivity in the sample tubes with a γ counter (Beckman Instruments, Irvine, CA). The cell monolayers were placed on ice and rapidly washed twice with BSS plus 0.2% BSA and incubated for 4 min at 4 °C with either a pH 7.4 or a pH 4.0 glycine buffer (100 mM NaCl/50 mM glycine). The pH 4.0 buffer was used to remove surface-bound radiolabeled EGF as described by Carpenter (1985). The medium from these washes (pH 4.0 to 7.4) was pipetted into sample tubes for measurement of radioactivity. The cells were solubilized with 1 N NaOH, and the radioactivity from each well was measured. The total EGF binding was given by the radioactivity from the wells washed with the pH 7.4 buffer (corrected for nonspecific binding as well as the radioactivity which dissociated during the wash). Internalized ^{125}I-EGF was measured by the radioactivity from the cells washed with the pH 4.0 buffer (also corrected for nonspecific binding). The surface-bound radioactivity was given by the difference between the total specific binding (pH 7.4 wash) and the total internalized (pH 4.0 wash). Each data point is the average of duplicate wells corrected for nonspecific binding. From these measurements, we obtained the free ligand concentration, [L], the surface-bound ligand concentration, $[LR]_s$, and the internalized ligand concentration, $[LR]_i$.

Method 2. Cells grown to confluence were washed twice with room temperature DMEM plus 0.2% BSA. Next, 1 mL of prewarmed 37 °C binding medium was added to each well, and the plates were placed in a 37 °C water bath. After 15 min, unlabeled EGF (40–100-fold molar excess) and ^{125}I-EGF at concentrations ranging from 0.2 to 50 ng/mL were added to the wells, and the cells were incubated for various times, 1–60 min. The free ligand concentration, the surface-bound ligand concentration, and the internalized ligand concentration were all determined as in method 1. In method 2, all of the ^{125}I-EGF molecules are initially in the medium, whereas in method 1 all of the ^{125}I-EGF molecules are initially surface-bound.

Sensitivity Analysis. We evaluated the sensitivity of predicted concentrations to changes in parameters in order to determine which type of experiment was best for measuring each parameter and to clarify what variables should be measured. Such an analysis is performed by calculating "sensitivity functions". In general, if such a function is nonzero for a given variable and parameter, that variable will be useful in determining the parameter. Conditions under which a sensitivity function is far from zero are promising regions for confident identification of a parameter. We calculated Bode's sensitivity function (Frank, 1978) over time using model simulations of eq 2–5 given under Model and Analysis. This function can be defined by an example: the sensitivity of the variable $[LR]_s$ to changes in the parameter k_e is given by

$$S_{k_e}^{[LR]_s} \equiv \frac{k_e^*}{[LR]_s^*} \frac{\delta[LR]_s}{\delta k_e} \qquad (1)$$

where the derivatives are evaluated at the nominal or best-fit values, k_e^* and $[LR]_s^*$. We perturbed each parameter by 10% and evaluated the sensitivity over the time course of the experiments for $[LR]_s$, $[LR]_i$, [L], and $[R]_s$. Sensitivity functions for the free receptor concentration, $[R]_s$, were evaluated to determine the importance of measuring this variable, even though we did not measure it in our studies.

Results

Model and Analysis. We used the basic model of ligand binding and internalization of Wiley and Cunningham (1981) but analyzed the data without steady-state assumptions [see also Lauffenburger et al. (1987)]. Our model does not include a step for coated-pit trapping of the ligand–receptor complex; we have assumed that trapping is not a rate-limiting step and can be included in the endocytic step. Also implicit in our formulation is an inclusion of diffusional resistance in the association and dissociation steps. Figure 1 is a schematic drawing of the model, and the following four mass balance equations describe the system:

$$d[R]_s/dt = V_r - k_t[R]_s - k_a[L][R]_s + k_d[LR]_s \qquad (2)$$

$$d[LR]_s/dt = k_a[L][R]_s - k_d[LR]_s - k_e[LR]_s \qquad (3)$$

$$d[LR]_i/dt = k_e[LR]_s - k_h[LR]_i \qquad (4)$$

$$d[L]/dt = k_d[LR]_s - k_a[L][R]_s \qquad (5)$$

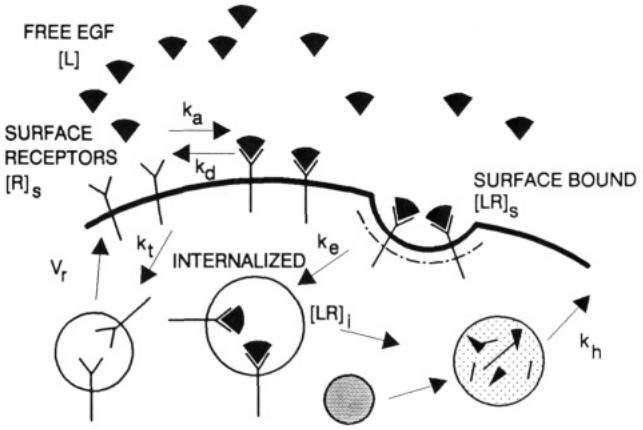

FIGURE 1: Schematic diagram of a model for the receptor-mediated endocytosis of EGF. EGF is represented by the dark triangles, the EGF receptor by the Y-shaped figures. The rate constants are described in the text.

The four dependent variables in these equations are the concentration of free surface receptors, $[R]_s$, the concentration of ligand–receptor complexes on the surface, $[LR]_s$, the concentration of ligand–receptor complexes inside the cell, $[LR]_i$, and the free ligand concentration, [L]. The parameters are defined as k_a = the association rate constant for binding of the ligand to the receptor, k_d = the rate constant for dissociation of the ligand from the receptor, k_e = the rate constant for endocytosis of ligand–receptor complexes, k_h = the "hydrolysis" rate constant for elimination of degraded ligand from the cells, k_t = the turnover rate constant for internalization of free receptors, and V_r = the insertion rate of free receptors onto the cell surface. To the extent that the assumed reaction scheme is correct in order and molecularity, the rate constants should be independent of time and of concentrations $[LR]_s$, $[LR]_i$, [L], and $[R]_s$.

We wanted to test the ability of the model to describe two kinds of experiments, and to maximize the confidence with which the parameters were identified. Accordingly, we chose not to invoke an assumption of steady state, and we measured the free ligand concentration [L] as well as the surface-bound $[LR]_s$ and internalized $[LR]_i$ concentrations. We normalized each variable to the total amount of radioactivity at each time point and then solved eq 2–5 numerically using Adams–Bashforth second-order integration in a commercial software package (TUTSIM, Palo Alto, CA). We used the Simplex algorithm to minimize the sum of squared differences between the predicted curves and the data, and we obtained best fits for normalized versions of eq 3–5. In fitting the free ligand curve, we corrected for the fact that degraded ligand from the cells shows up as radioactivity in the medium. From these best-fit results, we obtained the six model parameters k_a, k_d, k_e, k_h, k_t, and V_r. These parameters were measured over a range of ^{125}I-EGF concentrations by using two different experiments with different sets of initial conditions. For method 1, the initial conditions are as follows: at $t = 0$, [L] = 0 (no free ligand), $[LR]_s = 1$, and $[LR]_i = 0$ (all radioactivity is on the cell surface). Initial conditions for method 2 are as follows: at $t = 0$, [L] = 1 (all radioactivity is present as free hormone), $[LR]_s = 0$, and $[LR]_i = 0$.

A fourth initial condition, necessary for both methods, was the specification of the total number of potentially available surface receptors. We used a value of 25 500 surface receptors per cell, as measured using Scatchard analysis by Oberg et al. (1988) for FRL cells.

Figure 2 shows the influence of incubation time at 37 °C on the number of surface-bound EGF–receptor complexes

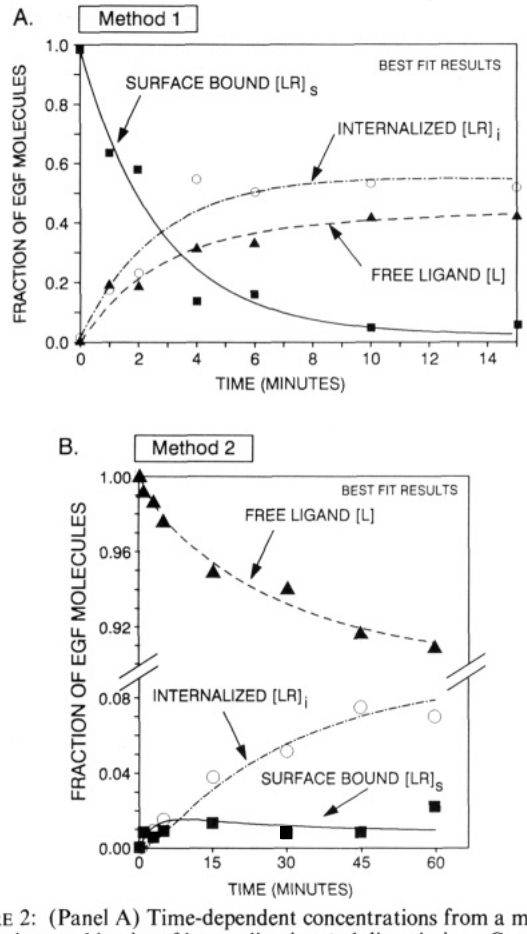

FIGURE 2: (Panel A) Time-dependent concentrations from a method 1 experiment: kinetics of internalization and dissociation. Confluent cultures of fetal rat lung cells were incubated at 4 °C for 2.5 h with ^{125}I-EGF (10 ng/mL, specific activity 59 000 cpm/ng). At the end of the 4 °C incubation, the cells were washed of free ligand and then incubated for various times at 37 °C. The initial surface receptor occupancy was 9300 receptors per cell. At the specified times, the cells were removed from the 37 °C incubator, and the amount of radioactivity present as free ligand (▲), surface-bound ligand (■), and internalized ligand (○) was determined as described under Materials and Methods. The concentrations were corrected for nonspecific binding and normalized to the total amount of radioactivity at each time. The curves represent the best-fit predictions of the model. (Panel B) Time-dependent concentrations from a method 2 experiment: binding and internalization kinetics. Confluent cultures of fetal rat lung cells were incubated at 37 °C with ^{125}I-EGF (5 ng/mL, specific activity 98 000 cpm/ng) in DMEM supplemented with 20 mM HEPES and 0.2% BSA. At the specified times, the amount of radioactivity present as free ligand (▲), surface-bound ligand (■), and internalized ligand (○) was determined as described under Materials and Methods. Concentrations were corrected for nonspecific binding and normalized to the total amount of radioactivity at each time. The curves represent the best-fit predictions of the model.

$[LR]_s$, internalized complexes $[LR]_i$, and free ligand [L]. When method 1 was employed (panel A), the amount of ligand dissociating from the cells at 37 °C was equivalent to 40% of the total ligand prebound to the surface at 4 °C. The remaining surface-bound ligand was rapidly and extensively internalized within 10 min after the temperature shift. This protocol allows one to temporally distinguish ligand binding (which occurs at 4 °C) from internalization (which occurs only after the shift to 37 °C). When this experiment was performed as described in method 2—which, while simulating physiological conditions, does not temporally separate binding and internalization—the results shown in Figure 2B were obtained. The free ligand concentration remained high over the course of the experiment, internalized ligand was seen to accumulate, and maximal $[LR]_s$ values were achieved within 10 min.

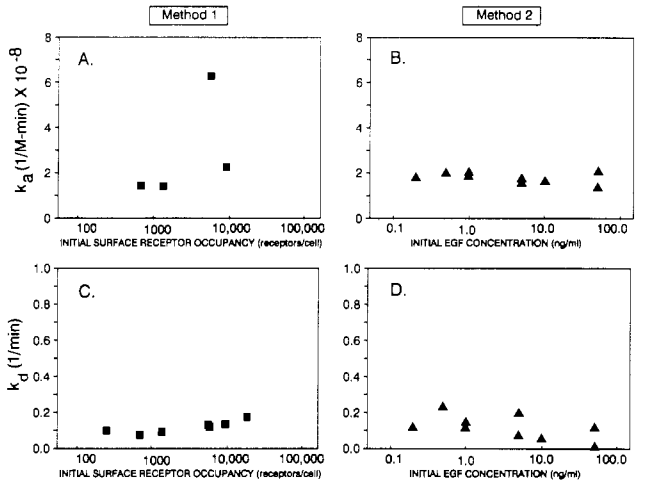

FIGURE 3: (Panels A and B) Measured association rate constant k_a does not change with the initial free ligand concentration. (A) Method 1 experiments (■): the k_a's were evaluated from best fits of the model to the data as described under Model and Analysis. (B) Method 2 experiments: (▲) represents our results evaluated as described under Model and Analysis. (Panels C and D) The measured dissociation rate constant k_d does not change with the initial surface receptor occupancy. Panel C shows results of method 1 experiments (■), while panel D shows the results of the method 2 approach (▲).

Association and Dissociation Rate Constants: k_a and k_d. The initial step in the endocytic pathway, the formation of ligand–receptor complexes, is characterized by the association rate constant k_a, while the reverse step is characterized by the dissociation rate constant k_d. These constants were determined as shown in Figure 3. By use of method 1, k_a (Figure 3A) and k_d (Figure 3C) were measured at increasing levels of receptor occupancy. Similar estimates using method 2 were made at increasing concentrations of free ^{125}I-EGF; k_a is shown in Figure 3B and k_d in Figure 3D. The estimates of k_d obtained by method 1 (Figure 3C) were independent of receptor occupancy (200–18 000 receptors per cell). Results from method 2 (Figure 3D), though more scattered, suggest that k_d is independent of the initial free ^{125}I-EGF concentration (0.2–50 ng/mL). In contrast, Figure 3A,B appears to show the discrepant dependence of k_a on the initial receptor occupancy (Figure 3A) and the initial free ^{125}I-EGF concentration (Figure 3B). The results of method 1 suggest k_a depends on receptor occupancy while results of method 2, indicate constant k_a. Sensitivity analysis helps resolve this apparent discrepancy. The relevant sensitivity functions are shown in Figure 4. The analysis shown in Figure 4A suggests that one can have little confidence in measurements of k_a by method 1; i.e., experiments in which free ligand concentration was initially zero are ill-suited to the identification of association rate constants, and the apparent dependence of k_a on receptor occupancy is spurious. The analysis illustrated in Figure 4B indicates that somewhat more confidence can be placed in method 2 (high initial free ligand concentration) for measuring k_a, particularly if data are acquired at early times. Figure 4C,D indicates that method 1 is clearly the better approach for identification of the dissociation constant k_d; the scatter seen in Figure 3D is not surprising under the circumstances. Taken together, Figures 3 and 4 show that the best average k_a value for ^{125}I-EGF binding (method 2) is $(1.8 \times 10^8) \pm (0.2 \times 10^8)$/

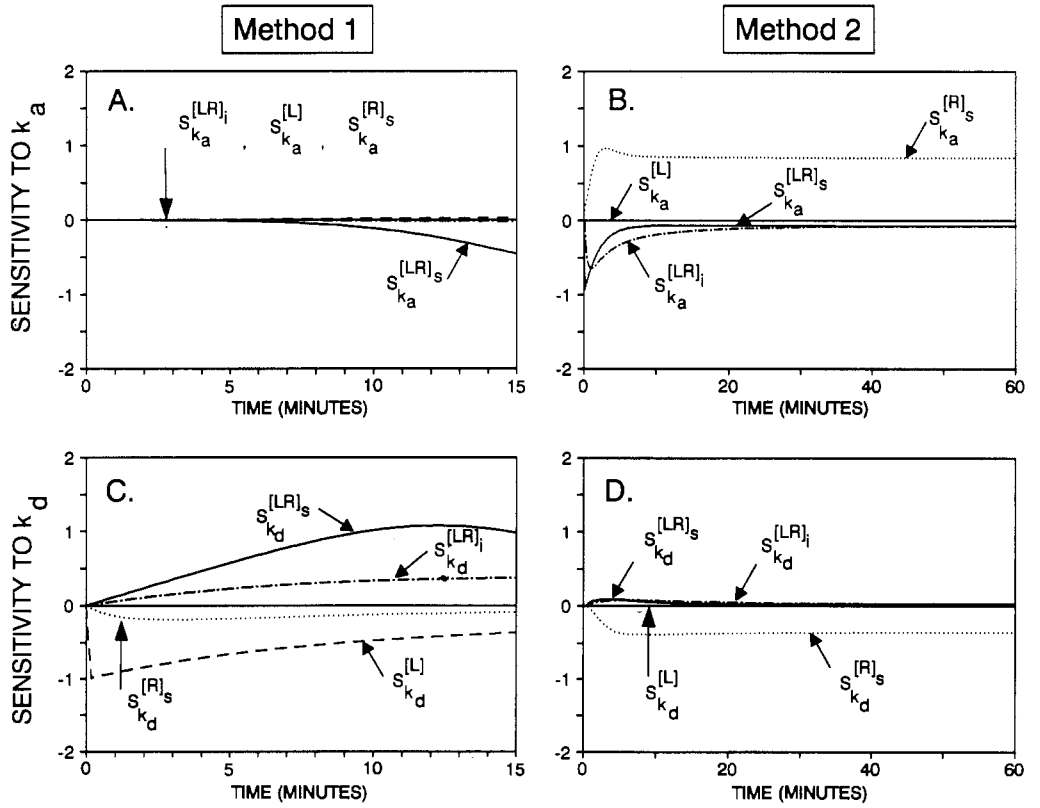

FIGURE 4: Sensitivity functions for the association and dissociation rate constants. Each sensitivity function expresses the change in a predicted concentration when a parameter is perturbed by 10%. For example, $S^{[LR]_s}_{k_a}$ represents the sensitivity of the concentration of the surface-bound ligand–receptor complexes $[LR]_s$ to a 10% change in k_a. The time-dependent functions were calculated by using Bode's definition of sensitivity as given in eq 5 in the text. Nonzero sensitivities indicate that comparison of measured and predicted values of the relevant dependent variable would be useful for determining the relevant rate constant. [L] represents the free ligand concentration; $[LR]_s$ represents the concentration of surface-bound ligand; $[LR]_i$ represents the concentration of internalized ligand. Although the free surface receptor concentration $[R]_s$ was not measured in our experiments, we have presented the predicted functions for that dependent variable. (Panel A) Sensitivity functions for k_a using method 1. (Panel B) Sensitivity functions for k_a using method 2. (Panel C) Sensitivity functions for k_d using method 1. (Panel D) Sensitivity functions for k_d using method 2.

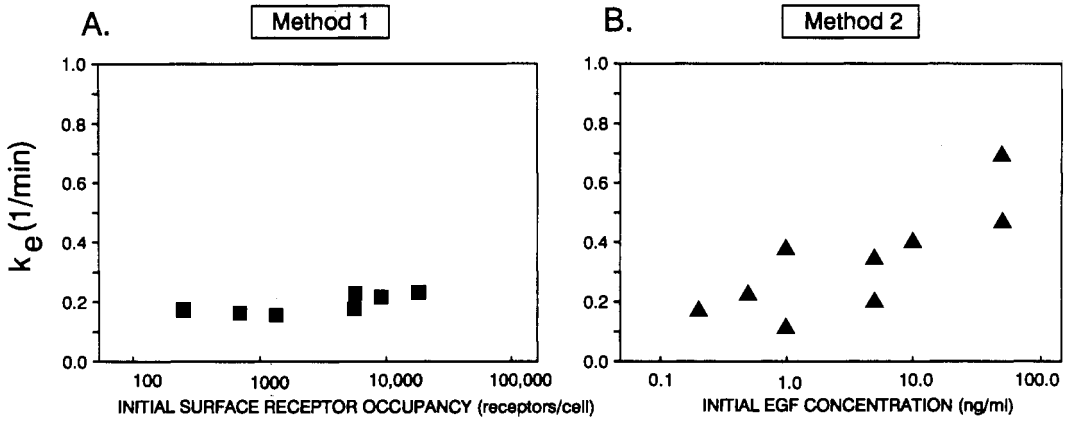

FIGURE 5: Measured endocytic rate constant k_e does not change with the initial surface receptor occupancy but seems to increase with the initial free ligand concentration. (Panel A) Method 1 experiments: the k_e's were evaluated by the best fit of the model to concentration histories like those in Figure 2A as described under Model and Analysis (■). (Panel B) Method 2 experiments: (▲) represents our results evaluated by the best fit of the model to concentration histories like those in Figure 2B, as described under Model and Analysis.

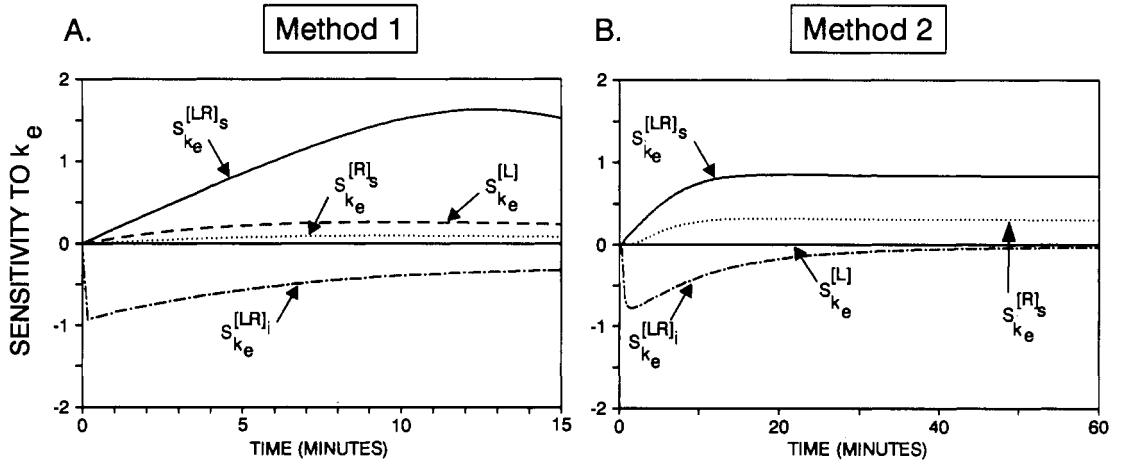

FIGURE 6: Sensitivity functions for the endocytic rate constant k_e. The function $S_{k_e}^{[LR]_s}$ represents the sensitivity of the concentration of surface-bound ligand–receptor complexes $[LR]_s$ to changes in k_e. Functions describing the sensitivity of [L], $[LR]_i$, and $[R]_s$ are defined analogously. The sensitivity functions were calculated by using Bode's definition of sensitivity as given in eq 5 in the text. Each curve was predicted based on a 10% perturbation of k_e. Nonzero sensitivities indicate that the dependent variable would be useful for determining k_e. [L] represents the free ligand concentration; $[LR]_s$ represents the concentration of surface-bound ligand; $[LR]_i$ represents the concentration of internalized ligand. Note that the free surface receptor concentration $[R]_s$ was not measured in our experiments, although we present the predicted sensitivity functions. (Panel A) Sensitivity functions for k_e using method 1. (Panel B) Sensitivity functions for k_e using method 2.

(M·min), and the best average k_d value (method 1) is 0.12 ± 0.03/min.

Endocytic Rate Constant k_e. This parameter characterizes the rate at which EGF–receptor complexes are endocytosed. It is reasonable to assume that any conditions that alter k_e may affect the nature of the endocytic step. Figure 5 shows the results of experiments in which we measured k_e as a function of the range of initial surface receptor occupancy (method 1, Figure 5A) and the free ^{125}I-EGF concentration (method 2, Figure 5B). Results from method 1 indicate that k_e is constant at an average value of 0.19 ± 0.03/min, suggesting that the rate coefficient for endocytosis is independent of initial receptor occupancy. However, Figure 5B suggests that at very high initial free ligand concentrations the parameter increased somewhat. Sensitivity analysis of these experiments is shown in Figure 6. For each method, the sensitivity was high, justifying confidence in these values of k_e.

Discussion

We have shown that at 37 °C the dissociation rate constant k_d and the association rate constant k_a are independent of surface receptor occupancy and free ligand concentration, respectively. The constancy of these parameters suggests that diffusion of free ligand to the cell surface is not a rate-limiting step in the endocytic process under the circumstances of our experiments. According to diffusion theory (Erickson et al., 1987), k_a and k_d should depend on the number of receptors on the cell surface if diffusion affects the overall reaction rate. Since we observed no dependence of k_a and k_d on surface occupancy, we conclude that binding and dissociation of EGF are not limited by the diffusion of free EGF over the ranges of concentrations presented in this study.

We have calculated a receptor affinity constant at 37 °C, $K_D = k_d/k_a = (0.67 \times 10^{-9}) \pm (0.15 \times 10^{-9})$ M, without assuming equilibrium conditions. Due to rapid internalization of ^{125}I-EGF at 37 °C, most measurements of affinity constants have been with cells maintained at 4 °C. Earlier (Oberg et al., 1988), we reported values of $K_D = 0.22 \times 10^{-9}$ M for high-affinity receptors and 2.6×10^{-9} M for low-affinity receptors; these values were obtained for EGF and FRL cells at 4 °C using Scatchard analysis. Our present 37 °C value falls within the range observed at 4 °C. Since the physical properties of biological membranes can be expected to vary considerably between these two temperatures, it has never been clear heretofore that affinity constants measured at low temperatures are, in fact, similar to the values at 37 °C.

Our measured K_D at 37 °C did not change with receptor occupancy. This constancy implies a single affinity class of

Table I: Comparison of Rate Constants Reported for EGF in Different Cell Types[a]

cell type	k_a [1/(M·min)]	k_d (1/min)	k_e (1/min)	source
fetal rat lung		0.12	0.19	present study (method 1)
	1.8×10^8		0.11–0.69	present study (method 2)
A431	$(1.8–2.2) \times 10^6$	0.002	0.04–0.16	Wiley (1988)
human fibroblasts	1.7×10^8	0.74	0.14	Knauer et al. (1984)
human fibroblasts	3.06×10^8	3.3	0.078	Myers et al. (1987)
Balb/c 3T3			0.37	Gex-Fabry & DeLisi (1984)
Leydig tumor cells			0.16	Lloyd & Ascoli (1983)

[a] All values in this table were obtained at 37 °C. Three parameters from this model were not readily identifiable in the absence of measurements of free surface receptor concentration or degradation products: k_t, the rate constant for the endocytosis of unoccupied receptors; V_r, the rate of insertion of new receptors onto the cell surface; k_h, the rate constant for removal of degraded ligand from the cells. We did, however, obtain regression values for these parameters which were used in fitting the data: for method 1 experiments $k_t = 0.015/\text{min} \pm 0.006/\text{min}$, $V_r = 1300$ receptors/(cell·min) ± 2000 receptors/(cell·min), and $k_h = 0.026/\text{min} \pm 0.013/\text{min}$; for method 2 experiments, $k_t = 0.037/\text{min} \pm 0.029/\text{min}$, $V_r = 2500$ receptors/(cell·min) ± 860 receptors/(cell·min), and $k_h = 0.059/\text{min} \pm 0.029/\text{min}$.

receptors over the range of concentrations studied at 37 °C. The equilibrium binding measurements at 4 °C (Oberg et al., 1988) were interpreted to imply multiple affinity classes of receptors, as indicated by curved Scatchard plots. Equilibrium binding studies conducted at low temperatures, however, can give varying results depending on such factors as how fast the cells are cooled (Weigel & Oka, 1983), the potential loss of receptors into the medium at low temperatures (Kaplan & Keogh, 1982), or different levels of cell-surface exposure (affected by plating conditions). A nonuniform distribution of receptors at the cell surface might also affect the measured affinity. The cellular affinity of the ligand at equilibrium might be different from the dynamic affinity. Indeed, DeLisi and Weigel (1981) have predicted that surface receptor distribution could strongly affect the dynamics of binding yet have little effect on the equilibrium between bound and free ligand. (Since our measurements of k_a and k_d do not rely on equilibrium assumptions, surface receptor distribution may be unimportant.) Interestingly, Gex-Fabry and DeLisi (1984) showed that a curved Scatchard plot could in theory be obtained if there were a limiting amount of some cell-surface protein necessary for binding or coated-pit trapping. It is possible then that some membrane component affects the equilibrium binding at 4 °C, resulting in a curved Scatchard plot, but does not become limiting or affect binding at 37 °C.

Our method 1 results suggest that k_e is independent of initial surface concentration of EGF. Using an experimental approach similar to our method 2, Wiley (1988) observed a similar constancy of k_e in human fibroblasts. He calculated k_e using the method of Opresko and Wiley (1987) in which eq 4 is integrated numerically under the assumption that the $k_h[LR]_i$ term is negligible. Interestingly, for A431 cells, k_e decreased with an increase in the average number of occupied receptors. Wiley (1988) pointed out that the change in k_e might be due to the abnormally high number of EGF receptors on the A431 cells and a corresponding limiting level of some component necessary for endocytosis. In addition, occupied receptors might interfere with one another at high occupancy levels. We found no such effect at initial receptor occupancy levels as high as approximately 18 000 receptors per cell. The average value of k_e for the human fibroblasts and for the A431 cells at low surface receptor occupancies is only slightly lower than for the FRL cells (0.16/min compared to 0.19/min).

The fact that k_e does not vary with occupied receptor concentration suggests that clustering of ligand–receptor complexes in coated pits (Haigler et al., 1978, 1979; Schlessinger et al., 1978) is probably not a rate-limiting step. While it is possible to include a step for clustering in coated pits in models of ligand–receptor interactions (Gex-Fabry & DeLisi, 1984; Golstein et al., 1981), we found good agreement with observations when the endocytic step was assumed to be a simple first-order process.

Recent evidence has shown that EGF receptors can dimerize in coated pits (Boni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987a,b; Cochet et al., 1988; Fanger et al., 1989). On the assumption that dimerization of ligand–receptor complexes should be a second-order process, we evaluated the effect of replacing the first-order endocytic term $k_e[LR]_s$ by the second-order term $k_e[LR]_s^2$ in the model equations. As judged by the cumulative sum of squared differences, the resulting fit to experimental data was poorer than that obtained assuming first-order endocytosis. Therefore, though dimerization of ligand–receptor complexes may have occurred, the rate of endocytosis was first order in surface receptor occupancy. It has also been reported that dimerization affects the affinity of the receptor (Boni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987a). If such were the case, we should have seen a change in affinity at the higher EGF concentrations, and we did not. It should be noted, however, that the fit of our model to early-time data from method 2 was relatively poor at high free ligand concentrations, leaving open the possibility of dimerization or other phenomena not included in the model.

The apparent variation of k_e with the initial concentration of ligand in the medium as shown in Figure 5B is problematic. As just mentioned, at the highest EGF concentration, the ability of the mathematical model to describe the early (first 5 min) increase of surface-bound EGF–receptor complexes was poor; the measured rise in $[LR]_s$ was steeper than the model was able to accommodate. This discrepancy might be attributable to the existence of a second class of receptors. If a small fraction of receptors had a higher affinity than the rest, one would expect a quick, early rise of $[LR]_s$, poorly described by our model. Such an explanation is speculative, however, and we can do little more than draw attention to the possibility of unexplained phenomena at the highest levels of free EGF.

Table I compares our measured rate constants for FRL cells with rate constants reported for EGF in other cell types. With the exception of the value for A431 cells, the values for k_a are in good agreement. In most cases, the k_e values are similar; some of the variation may be differences in the approach to data analysis. For example, the technique of Opresko and Wiley (1987b) applied to our own model results in lower values of k_e than we report. This is probably because the method of Opresko and Wiley neglects ligand degradation and removal from the cell. Our model simulations indicate that if k_h is 0.012/min, k_e derived by using the Opresko–Wiley approach is approximately 0.02/min less than the value we would deduce. This discrepancy is about the size of the difference between k_e measured by using our approach and k_e measured by using the analysis of Opresko and Wiley (1987b).

Table I shows substantial variation among reported values

of the dissociation constant, k_d. While these differences may be due to differences in cell types, the various analytical techniques may also affect the measured values. Most of the k_d values were measured from experiments similar to our method 2 experiments, and we have shown that little confidence can be placed in values of k_d obtained in such fashion.

While others have reported values for the other parameters used in this model—V_r, k_t, and k_h—sensitivity analysis indicates that these parameters cannot be confidently measured from method 1 and method 2 experiments. However, the degradation and removal rate constant k_h could be identified if the actual amount of degraded EGF in the medium were evaluated by measuring the trichloroacetic acid soluble portion of the medium.

In the case of receptor-mediated endocytosis of EGF in FRL cells, we found that the association rate constant k_a and the dissociation rate constant k_d were both measurable with confidence and were constant with respect to surface receptor occupancy and free ligand concentration. The fact that k_a and k_d are constant indicates that the binding and dissociation of EGF are not diffusion-limited and allows us to calculate an affinity for the EGF–receptor at 37 °C. Despite some evidence that the endocytic rate constant k_e depends on free ligand concentration, the parameter appears to be independent of initial surface receptor occupancy, implying that endocytosis is first order and that any higher order steps such as dimerization must not have been rate limiting in our experiments.

Acknowledgments

We thank Kelly Adair, Cindy Hunt, and John Kuchta for their technical support.

References

Boni-Schnetzler, M., & Pilch, P. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7832–7836.

Brown, M. S., Anderson, R. G. W., & Goldstein, J. L. (1983) *Cell 32*, 663–667.

Carpenter, G. (1985) *Methods Enzymol. 109*, 101–110.

Carpenter G. (1987) *Annu. Rev. Biochem. 56*, 881–894.

Carpenter, G., & Cohen, S. (1976) *J. Biol. Chem. 7*, 159–171.

Cochet, C., Kashles, O., Chambaz, E. M., Borrello, I., King, C. R., & Schlessinger, J. (1988) *J. Biol. Chem. 263*, 3290–3295.

DeLisi, C., & Weigel, F. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5569–5572.

Dunn, W. A., Connolly, T. P., & Hubbard, A. L. (1986) *J. Cell Biol. 102*, 24–36.

Erickson, J., Goldstein, B., Holowka, D., & Baird, B. (1987) *Biophys. J. 52*, 657–662.

Fanger, B. O., Stephens, J. E., & Staros, J. V. (1989) *FASEB J. 3*, 71–75.

Frank, P. M. (1978) *Introduction to System Sensitivity Theory*, Academic Press, New York.

Gex-Fabry, M., & DeLisi, C. (1984) *Am. J. Physiol. 247*, R768–R779.

Gladhaug, I. P., & Christoffersen, T. (1988) *J. Biol. Chem. 263*, 12199–12203.

Goldstein, B., Wofsy, C., & Bell, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5695–5698.

Haigler, H. T., Ash, J. R., Singer, S. J., & Cohen, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3317–3321.

Haigler, H. T., McKanna, J. A., & Cohen, S. (1979) *J. Cell Biol. 81*, 382–395.

Hanover, C. J., & Dickson, R. B. (1985) in *Endocytosis* (Pastan, I., & Willingham, M. C., Eds.) pp 131–162, Plenum Publishers Corp., New York.

Kaplan, J., & Keogh, E. A. (1982) *J. Cell Biol. 94*, 12–19.

Knauer, D. J., Wiley, H. S., & Cunningham, D. D. (1984) *J. Biol. Chem. 259*, 5623–5631.

Lauffenburger, D. A., Linderman, J., & Berkowitz, L. (1987) *Ann. N.Y. Acad. Sci. 506*, 147–162.

Lloyd, C. E., & Ascoli, M. (1983) *J. Cell Biol. 96*, 521–526.

Murthy, U., Basu, M., Sen-Majumadr, A., & Das, M. (1986) *J. Cell Biol. 103*, 333–342.

Myers, A. C., Kovach, J. S., & Vuk-Pavlovic, S. (1987) *J. Biol. Chem. 262*, 6494–6499.

Oberg, K. C., Soderquist, A. M., & Carpenter, G. (1988) *Mol. Endocrinol. 2*, 959–965.

Opresko, L. K., & Wiley, H. S. (1987a) *J. Biol. Chem. 262*, 4109–4115.

Opresko, L. K., & Wiley, H. S. (1987b) *J. Biol. Chem. 262*, 4116–4123.

Savage, C. R., & Cohen, S. (1972) *J. Biol. Chem. 247*, 7609–7611.

Schlessinger, J., Shechter, Y., Willingham, M. C., & Pastan, I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2659–2663.

Teslenko, L. V., Kornilova, E. S., Sorkin, A. D., & Nikolsky, N. N. (1987) *FEBS Lett. 221* 105–109.

Weigel, P. H., & Oka, J. A. (1983) *J. Biol. Chem. 258*, 5089–5094.

Wiley, H. S. (1988) *J. Cell Biol. 107*, 801–810.

Wiley, H. S., & Cunningham, D. D. (1981) *Cell 25*, 433–440.

Wiley, H. S., & Cunningham, D. D. (1982) *J. Biol. Chem. 257*, 4222–4229.

Yarden, Y., & Schlessinger, J. (1987a) *Biochemistry 26*, 1434–1442.

Yarden, Y., & Schlessinger, J. (1987b) *Biochemistry 26*, 1443–1451.