Kinetic Analysis of Agonist-Receptor Interactions

MODEL FOR THE "IRREVERSIBLE" BINDING OF CHOLERAGEN TO HUMAN FIBROBLASTS

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Current studies on receptor-ligand interactions usually employ a "binding" step followed by extensive washing to remove free ligand. This procedure, by definition, removes reversibly bound ligand from the receptor; the equations used by most workers to analyze the data, however, require an equilibrium between free and bound ligand and are not applicable given the design of most binding assays. The assay, in fact, measures binding that is slowly reversible or irreversible. Assuming that ligand receptor interaction involves two stages with a reversible step followed by an "irreversible" event, the $K_r$ for the reversible reaction may be obtained from the rate of the irreversible step.

Choleragen binding to human fibroblasts was only slowly reversible at both 0 and 37 °C. The two-step model was, therefore, applied experimentally to determine the $K_r$ for the reversible step in [125I]-choleragen binding to human fibroblasts at 0 °C ($K_r = 1.9 \times 10^8$ M$^{-1}$) and at 37 °C ($K_r = 3.8 \times 10^8$ M$^{-1}$). As predicted by the two-step model for ligand binding, the addition of 50 µg/ml of unlabeled toxin enhanced the rate of release of radioactivity at 37 °C; the rate of radiolabel release remained low at 0 °C, even with unlabeled toxin present in the medium. The rate of release of previously incorporated [125I]-toxin was accelerated by 50 µg/ml of toxin $> 5$ µg/ml of toxin. The two-step model for ligand binding appears to be applicable to the study of [125I]-choleragen binding to fibroblasts and should be useful, in general, for the analysis of receptor-ligand interaction.

The initial event in the action of many agonists is the binding of the agent to cell surface receptors. Cellular response may be a direct result of formation of the agonist-receptor complex or require processing of the complex; processing may occur within the plane of the plasma membrane and, in some instances, result in internalization of the agonist (1-4). It is not clear to what extent the processing of the agonist-receptor complex is critical to the action of the agent or whether it may, for example, represent a clearance mechanism that terminates agonist action. Processing of the agonist does, however, remove some of the cell-associated agent from direct and freely reversible interaction with free agonist in the medium. Due to the processing of the agonist, and the nonequilibrium state of the cell-associated agonist with that free in the medium, analysis of total cell-associated agonist for determination of receptor sites by methods dependent on equilibrium of free agonist with that associated with the cell is no longer justified. Although the individual events involved in processing may, in fact, be reversible, they result in removal of a fraction of cell-associated agonist from direct contact with agonist in the medium and in a slowly reversible system may lead to a nonequilibrium situation.

The interaction of choleragen (cholera toxin) with human fibroblasts appears to be an example of "irreversible" binding (5). Free toxin binds to a cell surface receptor which is believed to be ganglioside GM₁ (6-9); toxin then causes the NAD-dependent activation of adenylate cyclase by ADP-ribosylation of a regulatory component of the cyclase system (6-13). Following binding, toxin has been shown to undergo patching, capping, and internalization (14-16). Prior studies on choleragen interaction with human fibroblasts have demonstrated that at 37 °C cell-associated toxin was not in equilibrium with the medium (5). In the present investigation, a model is presented which is generally applicable to the study of the irreversible interaction of an agonist with cells; the kinetic analysis is applied to the study of toxin binding to fibroblasts.

EXPERIMENTAL PROCEDURES

Methods

Tissue Culture—Human skin fibroblasts were maintained in a culture as described earlier (17, 18). For binding studies, fibroblasts were plated in 24-well Costar plates (50,000 cells/well) and grown to confluency with one feeding 24 h prior to the study.

Binding Studies—The wells were washed 5 times with 1 ml of Hanks' balanced salt solution containing 1% bovine serum albumin. One milliliter of solution was added with the indicated concentrations of [125I]-choleragen. Following the selected incubation times, the medium was removed and the fibroblasts washed 5 times with 1 ml of HBSS-BSA. The cell layer was incubated in 1 ml of 1 N NaOH; samples (0.5 ml) of the dissolved protein were counted in a Beckman gamma counter and used to determine the protein content. Similar wells were used to measure cell number.

Materials

[125I] carrier-free was obtained from Amersham. Choleragen was iodinated with chloramine-T by Dr. Peter H. Fishman (National Institutes of Health) as described earlier by Cuatrecasas (19). Choleragen was obtained from Schwarz/Mann; Hanks' balanced salt solution from Gibco (Grand Island, NY); bovine serum albumin from Reheis Chemical Co. (Phoenix, AZ); molecular weight standards from Pharmacia. Protein was determined by the method of Lowry et al. (20) using bovine serum albumin as a standard.

RESULTS

Incorporation of [125I]-Choleragen into Human Fibroblasts—The initial rate of uptake of [125I]-choleragen by human fibroblasts was dependent on toxin concentration at 0 and 37 °C; the uptake of toxin was measured by incubation of fibroblasts with [125I]-choleragen followed by rapid extensive

1 The abbreviations used are: GM₁, galactosyl-N-acetylgalactosaminyl(N-acetyleneuraminyl)-galactosylglucosylceramide; HBSS-BSA, Hanks' balanced salt solution-bovine serum albumin.

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washing in toxin-free medium. The radioactivity thus incorporated into the cell layer was not in equilibrium with free toxin but was, in fact, irreversibly bound given the time span of the assay. Ligand irreversibly bound to the cell layer increased initially as a linear function of time and under all conditions investigated reached a plateau by 120 min (Fig. 1). Initial rates were obtained from the linear portion of the binding isotherms (at least 7 time points for each case) and were analyzed, assuming that an initial reversible step is followed by a poorly reversible one (see "Appendix"). Kinetic analysis of the uptake data as outlined in the Appendix gave the rates of the irreversible reaction at different ligand concentrations.

Release of $^{125}$I-Choleragen from Human Fibroblasts—As noted, $^{125}$I-choleragen bound to fibroblasts remained incorporated within the cell layer after free $^{125}$I-choleragen was removed and the fibroblasts were extensively washed. Addition of unlabeled choleragen to medium overlaying the cells resulted in the release of $^{125}$I-choleragen from fibroblasts incubated at 37 °C but not at 0 °C (Fig. 3). Fibroblasts incubated with $^{125}$I-choleragen at 0 °C and then exposed to unlabeled toxin at 37 °C released $^{125}$I-choleragen (Fig. 4). In the absence of unlabeled toxin in the medium, the fibroblasts released <10% of the radiolabel (data not shown). Fibroblasts incubated with $^{125}$I-toxin at 37 °C did not release radiolabel when incubated with unlabeled toxin at 0 °C (Fig. 5). At 37 °C, incubation of fibroblasts for 90 min with unlabeled toxin resulted in the release of >80% of cell-associated radioactivity (Figs. 3–5).

The rate of release of $^{125}$I-toxin at 37 °C was greater with a higher concentration of unlabeled choleragen (Fig. 6). The radioactivity released with time from the fibroblasts at 37 °C by unlabeled toxin was precipitable with 5% trichloroacetic acid, as was >95% of the radioactivity remaining in the cell layer. The trichloroacetic acid-precipitable radioactivity in both the medium and cell layer was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21); two distinct peaks of radioactivity were observed with mobilities identical with those of the A$_1$ and B peptides of choleragen (Fig. 7). The ratio of A$_1$ and B in the released toxin was identical with that of A$_1$ and B in the cell layer (Fig. 7).

**DISCUSSION**

The data derived from analysis of $^{125}$I-choleragen fibroblast interaction can be interpreted in a two-step model for receptor-ligand interaction. The initial event is the reversible binding of ligand to receptor; the second step(s) is a relatively irreversible reaction (given the time scale studied) so that bound ligand is no longer in equilibrium with free. Based on the rates of the irreversible reaction at different ligand concentrations, it is possible to calculate the affinity of ligand for receptor. At both 0 and 37 °C, a $K_a$ for the reversible step is obtained which is similar to that for the irreversible interaction of toxin and G$_a$ oligosaccharide (22). The latter is believed to be the first step in the toxin-cell surface interaction. The use of the two-step model, therefore, gives the answer predicted...
with 'I-toxin at per panel) times by the procedure given in Fig. 3; medium for BSA at 37 °C for the indicated times. The percentage of total radioactivity in the cell layer and medium was determined at the indicated times by the procedure given in Fig. 3; medium (C), cell layer (O).

**Fig. 5 (center).** Effect of unlabeled toxin on the rate of release at 0 and 37 °C of radiolabel from fibroblasts incubated with 'I-toxin at 37 °C. Fibroblasts were incubated at 37 °C for 3 h with 'I-toxin (0.01 pg, 300,000 cpm in 1 ml of HBSS-BSA) for 3 h as indicated in Fig. 3. The fibroblasts were incubated at 0 °C for 12% polyacrylamide slab gels as described earlier (21) with control 'I-toxin and other proteins as molecular weight standards. The gels were sliced and counted. The migration of control A, and B subunits of 'I-toxin is indicated in the figure legend. The migration of molecular weight standards was: α-lactalbumin (14,000), fraction 35; trypsin inhibitor (20,000), fraction 30; carbonic anhydrase (30,000), fraction 21; ovalbumin (43,000), fraction 16; albumin (67,000), fraction 9; and phosphorylase B (94,000), fraction 7.

**Fig. 6 (right).** Effect of toxin concentration on rate of release of radiolabel from fibroblasts previously incubated with 'I-toxin at 37 °C. Fibroblasts were incubated at 37 °C for 3 h with 'I-toxin (0.01 pg, 300,000 cpm in 1 ml of HBSS-BSA). The cells were washed and incubated at 37 °C for the indicated times with 5 µg/ml of unlabeled choleragen (0.01 pg, 300,000 cpm in 1 ml of HBSS-BSA). The cells were washed and incubated at 37 °C for the indicated times with 5 µg/ml (A, Δ) or 50 pg/ml (O, O) of unlabeled choleragen. The percentage of total radioactivity in the medium (C, Δ) or cell layer (O, A) was determined as described in Fig. 3.

**Fig. 4 (left).** Effect of unlabeled toxin on the rate of release at 37 °C of 'I-choleragen previously bound to human fibroblasts at 0 °C. 'I-Choleragen was bound to fibroblasts at 0 °C as described in Fig. 3. The cells were washed 5 times with 1 ml of HBSS-BSA and then incubated with 50 pg of choleragen in 1 ml of HBSS-BSA at 37 °C for the indicated times. The percentage of total radioactivity in the cell layer and medium was determined at the indicated times by the procedure given in Fig. 3; medium (C), cell layer (O).

**Fig. 7** Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 'I-choleragen bound to fibroblasts (upper panel) and subsequently released in the presence of unlabeled toxin (lower panel). Fibroblasts were incubated in 24-well Costar plates with 'I-choleragen (0.01 pg, 300,000 cpm in 1 ml of HBSS-BSA) at 37 °C as outlined in Fig. 6. The fibroblasts were then washed 5 times with 1 ml of HBSS-BSA. To one sample, 1 ml of 5% trichloroacetic acid was added immediately; the cells were scraped and harvested by centrifugation at 2230 × g for 5 min. One milliliter of HBSS-BSA containing 50 µg/ml of choleragen was added to the remaining samples. At 120 min, 0.4 ml of medium was removed and mixed with 40 µl of 50% trichloroacetic acid. The pellet was harvested by centrifugation at 2230 × g for 5 min. In both cases, >95% of the radioactivity was in the trichloroacetic acid-insoluble fraction. The pellets were heated at 65 °C for 10 min in 50 mM dithiothreitol, 0.05% bromphenol blue, 0.5% sodium dodecyl sulfate, 50 mM Tris base (pH 11.0) and 50% glycerol (0.4 ml). The samples were applied to 12% polyacrylamide slab gels as described earlier (21) with control 'I-toxin and other proteins as molecular weight standards. The gels were sliced and counted. The migration of control A, and B subunits of 'I-toxin is indicated in the figure legend. The migration of molecular weight standards was: α-lactalbumin (14,000), fraction 35; trypsin inhibitor (20,000), fraction 30; carbonic anhydrase (30,000), fraction 21; ovalbumin (43,000), fraction 16; albumin (67,000), fraction 9; and phosphorylase B (94,000), fraction 7.

**Analysis of Irreversible Binding of Choleragen to Cells**

Based on in vitro binding studies, the model also predicts that addition of unlabeled ligand to the medium should accelerate the release of bound ligand. In the presence of unlabeled choleragen, >90% of bound 'I-choleragen which was released in 120 min, whereas <10% was released in its absence. This result is consistent with the ability of toxin which has been irreversibly bound (e.g., internalized) at 37 °C to enter into equilibrium with that in the medium; these data, therefore, suggest that the model is consistent with the recycling of ligand-receptor complex (1–3). Since the labeled toxin released at 37 °C contains subunits of native molecular weight, it appears not to have been degraded.

The observed release of labeled bound toxin by unlabeled toxin is based on exchange with the "irreversibly" bound ligand. If the two steps involved in binding are represented as

\[ L + R \overset{K_1}{\rightarrow} LR \overset{K_2}{\rightarrow} LR^* \]

where \( L^* \) represents irreversibly bound ligand, the extent of irreversible binding is determined by the ratio of \( K_2 \) to \( K_1 \). In the presence of unlabeled L, a finite value of \( K_2 \) with free exchange between LR and L would lead to a slow exchange of \( L \) and \( L^* \); in the case of toxin, this exchange would lead to a rapid release of bound labeled toxin. A finite value of \( K_2 \) is the major difference between the present kinetic treatment of ligand-cell interactions and that used by Sandö and Neufeld (23) for receptor-mediated uptake of α-1-iduronidase. The absence of significant exchange at 0 °C in the present system is consistent with a temperature-dependent change in the ratio of \( K_2 \) to \( K_1 \).

The model does not define LR or LR*. LR, in fact, is not identified experimentally. Its existence is postulated based on the known reversible interaction between toxin and GM1-ganglioside (22, 24). LR* may represent a heterogeneous group of complexes, which may be in equilibrium with each
Analysis of Irreversible Binding of Choleragen to Cells

The proposed model should be generally applicable to other systems. The model is particularly valuable for analysis of hormone-receptor interactions, since it permits the use of current methodology, which measures irreversible binding, to obtain information about preceding reversible events. The data in the literature are consistent with the occurrence of both reversible and irreversible events. The presence of an initial reversible agonist-receptor interaction has been documented in a number of cases, where receptor has been identified and partially purified (25, 26). A number of elegant studies have shown internalization of certain ligands at 37 °C (1-4, 27-33). In these cases, the reason that binding is apparently irreversible is obvious; ligand within the cell should not be in equilibrium with that in the medium. If there is with a given agonist a reversible followed by an irreversible event, then it should be possible to use the rate of the irreversible interaction of hormone with its target cell to determine the affinity of cell surface receptor for hormone.

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APPENDIX

The methods generally used to determine binding of hormones, metabolites, or toxins to cell surfaces involve an initial incubation with labeled compound, removal of the agent followed by exhaustive washing, and finally, counting of the cells to determine uptake. Since uptake is measured after exhaustive washing, which would presumably remove any agent in rapid equilibrium with the membrane, the counts remaining in the cell layer are, by definition, irreversibly bound. The interaction may, in fact, be slowly reversible if the dissociation is examined over times much greater than those used during the exhaustive washing. In a number of cases, particularly for choleragen, it is known that the binding of toxin to its receptor GM,, is reversible in vitro (22, 24). The irreversible step, therefore, follows a reversible interaction and may result from a number of processes, e.g. capping, patching, internalization, interaction with the bilayer. A mathematical model may be proposed assuming that the initial interaction of the agent with the cells may be reversible, with the subsequent event being irreversible or slowly reversible. If we assume that the assay measures irreversible binding, then it is possible to quantify the proposed reversible step by measuring the kinetics of the irreversible step.

Consider the following reaction scheme

\[
\begin{align*}
&K_1
\end{align*}
\]

(1A)

where \( L \) corresponds to the concentration of ligand which is free in the medium; \( R \) is the concentration of uncomplexed receptor; \( LR \) is the freely reversible ligand-receptor complex and \( LR^* \) is the poorly reversible ligand-receptor complex.

Assuming steady state conditions for \( LR \), one obtains

\[
\frac{d[LR]}{dt} = 0 = K_1[L][R] - K_{1-}[LR] - K_2[LR] + K_2[LR^*]
\]

(2A)

Rearranging:

\[
\frac{K_1}{K_{1-} + K_2} [L][R] = [LR] \cdot \frac{K_2[LR^*]}{(K_1 + K_2)}
\]

(4A)

The rate of irreversible (or slowly reversible) interaction, \( V_r \), is given by the following equation:

\[
V_r = K_2[LR] - K_2[LR^*]
\]

(6A)

Combining with Equation 5A and defining \( K' = K_2/(K_{1-} + K_2) \),

\[
V_r = K_2\left[ K'[L][R] + \frac{K_{1-}}{K_{1-} + K_2}[LR] \right] - K_2[LR^*]
\]

(7A)

This equation is general and can be used to describe the kinetics of irreversible or slowly reversible ligand-cell interactions. For initial rates, i.e. \( [LR^*] \approx 0 \), Equation 7A, when combined with conservation of mass considerations, reduces to the familiar Langmuir isotherm, Equation 8A.

\[
V_r = \frac{K_2K'[L][R]}{[R] + K'[L][R]}
\]

(8A)

This equation is linear and the slope of a plot of \( V_r/[L] \) versus \( V_r \) yields \( K' \). For cases in which the rate of dissociation of \( LR \) is much greater than the rate of irreversible interaction, i.e. \( K_{1-} \gg K_2 \), \( K' \) corresponds to the equilibrium constant for reversible interactions. Thus, in principle, one can estimate the affinity for reversible interactions by measuring subsequent irreversible interactions. It is emphasized that this method cannot be used alone to evaluate the total number of cell surface receptors; \( K_2 \) cannot be separated mathematically from \( R \).

This same general model has been used previously to account for desensitization at cholinergic receptors (35, 36) and the interaction between the \( \beta \)-adrenergic receptor and adenylate cyclase (37). The main difference between those treatments and that used in the present study is that we have separated experimentally the irreversible (high affinity) step from the initial reversible interaction and used a kinetic analysis to evaluate ligand-receptor interaction. The present model is also similar to the ternary complex model proposed by De Lean et al. (38) in that two forms of ligand-receptor complexes (high and low affinity) are postulated in both cases. Since we have no independent evidence of "additional membrane components" involved in the interconversion of the two forms of choleragen-receptor complexes, we have not specified the composition of the irreversible complex.

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