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Ligand/Receptor Internalization: A Spectroscopic Analysis and a Comparison of Ligand Binding, Cellular Response, and Internalization by Human Neutrophils

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We have compared the kinetics of the responses of neutrophils to the kinetics of ligand-receptor interaction and internalization, using as a model ligand the fluoresceinated hexapeptide N-CHO-Nle-Leu-Phe-Nle-Tyr-Lys-Fluorescein (Nle, norleucine). Cellular responses, ie, membrane depolarization, enzyme (elastase) secretion, and superoxide anion (O_2^-) generation, are all initiated within 10 sec of the exposure of cells to stimulus. In the cases of membrane depolarization and secretion (in cytochalasin B-treated cells), full responses are elicited by binding which occurs within 15 sec of peptide addition.

Ligand binding and internalization have been analyzed over the same time frame with new spectroscopic techniques. The association of ligand and receptor is monitored using an antibody to fluorescein. The antibody to fluorescein specifically quenches the ligand which is in solution, but receptor-bound ligand is inaccessible to the antibody. The internalization of the receptor-bound ligand is monitored by the accessibility of the fluoresceinated peptide to quenching by an external pH change (7.4 → 4.0). Ligand which is either outside or on the cell surface is instantaneously quenched while intracellular peptide (or intracellular fluorescein derived from fluorescein diacetate) is only slowly quenched. No internalization is observed until 1 min after binding begins and internalization proceeds at a rate of up to 5,000 receptors/min/cell following a near optimal stimulatory ligand concentration ($\sim$ 1 nM) while the occupied receptors are being cleared from the surface. A comparison of the kinetics of internalization and the cellular responses suggests that internalization of the ligand is too slow to be involved in the triggering of the cellular responses.

Key words: ligand-receptor interaction, neutrophils, cellular response, fluorescein, peptides

Receptor internalization typically follows the occupancy of cell-surface receptors by specific ligand molecules, and there is considerable interest in the potential biological roles that internalization may play. While it is already clear that receptor internalization is part of the process of receptor modulation, there is also evidence which suggests that long-term cellular responses, such as cell growth stimulated by

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peptide and steroid hormones, may require the transport of the ligand to the nucleus or other intracellular locations [1–3].

The internalization processes have generally been analyzed by identification of the ligand at an intracellular site using techniques such as EM (electron microscopic) autoradiography or subcellular fractionation. Recently it has become possible to examine the internalization process by fluorescence microscopy and microfluorometry. For example, the internalization of fluoresceinated bacteria by leukocyte has been examined using crystal violet as a quenching agent for fluorescein [4]. Completely phagocytosed bacteria in sealed membrane vesicles are inaccessible to quenching by the crystal violet. Moreover, when a phagocytosed particle or internalized ligand enters a lysosome or an intracellular compartment of altered pH, the spectral properties of the fluorescein are responsive to the pH of the enviroment [5; and references included therein].

With the availability of high-affinity synthetic N-formyl peptides which can be radiolabeled, photoaffinity-labeled, or labeled with fluorophores, we and others have used these ligands intensively in studies of ligand binding, cellular response, and cellular internalization of the receptor by neutrophils [6–18]. In some respects these studies may serve both as a model for receptor-mediated neutrophil activation and as a model for ligand/receptor processing by cells in general.

Previous studies by Niedel et al [12] using the rhodaminated N-formyl hexapeptide (TMR-FNLPNTL) detected internalized ligand by video intensification fluorescence microscopy within 2 min after neutrophils, exposed to the ligand at 4°C, were warmed to 37°C. Because of the relative rapidity of the internalization process we sought to develop a quantitative approach for examining this rapid internalization process. In this report we describe a quantitative, spectroscopic, and kinetic analysis of the internalization of a fluoresceinated N-formyl hexapeptide (FL-FNLPNTL) by neutrophils. We compare the kinetics of the peptide binding to the cellular receptor, the kinetics of several cellular responses, and the kinetics of internalization. The measurements are all performed on cells in suspension using spectroscopic methods which may have general applicability to different cell types stimulated by different ligands. In an accompanying paper we show, using subcellular fractionation experiments, that N-formyl peptide covalently attached to the receptor is internalized and detected in a Golgilike compartment after ~ 1 min of exposure of the cells to the ligand [19].

MATERIALS AND METHODS

Neutrophils

Neutrophils were obtained from human blood by the method of Henson and Oades [20] and prepared for these studies as described previously [6].

Reagents

The fluoresceinated chemotactic hexapeptide, FL-FNLPNTL (N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys), and high-affinity antibody to fluorescein were prepared and characterized as described [6].

Kinetic Assays of Neutrophil Response

The production of superoxide anion [6,7], elastase secretion [8] and the response of the membrane potential sensitive carbocyanine dye "DiS(C_3)-5" (3,3′ -dipropylthiodicarbocyanine) [6,7] were measured as described.

Fluorometric Assays of the Binding of the Peptide FL-FNLPNTL to Its Receptor and Internalization of the Peptide

The binding of the fluorescent peptide was monitored essentially as described [7,8] but the significant details are reemphasized. Cell suspensions (10^7/ml) were exposed to fluorescent peptide (~ 1 nM) in a plastic cuvette held in the stirred, thermostatted sample compartment of SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL) which is interfaced to an HP 9825 computer and HP 7225 XY plotter. The fluorescence of the peptide is monitored continuously, and after a defined time interval the extent of receptor-bound ligand is analyzed with a high-affinity antibody to fluorescein (20 nM). This antibody has the following properties: (1) it rapidly quenches ($> 95\%$) the fluorescence of the free peptide in a time interval of ~ 1 sec [7] and (2) the receptor-bound ligand is inaccessible to quenching by the antibody (see [9]).

Internalization of the bound peptide was examined under the same conditions with the use of an additional step. Following the examination of the extent of peptide binding to its receptors with the antibody to fluorescein, the pH of the cell suspension (1.5 ml) was adjusted from 7.4 (3 mM phosphate buffer) to 4.0 by the addition of 15 μl (100-fold dilution) 0.33N HCl. In these experiments the antibody addition and the pH change are accomplished in a period of 20 sec, during which time both the extent of ligand binding to the receptor and ligand internalization can be measured. Extracellular fluorescein or its derivatives are quenched essentially instantaneously ($\sim 99\%$) by this change in pH while intracellular fluorescein derived from fluorescein diacetate is only quenched after periods $>$ 30 sec following the extracellular pH change.

RESULTS AND DISCUSSION

Responses

The kinetics of several responses of neutrophils to the fluoresceinated N-formyl hexapeptide (FL-FNLPNTL) are summarized in Table I. We have examined the fluorescent response of the dye DiS(C_3)-5, which appears to reflect a membrane depolarization, the reduction of cytochrome C by superoxide anion [7], and the secretion of elastase (in cytochalasin B-treated cells) using the elastase-specific fluorescence substrate MeO-Succ-Ala-Ala-Pro-Val-MCA [8]. All of these responses are initiated within 10 sec of exposure of the cells to the stimulus. The maximal depolarization, the completion of the secretion, and the maximal rate of generation of superoxide anion occurs within 30 sec of the addition of the stimulus.

We have used a "pulse" format to analyze the dependence of the cellular response on the extent of receptor occupancy [7,8]. This pulse approach uses an antibody to fluorescein to interrupt the binding of the FL-FNLPNTL to the cellular receptor for N-formyl peptides. Since the complex between the fluorescent peptide and antibody forms within 2 sec and is essentially nonstimulatory, addition of the

TABLE I. Kinetics of Neutrophil Responses*

	"Depolarization" (DiS(C_3)-5)	Superoxide (cytochrome C)	Elastase secretion (MeO-Succ-Ala-Ala-Pro-Val-MCA)
Latency	5 sec	< 10 sec	5 sec
Maximum rate	30 sec[a]	< 30 sec[b]	30 sec[c]
Completion		< 3 min	
Binding time[d]	< 10 sec	> 60 sec	5 sec

*[6–8]. The materials in parentheses are used in the detection of the response.
[a]Maximum fluorescence response.
[b]Maximal rate of superoxide generation.
[c]Maximal rate of substrate cleavage detected.
[d]The time required in a "pulse" experiment for the binding of a near-optimal stimulus dose (ie, 1–2 nM FL-FNLPNTL) to elicit the complete cellular response. A pulse experiment involves exposure of the cells to the FL-FNLPNTL, followed at the indicated time, by the high-affinity antibody to fluorescein.

antibody serves to inhibit further cellular stimulation by the free peptide. We find that for a near optimal stimulus dose (1–2 nM), the entire secretory and "depolarization" responses are elicited by the binding of stimulus to the receptor which occurs in less than 5 sec [7,8]. In contrast, more than 1 min of continuous binding is required to elicit the entire superoxide response [7].

Binding

All of these data indicate that the responses of the cells to the fluorescent peptide depend upon relatively short periods of interaction between the peptide and its receptors. In order to understand quantitatively how the responses depend upon binding and internalization of the peptide, we developed rapid spectroscopic methods to analyze in real time the kinetics of these processes [7,9].

How rapid is the binding of the peptide to the receptor? The pulse format of stimulation may be used in an independent way to examine the kinetics of the association of the fluorescent peptide with the cellular receptor. High-affinity antibodies to fluorescein ($K_A > 10^{10} M^{-1}$) have the general property of quenching fluorescein fluorescency by > 95%. Since the FL-FNLPNTL which is bound to the receptor is inaccessible to the antibody [see ref. 9], the antibody rapidly quenches the fluorescence of those ligand molecules which are free in solution (Fig. 1). Since the fluorescence of the peptide is not altered by its binding to the receptor* the residual fluorescein fluorescence after antibody addition to a cell suspension exposed to FL-FNLPNTL is proportional to the fraction of the ligand bound to the receptor. Analyses of the binding data from several experiments indicates that the association rate constant $K_{ON} \sim 3\text{–}4 \times 10^8 M^{-1} min^{-1}$ (Fig. 2). The data points in Figure 2 were obtained from a fluorometry experiment using the antibody to fluorescein on a single donor; the solid lines represent a theoretical fit to data obtained in a parallel experiment using the fluorescence activated cell sorter according to [9].

Steady-state binding analyses using the antibody technique performed at 4–15°C are consistent with a value of 50,000 for the average number of receptors for this

*The total fluorescence intensity of the peptide in the cell suspension is the same whether binding to the receptor has occurred or whether the binding has been blocked by excess unlabeled FNLPNTL.

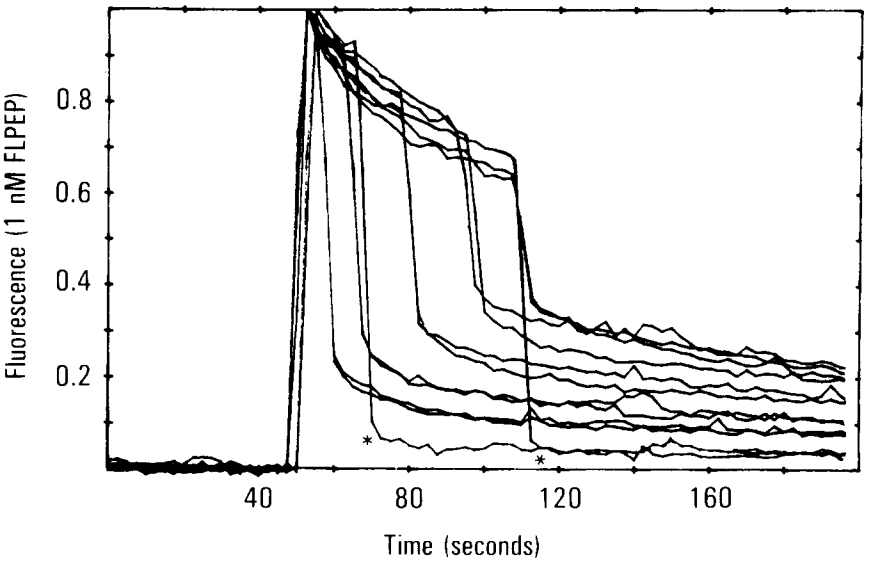

Fig. 1. Assay of the kinetics of binding of 1 nM FL-FNLPNTL (FLPEP) to human neutrophils at 37°C: The data are plotted as fluorescence intensity of the FL-FNLPNTL as a function of time. The cell suspension contained 10^7 cells/ml in buffer without added protein (see Fig. 3). After 50 sec, 1 nM FL-FNLPNTL was injected into the cell suspension. At 7.5, 15, 30, 45, or 60 seconds later, antibody to fluorescein (20 nM) was injected into the suspension. In the traces indicated by an asterisk, binding of the FL-FNLPNTL to the cells was blocked by the presence of 1 μM unlabeled hexapeptide. In this case, antibody quenched the fluorescence essentially to baseline, indicating that the receptors had not bound the FL-FNLPNTL. The spectroscopic conditions used excitation at 490 nm (slit 8 nm; 490 nm three-cavity interference filter; Corion, Holiston, MA) and emission at 520 nm (slit 8 nm; Corning 3-70 band pass filter).

ligand on human neutrophils from normal human donors and a K_D ~0.5–1 nM.* Based on the kinetics of binding (Fig. 2) and the dependence of the response on the period of uninterrupted binding [7,8], we calculate that more than 80% of the secretory or depolarization responses are elicted by occupancy of only 5% of the receptors in a time period of up to ~10 sec [8]. In order to generate 80% of the superoxide response, ~50% of the receptors must be occupied (Sklar, unpublished data).

Internalization of the Ligand

The internalization of the ligand is examined using a "pulse" analysis consisting of two steps (Fig. 3). The first component of the analysis uses the antibody to fluorescein to determine the fraction of receptor-bound ligand as previously shown in Figure 1. The second component in the analysis uses a mild pH change (7.4→4.0) 10 to 15 sec following the addition of antibody. Extracellular fluorescein, free fluorescent

*Steady-state binding experiments with FL-FNLPNTL-^{125}I-TL indicate that the bound cellular fluorescence is proportional to the bound radiolabel and the K_D is ~0.75 nM (J. Niedel, personal communication). The K_D of FNLPN-^{125}I-TL is ~1–2nM [12]. While no association rate constants for the iodinated ligands have been published we estimate a value of ~10^8 M^{-1} min^{-1} based on the initial association rate for binding from [12].

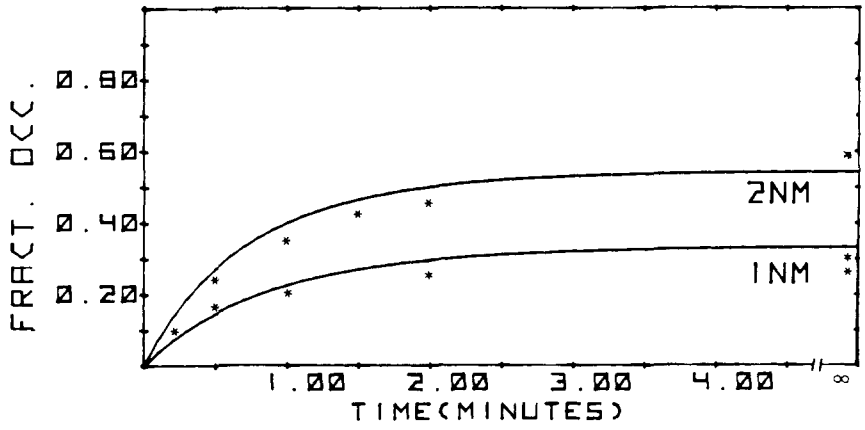

Fig. 2. Summary of binding kinetics from spectrofluorimetric and flow cytometric experiments for neutrophils from a single human donor: The data are plotted as fractional receptor occupancy vs time (at 37°C). Equilibrium binding experiments ([*], time "∞", 15°) indicated that the cells of this particular donor had ~90,000 receptors per cell for the N-formyl peptide. The individual data points were obtained from experiments performed as in Figure 1; the solid traces were calculated from a computerized analysis (Sklar, L.A., McNeil, V.M., and Finney, D.A., unpublished) of flow cytometric binding data obtained in a parallel experiment according to the methods of Sklar and Finney [9]. The curves were calculated using values of $K_{ON}=4 \times 10^8\ M^{-1}min^{-1}$ and $K_{OFF}^{APP} = 0.4/min$. Typical K_{ON} values are in the range of $3–4 \times 10^8\ M^{-1}min^{-1}$ at 25°C or 37°C; K_{OFF}^{APP} values are in the range of 0.2–0.4/min and may be a function of temperature, the length of time of occupancy, and the number of receptors occupied. In addition, true K_{OFF} values must take into account the extent and rate of receptor internalization.

peptide and extracellular, receptor-bound fluorescent peptide (as determined at 15°C) are quenched instantaneously greater than 99% by this pH change; in contrast, intracellular fluorescein derived from fluorescein diacetate is only slowly quenched and only at times longer than 30 sec following the pH change (data not shown). Thus the extent of residual peptide fluorescence (compared to peptide fluorescence in a cell suspension in which receptor binding and receptor-mediated internalization of the fluorescent peptide have been blocked by the presence of excess unlabelled peptide) represents the fraction of the fluorescent peptide which is inaccessible to the extracellular pH change. We view this component to represent intracellular or internalized ligand which is present in a relatively impermeable compartment.

The fraction of internalized fluorescence increases with time at 37°C (Figs. 3,4). After 30 to 45 sec following the onset of binding less than 1% of the ligand bound to receptors is internalized, by 3 min more than 60% is inaccessible to the extracellular pH change. For an optimal stimulus dose, receptor internalization occurs at a relatively constant rate of ~5,000 receptors/cell/min after a 1-min delay until most if not all of the receptors are cleared from the surface. No ligand inaccessible to the pH change is detected when these experiments are performed at 4°C or 15°C.

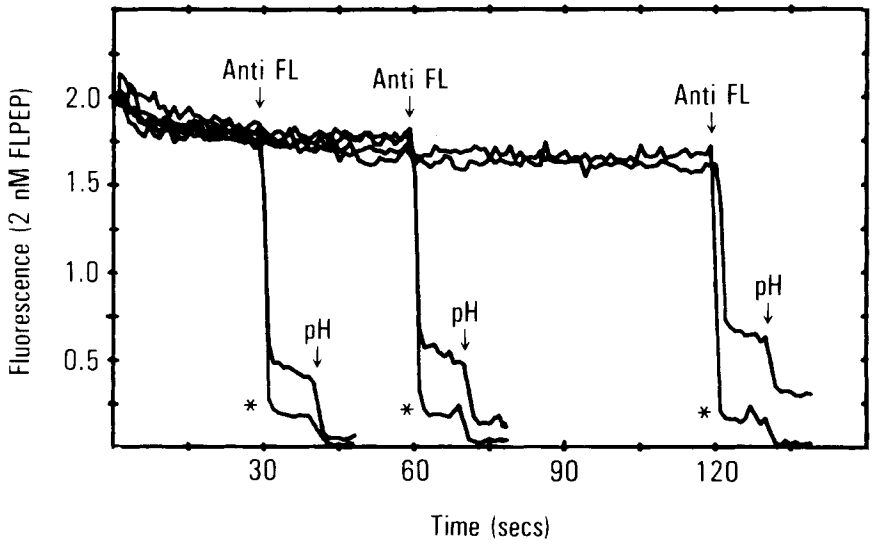

Fig. 3. Assay of the kinetics of receptor-mediated internalization of FL-FNLPNTL (FLPEP) by human neutrophils at 37°C: The data are plotted as the fluorescence intensity of 2 nM FL-FNLPNTL in the cell suspension (1.5 ml) as a function of time; data in Figure 2 are derived from the same donor. Spectroscopic conditions are as in Figure 1. At 0 time, the cells were exposed to 2 nM FL-FNLPNTL; at 30, 60, or 120 sec, 20 nM anti-fluorescein was added. Ten seconds following the antibody addition (ie, 40, 70, or 130 sec elapsed), 15 μl of 0.33 nM HCl was added to the suspensions (final pH 4.0). In the traces indicated by asterisks, the binding and internalization of FL-FNLPNTL was blocked by 1 μM unlabeled peptide. In these experiments the residual fluorescence after antibody addition, compared to the blocked controls, represents binding of the FL-FNLPNTL to the receptors; the residual fluorescence after the pH change, compared to the blocked controls, represents internalized FL-FNLPNTL. In these experiments, the cell suspension contained 0.1 mg/ml added protein which inhibits the time-dependent decrease of the total fluorescence intensity of FL-FNLPNTL. For contrast, note that protein is excluded in the experiments of Figure 1. While any of the proteins tested (bovine serum albumin, ovalbumin, and superoxide dismutase) are useful in this regard, it should be noted that (1) bovine serum albumin competes with the receptor for FL-FNLPNTL; (2) ovalbumin fluoresces strongly at pH 4; and (3) superoxide dismutase is the protein of choice because it neither competes for FL-FNLPNTL, nor fluoresces at pH 4. It has the advantage of providing additional protection of the fluorophore against potential destruction by the free radicals produced by the cells.

Comparison of Binding, Response, Internalization

There is essentially no internalization of the peptide on a time scale in which the responses we have measured are elicited. In addition to these rapid responses described above (which include an apparent depolarization, superoxide anion, and elastase secretion), intracellular calcium dislocation [21], secretion of proteases in cytochalasin B-treated cells [17], and even cellular reorientation within a chemotactic gradient [22] all appear to be too rapid to require internalization of the ligand. Since neutrophils can respond chemotactically for periods of several hours, it has been suggested that internalization of the ligand-receptor complex and recycling of the unoccupied receptor is required to maintain prolonged chemotactic responsiveness [13].

It is not known whether these internalization and recycling processes serve additional roles. It is conceivable that for the rapid responses we measure, internal-

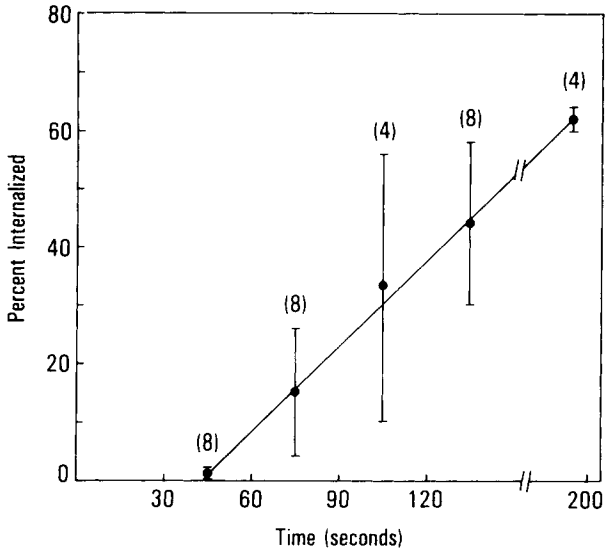

Fig. 4. Summary of the kinetics of internalization of FL-FNLPNTL (1 or 2 nM) by human neutrophils at 37°C: Data are plotted as the fraction of occupied receptors internalized vs time; the data are obtained from four experiments (three donors), with duplicate determinations in each experiment. The numbers in parentheses represent the number of determinations. Methods are as in Figure 3. The errors (± SD) appear to arise from donor variability rather than uncertainty in a given experiment. In all cases $< 1\%$ of the occupied receptors were internalized in the time interval of 30–45 sec following the exposure of cells to FL-FNLPNTL.

ization only contributes to the down-regulation of the cellular responses. A major role may be to remove the stimulatory ligand-receptor complex from continued contact with effector systems which may be present in the cell membrane and which participate in triggering the cellular responses.

The Generality of the Spectroscopic Procedures

The fluoresceinated N-formyl peptide may be unique in that while the free peptide in solution is recognized and quenched by antibody to fluorescein, the receptor-bound peptide is inaccessible to antibody. This specificity of recognition probably arises because of steric considerations at the receptor. At least four of the six amino acid residues of the bound peptide are located in the binding site at the receptor [16] and the fluorescein probably protrudes no more than ~ 10 Å from the binding pocket. This fluorescein is inaccessible to the antifluorescein IgG and its fragments [7]. The inaccessibility of the fluorescein may be due either to the proximity of the fluorescein to the binding pocket, the protection of the fluorescein in a pocket by the overall receptor structure, or steric hindrance from receptor components such as covalently bound carbohydrates which could limit the approach of the antibody.

The technique which uses fluorescein-labeled ligands and antibody to fluorescein to distinguish free from receptor-bound ligand may not be perfectly general because there is no reason, a priori, that any large ligand molecule labeled with fluorescein could not be simultaneously bound to its receptor and to an antibody to

fluorescein.* However, there are a large number of small ligand molecules—peptide and steroid hormones—which have been labeled with fluorescein and which may behave in a similar fashion to the N-formyl hexapeptide. For fluorimetric assays of the type described here, the product of the binding affinity of the ligand and the receptor concentration must approach unity so that a reasonable fraction of the ligand molecules are bound to the receptor. (If $K_A = 10^9 M^{-1}$, then the receptor concentration should be about 1 nM. For example, if there are 60,000 receptors per cell, a cell suspension of 10^7 cell/ml is required.) In our experiments with the spectrofluorometer, we are able to analyze binding at ligand concentrations down to ~0.5 nM. The limiting factor is the contribution of the background fluorescence and scattered light from the cell suspension. In complementary fluorescence flow cytometry experiments [9], it is possible to detect cell bound ligand even at 0.1 nM ligand.

The evaluation of the internalization of ligand by the variation of the external pH may prove to be a general technique. In principle, in any system where fluoresceinated ligand and its association with cells may be quantitated (ie, by fluorescence spectroscopy, microspectrofluorometry, or fluorescence flow cytometry), an extracellular pH change may distinguish external from internal ligand. This approach is, however, subject to two cautions which must be evaluated in each experimental system.

First, the external pH change must not affect intracellular pH at least over the time frame associated with the measurement of residual fluorescence of the intracellular ligand. As noted, in our experimental system using continuous analyses, less than 10 sec are required to measure the intracellular ligand fluorescence after the external pH has been changed from pH 7.4 to 4.0. In contrast, it takes more than 30 sec for intracellular fluorescein (derived from fluorescence diacetate) to even begin to be quenched by this external pH change.

The second caution in the application of the pH technique involves the intracellular environment of the ligand molecules. Many internalized ligands appear to end up in a lysosomal compartment of lowered pH. In fact, the spectral properties of fluorescein-labeled ligand molecules have been used by other investigators to ascertain the rate and extent of the ligand compartmentalization [5 and references therein]. In the measurements shown here, at times following the onset of ligand binding by only 2 or 3 min, the ligand has apparently not yet encountered intracellular environments of lower pH. In such environments, its fluorescence, when excited at 490 nm, would be strongly diminished.† Rather, as seen in Figure 3, the total fluorescence intensity of the fluorescent peptide (prior to antibody addition or pH change) in cell suspensions is not significantly altered (as a function of time) whether or not excess unlabeled peptide is present. These results imply that over this 2–3-min time scale, the peptide fluorescence is the same whether or not binding or internalization has occurred. Moreover, the constancy of the total peptide fluorescence over this period

*For example, antifluorescein antibodies quench to a great extent the fluorescence of fluoresceinated IgG in solution (L.A. Sklar, unpublished data). Moreover, these antibodies quench the fluorescence of floresceinated transferrin even when it is receptor-associated (R.D. Klausner, personal communication).

†The quenching profile for fluoresceinated peptide vs pH is such that when excited at 490 nM, the relative intensities (compared to pH 8 or above) are > 90% at pH 7.5, ~25% at pH 6, ~10% at pH 5, and < 1 % at pH 4.

implies that the ligands have not yet reached a compartment of lower pH. Indeed, results discussed in [19] imply that the peptide is internalized via a pathway which involves the Golgi and ends up in the cytosol either without encountering the lysosome or with only a small fraction of the peptide resident in a lysosomal compartment for a short period of time.

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