Characterization of a Fluorescent Substance P Analog

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ABSTRACT: We describe the development and characterization of substance P labeled at Lys^3 with fluorescein ([fluorescein Lys^3]SP) as a fluorescent probe for the neurokinin 1 (NK1) receptor. [fluorescein Lys^3]SP is an agonist at the human NK1 receptor, with an affinity for both the high-affinity and low-affinity binding states of the receptor approximately 6-fold lower than that of substance P. Binding of the probe to the human NK1 receptor expressed in Sf9 insect cells was observed directly by monitoring either a decrease in fluorescence intensity or an increase in anisotropy of the [fluorescein Lys^3]SP. Detection by anisotropy gave the larger signal and thus was used to characterize the interaction of [fluorescein Lys^3]SP with the receptor. The anisotropy of the bound ligand was 0.17, compared to 0.04 for the free ligand. The fluorescence was quenched by about 15% upon binding to the receptor. Bound [fluorescein Lys^3]SP was displaced by unlabeled SP and by the quinuclidine antagonist L-703,606. As expected for an agonist, binding was also reduced by the addition of the nonhydrolyzable guanine nucleotide analog GppNHp. [fluorescein Lys^3]SP should provide a useful structural and kinetic probe for the NK1 receptor.

G protein coupled receptors are a widely distributed class of cell surface proteins that respond to a variety of ligands ranging from small biogenic amines such as catecholamines to larger peptide ligands such as glucagon. The receptors are composed of a single polypeptide chain that traverses the membrane seven times. The signal is transduced from the ligand-activated receptor to effector proteins such as adenylyl cyclase or phospholipases *via* the action of the guanine nucleotide binding regulatory proteins (G proteins; Gilman, 1987). The binding sites of many small ligands, such as catecholamines and retinal, in the receptors have been well characterized by various analytical methods, including site-directed mutagenesis and fluorescence spectroscopy (Tota et al., 1991). It is apparent that many small ligands interact within the transmembrane region of the receptor. However, the binding interactions for peptide ligands in G protein coupled receptors remain less well defined.

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, SP)[1] is an 11 amino acid neurotransmitter that plays an important role in neuromodulation through activation of the neurokinin 1 (NK1) receptor (Nakanishi, 1991). To probe the structure of the SP binding site in the NK1 receptor, we have prepared a fluorescent analog of SP labeled with fluorescein at the free amino group of Lys^3. The human NK1 receptor was expressed in Sf9 cells using the baculovirus system and probed with [fluorescein Lys^3]SP. Binding of the peptide could be detected by changes in fluorescence anisotropy, by accessibility of the fluorophore to an anti-fluorescein antibody, and by a decrease in fluorescence intensity upon binding to the receptor. The availability of [fluorescein Lys^3]SP will facilitate rapid kinetic and structural analysis of the NK1 receptor.

MATERIALS AND METHODS

Synthesis of [fluorescein Lys^3]SP. SP (Cambridge Research Biochemicals) was dissolved in 0.1 M bicarbonate buffer (pH 9.0) at 1–5 mg/mL. The succinimidyl ester of 5- (and 6-) carboxyfluorescein (Molecular Probes) was dissolved in acetonitrile as a 10× stock. One equivalent of the succinimide was added slowly, under argon, to the SP while being stirred. The reaction was allowed to continue for 1 h at room temperature in the dark. The reaction was monitored by TLC using Baker-flex silica gel IB2-F sheets and a solvent system of 4:1:1:2 1-butanol–pyridine–acetic acid–water (Liang & Cascieri, 1980). An orange precipitate formed and was dissolved in 40% acetonitrile–H_2O with 0.05% TFA. The monolabeled SP was isolated by HPLC using a C-18 column with a 40–95% acetonitrile gradient in H_2O with 0.05% TFA. Mass spectroscopic analysis gave a weight consistent with incorporation of a single fluorescein, and sequence data showed no lysine in position 3, indicating modification at that residue. The peptide was stored dry or as a 1 mM solution in ethanol.

Since the synthesis of [fluorescein Lys^3]SP was performed on a small scale and the yield was low (<10%), the extinction coefficient of the [fluorescein Lys^3]SP was estimated as follows: The extinction coefficient of the succinimidyl ester of 5- (and 6-) carboxyfluorescein reacted with an excess of lysine was 55 000 cm^{-1} M^{-1} measured at 495 nM in buffer A (buffer A: 50 mM Tris, pH 7.5, 150 mM NaCl, 0.02% BSA, 5 mM $MnCl_2$, 4 μg/mL leupeptin, 40 μg/mL bacitracin, 10 μM phosphoramidon, and 0.1 mM PMSF). This was similar to the reported value of the succinimide ester (68 000 cm^{-1} M^{-1} at 494 nm; Huagland, 1992). The value of 55 000 cm^{-1} M^{-1} was therefore used as the extinction coefficient of [fluorescein Lys^3]SP to estimate the concentration of the peptide for all further studies.

Anti-Fluorescein Antibody. Anti-fluorescein antibody was from Molecular Probes (Eugene, OR). The antibody was supplied at a concentration such that 10 μL would quench

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[1] Abbreviations: SP, substance P; NK1, neurokinin 1; GppNHp, 5′-guanylyl imidodiphosphate.

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90% of the fluorescence of 5 nM fluorescein at pH 8.0. The antibody was exchanged into 50 mM Tris-HCl and 150 mM NaCl by gel filtration prior to use.

Expression and Binding of the Human NK1 Receptor in Sf9 Cells. The cDNA encoding the human NK1 receptor was fused with the antigenic 14 amino acid carboxy tail of the hamster β_2AR and expressed in Sf9 cells as previously described (Mazina et al., 1993). Membranes were prepared and stored frozen in 0.25 M sucrose, 25 mM HEPES, pH 7.4, 5 mM EGTA, and 5 mM $MgCl_2$ as described (Mazina et al., 1993). Radioligand binding was performed on the membranes with either ^{125}I L-703,606 (Cascieri et al., 1992), ^{125}I Bolton–Hunter SP (New England Nuclear), or [^{3}H]SP (New England Nuclear) in buffer A at room temperature. The reaction was incubated for 1 h at room temperature and then filtered over GF/C paper. [^{3}H]SP assays were chilled on ice before filtration. The filters were washed with 3 × 4 mL of ice-cold 50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $MnCl_2$. Specific activities for the human NK1 receptor in the membrane preparations were usually between 5 and 10 pmol of [^{3}H]SP binding/mg of protein.

Data Analysis. Data were analyzed using the equations described by Munson and Rodbard (1980) or Schimerlik (1980) using the Marquardt's algorithm as described by Press et al. (1988). The method of Scatchard (1949) was used to determine the B_{max} and K_d for single populations of binding sites. Anisotropy titrations were fit by nonlinear regression to eq 3 (see Results) where the amount of free ligand was determined by the method of Munson and Rodbard (1980). Theoretical and experimental anisotropies were used for calculating the sum of squares.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a SLM 48000 spectrofluorometer. All measurements were performed at 20 °C in buffer A, unless indicated. For routine measurements, the instrument was configured in a T format; most measurements bypassed both the excitation and emission monochrometers. Excitation was achieved with an Ar laser, where the 488-nm line was selected by a sharp band-pass filter and attenuated with a 1 OD neutral density filter. In order to obtain a correction factor to compensate for differences between photomultipliers, the sample was excited with both vertical and horizontal light. This was achieved by first depolarizing the laser and then selecting either vertical or horizontal light with a polarizer. Emission light was selected with a 530-nm band-pass filter (Corion), followed by either a vertical or horizontal polarizer. Using the T format, vertical and horizontal emissions were collected simultaneously. Single point polarization was collected using a routine supplied by SLM which repeatedly checked the background and adjusted the correction factor.

Kinetic assays were set up to simultaneously record vertical and horizontal light. Background and correction factors were recorded immediately prior to the relevant portion of the data collection. All signals were recorded in the ratio mode using rhodamine as the standard. The background fluorescence (membranes without labeled peptide) was subtracted from each signal, and then the correction factor was applied to the vertical intensity. The data were then analyzed as follows:

$$\text{anisotropy} = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (1)$$

$$\text{intensity} = (I_{\parallel} + 2I_{\perp}) \quad (2)$$

Fluorescence lifetime and rotational dynamic measurements were performed using the phase/modulation technique (Lakowicz, 1983) and analyzed with software supplied with the SLM 48000.

The quantum yield of [fluorescein Lys^3]SP was determined by comparison of the integrated emission spectrum to that of fluorescein in 0.1 N NaOH using the method described by Eaton (1988). A quantum yield of 0.92 was used for fluorescein in 0.1 N NaOH (Weber & Teale, 1957).

Accuracy and Precision of Anisotropy Measurements. Anisotropy recorded in the steady-state mode had the advantage of repeated collection of the blank values and adjustment of the polarization correction factor. The blank value was determined as the vertical and horizontal intensities of the membrane sample in the absence of [fluorescein Lys^3]-SP. In practical terms, the accuracy of the anisotropy readings was then dependent on the signal to noise ratio of the sample. Anisotropy recorded in the kinetic mode did not continuously record a polarization correction factor. However, this correction factor only changed by a few percent over the course of data collection. In the kinetic mode, a background measurement for each sample was recorded prior to the addition of [fluorescein Lys^3]SP. For these reasons, the anisotropy value obtained from the steady-state and kinetic modes may differ slightly, usually by less than 0.02.

For either method of data collection, a high concentration of membranes produced a very turbid solution which could theoretically result in a decrease in the observed anisotropy signal. The anisotropy of the light scatter of the membrane suspension (1 mg/mL) was examined by exciting at 485 nM with no emission filters and determined to be 0.85. A 10-fold dilution of the membranes gave an anisotropy of 0.90. A large dependence on concentration would indicate that the high turbidity was interfering with anisotropy measurements. Therefore, the effects of turbidity seem to be modest under the conditions used for these studies. The anisotropy of [fluorescein Lys^3]SP measured in buffer (0.04) was similar to that measured in the presence of membranes where the binding site of the receptor was blocked with L-703,606 (0.035, Figure 10).

RESULTS

Synthesis of [fluorescein Lys^3]SP. Reaction of substance P with the succinimidyl ester of 5- (and 6-) carboxyfluorescein resulted in the formation of a peptide with a mass consistent with the addition of one fluorescein to each molecule of SP. Amino acid sequencing of the derivatized SP revealed no lysine at position 3, indicating Lys^3 to be the point of attachment of the fluorescein. Since synthesis was performed on a small scale, and the yield was low (<10%), the extinction coefficient of the [fluorescein Lys^3]SP was estimated to be 55 000 cm^{-1} M^{-1}, as described in Materials and Methods. The value of 55 000 cm^{-1} M^{-1} was used to estimate the concentration of the peptide for all further studies.

The fluorescence lifetime of [fluorescein Lys^3]SP was determined from phase/modulation studies to be 3.9 ns (Figure 1), consistent with the reported value of fluorescein (4 ns; Canter & Schimmel, 1980). The similarity of the measured lifetime of [fluorescein Lys^3]SP to that of fluorescein alone suggests that the fluorescence is not quenched by its attachment to SP. Indeed, the quantum yield of [fluorescein Lys^3]SP was estimated to be 73% (data not shown). The absence of quenching of the fluorescein fluorescence may indicate that the fluorescein does not interact with the remainder of the SP peptide.

The anisotropy of [fluorescein Lys^3]SP in buffer A was determined to be 0.0397 ± 0.0006. In order to explore the rotational dynamics of [fluorescein Lys^3]SP in solution, the viscosity of the solution was increased by adding glycerol and the data were analyzed by the method of Perrin (1926) (Figure

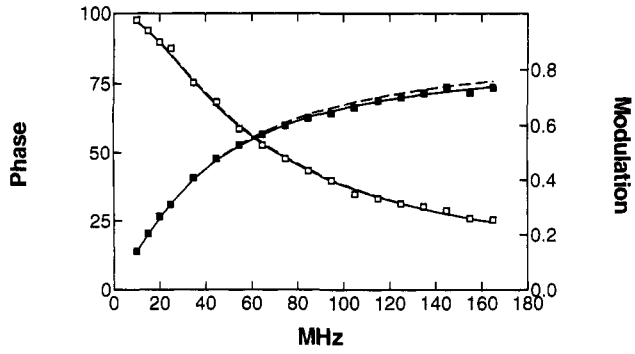

FIGURE 1: Phase (■) and modulation (□) data for 80 nM [fluorescein Lys³]SP. Shown is a fit to one component (- - -, 3.8 ns, X^2 = 3.44) or two components where 98.3% was fit with a lifetime of 3.9 ns and the remaining 1.7% was fit with a lifetime of 660 ps (—, X^2 = 2.92).

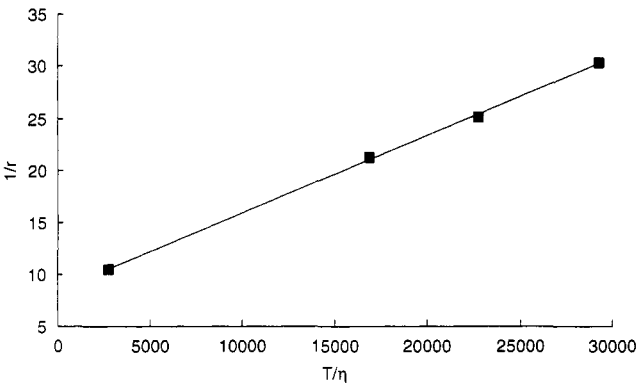

FIGURE 2: Perrin plot of 100 nM [fluorescein Lys³]SP. Anisotropy was measured in increasing concentrations of glycerol in buffer A at 293 K (20 °C). The limiting anisotropy was estimated from the inverse of the y intercept to be 0.29. The slope was 0.000752 P/deg.

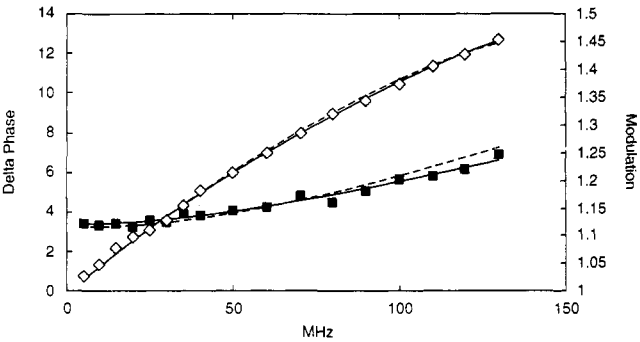

FIGURE 3: Phase (◇) and modulation (■) data for the rotational properties of 100 nM [fluorescein Lys³]SP. The delta phase and modulation for vertical and horizontal polarized light were measured as a function of frequency. When the data were fit to one class of rotators, the limiting anisotropy was 0.227 and the rotational correlation time was 762 ps (- - -, X^2 = 0.48). A two-component fit gave a limiting anisotropy of 0.333 and a rotational correlation time of 348 ps with a fractional loss of 88%. A longer component of 2.2 ns with a fractional loss of 12% was also observed (—, X^2 = 0.255).

2). The y intercept of this plot gave a limiting anisotropy of 0.29. The theoretical limiting anisotropy of fluorescein is 0.37 (Weber & Shinitzky, 1970). The lower value of 0.29 determined from the Perrin plot suggests a significant degree of local chromophore motion in the peptide. Rotational correlation times of [fluorescein Lys³]SP were measured directly by the phase/modulation technique, as shown in Figure 3. The lifetime anisotropy decay data were best described by two rotational correlation times of 348 ps and 2.2 ns and a limiting anisotropy of 0.33. These data are consistent with a significant degree of local rotational freedom of the fluorescein chromophore on the SP and indicate that the fluorescein does not interact to a significant extent with the remainder of the peptide itself.

Displacement of Radiolabeled SP by [fluorescein Lys³]-SP. Both [³H]SP and ¹²⁵I Bolton–Hunter SP were used for assaying the NK1 receptor expressed in Sf9 cells. [³H]SP could be used at a higher chemical concentration than ¹²⁵I Bolton–Hunter SP and thus was more likely to saturate all of the binding sites on the receptor. Scatchard analysis of [³H]SP binding to the membrane-bound NK1 receptor was nonlinear (Figure 4C), indicating more than one class of binding sites. In the presence of 100 μM GppNHp, the Scatchard plot was fit by a straight line, indicating conversion to a single population of sites (Figure 4D). Nonlinear regression analysis of these data, using the K_d value determined by Scatchard analysis in the presence of GppNHp as a constraint, gave a fit in which 7.5% of the total sites had a K_d of 0.14 nM. This value agreed with the high affinity K_d determined by ¹²⁵I Bolton–Hunter SP binding (Table 1). Only a small fraction of the total [³H]SP binding sites were high affinity and sensitive to GppNHp.

Competition curves of [fluorescein Lys³]SP against both [³H]SP and ¹²⁵I Bolton–Hunter SP were performed, and the data are summarized in Table 1. The addition of fluorescein to SP to form [fluorescein Lys³]SP resulted in approximately a 6-fold reduction in affinity for both the high- and low-affinity sites. Measurements of changes in phosphoinositol turnover in CHO cells expressing the human NK1 receptor showed that [fluorescein Lys³]SP was a full agonist since the maximum increase in phosphoinositol turnover was identical to that evoked by SP itself (data not shown). Like SP itself, [fluorescein Lys³]SP showed the highest affinity for the NK1 receptor subtype and lower affinity for the NK3 receptor, followed by the NK2 receptor (M. A. Cascieri, personal communication).

Binding of [fluorescein Lys³]SP Detected by Fluorescence. Fluorescence-activated cell sorting was used to demonstrate binding of [fluorescein Lys³]SP to intact Sf9 cells expressing the human NK1 receptor (Figure 5A). Addition of [fluorescein Lys³]SP to these cells resulted in the appearance of a population of cells exhibiting a large increase in fluorescence at 530 nm over the background levels. This fluorescence was blocked by the addition of 10 μM SP. Only a small amount of nonspecific binding (not blocked by SP) was observed. Figure 5B shows the binding of [fluorescein Lys³]SP to uninfected Sf9 cells. These experiments provide direct evidence that [fluorescein Lys³]SP binds to the NK1 receptor on the surface of Sf9 cells.

The binding of [fluorescein Lys³]SP to membranes prepared from Sf9 cells was measured using an SLM 48000 spectrofluorometer. Binding to the receptor could be detected by either a small (approximately 2%) decrease in fluorescence intensity at 530 nm or a larger increase in anisotropy at 530 nm (Figure 6). Addition of the quinuclidine antagonist L-703,606 to the mixture resulted in a displacement of the bound [fluorescein Lys³]SP from the receptor as detected by a return of intensity and anisotropy to initial values. The change in fluorescence intensity of the bound [fluorescein Lys³]SP appeared to be more susceptible to baseline drift than the anisotropy, propably due to photobleaching. Anisotropy measurements appeared to be a more robust means of detecting [fluorescein Lys³]SP binding. The interaction with the receptor resulted in approximately a 2-fold increase in anisotropy (depending on the concentration of receptor and ligand), and this parameter was less susceptible to baseline drifts. However, it was often necessary to correct anisotropy

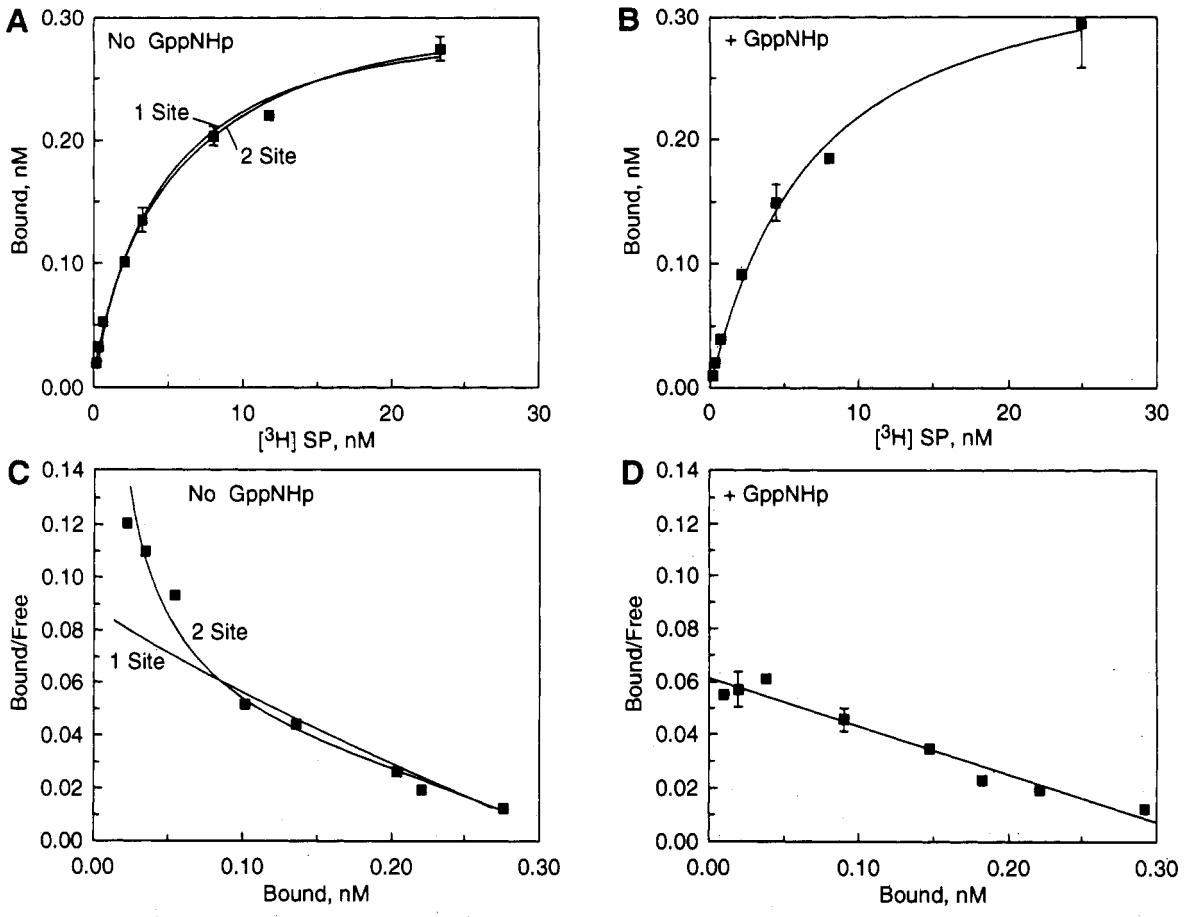

FIGURE 4: Binding of [^{3}H]SP to membranes from Sf9 cells expressing the human NK1 receptor. (A) Membranes were diluted to a final concentration of 0.03 mg/mL of protein in buffer A. The data were fit to one or two classes of binding sites as indicated. The fitted values for one site were B_{max} = 0.32 nM and K_d = 4.2 nM (X^2 = 0.006). Alternatively, the data were fit to a two-site model using the low-affinity K_d from the Scatchard analysis (5.6 nM, panel D) as a constrained parameter resulting in a fit of 0.306 nM low-affinity sites and 0.025 nM high-affinity sites with a K_d of 0.14 nM (X^2 = 0.0002). (B) Membranes were diluted in the same manner except buffer A contained 100 μM GppNHp. The fitted values were B_{max} = 0.37 nM and K_d = 6.8 nM (X^2 = 0.0005) (C) Scatchard-type plots of the data in panel A. The curves represent the theoretical values generated from the nonlinear regression described in panel A. (D) Scatchard analysis of the data in panel B. Binding in the presence of GppNHp was fit to one class of sites with a K_d of 5.6 nM and a B_{max} of 0.33 nM by using a linear regression of the Scatchard transformed data.

Table 1: Binding Constants for SP Peptide Ligands in Sf9 Membranes

ligand	K_d, nM	
	K_H[a]	K_L[b]
SP	0.22	8.9
[fluorescein Lys^3]SP	1.20	48.6

[a] Determined by competition against ^{125}I Bolton–Hunter SP. The K_d of ^{125}I Bolton–Hunter SP was determined to be 0.078 nM by Scatchard analysis. [b] Determined by competition against [^{3}H-Pro^2]SP. The K_d of [^{3}H]SP was determined to be 5.6 nM by Scatchard analysis.

recordings to compensate for an apparent linear baseline decay. This linear decay was observed both before and after the addition of competing ligand to remove the [fluorescein Lys^3]-SP from the receptor, indicating either an artifact due to photobleaching or a loss of stability of the receptor over extended periods of time (Figure 7). The slope of the decay was on the order of -1×10^{-6}/s.

Figure 7 also demonstrates that bound [fluorescein Lys^3]-SP was sensitive to the addition of both the small molecule antagonist L-703,606 and the nonhydrolyzable guanine nucleotide analog GppNHp. Both of these agents reduced the anisotropy of [fluorescein Lys^3]SP, consistent with a reduction in the fraction of ligand bound to the receptor. GppNHp would allosterically convert high-affinity sites to low-affinity sites, whereas L-703,606 would compete directly for [fluorescein Lys^3]SP binding sites on the receptor. Bound [fluorescein Lys^3]SP was displaced by both SP or L-703,606 with similar kinetics (Figure 8). In both cases, the dissociation kinetics of [fluorescein Lys^3]SP were best fit by a biphasic decay, with a fast phase of 0.07 ± 0.07 s^{-1} and a slow phase of 0.004 ± 0.003 s^{-1} (average ± standard deviation of 8 experiments). The fast phase and the slow phase are believed to correspond to the low- and high-affinity sites, respectively (Figure 8C,D). The addition of GppNHp initiated a predominantly monophasic dissociation of [fluorescein Lys^3]-SP from the receptor with a rate similar to that of the fast phase initiated by SP or L-703,606 (Figure 7, 0.07 ± 0.03, n = 6).

Exposure of [fluorescein Lys^3]SP to Anti-Fluorescein Antibody. Antibodies have been described which bind with high affinity to fluorescein and quench its fluorescence (Lopatain & Voss, 1971; Watt & Voss, 1977). Exposure of [fluorescein Lys^3]SP to anti-fluorescein antibody resulted in rapid quenching of the fluorescence (Figure 9). Incubation of Sf9 membranes with [fluorescein Lys^3]SP resulted in a partial protection of fluorescein from quenching by the antibody. The kinetics of quenching were complex and could be fit to two to three exponentials. The first exponential probably represents quenching of the fluorescein of the free ligand by the antibody. The protection from quenching by the antibody was reversed by the addition of 10 μM L-703,-

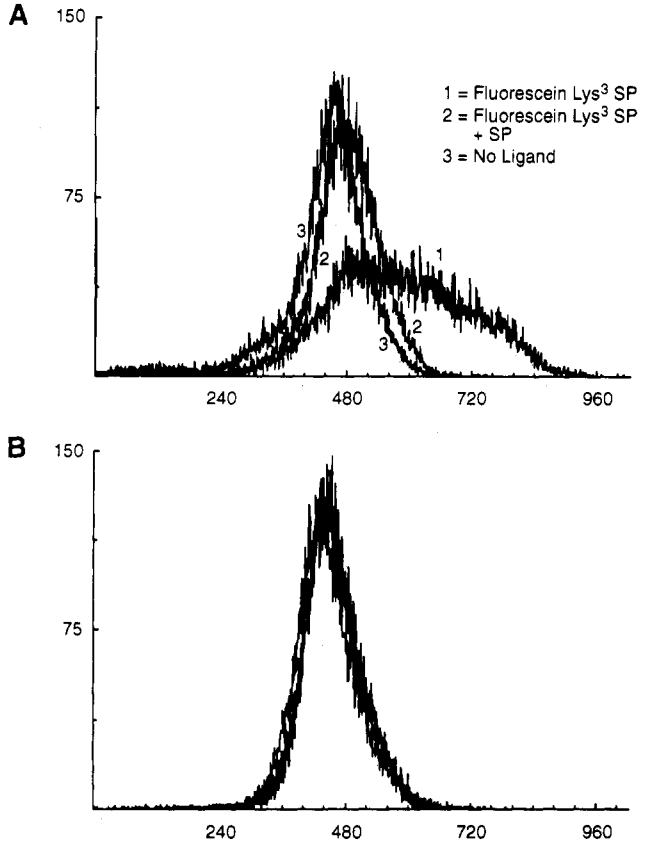

FIGURE 5: Fluorescence-activated cell sorting of Sf9 cells using 5 nM [fluorescein Lys³]SP. (A) Sf9 cells infected with baculovirus containing the gene for the human NK1 receptor. (B) Uninfected Sf9 cells. The number of cells is plotted as a function of the logarithm of fluorescence intensity. In both panels trace 1 = cells incubated with 5 nM [fluorescein Lys³]SP, trace 2 = cells incubated with 5 nM [fluorescein Lys³]SP and 10 μM SP, and trace 3 = cells with no ligand.

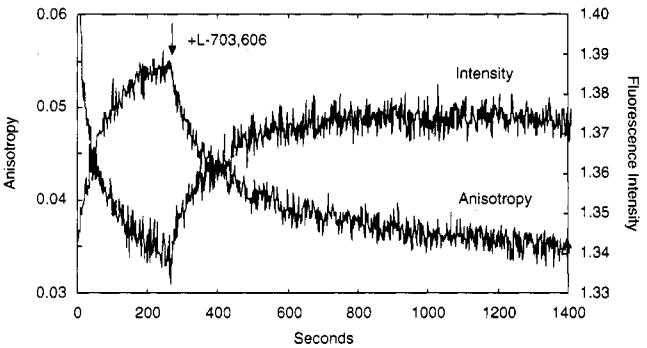

FIGURE 6: Binding and displacement of [fluorescein Lys³]SP detected by both a change in intensity and a change in anisotropy. Two nanomolar [fluorescein Lys³]SP was added to membranes containing the NK1 receptor diluted to a concentration of 2 nM, as determined by [³H]SP binding. At the indicated time, 10 μM L-703,606 was added from a 1000× stock. Data were recorded at 2-s intervals.

606. The anti-fluorescein quenching data obtained in the presence of L-703,606 were fit to obtain a rate constant for the quenching of free [fluorescein Lys³]SP. This rate constant was then used as a fixed value for evaluating the antibody-mediated quenching of [fluorescein Lys³]SP bound to the receptor. The slow sites (0.035 and 0.0017 s^{-1}, Figure 9) were on a time scale consistent with the dissociation of [fluorescein Lys³]SP from the receptor as detected by anisotropy. In analyzing the data, the amplitude of the rapid phase should be equal to the percentage of free [fluorescein Lys³]SP in the presence of the receptor. Since the anisotropy of the fluorescence was recorded in addition to intensity, it is

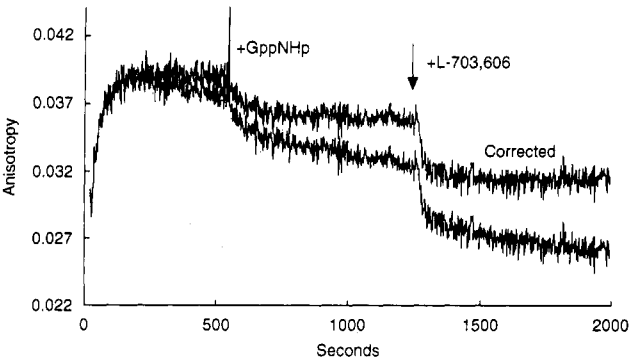

FIGURE 7: Effect of GppNHp and L-703,606 on binding of [fluorescein Lys³]SP to the NK1 receptor and application of a correction factor. Five nanomolar [fluorescein Lys³]SP was added to the NK1 receptor diluted to a concentration of 2 nM, as determined by [³H]SP binding. At the indicated time, 100 μM GppNHp or 10 μM L-703,606 was added from a 1000× stock. In the bottom tracing the data were corrected for a overall downward slope of -2.74×10^{-6}.

possible to estimate the anisotropy of the receptor-bound fraction of [fluorescein Lys³]SP using the equation (Lakowicz, 1983):

$$r_{av} = r_{bound}(\text{fraction of ligand bound}) + r_{free}(\text{fraction of ligand free}) \quad (3)$$

Since the quenching of [fluorescein Lys³]SP upon binding to the receptor is small, eq 3 was used directly to estimate the anisotropy of [fluorescein Lys³]SP bound to the NK1 receptor. The anisotropy value used for the free ligand was determined for each experiment by blocking the receptor with L-703,606. An estimate of the anisotropy of [fluorescein Lys³]SP bound to the NK1 receptor was 0.17 ± 0.01 (n = 3 separate experiments, where the fraction of ligand bound was between 22% and 42%).

Titration of [fluorescein Lys³]SP Binding Detected by Anisotropy. Increasing concentrations of [fluorescein Lys³]-SP were added to Sf9 membranes containing the NK1 receptor, resulting in a decrease in the observed anisotropy. This decrease would be expected from eq 3 because as the concentration of total ligand increases, the fraction of bound ligand decreases. The data were fit using the bound anisotropy determined above and assuming two classes of binding sites (Figure 10). The calculated values for the high- and low-affinity sites (0.36 and 48.9 nM) and the proportion of the two sites (8% high affinity) were similar to values obtained from radioligand binding assays (Table 1 and Figure 4).

Quenching of [fluorescein Lys³]SP Fluorescence as a Function of pH. Changes in the fluorescence intensity of [fluorescein Lys³]SP were examined at pH 7.5 and pH 9.1. Figure 11 examines the changes in the fluorescence intensity as [fluorescein Lys³]SP is being released from the receptor after the addition of L-703,606 and GppNHp. L-703,606 and GppNHp were added simultaneously to induce a rapid dissociation. The increase in fluorescence intensity observed at pH 7.5 when the ligand was released (Figures 6 and 11A) was not seen at pH 9.1 (Figure 11B). Instead, a slight fluorescence decrease was seen upon dissociation of [fluorescein Lys³]SP from the receptor at pH 9.1. The extent of the increase in anisotropy observed upon binding of [fluorescein Lys³]SP at pH 9.1 was within 20% of that determined at pH 7.5 (data not shown). These results demonstrate that [fluorescein Lys³]SP bound to the receptor at pH 9.1, but this binding was not accompanied by a quenching of the fluorescence of the ligand.

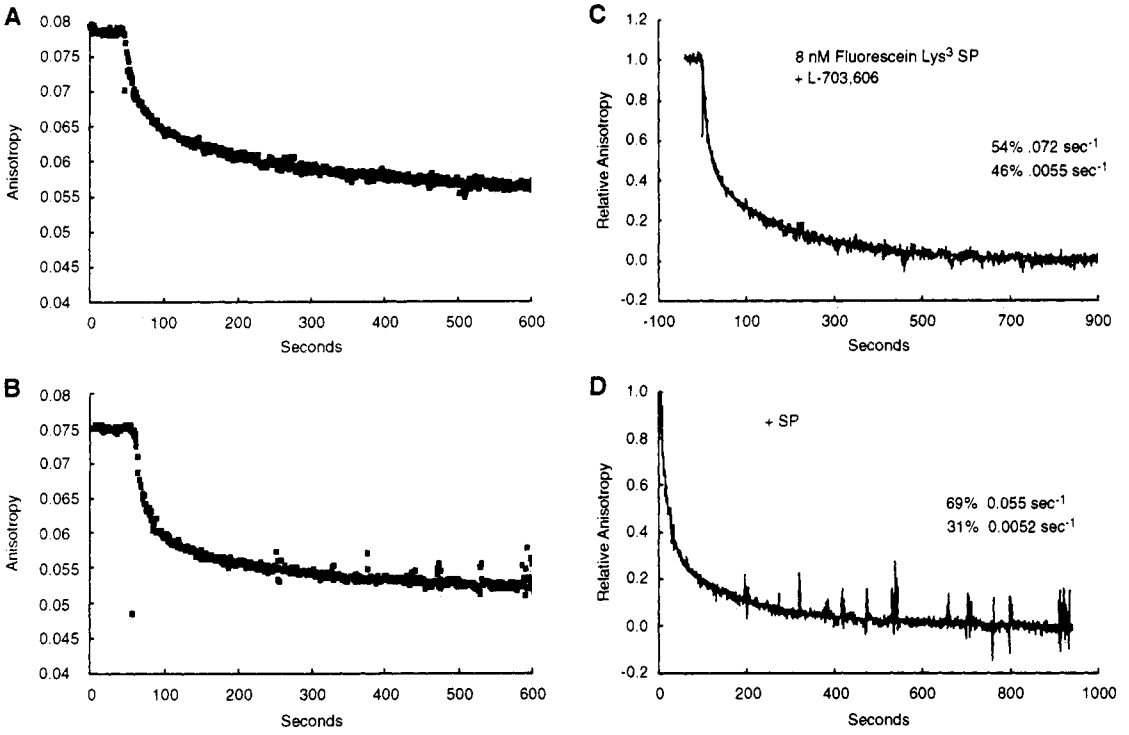

FIGURE 8: Dissociation of 8 nM [fluorescein Lys^3]SP initiated by either L-703,606 (A and C) or SP (B and D). The data in panels C and D were corrected for a linear decay as described in Figure 7 and fit to a biexponential decay using the equation Ampl × $e^{-k_1 t}$ + Amp2 × $e^{-k_2 t}$ + end point. For this fit, the amplitudes, rates, and end point were allowed to vary. The amplitudes and rates are given above for dissociation initiated by either L-703,606 (C) or SP (D).

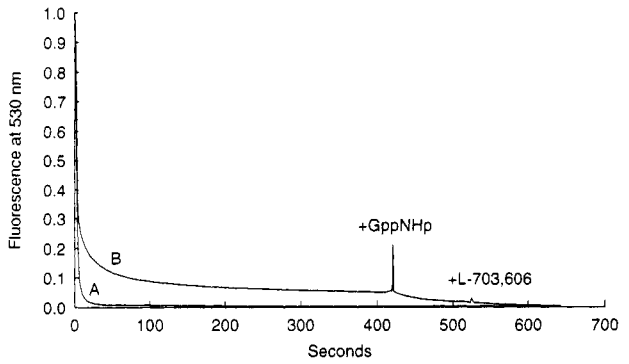

FIGURE 9: Antibody quenching of [fluorescein Lys^3]SP. Five nanomolar [fluorescein Lys^3]SP was incubated with approximately 2 nM NK1 receptor in the presence (A) or absence (B) of 10 μM L-703,606. Anti-fluorescein antibody (10 μL in 50 mM Tris, 150 mM NaCl, pH 7.5) was added at time 0. At the indicated times, 100 μM GppNHp and 10 μM L-707,606 were added to the unblocked receptor in order to complete the dissociation of [fluorescein Lys^3]-SP. The L-703,606 blocked data were fit to a biexponential decay as described in Figure 8. The fast phase of 0.34 s^{-1} accounted for 98.6% of the amplitude. The data from the unblocked receptor were fit to three exponentials. The rate for the fastest phase was held constant at 0.34 s^{-1} and accounted for 73.3% of the amplitude. The remaining decay was fit with 17.3% at 0.035 s^{-1} and 9.5% at 0.0017 s^{-1}. The anisotropy of the sample before addition of the antibody was 0.071 in the absence of L-703,606 and 0.041 in the presence of L-703,606.

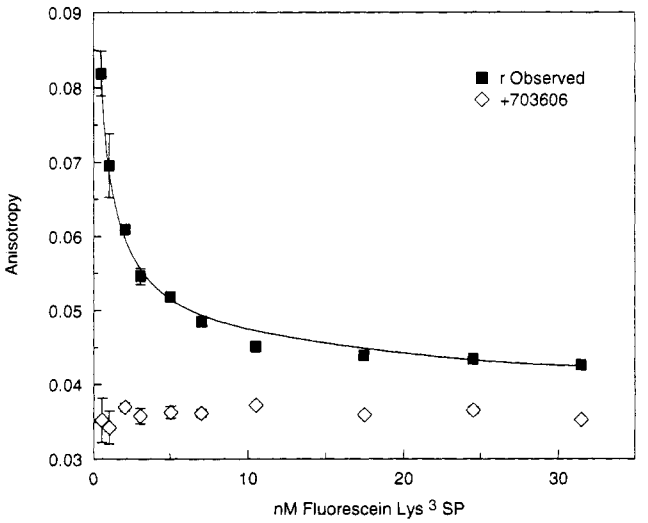

FIGURE 10: Titration of the NK1 receptor by [fluorescein Lys^3]SP. Increasing amounts of [fluorescein Lys^3]SP were added to Sf9 membranes containing the NK1 receptor at a concentration of 3 nM, as determined by [^{3}H]SP binding. The data were fit by constraining the free anisotropy (0.035) and the bound anisotropy (0.17). The fitted parameters were 3.8 nM total binding sites with 7.5% of the sites in the high-affinity state. The measured K_d for the high-affinity site was 0.36 nM and the measured K_d for the low-affinity site was 49 nM.

DISCUSSION

Dynamic Properties of [fluorescein Lys^3]SP. A Perrin plot of [fluorescein Lys^3]SP gave a limiting anisotropy of 0.29 (Figure 2). This value, along with the fluorescence lifetime (3.9 ns, Figure 1), could be used to estimate the rotational correlation time of the peptide from the equation:

$$r = r_0/(1 + \tau/\varphi) \quad (4)$$

where τ = the fluorescence lifetime and φ = the rotational correlation time. The rotational correlation time for [fluorescein Lys^3]SP was determined to be 624 ps. This is similar to the value obtained by the relationship:

$$\varphi = (\nu + h)\eta M/RT \quad (5)$$

where ν is the specific volume of the peptide, assumed to be 0.74, h is the hydration, assumed to be 0.2 g of H_2O/g of peptide, $\eta = 0.01$ P, $R = 8.314 \times 10^7$, and $T = 293$ K. Equation 5 yields a rotational correlation time of 658 ps, assuming that

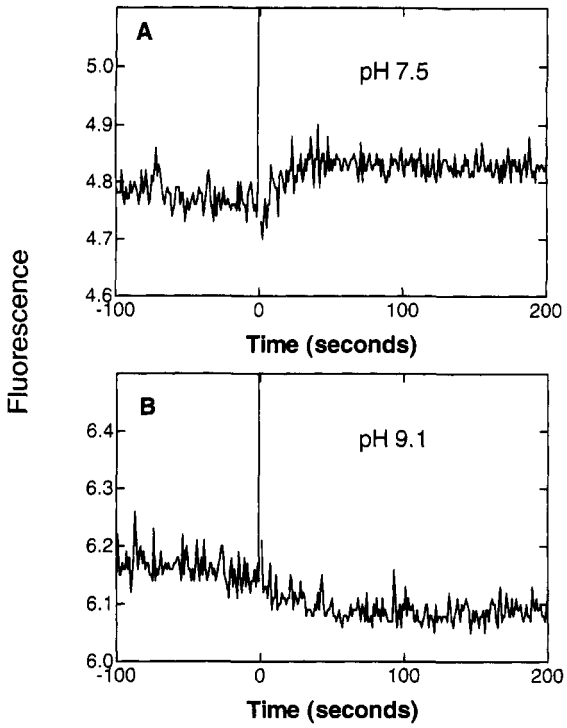

FIGURE 11: pH dependency of fluorescence quenching. One nanomolar [fluorescein Lys^3]SP was allowed to bind to membranes containing the HNK1 receptor at either pH 7.5 (A) or pH 9.1 (B). At the indicated time, 80 μM GppNHp and 2 μM L-703,606 were added to initiate a rapid dissociation of peptide. $MnCl_2$ was omitted for this experiment.

the peptide is globular (Lakowicz, 1983). The observation that the theoretical and derived rotational correlation times were similar might suggest that this assumption is valid and SP is a globular peptide. However, the limiting anisotropy of 0.29 for [fluorescein Lys^3]SP derived from the Perrin plot is lower than the 0.37 maximum value. This observation suggests that there is some local probe mobility that is not distinguished from macromolecular mobility by Perrin analysis, which can detect only the average rotational correlation time. Differential polarized phase fluorometry was therefore used to directly measure the rotational dynamics of the peptide (Figure 3). When the data were fit to a single component, the calculated rotational correlation time was 762 ps. However, the fit was improved by using a two-component fit with a short time of 348 ps that accounts for 88% of the decrease in anisotropy and a longer time of 2.2 ns accounting for the remaining 12%. The limiting anisotropy estimated by this method was 0.33. The shorter component is most likely due to local mobility of the fluorescein group, while the longer correlation time is probably due to the rotation of the entire peptide. Since this long component is much longer than the 658 ps calculated assuming a globular peptide (see above), these data suggest that [fluorescein Lys^3]SP is in an elongated state in aqueous solution. Indeed, NMR data have indicated that SP takes on an extended conformation in aqueous solution (Sumner et al., 1990).

Properties of Bound [fluorescein Lys^3]SP. Upon binding to the receptor, there is a decrease in fluorescence intensity and an increase in anisotropy of [fluorescein Lys^3]SP. Under the conditions in which the fluorescence was measured, most of the ligand in the sample was free, thus diminishing the magnitude of the spectral changes observed upon binding. The observed fluorescence quenching of the mixture of free and bound ligand was small, about 2%, while the anisotropy increased >200%, from 0.04 to values greater than 0.10. Thus, anisotropy was used for further analysis of the receptor–ligand interactions. In order to examine the accessibility of the bound fluorescein to the solvent phase, the receptor-bound peptide was exposed to an anti-fluorescein antibody. The antibody rapidly binds to and quenches free fluorescein and thus can be used to determine the solvent accessibility of the fluorophore. This anti-fluorescein antibody has been previously used to discriminate between fMet-Leu-Phe analogs that were free in solution and that bound to its receptor (Sklar et al., 1984, 1990). Addition of the antibody to a sample containing both receptor-bound and free [fluorescein Lys^3]SP rapidly quenched the fluorescence of the free ligand but only slowly quenched the fluorescence of the ligand bound to the receptor (Figure 9). Thus, the fluorescein moiety on the receptor-bound ligand was protected from interaction with the antibody, probably due to steric hindrance of antibody binding by the proximity of the fluorescein to the receptor. Future comparisons of neurokinin peptides labeled at multiple sites, or labeled with different spacer arms, should yield a more detailed description of the interaction between the fluoresceinated amino acid and the receptor.

The ability of the antibody to discriminate between bound and free ligand allowed an estimation of the anisotropy of the bound ligand, calculated to be 0.17. A potential problem in interpreting the anisotropy data would arise from the high turbidity of the membrane-containing solution. However, the anisotropy of the free ligand measured in buffer and measured in the membrane suspension was about the same (see Materials and Methods). At present, we feel the value of 0.17 is accurate to within a few percent, but a completely unambiguous value for the bound anisotropy could only be obtained in a nonturbid solution, in the absence of any free ligand.

This limiting anisotropy of bound ligand (0.17) is less than the theoretical limiting anisotropy of 0.37 and less than the values of 0.33 and 0.29 estimated for [fluorescein Lys^3]SP from rotational dynamic studies and from the Perrin plot, respectively. Therefore, the ligand in the binding site of the NK1 receptor possesses a significant degree of local mobility. This local mobility of the probe may arise from some rotational freedom of the fluorescein or from segmental mobility of the region where the ligand binds to the receptor. Rotational dynamic studies on the free peptide indicated a significant degree of local mobility of the fluorescein (Figure 3), and it is possible that this freedom is retained when the peptide binds to the receptor. A recent study of fluorescein-labeled EGF revealed that the fluorescein on that peptide also had a significant degree of local mobility. The anisotropy of the bound fluorescein-labeled EGF was 0.18 (Carraway & Cerione, 1993), which was very similar to our estimated value of 0.17. Perhaps, in both systems, this anisotropy arises from a combination of a very immobile peptide–receptor complex imbedded in a lipid bilayer and a relatively unhindered fluorescein attached to that peptide.

An estimation of the fraction of bound ligand should also allow an estimation of the maximum amount of quenching of the receptor-bound ligand. Since intensity and anisotropy were recorded simultaneously, these two parameters could be directly compared for the same sample. The observed anisotropy can be used in conjunction with the estimate of bound anisotropy, 0.17, to estimate the fraction of [fluorescein Lys^3]SP that is bound to the receptor (15%, Figure 6). Since there was a 2.2% change in the total fluorescence intensity, and 15% of the ligand was bound to the receptor, then the quenching upon binding would appear to be approximately 14.7% (0.022/0.15). These quenching data indicate that the fluorescein moiety of [fluorescein Lys^3]SP is interacting to some degree with the receptor. Two potential mechanisms

for fluorescein quenching arise from hydrophobic interactions and protonation. Hydrophobic quenching would arise from stacking of fluorescein with aromatic amino acids (Watt & Voss, 1977). Alternatively, protonation of fluorescein would result in a decrease in its absorbance and fluorescence intensity (Haugland, 1992). Both of these mechanisms have been postulated for formyl peptide receptors labeled with fluorescein at different positions (Fay et al., 1993). The pH dependence of quenching observed for [fluorescein Lys^3]SP in the present study indicates that protonation is occurring upon binding (Figure 11). Since no quenching was observed at pH 9.1 whereas a significant amount of binding was detected (by anisotropy), the protonation mechanism must not be required for binding.

Both the degree of quenching and the local freedom of the fluorescein at Lys^3 in the receptor-bound probe suggest that this position on the peptide is not deeply buried in the core of the receptor. A deeply buried fluorescein would have been expected to result in more highly quenched fluorescence or more hindered mobility of the fluorophore upon binding to the receptor. For example, quenching of the buried fluorescein on the pentapeptide formyl peptide receptor ligand was reported to be 45% (Fay et al., 1993). However, the 15% quenching does indicate that there is some interaction with the receptor. Together, these observations suggest that Lys^3 may be in a position to interact with amino acids on the surface of the NK1 receptor. Further studies using purified receptor in conjunction with mutagenesis studies should allow the identification of specific receptor–ligand interactions in the binding site.

Kinetics of Interaction of [fluorescein Lys^3]SP with the Human NK1 Receptor. Changes in anisotropy of [fluorescein Lys^3]SP were used to analyze the kinetics of the interaction between [fluorescein Lys^3]SP and the NK1 receptor. The dissociation of [fluorescein Lys^3]SP from the NK1 receptor was clearly biphasic (Figure 8), with rates that appear to reflect the dissociation of the ligand from the high- and low-affinity agonist binding sites. The ratio between the two rates of dissociation (19.2) was similar to the ratio of the affinities of the two binding sites (40.5, Table 1). The high-affinity binding site is believed to arise from receptor coupling to G protein. Consistent with this effect, the binding of [fluorescein Lys^3]SP was sensitive to the presence of the nonhydrolyzable guanine nucleotide analog GppNHp (Figure 7). It has been previously observed for the NK1 receptor (Kwatra et al., 1993; Mazina et al., 1994) and the β-adrenergic receptor (Kleymann et al., 1993) that Sf9 cells express G proteins which can couple to seven transmembrane receptors.

The addition of GppNHp caused a rapid decrease in anisotropy of the fluorescein, consistent with the dissociation of the [fluorescein Lys^3]SP upon conversion of the receptor to the low-affinity state. The rate of dissociation induced by GppNHp (0.07 ± 0.03 s^{-1}) was not significantly different from the fast phase of ligand release in the presence of SP (0.07 ± 0.07 s^{-1}). Thus, the reduction in the affinity of the receptor for the agonist upon addition of GppNHp was rapid in the time scale of the present experiment. Indeed, a recent stopped-flow study using fluorescent formyl peptides indicated that guanine nucleotides induced a rapid conversion of receptors to low affinity with very little lag time (Neubig & Sklar, 1993).

The changes in polarization of [fluorescein Lys^3]SP as it binds to the NK1 receptor should provide an excellent tool for further kinetic analysis of receptor–ligand interactions. Monitoring polarization changes may be the preferred method for analysis of receptor–ligand interaction in situations where intensity changes and fractional saturations are low. Such studies should provide insights into the molecular nature of both receptor–ligand interactions and receptor–G protein interactions.

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