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Mechanism of γ-Aminobutyric Acid/Benzodiazepine Receptor Turnover in Neuronal Cells: Evidence for Nonlysosomal Degradation

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SUMMARY

In a previous report we described the use of flunitrazepam as a photoaffinity label to monitor the turnover of the γ-aminobutyric acid/benzodiazepine receptor complex in primary brain and spinal cord cell cultures [*Science (Wash. D. C.)* **226**:857–860 (1984)]. In the present communication we have extended our studies on the kinetics of receptor turnover and have examined the mechanism of receptor degradation. There are approximately 60,000 irreversible binding sites per neuron, and photolabeling is stereospecific. Photolabeling does not demonstrably alter the kinetics of degradation, and a complete rate equation relating the kinetic constants for degradation to receptor number is described. The rapid phase of degradation is slowed at low temperature ($Q_{10} = 5$, which corresponds to an apparent energy of activation of 25 kcal/mol) and by inhibitors of ATP production (sodium azide, 2-deoxyglucose, and 2,4-dinitrophenol). The fast phase for the degradation of photolabeled receptor is not affected by lysosomotropic agents (methylamine, ammonium chloride, and chloroquine) or by elimination of horse serum and chick embryo extract from the growth medium. In contrast, overall protein degradation is inhibited by methylamine and enhanced in serum-free medium. The results suggest that the γ-aminobutyric acid receptor complex is degraded through an energy-dependent nonlysosomal pathway.

That the cellular response of many receptor systems may be controlled by the ability of neurons to alter receptor numbers and thereby regulate their sensitivity to substances such as neurotransmitters, hormones, and drugs is well known. For example, defective regulation of catecholamine receptor number has been implicated in various pathological states such as depression and schizophrenia. In this regard, the clinical effects of the antidepressants (and perhaps other classes of psychoactive drugs) may be mediated through their ability to adjust receptor number (1). Additionally, an increase in the number of glutamate receptors may underlie certain kinds of long term potentiation (2).

Receptor number is ultimately controlled by the kinetics of turnover (synthesis and degradation), and thus an understanding of such kinetics will help elucidate the molecular mechanism(s) by which receptor number is regulated. Although much is known about the turnover (synthesis and degradation) (3–6) and recycling (see Ref. 7 for review) of several cell surface proteins in non-neural cells, knowledge of neurotransmitter receptor turnover in the central nervous system has been limited due to the lack of defined preparations and specific irreversible probes. In particular, little is known about the molecular mechanisms by which neurotransmitter receptors are degraded.

The GABA-R provides an opportunity to study the turnover of a receptor localized to the nervous system. Many of the physiological actions of benzodiazepines are mediated through benzodiazepine binding sites (8–12) that, although distinct from the GABA binding sites, are located on the same macromolecular protein complex (13–15). Biochemical and electrophysiological experiments on primary monolayer cell cultures prepared from embryonic chick spinal cord and brain show that GABA exhibits properties characteristic of a neurotransmitter (16–18), that the action of GABA whether exogenously applied or synaptically released is enhanced by benzodiazepines (16, 19, 20), and that the high affinity benzodiazepine binding site corresponds to the functional benzodiazepine receptor (21–23).

In previous communications from our laboratory, flunitrazepam has been used as a photoaffinity label to demonstrate that 80% of the benzodiazepine receptors are on the cell surface

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ABBREVIATIONS: GABA-R, γ-aminobutyric acid/benzodiazepine receptor; ara-C, cytosine arabinoside; [^{3}H]β-CCM, [^{3}H]methyl-β-carboline-3-carboxylate; [^{3}H]DADLE, [^{3}H][D-Ala-D-Leu]-enkephalin; GABA, γ-aminobutyric acid; PBSS, phosphate buffered salt solution; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

with their ligand recognition sites facing extracellularly, whereas 20% are intracellular (24), and to monitor the kinetics of receptor turnover (25). Here, we report that 1) photolabeling does not alter the kinetics of receptor degradation, and that 2) the mechanism of degradation does not require acidic protease activity, consistent with a model in which the GABA-R is degraded via a nonlysosomal pathway.

Experimental Procedures

Materials. [^{3}H]Flunitrazepam (85 Ci/mmol), [^{3}H]muscimol (15 Ci/mmol), [^{3}H]β-CCM (84 Ci/mmol), [^{3}H]QNB (33 Ci/mmol), and [^{3}H] DADLE (44 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{35}S]Methionine (1000 Ci/mmol) was from Amersham (Arlington Heights, IL). The benzodiazepine drugs and levorphanol were a gift of Dr. W. Scott and Dr. P. Sorter of Hoffmann-La Roche (Nutley, NJ). All other chemicals were from commercial sources.

Cell culture. Brain cultures were prepared as previously described (17, 21, 25). Briefly, tissue from 7-day chick embryos (Shamrock Farms, NJ) was minced, incubated with trypsin (0.025%, 5 min), collected by centrifugation (900 rpm, 5 min), triturated with fire-polished Pasteur pipets, and added to collagen-coated dishes (Falcon) in complete medium (Eagle's minimal essential medium, supplemented with (final concentrations) 10% heat-inactivated horse serum (GIBCO, Grand Island, NY) 5% chick embryo extract, 2.4 mM glutamine, and penicillin-streptomycin. For degradation experiments, 6-well dishes (35 mm/well) were usually used; for recovery experiments 60-mm or 100-mm dishes were used. Ara-C (1 μM) was added after 1 day to control the proliferation of non-neuronal cells. Cultures were fed the following day and again 2–3 days later with medium identical to that used for plating but containing 24 mM K^+. Cultures were generally used after 1 week.

Reversible binding of [^{3}H]flunitrazepam. Cells were washed with PBSS (in mM: NaCl, 123; KCl, 5.4; NaH_2PO_4, 11; $MgSO_4$, 0.4; $CaCl_2$, 0.9; glucose, 22.2; pH 7.4), scraped with a rubber policeman, homogenized in a Dounce homogenizer and centrifuged (30,000 × g, 20 min). Pellets were rehomogenized in PBSS. Samples of homogenate (approximately 100 μg of protein) were added to assay tubes containing [^{3}H]flunitrazepam (final concentration, 5 nM) in a total volume of 0.5 ml, incubated on ice for 30–45 min, and filtered rapidly under vacuum through Whatman GF/B glass-fiber filters. Filters were washed four times with 5 ml of ice-cold PBSS and radioactivity was determined by scintillation counting. Nonspecific binding (approximately 10% of total) was determined in the presence of 1 mM flurazepam and was subtracted from total binding to yield specific binding. All determinations were performed in triplicate. Protein was determined by the method of Lowry *et al.* (26), using bovine serum albumin as standard.

Photoinactivation. To "photoinactivate," intact cell cultures were washed twice with PBSS, incubated with 100 nM flunitrazepam (4°, 30 min), and irradiated with long wavelength UV light (General Electric F40BLB black light bulb at a distance of approximately 2 cm; 4°; 30–45 min unless stated otherwise). The extent of photoinactivation in most experiments was approximately 75%. Control dishes were incubated with flunitrazepam but were not irradiated ("mock-inactivation"). Cells were then washed extensively with PBSS to remove trapped and reversibly bound flunitrazepam. For recovery experiments, cultures were returned to the incubator after the addition of complete medium.

Photolabeling. Photolabeling was conducted using a protocol identical to that for photoinactivation, except that 5 nM [^{3}H]flunitrazepam was used and the irradiation time was generally 20–30 min. In some dishes, 1 mM flurazepam was included to determine nonspecific photolabeling, which was approximately 40% of total. Unless indicated otherwise, all data represent "specific photolabeling" (total photolabeling minus nonspecific photolabeling).

Degradation of receptor. To monitor receptor degradation, cultures were photolabeled with [^{3}H]flunitrazepam as described above. Growth medium (containing inhibitors or lacking serum and chick embryo extract, as indicated in legends to tables and figures) was then added and cultures were returned to the incubator. At each time point, medium was withdrawn and replaced with fresh medium. The withdrawn medium was centrifuged at 1000 rpm to sediment cell fragments and radioactivity in the supernatant was determined. After the last time point, cells were scraped and the remaining cell-associated radioactivity was determined. The degradation rate of nonspecific photolabeling is multiphasic and variable but does not affect the kinetics of degradation of specific photolabeling.

Synthesis of general cellular protein. To determine the effect of cycloheximide on protein synthesis, cultures were incubated for 5 hr (37°) in the absence (control) or presence of cycloheximide (20 μg/ml), after which [^{35}S]methionine was added; in some cases, the cycloheximide was washed out before addition of isotope. Incubation was continued for 30 min (37°), and the cultures were then washed to remove nonincorporated radioactivity. SDS/Nonidet P-40/urea (0.2%/2%/8 M) was added (2 ml/dish), aliquots were removed, and TCA was added to a final concentration of 10%. The samples were incubated on ice for 30 min and TCA-precipitable radioactivity was determined by filtration through Whatman GF/B glass fiber filters, which were then washed under vacuum with PBSS containing 10% TCA. Radioactivity retained on the filters was determined by scintillation counting.

Degradation of cellular protein. Cultures were labeled with [^{35}S] methionine (24 hr, 37°), washed with complete medium containing 2 mM L-methionine (to prevent further incorporation of label), and incubated at 37° after the addition of medium containing unlabeled methionine (lacking serum or containing inhibitors, as indicated in legends to tables and figures). At each time point a small aliquot of medium was removed and replaced with fresh medium. TCA was added to the aliquots to a final concentration of 10%, the samples were incubated on ice for 30 min and centrifuged for 5 min in a Beckman microfuge, and radioactivity in the supernatant was determined by scintillation counting. Radioactivity remaining in the cells was determined at the end of the experiment.

Results

Photoaffinity labeling of the GABA-R. Photolabeling of intact brain cell cultures is inhibited in a stereospecific manner inasmuch as the dextrorotatory enantiomer Ro 11-6896 [B10(+)] inhibits photolabeling to a greater extent than the levorotatory isomer Ro 11-6893 [B10(−)] (Table 1). The specifically incorporated radioactivity migrates as bands of molecular weight 48,000 and 51,000 on SDS polyacrylamide gels (24, 25), similar to results obtained with homogenates of embryonic chick brain (22). Photolabeling is selective for neurons. Specific photolabeling per mg protein is much greater for neurons grown almost free of non-neuronal cells than for neurons grown with a monolayer of non-neuronal cells; there are approximately 60,000 irreversible sites per neuron with or without nonneuronal cells (Table 2).

GABA-R degradation. Fig. 1 shows the kinetics of GABA-R degradation in intact brain cultures, determined by monitor-

TABLE 1

Stereospecificity of photolabeling

Cultures were photolabeled in the presence or absence of Ro 11-6896 [B10(+)] or Ro 11-6893 [B10(−)] (100 nM or 1 μM). Results show specific photolabeling as a percentage of control (mean ± SE of two experiments).

Benzodiazepine	Photolabeling
	%
Control	100
B10(−), 100 nM	102 ± 3
B10(+), 100 nM	10 ± 6
B10(−), 1 μM	41 ± 1
B10(+), 1 μM	6 ± 6

TABLE 2

Specificity of photolabeling for neurons

Brain cultures containing neurons and few non-neuronal cells (ara-C treated), and cultures containing neurons and a monolayer of non-neuronal cells (not treated with ara-C) were photolabeled with 5 nM [^{3}H]flunitrazepam with or without 1 mM flurazepam (4° 1 hr UV). After extensive washing, bound radioactivity was determined. Neurons were counted in sister cultures (three dishes/condition, 25 high-power fields/dish, approximately 400 cells/dish). Results show specific photolabeling, mean ± standard error (three independent experiments).

Condition	Specific Photolabeling			
	neurons/dish × 10^5	*fmol/dish*	*fmol/mg of protein*	*sites/neuron* × 10^4
Neurons + few non-neuronal cells	9.2 ± 1.0	97 ± 27	74 ± 10	6.2 ± 1.5
Neurons + many non-neuronal cells	8.1 ± 0.2	77 ± 14	36 ± 3	5.6 ± 0.9

ing the release of radioactivity after photolabeling. Importantly, the efflux of radioactivity does not represent loss of reversibly bound drug, inasmuch as control experiments demonstrate that the standard wash procedure is adequate to remove greater than 99% of reversibly bound [^{3}H]flunitrazepam (18,900 ± 955 cpm/dish before washing, 100 ± 20 cpm/dish after washing). In turnover experiments, the specific photolabeling (2000–6000 cpm/dish) that persisted after washing was dependent on the cell density and dish size. Consistent with our earlier report, degradation is well described by a two-site model in which 42% of the receptors are degraded with a half-time of 3.8 ± 0.5 hr (k_1 = 0.182), whereas the remaining receptors are degraded approximately 8-fold more slowly (half-time = 32 ± 4 hr, k_2 = 0.02).

To determine the effect of temperature on GABA-R degradation, brain cultures were photolabeled and allowed to degrade for 6 hr at various temperatures; during this interval, degradation of the slow phase is negligible. The rate of degradation decreases as the temperature is lowered, exhibiting a Q_{10} of approximately 5 (which corresponds to an apparent energy of activation of 25 kcal/mol) (Fig. 2). Degradation is inhibited 18 ± 2% (10 determinations) by inhibitors of metabolic energy production, such as sodium arsenate, sodium azide, dinitrophenol, and 2-deoxyglucose (mean ± standard error, pooled results for data shown in Table 3).

It has previously been demonstrated that weak bases such as methylamine inhibit lysosomal proteolytic activity by increasing the pH of lysosomes, which are acidic (27, 28). Fig. 3 and Table 4 show that 10 mM methylamine inhibits the degradation of [^{35}S]methionine-labeled proteins approximately 30%. This result is similar to results obtained with skeletal muscle cultures (29) and suggests that some cellular protein is degraded in lysosomes. In addition, protein degradation is enhanced in medium lacking horse serum and chick embryo extract ("step-down" conditions), consistent with results obtained with cell cultures of skeletal muscle (29, 30) and fibroblasts (31). Methylamine inhibits degradation to a similar degree in both complete and step-down medium. Regulation of protein degradation by serum and methylamine is similar in cultures containing neurons and few non-neuronal cells (ara-C treated) and cultures that contain neurons and a dense monolayer of non-neuronal cells (not treated with ara-C) (Table 4).

In contrast to overall cellular protein, the fast phase of GABA-R degradation is not enhanced by step-down medium (Fig. 3; Table 4). Further, receptor degradation is not inhibited by methylamine (in complete or step-down medium) nor by two other lysosomotropic agents, chloroquine (10 μM) and ammonium chloride (10 mM).

Photoinactivation. We next used recovery of receptor after photoinactivation to monitor receptor synthesis. Intact brain cultures were incubated with 100 nM unlabeled flunitrazepam and then irradiated for various times with UV light. As shown in Fig. 4, the specific reversible binding of 5 nM [^{3}H]flunitrazepam to washed membrane homogenates decreases exponentially with a pseudo-first order rate constant for inactivation k_{obs} = 0.053/min ($t_{½}$, approximately 13 min). About 25% of the reversible binding persists even after prolonged irradiation. Photoaffinity labeling irreversibly links flunitrazepam to approximately 25% of the binding sites, whereas binding to the remaining sites is greatly reduced due to a reduction in the affinity for benzodiazepine positive modulators (23, 32, 33). Maximal inhibition of reversible [^{3}H]flunitrazepam binding was obtained with 100 nM unlabeled flunitrazepam, inasmuch as decreasing the concentration to 5 nM did not decrease the extent of photoinactivation (Table 5).

The following results indicate that the decrease in binding is not due to residual flunitrazepam: 1) no photoinactivation

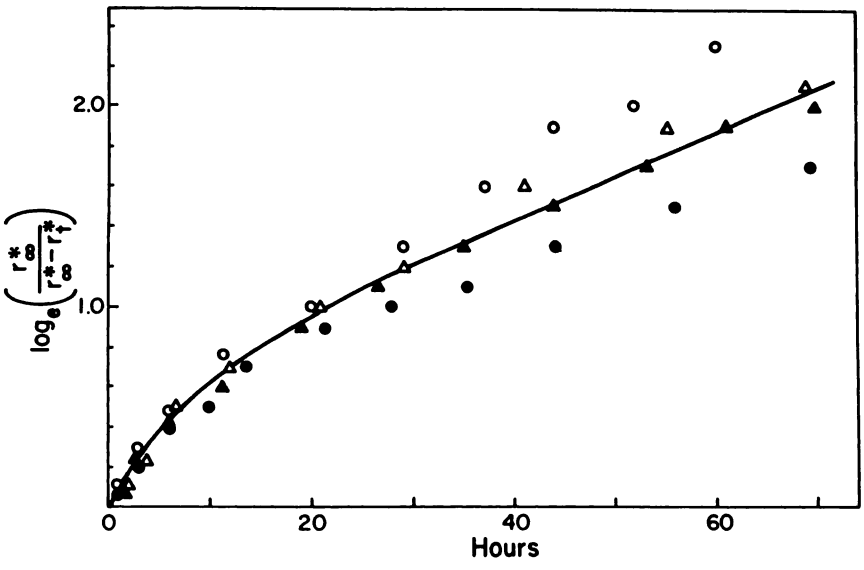

Fig. 1. Degradation kinetics of GABA-R. Brain cells were photolabeled and washed and the efflux of radioacitvity was monitored as described in Experimental Procedures. Data show the accumulation of released specifically bound radioactivity, where r^*_t = *specifically bound radioactivity released at time t* after photolabeling, and r^*_∞ = cumulative release of specifically bound radioactivity plus the specifically bound radioactivity that remains at the end of the experiment. The different symbols show the experimental values obtained in four independent experiments. The line is theoretical 2-exponential fit determined by nonlinear regression.

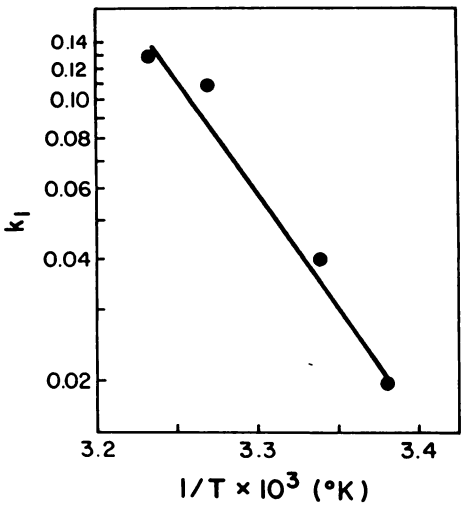

Fig. 2. Temperature dependence of GABA-R degradation. Brain cell cultures were photolabeled, washed, and incubated at the indicated temperatures in PBSS containing 10% horse serum and 2.5% chick embryo extract. Degradation during a 6-hr period was determined by monitoring the efflux of radioactivity as described in Experimental Procedures; k_1 is the degradation rate constant for the fast phase of degradation, as described in Appendix. The data represent the mean of three independent experiments, each performed in sextuplicate.

TABLE 3

Effect of metabolic inhibitors on GABA-R degradation

Brain cultures were photolabeled with 5 nM [^{3}H]flunitrazepam, washed, and incubated at 37° in the absence (control) or presence of the inhibitors. Degradation was determined by monitoring the efflux of radioactivity into the medium during a 6-hr period. Data are expressed as percentage of control degradation. Each value represents the mean ± standard error of two individual experiments (each performed in triplicate).

Condition	Degradation
	% of control
Control	100
Sodium arsenate (10 mM)	83 ± 9
Sodium azide (5 mM)	82 ± 5
2-Deoxyglucose (5 mM)	78 ± 7
Dinitrophenol (0.5 mM)	83 ± 2
Sodium azide (5 mM) + 2-Deoxyglucose (5 mM)	84 ± 3

occurs when cells are incubated with flunitrazepam but are not irradiated (Table 5); 2) decreasing the concentration of flunitrazepam from 100 to 5 nM does not decrease the extent of photoinactivation (Table 5); 3) when intact cultures are incubated with 100 nM flunitrazepam, the concentration of residual flunitrazepam in the reversible binding assay is only approximately 0.015 nM (Table 6), which is far below both the binding K_D (approximately 2 nM) and the concentration of [^{3}H]flunitrazepam (5 nM) used in the binding assay. Reversible [^{3}H] flunitrazepam binding is not reduced when 1 mM flurazepam is present during photoinactivation, suggesting that photoinactivation is a specific, receptor-mediated process (Table 5). The specificity of the reaction is also demonstrated by the finding that photoinactivation has little or no effect on the specific binding of ligands selective for GABA binding sites ([^{3}H]muscimol), β-carboline binding sites ([^{3}H]β-CCM), muscarinic cholinergic receptors ([^{3}H]QNB), or opiate receptors ([^{3}H] DADLE) (Table 7).

To monitor GABA-R reappearance ("recovery"), cultures were photoinactivated and allowed to recover as described in Experimental Procedures. In the experiment shown in Table 1,

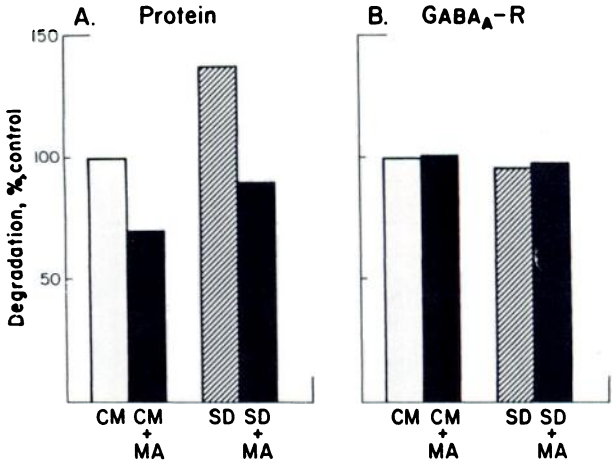

Fig. 3. Degradation of cellular protein, but not that of GABA-R, is altered by serum deprivation and methylamine. A, Cells were labeled with [^{35}S] methionine (2 nM, 24 hr, 37°), washed, and incubated in the absence (control) or presence of methylamine (*MA*) in either complete medium (*CM*) or medium lacking serum and chick embryo extract (step-down, *SD*). Degradation was determined as the efflux of 10% TCA-soluble radioactivity during a 6-hr period. Each *bar* represents the mean of at least two experiments, each performed in sextuplicate. B, Neurons were photolabeled with [^{3}H]flunitrazepam as described in Experimental Procedures, washed, and incubated as described in A. Degradation was determined by monitoring the efflux of radioactivity during a 6-hr period. Each *bar* represents the mean of at least two experiments, each performed in triplicate or sextuplicate. Symbols are the same as in A.

TABLE 4

Effect of lysosomotropic agents on the degradation of GABA-R and overall cellular protein

In experiment 1, brain cultures were photolabeled with 5 nM [^{3}H]flunitrazepam, washed, and incubated at 37° in the absence (control) or presence of inhibitors, in either complete medium or medium lacking serum and chick embryo extract (step-down), as indicated. Degradation was determined as the efflux of radioactivity into the medium during a 6-hr period. Data show the mean of the number of independent experiments shown in parentheses (1, if not indicated otherwise), expressed as percent of control degradation (complete medium, no inhibitors). Each experiment was performed in triplicate or sextuplicate. In experiment 2, brain cultures were labeled with [^{35}S]methionine (2 nM, 24 hr, 37°), washed, and incubated at 37° in the absence (control) or presence of methylamine in either complete or step-down medium. Degradation was determined as the efflux of 10% TCA-soluble radioactivity during a 6-hr period. Each experiment was performed in sextuplicate and the data are expressed as for GABA-R.

Condition	Degradation	
	Complete medium	Step-down medium
	% of control	
Expt. 1. GABA-R degradation		
No additions	100	96 ± 5 (4)
Methylamine (10 mM)	100 ± 1 (2)	98 ± 2 (2)
Chloroquine (10 μM)	97 ± 3 (2)	94 ± 4 (2)
Ammonium chloride (10 mM)	96 ± 2 (2)	ND*
Expt. 2. Cellular protein degradation		
Neurons + few non-neuronal cells		
No additions	100	138 ± 14 (2)
Methylamine (10 mM)	70 ± 9 (2)	90 ± 16 (2)
Neurons + many non-neuronal cells		
No additions	100	135
Methylamine (10 mM)	71	83

* ND, not determined.

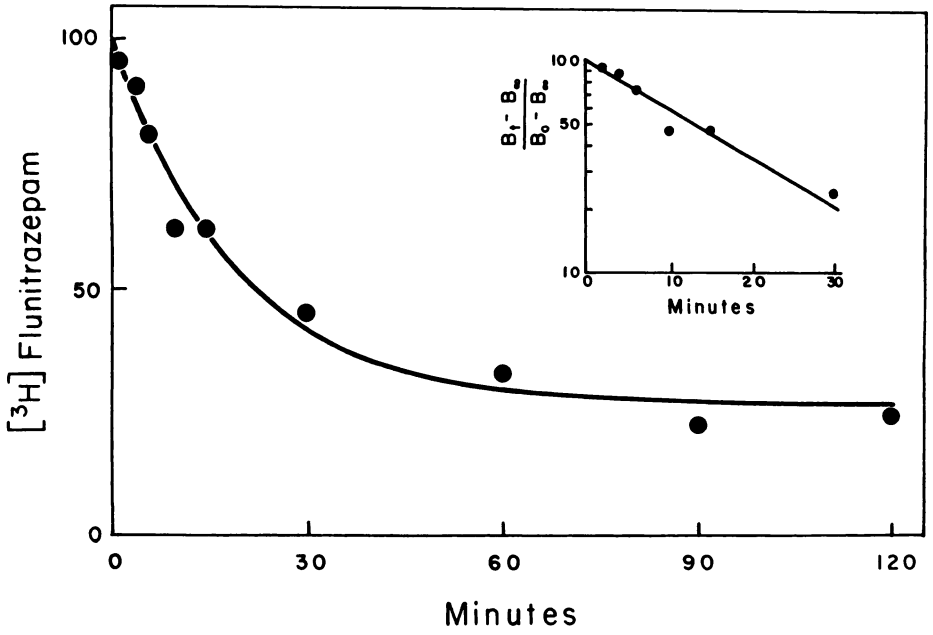

Fig. 4. Kinetics of GABA-R photoinactivation. Cells were incubated with 100 nM flunitrazepam, irradiated for the indicated times, washed and homogenized and the specific reversible binding of 5 nM [^{3}H]flunitrazepam was determined as described in Experimental Procedures. Data show specific [^{3}H]flunitrazepam binding expressed as percentage of control (mean of two experiments, each performed in triplicate). *Inset*, semilogarithmic plot of same data. B_t = binding at time t after photoinactivation, B_0 = binding in absence of photoinactivation, and B_∞ = binding resistant to photoinactivation.

TABLE 5

Effect of flunitrazepam concentration on photoinactivation and recovery

Cells were incubated in the absence or presence of the indicated concentrations of flunitrazapam or flurazepam and then irradiated or not with UV light as described in Experimental Procedures. After irradiation, cells were washed and either immediately, or after a 24-hr recovery period, homogenates were prepared. The reversible binding of 5 nM [^{3}H]flunitrazepam was measured as described in Experimental Procedures. Data show mean ± standard error of triplicate determinations, expressed as percentage of control binding.

Condition			Specific binding	
Flunitrazepam	Flurazepam	UV	1 hr	24 hr
nM	mM		% of control	
Expt. 1				
0	0	–	100 ± 13	130 ± 7
100	0	–	102 ± 7	99 ± 5[a]
100	0	+	31 ± 1	94 ± 4
5	0	+	31 ± 4	115 ± 10
Expt. 2				
100	0	–	100 ± 8	ND[b]
100	0	+	33 ± 4	ND
100	1	–	82 ± 5	ND
100	1	+	96 ± 6	ND

[a] In nine independent recovery experiments, reversible binding returned to 126 ± 23% of control (see text).
[b] ND, not determined.

TABLE 6

GABA-R reappearance after photoinactivation is not due to loss of free flunitrazepam

Cells were incubated with 100 nM [^{3}H]flunitrazepam (1 hr, 4°) and washed without prior exposure to UV light. Either immediately or after 24 hr (37°), cultures were washed again, scraped, homogenized, and centrifuged, and radioactivity in the pellets was determined. Results are means of two independent experiments.

Time	Radioactivity	
hr	fmol/dish	Final concentration,[a] nM
1	20	0.015
24	12	0.009

[a] Values based on the specific activity of [^{3}H]flunitrazepam corrected for counting efficiency and standard tissue dilution for reversible binding assays.

TABLE 7

Specificity of photoinactivation

Cells were exposed to flunitrazepam (100 nM), irradiated, and washed, and reversible binding was determined at the indicated ligand concentrations. Binding studies were performed on fresh tissue using 0.05 M Tris·HCl, pH 7.4, as the buffer. Binding was measured at 0–4° except with [^{3}H]DADLE, for which binding was at 21°. Ratios are based on two or three determinations that agreed within 20%, each of which was performed in triplicate.

Ligand	Photoinactivated / Mock inactivated
[^{3}H]Flunitrazepam (5 nM)[a]	0.1
[^{3}H]Muscimol (10 nM)[b]	0.8
[^{3}H]DADLE (7.5 nM)[c]	1.0
[^{3}H]QNB (1 nM)[d]	0.8
[^{3}H]β-CCM (2 nM)[e]	0.9

Nonspecific binding was determined with (*a*) 1 mM flurazepam, (*b*) 50 μM GABA, (*c*) 1 μM levorphanol, (*d*) 500 nM scopolamine, and (*e*) 1 mM flurazepam.

reversible binding returned to within error of control (noninactivated) levels 24 hr after inactivation. However, in nine independent experiments, reversible binding returned to 126 ± 23% of control (mean ± SEM), suggesting receptor accumulation. The data in Tables 5 and 6 demonstrate that residual drug does not contribute significantly to receptor reappearance (see values obtained at 24 hr).

To examine the rate of receptor recovery, cultures were photoinactivated and allowed to recover for various times. The reversible binding of [^{3}H]flunitrazepam to cellular homogenates increases almost linearly for up to 3 hr. Brain cultures recovered at a rate of 6 ± 1.5%/hr (mean ± SE of seven independent experiments), or 24 ± 6 fmol/mg/hr based on a B_{max} of 400 fmol/mg of protein.[2] Spinal cord cell cultures recover at a rate of 7%/hr and 9%/hr (two experiments). Reappearance of [^{3}H]flunitrazepam binding sites during a 6- or 7-hr recovery period is inhibited 79 ± 13% by cycloheximide (20 μg/ml) or puromycin (100 μg/ml) (four experiments, pooled data for both inhibitors) (Table 8). To examine the effect of cycloheximide on protein synthesis, cultures were exposed to [^{35}S]methionine after a 5-hr incubation in the presence or absence (control) of

[2] G. Schiller, unpublished data.

TABLE 8

Sensitivity of GABA-R recovery and protein synthesis to inhibition by cycloheximide and puromycin

In experiment 1, cells were photoinactivated as described in Experimental Procedures, washed, and allowed to recover at 37° in the absence (a) or presence of cycloheximide (b) or puromycin (c). At the indicated times, cells were scraped and homogenized and the reversible binding of 5 nM [^{3}H]flunitrazepam was determined. In experiment 2, cells were preincubated for 5 hr in the absence (a) or presence of cycloheximide (b), and then exposed to [^{35}S]methionine (70 nM, 0.5 hr 37°) in the continued presence of cycloheximide. In (c), cells were washed for 1 hr to remove cycloheximide and then exposed to [^{35}S]methionine as above. Determinations for each condition used five dishes per point. Data show mean ± standard error; values in parentheses are the number of independent experiments.

	Response
	% *control*
Expt. 1. GABA-R reappearance[a]	
a. Control (6.5 or 24 hr)	100
b. Cycloheximide	
1 μg/ml, 24 hr	3 (1)
20 μg/ml, 6.5 hr	24 ± 25 (2)
c. Puromycin	
100 μg/ml, 6.5 hr	18 ± 19 (2)
Expt. 2. Protein synthesis[b]	
a. Control	100
b. Cycloheximide (20 μg/ml)	3 ± 1 (2)
c. Cycloheximide (20 μg/ml) and then washed	92 ± 15 (2)

[a] Reappearance of specific [^{3}H]flunitrazepam (5 nM) binding was measured as the binding at the end of the recovery period in the absence (B_t) or presence (B_t') of inhibitor, minus the binding immediately after photoinactivation (B_0). Thus % reappearance = $(B_t' - B_0)/(B_t - B_0) \times 100$.

[b] [^{35}S]methionine incorporation measured as TCA-precipitable cpm.

TABLE 9

Lack of effect of photoinactivation on protein synthesis

After the photoinactivation procedure, cells were washed and incubated at 37° for the indicated times. Protein synthesis was then determined and results show mean ± standard error; values in parentheses are number of dishes.

Time	Control	Photoinactivated
hr	*cpm* × 10^5*/dish*	
0[a]	1.92 ± 0.08 (6)	1.96 ± 0.07 (6)
24[b]	4.90 ± 0.88 (5)	4.80 ± 1.21 (5)

[a] Experiment 1: incorporation of [^{35}S]methionine (30 nM, 30 min, 37°) into 10% TCA-precipitable material.

[b] Experiment 2: same as experiment 1 except 100 nM [^{35}S]methionine was used.

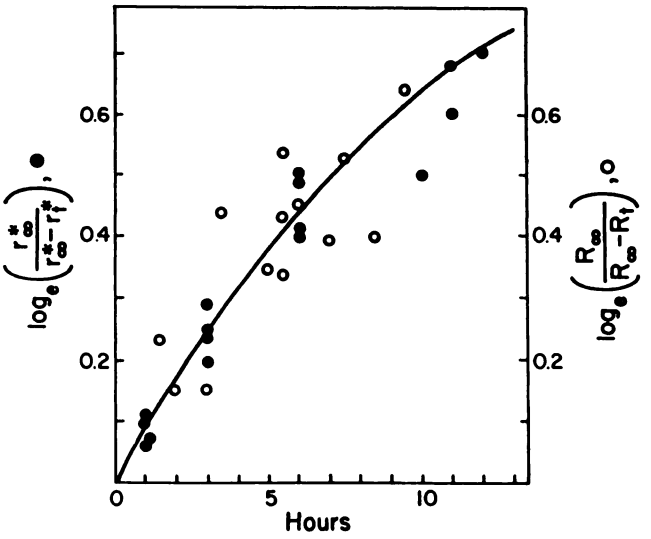

Fig. 5. Comparison of the kinetics of GABA-R recovery and degradation. Cells were subjected to the photoinactivation procedure and allowed to recover as described in Experimental Procedures. At the indicated times, specific reversible binding was determined (○). The *ordinate* shows the logarithmic transformation of the binding data, where R_t = binding at time t after photoinactivation and R_∞ = binding in control (noninactivated) cultures. Degradation data (●) were taken from Fig. 1. Line is theoretical exponential fit to data by nonlinear regression.

20 μg/ml cycloheximide. As shown in Table 8, cycloheximide inhibits by 97% the incorporation of radioactivity into TCA-precipitable material (mean of two experiments, five dishes/point). The effects of cycloheximide are reversible; when cycloheximide is washed out 1 hr before incubation with [^{35}S]methionine, incorporation returns to 92% of control (Table 8). The inhibition of receptor recovery (after photoinactivation) by the reversible protein synthesis inhibitors cycloheximide and puromycin suggest that receptor recovery requires *de novo* receptor synthesis. Alternatively, reappearance may result from synthesis of short-lived proteins that mediate processing of receptor precursors or unmasking of sequestered sites.

To determine whether the rapid rate of GABA-R turnover is due to photochemical damage of cell membranes, the following three experiments were performed. Two sets of sister cultures were incubated for 24 hr with [^{35}S]methionine and washed, and then one set was irradiated with UV light in the presence of 100 nM unlabeled flunitrazepam (photoinactivation). Protein degradation was followed for 48 hr, and no difference was observed between control and inactivated cultures at each of eight times examined (data not shown). Secondly, protein synthesis was unaffected when cultures were photoinactivated and protein synthesis was assayed either immediately or after a 24-hr recovery period (Table 9). In a third experiment, one of two sets of cultures was photoinactivated, and then both sets were washed and returned to the incubator. After 24 hr, neurons were counted (three dishes, 14 high power fields/dish) and no difference was observed between control (23 ± 4 cells/field) and photoinactivated (20 ± 2 cells/field), where values represent means ± SE.

The experiments described above demonstrate that photoaffinity labeling does not perturb general cellular function. To determine whether photolabeling selectively alters the kinetics of GABA-R degradation, the kinetics of receptor degradation and reappearance were compared. Fig. 5 demonstrates that the early rate of GABA-R reappearance agrees closely with the kinetics of GABA-R degradation, indicating that photolabeling does not measurably alter the kinetics of receptor degradation (see Appendix and Discussion).

Discussion

The phenomenon of receptor degradation can be thought of as a potential locus for the metabolic regulation of receptor number by the cell. But there is, at present, no all-inclusive model that satisfactorily explains the mechanism of neurotransmitter receptor degradation. Thus, whereas the nicotinic acetylcholine receptor of cultured skeletal muscle (29) and the prolactin receptor of mammary gland cells (34) are degraded lysosomally, it is impossible to predict how the neuronal GABA/benzodiazepine receptor will be degraded. For example, it is not yet known whether and how cell shape affects receptor degradation; the complex, extended geometry of the neuron is polarized, in that most synaptic contacts are located on dendrites, and few if any lysosomes can be found in dendrites under normal conditions.

Flunitrazepam as a photoaffinity label for the GABA-

R on living neurons in cultures. The stability of the bond between receptor and photoaffinity label is critical to the interpretation of these experiments. Reversal of the photolabeling reaction would result in the release of radioactivity into the medium, resembling receptor degradation. Similarly, if unlabeled flunitrazepam could dissociate from the GABA-R after photoinactivation, a time-dependent increase in the reversible binding of [^{3}H]flunitrazepam would result, resembling the appearance of new receptors. The following results show that the bond is stable over the time course of the turnover experiments. 1) The photolabeled receptor-ligand complex is stable in SDS containing 2-mercaptoethanol, as demonstrated by the ability to recover about 90% of the specific irreversible binding in the 48,000 and 51,000 bands after SDS-PAGE (24, 25). 2) Greater than 90% of the specifically bound radioactivity remains associated with [^{3}H]flunitrazepam-photolabeled membranes after dialysis in Triton X-100 (25). 3) After photolabeling, the radioactivity released into the medium does not comigrate with authentic flunitrazepam on thin layer chromatography (25). 4) The reappearance of reversible [^{3}H]flunitrazepam binding after photoinactivation is inhibited by cycloheximide and puromycin.

The specificity of the reaction for the benzodiazepine-sensitive modulator site on the functional GABA-R is indicated by the following observations. 1) Photoinactivation has no effect on the reversible binding of ligands for the GABA binding site, opiate receptor, or muscarinic receptor. 2) Photolabeling of the 48,000 and 51,000 bands is completely inhibited by 1 mM flurazepam. Additionally, no labeled bands are observed when cell cultures are photoinactivated before photolabeling (25). 3) Photolabeling favors neurons over the non-neuronal cells that coexist with neurons, consistent with the resistance of C6 glioma cells to photolabeling (35). 4) After photoinactivation, reversible binding and benzodiazepine-induced potentiation of GABA responses decrease to a similar degree (21, 25).

Additionally, photolabeling has no measurable effect on protein degradation, protein synthesis, cell number or morphology, nor on electrophysiological parameters such as membrane resting potential, input resistance, or GABA-induced conductance (21, 25).

Kinetics of GABA-R degradation and synthesis. When receptor is labeled irreversibly by [^{3}H]flunitrazepam and the cells are returned to the incubator, receptor degradation can be followed by monitoring either the decrease in cell-associated radioactivity or the release of radioactivity into the medium. The efflux of radioactivity corresponds to the disappearance of radioactivity in the 48,000 and 51,000 bands (25). Seventy percent of the released radioactivity is soluble in TCA and elutes after the total column volume on a Sephadex G-25 column (36), indicating that the release of radioactivity cannot be explained by dissociation of intact receptor into the medium. GABA-R degradation proceeds as a two-component time course in which 42% of the receptors are degraded with a fast half-time of 3.8 hr, whereas 58% of the receptors are degraded 8-fold more slowly. Biphasic degradation kinetics suggest the existence of receptor subtypes that are turned over at different rates. Importantly, the observation that the 48,000 and 51,000 species decrease proportionately (36), as do surface and internal sites (37), indicates that neither of these forms of molecular heterogeneity account for the biphasic degradation kinetics. Rather, they may result from heterogeneity of neuronal cell type or, by analogy with the nicotinic acetylcholine receptor in skeletal muscle, the presence of synaptic and extrasynaptic receptors (38). If the receptor exists in more than one state, then all such hypothetical states must undergo irreversible labeling at the same rate, as demonstrated by the linear first-order kinetics of photoinactivation. However, an initially heterogeneous population of binding sites could yield linear inactivation kinetics due to complex time-dependent rate changes induced by modifiers (39).

The biphasic degradation kinetics of the GABA-R are unusual in that monophasic kinetics have been observed for other neurotransmitter receptors, such as the adrenergic receptor on glandular tissue (40) and the nicotinic acetylcholine receptor on skeletal muscles in culture (41, 42), as well as for hormone receptors, such as the insulin receptor in hepatocytes (43) and adipocytes (4) and the epidermal growth factor receptor on fibroblasts and epidermoid carcinoma cells (6). We cannot say whether the kinetics of GABA-R degradation reflect the overall rate of neuronal membrane degradation or are unique to the GABA-R. In this regard, the kinetics of photolabeled receptor degradation are consistent with the kinetics of overall protein turnover, although the relative proportion of rapidly to slowly degrading receptor is greater than average.

Interestingly, the rapid phase of GABA-R degradation ($t_{1/2}$ = 3.8 hr) is similar to the rates of degradation found for other types of receptors during "down regulation" (6, 44, 45). Theory predicts that the kinetics of receptor recovery are determined by the rate constant(s) of degradation (46). Whereas degradation experiments reflect the degradation rate constant of photolabeled receptor, recovery experiments reflect the degradation rate constant of native receptor. If photolabeling alters the rate constant for degradation, the kinetics of degradation should not agree with the kinetics of recovery. Based on the kinetics of recovery, recovery would be expected to be 65% completed by 24 hr, and because 75% of the reversible binding is blocked initially, the binding at 24 hr should be 25% + (0.65 × 75%) = 74% of control. However, accumulation of GABA-R to 126% of control levels (see Results) would increase the apparent recovery rate above that predicted from degradation experiments. This is similar to the degree of receptor accumulation that has been observed for the acetylcholine receptor in skeletal muscle cultures (41).

To reduce the effect of receptor accumulation, we studied the early rate of receptor reappearance. We previously demonstrated that recovery of reversible binding corresponds to reappearance of the 48,000 and 51,000 bands (25). Fig. 5 demonstrates that the experimentally determined initial rate of GABA-R recovery agrees closely with the experimentally determined rate of GABA-R degradation. The rate of receptor synthesis, V_{syn} = 33 fmol/mg/hr, calculated from the kinetics of degradation is probably within error of the value (24 fmol/mg/hr) determined from the initial rate of GABA-R replacement (6%/hr), where both values are based on B_{max} = 400 fmol/mg for reversible binding to culture homogenates. These results indicate that the kinetics of disappearance of photolabeled receptor accurately reflect GABA-R degradation. Down regulation of the $GABA_A$/benzodiazepine receptor in rat brain cultures upon chronic treatment with GABA is reversed upon removal of GABA, and binding returns to near control within 24 hr (47), in good agreement with our results.

Mechanism of GABA-R degradation. By directly labeling the benzodiazepine-sensitive modulator site with [^{3}H]flunitra-

zepam, we report a first step toward understanding the mechanism of GABA/benzodiazepine receptor degradation. The fast phase of degradation is strongly dependent on temperature, exhibiting an apparent energy of activation of approximately 25 kcal/mol, similar to the value obtained for degradation of proteins microinjected into HeLa cells (48), for the ATP-dependent degradation of ubiquitin-lysozyme conjugates in reticulocyte lysates (49), and for degradation of the acetylcholine receptor in skeletal muscle cultures (29). Degradation is slowed by inhibitors of energy production to a degree similar in magnitude to that observed for degradation of cellular protein (50) or of the acetylcholine receptor in skeletal muscle cultures (29). However, it is not yet known whether degradation has a direct dependence on energy, or whether the slowing of degradation in the presence of the inhibitors represents an adaptive response to the depletion of energy stores in the cell or to a decrease in the rate of efflux of degradation products.

Lysosomes are an important site of protein degradation and previous studies with a variety of non-neuronal cells suggest that they are involved in the degradation of membrane proteins. Thus, it was of interest to determine whether the GABA-R is degraded in lysosomes. As little is known about protein degradation in neurons, we investigated the degradation of general cellular protein for comparison.

Whereas the degradation of GABA-R is not altered by medium lacking serum and embryo extract, the degradation of overall cellular protein is enhanced under such conditions. Interestingly, protein degradation in complete medium and under step-down conditions is inhibited to a similar degree (approximately 30%; Table 4) by methylamine, suggesting that step-down medium activates both lysosomal and nonlysosomal proteolytic systems. Moreover, the rapid phase of GABA-R degradation is not affected by lysosomotropic agents, suggesting that degradation proceeds through a nonlysosomal pathway in embryonic brain neurons maintained in primary monolayer cell cultures.

It is possible that the loss of radioactivity from [^{3}H]flunitrazepam-photolabeled neurons results from cleavage of the ligand binding domain on the cell surface (24), presumably by a protease in the plasmalemma. The fact that degradation occurs normally in the absence of serum and embryo extract indicates that proteolysis is cell mediated, although the possibility that a very active protease is secreted by the cultures cannot be excluded. If surface receptor were trimmed in this way, the unlabeled portion of the receptor could be degraded in lysosomes. Moreover, the possibility that native, nonphotolabeled receptor is entirely degraded in lysosomes cannot be excluded.

But precedent for nonlysosomal degradation of hormone and neurotransmitter receptors can be found from studies of non-neuronal and transformed cell lines; the insulin receptor of hepatocytes is not degraded lysosomally (51), and protein kinase C and calcium have been implicated in the degradation of the muscarinic acetylcholine receptor of neuroblastoma cells (52, 53), consistent with degradation by a nonlysosomal calcium-sensitive protease(s). Additionally, calpain may mediate the regulation of glutamate receptors during long term potentiation (2). The occurrence of apparent nonlysosomal degradation of receptors in three to four separate receptor systems in different cell types suggests that this phenomenon may represent a generally important mechanism for the degradation of neurotransmitter and hormone receptors.

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Appendix

If it is assumed that the concentration of receptors in the cell at any time ($[R]_t$) is governed by the zero-order rate of receptor synthesis (V_{syn}) and by the first-order rate of degradation ($V_{deg} = k[R]_t$) (46), where k is the degradation rate constant, then the rate of change in $[R]_t$ will be due to the difference between the rates of synthesis and degradation:

$$d[R]_t/dt = V_{syn} - k[R]_t, \quad (1)$$

which upon integration yields

$$[R]_t = [R]_\infty (1 - e^{-kt}) \quad (2)$$

In the steady state (i.e., as $t \rightarrow \infty$), the concentration of receptors is constant ($d[R]/dt = 0$), and thus $V_{syn} = k[R]$.

We evaluated Eq. 1 experimentally by determining the rate of receptor reappearance after photoinactivation and the kinetics of disappearance of photolabeled receptor. The first-order rate equation for degradation of photolabeled receptor is given by

$$-d[R^*]_t/dt = k^*[R^*]_0 \quad (3)$$

where $[R^*]_0$ is the initial concentration of labeled receptors, $[R^*]_t$ is the concentration remaining at time t after photolabeling, and k^* is the degradation rate constant for photolabeled receptor. Integration of this equation yields

$$[R^*]_t = [R^*]_0 e^{-k^*t} \quad (4)$$

The amount of radioactivity released into the medium at any time ($[r^*]_t$) is given by Eq. 5.

$$[r^*]_t = [r^*]_\infty (1 - e^{-kt}) \quad (5)$$

where $[r^*]_t = [R^*]_0 - [R^*]_t$ and $[r^*]_\infty = [R^*]_0$. The logarithmic expression of Eq. 5 is

$$\log_e[([r^*]_\infty - [r^*]_t)/[r^*]_\infty] = -k^*t \quad (6)$$

Thus, a plot of the logarithm (base e) of the fractional release of radioactivity against time should give a straight line with a slope of $-k^*$. As described in results, the experimental data were inconsistent with a single exponential fit as described in Eqs. 5 and 6 but could be described by a model in which two independent sites are labeled:

$$[r^*]_t = [r_1{}^*]_\infty (1 - e^{-k_1t}) + [r_2{}^*]_\infty (1 - e^{-k_2t}) \quad (7)$$

where R_1 is degraded more rapidly than R_2 (see Results for experimentally determined values).

After photoinactivation, newly synthesized receptors are not irreversibly bound to flunitrazepam. Thus, the rate of receptor appearance is described by Eq. 2. As shown in Fig. 5, the time course of receptor reappearance agrees well with the kinetics of degradation of photolabeled receptor, indicating that photolabeled receptor is degraded at the same rate as native receptor ($k = k^*$). Thus

$$[R]_t = [R]_\infty [0.42 (1 - e^{-0.182t}) + 0.58 (1 - e^{-0.022t})] \quad (8)$$

is the complete rate equation describing the kinetics of receptor turnover.

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