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Identification of an Intracellular Pool of γ-Aminobutyric Acid$_A$/Benzodiazepine Receptors en Route to the Cell Surface of Brain Neurons in Culture

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SUMMARY

We have previously shown that approximately 20% of the γ-aminobutyric acid (GABA)$_A$/benzodiazepine receptors in intact neurons are intracellular [*J. Neurosci.* 6:2857–2863 (1986); *Mol. Pharmacol.*, 35: 75–84], but the nature of this pool remained unknown. In this report, we describe the synthesis, appearance in the plasma membrane, and degradation of the GABA$_A$/benzodiazepine receptor complex in nerve cells derived from embryonic chick brain and grown in primary monolayer cell culture. Irreversible photoaffinity labeling of the benzodiazepine-sensitive modulator site on the GABA$_A$/benzodiazepine receptor using [^{3}H]flunitrazepam as a permeable probe was used in conjunction with exhaustive trypsinization of intact cells and competition binding using Ro7-0213 (a benzodiazepine bearing a charged quarternary ammonium moiety). Newly synthesized intracellular receptors that are transported to the surface membrane comprise at most only 4% of total receptors and up to one fifth of the steady state intracellular pool. The kinetics of receptor recovery after photoaffinity blockade are consistent with a model in which newly synthesized receptors first appear within the cell and then undergo intracellular transit before appearing on the cell surface. This suggests a "precursor-product" relationship between newly synthesized intracellular receptors and surface receptors. However, the majority of intracellular receptors are not transported to the cell surface and may represent a "nonprecursor" pool. The kinetics of degradation for intracellular receptors are consistent with the overall kinetics for total (surface plus intracellular) receptors. Intracellular and surface receptors are pharmacologically similar with regard to their sensitivity to methyl-β-carboline-3-carboxylate binding and their affinity for flunitrazepam. Degradation of the individual receptor subunits (with apparent molecular weights of 51,000 and 48,000) was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and both photoaffinity-labeled subunits degrade with indistinguishable biphasic kinetics. From these results, and the results of our previous studies, we propose a minimal model describing the dynamic cellular pathway for GABA$_A$/benzodiazepine receptor metabolism in central nervous system neurons.

The neuron synthesizes about 10^5 protein molecules per minute but little is known about the cellular mechanisms involved in neurotransmitter receptor assembly, targeting, and turnover in the CNS. The GABA receptor is a large multimeric protein that contains binding sites for GABA and benzodiazepines as well as diverse pharmacological agents such as barbiturates. We have previously described the turnover kinetics of the GABA-R in embryonic chick brain cell cultures, using [^{3}H] flunitrazepam as an irreversible photoaffinity label for the benzodiazepine-sensitive modulator site located on the functional GABA-R (1). The photolabeled receptor complex is degraded through an energy-dependent nonlysosomal mechanism (2). Moreover, the sensitivity of surface GABA-Rs to trypsin attack can be used to determine the fraction of the receptors that are located on the cell surface (3, 4). A total of 80% of the GABA-Rs are sensitive to trypsin cleavage and are cell surface receptors, whereas 20% of the receptors are insensitive to trypsin and are intracellular and/or membrane sequestered.

We have previously reported that benzodiazepine binding sites located on surface receptors face extracellularly and can be separated into two populations based upon their differential sensitivity to inactivation by trypsin (3, 4). Whereas 75% of the surface sites are inactivated by trypsin, 25% are spared and are associated with a cleaved fragment of the receptor (M_r 24,000), that spans the plasma membrane (xRF24). Thus, 40%

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GABA-R, GABA$_A$/benzodiazepine receptor complex; PBSS, phosphate-buffered salt solution; Ro7-0213, quaternized derivative of the benzodiazepine diazepam; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CNS, central nervous system; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; xRF24, trypsin-generated transmembrane fragment of M_r = 24,000.

of total cellular GABA and benzodiazepine binding sites are resistant to trypsin attack.[2]

The present study focuses on two related aspects of GABA-R metabolism, the incorporation of newly synthesized receptor into the surface membrane and the identification and functional significance of the observed intracellular pool of GABA-Rs. We provide evidence that newly synthesized receptors pass through a trypsin-resistant intracellular transport pool before incorporation into the nerve cell plasma membrane. Strikingly, most of the intracellular receptors are not precursors of cell surface receptors and, thus, are not destined for incorporation into a pool of constitutively recycled membrane vesicles. By utilizing Ro7-0213 (a benzodiazepine that bears a quaternary ammonium moiety) as a probe for surface receptors, we determined the rates for the degradation of intracellular GABA-Rs. These rates were found to be consistent with those of the total population of GABA-Rs. This study is an important step towards understanding the cellular pathways for the synthesis, assembly, and degradation of the functional $GABA_A$/benzodiazepine receptor in the CNS.

Materials and Methods

The benzodiazepines flunitrazepam, flurazepam, and Ro7-0213, as well as the methyl derivative of β-carboline-3-carboxylate, were kindly provided by Dr. P. Sorter of F. Hoffman-LaRoche (Nutley, NJ). All other compounds were obtained from commercial suppliers.

Primary brain cell cultures were prepared from 7-day chick embryos, as described previously (5, 6). Cells were maintained in culture for 1 week before use.

To measure the reversible binding of [^{3}H]flunitrazepam, aliquots of membrane homogenates prepared from intact cell cultures were incubated with 5 nM [^{3}H]flunitrazepam (80–86 Ci/mmol; New England Nuclear, Boston, MA) for 45 min at 4° in PBSS (pH 7.4). The incubations were terminated by filtration through Whatman GF/B glass fiber filters, which were then washed four times with a total of 20 ml of PBSS. The radioactivity associated with the filters was determined by liquid scintillation counting.

Irreversible photoaffinity labeling of benzodiazepine binding sites in intact brain cultures was carried out according to procedures outlined previously (1–4). Briefly, cultures were rinsed two times with ice-cold PBSS, equilibrated with 5 nM [^{3}H]flunitrazepam, and then irradiated with long-wave UV light for 45 min at 4° unless otherwise indicated. Intact cells were washed five times with ice-cold PBSS, scraped from culture dishes, homogenized in buffer, and centrifuged (30,000 × g for 20 min). The resulting pellets were resuspended in 1 mM flurazepam in PBSS and incubated 45 min, and aliquots either were used to measure irreversible cell-associated radioactivity by filtration assay or were solubilized for SDS-PAGE.

To photoaffinity label intracellular benzodiazepine binding sites, cultures were incubated with 5 nM [^{3}H]flunitrazepam in the presence of 30 μM Ro7-0213 for 45 min, irradiated with UV light for 5–10 min, and processed as described above. Photoaffinity labeling of benzodiazepine receptors with [^{3}H]flunitrazepam is an irreversible reaction. If given a long enough time, the reaction will go to completion and [^{3}H]flunitrazepam will label surface receptors even in the presence of Ro7-0213. Thus, to specifically label the intracellular pool of receptors, cells were irradiated for only 5–10 min. This is a kinetic rather than an equilibrium experiment.

To study GABA-R degradation, cells were photoaffinity labeled with 5 nM [^{3}H]flunitrazepam and washed, fresh medium was added, and the cultures were returned to the incubator for varying periods of time, then processed for either SDS-PAGE or filtration assay.

For photoaffinity blockade and recovery experiments, cultures were incubated with 100 nM unlabeled flunitrazepam (30 min at 4°) followed by exposure to UV light (45 min at 4°). Cells were washed five times with ice-cold PBSS and either used immediately or returned to the incubator with fresh medium. Control cultures were incubated with 100 nM flunitrazepam but were not irradiated with UV light.

In all experiments, 1 mM flurazepam was used to determine nonspecific binding. This was subtracted from total binding to yield the specific component.

SDS-PAGE was performed as previously described (1, 3). The amount of radioactivity associated with the separated proteins was determined by cutting each gel lane (approximately 90 mm) into 1 mm slices, dissolving the slices with 0.5 ml of 50% hydrogen peroxide overnight (43°), and determining the radioactivity associated with each slice by liquid scintillation counting. When photoaffinity labeling is performed in the presence of a variety of protease inhibitors, both the M_r = 51,000 and 48,000 bands are observed. In addition, both bands are stable to reelectrophoresis. These results suggest that the observation of two labeled bands is not a result of proteolysis.

Intact cells were trypsinized as outlined previously (3). Briefly, cells were incubated with trypsin (0.5 mg/ml) in a HEPES buffer for 90 min at 37°. To terminate trypsin activity, cells were placed on ice and soybean trypsin inhibitor (0.5 mg/ml, final concentration) was added. Cells were scraped from culture dishes, homogenized, and centrifuged (30,000 × g, for 20 min). The resulting pellets were resuspended in PBSS and the reversible binding of [^{3}H]flunitrazepam was determined as described above.

Results

Characterization of intracellular GABA-Rs. Competition binding curves for displacement of [^{3}H]flunitrazepam binding by β-carbolines are characterized by Hill coefficients of less than 1, suggesting that there are two pharmacologically distinct benzodiazepine binding subtypes, BZ_1 and BZ_2 (7, 8). To determine whether exhaustive trypsinization of intact cells selectively inactivates either of these binding sites, β-carboline binding was examined both before and after trypsin treatment of intact cells. After trypsinization, the Hill coefficient of displacement of [^{3}H]flunitrazepam binding by methyl-β-carboline-3-carboxylate is unchanged (Table 1), indicating that the benzodiazepine binding sites associated with intracellular GABA-Rs and the trypsin-generated transmembrane receptor fragment xRF24 consist of both BZ_1 and BZ_2 sites.

Evidence for an intracellular pool of "nonprecursor" GABA-Rs. To determine whether internal receptors are precursors to cell-surface receptors, we monitored the trypsin sensitivity of photoaffinity-labeled intracellular benzodiazepine binding sites after the treated cells were returned to the incu-

TABLE 1

Trypsin inactivates both BZ_1 and BZ_2 benzodiazepine binding site subtypes

The displacement of [^{3}H]flunitrazepam binding by methyl-β-carboline-3-carboxylate to homogenates prepared from untreated (control) and trypsin-treated brain cells was measured. Cells were incubated in the absence (control) or presence of 0.5 mg/ml trypsin for 90 min at 37° in HEPES buffer. Binding assays were performed as described in Materials and Methods using 5 nM [^{3}H]flunitrazepam. Values represent the calculated Hill coefficients ± standard errors. Linear regression coefficients for Hill plots averaged 0.981 ± 0.004. Numbers in parentheses represent the number of individual experiments.

Treatment	Hill Coefficients
Control	−0.65 ± 0.05 (2)
Trypsin	−0.53 ± 0.01 (2)

[2] Half of the trypsin-resistant benzodiazepine binding sites (20% of total) are associated with uncleaved intracellular and/or membrane sequestered receptors, whereas the remaining sites (20% of total, 25% of surface) are associated with the trypsin-cleaved receptor fragment.

bator for 4 or 8 hr. Immediately after the selective photolabeling of intracellular sites, virtually all of these sites remain resistant to attack by extracellular trypsin (Table 2). Significantly, there is little increase in the trypsin sensitivity with time, suggesting that most (at least 80%) of the photolabeled intracellular sites are not transported rapidly to the cell surface.

GABA-R biosynthesis and incorporation into the plasma membrane. GABA-R synthesis in intact brain cell cultures can be measured by photoaffinity blocking existing benzodiazepine binding sites with unlabeled flunitrazepam and at various times thereafter monitoring the recovery of the reversible binding of [^{3}H]flunitrazepam (1, 2). Although maximum irreversible binding of flunitrazepam is typically only 25% of maximum reversible binding, the remaining reversible binding sites are converted to much lower affinity (9–11). Thus, by examining the recovery of the reversible binding of 5 nM [^{3}H]flunitrazepam to high affinity binding sites after photoaffinity blockade, the synthesis of new receptors can be monitored.

Initially, the rate of receptor recovery was determined. Cells were photoaffinity blocked and allowed to recover for various times at 37°. Immediately after blockade, the reversible binding of 5 nM [^{3}H]flunitrazepam is 16 ± 1% (13 experiments) of control. Thereafter, [^{3}H]flunitrazepam binding increases in a time-dependent fashion with an initial rate, expressed as a percent of control binding, of 4%/hr (Fig. 1). After a 24-hr recovery period, [^{3}H]flunitrazepam binding returns to 90 ± 9% (three experiments) of control values.

To monitor GABA-R incorporation into the plasma membrane, we examined at various times the trypsin sensitivity of newly synthesized receptors. Immediately after photoaffinity blockade, approximately 40% of the binding sites for [^{3}H] flunitrazepam are resistant to exhaustive trypsin treatment in intact cells (Fig. 2). At 2 and 3 hr after photoaffinity blockade, the percentage of trypsin-resistant [^{3}H]flunitrazepam binding sites increases. After 4 hr, the percentage of trypsin-resistant [^{3}H]flunitrazepam binding sites returns to within error of control levels (approximately 40% resistant), suggesting a return to steady state. The initial rise and subsequent fall in the percentage of trypsin sensitivity would be expected if a pool of newly synthesized intracellular GABA-Rs were in transit to the cell surface.

Kinetics of intracellular GABA-R degradation. We have shown previously (1, 2), that GABA-R degradation is described by a two-site model in which 42% of the receptors are degraded with a half-time of 3.8 hr, whereas the remaining

TABLE 2

Trypsin resistance of photoaffinity-labeled intracellular GABA-Rs over time at 37°

Intact cells were photoaffinity labeled with 5 nM [^{3}H]flunitrazepam in the presence of 30 μM Ro7-0213 (to label only intracellular receptors) and returned to the incubator. At the indicated times, the cells were incubated in the presence or absence (control) of trypsin as described in Materials and Methods and the specific irreversible cell-associated radioactivity was determined. Values represent means ± standard errors of triplicate determinations, normalized to control. Numbers in parentheses represent the number of individual experiments.

Incubation Time	Trypsin Resistance of [^{3}H]Flunitrazepam Remaining Bound
hr	%
0	99 ± 6 (8)
4	94 ± 14 (3)
8	89 (1)

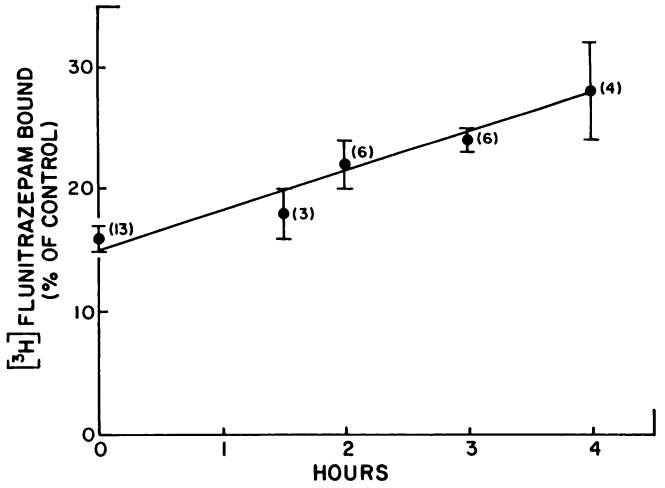

Fig. 1. Time course for recovery of [^{3}H]flunitrazepam binding after photoaffinity blockade. Cultures were photoaffinity blocked with 100 nM unlabeled flunitrazepam, washed, and returned to the incubator. At the indicated times, cells were scraped from culture dishes and homogenized and the specific reversible binding of 5 nM [^{3}H]flunitrazepam was measured as described in Materials and Methods. *Data points* represent the means ± standard errors of triplicate determinations, normalized to control. *Numbers in parentheses* represent the number of individual experiments.

sites are degraded more slowly, with a half-time of 32 hr. To determine the kinetics of intracellular GABA-R degradation, intact cells were irreversibly photoaffinity labeled with 5 nM [^{3}H]flunitrazepam in the presence of excess Ro7-0213 (a permanently charged benzodiazepine) to selectively label intracellular benzodiazepine binding sites (3). As shown in Fig. 3, the loss of specific irreversibly bound radioactivity from both intracellular and total GABA-Rs proceeds with a similar time-course ($t_{½}$ = 3.8 and 32 hr). This shows that the kinetics of degradation of intracellular receptors are the same as for the total population of receptors (surface plus intracellular).

The M_r = 51,000 and 48,000 species are degraded with similar rates. SDS-PAGE of radioactivity from cells photoaffinity labeled with [^{3}H]flunitrazepam reveals two labeled subunits with M_r = 51,000 and 48,000 (3, 8). By utilizing SDS-PAGE, the kinetics of degradation for the individual species were determined. Cells were photoaffinity labeled with [^{3}H]flunitrazepam and at various times thereafter subjected to SDS-PAGE. Fig. 4 shows that both labeled species disappear with similar kinetics, indicating that the biphasic kinetics of degradation do not result from differential degradation of these individual labeled subunits.

Discussion

At chemical synapses, neurotransmitters such as GABA alter conductance of the neuronal membrane through receptor-operated ion channels located within the cell surface membrane. Receptor regulation depends on the rates of receptor metabolism and on the partitioning and intracellular transport of receptor between surface and intracellular membranous compartments. Recently, it has been shown that the number of GABA-Rs is subject to down-regulation by chronic exposure to GABA agonists (12, 13), whereas the allosteric interactions between the GABA and benzodiazepine binding sites become uncoupled upon chronic exposure to homologous (benzodiazepine) and heterologous (methylxanthine) ligands (14, 15). The

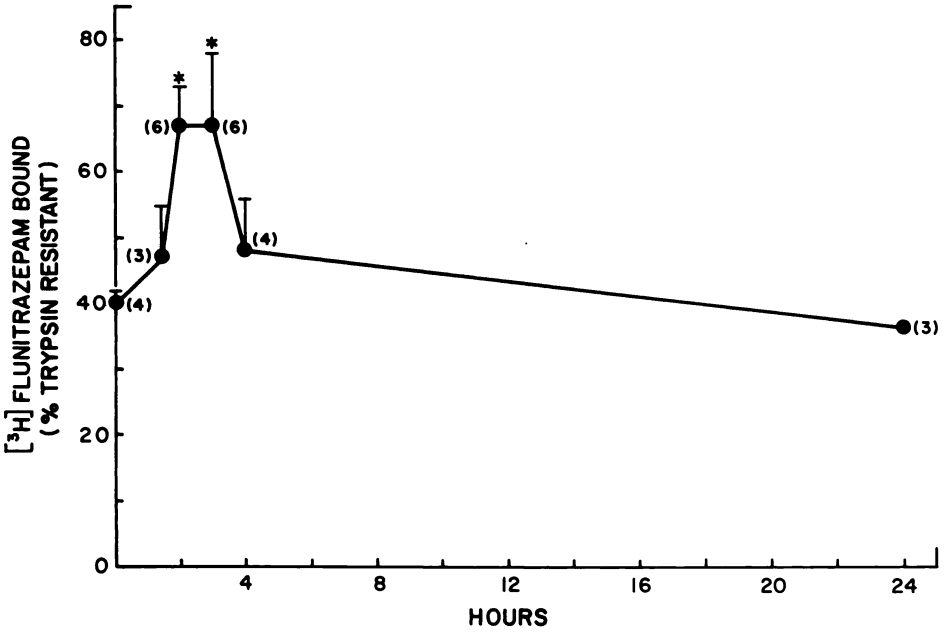

Fig. 2. Evidence for an intracellular pool of newly synthesized GABA-Rs in transit to the cell surface. Cultures were photoaffinity blocked with 100 nM unlabeled flunitrazepam, washed, and returned to the incubator. At the indicated times, intact cultures were incubated in the presence or absence (control) of trypsin as described in Materials and Methods. After trypsin treatment, cells were scraped from culture dishes, centrifuged, and resuspended in PBSS and the specific reversible binding of 5 nM [^{3}H]flunitrazepam was measured. *Data points* represent the means ± standard error of triplicate determinations, normalized to control. Number of individual experiments is indicated in parentheses. * $p < 0.005$.

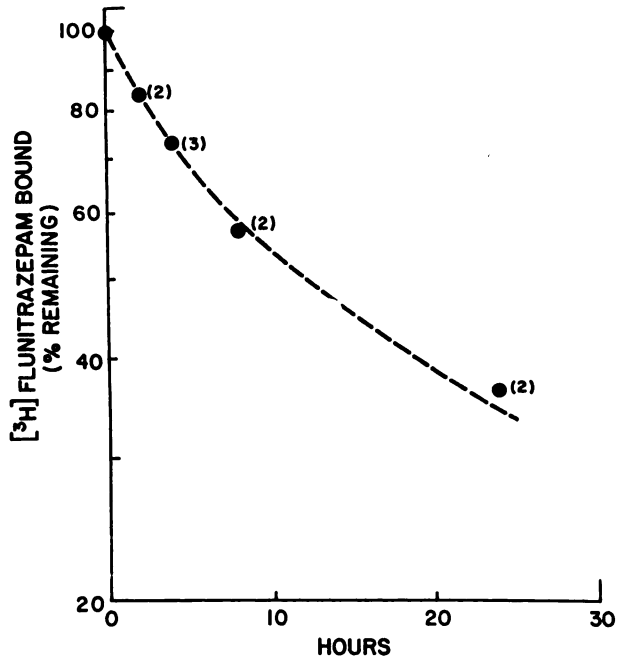

Fig. 3. Intracellular GABA-Rs degrade with biphasic kinetics similar to those of total (surface plus intracellular) receptors. Intact cells were photoaffinity labeled with 5 nM [^{3}H]flunitrazepam in the presence of 30 μM Ro7-0213 (to spare surface sites). Cells were washed and returned to the incubator for various times and then homogenized. The specific cell-associated radioactivity remaining at each of the time points was determined by filtration. The results are plotted semilogarithmically and each *data point* represents the mean of quadruplicate determinations. The *numbers in parentheses* represent the number of individual experiments. The *dashed line* represents a theoretical curve based on the biphasic degradation of total (surface plus intracellular) receptors ($t_{½}$ = 3.8 and 32 hr) as previously described by Borden *et al.* (1, 2).

major conclusions of this paper relate to understanding the cellular mechanisms involved in neurotransmitter receptor regulation.

We previously identified an intracellular pool of GABA-Rs in intact brain neurons (3, 4). The intracellular pool may consist of newly synthesized and/or internalized receptors or a pool of receptors that are distinct from surface GABA-Rs. We have measured directly the degradation of intracellular GABA-Rs as well as the synthesis and incorporation of the GABA-R into the surface plasmalemma.

The benzodiazepine binding sites associated with intracellular GABA-Rs are pharmacologically similar to the sites associated with surface receptors. The binding affinity for [^{3}H] flunitrazepam is unaltered after trypsin treatment and intracellular receptors contain the same M_r = 51,000 and 48,000 receptor subunits as total receptor (3). Moreover, the Hill coefficient for methyl-β-carboline-3-carboxylate displacement of [^{3}H]flunitrazepam binding is unchanged by trypsinization. Thus, surface trypsinization does not selectively destroy either BZ_1 or BZ_2 benzodiazepine binding site subtypes.

The majority of intracellular receptors do not appear to be en route to the cell surface. After a 4- or 8-hr incubation at 37°, less than 20% of the intracellular benzodiazepine binding sites become trypsin sensitive (Table 2), suggesting that most of the internal GABA-Rs are not rapidly transported to the cell surface and recycled. These receptors may represent a "nonprecursor" pool of receptors and/or GABA-Rs that are destined for degradation. Alternatively, it is possible that the photoaffinity-labeled receptor may not be capable of normal incorporation into the surface membrane. This seems unlikely, because photolabeling does not alter receptor number or degradation (2, 8).

The notion that neurotransmitter receptors may be located intracellularly but fail to be inserted in the plasmalemma is not restricted to the GABA-R. Intracellular nicotinic acetylcholine receptors constitute one fifth to two thirds of the total present in chick myotubes, BC3H-1 cells, or chick ciliary ganglion cells in culture (16–21). Interestingly, Stollberg and Berg (21) reported that only a small fraction (5–20%) of the intracellular nicotinic acetylcholine receptors are actually transported to the cell surface. These similar results in two separate receptor systems suggest that the presence of large nonprecursor intra-

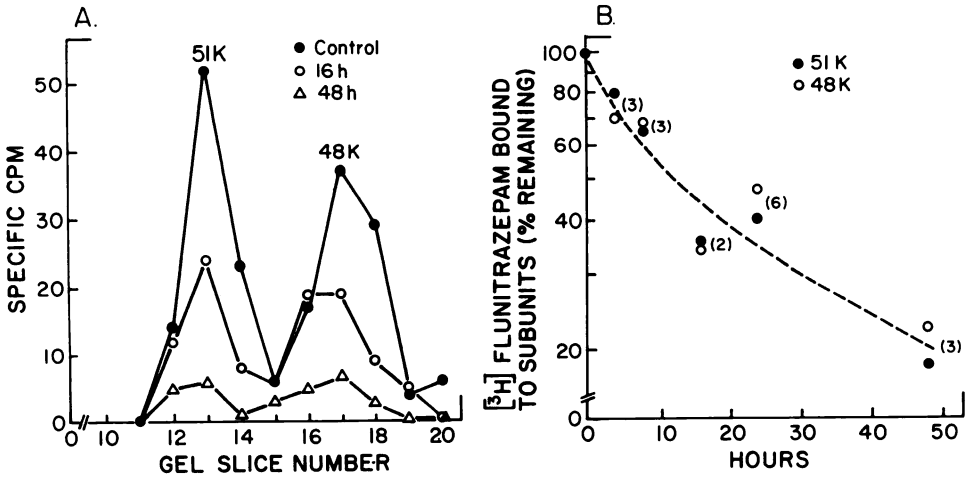

Fig. 4. The M_r = 51,000 and 48,000 benzodiazepine binding species degrade with similar rates and with biphasic kinetics. Cells were photoaffinity labeled with 5 nM [^{3}H]flunitrazepam, washed, and returned to the incubator. At various times SDS-PAGE was performed using 90-mm-long gels. Gels were then sliced into 1-mm segments and the radioactivity associated with the M_r = 51,000 and 48,000 species was determined as described in Materials and Methods. Each peak of radioactivity is about 3 mm wide. A, The distribution of radioactivity in the M_r = 51,000 and 48,000 peaks is shown immediately (control), and 16 and 24 hr after photoaffinity labeling. B, The radioactivity remaining associated with the M_r = 51,000 (●) and 48,000 (○) subunits is plotted semilogarithmically and is expressed as percentage of control. The *numbers in parentheses* are the number of individual experiments. The *dashed line* represents a theoretical curve of the degradation of GABA-Rs based on $t_{½}$ = 3.8 and 32 hr, as described by Borden *et al.* (1, 2).

cellular pools of receptor may be indicative of a cellular mechanism of general biological significance.

The result that intracellular GABA-Rs degrade with kinetics ($t_{½}$ = 3.8 and 32 hr) similar to those of total (surface plus intracellular) receptors provides additional support for the argument that the biosynthesis of intracellular and surface receptors is similar. Theory predicts that the kinetics of receptor synthesis are determined by the rate constant(s) of degradation (2, 22). As seen in Fig. 4, both the M_r = 51,000 and 48,000 subunits degrade with indistinguishable biphasic rates. Thus, the biphasic kinetics of GABA-R degradation cannot be explained by the existence of two pools of receptor (surface and intracellular) or by the presence of two receptor subunits.

To examine the fate of the internal pool of GABA-Rs, we monitored the trypsin sensitivity of newly synthesized receptors. This approach utilizes photoaffinity blockade of existing sites with unlabeled flunitrazepam, coupled with subsequent reversible [^{3}H]flunitrazepam binding to monitor the appearance of new sites (1, 2). The results of Fig. 2 show that photoaffinity blockade does not alter the fraction of the binding that is resistant to exhaustive trypsin attack. When cells are returned to the incubator after photoblockade of existing receptors, the percentage of trypsin-resistant [^{3}H]flunitrazepam binding sites increases transiently and then returns to steady state levels (Fig. 2). This observation is consistent with a kinetic model in which the rate of receptor synthesis exceeds the rate of transit to the cell surface. The transit time for insertion of newly synthesized receptor into the neuronal plasmalemma would then be from 3 to 4 hr. These results are in agreement with those obtained for the acetylcholine receptor of cultured chick myotubes (17, 18) and ciliary ganglion neurons (21), as well as for the insulin receptor of adipocytes (23). For these receptors, up to 3 hr is required to reach the plasma membrane after biosynthesis.

In summary, we propose a minimal model describing the cellular pathway of the GABA-R (Fig. 5). Of the total GABA-Rs in chick brain, 20% are intracellular. Of these, at most 20%

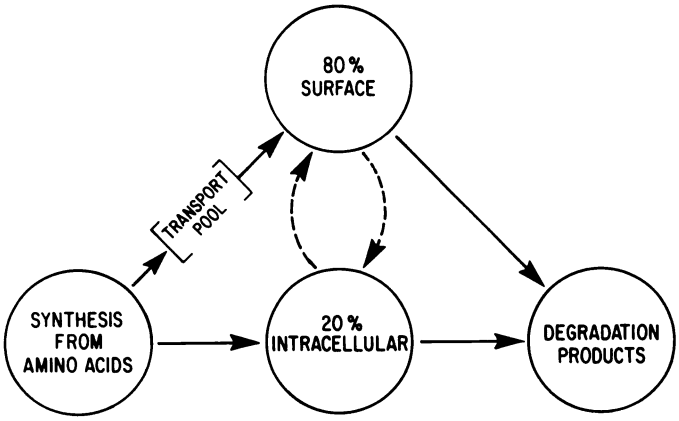

Fig. 5. Summary of the subcellular pools of GABA-R identified in embryonic brain cell culture. The results are consistent with a model in which newly synthesized receptor arrives at the cell surface via a small intracellular transport pool. Surface and internal intracellular receptors are degraded with similar rates. The *dashed lines* indicate potential routes for the slow transit of receptor. Photoaffinity-labeled receptor does not undergo recycling, on the time scale of minutes to several hours. The model is not intended to imply that receptors in transit to the cell surface do not first go to the internal pool and then to the surface or that the internal pool can not be derived from the surface pool.

are newly synthesized receptors in transit to the cell surface. The majority of the intracellular GABA-Rs are not destined for incorporation into cell surface membrane and may represent a nonprecursor pool of receptors and/or receptors destined for degradation. Both intracellular and surface GABA-Rs appear to be pharmacologically similar and exhibit similar degradation kinetics. By understanding the mechanisms of GABA-R synthesis, assembly, and degradation, the general principles that govern the regulation and turnover of neurotransmitter receptors in the CNS may be elucidated.

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