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Transmembrane Topology of the γ-Aminobutyric Acid$_A$/ Benzodiazepine Receptor: Subcellular Distribution and Allosteric Coupling Determined *In Situ*

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SUMMARY

The subcellular distribution, allosteric coupling, and topology of the γ-aminobutyric acid$_A$ (GABA)/benzodiazepine receptor were investigated *in situ*. We have shown previously that a transmembrane fragment (M_r = 24,000) of the benzodiazepine receptor remains in the plasma membrane after trypsin treatment of embryonic brain neurons maintained in primary monolayer cell culture. Here we report a study of the effects of exhaustive trypsinization on the binding of ligands to the GABA recognition site. Approximately 60% of the binding sites for [^{3}H]muscimol in intact cells are inactivated by extracellular trypsin and, therefore, are associated with cell surface GABA receptors, whereas 40% of the sites are trypsin resistant. GABA potentiates [^{3}H]flunitrazepam binding to intact cells and trypsin-treated intact cells. GABA-induced enhancement of [^{3}H]flunitrazepam binding to intact cells is eliminated when cell-surface benzodiazepine receptors are blocked with Ro7-0213 (a charged benzodiazepine), but some enhancement remains after extracellular trypsinization. This provides indirect evidence for the existence of a population of trypsin-resistant GABA recognition sites associated with cell-surface receptors and indicates that trypsin-resistant cell-surface GABA recognition sites are allosterically linked to trypsin-resistant cell-surface benzodiazepine recognition sites, which we have shown previously to be associated with the trypsin-generated M_r 24,000 transmembrane fragment. The results are discussed in terms of a model of the transmembrane topology of the GABA/benzodiazepine receptor.

A fundamental question in molecular neuroscience is the relationship of neurotransmitter receptor structure to receptor function at chemical synapses in the CNS. How does the nerve cell assemble neurotransmitter receptors and what is the role of neuromodulation in the control of synaptic activity? Most of what is known about the transmembrane topology and subcellular distribution of neurotransmitter receptor-gated ion channels is derived from studies of the nicotinic acetylcholine receptor-gated Na^+/K^+ channel of muscle. Much less is known about the GABA-R, an important inhibitory neurotransmitter receptor in the CNS (1, 2). The GABA-R is a receptor-effector system that contains an integral Cl^- channel and is modulated by a variety of centrally acting compounds such as barbiturates, benzodiazepines, and convulsants (3–8). From a variety of pharmacological and ligand binding studies (see Ref. 9 for review) it has been concluded that the binding sites for GABA agonists, benzodiazepines, β-carbolines, barbiturates, and picrotoxin are located on the same macromolecular protein complex (10–12).

We have shown previously (13), using exhaustive trypsinization of intact and saponin-permeabilized chick brain cell cultures, that it is possible to distinguish cell surface benzodiazepine binding sites from intracellular or membrane-sequestered sites. Approximately 80% of the [^{3}H]flunitrazepam binding sites of intact cells are present on the cell surface facing the extracellular medium, whereas the remaining 20% are inaccessible to trypsin or to Ro7-0213 (a membrane-impermeant benzodiazepine used to distinguish cell surface sites) and are presumed to be associated with intracellular and/or membrane-sequestered GABA-R. Surface benzodiazepine binding sites are differentially sensitive to trypsin in that 75% (of surface) are inactivated, whereas the remaining 25% retain binding activity and are found on SDS-PAGE to be associated with a trypsin-cleaved transmembrane receptor fragment with an approximate M_r of 24,000 (xRF24).

In the present study, we report the effects of trypsin treatment on the GABA recognition site. The results suggest that

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ABBREVIATIONS: CNS, central nervous system; GABA-R, γ-aminobutyric acid$_A$ receptor; GABA, γ-aminobutyric acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethy)-1-piperazinethanesulfonic acid; PBSS, phosphate-buffered Earle's balanced salt solution.

trypsin-resistant cell-surface GABA recognition sites are associated with the same population of receptors as trypsin-resistant cell-surface benzodiazepine recognition sites. The significance of these findings with respect to the transmembrane topology of the GABA-R and the locations of the GABA and benzodiazepine binding sites on the receptor complex is discussed.

Experimental Procedures

Materials. Bovine pancreatic trypsin and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). Dialysis tubing was purchased from Spectrum (Los Angeles, CA) and had a molecular weight cutoff of 15,000. All other chemicals were obtained from commercial sources. Benzodiazepines were a gift of Dr. P. Sorter of F. Hoffman-La Roche (Nutley, NJ). [*methyl*-^{3}H(N)]Muscimol (15.4 Ci/mmol) and [*methyl*-^{3}H]flunitrazepam (70–80 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The purity of the benzodiazepine Ro7-0213 was verified by thin layer chromatography. The chromatogram was developed with chloroform/methanol/acetic acid (2:1:0.02, v/v) and only one major spot was visualized with iodine vapor or UV light ($R_f = 0.2$).

Preparation of neuronal cultures. Primary brain cell cultures were prepared from 7-day chick embryos as described (14, 15) and were maintained for 6 to 8 days before use. To control the proliferation of nonneuronal cells, cytosine arabinoside (1 μM) was added 24 hr after plating. Cultures were fed with fresh medium on the following day and at 2–3-day intervals thereafter.

Trypsinization. Exhaustive trypsin treatment of intact brain cultures was carried out as described previously (13). Briefly, cells were washed with 25 mM HEPES buffer (pH 7.2, 270 mOsm) containing 5.4 mM KCl, 0.9 mM $CaCl_2$, 0.4 mM $MgSO_4$, and 190 mM sucrose and incubated in trypsin (0.5 mg/ml) for 90 min at 37° in the same buffer. After the incubation, cultures were placed on ice and soybean trypsin inhibitor was added (0.5 mg/ml, final concentration) to terminate trypsin activity. Cells were scraped from culture dishes, homogenized, and centrifuged at 30,000 × *g* for 20 min. The resulting pellets were resuspended in PBSS, (pH 7.4, 294 mOsm) containing 123 mM NaCl, 5.4 mM KCl, 11 mM NaH_2PO_4, 0.9 mM $CaCl_2$, 0.4 mM $MgSO_4$, and 22.2 mM glucose unless indicated otherwise. To permeabilize the plasmalemma of intact brain cells, cultures were incubated for 3 min with 0.5% (w/v) saponin in 20 mM sodium phosphate buffer (pH 7.2) containing 300 mM sucrose. Culture homogenates and saponin-permeabilized cells were trypsinized as outlined for intact cells.

Preparation of culture homogenates. To remove endogenous GABA, culture homogenates were extensively washed by dialysis before being assayed for either 1) [^{3}H]muscimol binding; 2) enhancement of benzodiazepine binding by GABA; or 3) enhancement of [^{3}H]muscimol binding by benzodiazepines. Membrane pellets were resuspended in 1 mM EDTA and 1 mM phenylmethylfluorosulfate and dialyzed against 25 mM potassium phosphate buffer (4°, 24 hr), with at least four changes of dialysis buffer. For experiments in which the reversible binding of [^{3}H]muscimol was to be measured, 50 mM Tris·HCl, pH 7.2, was used for the final buffer change. Dialyzed membranes were either used immediately or stored at −70° for later use.

[^{3}H]Muscimol binding to cellular homogenates. To measure GABA-R binding, aliquots of dialyzed culture membrane homogenate were incubated with [^{3}H]muscimol for 60 min at 4° in 50 mM Tris·HCl. The incubation was terminated by filtration through Whatman GF/B glass fiber filters, which were then washed three times with a total of 15 ml of buffer. The radioactivity associated with the filters was measured by liquid scintillation counting. Nonspecific binding was determined in the presence of 1 mM GABA and was subtracted from total binding to yield the specific component. To measure the effect of benzodiazepines on reversible binding of [^{3}H]muscimol, aliquots of dialyzed culture homogenates were incubated with 8 nM [^{3}H]muscimol in the presence and absence of various benzodiazepines for 20 min at 37° (16) and for 40 min at 4° in 50 mM Tris·HCl and then filtered as described above. Control binding of [^{3}H]muscimol determined in the absence of benzodiazepine by this method was equivalent to that determined with a single 60-min incubation at 4°.

[^{3}H]Flunitrazepam binding to intact cells. Irreversible photoaffinity labeling of intact cells was carried out as described previously (13, 14, 17). Cells were washed with PBSS and photoaffinity labeled by incubation with 1 nM [^{3}H]flunitrazepam (45 min, 4°, PBSS) followed by irradiation with long wavelength UV light (General Electric F40 BLB bulb) for 5–10 min at 4°. Cells were washed with cold PBSS, scraped from culture dishes, and homogenized in buffer. The homogenate was centrifuged at 30,000 × *g* for 20 min, the pellets were resuspended in PBSS containing 1 mM flurazepam and incubated 45 min at 4° to permit dissociation of reversibly bound [^{3}H]flunitrazepam, and irreversible cell-associated radioactivity was determined by filtration assay. Nonspecific photoaffinity labeling was determined by labeling in the presence of 1 μM flunitrazepam. To determine the effect of GABA on irreversible photoaffinity labeling, cultures were rinsed extensively with PBSS, incubated with 1 nM [^{3}H]flunitrazepam in the presence of 10 μM GABA (45 min, 4°, PBSS), and irradiated with UV light (5–10 min, 4°).

[^{3}H]Flunitrazepam binding to cellular homogenates. To measure reversible [^{3}H]flunitrazepam binding, aliquots of culture homogenates were incubated with 5 nM [^{3}H]flunitrazepam for 60 min at 4° in PBSS. The incubation was terminated by filtration through Whatman GF/B glass fiber filters, which were then washed four times with a total of 20 ml of buffer. Radioactivity remaining on the filters was determined by liquid scintillation counting. Flurazepam (1 mM) was used to determine nonspecific binding, which was subtracted from total binding to yield the specific component. To measure the effect of GABA on reversible binding of [^{3}H]flunitrazepam, aliquots of dialyzed culture homogenate were incubated with 0.75–1 nM [^{3}H]flunitrazepam in the presence of 10 μM GABA (60 min, 4°, PBSS) and filtered as described above. Protein was measured by the method of Lowry *et al.* (18).

Analysis of binding data. Estimates of K_D and B_{max} were derived from binding data by computer-aided nonlinear regression of untransformed data. Results were fitted to both one-site and two-site models. To determine whether the fit was significantly improved by the use of the two-site model, an F test was carried out on the residuals (19).

Results

Identification and characterization of [^{3}H]muscimol binding to the GABA-R. Fig. 1 shows the specific binding of [^{3}H]muscimol, a GABA-R agonist, to cellular homogenates. Results are consistent with a single binding site with a K_D of 28.8 ± 8 nM and a B_{max} of 0.25 ± 0.08 pmol/mg of protein (nine experiments). Fig. 2 shows the stereospecific displacement of specifically bound [^{3}H]muscimol by GABA-R agonists and antagonists, as well as other ligands. The relative potencies are as follows: muscimol > GABA > (+)-bicuculline > (−)-bicuculline methylbromide > β-alanine > strychnine > glycine. The potencies and rank order for inhibition of [^{3}H]muscimol binding in cell culture homogenates are in agreement with their reported potencies in displacing [^{3}H]GABA binding in mammalian brain homogenates (20, 21). All the benzodiazepines tested increased significantly the specific binding of [^{3}H]muscimol to cellular homogenates (Fig. 3), as expected for a functional GABA-R.

Effect of trypsin on [^{3}H]muscimol binding: evidence for a membrane-protected pool of GABA-Rs. Exhaustive trypsinization of intact cells reduced the subsequent reversible

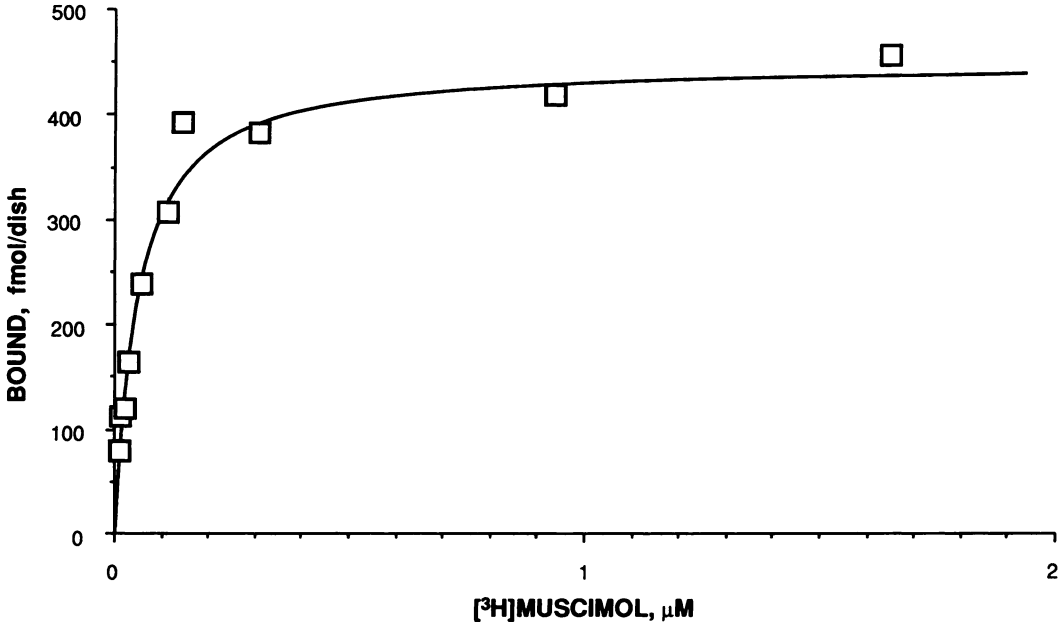

Fig. 1. [^{3}H]Muscimol binds to a single class of sites in homogenates of brain cell cultures. Saturation binding of [^{3}H]muscimol (7 nM–1.8 μM; nonspecific binding determined in 1 mM GABA) was performed as described in Experimental Procedures. *Points* are the means of triplicate determinations. Results are from a typical experiment, which was repeated nine times with similar results. The *solid line* is the theoretical curve of the best computer fit to a single site binding model, which for this particular experiment yielded a K_D of 47.6 nM. The fit was not significantly improved when a two-site model was used.

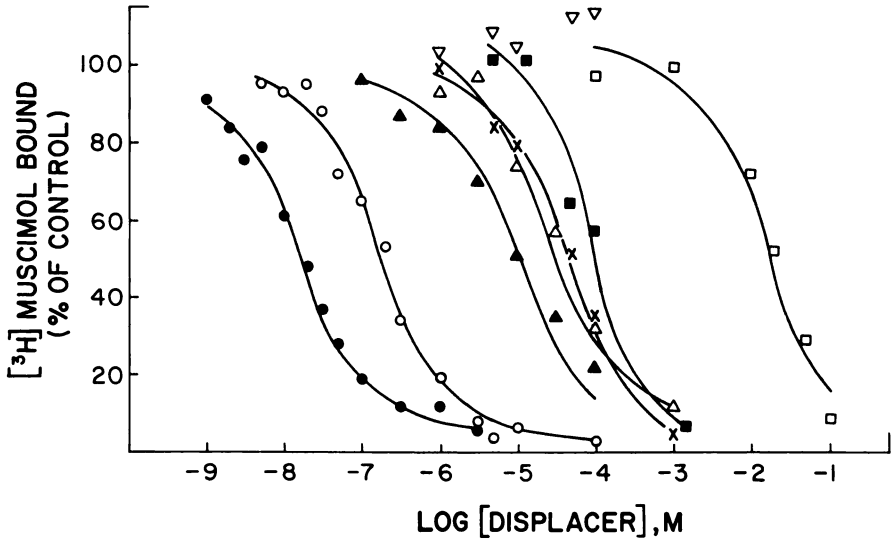

Fig. 2. [^{3}H]Muscimol binds to a stereospecific site with the pharmacology of a $GABA_A$ receptor. Displacement of [^{3}H]muscimol binding to homogenates prepared from brain cell cultures by various ligands is shown. Culture membranes were incubated with 8 nM [^{3}H]muscimol and various concentrations of muscimol (●), GABA (○), (+)-bicuculline (▲), (−)-bicuculline methylbromide (△), β-alanine (×), strychnine (■), glycine (□), and diaminobutyric acid (▽). Binding assays were performed as described in Experimental Procedures. Results are expressed as percent of [^{3}H]muscimol binding in the absence of competitor. Each *data point* is the mean of triplicate determinations.

binding of 10 nM [^{3}H]muscimol to dialyzed cellular homogenates to 42 ± 5% (eight experiments) of binding to dialyzed homogenates from nontrypsinized sister cultures. Thus, 58% of the binding is inactivated when intact cells are exposed to trypsin and then homogenized. To determine to what extent trypsin resistance reflects protection by the intact plasma membrane, cells were homogenized or permeabilized with the detergent saponin before trypsinization. When cells were disrupted by extensive homogenization (100 strokes of dounce) and then trypsinized, the fraction of trypsin-resistant [^{3}H]muscimol binding decreased to 16% (two experiments). Similarly, when cells were permeabilized with 0.5% saponin before trypsinization, the fraction of trypsin-resistant binding decreased to 10 ± 4% (three experiments; Fig. 4). The observation that disruption of the plasma membrane reduces the percentage of trypsin-resistant [^{3}H]muscimol binding suggests the presence of an internal and/or membrane-protected pool of receptors.

To determine whether the decrease in [^{3}H]muscimol binding after trypsin treatment is due to a loss of muscimol binding sites or to a change in binding affinity, saturation experiments using [^{3}H]muscimol were performed on homogenates prepared from trypsinized intact and saponin-permeabilized brain cells. Data from saturation binding experiments are consistent with a single class of GABA-R sites (Figs. 1 and 5). Binding affinity for muscimol is not significantly altered by trypsinization (K_D = 20.5 ± 5 nM, five experiments), indicating that the decrease in [^{3}H]muscimol binding after trypsinization reflects a real decrease in the number of binding sites (B_{max} = 44 ± 9% of control). Interestingly, this result is similar to our previous finding that 40% of the [^{3}H]flunitrazepam binding sites of intact cells are resistant to trypsin attack from the extracellular solution (13). We have since found that the proportion of protease-resistant [^{3}H]flunitrazepam binding sites in intact cells is similar for two other proteases, chymotrypsin and proteinase K (0.2 to 1 mg/ml).

Ro7-0213-sensitive [^{3}H]flunitrazepam binding sites

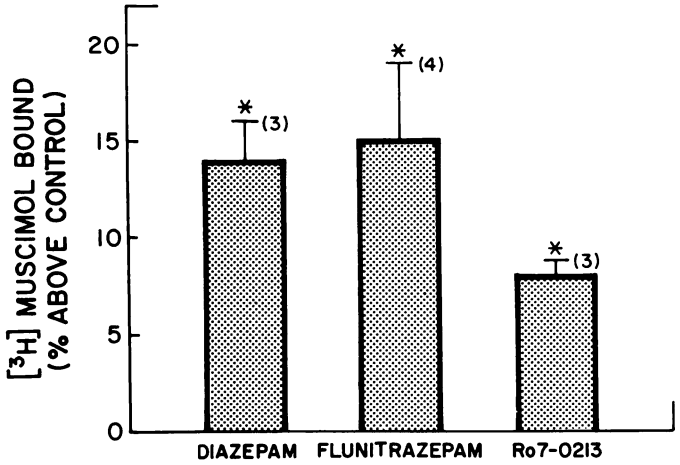

Fig. 3. Benzodiazepines potentiate [^{3}H]muscimol binding. The enhancement of specific binding of [^{3}H]muscimol (8 nM) to dialyzed culture membrane homogenates by diazepam (10 μM), flunitrazepam (1 μM), and Ro7-0213 (30 μM) was measured as described in Experimental Procedures. Results are mean values ± standard error from three or four experiments (each in quadruplicate) for the specific binding of [^{3}H]muscimol in the presence of added benzodiazepine, expressed as a per cent increase over [^{3}H]muscimol binding in the absence of benzodiazepine (control). *, increase in binding is statistically significant ($p < 0.05$).

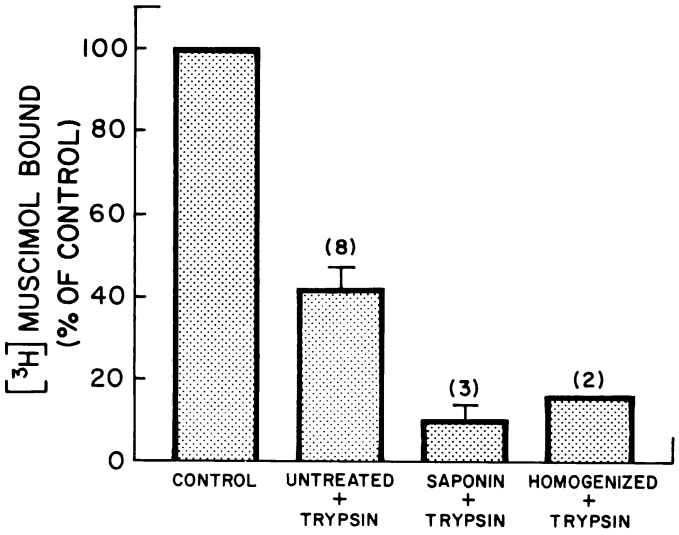

Fig. 4. Disruption of cells by saponin or by homogenization exposes previously inaccessible [^{3}H]muscimol binding sites to trypsin attack. Inactivation of the specific reversible binding of [^{3}H]muscimol by trypsinization of intact, saponin-permeabilized, and homogenized brain cell cultures is shown. Untreated and saponin-treated cells as well as homogenates prepared from intact cells were incubated in the absence (control) or presence of 0.5 mg/ml trypsin for 90 min at 37°. Cultures were then homogenized and dialyzed, and reversible binding of 10 nM [^{3}H]muscimol was measured as described in Experimental Procedures. Within an experiment, means were calculated from triplicate determinations and normalized to control. Numbers in parentheses represent the number of separate experiments.

of intact neurons are on the cell surface and are coupled allosterically to GABA recognition sites. Ro7-0213 is a quaternary ammonium derivative of the benzodiazepine diazepam that bears a full positive charge and is therefore expected to diffuse across the intact plasma membrane much more slowly than flunitrazepam, which is highly lipophilic. Ro7-0213, like diazepam and flunitrazepam, increases [^{3}H]muscimol binding

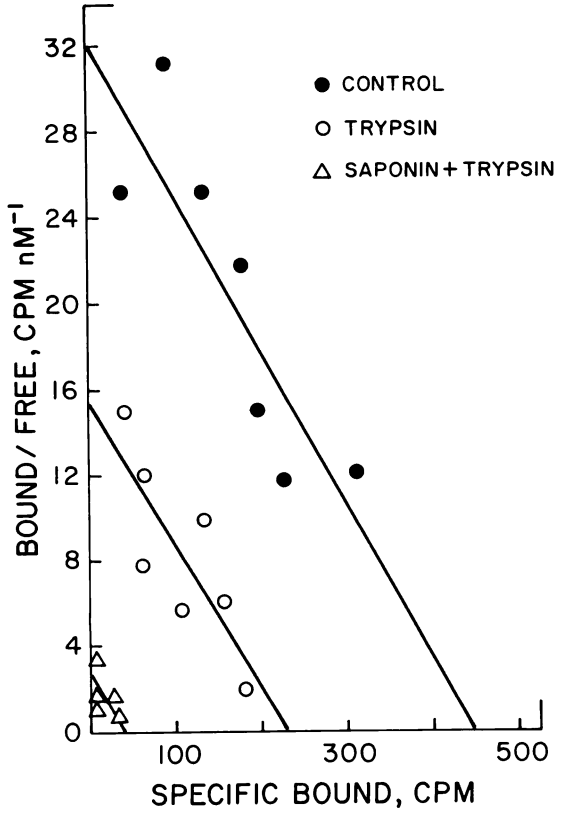

Fig. 5. Surface trypsinization reduces the number of [^{3}H]muscimol binding sites, and saponin treatment permits digestion of virtually all sites. Saturation binding of [^{3}H]muscimol to homogenates prepared from control (●), trypsin-treated (○), and saponin plus trypsin-treated (△) intact brain cell cultures is shown. Each *data point* represents the mean of triplicate determinations. Scatchard plots of the data are shown for graphical presentation. The *lines* represent the best fit to a single-site model, as determined by nonlinear regression of untransformed data, which for this particular experiment yielded the following parameters: control, K_D = 14 nM, B_{max} = 455 cpm; trypsin-treated, K_D = 15 nM, B_{max} = 235 cpm; saponin + trypsin-treated, K_D = 14.2 nM, B_{max} = 40 cpm. In this experiment, the B_{max} for trypsin-treated cells was 52% of control, which was slightly greater than average; mean of five experiments was 44 ± 9% trypsin-resistant. B_{max} data are expressed in cpm because trypsinization causes a loss in general cell protein as well as a loss of GABA receptors and expressing the data on a per milligram of protein basis would not reflect the absolute trypsin sensitivity of the GABA receptor.

to cellular homogenates, presumably by increasing the affinity of the GABA recognition site for [^{3}H]muscimol (Fig. 3). Importantly, Ro7-0213 potentiates the electrophysiologically measured GABA-mediated chloride conductance in chick spinal cord cultures,[3] indicating that Ro7-0213 binds to a benzodiazepine recognition site that is associated with the functional GABA-R.

As shown in Fig. 6A, Ro7-0213 displaces the reversible binding of 5 nM [^{3}H]flunitrazepam to cellular homogenates with a K_I of 229 ± 6 nM (three experiments). Ro7-0213 is similarly potent in inhibiting irreversible [^{3}H]flunitrazepam photoaffinity labeling of intact cells (Fig. 6B; Table 1), but about 20% of the specific irreversible photoaffinity labeling is insensitive to Ro7-0213 These sites are also resistant to extracellular trypsin; 100 ± 3% (eight experiments) of the Ro7-0213 resistant

[3] D. Mierlak and D. H. Farb, unpublished results.

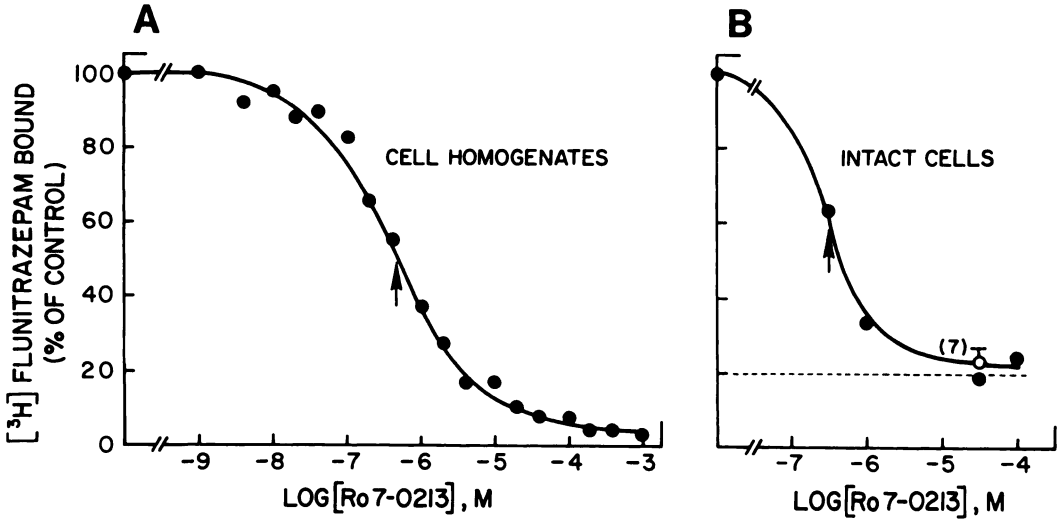

Fig. 6. Ro7-0213 completely displaces [^{3}H]flunitrazepam binding to cellular homogenates whereas in intact cells a significant amount of binding is resistant to Ro7-0213. Competitive binding of Ro7-0213 and [^{3}H]flunitrazepam is shown. A, Displacement by Ro7-0213 of the reversible binding of [^{3}H]flunitrazepam to homogenates prepared from intact cells. Binding assays were performed as described in Experimental Procedures using 5 nM [^{3}H]flunitrazepam. Controls for nonspecific binding contained 1 mM flurazepam. Each *data point* is the mean of triplicate determinations. K_i = 223 nM for Ro7-0213. K_i, the equilibrium dissociation constant for competitor, was calculated as described by Cheng and Prusoff (44): $K_i = IC_{50}/(1 + L^*/K_D^*)$, where IC_{50} is the concentration of competing ligand at 50% displacement, L^* is the concentration of radioactive ligand, and K_D^* is its dissociation constant (2 nM). Results are from a single experiment, out of a total of three independent experiments that produced similar results. B, Inhibition of the irreversible binding of [^{3}H]flunitrazepam to intact cells by Ro7-0213. Intact cells were irreversibly photoaffinity labeled with [^{3}H]flunitrazepam (5 nM) in the presence of various concentrations of Ro7-0213 and the specific irreversible binding was measured by filtration assay. Values represent the specific irreversible binding measured ± standard error, expressed as a percentage of binding in the absence of Ro7-0213. The *dashed line* represents the maximal displacement of [^{3}H]flunitrazepam binding by Ro7-0213 in intact cells. Number in parentheses represents the number of separate experiments.

TABLE 1

Ro7-0213 blocks cell surface benzodiazepine receptors

Quadruplicate intact and saponin-treated cultures were irreversibly photoaffinity labeled with [^{3}H]flunitrazepam (0.75–1 nM) in the absence and presence of 30 μM Ro7-0213. After photoaffinity labeling, some cultures were incubated in the presence of trypsin (0.5 mg/ml, 90 min, 37°). The specific irreversible binding of [^{3}H]flunitrazepam was measured by filtration assay. Values represent specific irreversible binding ± standard error, normalized to control. The number of individual experiments is shown in parentheses.

Treatment	[^{3}H]Flunitrazepam binding
	%
None (control)	100
Ro7-0213	23 ± 2 (7)
Ro7-0213 + Trypsin	23 ± 2 (8)
Saponin + Ro7-0213	4 ± 4 (3)

TABLE 2

Potentiation by GABA of specific irreversible [^{3}H]flunitrazepam binding to intact cells: effects of trypsin and Ro7-0213

Intact cultures were incubated (90 min, 37°) in the absence or presence of trypsin (0.5 mg/ml) and then were photoaffinity labeled with [^{3}H]flunitrazepam (0.75–1 nM) in the absence or presence of 30 μM Ro7-0213 and in the absence or presence of 10 μM GABA. The specific irreversible binding of [^{3}H]flunitrazepam was determined by filtration assay. Values represent the enhancement of specific irreversible binding ± standard error, expressed as a percentage of specific irreversible binding to sister cultures that were treated identically except for the omission of GABA. The number of individual experiments is shown in parentheses. Typical results from one such experiment were as follows (specific cpm/assay): control, 795 ± 42; control + GABA, 1020 ± 57 (28% increase); trypsin, 327 ± 51; trypsin; + GABA, 429 ± 36 (31% increase); Ro7-0213, 151 ± 20; Ro7-0213 + GABA, 145 ± 20 (4% decrease).

Addition	[^{3}H]Flunitrazepam binding
	% *increase*
10 μM GABA	36 ± 6 (5)
Trypsin + 10 μM GABA	34 ± 5 (3)
Ro7-0213 + 10 μM GABA	−5 ± 6 (3)

binding sites are protected from trypsin attack in intact cells (Table 1). When cells are permeabilized with saponin before trypsinization, virtually all sites are sensitive to trypsin (13) and to competition by Ro7-0213 (Table 1). This result argues that trypsin and Ro7-0213 do not readily enter untreated cells but are allowed to enter through saponin-generated fenestrae. Thus, in the presence of excess Ro7-0213, [^{3}H]flunitrazepam selectively labels the intracellular pool of benzodiazepine binding sites in intact cells.

In intact cells, 10 μM GABA potentiates [^{3}H]flunitrazepam photoaffinity labeling by 36%. In contrast, GABA does not potentiate photoaffinity labeling when 30 μM Ro7-0213 is present to block surface benzodiazepine binding sites (Table 2). Thus, binding of [^{3}H]flunitrazepam to intracellular sites is not enhanced when GABA is added to the extracellular medium.

Is the benzodiazepine binding site on xRF24 allosterically coupled to the GABA recognition site? To determine whether the benzodiazepine binding site of the trypsin-generated receptor fragment xRF24 is coupled to a GABA recognition site, we examined the ability of GABA to enhance [^{3}H]flunitrazepam photoaffinity labeling of intact trypsin-treated cells. GABA (10 μM) increases [^{3}H]flunitrazepam photoaffinity labeling of trypsinized intact cells to the same extent as labeling of nontrypsinized intact cells (Table 2). Because extracellularly applied GABA does not enhance binding to intracellular sites (see above), and because the only cell surface [^{3}H]flunitrazepam binding sites that remain after trypsinization are those associated with xRF24 (13), this result indicates that the cell surface

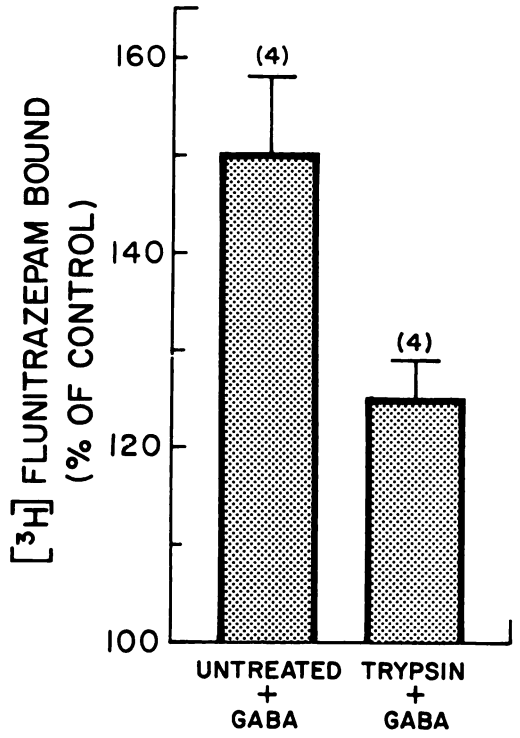

Fig. 7. Trypsinization of the cell surface reduces by 50% the ability of GABA to potentiate [^{3}H]flunitrazepam binding to homogenates of cells. GABA enhancement of specific reversible [^{3}H]flunitrazepam binding to homogenates prepared from control and trypsin-treated intact cells is shown. Cells were incubated in the absence or presence of trypsin and enhancement of specific reversible binding of 1 nM [^{3}H]flunitrazepam by 10 μM GABA was measured as described in Experimental Procedures. Values represent the enhancement of specific reversible binding of [^{3}H] flunitrazepam by GABA expressed as a percentage of the binding observed in the absence of GABA (control). Numbers in parentheses represent the number of separate experiments.

benzodiazepine binding site associated with xRF24 retains its allosteric coupling to a GABA recognition site.

It seems possible, however, that binding of [^{3}H]flunitrazepam to intact cultures might be influenced to some degree by residual GABA, so the degree of enhancement produced by added GABA should be interpreted cautiously. Thus, we examined the ability of GABA to enhance the reversible binding of [^{3}H]flunitrazepam to dialyzed membrane homogenates prepared from control and trypsin-treated intact cells. For homogenates of untreated cells, GABA (10 μM) increases the binding of [^{3}H]flunitrazepam (0.75–1 nM) by 50 ± 8% (four experiments; Fig. 7). For homogenates of trypsinized cells, GABA potentiates [^{3}H]flunitrazepam binding by only 25 ± 4% (four experiments), a striking 50% reduction of potentiation.

Discussion

Elucidating the cellular mechanisms underlying the coupling between neuromodulatory and neurotransmitter receptors is important to understanding CNS function. The GABA-R is a good model for studying these interactions because a variety of compounds, themselves without effect on membrane permeability, act as modulators of GABA-R function. Biochemical studies have revealed that GABA enhances benzodiazepine binding (22) and that benzodiazepines enhance GABA binding (23), indicating that the binding sites for benzodiazepines and GABA are allosterically coupled. Although the actual subunit composition has not been firmly established, it is believed that there are two subunits, α and β, and that the native receptor exists as a tetramer with the stoichiometry of $\alpha_2\beta_2$ (11, 24). Recent reports (25, 26) have concluded that the α subunit is specifically photoaffinity labeled with [^{3}H]flunitrazepam whereas the β subunit is labeled with [^{3}H]muscimol. Others (12, 27–30) have reported that two or more subunits are photoaffinity labeled by [^{3}H]flunitrazepam. These bands may represent different isoforms of the α subunit (31, 32) or may represent benzodiazepine binding sites located on both α and β subunits. These additional photoaffinity-labeled proteins may or may not also contain a GABA binding site. Thus, it is unclear whether or not the binding sites for GABA and benzodiazepines are located on the same or different polypeptide chains, nor is it known how GABA-R structure is related to the functional coupling observed between the GABA and benzodiazepine recognition sites.

As a first step toward relating the primary sequence of a receptor to its function, it is necessary to establish its transmembrane topology. This problem may be approached either theoretically, using hydropathy analysis and molecular modeling, or experimentally, using functional mapping techniques such as selective proteolytic cleavage in conjunction with group-selective probes, antibodies against identified portions of the sequence, and membrane-impermeant reagents. For example, whereas genetic cloning methodology has yielded the primary amino acid sequence of the subunits of the nicotinic acetylcholine receptor (33), functional mapping studies have been instrumental in resolving differences between theoretical models based upon hydropathy analysis of the amino acid sequences of the receptor subunits (34).

Formation of xRF24. We have shown previously that exhaustive trypsinization of intact cells results in formation of xRF24, a transmembrane receptor fragment with an apparent M_r of 24,000 that contains a benzodiazepine recognition site (13). It is useful to reexamine this result in the light of a model (Fig. 8) proposed by Schofield *et al.* (35) on the basis of hydropathy analysis of deduced amino acid sequences for the putative α and β subunits of the bovine GABA-R. According to this model, each subunit includes four membrane-spanning helices and an extended extracellular amino-terminal domain. The benzodiazepine and GABA binding sites are suggested to reside on the extended extracellular amino-terminal portions of the α and β subunits, respectively. Interestingly, expression of the cloned genes by mRNA injection into *Xenopus* oocytes yields a receptor that responds to GABA but that is unresponsive to benzodiazepines (32). This may indicate that an additional subunit or some form of posttranslational modification is required for benzodiazepine sensitivity.

To what extent is the proposed structure of the GABA-R consistent with the results of proteolysis experiments? Inspection of the amino acid sequence of the putative α subunit reveals numerous potential sites of trypsin cleavage (Fig. 8, *circles*). Assuming that the chick α subunit is similar, xRF24 could be generated either 1) by trypsin attack on the short extracellular loop between M2 and M3 of the α subunit or 2) by cleavage primarily at the amino end of the protein (Fig. 8, *eTS*). The former mechanism seems unlikely, because it cannot easily account for the fact that xRF24 vanishes when trypsin

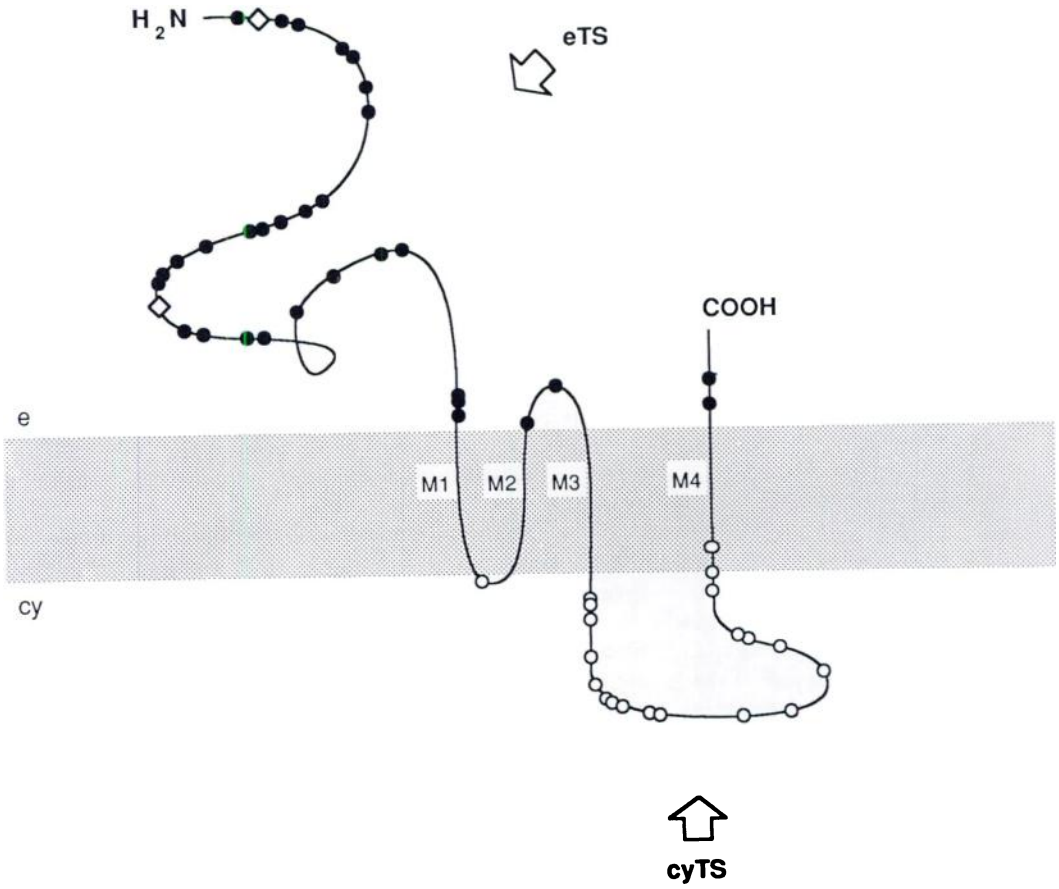

Fig. 8. Theoretical model of the transmembrane orientation of the bovine GABA-R α subunit, as proposed by Schofield *et al.* (35) on the basis of hydropathy analysis of the deduced amino acid sequence. Potential trypsin cleavage sites (lysine and arginine residues) are indicated by *solid circles* if located facing extracellularly, or by *open circles* if located facing cytoplasmically. Of these, there must be at least one extracellularly facing trypsin-sensitive site (*eTS*), and at least one cytoplasmic site (*cyTS*), such as we have described previously for the benzodiazepine receptor (13). The data in this paper would be consistent with this model if eTS and cyTS were located in the regions indicated by the *arrows*, and if xRF24 includes the cytoplasmic loop between M3 and M4. Possible glycosylation sites are indicated by *diamonds*.

is permitted access to the inner surface of the membrane (13). With the first mechanism, the only potential point of intracellular attack on xRF24 is the short loop between M1 and M2, which would still leave a large enough fragment (approximately 20,000) to be detectable by SDS-PAGE under our conditions, yet no such fragment is found. This is not a problem with the latter mechanism, because destruction of xRF24 could occur by attack on the large intracellular loop between M3 and M4 (Fig. 8, *cyTS*). This region contains many potential trypsin cleavage sites, so that it would not be surprising if the digestion products were too small to be detected by SDS-PAGE under our conditions. Similar arguments apply if xRF24 is assumed to be derived from the additional α subunits described by Levitan *et al.* (32) or from the β subunit described by Schofield *et al.* (35), except that the latter has no potential intracellular trypsin cleavage sites between M1 and M2, further strengthening the case for formation of xRF24 by amino-terminal cleavage.

The location of the photoaffinity-labeled benzodiazepine recognition site on xRF24 cannot yet be unambiguously identified. The preceding considerations argue that residues that are far out on the amino terminal portion do not contribute significantly to binding, because much of this region is probably lost during trypsinization, yet affinity for [^{3}H]flunitrazepam is unaltered (13). It remains possible that the benzodiazepine recognition site is on the amino-terminal region near the point at which the polypeptide chain enters the membrane; however, in view of the lipophilic nature of the benzodiazepines, it is also possible that the benzodiazepine recognition site is at the external membrane/solution interface, associated with one of the transmembrane segments M1–M4.

[^{3}H]Muscimol binding sites of embryonic neurons in culture are homogeneous. Saturation (Fig. 1) and competition (Fig. 2) binding studies of [^{3}H]muscimol to dialyzed homogenates prepared from embryonic chick brain cell cultures are consistent with a single homogeneous class of binding sites. The data do not exclude the existence of additional sites of very low affinity or low abundance, which would escape detection. Our results are in agreement with those reported using chick cerebral cells cultured for 10 days (36), as well as rat cerebral cortex neurons cultured for 3 days (37). In contrast, rat cerebral cortex neurons cultured for 3 weeks have been shown to exhibit both a low and a high affinity binding site for GABA (38). Interestingly, cerebellar granule cells grown in culture in the absence of GABA exhibit only a single binding component, whereas cells grown in the presence of GABA exhibit two components (39). Studies of GABA and muscimol binding to membrane homogenates prepared from adult rat, mouse, and chicken brain have demonstrated the presence of two distinct GABA/muscimol binding sites of different affinities, both of which exhibit receptor-like pharmacological specificity (20, 36, 40, 41). Nevertheless, the rank order and potencies of several GABA-R agonists and antagonists in inhibiting [^{3}H]muscimol binding in chick culture membrane homogenates (Fig. 2) correlate well with their ability to inhibit [^{3}H]GABA binding in adult mammalian brain tissue. Importantly, benzodiazepines significantly potentiate the specific reversible bind-

ing of [^{3}H]muscimol to cellular homogenates (Fig. 3), indicating that an allosteric coupling exists between benzodiazepine binding sites and muscimol binding sites. Thus, the [^{3}H]muscimol binding sites detected in embryonic brain cell cultures probably represent functional GABA-Rs.

[^{3}H]Muscimol binding sites and [^{3}H]flunitrazepam binding sites exhibit similar trypsin sensitivity. Approximately 40% of the specific binding of [^{3}H]muscimol is resistant to exhaustive trypsinization of intact cells (Fig. 4). The decrease in binding reflects a reduced number of receptors rather than a change in binding affinity (Fig. 5). Thus, at least 60% of the muscimol binding sites (those inactivated by trypsin) are facing extracellularly on cell surface GABA-Rs, whereas the remaining 40% of the muscimol binding sites are protected from trypsin attack. The trypsin-resistant muscimol binding sites could conceivably be associated with intracellular or membrane-sequestered GABA-Rs that are inaccessible to trypsin, with cell surface GABA-Rs that are cleaved by trypsin but retain their binding sites for muscimol, or with a combination of both.

The percentage of trypsin-resistant [^{3}H]muscimol binding sites is in good agreement with the percentage of trypsin-resistant benzodiazepine binding sites. When intact neurons are exposed to trypsin, 60% of the benzodiazepine binding sites are destroyed. Of the 40% of benzodiazepine binding sites that survive after trypsin treatment, half (20% of total) face extracellularly and are associated with xRF24, a receptor fragment, whereas the remaining half (20% of total) are associated with undigested receptors that are probably intracellular (13). This interpretation is in agreement with the finding that 80% of the benzodiazepine binding sites of intact neurons are accessible to the charged benzodiazepine Ro7-0213, whereas the remaining 20% may be rendered accessible to Ro7-0213 by treatments that disrupt the plasma membrane, such as extensive homogenization or treatment with saponin (13). Thus, it is reasonable to hypothesize that, of the 40% trypsin-resistant [^{3}H]muscimol binding sites, half are associated with intracellular and/or membrane-sequestered GABA-Rs and half are associated with xRF24 or another cleaved receptor fragment.

Trypsin-resistant cell surface GABA and benzodiazepine recognition sites reflect a common receptor population. In view of the numerous potential trypsin cleavage sites in the sequences of the putative α (Fig. 8) and β subunits of the GABA-R (35), it is somewhat remarkable that any cell-surface GABA or benzodiazepine recognition sites survive after trypsinization. Indeed, most surface sites are destroyed by trypsin; however, a minority are trypsin-resistant. Resistance to trypsin does not reflect failure of trypsin to gain access to the receptor, because partial cleavage does occur (resulting in formation of xRF24). These trypsin-resistant sites could be due to subunit heterogeneity or to differences in conformation, glycosylation, or association with other proteins. The existence of GABA-R heterogeneity is supported by the isolation by Levitan *et al.* (32) of two additional GABA-R cDNAs homologous to the α subunit sequence previously reported by Schofield *et al.* (35). The fact that the proportion of trypsin-resistant [^{3}H]muscimol binding sites is the same as the proportion of trypsin-resistant [^{3}H]flunitrazepam binding sites suggests that these two classes of trypsin-resistant sites may reflect a common population of receptors. This is substantiated by the finding that GABA potentiates [^{3}H]flunitrazepam binding to trypsinized intact cultures, indicating that the benzodiazepine binding site on xRF24 is allosterically coupled to a GABA recognition site. Thus, xRF24 may bear both a benzodiazepine binding site and a GABA recognition site. Another interesting possibility is that the GABA recognition site is on a separate unlabeled fragment or subunit that remains in the membrane after trypsinization and is coupled to xRF24 by noncovalent interactions. The fact that both the GABA and benzodiazepine recognition sites are protected suggests that these sites are in close physical proximity. Alternatively, resistance to trypsin could be due to some global characteristic of the receptor, such as synaptic versus extrasynaptic localization, in which case no such close physical association would be necessary.

Are intracellular GABA and benzodiazepine recognition sites functionally coupled? We have shown previously that the degree of allosteric coupling between the GABA and benzodiazepine binding sites of the GABA/benzodiazepine receptor in culture is subject to alteration in response to chronic exposure of cultures to benzodiazepines (42) or methylxanthines (43). The finding that the interaction between these sites is subject to regulatory influences, rather than being an invariant property of the receptor, raises the interesting question of whether GABA and benzodiazepine binding sites of intracellular receptors are coupled or whether this coupling is conferred only upon insertion into the plasma membrane. To determine whether intracellular benzodiazepine binding sites are coupled to GABA binding sites, we studied the ability of GABA to potentiate the irreversible binding of [^{3}H]flunitrazepam to intact cultures in the presence of a saturating concentration of Ro7-0213, which prevents [^{3}H]flunitrazepam from binding to surface sites. Thus, in the presence of excess Ro7-0213, [^{3}H]flunitrazepam specifically labels intracellular sites. We find that binding of [^{3}H]flunitrazepam to intracellular receptors of intact neurons is unaffected by exogenous GABA. This is consistent with the view that intracellular GABA and benzodiazepine binding sites are allosterically uncoupled; however, it is also possible that externally added GABA is unable to gain access to intracellular receptors. Although many neurons accumulate GABA through high and low affinity uptake systems (15), uptake of GABA will be slow under the experimental conditions (4°). Moreover, intracellular receptors may be sequestered in a compartment from which GABA is excluded or, conversely, in a compartment in which the endogenous GABA concentration is so high that [^{3}H]flunitrazepam binding is already maximally potentiated.

Interestingly, GABA potentiates [^{3}H]flunitrazepam binding to dialyzed homogenates of trypsinized cultures by only half as much as binding to dialyzed homogenates of control cultures. Because the benzodiazepine binding site on xRF24 is allosterically coupled to a GABA recognition site and because trypsin treatment reduces the ratio of surface to intracellular benzodiazepine binding sites from 80:20 to 50:50, this result is consistent with the hypothesis that intracellular benzodiazepine binding sites are not functionally coupled to GABA recognition sites. Alternatively, xRF24 may remain allosterically coupled to a GABA recognition site, but the "strength" of coupling may be less than for the native receptor. This seems relatively unlikely, because enhancement of [^{3}H]flunitrazepam binding to intact cells, which is due to the effect of GABA on presumed surface receptors, is similar for control and trypsin-treated cells (Table 2). It is possible, however, that trypsinization could alter the positioning of the various components in the membrane

sufficiently to have a subsequent washing step decrease the coupling between cell surface benzodiazepine binding sites and GABA binding sites.

In summary, we have shown that at least 60% of the GABA recognition sites are exposed on the cell surface. By association with benzodiazepine binding sites, it is probable that the actual percentage of surface GABA recognition sites is 80%. Additionally, the benzodiazepine binding sites associated with the receptor fragment xRF24, which is generated by trypsinization of intact cells, not only retain their ability to bind benzodiazepine ligands but also retain their ability to be modulated by GABA. This result indicates that xRF24 is derived from the GABA-R and that there is a fraction of cell surface GABA-Rs for which both the GABA and benzodiazepine binding sites are protected from destruction by trypsin. It is clear that xRF24 must carry at least a benzodiazepine binding site, because it can be irreversibly labeled by [^{3}H]flunitrazepam after trypsinization. The GABA binding site could be either on xRF24 or on a separate unlabeled fragment or subunit that is coupled with xRF24 by noncovalent interactions. Nevertheless, xRF24 is clearly an integral component of the GABA-R and contains the structural information necessary for the functional coupling of GABA and benzodiazepine recognition sites. Experiments such as those described in this report provide a powerful tool for testing hypotheses regarding the structural features of the GABA-R and the functional role of its parts.

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