Transmembrane Topology and Subcellular Distribution of the Benzodiazepine Receptor

Cynthia Czajkowski and David H. Farb

Department of Anatomy and Cell Biology, The State University of New York, Downstate Medical Center, Brooklyn, New York 11203

The distribution and transmembrane topology of benzodiazepine receptors were investigated *in situ* using intact and saponin-treated neurons of brain cell cultures. Reversible ligand binding and photoaffinity labeling, using ^3H -flunitrazepam as a probe, were employed in conjunction with trypsin-induced exhaustive proteolysis and competition binding using a novel benzodiazepine (Ro7-0213) that contains a quaternary ammonium moiety bearing a full positive charge. About 80% of the benzodiazepine receptors, with apparent subunit molecular weights of 48,000 and 51,000, are located in the surface membrane and contain one or more trypsin-sensitive sites exposed at the extracellular surface. The ligand recognition sites of the surface receptors are orientated toward the extracellular space and are sensitive to both competition by Ro7-0213 and trypsin attack. Approximately 20% of the receptors are intracellular or sequestered and are insensitive to both trypsin and competition by Ro7-0213 in intact cells. Strikingly, all of the ^3H -flunitrazepam photolabeled sites inaccessible to competition by Ro7-0213 are also resistant to extracellular trypsin, but are sensitive to trypsin and Ro7-0213 in saponin-treated cells. This result provides strong evidence for protected receptors. Surface receptors are differentially sensitive to trypsin-induced inactivation such that 57% (of total) are inactivated, while 24% (of total) are cleaved but, surprisingly, retain the capacity to bind ^3H -flunitrazepam. The observation that the ability of the receptor fragment to bind ligand is unaltered demonstrates that the integrity of the benzodiazepine binding site is not contingent upon the presence of about half of the intact polypeptide chain. The receptor fragment, with an apparent molecular weight of 24,000, is produced upon exposure to extracellular trypsin and remains anchored in the plasma membrane. The fragment also contains at least one trypsin-sensitive site that is exposed at the intracellular face of the plasma membrane. The results indicate that this receptor fragment is a transmembrane segment of the GABA/benzodiazepine receptor complex, in which the ligand recognition site faces extracellularly.

A question of central importance to understanding the structure and function of the GABA/benzodiazepine receptor complex (GABA/BZD-R) concerns its transmembrane topology and subcellular distribution. It is known that benzodiazepine drugs potentiate GABA responses determined on intact neurons in culture (Chan and Farb, 1985; Choi et al., 1977; Macdonald and Barker, 1978) or on isolated patches of adrenal chromaffin cell surface membrane (Bormann and Clapman, 1985) and that

postsynaptic densities fractionated from bovine brain are enriched in Type I benzodiazepine binding sites (Trifletti and Snyder, 1985), indicating that some functional receptors are located in the plasma membrane. Similarly, it is known that photoaffinity labeling of intact brain cells with ^3H -flunitrazepam yields labeled proteins with apparent molecular weights (M_r) of 48,000 and 51,000 (Borden et al., 1984), similar to those observed in photolabeled membrane homogenates (Farb et al., 1984; Mohler et al., 1980; Sieghart and Karobath, 1980; Thomas and Tallman, 1981). However, it cannot be concluded that the benzodiazepine recognition site is exposed at the extracellular surface of the plasma membrane since benzodiazepines are lipophilic and would be expected to pass rapidly across the membrane.

Proteolytic enzymes have proved to be useful tools in studying the transmembrane orientation of membrane proteins by the simple fact that they are macromolecules that do not cross biological membranes. This approach has been used to gain an understanding of the structure and function of the nicotinic ACh receptor (Wennogle and Changeux, 1980) and the beta-adrenergic receptor (Strulovici and Lefkowitz, 1984). Similarly, compounds that carry a nonionizable full positive charge are also excluded by biological membranes and have been used to distinguish cell-surface from intracellular receptors for muscarinic ACh receptors (Maloteaux et al., 1983) and beta-adrenergic receptors (Hertel et al., 1983). As a first step toward elucidating the transmembrane topology and subcellular distribution of the GABA/BZD-R, we report here the results of experiments utilizing differential trypsinization of BZD-Rs *in situ* to determine (1) the distribution of membrane-associated BZD-Rs between the cell-surface and intracellular membranes and (2) aspects of the transmembrane topology of the BZD-R. Additionally, we have used Ro7-0213, a positively charged BZD, to distinguish between cell-surface and intracellular BZD-Rs. We have determined that approximately 80% of the BZD-Rs are surface receptors with their ligand binding sites facing extracellularly. We have also identified a low-molecular-weight ($M_r = 24,000$) fragment of BZD-R that contains the intact ligand recognition site and spans the plasma membrane, such as might be expected for a transmembrane segment of receptor.

Materials and Methods

Benzodiazepines were a gift of Dr. P. Sorter of F. Hoffman-La Roche (Nutley, NJ). All other chemicals were obtained from commercial sources.

Neurons were dissociated from brain of 7 d chick embryos, plated on collagen-coated 100 mm plastic tissue culture dishes and maintained in culture for 1 week as described previously (Borden et al., 1984; Farb et al., 1979). Membrane homogenates prepared from 20 d embryonic chick brains (20 d P₂) were prepared as outlined in Chan et al. (1983) and Gibbs et al. (1985). For trypsinization, cultures were washed twice in 25 mM HEPES buffer containing 5.4 mM KCl, 0.9 mM CaCl₂, 0.4 mM MgSO₄·7H₂O, and 190 mM sucrose (pH 7.2). To trypsinize extracellular binding sites on intact cells, cultures were incubated for 90 min

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Correspondence should be addressed to Dr. David H. Farb, Department of Anatomy and Cell Biology, Box 5, State University of New York, Downstate Medical Center, Brooklyn, NY 11203.

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Figure 1. Effect of saponin on the sensitivity of BZD-R to trypsinization *in situ*. Data show the specific reversible and irreversible binding of ^3H -flunitrazepam to homogenates prepared from trypsinized intact and saponin-treated cells in culture. Intact and saponin-treated brain cell cultures were incubated in the absence (control) or presence of 0.5 mg/ml trypsin in HEPES buffer, pH 7.2, for 90 min at 37°C, soybean trypsin inhibitor was added, and cells were scraped from the culture dishes, centrifuged, resuspended in PBSS and the specific reversible (open bars) and irreversible (hatched bars) binding of 5 nM ^3H -flunitrazepam was measured as described in Materials and Methods. Saponin treatment alone did not affect either the reversible ($101 \pm 7\%$, $n = 7$) or irreversible ($105 \pm 14\%$, $n = 5$) binding. Data points represent the mean $\pm$ SEM of triplicate determinations, normalized to control. Numbers in the parentheses represent the number of separate experiments.

Figure 2. Time course for trypsin-induced inactivation of the specific reversible binding of ^3H -flunitrazepam to trypsin-treated intact cells and trypsin-treated homogenates. Intact cells (●) and membrane homogenates prepared from either brain cell cultures (○) or 20 d P₂ (△) were incubated in the absence (control) or presence of 0.5 mg/ml trypsin at 37°C in HEPES buffer for the indicated times and the reversible binding of 5 nM ^3H -flunitrazepam was measured as described in Materials and Methods. The numbers in the parentheses represent the number of separate experiments.

(unless otherwise indicated) at 37°C with or without 0.5 mg/ml bovine pancreas trypsin (Sigma) in the above buffer. To expose trypsin-resistant binding sites, cells were either (1) scraped from culture dishes, homogenized, and then incubated in trypsin, or (2) treated for 3 min with 0.5% saponin in 20 mM sodium phosphate buffer, pH 7.2, with 300 mM sucrose (Fambrough and Devreotes, 1978) and washed twice with HEPES buffer prior to incubation in trypsin. Trypsin activity was terminated by placing the incubations on ice and adding soybean trypsin inhibitor (0.5 mg/ml, final concentration). Cells were scraped from culture dishes, homogenized, and centrifuged at $30,000 \times g$ for 20 min. The resulting pellets were resuspended in a phosphate-buffered Earle's balanced salt solution (PBSS; pH 7.4) and the reversible and irreversible binding of 5 nM ^3H -flunitrazepam determined as described below. In a control experiment, 0.5 mg/ml soybean trypsin inhibitor was added to intact cultures simultaneously with trypsin, and the cultures were incubated for 90 min at 37°C. The soybean trypsin inhibitor prevented the cleavage of the photolabeled subunits, and no change in binding of 5 nM ^3H -flunitrazepam was observed. These results indicate that this amount of inhibitor prevented trypsin action.

To measure reversible binding, aliquots of culture homogenates were incubated with 5 nM ^3H -flunitrazepam (New England Nuclear) for 60 min at 4°C in PBSS. The incubation was terminated by filtration through Whatman GF/B glass fiber filters, which were then washed 4 times with a total of 20 ml of buffer. Radioactivity remaining on the filters was determined by liquid scintillation counting. In all experiments, 1 mM flurazepam was used to determine nonspecific binding, which was subtracted from total binding to yield the specific component.

Photoaffinity labeling of intact cultures and culture homogenates with flunitrazepam was carried out according to procedures described previously (Borden et al., 1984; Chan et al., 1983). Briefly, cultures were rinsed 2 times with ice-cold PBSS using a total volume of 10 ml and photolabeled by incubation with 5 nM ^3H -flunitrazepam (30 min, 4°C, in PBSS) followed by irradiation with long-wavelength ultraviolet light (General Electric F40 BLB bulb) for 45 min at 4°C. Intact cells were washed with ice-cold PBSS, scraped from culture dishes, and homogenized in buffer. The homogenates were then centrifuged at $30,000 \times g$ for 20 min, and the resulting pellets were resuspended in PBSS containing 1 mM flurazepam, incubated for 45 min; and aliquots were either used to measure cell-associated radioactivity by filtration assay through Whatman GF/B filters, or solubilized in SDS "stop solution" containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercapto-

ethanol, and 0.001% bromophenol blue (tracking dye). Photolabeling of culture homogenates was carried out according to a similar protocol. In some samples, 1 mM flurazepam was included during the photolabeling procedure to determine nonspecific photolabeling.

SDS-PAGE was performed according to the method of Laemmli (1970). The samples were boiled for 3 min in SDS stop solution before application to 10% polyacrylamide gels. The following proteins, obtained from Bio-Rad, were used as molecular-weight standards: phosphorylase B ($M_r = 92,000$), BSA ($M_r = 66,200$), ovalbumin ($M_r = 45,000$), carbonic anhydrase ($M_r = 31,000$), soybean trypsin inhibitor ($M_r = 21,500$), and lysozyme ($M_r = 14,400$). Following electrophoresis, gels were processed by either of 2 methods:

Method a. Gels were fixed and stained with a solution of 0.4% Coomassie brilliant blue solution in 50% methanol–7% acetic acid and destained by repeated washings in 7% acetic acid. The amount of radioactivity associated with the separated proteins was determined by cutting each gel lane into 1 mm slices, dissolving the slices with 0.5 ml of 50% hydrogen peroxide overnight at 43°C, and determining the radioactivity associated with each slice by liquid scintillation counting.

Method b. Immediately following electrophoresis, individual gel lanes were cut out of the slab gel, frozen with dry ice, and stored at -70°C prior to cutting into 1 mm slices. Radioactivity was eluted from the gel slices with 0.5 ml of a solution containing 1% SDS, 0.025 M Tris, and 0.0625 M glycine (pH 8.3). The radioactivity associated with each slice was determined by liquid scintillation counting. The distribution of radioactivity within the gels was similar for the 2 processing methods.

Results

Effect of trypsin on BZD-R binding

Following trypsin-induced exhaustive proteolysis of intact brain cell cultures, the binding of ^3H -flunitrazepam was reduced significantly. There was a similar reduction in reversible and UV-induced irreversible ^3H -flunitrazepam binding, with $43 \pm 7\%$ ($n = 11$) of the reversible and $41 \pm 7\%$ ($n = 5$) of the irreversible binding remaining resistant to trypsin (Figs. 1, 2) following a 90 min incubation. Thus, about 42% of the binding is protected from trypsin attack. Addition of fresh trypsin after 60 min of incubation did not change the amount of trypsin-resistant binding. No further reduction was observed when trypsin treatment was extended to 3 hr. Thus, a 90 min incubation time for trypsin treatment was routinely chosen to ensure that trypsinization was exhaustive. Soybean trypsin inhibitor (0.5 mg/ml, final concentration) completely blocked the effect of trypsin.

Figure 3. Saturation binding of ^3H -flunitrazepam to homogenates prepared from control (●) and trypsin-treated (○) brain cell cultures. Cells were incubated in the absence or presence of 0.5 mg/ml trypsin in HEPES buffer for 90 min at 37°C. Binding was determined as described in Materials and Methods. Scatchard plots of the data are shown. Lines through the data points represent the best fit determined by linear-regression analysis, and each data point represents the mean of triplicate determinations. For control: $K_D = 4.4$ nM, $B_{\max} = 6800$ cpm; in trypsin-treated: $K_D = 4.8$ nM, $B_{\max} = 3200$ cpm. $B_{\max}$ data are not expressed on a per milligram protein basis since trypsinization causes a loss in general cell protein as well as a loss of BZD-R. Thus, expressing the data on a per milligram basis would not reflect the absolute sensitivity of the BZD-R to trypsin.

In order to determine whether the loss in binding following trypsinization of intact cells reflected a real loss in receptor number or a change in binding affinity for flunitrazepam, we performed both saturation and competition binding experiments on membrane homogenates derived from trypsinized intact cells. As demonstrated in Figure 3, trypsin-induced cleavage of the BZD-R of intact cells reduced the number of ^3H -flunitrazepam binding sites ($B_{\max}$; control = 6800 cpm; trypsinized = 3200 cpm), whereas binding affinity was virtually unchanged (Fig. 4). In addition, GABA stimulation of reversible ^3H -flunitrazepam binding was observed both before and after treatment of cells with trypsin.

The reduction in BZD-R number observed following trypsin treatment is probably not due to cell death or plasmalemma disruption since trypsin itself does not appear to cause cell death or membrane leakiness. When cultures were first treated with trypsin and then exposed to trypan blue, a vital dye, the number of cells that excluded dye in treated cultures ($86 \pm 1\%$, $n = 2$, 530 cells counted) was identical to untreated cultures (86% , $n = 2$, 465 cells counted).

To determine if the intact plasma membrane protected intracellularly directed binding sites of surface receptors and/or binding sites of intracellular receptors, cells were homogenized prior to trypsinization. Figure 2 shows that prior homogenization exposed additional (27%) benzodiazepine receptors to enzymatic digestion. The trypsin-resistant reversible binding was $22 \pm 1\%$ ($n = 5$) when cells were first homogenized (20 strokes of Dounce) and then trypsinized (90 min); 15% ($n = 2$) when homogenates of cells were frozen, thawed, and then tryp-

Figure 4. Competitive binding of flunitrazepam and ^3H -flunitrazepam to homogenates prepared from control (●) and trypsin-treated (○) brain cells. Cells were incubated for 90 min at 37°C in the absence or presence of 0.5 mg/ml trypsin in HEPES buffer. Binding assays were performed as described in Materials and Methods using 5 nM ^3H -flunitrazepam (nonspecific binding in 1 mM flurazepam was subtracted). Each data point is the mean of triplicate determinations. K_i 's = 5.4 nM and 6.2 nM for control and trypsin-treated cultures, respectively.

sinized; and $16 \pm 2\%$ ($n = 11$) when cells were homogenized more extensively (100 strokes) before exposure to trypsin. Similar results were obtained with P_2 homogenates of 20 d embryonic brain ($12 \pm 2\%$ resistant, $n = 2$). Irreversible binding of ^3H -flunitrazepam to trypsin-treated culture homogenates likewise was reduced, with only 16% of the binding remaining resistant.

Permeabilization of the plasmalemma with saponin also increased the effect of trypsin, rendering most of the previously trypsin-resistant binding trypsin-sensitive; saponin alone had no effect on binding (Fig. 1, legend). Once again, reversible and irreversible binding were reduced proportionately, with 11% of the irreversible and 7% of the reversible binding resistant to inactivation by treatment with saponin and trypsin.

The observation that trypsinization of both saponin-treated cells and cellular homogenates results in the inactivation of most of the benzodiazepine binding sites argues that the amount of trypsin-resistant binding seen in intact cells is not a result of inactive (autodigested) trypsin.

FLUNITRAZEPAM (Ro5-4200)

QUATERNIZED BZD (Ro7-0213)

Figure 5. Structures of flunitrazepam and the quaternized benzodiazepine, Ro7-0213.

Figure 6. A quaternized BZD (Ro7-0213) gains access to previously inaccessible BZD-Rs after exposure of cells to saponin. Intact (A, B) and saponin-treated (C) cells were photolabeled with 5 nM ^3H -flunitrazepam (10 min UV) in the absence (A) and presence of 30 μM Ro7-0213 (B, C) and prepared for SDS-PAGE. The distribution of radioactivity in the $M_r = 51,000$ and 48,000 subunits is shown. This experiment was repeated 2 times with similar results.

Effect of Ro7-0213 on ^3H -flunitrazepam binding

As a further test of the hypothesis that there is a population of BZD binding sites that is inaccessible from the extracellular medium, experiments were carried out using the quaternized benzodiazepine, Ro7-0213, to determine directly the amount of cell surface BZD-Rs. Since Ro7-0213 bears a full positive charge, it would not be expected to cross the plasma membrane (Fig. 5). Photoaffinity labeling of intact cells with 5 nM ^3H -flunitrazepam in the presence of 30 μM Ro7-0213 ($K_i = 300$

nM) resulted in a $78.5 \pm 0.5\%$ ($n = 2$) reduction in the amount of specific irreversible binding (Fig. 6). Thus, about 20% of the specific irreversible binding is protected from competition by Ro7-0213 in intact cells. All of these sites were also resistant to extracellular trypsin. When cultures were first treated with saponin and then photolabeled in the presence of 30 μM Ro7-0213, virtually all of the irreversible binding sites were sensitive to competition by the quaternized benzodiazepine ($93.5 \pm 5.5\%$, $n = 2$).

SDS-PAGE of the trypsinized BZD-R

The discrepancy observed between the fraction of ^3H -flunitrazepam binding resistant to trypsin (ca. 40%) and the fraction of irreversible binding inaccessible to competition by Ro7-0213 (ca. 20%) suggested that not all of the trypsin-resistant binding measured by filtration was intracellular. Thus, either 20% of the sites were surface receptors and protected from trypsin but accessible to Ro7-0213, or 20% of the sites were cleaved by trypsin but retained their ability to bind ^3H -flunitrazepam. To distinguish these hypotheses, homogenates derived from trypsinized cells were photolabeled and subjected to SDS-PAGE. Trypsinization of intact cells generated a new, lower molecular weight ($M_r = 24,000$) peak in addition to the remaining $M_r = 48,000$ and 51,000 proteins (Fig. 7, $n = 3$). The same pattern of radiolabeled proteins was obtained when cultures were first photolabeled and then treated with trypsin ($n = 3$). It will be interesting to determine whether the $M_r = 24,000$ trypsin-generated fragment is derived from one or both of the $M_r = 51,000$ and 48,000 labeled proteins. The recovery of the specific photolabeling in these 3 proteins ($M_r = 51,000$, 48,000, and 24,000) was $43 \pm 5\%$ ($n = 6$) of that present initially in the $M_r = 48,000$ and 51,000 subunits. This compares favorably with the value of $41 \pm 7\%$ (5 experiments in triplicate) obtained for trypsin-resistant photolabeling as measured by filter binding (Fig. 1). Thus, the binding measured by filtration is composed of 2 components: binding associated with a trypsin-generated fragment of the BZD-R and binding associated with intact BZD-Rs. Significantly, the specific radioactivity remaining in the $M_r = 51,000$ and 48,000 BZD-R proteins following trypsinization was $19 \pm 4\%$ ($n = 6$) of control. This is similar to the amount of irreversible binding resistant to competition by the quaternized BZD, Ro7-0213 (ca. 20%, Fig. 6). The $M_r = 24,000$ trypsin-generated fragment contained $24 \pm 2\%$ ($n = 6$) of the radioactivity initially present in the $M_r = 48,000$ and 51,000 proteins (Table 1). Finally, either homogenization or exposure of neuronal cells to saponin before trypsinization decreased substantially (ca. 59%) the amount of radioactivity that migrated with a $M_r = 24,000$ (Figs. 7, 8). The above results indicate that trypsinization generates a receptor fragment of $M_r = 24,000$ and that this fragment contains the ligand recognition site and has a transmembrane domain. Additionally, in intact cells, a similar fraction of BZD-Rs are inaccessible to both trypsin and the

Table 1. Trypsin sensitivity of the benzodiazepine receptor (specific binding remaining as percentage of control)

Treatment	Filtration assay		SDS-PAGE		
	Reversible	Irreversible	51 + 48 + 24 ^a	51 + 48	24
None (control)	100%	100%	100%	100%	0%
Trypsin	$43 \pm 2\%$ (11)	$41 \pm 7\%$ (5)	$43 \pm 5\%$ (6)	$19 \pm 4\%$ (6)	$24 \pm 2\%$ (6)
Saponin + trypsin	$7 \pm 1\%$ (7)	$11 \pm 5\%$ (3)	$15 \pm 4\%$ (5)	$5 \pm 3\%$ (5)	$10 \pm 3\%$ (5) ^b

Intact and saponin-treated cells were incubated in the absence (control) and presence of 0.5 mg/ml trypsin (90 min, 37°C) and the specific reversible and irreversible binding of 5 nM ^3H -flunitrazepam was measured by filtration assay and SDS-PAGE. Values represent the specific binding observed relative to that observed in the absence of trypsin, expressed as a percentage. Numbers in the parentheses represent the number of individual experiments.

^a 51, 48, and 24 represent $M_r = 51,000$, 48,000, and 24,000, respectively.

^b Percentages were determined from the summed cpm in gel slices corresponding to the position of the $M_r = 24,000$ peak. However, counts appeared uniformly distributed above baseline and did not form a true peak.

Figure 7. Trypsinization of intact cells and saponin-treated cells reveals membrane-protected BZD-Rs. Data shown are the results of SDS-PAGE of photolabeled and trypsinized cells. Cells were incubated for 90 min at 37°C in the absence (A) or presence (B) of 0.5 mg/ml trypsin in HEPES buffer. Trypsin activity was terminated by placing the dishes on ice and adding soybean trypsin inhibitor. The cells were homogenized and centrifuged, and the pellet was resuspended in PBSS, photolabeled with 5 nM ^3H -flunitrazepam and then subjected to SDS-PAGE. The gels were sliced into 1 mm slices and eluted with H_2O ; the radioactivity associated with each slice was determined by scintillation counting. In control cultures (A), no trypsin was present during the incubation, and 2 peaks of $M_r = 51,000$ and $48,000$ were seen. Following trypsin treatment of intact cells (B), a third peak, $M_r = 24,000$, was also present. When cells were treated with saponin (0.5%) and then trypsinized (C) no peaks were detectable. Results are from a single experiment, which was repeated 3 times with similar results. The same results were observed when cells were photolabeled and then treated with trypsin ($n = 3$). Numbers represent $M_r \times 10^{-3}$. The peaks observed at $M_r = 51,000$, $48,000$, and $24,000$ were seen in every experiment ($n = 6$). The small positive and negative deflections from the baseline observed were seen inconsistently from experiment to experiment and represent random "noise."

quaternized benzodiazepine, Ro7-0213, suggesting that in both methods the same population of BZD-Rs is being examined.

Discussion

Little is known about the organization of neurotransmitter receptors at central synapses, and determining the relationship of receptor structure and transmembrane topology to receptor function is important to understanding the fundamental mechanisms of chemical synaptic transmission. Until recently, only 2 general kinds of receptor-effector systems were known, as exemplified by (1) the nicotinic ACh receptor, which contains an integral effector, and (2) the muscarinic ACh receptor and beta-adrenergic receptor, which do not have integral effector mechanisms but are coupled to transducing systems such as the enzyme adenylate cyclase. The GABA receptor, which contains a benzodiazepine-sensitive modulator site that may be on the same polypeptide chain as the GABA site (Schoch et al., 1985; Sigel et al., 1982), represents a novel receptor system, and the question of the relationship of receptor structure to function is an important one.

Figure 8. Trypsinization of cell homogenates reveals no labeled BZD-Rs. Results of SDS-PAGE of photolabeled, trypsinized culture homogenates are shown. Membrane homogenates prepared from brain cell cultures were incubated in 0.5 mg/ml trypsin for 90 min at 37°C in HEPES buffer. The enzyme reaction was terminated by placing the incubations on ice and adding soybean trypsin inhibitor. The homogenates were centrifuged and the pellets were resuspended in PBSS, photolabeled with 5 nM ^3H -flunitrazepam, and then subjected to SDS-PAGE. No detectable peaks were observed.

The main conclusions of this study relate to the transmembrane topology and subcellular distribution of the GABA/BZD-R. Exhaustive proteolysis by trypsin of intact neuronal cell cultures from embryonic chick brain demonstrates that 43% of the benzodiazepine binding sites are resistant to, while 57% are inactivated by, trypsinization at the extracellular surface of the plasma membrane. This reduction in binding is due to a decrease in receptor number (Figs. 3, 4). Two types of cell surface receptors can be distinguished based on their differential sensitivity to inactivation by trypsin: those that are inactivated completely by trypsin (57% of total) and others that are cleaved (24% of total) but retain binding activity. The fact that some of the surface binding sites are inactivated quickly while others are cleaved but not inactivated could be due to heterogeneity among the surface benzodiazepine receptors, heterogeneity of receptor subunits, or heterogeneity of cells. In primary neuronal cell cultures different neuronal cell types are present. It is not yet clear whether this difference in the sensitivity to trypsin derives from a single kind of receptor with multiple nonidentical or asymmetrical sites, or from preexisting differences among surface receptors. These results, suggesting receptor heterogeneity, may explain the biphasic kinetics of BZD-R degradation (Borden et al., 1984), as well as other forms of receptor heterogeneity reported (Lo et al., 1982; Sieghart et al., 1983).

The observation that cell disruption by either extensive homogenization or saponin treatment causes some of the previously trypsin-resistant ^3H -flunitrazepam binding to become trypsin-sensitive supports the argument that a large fraction of the potentially trypsin-sensitive benzodiazepine binding sites are protected by the plasmalemma. There are at least 3 possible explanations for these results: (1) the neurons might contain an internal pool of receptors; (2) the segment of receptor on which the ligand recognition site resides might contain a membrane-embedded domain, spanning the membrane and sensitive to trypsin attack at its cytoplasmic face; or (3) trypsinization might stimulate sequestration or internalization of BZD-Rs, protecting them from further proteolysis.

Competition studies performed on intact cells, using the quaternized benzodiazepine Ro7-0213, also suggest the existence of both surface and internal benzodiazepine binding sites (Fig. 6). In the presence of a saturating concentration of Ro7-0213, approximately 20% of the specific irreversible ^3H -flunitrazepam binding is resistant to competition and may represent internal and/or sequestered BZD-Rs. These sites are also trypsin-resistant. This result indicates that trypsin does not cross the plasma membrane. The observation that 80% of the binding is sensitive to competition by the quaternized benzodiazepine strongly suggests that these sites face extracellularly and are associated with cell-surface BZD-Rs. In electrophysiological experiments, Ro7-

Figure 9. Model of the subcellular distribution of the BZD-R is shown. Eighty percent of the BZD binding sites are susceptible to both trypsin attack and competition by the quaternized BZD, Ro7-0213, and are represented as cell-surface BZD-Rs containing their ligand recognition sites facing extracellularly. Trypsin and Ro7-0213 cannot readily cross the plasma membrane and have access only to these extracellularly directed binding sites. Twenty percent of the binding sites are protected by the intact plasma membrane from both trypsin and Ro7-0213 and probably represent BZD-Rs associated with intracellular membranes. Flunitrazepam (FNZ), a typical BZD, is lipophilic and has access to both sides of the plasmalemma. Following saponin treatment, the plasma membrane is disrupted, and trypsin, Ro7-0213, and flunitrazepam all have access to both sides of the plasma membrane.

0213 potentiates the GABA-mediated chloride conductance in chick spinal cord neurons in culture (D. Mierlak, unpublished observations), further pointing to the presence of functional cell-surface BZD-Rs. Following permeabilization of the plasmalemma with saponin, all of the BZD binding sites become sensitive to competition by Ro7-0213. These results are in agreement with those obtained from trypsinization experiments using cell homogenates and saponin-treated cells and provide additional evidence that saponin is affecting the integrity of the plasmalemma and not directly affecting the BZD-R.

SDS-PAGE of trypsinized and photolabeled intact cells reveals a decrease in labeling of the normally observed $M_r = 51,000$ and $48,000$ receptor subunits (approximately 20% remaining) and the appearance of a smaller receptor fragment that retains its recognition site for flunitrazepam and can be photolabeled subsequent to trypsin treatment (approximately 20% of control labeling; Table 1, Fig. 7). Thus, the reversible and irreversible binding measured by filtration following trypsinization of intact cells (approximately 40% of control) represent both intact benzodiazepine receptors and a membrane-associated trypsin-generated fragment of the receptor that retains the ligand binding site. Following disruption of the plasmalemma, the trypsin-resistant binding sites become trypsin-sensitive and the $M_r = 24,000$ fragment is not observed (Figs. 7, 8). This suggests that the receptor fragment is a transmembrane segment of the BZD-R.

A similar trypsin-generated fragment has been reported when photolabeled rat brain homogenates were exposed to proteolytic enzymes (Eichinger and Sieghart, 1985; Klotz et al., 1984). In these studies, trypsin only partially inactivated the benzodiazepine binding sites. This is in contrast to the results reported here using both trypsinized culture homogenates and homogenates

Figure 10. Model depicting the generation of the $M_r = 24,000$ transmembrane BZD-R fragment. Following trypsinization of intact cells, a $M_r = 24,000$ receptor fragment is generated from either one or both of the original receptor subunits ($M_r = 51,000$ and $48,000$), indicating the presence of at least one extracellular trypsin-sensitive site (eTS) on the subunits. The fragment retains BZD binding activity and remains associated with the plasma membrane. The $M_r = 24,000$ receptor fragment is not observed following trypsin treatment of saponin-treated cells or cell homogenates, indicating that it spans the plasma membrane and contains a cytoplasmic trypsin-sensitive site ($cyTS$). The solid shading represents receptor proteins that exhibit binding activity and are measured under our electrophoretic conditions; stippling represents the trypsin-cleaved fragments of the BZD-R that are not measured by our techniques and may or may not be associated with the plasma membrane; e and c represent the extracellular and cytoplasmic side of the plasmalemma, respectively.

prepared from 20-d-old chick brains. The discrepancy may be due to differences in experimental conditions, such as homogenization techniques, membrane protein concentration, buffer, temperature, trypsin concentration, and time of incubation in trypsin. In principle, purified membranes form closed vesicles *in vitro* and proteases attack the proteins exposed on the outside of these vesicles exclusively. The vesicles, once formed, can be disrupted to varying degrees by different experimental conditions, e.g., warming, vigorous mechanical agitation, sonication, freezing-thawing, and hypo-osmotic treatment (Whittaker, 1969). Thus, the difference in protease sensitivity observed by Eichinger and Sieghart (1985) using rat brain homogenates compared to our results using culture and 20-d-old chick brain homogenates may be due to different experimental conditions during membrane homogenate preparation, resulting in varying amounts of sealed and unsealed vesicles. In the present study, exhaustive trypsinization of intact cells, saponin-treated cells, and homogenates prepared from cell cultures was examined. By using intact cells in culture, the condition of the vesicles formed by homogenization is not an issue, and a closer approximation to the *in vivo* situation is achieved.

In summary, we conclude that approximately 80% of the BZD-Rs in intact cells are susceptible to trypsin attack and may represent surface receptors, while 20% are inaccessible to trypsin or Ro7-0213 and may represent sequestered or intracellular receptors (Fig. 9). Following trypsin treatment of intact cells, a cleaved receptor fragment of $M_r = 24,000$ is generated. It remains anchored in the plasma membrane and retains the ability to bind 3H -flunitrazepam reversibly and irreversibly. After either saponin treatment or extensive homogenization, trypsin significantly degrades the fragment, indicating that it has a cytoplasmic domain as well as a cell-surface domain, as expected for a transmembrane segment of the BZD-R (Fig. 10). *In situ* trypsinization combined with the use of membrane-impermeable recognition site ligands for the benzodiazepine receptor should

provide a useful tool for probing the structural features of the GABA/BZD-R involved in the processes of ligand binding and transduction as well as receptor turnover, assembly, and processing.

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