Localization of the Strychnine Binding Site on the 48-Kilodalton Subunit of the Glycine Receptor†

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ABSTRACT: Amino acid residues that participate in antagonist binding to the strychnine-sensitive glycine receptor (GlyR) have been identified by selectively modifying functional groups with chemical reagents. Moreover, a region directly involved with strychnine binding has been localized in the 48-kDa subunit of this receptor by covalent labeling and proteolytic mapping. Modification of tyrosyl or arginyl residues promotes a marked decrease of specific [^{3}H]strychnine binding either to rat spinal cord plasma membranes or to the purified GlyR incorporated into phospholipid vesicles. Occupancy of the receptor by strychnine, but not by glycine, completely protects from the inhibition caused by chemical reagents. Furthermore, these tyrosine- or arginine-specific reagents decrease the number of binding sites (B_{max}) for [^{3}H]strychnine binding without affecting the affinity for the ligand (K_d). These observations strongly suggest that such residues are present at, or very close to, the antagonist binding site. In order to localize the strychnine binding domain within the GlyR, purified and reconstituted receptor preparations were photoaffinity labeled with [^{3}H]-strychnine. The radiolabeled 48-kDa subunit was then digested with specific chemical proteolytic reagents, and the peptides containing the covalently bound radioligand were identified by fluorography after gel electrophoresis. *N*-Chlorosuccinimide treatment of [^{3}H]strychnine-labeled 48K polypeptide yielded a single labeled peptide of M_r ~7300, and cyanogen bromide gave a labeled peptide of M_r 6200. Examination of the cleavage sites of these proteolytic reagents in the known primary sequence of the 48-kDa subunit [Grenningloh et al. (1987) *Nature 328*, 215–220] indicates that the [^{3}H]strychnine binding site is located within amino acids 171–220 of this protein and probably involves tyrosine-197 or tyrosine-202. The proposed antagonist binding site corresponds to an extracellular domain close to the first transmembrane region of the 48-kDa subunit. A similar location of ligand binding sites has been reported for the α-subunits of nicotinic acetylcholine receptors. Thus, our results further reinforce the structural homologies observed between members of the ligand-gated receptor-channel family.

The glycine receptor (GlyR)[1] is a multimeric membrane glycoprotein that transiently opens a chloride channel in response to ligand binding, thus producing postsynaptic membrane hyperpolarization (Aprison & Daly, 1978; Betz, 1987). Glycine-mediated inhibitory actions are blocked by the convulsive alkaloid strychnine, and this selective antagonist has been utilized as a tool for GlyR purification and characterization (Young & Snyder, 1973; Pfeiffer et al., 1982; Marvizón et al., 1986; Ruiz-Gómez et al., 1989a). Purified GlyR preparations from spinal cord of different mammals have been reported to contain two types of glycosylated membrane polypeptides of M_r 48 000 and 58 000, in addition to a 93 000-dalton receptor-associated peripheral membrane protein (Pfeiffer et al., 1982; Schmitt et al., 1987). The 48- and 58-kDa polypeptides are assumed to be homologous on the basis of immunological cross-reactivity and related peptide maps, and have been proposed to be assembled in a pentameric core structure which forms the glycine-gated chloride channel (Pfeiffer et al., 1984; Langosch et al., 1988). Recent cloning of the cDNA of the strychnine binding subunit (48 kDa) of the GlyR has revealed that it shares significant sequence and structural homologies with nicotinic acetylcholine receptor proteins and with the subunits of the $GABA_A$ receptor, another chloride channel coupled receptor (Grenningloh et al., 1987; Schofield et al., 1987; Barnard et al., 1987).

A better understanding of the mechanisms of action of these receptors requires the identification of the functional groups and domains of the protein implicated in the specific interaction with ligands. In the case of the nicotinic acetylcholine receptor, substantial progress has been made in the elucidation of the role of relevant functional groups in receptor function, as well as in the localization of ligand binding sites within the receptor structure (Kao et al., 1984; Dennis et al., 1988; Abramson et al., 1989; Steinbach & Ifune, 1989). However, little is known about the characterization and localization of ligand binding sites in the GlyR. Functional expression of the RNA encoding the 48-kDa subunit of the glycine receptor in Xenopus oocytes and mammalian cells has demonstrated that this polypeptide carries glycine and strychnine binding sites (Schmieden et al., 1989; Sontheimer et al., 1989). Photoaffinity labeling experiments have also localized the strychnine binding site of the GlyR on the 48-kDa subunit (Graham et al., 1981, 1983), although the 58-kDa polypeptide is also labeled when purified GlyR preparations are used (García-Calvo et al., 1989). Previous pharmacological studies have suggested that glycine and strychnine bind to different, mutually interactive sites of the GlyR (Young & Snyder, 1974; Marvizón et al., 1986). Finally, recent chemical modification

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[1] Abbreviations: DS, diazotized sulfanilate; GlyR, glycine receptor; NAI, *N*-acetylimidazole; NCS, *N*-chlorosuccinimide; PG, phenylglyoxal; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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experiments from our laboratory have confirmed the location of glycine recognition site(s) in the 48-kDa polypeptide and provided further evidence for the presence of distinct binding sites for glycine and strychnine at the GlyR (Ruiz-Gómez et al., 1989b).

In this paper, we have used a chemical modification approach in order to identify specific amino acid residues in the GlyR involved in its interaction with the antagonist strychnine. Further, we have utilized photoaffinity labeling and peptide mapping of the purified and reconstituted GlyR (García-Calvo et al., 1989) to localize regions of the 48-kDa polypeptide that are involved with strychnine binding. Our results indicate that tyrosine and arginine residues are implicated in the binding of the antagonist to the GlyR and suggest that the strychnine recognition site might be located in an extracellular domain close to the first transmembrane region of the 48-kDa subunit. The relative position of such a domain resembles the location of binding sites at the α-subunits of the nicotinic acetylcholine receptor (Steinbach & Ifune, 1989), thus reinforcing the homology observed between these receptor proteins.

Experimental Procedures

Materials. Most chemicals were from sources previously described (García-Calvo et al., 1989). [^{3}H]Strychnine (15–30 Ci/mmol) was obtained from Amersham. Dithiothreitol, diethyl pyrocarbonate, and *N*-chlorosuccinimide were purchased from Sigma. *N*-Acetylimidazole was obtained from Aldrich, phenylglyoxal from Fluka, diazotized sulfanilate from Calbiochem, and cyanogen bromide from Merck. Molecular weight standards were purchased from Bio-Rad.

Glycine Receptor Preparations. Chemical modification experiments were performed using either GlyR-enriched membranes or purified GlyR incorporated into phospholipid vesicles. Synaptic plasma membranes from medulla oblongata and spinal cord of male Wistar rats were prepared as described (Ruiz-Gómez et al., 1989b). The final membrane pellets were resuspended in 25 mM potassium phosphate buffer, pH 7.4, and stored at −60 °C until used. GlyR was purified from rat spinal cord by affinity chromatography on 2-aminostrychnine–agarose (Schmitt et al., 1987; García-Calvo et al., 1989) and reconstituted into phosphatidylcholine vesicles as recently described by our laboratory (García-Calvo et al., 1989). The reconstituted GlyR receptor preparations bound ~3 nmol of [^{3}H]strychnine/mg of protein, displayed two protein bands of M_r 48 000 and 58 000 when subjected to SDS–PAGE, and showed pharmacological and functional characteristics similar to those observed in native membranes (García-Calvo et al., 1989). Such reconstituted preparations consist of small diameter lipid vesicles in a medium containing 120 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 25 mM potassium phosphate, pH 7.4, and protease inhibitors (1 mM benzamidine, 17 milliunits/mL aprotinin, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1 mM benzetonium chloride), except when indicated otherwise. Reconstituted GlyR was used within 3 h of preparation from the purified receptor.

Reaction with Group-Specific Reagents. Except where indicated otherwise, spinal cord membranes were suspended at a protein concentration of 1–2 mg/mL in 200 mM KCl/25 mM potassium phosphate buffer, pH 7.5 (buffer A), and incubated in the presence of the desired final concentrations of different protein-modifying reagents, added from fresh concentrated solutions. Stock solutions of phenylglyoxal and diethylpyrocarbonate were made in ethanol and those of diazotized sulfanilate and *N*-acetylimidazole in buffer A. The following incubation conditions were used to achieve modification specificity (Takashaki, 1968; Burch et al., 1983; Peerce & Wright, 1985): phenylglyoxal, 37 °C; diazotized sulfanilate, 37 °C in the dark; diethyl pyrocarbonate, 4 °C, the pH of buffer A being changed to 6.0; *N*-acetylimidazole, 25 °C. The mixtures were incubated with gentle shaking for the time indicated in the figures. The reactions were terminated by addition of 10 volumes of ice-cold buffer A. Unreacted reagent was removed by centrifugation at 48000*g* for 20 min. The resulting pellets were washed 3 times by repeated centrifugation and resuspension in the same medium, and finally resuspended in a small volume of buffer A and used in binding studies (see below).

To test the ability of agonist or antagonist ligands to protect against the effects of the different reagents, glycine or strychnine was coincubated with the membranes during the treatments. Control membrane suspensions were taken through the same steps of reagent-treated membranes in each experiment.

Other chemical modification experiments were performed in reconstituted GlyR preparations. GlyR (4–8 pmol of [^{3}H]strychnine binding sites) was incubated with gentle shaking with the desired concentrations of phenylglyoxal or diazotized sulfanilate at 37 °C in the dark in reconstitution buffer (see above). Experiments were performed in either the presence or the absence of 5 mM DTT in the incubation and reconstitution buffers. Protection experiments were carried out by coincubation with the desired ligand. The reactions were terminated by rapidly separating the receptor from the excess reagent (and the protecting ligand, when present) by using a Sephadex G-50 minicolumn centrifugation technique (Vázquez et al., 1988). The eluted proteoliposomes were assayed for [^{3}H]strychnine binding activity. Control aliquots of reconstituted GlyR were subjected to the same process in the absence of reagents.

Binding Assays. [^{3}H]Strychnine binding to control or treated synaptic membranes suspended in buffer A was determined as previously described (Ruiz-Gómez et al., 1989b). Displacement of [^{3}H]strychnine binding by glycine was investigated by using 2 nM [^{3}H]strychnine. The density of binding sites (B_{max}) and the equilibrium dissociation constant for [^{3}H]strychnine were determined by Scatchard analysis. Binding to the reconstituted GlyR was performed by a similar method, except that the radioligand concentration was 25 nM and the Millipore HAWP 02500 filters were used instead of Whatman GF/B glass fiber filters (García-Calvo et al., 1989). All binding assays were performed in triplicate. Nonspecific binding was defined in the presence of either 100 μM strychnine or 10 mM glycine.

Photoaffinity Labeling. The reconstituted GlyR was incubated with 40 nM [^{3}H]strychnine for 30 min at 4 °C and subjected to photoaffinity labeling as described (Graham et al., 1985; García-Calvo et al., 1989). The source of ultraviolet light was an Osram-HBO 100w/2 lamp filtered through a chloroform solution. After the illumination period, samples were freezed in liquid nitrogen, lyophilized, and disolved in SDS buffer (8% SDS, 10% glycerol, 5% β-mercaptoethanol, and 25 mM Tris-HCl, pH 6.5). Photolabeled GlyR samples were analyzed by 10% SDS–PAGE and processed for fluorography (Bonner & Laskey, 1974). Dried gels were exposed to Hyperfilm-MP at −70 °C. In other experiments, gels were processed for Coomassie Blue staining.

Chemical Cleavage and Peptide Mapping of the Photoaffinity-Labeled 48-kDa Subunit of the GlyR. After SDS–PAGE in 10% polyacrylamide slab gels and fluorography, an autoradiogram was used as a guide to cut out the 48-kDa subunit photoaffinity labeled with [^{3}H]strychnine. The 48-kDa

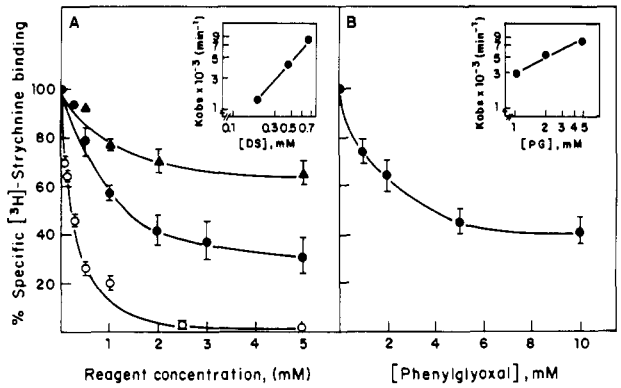

FIGURE 1: Effects of different protein-modifying reagents on [^{3}H]-strychnine binding to rat spinal cord synaptic plasma membranes. Suspensions of synaptic membranes were pretreated with the indicated concentrations of chemical reagents for 30 min and incubated with 2 nM [^{3}H]strychnine as detailed under Experimental Procedures. The values are the mean ± SEM of four to seven independent determinations performed in triplicate. Specific [^{3}H]strychnine binding to untreated membranes (800–1000 pmol/mg of protein) was taken as 100% binding in each experiment. The following protein-modifying reagents were tested: (A) diethylpyrocarbonate (▲), *N*-acetylimidazole (●), and diazotized sulfanilate (○); (B) phenylglyoxal (●). The time course (5–60 min) of inhibition of [^{3}H]strychnine binding was studied at different diazotized sulfanilate and phenylglyoxal concentrations, and the observed inactivation rate constants (K_{obs}) were determined as described (Cheng & Nowak, 1989). The insets to panels A and B show the double logarithmic plot of K_{obs} versus DS and PG concentration, respectively.

band was excised and extracted from the dried gel, as previously reported (Kawata et al., 1989), and chemically hydrolyzed at tryptophan residues with *N*-chlorosuccinimide (NCS) as described (Lischwe & Ochs, 1982) or digested at methionine residues with cyanogen bromide as reported (Lam & Kasper, 1980). In some experiments, cleavage was performed directly in the gel. The proteolytic fragments were separated by gel electrophoresis on a 12–20% linear gradient of polyacrylamide according to the method of Cleveland for peptide mapping (Cleveland et al., 1977) or by gel electrophoresis in 20% polyacrylamide, 0.5% bis(acrylamide), and 10% glycerol as previously described; this method allows an accurate determination of the molecular weight of low molecular weight peptides (Giulian et al., 1983). In either case, the labeled peptides generated by chemical cleavage were identified by fluorography as described above, and their apparent molecular weight was determined on the basis of the relative mobility of an appropriate set of standards.

Results

Pretreatment of synaptic plasma membranes with low concentrations of diazotized sulfanilate (DS) markedly inhibited specific [^{3}H]strychnine binding (Figure 1A). Such an effect could be due to chemical modification of tyrosine or histidine residues (Farroqui, 1976). In order to distinguish between these possibilities, similar experiments were performed using *N*-acetylimidazole or diethyl pyrocarbonate, that would preferentially react with tyrosine and histidine residues, respectively (Peerce & Wright, 1985; Burch et al., 1983). Data in Figure 1 indicate that the tyrosine-specific reagent *N*-acetylimidazole promotes a marked decrease in [^{3}H]strychnine binding, whereas diethyl pyrocarbonate has little effect, suggesting that DS abolished specific antagonist binding to the GlyR by modifying tyrosine functional groups in the receptor protein. The time course of inhibition of [^{3}H]strychnine binding by different DS reagent concentrations was also studied. Semilog plots of remaining binding activity versus time are linear (data not shown), indicating that inactivation is pseudo first order and likely due to a simple chemical event. Furthermore, the loss of [^{3}H]strychnine binding is consistent with the modification of a single tyrosine residue, since the double logarithmic plot of the observed inactivation rate constants (K_{obs}) versus inhibitor concentration is linear (Figure 1A inset) and displays a slope of 0.85 (Cheng & Nowak, 1989).

Table I: Effect of Diazotized Sulfanilate and Phenylglyoxal on [^{3}H]Strychnine Binding to the Glycine Receptor: Specific Protection by Strychnine[a]

membrane pretreatment	[^{3}H]strychnine-specific binding (% of control)
none	100
glycine (1 mM)	102 ± 4
strychnine (10 μM)	93 ± 3
phenylglyoxal (5 mM)	46 ± 3[b]
phenylglyoxal (5 mM) + glycine (1 mM)	59 ± 4[b]
phenylglyoxal (5 mM) + strychnine (0.1 μM)	78 ± 2[c]
phenylglyoxal (5 mM) + strychnine (1 μM)	82 ± 3[c]
phenylglyoxal (5 mM) + strychnine (10 μM)	89 ± 4[c]
diazotized sulfanilate (0.1 mM)	63 ± 3[b]
diazotized sulfanilate (0.1 mM) + glycine (1 mM)	62 ± 3[b]
diazotized sulfanilate (0.1 mM) + strychnine (10 μM)	100 ± 8[c]
diazotized sulfanilate (0.2 mM)	44 ± 0.5[b]
diazotized sulfanilate (0.2 mM) + strychnine (1 μM)	78 ± 7[c]

[a] Membranes were pretreated in the presence of the indicated reagents and glycine receptor ligands as described under Experimental Procedures. Binding assays were performed as in Figure 1. The values are the mean ± SEM of at least three independent determinations performed in triplicate. [b] $p < 0.001$ when compared to control (no pretreatment). [c] $p < 0.01$ when compared to reagent alone.

A similar set of experiments was performed using phenylglyoxal, an arginine-specific reagent (Takashaki, 1968). Phenylglyoxal inhibits [^{3}H]strychnine-specific binding in a concentration- (Figure 1B) and time-dependent way; the plot of K_{obs} versus reagent concentration is also linear (Figure 1B inset) and displays a slope of 0.95, suggesting that one arginine residue is modified to promote inhibition of [^{3}H]strychnine binding. The effect of the simultaneous presence of both types of reagents on [^{3}H]strychnine binding was also investigated. Pretreatment of synaptic membranes with 0.1 mM DS and 1 mM PG promoted a 51 ± 4% decrease in antagonist binding. Such loss of binding activity is more marked than that promoted by the same concentrations of the reagents acting independently (36 ± 3% and 25 ± 4% for DS and PG, respectively; values are the mean ± SEM of three independent experiments in all cases).

The role of these essential tyrosine and arginine residues in [^{3}H]strychnine binding was further studied in protection experiments. Table I shows that occupancy of the receptor by strychnine prevented the effect of either DS or PG, whereas the presence of the agonist glycine resulted in no protection. The characteristics of inhibition were not dependent (data not shown) on the presence or absence in the incubation media of anions (Cl^-, Br^-, I^-, etc.) known to interact with the GlyR (Marvizón et al., 1986). The specific protection afforded by strychnine could be detected at very low antagonist concentrations (0.1 μM) and was almost total at 10 μM strychnine. Taken together, these results indicate that the tyrosine and arginine residues modified by DS and PG are located in or near the strychnine binding site in the glycine receptor.

Moreover, when the saturation curves for [^{3}H]strychnine binding in control conditions and in membranes pretreated with different protein modifying reagents were compared, the Scatchard plots (Figure 2) indicate that the effects of *N*-

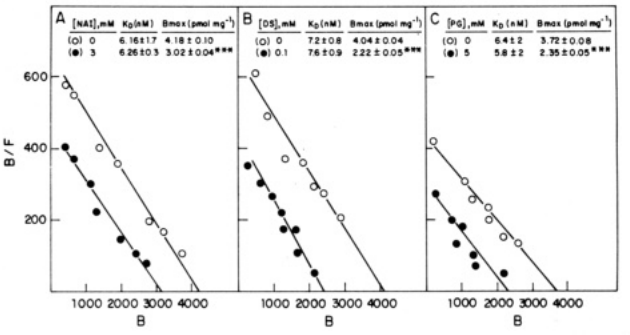

FIGURE 2: Scatchard plots from the equilibrium binding data of [^{3}H]strychnine to control and treated membranes. Saturation experiments of [^{3}H]strychnine binding (1–40 nM) were performed using membranes pretreated for 30 min as detailed under Experimental Procedures with the indicated concentrations of *N*-acetylimidazole [(●) panel A], diazotized sulfanilate [(●) panel B], or phenylglyoxal [(●) panel C], and untreated preparations [(○) panels A–C]. K_d and B_{max} values are presented as the mean ± SEM of three independent experiments performed in triplicate. A representative plot is shown for each experimental condition. (B) is expressed as femtomoles of [^{3}H]strychnine specifically bound per milligram of protein, and F is the concentration of free [^{3}H]strychnine in nanomolar. (***) $p <$ 0.001 when compared to control (no treatment).

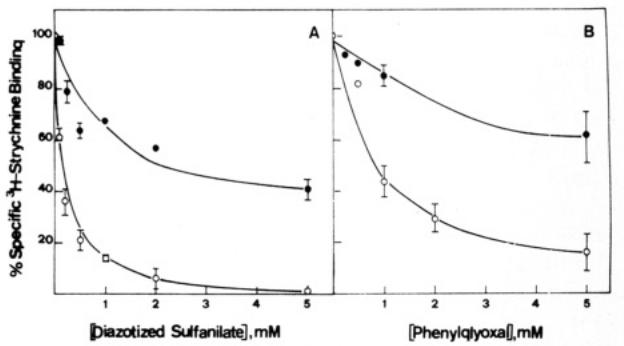

FIGURE 3: Effect of modification of the purified and reconstituted glycine receptor with diazotized sulfanilate or phenylglyoxal on [^{3}H]strychnine binding. Preparations of purified GlyR incorporated into phospholipid vesicles were pretreated for 30 min with the indicated concentrations of diazotized sulfanilate (A) or phenylglyoxal (B) in the absence (○) or presence (●) of dithiothreitol and assayed for specific [^{3}H]strychnine binding, as detailed under Experimental Procedures. The values shown are the mean ± SEM of three to five independent experiments performed in triplicate.

acetylimidazole, diazotized sulfanilate, and phenylglyoxal are due to a significant decrease in the number of antagonist binding sites (B_{max}) in the GlyR, with no changes in affinity (K_d). These results strongly suggest that the tyrosine and arginine residues are directly involved in the interaction with strychnine. In contrast, treatment of the membranes with diethylpyrocarbonate resulted in a 2-fold decrease in affinity without significant changes in B_{max} (data not shown).

Similar results were obtained in chemical modification experiments performed with purified and reconstituted glycine receptor (García-Calvo et al., 1989). Figure 3 shows that treatment of such preparations with increasing diazotized sulfanilate or phenylglyoxal concentrations produced a loss of specific [^{3}H]strychnine binding sites similar to that observed in spinal cord membranes, although the extent of the effect of phenylglyoxal modification is better assessed in the reconstituted preparation (compare the 90% inhibition shown in Figure 3B for 5 mM PG with the 60% inhibition detected in membranes at the same reagent concentration). Preincubation of the reconstituted receptor with 10 μM strychnine completely prevented the effects of either DS or PG on the binding of the antagonist ligand, whereas glycine did not afford protection

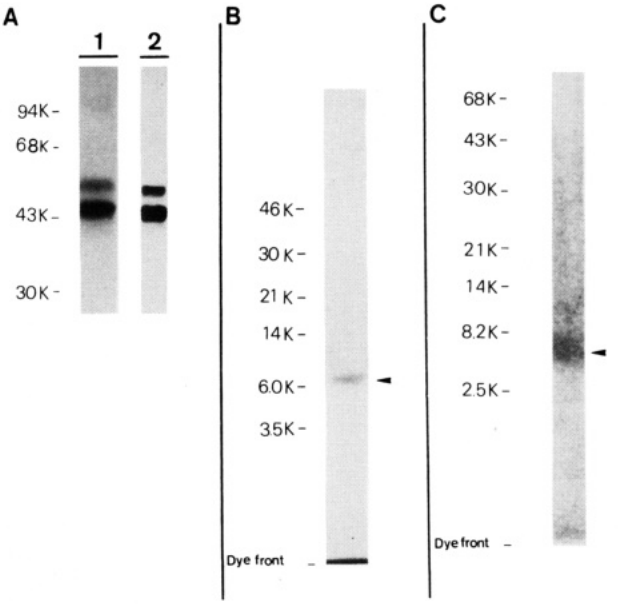

FIGURE 4: Photoaffinity labeling of purified and reconstituted glycine receptor by [^{3}H]strychnine and chemical cleavage of the labeled 48-kDa subunit. (A) Lane 1, SDS–PAGE of a reconstituted GlyR preparation (corresponding to ~8 pmol of [^{3}H]strychnine binding sites) used for photoaffinity labeling studies as visualized by Coomassie Blue staining. Lane 2, representative photoaffinity labeling of the reconstituted GlyR by [^{3}H]strychnine. Reconstituted preparations (6–8 pmol of strychnine binding sites) were incubated with 40 nM [^{3}H]strychnine and subjected to ultraviolet light, SDS–PAGE, and fluorography as detailed under Experimental Procedures. The positions of the molecular weight standards are shown on the left. The other panels show fluorographs of labeled peptides generated by complete proteolysis of the [^{3}H]strychnine-labeled 48-kDa subunit of the GlyR with *N*-chlorosuccinimide (B) or with cyanogen bromide (C). After photoaffinity labeling, the 48-kDa band was extracted from the gel and treated with NCS or cyanogen bromide as described under Experimental Procedures. The [^{3}H]strychnine-labeled fragments generated were resolved in 20% polyacrylamide, 0.5% bis(acrylamide), and 10% glycerol gels and visualized by fluorography. Results are representative of three experiments. The positions of relative molecular mass markers and of the dye fronts are shown on the left.

(data not shown). When the reconstituted GlyR was treated with DS or PG in the presence of the disulfide-reducing agent dithiothreitol (Figure 3, solid circles), the effects of the former reagents on [^{3}H]strychnine binding were significantly altered. This "masking" effect of dithiothreitol would suggest that the accessibility of the tyrosine and arginine residues could be regulated by sulfhydryl group modification (see below).

In the next set of experiments, we used purified and reconstituted GlyR preparations to localize the strychnine binding site within the receptor structure. Previous studies have established the utility of using [^{3}H]strychnine to photoaffinity label the GlyR (Graham et al., 1981, 1983; García-Calvo et al., 1989). Since strychnine lacks typical photoactivable groups, the reaction has been suggested to involve energy transfer from aromatic amino acids in the receptor (Graham et al., 1983), in agreement with our results (this paper) showing the presence of a tyrosine residue in the binding site of strychnine. Thus, we have used photoaffinity labeling to map this binding site in the 48-kDa polypeptide of the GlyR. Considering the limited quantities of purified GlyR available, we have mapped the site of photoaffinity labeling by using two chemical proteolytic reagents of differing substrate specificities. Because the primary structure of the 48-kDa subunit is known (Grenningloh et al., 1987) and the proteolytic reagents are highly sequence-specific, the site of label incorporation may be deduced from the sizes of the labeled fragments eventually generated.

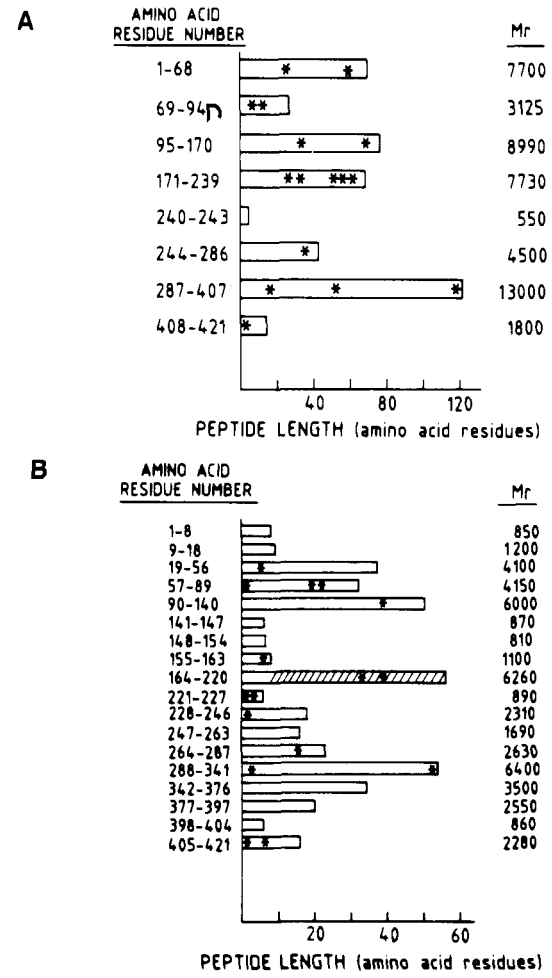

FIGURE 5: Histogram of the peptides that could theoretically be generated from a complete digest of the 48-kDa subunit of the rat spinal cord glycine receptor by *N*-chlorosuccinimide (A) or cyanogen bromide (B). The different limit peptides are arranged according to their position in the complete amino acid sequence of this protein (Grenningloh et al., 1987) and are identified by the amino acid residue numbers shown on the left. The lengths of the peptides are shown on the *x* axis, and the calculated molecular molecular weights of the peptides are shown on the right. Molecular weights were calculated on the basis of the actual amino acid composition and are corrected for peptide bonds. These histograms are based on those used by Matsui et al. (1989) for analyzing similar experiments performed with the α_2-adrenergic receptor. Asterisks indicate the position of tyrosine residues, and the shaded area in peptide 164–220 of scheme B denotes the region of the 48-kDa subunit (amino acids 171–220) that would be labeled by [^{3}H]strychnine according to the data provided by the two proteolytic treatments.

Purified and reconstituted GlyR preparations consisting of polypeptides of 48 and 58 kDa (Figure 4A, lane 1) were photoaffinity labeled with [^{3}H]strychnine as described under Experimental Procedures. Since the radioligand is also incorporated to some extent to the 58-kDa subunit (Figure 4A, lane 2), the 48-kDa band was excised from the gel, extracted, and specifically cleaved at tryptophan residues with *N*-chlorosuccinimide (Lischwe & Ochs, 1982). Figure 4B shows that complete digestion of the 48-kDa subunit by NCS generates a single labeled peptide of M_r ~7.3K. Shorter periods of incubation with NCS resulted in partial cleavage of the photolabeled 48-kDa polypeptide; the lower molecular weight labeled peptide migrated on 12–20% acrylamide gradient gels with a molecular weight of ~7.6K, and other bands of M_r 8600, 11 200, and 12 700 were also observed in the lower part of the gel (data not shown). Figure 5A shows the peptides that could theoretically be generated from a complete digest of the 48-kDa subunit of the GlyR by NCS. The analysis of these predicted products of cleavage indicates that the labeled band of ~7.3 kDa most likely represent the peptide corresponding to amino acids 171–239 of the 48-kDa sequence (predicted M_r 7730). In addition to the similarity in apparent molecular weight, which is also displayed by peptide 1–68 (predicted M_r 7700), [^{3}H]strychnine labeling of peptide 171–239 could best explain the appearance of other labeled peptides generated as partial cleavage products. Moreover, labeling of peptide 1–68 could not account for the labeled fragment obtained after CNBr digestion (see below). In addition, it has been previously described that trypsin treatment that removes a fragment of ~11 kDa from the 48-kDa subunit (i.e., approximately 100 amino acids) does not affect the photoaffinity label of the GlyR subunit (Graham et al., 1983).

In order to further characterize the site of strychnine binding, cyanogen bromide was also used to localize the region of the 48-kDa subunit of the GlyR photolabeled by [^{3}H]-strychnine. Cyanogen bromide is specific for methionine residues and cleaves on the carboxyl terminal of the peptide bond. Figure 4C shows that an 18-h treatment with this proteolytic reagent generates a major labeled peptide of M_r 6.2K; another band migrating at ~7.45 kDa could also be seen in some experiments (not shown). There are three limit fragments of ~6.2 kDa predicted by the specific cleavage of methionine residues in the 48-kDa subunit of the GlyR (see Figure 5B), corresponding to amino acid residues 90–140 (M_r 6K), 164–220 (M_r 6.2K), and 288–341 (M_r 6.4K). The fact that complete digestion of the labeled receptor subunit with the other proteolytic reagent (NCS) generates a single tritium-labeled fragment of M_r ~7.3K (corresponding to peptide 171–239, see above) rules out peptides 90–140 and 288–341 as possible labeled products of CNBr cleavage (see Figure 5A,B), indicating that strychnine labels fragment 164–220 of the 48-kDa protein sequence. Such a peptide contains two tyrosine residues at positions 197 and 202. In conclusion, our results strongly suggest that the [^{3}H]strychnine binding site is located within amino acids 171–220 of the 48-kDa subunit of the GlyR, which is the fragment contained in both of the limit-labeled peptides 171–239 and 164–220 (see shaded area in Figure 5B).

Discussion

We have observed that the ligand binding properties of the GlyR can be altered when synaptic plasma membranes are pretreated with different chemical reagents that modify specific amino acid residues. Lysine-specific reagents such as fluorescein isothiocyanate markedly decreased the ability of glycine for displacing specifically bound [^{3}H]strychnine, without affecting [^{3}H]strychnine binding itself (Ruiz-Gómez et al., 1989b). Although sulfhydryl group reagents did not modify the affinity of strychnine or glycine for the GlyR, they showed a remarkable effect on the allosteric interaction between the strychnine and glycine binding sites (Ruiz-Gómez et al., submitted for publication).

Treatment of synaptic plasma membranes from rat spinal cord or preparations of purified and reconstituted GlyR (García-Calvo et al., 1989) with chemical reagents highly selective for the modification of tyrosyl (diazotized sulfanilate, *N*-acetylimidazole) or arginyl (phenylglyoxal) residues promoted a marked decrease of specific [^{3}H]strychnine binding to the GlyR in a time- and dose-dependent way. The kinetic analysis of the inhibition reaction suggests that only one tyrosine residue and one arginine residue have to be modified in order to prevent radioligand binding. On the other hand, the observations that either diazotized sulfanilate or phenyl-

glyoxal alone can completely abolish specific [^{3}H]strychnine binding to the GlyR and that the effects of these reagents are additive indicate that both tyrosine and arginine residues are necessary for the interaction of strychnine with the receptor. Further, the specific protection afforded by strychnine against diazotized sulfanilate or phenylglyoxal-induced modification and the fact that these reagents decrease the number of binding sites (B_{max}) for [^{3}H]strychnine without affecting ligand affinity (K_d) indicate that such tyrosine and arginine residues are at or very close to the binding site of the antagonist and strongly suggest their direct involvement in the interaction with strychnine.

A role for tyrosine residues in strychnine binding has been previously suggested by preliminary chemical modification experiments (Young & Snyder, 1974) and by the mechanism of photoaffinity labeling of the GlyR by [^{3}H]strychnine that seems to involve energy transfer from aromatic amino acids (Graham et al., 1983; Grenningloh et al., 1987). Although detailed structure–activity studies on the binding of strychnine derivatives to the GlyR are lacking, it seems that the basic nitrogen at position 19 (largely protonated at physiological pH) and the carbonyl group at position 10 in ring III are important determinants for recognition of strychnine by the GlyR (Mackerer et al., 1977; Marvizón et al., 1986; Phelan et al., 1989; García-Calvo, unpublished results). These functional groups in the strychnine molecule could interact with tyrosine or arginine residues in the receptor by hydrogen bonding or by means of saline bridges or partial electrostatic interactions. However, the role of other residues of the GlyR in the recognition of the antagonist or in the conformation of the binding site cannot be disregarded. For instance, our results indicate that the modification of histidine residues does slightly affect strychnine binding affinity and that sulfhydryl group modification modulates the accessibility of the strychnine binding site. The results presented in this paper, together with others recently reported by our laboratory (Ruiz-Gómez et al., 1989b) demonstrating the specific involvement of lysine residues in the interaction of the GlyR with glycine, confirm that glycine and strychnine bind to different although mutually interactive sites of the receptor protein, as have been suggested by previous studies (Young & Snyder, 1974; Braestrup et al., 1986; Marvizón et al., 1986; Ruiz-Gómez et al., 1989a).

In order to further characterize and localize the strychnine binding site, and given the limited quantities of purified GlyR available, we have taken advantage of the fact that the primary structure of the 48-kDa subunit of the receptor is known (Grenningloh et al., 1987), together with the ability of [^{3}H]strychnine to covalently label this protein. Thus, we have subjected the purified GlyR reconstituted into phospholipid vesicles (García-Calvo et al., 1989) to photoaffinity labeling by [^{3}H]strychnine and analyzed the molecular weight of the labeled peptides generated after chemical cleavage of the 48-kDa subunit with two different, highly sequence-specific proteolytic reagents. As discussed in detail under Results, the labeled peptide (M_r ~7.3K) generated as a result of complete digestion with NCS (Figure 4B) and the overall peptide labeling pattern obtained after partial digestion with this agent, as well as the major labeled peptide of 6.2 kDa generated after continued treatment with cyanogen bromide, are consistent with the site of [^{3}H]strychnine binding being located within amino acids 171–220 of the 48-kDa protein sequence (Figure 5B). According to the current topological models for the subunits of chloride channel coupled receptors (Schofield et al., 1987), the proposed antagonist binding region would be located in an extracellular domain close to the first membrane-spanning region (see scheme in Figure 6).

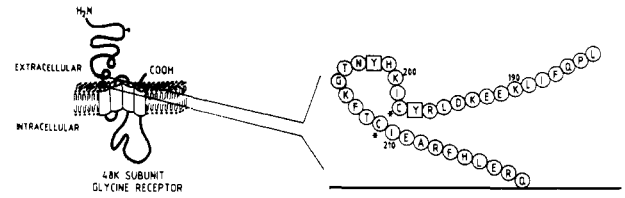

FIGURE 6: Proposed location of the strychnine binding site in the 48-kDa subunit of the glycine receptor. The figure on the left shows a schematic model for the topology in the neuronal membrane of the 48-kDa subunit of the GlyR, based on the model suggested for the $GABA_A$ receptor subunits by Schofield et al. (1987) and data from Grenningloh et al. (1987). The arrowhead indicates a potential N-glycosylation site. The scheme on the right details the amino acid sequence of the region proposed to contain the strychnine binding site. The structure of this extracellular domain close to the first putative membrane-spanning segment is drawn in an arbitrary manner. The numbers indicate the position of the amino acids in the sequence of the 48-kDa protein. Tyrosines at positions 197 and 202 are denoted as squares, and cysteines at positions 198 and 209 are marked with asterisks. Note the presence of arginine residues at positions 196, 213, and 218.

The proposed strychnine binding domain displays several interesting features. First, in agreement with the chemical modification data that suggest the direct involvement of one tyrosine in the GlyR interaction with strychnine, this region contains two tyrosine residues at positions 197 and 202. The essential tyrosine residue would need to be close enough to an arginine residue in the active conformation of the 48-kDa subunit in order to bind the antagonist. Although this particular arginine residue could be contributed by other different regions of the primary structure of the receptor, it is worth noting that several arginines are present in the vicinity of tyrosines 197 and 202 (see Figure 6). However, the localization of the arginine residue involved in strychnine binding has not been attempted and awaits further investigation.

The peptide shown in Figure 6 also contains two closely spaced cysteines. Given the observed effect of sulfhydryl reagents in the accessibility of the tyrosine and arginine residues implicated in strychnine recognition (Figure 3), it is tempting to speculate, in agreement with the suggestion of Betz and collaborators (Grenningloh et al., 1987), that cysteines-198 and -209 may form a disulfide bond loop which adequately exposes the amino acid side chains involved in ligand binding. Hence, the reduction of such a disulfide bond with dithiothreitol would alter the conformation of the strychnine binding site. However, it is worth noting that sulfhydryl reagents do not inhibit strychnine or glycine binding to the GlyR but modulate the allosteric interaction between the agonist and antagonist binding sites instead (Ruiz-Gómez et al., submitted for publication). The role in such processes of other highly conserved Cys-Cys domain present in the extracellular region of the 48-kDa protein (Grenningloh et al., 1987) cannot be disregarded.

Other interesting feature is that the proposed strychnine binding site would be very near the binding region suggested for the agonist glycine. Previous chemical modification and peptide mapping data (Ruiz-Gómez et al., 1989b), as well as sequence analysis (Grenningloh et al., 1987), are consistent with the participation of one or several lysine residues within positions 190–206 of the 48-kDa subunit in glycine binding to the receptor. However, the location of such a site needs to be confirmed in future experiments.

It would be also necessary to investigate the role of the other subunit of the GlyR, the 58-kDa polypeptide, in ligand binding and receptor function. Injection of the synthetic mRNA

corresponding to the full-length cDNA of the 48-kDa subunit in Xenopus oocytes or transfection of mammalian cells with such a cDNA (Schmieden et al., 1989; Sontheimer et al., 1989) led to functional expression of glycine-activated, strychnine-blocked chloride channels. This demonstrates that the 48-kDa subunit contains active binding sites for agonists and antagonists. However, the formation of functional receptor channels, specially those of high affinity for strychnine, occurs with low efficiency in these conditions, thus suggesting an important role for additional subunits (Sontheimer et al., 1989). This is also consistent with the proposed heteropentameric structure of the GlyR (Langosh et al., 1988) and with the observation that [^{3}H]strychnine also covalently labels the 58-kDa subunit of the receptor in photoaffinity labeling experiments (see Figure 4 of this paper). The high structural homology predicted for the 48- and 58-kDa subunits (Pfeiffer et al., 1984; Schmieden et al., 1989) and the amino acid requirements for strychnine interaction shown in this paper suggest the presence of a similar binding domain for strychnine in the 58K protein.

Finally, it is interesting to remark that the relative position of the proposed strychnine binding domain within the structure of the 48-kDa subunit of the GlyR resembles the location of agonist and competitive antagonist binding sites in the α-subunits of the nicotinic acetylcholine receptor [see Steinbach and Ifune (1989) and references cited therein]. This fact further reinforces the homology between these receptor proteins and suggests a similar localization of binding sites in the subunits of the $GABA_A$ receptor, another member of the receptor channel superfamily on which information regarding ligand binding domains is lacking. A better knowledge of the characteristics and localization of the ligand binding sites is essential for understanding the molecular mechanisms of receptor function and for the design of specific and active drugs acting on these important receptor proteins. Determining the exact role of the different domains and functional groups implicated in the interaction with agonists, antagonists, modulators, and ions and in the subsequent conformational changes involved in channel gating would require further analysis involving a combination of biochemical and genetic approaches.

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Existence of a Low-Affinity ATP-Binding Site in the Unphosphorylated Ca^{2+}-ATPase of Sarcoplasmic Reticulum Vesicles: Evidence from Binding of 2′,3′-*O*-(2,4,6-Trinitrocyclohexadienylidene)-[^{3}H]AMP and -[^{3}H]ATP†

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ABSTRACT: ATP-binding sites in the unphosphorylated Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles were titrated with 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-[^{3}H]AMP (TNP-AMP) or -[^{3}H]ATP (TNP-ATP) in the absence of Ca^{2+} at pH 7.0 and 0 °C by using a centrifugation procedure. In some measurements, the bound TNP-nucleotides were chased with ATP. The data were analyzed by best-fit computer programs as well as by Scatchard plots. The results showed the existence of 1 mol of TNP-AMP binding sites with high affinity (K_d = 7.62 nM) per mole of phosphorylatable sites. The affinity of these sites for ATP (K_d = 10.1 μM) agreed with that of catalytic sites for ATP in the absence of Ca^{2+}. The results further showed the existence of 2 mol of TNP-ATP binding sites with uniform affinity (K_d = 156 nM) per mole of phosphorylatable sites. Half of the bound TNP-ATP was fully chased by low concentrations of ATP. The affinity of this class of the sites for ATP (K_d = 8.9 μM) again agreed with that of catalytic sites for ATP. The other half of the bound TNP-ATP was fully chased only by much higher concentrations of ATP. Thus, the affinity of this class of the sites for ATP (K_d = 791 μM) was much lower than that of catalytic sites for ATP. Similar measurements were performed with sarcoplasmic reticulum vesicles pretreated by *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine. Although the affinities for TNP-ATP and for ATP were appreciably altered by this pretreatment, the results were essentially the same as those obtained with native vesicles. These results demonstrate that, in the unphosphorylated enzyme, there exists 1 mol of low-affinity ATP-binding sites as well as 1 mol of high-affinity ATP-binding sites (catalytic sites) per mole of phosphorylatable sites.

The membrane-bound Ca^{2+}-ATPase of sarcoplasmic reticulum catalyzes the active Ca^{2+} transport coupled to ATP hydrolysis (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962). The enzyme consists of a 110-kDa single polypeptide chain (Brandl et al., 1986) that has one catalytic site with a high affinity for ATP (Meissner, 1973; Dupont, 1977; Mitchinson et al., 1982; Inesi et al., 1982).

The ATPase activity is modulated by ATP in a complex manner, so that it exhibits non-Michaelian behavior with respect to ATP (Inesi et al., 1967; Yamamoto & Tonomura, 1967; Dupont, 1977; Verjovski-Almeida & Inesi, 1979). It has been shown that several steps in the catalytic cycle are accelerated by ATP binding to a putative regulatory site(s) at concentrations higher than those required for saturation of the catalytic site (Yamamoto & Tonomura, 1967; de Meis & de Mello, 1973; Froehlich & Taylor, 1975; Scofano et al., 1979; McIntosh & Boyer, 1983). These regulations are due to nonhydrolytic effects of ATP because the accelerations can be also induced by nonhydrolyzable ATP analogues (Dupont, 1977; Taylor & Hattan, 1979; McIntosh & Boyer, 1983; Dupont et al., 1985; Champeil et al., 1988; Seebregts & McIntosh, 1989).

It has been well documented that hydrolysis of EP[1] is modulated by ATP binding to the catalytic site of EP after the departure of ADP (McIntosh & Boyer, 1983; Bishop et al., 1987; Champeil et al., 1988; Seebregts & McIntosh, 1989). On the other hand, there are accumulating observations to suggest that ATP-induced regulations may be due to interaction between two distinct classes of ATP-binding sites or to dimeric interaction between two catalytic sites in the subunits (Ikemoto, 1982; Dupont et al., 1982; Carvalho-Alves et al., 1985; Dupont et al., 1985; Ferreira & Verjovski-Almeida, 1988). However, no convincing evidence for the existence of regulatory ATP-binding sites other than the catalytic site of EP has been presented so far.

One of the main obstacles to conclusive evidence for these regulatory sites is the difficulty of direct titration by ATP due

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[1] Abbreviations: EP, phosphoenzyme; SRV, sarcoplasmic reticulum vesicles; TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-AMP; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)-ATP; I-EDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; EDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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