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CHEMICAL MODIFICATION OF THE GLYCINE RECEPTOR WITH FLUORESCEIN ISOTHIOCYANATE SPECIFICALLY AFFECTS THE INTERACTION OF GLYCINE WITH ITS BINDING SITE

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Summary.- Fluorescein 5'-isothiocyanate (FITC) was used to modify lysine residues of the strychnine-sensitive glycine receptor. Pretreatment of rat spinal cord synaptic plasma membranes with FITC specifically affected the ability of glycine to displace [^{3}H]strychnine binding. Glycine completely prevented the effect of FITC modification, suggesting the existence of lysine group(s) either at or in the vicinity of the agonist binding site. Labeling of purified glycine receptor with FITC indicates that such lysine residue(s) are located in the 48,000 daltons polypeptide. Chemical cleavage of the FITC-labeled 48-kilodalton subunit with N-chlorosuccinimide reveals two major labeled fragments of Mr 13.9 kilodalton and 8.5-kilodalton, respectively, the labeling of each being protected by glycine.

The glycine receptor (GlyR) is a neurotransmitter-gated chloride channel protein that mediates postsynaptic inhibition in spinal cord and other areas of the central nervous system (1). Purified GlyR preparations from spinal cord of different mammals have been reported to contain two glycosylated membrane polypeptides of Mr 48,000 and 58,000 daltons and an additional 93,000 daltons subunit which is thought to be a peripheral protein associated to cytoplasmic domains of the receptor (2,3). The 48 kDa 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 constitute the channel-containing transmembrane structure of the GlyR (4). Photoaffinity labeling experiments have shown that the 48 KDa protein contains the binding site for strychnine, a convulsive

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Abbreviations used: FITC, fluorescein isothiocyanate; GlyR, glycine receptor; NCS, N-chlorosuccinimide; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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alkaloid that specifically antagonizes the action of glycine (5). Recent cDNA cloning of the 48 KDa polypeptide has revealed that it shares significant sequence and structural homologies with subunits of the nicotinic acetylcholine receptor and the $GABA_A$ receptor, other chemically gated ion channel proteins (6,7).

A better understanding of the mechanisms of action of the GlyR requires the identification of the functional groups and domains of the protein implicated in the specific interaction with ligands. Previous studies have suggested that glycine and strychnine bind to different, mutually interactive sites of the receptor (8-12). However, a more detailed characterization and localization of the ligand binding sites is lacking. In the present report we use a chemical modification approach to investigate the amino acids of the GlyR that may be involved in the reversible binding of the agonist glycine. Our results suggest that the 48 KDa subunit of the GlyR contains fluorescein-isothiocyanate (FITC)- sensitive lysine group(s) involved in the interaction of glycine with the receptor protein.

Experimental

Materials.- Most chemicals were from sources previously described (11,12). Fluorescein isothiocyanate was obtained from Sigma.
Glycine receptor preparations.- The chemical modification experiments were performed either using 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 (12). GlyR from rat spinal cord was purified by affinity chromatography on 2-amino-strychnine agarose as reported (3) and reconstituted into phosphatidylcholine vesicles as will be described in detail elsewhere (M. García-Calvo, A. Ruíz-Gómez, J. Vázquez, E. Morato, F. Valdivieso and F. Mayor, jr., submitted).
Reaction with FITC.- Membranes were suspended at a protein concentration of 1-2 mg/ml in 50 mM Tris-HCl, pH 8.5 and incubated at 30ºC in the dark for 10 min in the presence of the desired final concentration of FITC (10-500 μM), added from a fresh stock solution in dimethyl formamide, as described (13,14). The reactions were terminated by addition of ten volumes of 200 mM KCl, 25mM potassium phosphate buffer, pH 7.5, and centrifugation at 48,000 xg for 20 min. The resulting pellets were washed three times by repeated centrifugation and resuspension in the same medium and finally resuspended in the same buffer and utilized for binding studies. To test the ability of the presence of agonist or antagonist ligands to protect against the effect of FITC, membranes were coincubated with the desired compounds and FITC. Control membrane suspensions were taken through the same steps of FITC-treated membranes in each experiment. For labeling experiments, purified GlyR (10-30 pmol of [^{3}H]-strychnine binding sites) was incorporated into phospholipid vesicles in a medium containing 50mM Tris-HCl pH 8.8, 5mM EDTA, 5mM EGTA, 2.5mM iodoacetamide, 1mM benzamidine, 17mU/ml aprotinin, 0.1mM phenylmethylsulphonylfluoride, 0.1mM benzotonium chloride, and the reconstituted receptor incubated with FITC as described above. Protection experiments were carried out by co-incubation with the desired compound. 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 (15). The eluted proteoliposomes were used for binding or subjected to SDS-PAGE on 10% homogeneus slab gels as reported (16). FITC labeling was visualized under short-wavelength UV irradiation, photographed and analyzed by laser densitometer scanning of the negative. In some instance, gels were further processed for Coomassie-blue staining or chemical cleavage of the desired bands.

Chemical cleavage of the FITC-labeled 48 KDa subunit of the GlyR.- After SDS-PAGE in 10% polyacrylamide slab gels, FITC-labeled 48 KDa polypeptide bands were detected by UV transillumination, excised from the gel, washed and chemically hydrolyzed at tryptophan residues with N-chlorosuccinimide (NCS) as described (17). The cleavage fragments were resolved by gel electrophoresis on a 12-20% linear gradient of polyacrylamide according to the method of Cleveland (18) and visualized as above.

Binding assays.- [^{3}H]-strychnine binding to synaptic plasma membranes or purified receptor preparations was performed as reported (2,12).

Results and Discussion

The effect of pretreating synaptic membranes with FITC (which labels the ε-amino group of lysine residues under our experimental conditions) on the ligand binding properties of the GlyR was studied. Although [^{3}H]-strychnine specific binding was not affected (99±7% of control values a100 μM FITC, mean ± SEM of five independent determinations), a marked decrease in the potency of glycine for displacing specifically bound [^{3}H]-strychnine was detected even at low concentrations of FITC (Fig. 1). Pretreatment of the membranes with 10μM and 100μM FITC increased 3-fold and 5-fold, respectively, the IC_{50} for glycine displacement of [^{3}H]strychnine binding. These data suggested that FITC modification specifically affected the interaction of glycine with the receptor. Moreover, occupancy of the receptor by glycine completely prevented the effect of FITC, whereas the presence of the antagonist strychnine resulted in no protection (Table I). The protection afforded by glycine seems to be a consequence of its interaction with the GlyR and not a non-specific effect due to reaction of FITC with excess amino groups of the glycine molecule, since ethanolamine, a similar compound that do not binds to the GlyR, does not prevent the effect of FITC modification (Table I). Table I also shows that the Hill coefficient for the displacement of [^{3}H]strychnine binding by glycine is not affected by the pretreatment of the membranes with FITC. Taken together, these results indicate the existence of lysine residue(s) implicated in the interaction of the agonist with the GlyR, located in or near the glycine-binding domain.

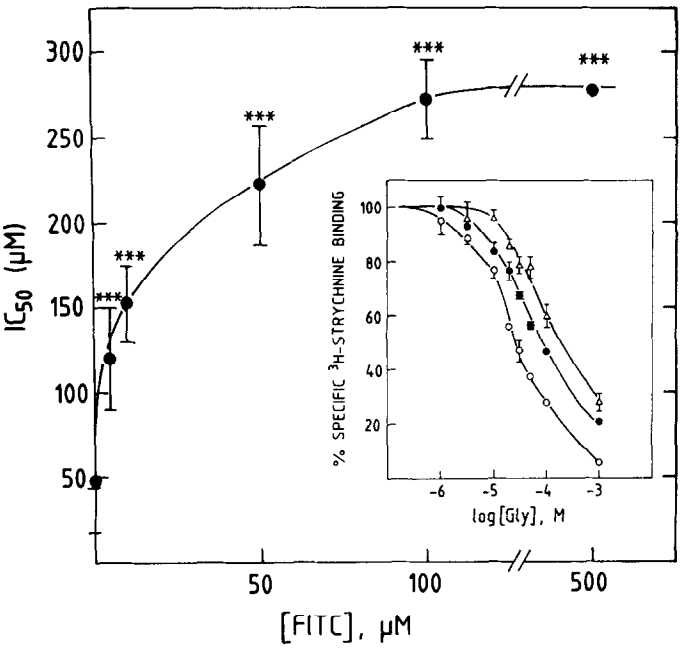


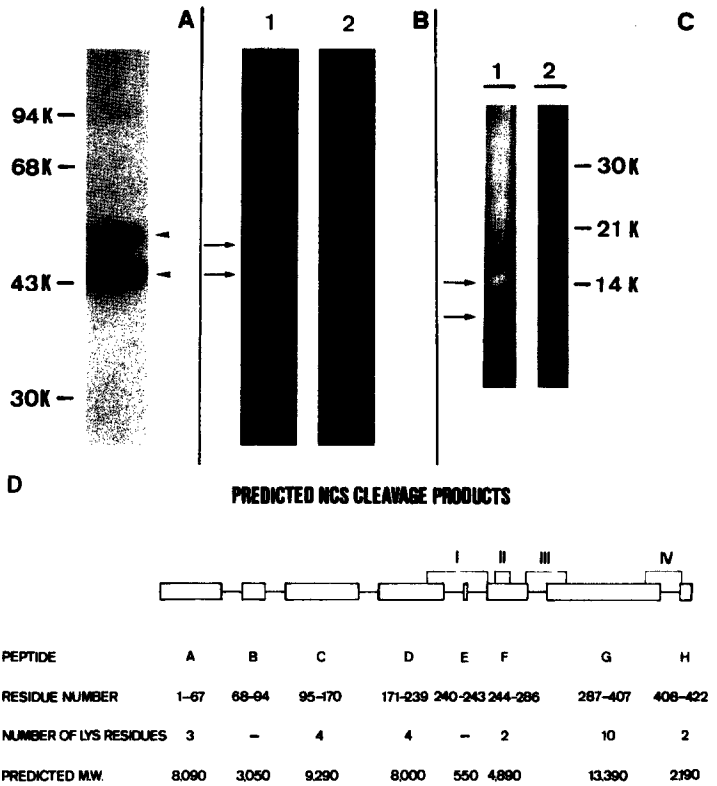
Figure 1 - Effect of FITC modification on the displacement by glycine of [^{3}H]-strychnine binding. Suspensions of synaptic membranes were pretreated with different concentrations of FITC as detailed in Material and Methods. Membranes were incubated with 2nM [^{3}H]strychnine and eight different concentrations of glycine. Non-specific binding was determined in the presence of 0.1 mM strychnine. IC_{50} values were calculated by least square fitting of Hill plots obtained from the displacement curves. The values are the mean ± S.E.M. of at least three independent determinations performed in triplicate. Insert, displacement curves obtained for membranes pretreated with no FITC(o), FITC 10μM(o) or FITC 100μM. The presence of dimethyl formamide (solvent for FITC) caused a slight increase in the IC_{50} of control from 33±5μM to 49±3μM.

The next set of experiments were aimed at the localization of the site of incorporation of FITC. In these experiments, a preparation of purified and reconstituted GlyR (consisting of 48

Table I - Effect of FITC modification on the displacement by glycine of [^{3}H]-strychnine binding. Specific protection by glycine

Membrane pretreatment	IC_{50}(μM)	n_H
None	49±3	0.74±0.03
FITC	153±22[a]	0.73±0.03
FITC + glycine	48±5	0.72±0.04
FITC + strychnine	186±20[a]	0.76±0.03
FITC + ethanolamine	157±15[a]	0.72±0.04

Membranes were pretreated in the presence of FITC 10μM and the indicated compounds as described in Materials and Methods. Glycine and ethanolamine were tested at 1mM, and strychnine at 10μM. Binding assays were performed as in Figure 1. The values are the mean ± S.E.M. of at least three independent determinations, performed in triplicate.
a, $p<0.0005$ when compared to control (no pretreatment).



PEPTIDE	A	B	C	D	E	F	G	H
RESIDUE NUMBER	1-67	68-94	95-170	171-239	240-243	244-286	287-407	408-422
NUMBER OF LYS RESIDUES	3	-	4	4	-	2	10	2
PREDICTED M.W.	8090	3050	9290	8000	550	4890	13390	2190

Figure 2 - Labeling of purified glycine receptor with FITC and chemical cleavage of the labeled 48KDa subunit
A. SDS-PAGE of a purified receptor preparation (corresponding to 10pmol of [^{3}H]strychnine binding sites) used for labeling studies as visualized by Coomassie-Blue staining. The position of relative molecular mass markers is shown. B. Labeling of the glycine receptor with FITC. 15 pmol of [^{3}H]strychnine binding sites of purified glycine receptor were incubated for 10 min with 50μM FITC in the absence (lane 1) or presence (lane 2) of 1mM glycine. The labeled bands were resolved by SDS-PAGE and visualized as detailed in Materials and Methods. C. Chemical cleavage of the labeled 48 KDa subunit. Purified glycine receptor (15 pmol of [^{3}H]strychnine binding sites) were treated with FITC in the absence (lane 1) or presence (lane 2) of glycine as described in B, and the labeled 48 KDa protein excised from the gel and subjected to chemical cleavage by N-chlorosuccinimide as described under Materials and Methods. The position of relative molecular mass markers is shown. D. Predicted sizes of the NCS cleavage products. Sequence data and the situation of the proposed transmembrane regions are taken from Grenningloh et al. (6). Predicted molecular weights were calculated assuming only one FITC molecule incorporated per peptide, when applicable.

KDa and 58KDa polypeptides, see Fig. 2A) was subjected to labeling with FITC in the absence or presence of different glycinergic ligands. As shown in Figure 2B, FITC preferentially labeled the 48KDa subunit and the incorporation of fluorescence on this protein was markedly inhibited by the presence of glycine. Neither strychnine nor ethanolamine affected the FITC

labeling pattern (data not shown). Thus, it can be concluded that the lysine residues implicated in the interaction of glycine are located in the 48KDa subunit of the GlyR.

In an attempt to further localize such residues in the structure of the 48KDa polypeptide, and considering the limited quantities of purified GlyR available, we chose to proteolitically map the labeled GlyR with N-chlorosuccinimide. Because the primary structure of the 48KDa subunit is known (6) and this proteolytic reagent is highly sequence-specific, the site(s) of FITC incorporation may be deduced from the sizes of the labeled fragments. Proteolytic digestion of the labeled polypeptide with NCS reveals two major labeled fragments of Mr 13.9KDa and 8.5KDa, respectively. The labeling of each of these fragments was inhibited in the presence of glycine (Fig. 2C). According to the predicted NCS cleavage products shown in Fig. 2D, the size of the smallest labeled fragment could correspond either to peptide A or peptide D of the sequence (assuming one or two FITC incorporated per peptide) or even to the partial cleavage product D+E. However, an unambigous assignation of the modified residue(s) either to peptide A or D is not possible.

The existence of highly reactive lysine residue(s) important for the interaction of glycine with its synaptic receptor provides further evidence for the presence of distinct binding sites for glycine and strychnine at the GlyR. These data are also consistent with preliminary experiments suggesting that amino group modification alter the ability of glycine to displace [^{3}H]strychnine binding (8,11). The specific protection to FITC modification provided by glycine suggests that the labeled lysine residues are directly involved in the interaction with the agonist. However, it cannot be ruled out that the lysine residues that are modified by FITC are masked as a result of glycine-induced changes in the receptor conformation. The lack of a suitable radioligand for the glycine binding site has prevented so far a more detailed investigation regarding labeling stoichiometry, and the mechanisms of the FITC effect (i.e., if the affinity or the number of glycine binding sites are affected). However, the participation of positively charged protein residues in glycine binding is to be expected, since structural analogs of glycine require a negatively charged functional group in order to interact with the glycine receptor (García-Calvo et al., unpublished results).

The location of the glycine binding site in the 48KDa subunit is in agreement with previous suggestions and data

recently obtained by H. Betz and collaborators using the Xenopus oocyte system, where the injection of mRNA obtained from cDNA corresponding to the 48KDa polypeptide is sufficient to elicit glycine-sensitive chloride channel activity (4,6). The same group has tentatively proposed amino acid residues 190-196 as the agonist binding domain of the GlyR, on the basis of the abundance of acidic and basic amino acids and analogies to the binding region of the homologous acetylcholine receptor (6). Although they are not conclusive, our labeling and proteolytic data are consistent with such interpretation. Indeed, FITC labeling of peptide D (amino acids 171-239) in one or several of the four existing lysines (residues 190, 193, 200 and 206) is coherent with the size of the smallest labeled fragment obtained. Such location would also account for the other major labelled peptide as a partial cleavage product (see scheme in Fig. 2D). However, determining the exact role of each of the individual residues that conform the glycine binding site would require further analysis involving a combination of biochemical and genetic approaches. This report provides a framework for a more detailed characterization of the structural determinants of the interaction of ligands with the glycine synaptic receptor.

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