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The M_r 93 000 Polypeptide of the Postsynaptic Glycine Receptor Complex Is a Peripheral Membrane Protein†

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ABSTRACT: The glycine receptor of mammalian spinal cord is an oligomeric membrane protein that, after affinity purification on aminostrychnine–agarose or immobilized antibody, contains three polypeptides of M_r 48 000, 58 000, and 93 000. Here, the association and the properties of the polypeptides of the rat glycine receptor were investigated. Upon phase partitioning in the nonionic detergent Triton X-114, the three receptor polypeptides behaved as a hydrophilic protein complex exhibiting phospholipid binding. Sucrose gradient centrifugation or gel filtration in the presence of dithiothreitol and Triton X-100 separated the M_r 93 000 polypeptide from the M_r 48 000 and 58 000 polypeptides, which harbor the antagonist binding site of the glycine receptor. Alkaline or dimethylmaleic acid anhydride treatment of crude synaptic membrane fractions resulted in extraction of the M_r 93 000 polypeptide. Lectin binding was observed for the M_r 48 000 and 58 000 glycine receptor subunits but not the M_r 93 000 polypeptide. These results indicate that the M_r 93 000 polypeptide is a peripheral membrane protein that is located at the cytoplasmic face of the postsynaptic glycine receptor complex.

Glycine is a major inhibitory neurotransmitter in spinal cord and other regions of the central nervous system (Werman et al., 1967; Curtis et al., 1968; Aprison & Daly, 1978). Binding of this amino acid to the postsynaptic glycine receptor (GlyR)[1] increases the chloride conductance of the neuronal membrane and thus produces a hyperpolarization, i.e., inhibition of the postsynaptic neuron. The convulsive alkaloid strychnine an-

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[1] Abbreviations: DMMA, dimethylmaleic acid anhydride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GlyR, glycine receptor; HRP, horseradish peroxidase; IgG, immunoglobulin G; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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tagonizes glycine-mediated inhibition, and glycine-displaceable [^{3}H]strychnine binding has been widely used to investigate and localize the GlyR in the nervous system of different species (Young & Snyder, 1973, 1974; Synder & Bennett, 1976).

Electrophysiological and biochemical evidence indicates that the GlyR is an integral membrane protein that contains a chemically gated chloride channel (Hamill et al., 1983; Betz, 1985). Upon solubilization with different detergents, the GlyR behaves as a large glycoprotein of M_r 250000 (Pfeiffer & Betz, 1981; Pfeiffer et al., 1982). After affinity purification on aminostrychnine–agarose, three polypeptides of M_r 48000, 58000, and 93000 are detected (Pfeiffer et al., 1982; Graham et al., 1985; Becker et al., 1986). The M_r 48000 and, to a much lesser extent, the M_r 58000 polypeptides can be covalently labeled with [^{3}H] strychnine upon UV illumination and thus contain the antagonist binding site of the GlyR (Graham et al., 1981, 1983, 1985). The function of the M_r 93000 polypeptide is unknown.

The structure and membrane topology of the GlyR polypeptides have been investigated with proteolytic enzymes and monoclonal antibodies (mAbs) (Graham et al., 1983; Pfeiffer et al., 1984). Also, different immunogenic determinants of the M_r 48000 and 93000 polypeptides have been colocalized in the postsynaptic membrane by immunoelectron microscopy (Triller et al., 1985). In addition, these experiments have shown that the M_r 93000 polypeptide possesses a large intracellular domain that protrudes from the postsynaptic membrane into the cytoplasm (Triller et al., 1985; Altschuler et al., 1986). Here, we describe properties of the M_r 93000 polypeptide that classify it as a peripheral membrane protein associated with the cytoplasmic domains of the GlyR.

Materials and Methods

Materials. Triton X-114 and 2,6-di-*tert*-butyl-*p*-cresol were purchased from Fluka, and Triton X-100 was from Serva. Horseradish peroxidase (HRP), concanavalin A, HRP-coupled wheat germ agglutinin, soybean phosphatidylcholine (type II-S), phenylmethanesulfonyl fluoride, aprotinin, poly(vinylpyrrolidone), dithiothreitol (DTT), and iodoacetamide were from Sigma. Anti-mouse IgG antibodies coupled to HRP and [^{3}H]strychnine (29 Ci/mmol) were from Amersham Buchler. Anti-mouse IgG antibodies coupled to alkaline phosphatase were from Promega (Protoblot system). All other chemicals were of analytical grade.

Purification of GlyR. GlyR from rat, pig, or mouse spinal cord was purified by affinity chromatography on aminostrychnine–agarose as described previously (Pfeiffer et al., 1982; Graham et al., 1985; Becker et al., 1986). Immunopurification of the GlyR from rat spinal cord was performed with polyclonal antibodies coupled to protein A–Sepharose according to Schneider et al. (1982). The antibodies used were raised in rabbits against a synthetic decapeptide corresponding to the amino-terminal amino acids of the M_r 48000 GlyR polypeptide from rat (B. Schmitt et al., unpublished results). This antiserum precipitated [^{3}H]strychnine binding sites from detergent extracts of rat spinal cord membranes and from affinity-purified GlyR preparations; also, it bound to the M_r 48000 polypeptide on immunoblots (B. Schmitt et al., unpublished results). Rat spinal cord membranes were solubilized as described by Pfeiffer et al. (1982), and the detergent extract was incubated overnight with antibody–protein A–Sepharose at 4 °C after dilution with 1 volume of 25 mM potassium phosphate, pH 7.4. Following transfer to a column, the beads were washed with 20 volumes of 50 mM Tris-HCl, pH 8.0, containing 0.05% (w/v) Triton X-100 and 200 mM NaCl. Antigen was eluted with 3.6 M $MgCl_2$ in 50 mM sodium citrate, pH 6.0, containing 0.05% (w/v) Triton X-100. Unless stated otherwise, protease inhibitors and DTT were included throughout all purification steps as described previously (Pfeiffer et al., 1982; Graham et al., 1985).

Phase Separation Experiments. For Triton X-114 phase partitioning according to Bordier (1981), the purification of the GlyR was modified as follows: Triton X-100 extracts of spinal cord membranes were applied to aminostrychnine–agarose, the column was washed with 1% (w/v) Triton X-114 (precondensed 2 times) in 10 mM Tris-HCl, pH 7.4, containing 150 mM KCl, a mixture of protease inhibitors (Pfeiffer et al., 1982), and, where indicated, 0.18% (w/v) phosphatidylcholine, and the GlyR was eluted in the same buffer containing 0.2 M glycine.

Sucrose Gradient Centrifugation. Affinity-purified GlyR (250 μL containing 5–10 pmol of [^{3}H]strychnine binding sites) was applied to 5–20% sucrose gradients (Pfeiffer et al., 1982). After centrifugation in a SW 40 rotor at 28000 rpm for 36 h at 4 °C, fractions of 500 μL were collected. The following marker proteins were detected in a parallel gradient by their enzymatic activity: pig heart mitochondrial malic dehydrogenase (4.32 S), rabbit muscle lactic dehydrogenase (6.95 S), bovine liver catalase (11.3 S), and, in some experiments, β-galactosidase (15.93 S).

Gel Exclusion Chromatography. Gel filtration of purified rat GlyR was performed as described (Pfeiffer & Betz, 1981; Pfeiffer et al., 1982), except that a Sephacryl S-300 column (1 × 110 cm) was used. A total of 10–20 pmol of purified GlyR (500 μL) was applied to the column.

Alkaline, DMMA, and High-Salt Treatments of Crude Synaptic Membranes. Crude synaptic membranes were prepared as described by Graham et al. (1982). For alkaline extraction (Neubig et al., 1979), the membranes were suspended at a protein concentration of about 0.5 mg/mL in 10 mM potassium phosphate buffer, pH 7.4, containing 0.2 mM EDTA and protease inhibitors. The pH of the suspension was adjusted to 11.0 by dropwise addition of 1 M NaOH. After incubation for 60 min at 4 °C, the membranes were sedimented at 180000*g* for 90 min at 4 °C. Preparations from which NaOH was omitted served as controls. The pellets were resuspended at a protein concentration of 3–10 mg/mL in 25 mM potassium phosphate buffer, pH 7.4, containing 0.2 M KCl, 0.2 mM EDTA, and protease inhibitors. The supernatants were neutralized by addition of 0.1 volume of 1 M potassium phosphate buffer, pH 7.4. For high-salt treatment, crude synaptic membranes were washed twice with 2 M KCl in 10 mM potassium phosphate buffer, pH 7.4, containing 0.2 mM EDTA and protease inhibitors. After centrifugation at 24000*g* for 45 min at 4 °C, the pellet was resuspended as described above. DMMA treatment of crude synaptic membranes was performed after two washes of the membranes with 5 mM sodium phosphate buffer, pH 8.0, as described by Steck and Yu (1973).

Immunoassay of GlyR Polypeptides in Crude Synaptic Membrane Fractions. GlyR polypeptides in crude synaptic membranes were quantitated by an enzyme-linked immunosorbent assay that will be described in detail elsewhere (C.-M. Becker et al., submitted for publication). Briefly, crude synaptic membranes (or pellets, or supernatants from alkali- or DMMA-treated membranes) were solubilized and adsorbed to nitrocellulose in a dot-blot chamber (Schleicher & Schüll). Different antigenic sites of the GlyR were assayed with specific mAbs (Pfeiffer et al., 1984) followed by HRP-coupled anti-mouse IgG antibodies. HRP-catalyzed color development with *o*-phenylenediamine was determined by transfer of nitro-

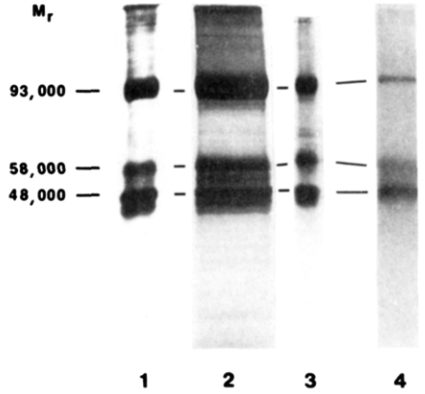

FIGURE 1: SDS–polyacrylamide gel electrophoresis of affinity-purified GlyR. (Lanes 1–3) GlyR purified on 2-aminostrychnine–agarose from different species. For silver staining, aliquots containing 2–4 pmol (0.5–1 μg) of purified GlyR from rat spinal cord (lane 1), pig spinal cord (lane 2), and mouse spinal cord (lane 3) were applied to the gels. (Lane 4) GlyR from rat spinal cord purified by immunoaffinity chromatography as described under Materials and Methods. Because of contaminating immunoglobulin, an immunoblot reacted with a mixture of the monoclonal antibodies GlyR 2b, GlyR 4a, GlyR 5a, and GlyR 7a (Pfeiffer et al., 1984) is shown; the different staining intensities of the polypeptides as compared to the silver staining shown in lanes 1–3 relate to this immunological detection procedure. The apparent molecular weight values of the GlyR polypeptides are indicated on the left side of the figure.

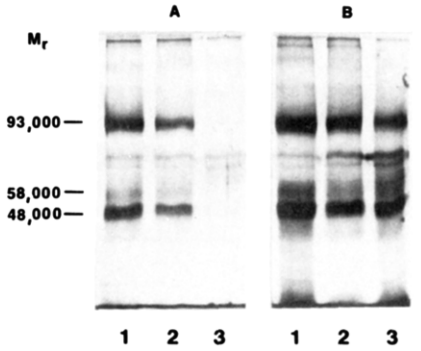

FIGURE 2: Triton X-114 phase partitioning of GlyR polypeptides. For all partitioning experiments and controls, 4 pmol, each, of rat GlyR purified in the presence of DTT (5 mM) was used. The phase distribution of the receptor polypeptides was analyzed by SDS–polyacrylamide gel electrophoresis and silver staining. In each display, lane 1 represents the GlyR preparation prior to phase partitioning, lane 2 the aqueous phase, and lane 3 the detergent phase after phase partitioning. (A) GlyR eluted from the affinity column in the absence of phosphatidylcholine. (B) GlyR eluted in the presence of soybean phosphatidylcholine. The positions of the GlyR polypeptides are indicated by their apparent molecular weight values.

cellulose pieces containing the samples into microtiter plates and measuring the adsorbance at 492 nm.

Gel Electrophoresis and Related Techniques. Electrophoresis on 10% SDS–polyacrylamide gels was performed according to Laemmli (1970). For gel electrophoresis, protein samples were precipitated according to Wessel and Flügge (1984). Protein was detected by silver staining (Merril et al., 1982). Where indicated, protein bands were scanned with a Hirschmann Elscript 400 densitometer, and computer-calculated area increments were used for quantitative determination of GlyR-specific bands. Proteins separated on slab gels were transferred to nitrocellulose according to Towbin et al. (1979) and stained with Poinceau S for the visualization of protein bands. Immunoblotting was done as described by Pfeiffer et al. (1984). For immunodetection of GlyR polypeptides in crude synaptic membranes, alkaline phosphatase-linked anti-mouse IgG (Promega) was used. Carbohydrate staining of nitrocellulose blots with HRP-coupled wheat germ agglutinin was performed according to Bartles & Hubbard (1984) and Morei and Jung (1984). Concanavalin A staining followed the protocol of Parish et al. (1977), but nitrocellulose blots were used.

Results

Copurification of M_r 48 000, 58 000, and 93 000 Polypeptides during Isolation of GlyR. As shown previously (Pfeiffer et al., 1982), affinity purification of the GlyR from detergent extracts of rat spinal cord membranes on 2-aminostrychnine–agarose produced three major polypeptides with relative molecular masses of 48 000, 58 000, and 93 000 (Figure 1, lane 1). The same polypeptide pattern was also found upon isolation of the GlyR from pig and mouse (Figure 1, lanes 2 and 3). Furthermore, using covalently bound antibodies directed against the N-terminal region of the M_r 48 000 polypeptide as an affinity adsorbent yielded preparations containing the same three M_r 48 000, 58 000, and 93 000 polypeptides. Figure 1, lane 4, shows the immunoblot of an immunopurified GlyR preparation from rat spinal cord. Also, chromatography of affinity-purified GlyR on wheat germ agglutinin–Sepharose did not alter the polypeptide composition of the receptor preparation [not shown; see Pfeiffer et al. (1982)].

Phase Separation Experiments. Affinity-purified rat GlyR was subjected to the phase separation procedure developed by Bordier (1981), which exploits the properties of the nonionic detergent Triton X-114 for distinguishing hydrophilic from hydrophobic, i.e., putative membrane, proteins. After elution from the affinity column in the absence of phosphatidylcholine, a lipid known to be essential for stabilizing the strychnine binding conformation of the GlyR (Pfeiffer & Betz, 1981), the three GlyR polypeptides were mainly enriched in the aqueous phase and thus exhibited hydrophilic properties (Figure 2A). After elution in the presence of phosphatidylcholine, the three polypeptides were also found in the detergent phase (Figure 2B). Thus, the GlyR polypeptides cofractionated upon phase separation; their relative phase distribution, however, was affected by phospholipid.

Separation by Sucrose Gradient Centrifugation of M_r 93000 Polypeptide from Other GlyR Subunits. When purified rat GlyR was subjected to centrifugation on 5–20% sucrose gradients (Figure 3A), the M_r 93 000 polypeptide did not fractionate together with the M_r 48 000 and 58 000 polypeptides but was enriched as a broad peak at the lower fractions of the gradients (apparent sedimentation constant 12–15 S). The [^{3}H]strychnine binding activity (Figure 3B) on the other hand cosedimented with the M_r 48 000 and 58 000 polypeptides, which are known to harbor the strychnine binding site of the GlyR (Graham et al., 1981, 1983, 1985). The apparent s value (8.1 S) of the strychnine binding site was similar to that reported previously (Pfeiffer & Betz, 1981; Pfeiffer et al., 1982).

Separation of the M_r 93 000 polypeptide from the M_r 48 000 and 58 000 polypeptides was observed only after purification and/or fractionation of the GlyR in the presence of the reducing agent DTT, a standard addition in our purification procedure (Pfeiffer et al., 1982). The gel scanning data shown in Figure 4 demonstrate that omission of DTT during purification and centrifugation produced a cosedimentation of both the M_r 93 000 and a large portion of the M_r 48 000 (and M_r 58 000; not shown) polypeptides at high apparent s values (>15 S). However, we never found that all of the M_r 48 000 subunit cofractionated with the M_r 93 000 polypeptide under these conditions. This might be due to partial dissociation and/or proteolysis of the GlyR. Sensitivity of the GlyR polypeptides

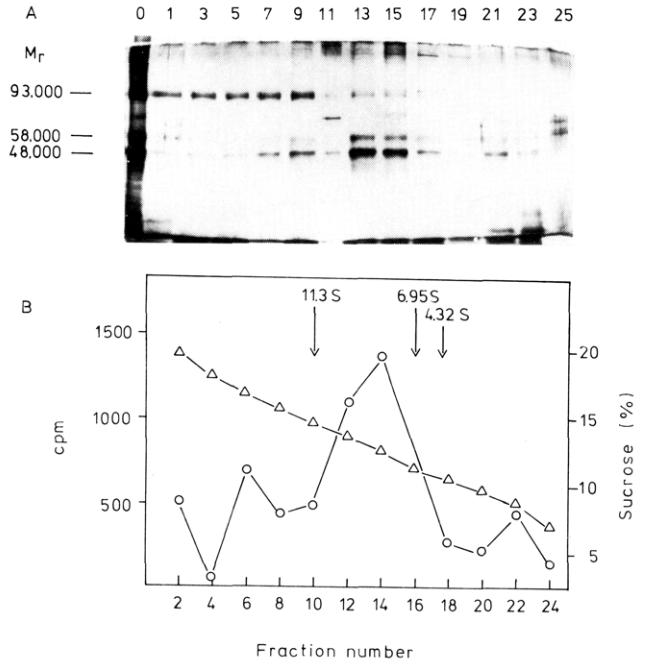

FIGURE 3: Separation of GlyR polypeptides by sucrose gradient centrifugation. (A) SDS–polyacrylamide gel electrophoresis of 5–20% sucrose gradient fractions of affinity-purified rat GlyR. The KCl and DTT concentrations in the gradient medium were 0.2 M and 5 mM, respectively. (Lane 0) Unfractionated GlyR (2 pmol); (lanes 1–25) aliquots of fractions of a sucrose gradient loaded with 7 pmol of the GlyR preparation shown in lane 0; numbering starts with the bottom fractions. Apparent molecular weight values are indicated on the left. (B) [^{3}H]Strychnine binding activity of the sucrose gradient fractions. Glycine-displaceable [^{3}H]strychnine binding in 60-μL aliquots of the fractions (○) was determined as described by Pfeiffer et al. (1982). The recovery of [^{3}H]strychnine binding sites after centrifugation was >40%. The sucrose concentration of the fractions was determined by their refraction indices (△). The positions of the marker proteins described under Materials and Methods are indicated by their S values. Odd-number fractions were used for (A) and even-number fractions from the same gradient for (B).

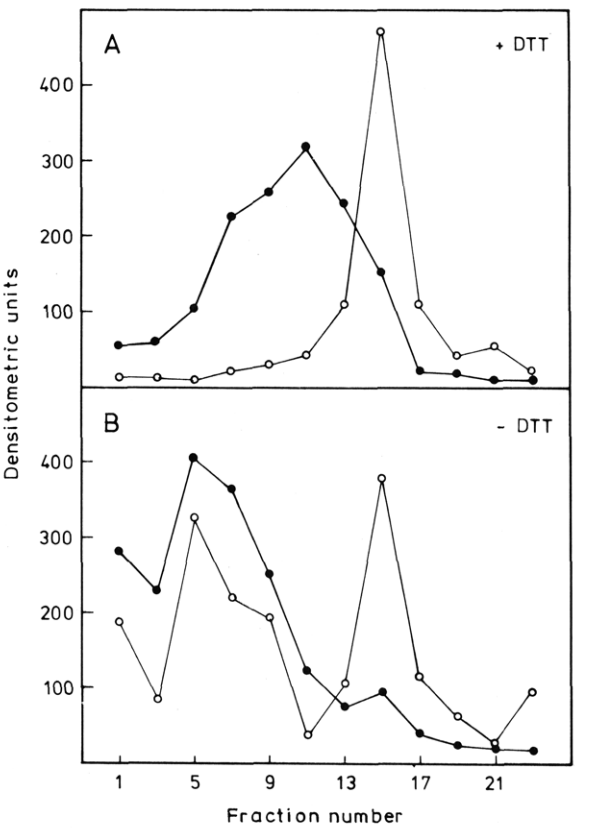

FIGURE 4: Effect of DTT on sedimentation behavior of GlyR polypeptides. Densitometric area values of the M_r 93 000 (●) and 48 000 (○) polypeptide bands in silver-stained SDS–polyacrylamide gels of 5–20% sucrose gradient fractions were determined as described under Materials and Methods. (A) Sucrose gradient centrifugation of GlyR purified in the presence of DTT. (B) Parallel gradient of GlyR purified in absence of DTT. The medium of both gradients contained 1 M KCl. Gradient A, but not gradient B, also contained DTT. Fraction numbering starts with bottom fraction. In both gradients the M_r 58 000 polypeptide showed the same distribution profile as the M_r 48 000 polypeptide (not shown).

to contaminating protease has been described (Graham et al., 1985; Becker et al., 1986) and is illustrated by samples 11 and 25 of the gel shown in Figure 3A.

In order to substantiate these sucrose gradient centrifugation data, control experiments were performed: (i) SDS–polyacrylamide gel electrophoresis revealed that the polypeptide patterns of GlyR preparations purified in either the absence or presence of DTT were indistinguishable (not shown). (ii) The separation by centrifugation of the M_r 93 000 polypeptide from the strychnine binding site of the GlyR was not influenced by addition of the ligands glycine (1 mM) or strychnine (1 μM), by different concentrations of KCl (0.2 or 1.0 M) to the gradient medium, or by use of glycerol gradients. (iii) Gel permeation chromatography of the GlyR on a Sephacryl S-300 column also produced a separation of the M_r 48 000 and 93 000 polypeptides whenever DTT was included during purification and/or chromatography (data not shown).

Extraction of M_r 93 000 Polypeptide from Synaptic Membrane Fractions. The interaction of the GlyR polypeptides was also investigated in crude synaptic membrane fractions:

(i) Washing spinal cord membranes with 2 M KCl prior to solubilization and affinity purification resulted in the loss of about 20% of the [^{3}H]strychnine binding sites. The GlyR purified from these high salt washed membranes exhibited the usual polypeptide composition (not shown).

(ii) Alkaline treatment at pH 11.0 is known to remove peripheral membrane proteins from erythrocyte membranes (Steck & Yu, 1973). After incubation of rat spinal cord membranes at pH 11.0, >80% of the [^{3}H]strychnine binding sites were still present. Upon solubilization, however, ≥90% of the sites were lost. Thus the polypeptide composition of the GlyR from pH 11.0 treated membranes could not be determined after affinity purification. Alkali-treated membranes and extracts thereof were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting with mixtures of mAbs recognizing the M_r 48 000 (mAbs 2b and 4a) and the M_r 93 000 (mAbs 7a and 9a) polypeptides, respectively. Figure 5A shows that alkaline treatment removed a substantial portion of the M_r 93 000 but not the M_r 48 000 (and M_r 58 000; not shown) polypeptides from the membranes. A quantitative evaluation of the pH 11.0 treatment by an immunoassay dot-blot procedure is given in Figure 6. After incubation of the membranes at pH 7.4, the epitopes recognized by mAbs GlyR 2b, 4a, 5a, and 7a were recovered (>98%) in the membrane pellet. In contrast, treatment at pH 11.0 led to a preferential extraction of the epitopes recognized by mAbs GlyR 5a and 7a,[2] which are known to be located on the M_r 93 000 polypeptide. The comparatively lower extraction of the mAb GlyR 5a epitope might reflect a higher sensitivity

[2] Recent experiments have shown that mAb GlyR 7a binds specifically only to the M_r 93 000 polypeptide (B. Schmitt and A. Rienitz, unpublished data). The immunoreactive material of apparent M_r 45 000–48 000 observed by Pfeiffer et al. (1984) does not represent the GlyR M_r 48 000 subunit but may correspond to a degradation product of the M_r 93 000 polypeptide.

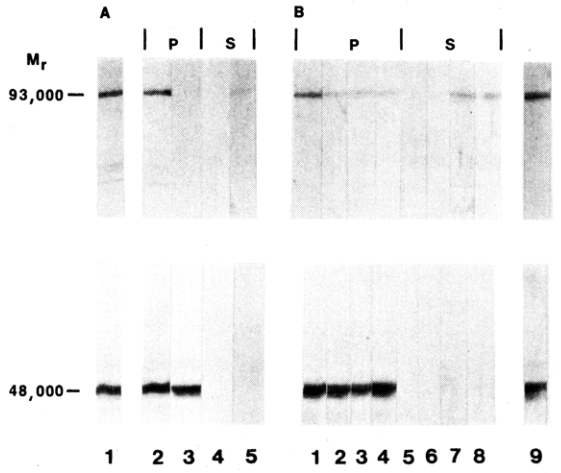

FIGURE 5: Release of M_r 93 000 GlyR polypeptide from crude synaptic membranes. Crude synaptic membranes were treated at pH 11.0 (A) or with DMMA (B) as described under Materials and Methods. The proteins of the membrane pellets (P) and the supernatants (S) were transferred to nitrocellulose after SDS–polyacrylamide gel electrophoresis and immunostained with mAbs recognizing either the M_r 93 000 (mAbs 7a and 9b; upper part of figure) or the M_r 48 000 (mAbs 2b and 4a; lower part of figure) GlyR polypeptides. (Panel A) (Lane 1) Untreated membranes (80 μg of protein); (lane 2) membranes treated at pH 7.4; (lane 3) membranes treated at pH 11.0; (lanes 4 and 5) supernatants of membranes shown in lanes 2 and 3, respectively. (Panel B) (Lanes 1–4) Membranes treated with 0, 2.5, 12.5, and 50 mM DMMA; (lanes 5–8) supernatants of membranes shown in lanes 1–4, respectively; (lane 9) 100 ng of affinity-purified GlyR.

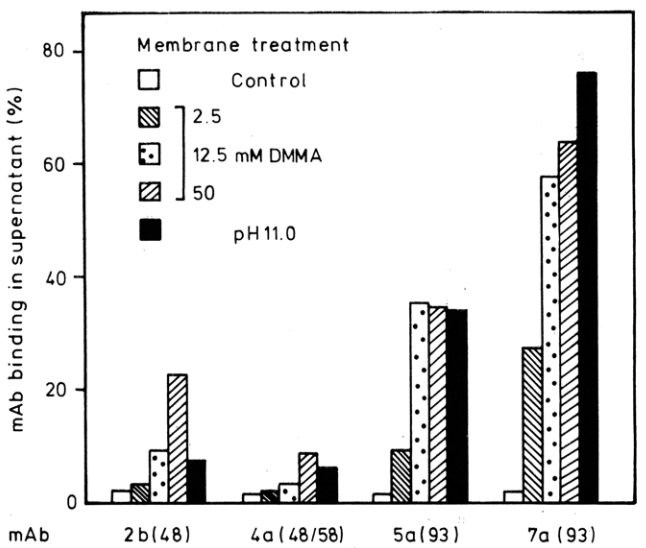

FIGURE 6: Quantitative determination of mAb GlyR binding sites in supernatants of alkali- and DMMA-treated synaptic membranes. Synaptic membrane fractions were treated at pH 11.0 and with DMMA as described under Materials and Methods. Binding of mAbs GlyR 2b, 4a, 5a, and 7a to the membrane pellets and the supernatants thereof was determined as described under Materials and Methods. The percentage of antigenic epitopes in the supernatants was calculated from the total number of mAb binding sites found in pellet and supernatant. After treatment at pH 11.0, up to 28% of the total antigenic sites present in untreated membranes were lost depending on the mAb used while more than 80% of the total [^{3}H]strychnine binding sites were recovered in the pellets. The GlyR polypeptides ($M_r \times 10^{-3}$) recognized by the different mAbs are given in brackets. Controls are supernatants of membranes treated at pH 7.4.

to the extraction procedures used. Further experiments indicated that the addition of DTT during both neutral and alkaline treatment did not affect the extraction of the mAb GlyR 5a and 7a epitopes (data not shown).

(iii) The protein modifying reagent DMMA has been shown to efficiently extract peripheral membrane proteins (Steck & Yu, 1973). Incubation with increasing concentrations of the reagent removed the M_r 93 000 but not the M_r 48 000 polypeptide from rat spinal cord membranes as revealed by immunoblot analysis (Figure 5B). Simultaneously, increasing amounts of the mAb GlyR 5a and 7a but only small amounts of the mAb 2b and 4a epitopes were recovered in the supernatants of the membranes (Figure 6).

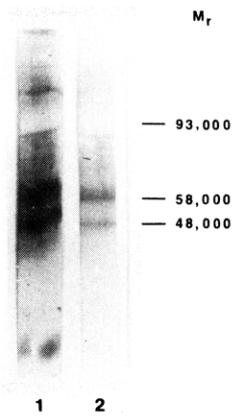

FIGURE 7: Carbohydrate staining of GlyR polypeptides. Purified rat GlyR (2 pmol per lane) was transfered to nitrocellulose after SDS–polyacrylamide gel electrophoresis and incubated sequentially with concanavalin A and HRP (lane 1) or with HRP-coupled wheat germ agglutinin (lane 2) as described under Materials and Methods. The positions of the GlyR polypeptides are indicated by their apparent molecular weight values.

Miscellaneous Properties of GlyR Polypeptides. Previously, the lectin concanavalin A that recognizes mannose and glucose sugars was shown to bind to the M_r 48 000 and 58 000 subunits of the pig GlyR but not the M_r 93 000 polypeptide (Graham et al., 1985). HRP-coupled wheat germ agglutinin, which recognizes *N*-acetylglucosamine residues, and concanavalin A in conjunction with HRP were applied to purified rat GlyR blotted onto nitrocellulose paper. Both lectins bound to the M_r 48 000 and 58 000 but not to the M_r 93 000 polypeptides (Figure 7). Thus, in contrast to the M_r 48 000 and 58 000 GlyR polypeptides, the M_r 93 000 polypeptide is not, or only poorly, glycosylated.

Amino acid analysis may provide information about the polarity properties of proteins (Capaldi & Vanderkooi, 1972). None of the GlyR polypeptides has a particularly low content of hydrophilic amino acids, i.e., a polarity index <40% (Table I). A significant variation between the different polypeptides concerns the number of proline residues. In contrast to the M_r 48 000 and 58 000 GlyR subunits, the M_r 93 000 polypeptide has a comparatively high proline content.

Discussion

The data presented in this paper indicate that the M_r 93 000 polypeptide can be classified as a peripheral membrane protein of the postsynaptic GlyR complex. Several lines of evidence show that this polypeptide is tightly associated with the M_r 48 000 and 58 000 GlyR subunits, which harbor the antagonist binding site of the receptor: (i) Immunocytochemical studies have shown that the M_r 93 000 polypeptide is localized at the same synaptic regions stained by mAb specific for the M_r 48 000 GlyR subunit (Triller et al., 1985; Altschuler et al., 1986). (ii) It cofractionates with the M_r 48 000 and 58 000 polypeptides on different affinity columns and in phase separation experiments. (iii) In the absence of reducing agent, it cosediments with the strychnine binding polypeptides upon sucrose gradient centrifugation. (iv) It cannot be extracted from synaptic membrane fractions by use of high ionic strength conditions or the reducing agent DTT. However, two con-

Table I: Amino Acid Composition of GlyR Polypeptides[a]

amino acid	GlyR polypeptide (mol %)		
	M_r 48 000	M_r 58 000	M_r 93 000
Asx	10.7	6.1	7.7
Thr	4.8	2.7	6.4
Ser	9.9	3.8	6.7
Glx	10.3	15.9	10.0
Pro	<1	4.0	10.1
Gly	15.2	13.6	8.4
Ala	8.8	10.8	8.0
Val	4.6	8.7	7.7
Met	1.9	1.3	2.2
Ile	4.9	6.3	5.4
Leu	9.2	9.5	10.4
Tyr	3.5	2.1	1.4
Phe	4.7	2.7	2.2
Lys	5.1	7.6	3.8
His	2.0	1.7	3.2
Arg	4.5	3.6	6.5
polarity index	47.3	41.4	44.3

[a] The subunits of purified rat GlyR (60 pmol) were separated on a 10% SDS–polyacrylamide gel and cut out from the gel after rapid Coomassie blue staining. After repeated washing of the gel slices with double-distilled water, the proteins were electroeluted, hydrolyzed in boiling 6 N HCl for 24 h in vacuo, and subjected to amino acid analysis. The polarity index was calculated according to Capaldi and Vanderkooi (1972).

ditions known to remove peripheral membrane proteins from the erythrocyte membrane, e.g., alkaline and DMMA treatment (Steck & Yu, 1973), extract the M_r 93 000 polypeptide from synaptic membrane fractions. Also, a separation of the M_r 93 000 polypeptide from the other subunits of the affinity-purified GlyR was obtained upon sucrose gradient centrifugation or gel filtration in the presence of the reducing agent DTT. This separation may reflect weakening of interactions between the GlyR polypeptides by opening of a critical disulfide bond. The latter, in turn, may facilitate the dissociation during centrifugation/gel filtration of the M_r 93 000 polypeptide from the "core" of the GlyR consisting of the M_r 48 000 and 58 000 subunits. Alternatively, the artifactual formation of an interchain disulfide bridge between the M_r 93 000 and one of the other GlyR polypeptides during receptor purification may explain the experimental data.

In the postsynaptic membrane, both protein–protein and protein–lipid interactions may stabilize the large GlyR complex containing all three polypeptides (s value >15 S). Our phase separation experiments employing Triton X-114 showed that the distribution of the GlyR is affected by phospholipids, thus providing evidence for the existence of lipid binding domains on this receptor. In the absence of phosphatidylcholine, however, the GlyR behaves as a hydrophilic protein, a result consistent with the amino acid composition of the individual GlyR polypeptides. The hydrophilic properties of the GlyR resemble those of the nicotinic acetylcholine receptors of *Torpedo* electric organ (Maher & Singer, 1985) and chick brain (Schneider et al., 1985).

By its biochemical properties, the M_r 93 000 polypeptide can be clearly distinguished from the other GlyR subunits: (i) In contrast to the ligand binding subunits, the M_r 93 000 polypeptide appears not to be glycosylated. This peripheral membrane protein thus can be assumed to be located at the cytoplasmic face of the glycinergic postsynaptic membrane, a conclusion consistent with the immunocytochemical data of Triller et al. (1985) and Altschuler et al. (1986). (ii) The M_r 93 000 polypeptide has a higher proline content than the other GlyR polypeptides. (iii) Upon DTT reduction and gradient centrifugation, the M_r 93 000 polypeptide behaves as a molecule that is larger (apparent sedimentation coefficient 13 S) than the strychnine binding site of the GlyR (8.1 S). The M_r 93 000 polypeptide thus can form homooligomeric complexes. This property cannot be attributed to isoelectric aggregation (pI of M_r 93 000 polypeptide 5.6–5.8; F. Pfeiffer and H. Betz, unpublished data) but may relate to yet unknown functions of the polypeptide or to its partial unfolding upon reduction.

These biochemical data raise the question whether the M_r 93 000 polypeptide is an integral component of the GlyR complex or a receptor-associated protein comparable to the M_r 43 000 protein of the postsynaptic membrane of *Torpedo* electric organ (Wennogle & Changeux, 1980; Sealock et al., 1984). The latter protein has been shown to codistribute with the nicotinic acetylcholine receptor on the cytoplasmic face of the subsynaptic membrane. It is extracted at pH 11.0, binds to phospholipid vesicles as well as actin, and has been implicated in various regulatory functions including the postsynaptic anchoring of the acetylcholine receptor via interactions with the cytoskeleton (Neubig et al., 1979; Wennogle & Changeux, 1980; Barrantes et al., 1983; Walker et al., 1984; Porter & Froehner, 1985). By analogy, the M_r 93 000 polypeptide might be involved in regulating and anchoring the GlyR at central synapses. On the other hand, our data do not exclude that the M_r 93 000 polypeptide has domains important for GlyR chloride channel function. Chloride transport studies on reconstituted GlyR preparations containing and lacking the M_r 93 000 polypeptide might clarify this issue.

The data presented here imply that our previously postulated structural model of the GlyR has to be revised. From protein staining of SDS–polyacrylamide gels and an estimated M_r of 250 000 (Pfeiffer et al., 1982), we have suggested that the GlyR may contain two copies of the M_r 48 000 polypeptide and one copy of each of the M_r 58 000 and 93 000 polypeptides (Betz et al., 1983, 1985; Betz, 1984, 1985). This model was based on the assumption that the M_r 93 000 polypeptide cosediments with the strychnine binding site upon sucrose density gradient centrifugation. Here, we have shown that this assumption is not correct; rather, the molecular characteristics determined previously for the strychnine binding activity of the GlyR (Pfeiffer et al., 1982) relate to a macromolecule that does not contain the M_r 93 000 polypeptide. In the neuronal membrane, the postsynaptic "GlyR complex" as defined by the M_r 48 000, 58 000, and 93 000 polypeptides must be considerably larger (M_r >400 000). An accurate evaluation of the stoichiometry of the different GlyR polypeptides in the postsynaptic membrane should help to provide a more detailed picture of the molecular organization of this ligand-gated neuronal anion channel.

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