Phosphorylation of the 48-kDa Subunit of the Glycine Receptor by Protein Kinase C*

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The postsynaptic glycine receptor purified from rat spinal cord is rapidly and specifically phosphorylated by protein kinase C. The target for phosphorylation is the strychnine-binding subunit of the receptor (molecular mass of ~48 kDa), which is phosphorylated on serine residues to a final stoichiometry of ~1 mol of phosphate/mol of subunit. The 48-kDa phosphoprotein was analyzed by proteolytic cleavage and peptide mapping in order to localize the site of phosphorylation within the receptor molecule. Examination of the 32P-labeled receptor fragments generated by digestion with N-chlorosuccinimide, cyanogen bromide, and endoproteinase lysine C and of the deduced amino acid sequence of the 48-kDa protein (Grenningloh, G., Rienitz, A., Schmitt, B., Mehfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E. D., and Betz, H. (1987) Nature 328, 215–220) indicates that the phosphorylation site is located in a region corresponding to the major intracellular loop of the predicted structure of the glycine receptor subunit and suggests serine 391 as the phosphorylated residue. In fact, synthetic peptides corresponding to residues 384–392 of the 48-kDa subunit was specifically phosphorylated by protein kinase C. Moreover, tryptic digests of this phosphopeptide and of the phosphorylated 48-kDa subunit of the glycine receptor migrated to the same position in twodimensional peptide mapping. Furthermore, antibodies elicited against peptide 384–392 were shown to inhibit the protein kinase C-dependent phosphorylation of the 48-kDa polypeptide. Interestingly, the relative position of the phosphorylated domain is similar to those known or proposed to be phosphorylated in other ligand-gated ion channel receptor subunits, thus suggesting further the existence of a homologous regulatory region in these receptor proteins.

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The glycine receptor is a chemically gated ion channel that mediates postsynaptic inhibition in spinal cord and other regions of the central nervous system. The interaction of the agonist glycine with the glycine receptor induces an increase in the chloride permeability of the postsynaptic membrane, thus producing a hyperpolarization (1). Glycine-mediated inhibitory actions are selectively antagonized by the convulsive alkaloid strychnine, and this compound has been utilized in pharmacological, biochemical, and localization studies of the glycine receptor (2–5). Purified glycine receptor preparations from spinal cord of different mammals have been reported to contain two glycosylated membrane polypeptides of M, 48,000 and 58,000 and an additional 93,000 subunit that is thought to be a peripheral protein associated with cytoplasmic domains of the receptor (6, 7). The 48-kDa polypeptide has been shown to contain agonist and antagonist binding sites (8–12). Recent cloning of this subunit (13) has revealed that it shares significant sequence and structural homologies with the nicotinic acetylcholine receptor proteins and with the subunits of the GABAγ receptor, another chloride channel-coupled receptor (14). However, little is known about the molecular mechanisms of glycine receptor function and regulation.

Recent studies have suggested a key role for the phosphorylation of plasma membrane receptors in the regulation of receptor function and the modulation of the sensitivity of cells to extracellular signals (15, 16). For instance, β-adrenergic and other adenylyl cyclase-coupled receptors have been shown to be phosphorylated and modulated by different kinases such as cAMP-dependent protein kinase (PKA), protein-kinase C (PKC), and a specific, agonist-dependent β-adrenergic receptor kinase (15, 17, 18). Regarding channel-coupled receptors, the nicotinic acetylcholine receptor (AChR) has been reported to be directly phosphorylated by at least three protein kinases (PKA, PKC, and an endogenous tyrosine kinase) on seven phosphorylation sites located in different subunits (16). In some instances, AChR phosphorylation has been shown to increase its rate of desensitization (16, 19). Since there is little information in the involvement of second messenger systems and protein phosphorylation in the regulation of chloride channel-coupled receptors, the present study was undertaken to explore directly the ability of protein kinases to phosphorylate the purified glycine receptor. Our results show for the first time that PKC phosphorylates the 48-kDa subunit of the purified glycine receptor. In agreement with a possible physiological role for inhibitory amino acid receptor phosphorylation, different reports have appeared during the course of our work showing in vitro phosphorylation of the GABAγ receptor (20–22) and suggesting a role for phosphorylation mechanisms in the modulation of GABAγ receptor functions (23–25). Further, we show that the PKC phosphorylation site in the glycine receptor is located in the major intracellular loop of the 48-kDa subunit in a
functional domain homologous to those known to be phosphorylated in the different subunits of the nicotinic acetylcholine receptor, and also to the region corresponding to the β-subunit of the GABA<sub>B</sub> receptor which contains the PKA consensus sequence (13). This suggests a common mechanism of regulation for the members of this receptor superfamily. Some of the findings presented in this paper have been reported in preliminary form (26).

**EXPERIMENTAL PROCEDURES**

**Materials**—[H]<sup>3</sup>Strychnine (15-30 Ci/mmol) and [γ-<sup>32</sup>P]ATP were obtained from Amersham Corp. N-Chlorosuccinimide, histones, phosphatidylethanolamine, phosphatidylcholine, sodium cholate, protease inhibitors, TPCK-treated trypsin, protein A-Sepharose, and 12-O-tetradecanoylphorbol-13-acetate were purchased from Sigma. CNBr was from Merck; endoproteinase lysine C, from Boehringer Mannheim; AG 1-8X columns, from Bio-Rad; keyhole limpet hemocyanin, from Calbiochem; and molecular weight standards, from Pharmacia LKB Biotechnology Inc. All other chemicals were of the highest purity commercially available.

**Purification of Glycine Receptor**—Glycine receptor was purified to homogeneity by affinity chromatography on 2-aminomethyl-propanesulfonic acid agarose as reported (6, 27). The glycine receptor was eluted from the column with 120 mM KC1, 5 mM EDTA, 5 mM EGTA, 5 mM dihydrothreitol, 1 mM benzamidine, 17 millimolar/ml aprotinin, 2.5 mM leupeptin, 25 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamid enzyme, 1% sodium dodecyl sulfate, 0.12 mg/ml phosphatidylserine, and the protease inhibitors. The purified receptor bound 3.2 nmol of [3H]strychnine/mg of protein, representing a 3,000-fold purification, and displayed two protein bands of M<sub>r</sub> 48,000 and 38,000 when subjected to SDS-PAGE (27).

**Protein kinase C**—The protein kinase C was purified from rat brain by sequential chromatography in DE-52, hydroxylapatite, and phosphatidylserine-agarose columns as described (28-30). In some experiments, PKC purified from pig brain by Dr. Michel Bouvier (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC) as described (31) or from rat brain (32) by Dr. Lisardo Bosca (Facultad de Farmacia, Universidad de Madrid) was also used. Similar quantitative results were obtained with the different preparations. PKC activity was determined by histone (H1) phosphorylation as reported previously (33). One unit was defined as the amount of enzyme necessary to incorporate 1 pmol of phosphate in histones/min at 30 °C.

**Phosphorylation Assays**—The pure receptor (1-4 pmol of [H]<sup>3</sup>strychnine binding sites) was phosphorylated in a total volume of 50 μL with 5.10<sup>-10</sup>-5.10<sup>-11</sup> μg of PKC in a buffer containing 40 mM Tris-HCl, 8-16 mM potassium phosphate, pH 7.4, 3 mM EGTA, 3 mM EDTA, 0.13% (w/v) sodium cholate, 52 mM NaCl, 6 mM glycine, 1-2 mM β-mercaptoethanol, 14 mM MgCl<sub>2</sub>, 5 mM NaF, 7-9 mM CaCl<sub>2</sub>, 0.65-0.01% (w/v) phosphatidylcholine, 0.1 mg/ml phosphatidylserine, 10 μM 12-O-tetradecanoylphorbol-13-acetate, 200 μg/ml bacitracin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 50 μM phenylmethylsulfonyl fluoride, 50 μM benzamidine, and 50 μM N-ethylmaleimide in a buffer (see below). In some experiments, the incubation was terminated by dilution with ice-cold glycine receptor purification medium (buffer A), the mixture applied to Sephadex G-50 minicolumns (35), and the phosphorylated glycine receptor repurified from the eluate on 200-μl aminomethyl-propanesulfonic acid agarose columns essentially as described (27). The [γ-<sup>32</sup>P]ATP was eluted specifically with glycine, lyophilized, and subjected to SDS-PAGE and autoradiography.

**Synthesis of Phosphorylated Peptides**—Peptides were synthesized on a solid phase by the Merrif method on insulin (36). The amino acid composition of the synthetic peptide was verified by amino acid analysis. The peptide was coupled to keyhole limpet hemocyanin with NCS hydroxylate, and the phosphorylated peptide was separated by reversed-phase high pressure liquid chromatography in DE-52, hydroxylapatite, and phosphatidylserine-agarose columns (37). The [3H]strychnine concentration used was 25 nM.

**Preparation of Synthetic Peptide and Antibody**—The synthetic peptide CAKKIDKISR, corresponding to residues 384-392 of the 48-kDa subunit of the glycine receptor, was synthesized on a solid phase method on the Merrif method. The N-terminal carboxyl was blocked with ammonia and the amino terminus, was synthesized by the solid-phase method on the Merrif method. The amino acid composition of the synthetic peptide was verified by amino acid analysis. The peptide was coupled to keyhole limpet hemocyanin with NCS hydroxylate, and the phosphorylated peptide was separated by reversed-phase high pressure liquid chromatography in DE-52, hydroxylapatite, and phosphatidylserine-agarose columns (31) or from rat brain (32) by Dr. Lisardo Bosca (Facultad de Farmacia, Universidad de Madrid) as described (31). The peptide phosphorylation reaction was terminated by the addition of 50 μL of 30% acetic acid and the phosphorylated peptide separated from radioactive ATP and other phosphopeptides by using anion exchange (AG 1-8X and Sephadex G-25 columns (1 x 10 cm and 1.5 x 30 cm, respectively).

**Phosphorylation of Glycine Receptor by Antibodies**—Glycine receptor or the appropriate controls were preincubated for 45 min at 30 °C with 20-40 μg of the immunoglobulin fraction prior to the phosphorylation reaction, essentially as described for similar experiments with the nicotinic acetylcholine receptor (38).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Gel electrophoresis was performed by the method of Laemmli (39). SDS sample buffer consisted of 8% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.5, 0.003% bromphenol blue. The molecular mass of the resolved peptides was determined using polypeptide standards that were coelectrophoresed with the samples. [γ-<sup>32</sup>P]ATP was localized by autoradiography at -70 °C. The radioactive content of the 48-kDa bands excised from the dried gel was determined by Cerenkov spectroscopy. Since the 48-kDa subunit contains one strychnine binding site (2, 5, 12), the number of 48-kDa polypeptides in each experiment can be calculated by ligand binding (see below) and the phosphorylation stoichiomtery (mole of phosphate incorporated/nmol of 48-kDa polypeptide) determined.

**Ligand Binding Assay**—Glycine-displaceable [H]<sup>3</sup>strychnine binding to the purified glycine receptor was determined as described (27). The [H]<sup>3</sup>strychnine concentration used was 25 nM.

**One-dimensional Phosphopeptide Maps of the Phosphorylated 48-kDa Subunit of the Glycine Receptor**—After SDS-PAGE in 10% polyacrylamide slab gels, an autoradiogram was used as a guide to cut out the protein bands of interest. These were then excised from the gel, washed, and hydrolyzed at trypticin phosphases by treating for 30-120 min with N-chlorosuccinimide (NCS) as described (38). The phosphopeptide maps were run by cellulose acetate (20 cm) and the phosphorylated peptides were revealed as yellow bands (20-40 cm) using a 12-20% linear gradient of polyacrylamide according to the method of Cleveland et al. (39) for peptide mapping. The [γ-<sup>32</sup>P]ATP-labeled fragments from NCS hydrolysis were identified by autoradiography. Some of these phosphopeptides were cut out from the gel and the reawaken gel pieces digested further at methionine residues with CNBr as reported (40). The final products of cleavage were again resolved by SDS-PAGE as described (39), using a 12-20% linear gradient of polyacrylamide, and identified by autoradiography of the dried gel. In some other experiments, the SDS-polyacrylamide gel slice containing radioactive 48-kDa protein was incubated for 18 h at 37 °C in 600 μL of a solution containing 0.1 mg/ml endoproteinase lysine C. The gel fragments were removed by centrifugation and the supernatant containing the digested peptides lyophylized. The proteolytic fragments were then resolved by gel electrophoresis in 20% polyacrylamide, 0.5% bisacrylamide, 10% glycerol (a method that allows an accurate determination of the M<sub>r</sub> of low molecular weight peptides (41)) and phosphopeptides identified by autoradiography. In all cases, the M<sub>r</sub> of the labeled proteolytic fragments was determined on the basis of the relative mobility of an appropriate set of standards. Since the amino acid sequence of the rat 48-kDa glycine receptor subunit is known (38), the M<sub>r</sub> of labeled fragments were compared with the molecular weights of the peptides that could theoretically be generated as a result of the specific cleavage of the 48-kDa polypeptide by each of the proteolytic reagents.

**Phosphoamino Acid Analysis**—The phosphorylated band corresponding to the 48-kDa polypeptide was excised from the gel and hydrolyzed in 6 N HCl for 3 h at 110 °C in a sealed tube. The hydrolysate and the appropriate phosphoamino acid stand-
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RESULTS

Phosphorylation of Purified Glycine Receptor—Fig. 1 illustrates the phosphorylation of the purified glycine receptor by protein kinase C. In the initial experiments it was observed that when the glycine receptor was incubated with partially purified protein kinase C preparations under phosphorylating conditions, a protein band of Mr = 48,000 was consistently labeled with 32P. Glycine receptor preparations did not show endogenous kinase activity. Under similar experimental conditions, the phosphorylation of the 48-kDa band was not observed when the catalytic subunit of cAMP-dependent protein kinase was included instead of protein kinase C (data not shown). The phosphorylated 48-kDa protein comigrated in SDS-PAGE gels with the 48,000 subunit of the iodinated purified glycine receptor. Moreover, the 48,000 phosphoprotein could be separated from the other phosphorylated peptides of the mixture by affinity chromatography on 2-aminostrychnine-agarose minicolumns and specifically eluted with glycine, thus allowing the identification of this phosphorylated band with the strychnine-binding subunit of the glycine receptor (data not shown).

Fig. 1 also shows that the phosphorylation of the 48-kDa subunit of the glycine receptor by protein kinase C is markedly increased (~25-fold) in the presence of protein kinase C activators in the incubation medium, thus rendering unlikely the possibility that the receptor phosphorylation is due to a contaminating kinase activity. Phosphorylation of the 48-kDa subunit was also observed when phosphorylation assays were performed in the absence of glycine (i.e. with previously dialyzed glycine receptor preparations), thus suggesting that the presence of agonists is not strictly required for the reaction to take place.

Rate and Stoichiometry of Phosphorylation—The time course of receptor phosphorylation by protein kinase C is shown in Fig. 2. The phosphorylation of the 48-kDa polypeptide increased rapidly during the first minutes and was apparently complete within 30–60 min. The stoichiometry of phosphorylation was ~0.4 pmol of phosphate incorporated per pmol of strychnine binding sites at 5 min of incubation whereas at the 30–60 min time points a stoichiometry of ~0.8 pmol of 32P/pmol of receptor is attained. Our data indicate that the phosphorylation rate is comparable to those observed for other receptor systems (31, 44) and suggest that the 48-kDa subunit of the glycine receptor is phosphorylated by protein kinase C at a single site (see below). Phosphoamino acid analysis of 32P-labeled receptor revealed that only serine residues were phosphorylated (data not shown).

Location of the Phosphorylation Site in the Receptor—In the next set of experiments we attempted to localize the phosphorylation site in the structure of the 48-kDa subunit. Considering the limited quantities of starting material available (i.e. 32P-labeled glycine receptor) in order to perform phosphopeptide sequencing, we chose to use sequence-specific proteolytic agents to cleave the protein and generate phosphopeptide maps. Because the primary structure of the 48-kDa polypeptide is known (13), the site(s) of phosphorylation may be deduced from the sizes of the labeled fragments.

In a first step, glycine receptor was subjected to phosphorylation by protein kinase C, the phosphorylated 48-kDa band identified by autoradiography, excised from the gel, and cleaved at tryptophan residues with NCS for 30 min as
described under "Experimental Procedures" (Fig. 3A, lane 2), 2 h (Fig. 3A, lane 3), or not treated (Fig. 3A, lane 1). NCS digestion for 30 min produced a lower molecular weight phosphopeptide that migrates on SDS-PAGE gels with a M₀ of 13,000. Other phosphopeptides of apparent molecular weight 15,000, 20,000, 23,000, 29,000, 35,000, and 40,000 were also found as a result of partial cleavage of the 48-kDa polypeptide. Longer periods of incubation with NCS resulted in an increased labeling of the M₀ ∼ 13,000 and ∼ 15,000 phosphopeptides (Fig. 3A, lane 3). The analysis of the theoretical products of complete cleavage of the glycine receptor subunit by CNBr (see Fig. 4A) indicates that the lower molecular weight phosphopeptide detected and the overall phosphoprotein pattern are only consistent with the phosphorylation site(s) being located in the fragment corresponding to amino acids 287-407 of the 48-kDa sequence (predicted M₀ of 13,000). According to the predicted structural model of the 48-kDa protein (13), peptide 287-407 would include most of the third and fourth membrane-spanning regions and the major intracellular loop between such transmembrane domains. Peptide 287-407 contains 7 serine residues, at positions 296, 308, 337, 353, 370, 372, and 391. To characterize further the phosphorylation site(s), the two major phosphopeptides obtained from NCS cleavage (those of M₀ ∼ 13,000 and ∼ 15,000, probably corresponding to fragments 287-407 and 287-421, respectively), were excised from the gel and analyzed by a second digestion with cyanogen bromide. In our experimental conditions, a single phosphopeptide with an apparent molecular weight of 3,700 was obtained (Fig. 4B). According to the predicted CNBr cleavage products of these peptides shown in Fig. 4B, this result discards the 3 serines contained within the fragment 287-341 as the phosphorylation site(s). The size of the labeled peptide could correspond either to fragment 342-375 (3,500 predicted molecular weight, containing serine residues 353, 370, and 372) or the partial cleavage product 376-404 (3,650 predicted molecular weight, containing serine residue 391).

In a different group of experiments, endoproteinase lysine C was also used to localize the region of the 48-kDa glycine receptor subunit phosphorylated by protein kinase C. Endoproteinase lysine C is specific for lysine residues and cleaves on the carboxyl terminus of the peptide bond. Fig. 3C shows that an 18-h treatment of the phosphorylated 48-kDa polypeptide with this proteolytic reagent generates a single labeled peptide of M₀ ∼ 2,800 when the digestion products were separated by gel electrophoresis in 20% polyacrylamide, 0.5% bisacrylamide, and 10% glycerol, a method reported to allow an accurate determination of low molecular weight peptides (41). Interestingly, a limit fragment of 2,770 daltons is predicted by the specific cleavage of the 48-kDa polypeptide by endoproteinase lysine C, corresponding to amino acid residues 290-411 and containing serine 391 (see Fig. 4C), one of the four possible phosphorylation sites according to the results from NCS and cyanogen bromide digestions (see above). In fact, inspection of the sequence (13) around the four possible phosphorylation sites indicates that serine 391 is the only candidate residue that is flanked by basic amino acids at both sides, a condition known to have an important influence in the site specificity of protein kinase C (45).

Therefore, we synthesized the peptide CAKKIDKISR, corresponding to residues 384-392 of the 48-kDa subunit of the rat glycine receptor (13), with a cysteine added to the amino terminus. This sequence would be located in a cytoplasmic domain close to the fourth membrane-spanning region, according to the model predicting the transmembrane orientation of the glycine receptor subunit (13), and contains serine 391, the proposed phosphorylation site by protein kinase C. As shown in Fig. 5A, the synthetic peptide is specifically phosphorylated by PKC. The phosphorylated synthetic peptide and the phosphorylated 48-kDa glycine receptor subunit were completely digested by TPKC-treated trypsin in order to compare their tryptic phosphopeptide maps. Fig. 5B shows that the labeled products of tryptic digestion of the synthetic peptide (left panel) and the 48-kDa subunit of the glycine receptor (center panel) migrated to a similar position when subjected to two-dimensional thin layer chromatography. An identical peptide map was also obtained when a mixture of equal radioactive quantities of the two samples were subjected together to two-dimensional peptide mapping (Fig. 5, right panel). Moreover, we tested the effect of antibodies elicited against the synthetic peptide on glycine receptor phosphorylation by protein kinase C. Fig. 6 shows that preincubation of the receptor with antipeptide antibodies caused a dose-dependent, marked inhibition of phosphorylation of the 48-kDa subunit of the receptor (compare lanes 2, 4, and 5 of Fig. 6) without affecting PKC autophosphorylation (Fig. 6, lanes 1 and 3). Although indirect steric or conformational effects as a consequence of the receptor-antibody interaction cannot be ruled out, this result is consistent with the phosphorylated site being located within peptide 384-392. Taken together, our results strongly suggest that the 48-kDa subunit is phosphorylated by protein kinase C at a single site and that serine 391 is the phosphorylated residue.

**Discussion**

The results presented in this paper indicate clearly for the first time that the 48-kDa subunit of the purified glycine receptor is stoichiometrically phosphorylated (~0.8 mol/mol) by protein kinase C. These results add this chloride channel-
coupled receptor to a growing number of plasma membrane receptors (epidermal growth factor, β-adrenergic, α₁-adrenergic, insulin, transferrin, and nicotinic acetylcholine receptors) that are known to serve as substrates for protein kinase C in vitro (for a review see Ref. 15). As for most of these receptors, the physiological significance of the glycine receptor phosphorylation has yet to be established. However, the phosphorylation of the 48-kDa subunit of the glycine receptor by protein kinase C seems to fulfill some of the requirements of a potentially important in vivo modification; the reaction proceeds rapidly and stoichiometrically, and the phosphorylation site is located on an intermembrane loop which, according to the structural model predicted for ligand-gated receptor channels, is present on the cytoplasmic side of the membrane (13, 14).

Several lines of evidence suggest strongly that phosphorylation takes place at serine 391, close to the fourth predicted transmembrane segment. First, the phosphopeptide pattern generated by proteolytic cleavage with NCS, cyanogen bromide, and endoproteinase lysine C is only consistent with the location of the phosphorylation site within amino acids 287-407 and suggests serine 391 as the phosphorylation site on
the basis of the sizes of the labeled products of cleavage.  

Second, serine 391 shows a flanking sequence similar to those recognized by protein kinase C in several other substrates (45). Third, a synthetic peptide containing serine 391 (corresponding to amino acid residues 384–392 of the 48-kDa protein) is specifically phosphorylated by protein kinase C, and its tryptic digest comigrates with the single labeled spot generated by complete tryptic digestion of the phosphorylated 48-kDa subunit of the glycine receptor (Fig. 5). Finally, antipeptide antibodies inhibit PKC phosphorylation of the glycine receptor (Fig. 6). Interestingly, serine 391 and its flanking sequence in the rat glycine receptor subunit are conserved in the two isoforms of the human 48-kDa protein cloned recently by Betz and colleagues (46), a fact consistent with an important regulatory role for this phosphorylation reaction.

Fig. 7 presents a model of the position of the phosphorylation site with respect to the functional domains of the 48-kDa subunit of the glycine receptor. It is worth noting that the proposed phosphorylated region in the glycine receptor is homologous to that containing a cAMP-dependent protein kinase consensus sequence in the β-subunit of the GABA_A channel (14). Although recent reports have shown in vitro phosphorylation of this receptor by protein kinases A and C (20–22), no attempt has been made to localize the phosphorylated residue(s).

The possible location of phosphorylated residues in chloride channel-coupled receptor subunits shown in Fig. 7 is also similar to those reported for different protein kinases in each of the subunits of the nicotinic acetylcholine receptor. These subunits are phosphorylated in a region of the major intracellular loops adjacent to the membrane-spanning α-helices.
that are proposed to form the sodium channel in the receptor models (16, 19). Thus, our results would reinforce the homology existent among these receptor proteins (14). It is tempting to speculate that, as suggested for G protein-coupled receptors (47), the ligand-gated receptor channels might contain a homologous regulatory domain, target of protein kinases implicated in modulating its function. In fact, the proposed location of this domain may be advantageous for regulating the activity of the channel or in modulating its interaction with other membrane or cytoskeletal proteins.

Little is known, however, about the effects of phosphorylation of ligand-gated channels on receptor function in vivo. In other transduction systems, receptor phosphorylation has been implicated in the molecular mechanisms of desensitization (15, 43, 48). Whereas the phenomenon of desensitization to GABA, glycine, and nicotinic acetylcholine agonists has been described (49–51), its mechanisms and biological function remains unclear although it may serve to regulate neuronal responses that involve persistent synaptic activity. In this regard, Huganir et al. (52) have shown in vitro that phosphorylation of the nicotinic acetylcholine receptor by the cAMP-dependent protein kinase increases the rate of receptor desensitization. Studies performed in intact muscle preparations have also shown that forskolin, an activator of adenylate cyclase, augments the desensitization of the nicotinic receptor (53) although other authors argue that such modulation is independent of cAMP (54). Calcinion gene-related peptide has been shown to enhance the rate of desensitization of the nicotinic acetylcholine receptor in cultured rat muscle cells by a mechanism that involves cAMP-dependent phosphorylation of the receptor (55). On the other hand, it has been reported recently that agents that activate protein kinase C regulate the rate of desensitization of nicotinic acetylcholine receptor in cultured sympathetic ganglion neurons (56).

Regarding chloride channel-coupled receptors, it has been shown recently that GABAA receptor function in hippocampal cells is maintained by phosphorylation factors (23). In experiments conducted in Xenopus oocytes, protein kinase C activators decrease the amplitude of the GABA-activated current (24), thus suggesting that the activity of these receptors is under the control of this kinase and that neurotransmitters that activate protein kinase C could profoundly affect neuronal signaling. In addition to or instead of regulating receptor activity, glycine receptor phosphorylation by protein kinase C might regulate its rate of internalization and metabolic disposition, as suggested for other types of receptors (15).

In conclusion, our results suggest that phosphorylation of the glycine receptor by protein kinase C may contribute to cross-communication between transmission pathways. Further studies will be necessary to provide information concerning the molecular mechanisms of functional regulation of the glycine receptor in vivo.

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REFERENCES
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A

B

C

FIG. 4. Histograms of the peptides that could theoretically be generated from a complete digestion of the 48-kDa subunit of the rat spinal cord glycine receptor by N-chlorosuccinimide (A), cyanogen bromide (B), and endoproteinase lysine C (C). The different limit peptides are arranged according to their positions in the complete amino acid sequence of the 48-kDa polypeptide (13) 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 weights of the peptides are shown on the right. Molar weights were calculated on the basis of the actual amino acid composition and are corrected for peptide bonds. Panel B only shows the theoretical cyanogen bromide digestion products of the carboxyl-terminal part of the protein. Asterisks indicate the position of serine residues.

The physiological significance of the glycine receptor phosphorylation has yet to be established. However, the phosphorylation of the 48-kDa subunit of the glycine receptor by protein kinase C seems to fulfill some of the requirements of a potentially important in vivo modification; the reaction proceeds rapidly and stoichiometrically, and the phosphorylation site is located on an intermembrane loop which, according to the structural model predicted for ligand-gated receptor channels, is present on the cytoplasmic side of the membrane (13, 14).

Several lines of evidence suggest strongly that phosphorylation takes place at serine 391, close to the fourth predicted transmembrane segment. First, the phosphopeptide pattern generated by proteolytic cleavage with NCS, cyanogen bromide, and endoproteinase lysine C is only consistent with the location of the phosphorylation site within amino acids 287-407 and suggests serine 391 as the phosphorylation site on
Glycine Receptor Phosphorylation by Protein Kinase C

![Image of a diagram showing peptide phosphorylation and autoradiography results.](image)

**Fig. 5.** Localization of the phosphorylation site in the 48-kDa subunit of the glycine receptor. A, phosphorylation of synthetic peptide 384-392 by protein kinase C. The synthetic peptide containing the suggested phosphorylated residue serine 391 (corresponding to amino acid residues 384-392 of the 48-kDa subunit of the rat glycine receptor with an amino-terminal cysteine) was incubated with protein kinase C and separated by thin layer chromatography as described under "Experimental Procedures," and the dried plate was subjected to autoradiography for the detection of phosphorylation. B, comparison between tryptic phosphopeptide maps of the 384-392 peptide and of the 48-kDa subunit of the glycine receptor. The affinity-purified glycine receptor and the purified synthetic peptide were phosphorylated by PKC as detailed under "Experimental Procedures." The phosphorylated 48-kDa subunit and the phosphorylated synthetic peptide were digested completely with TPCK-treated trypsin and the phosphopeptides resolved by electrophoresis in the first dimension followed by ascending chromatography as detailed under "Experimental Procedures." The resulting autoradiograms are shown. Left and center panels, tryptic phosphopeptide maps of the synthetic peptide and of the 48-kDa glycine receptor subunit, respectively; right panel, mixtures of equal radioactive quantities of the two samples, demonstrating comigration of the labeled cleavage fragments (indicated with an arrow).

**Fig. 6.** Inhibition of glycine receptor phosphorylation by antibodies to peptide 384-392. Affinity-purified glycine receptor (~1.5 pmol of [H]strychnine binding sites) was preincubated for 45 min at 30°C with 20 μg (lane 4) or 40 μg (lane 5) of antipeptide 384-392 immunoglobulins or in the absence of antibodies (lane 2). Subsequently, the glycine receptor was incubated with PKC as described under "Experimental Procedures," and the phosphorylated bands were detected by gel electrophoresis followed by autoradiography. Lanes 1 and 3 show the autoradiophosphorylation of PKC preincubated in the absence (lane 1) or in the presence (lane 3) of 40 μg of antipeptide immunoglobulins. The autoradiogram obtained was subjected to densitometry in the area corresponding to the phosphorylated 48-kDa subunit of the glycine receptor, using a Molecular Dynamics 300A computer densitometer.

The possible location of phosphorylated residues in chloride channel-coupled receptor subunits shown in Fig. 7 is also similar to those reported for different protein kinases in each of the subunits of the nicotinic acetylcholine receptor. These subunits are phosphorylated in a region of the major intracellular loop adjacent to the membrane-spanning α-helices.
that are proposed to form the sodium channel in the receptor models (16, 19). Thus, our results would reinforce the homology existing among these receptor proteins (14). It is tempting to speculate that, as suggested for G protein-coupled receptors (47), the ligand-gated receptor channels might contain a homologous regulatory domain, target of protein kinases implicated in modulating its function. In fact, the proposed location of this domain may be advantageous for regulating the activity of the channel or in modulating its interaction with other membrane or cytoskeletal proteins.

Little is known, however, about the effects of phosphorylation of ligand-gated channels on receptor function in vivo. In other transduction systems, receptor phosphorylation has been implicated in the molecular mechanisms of desensitization (15, 43, 48). Whereas the phenomenon of desensitization to GABA, glycine, and nicotinic acetylcholine agonists has been described (49–51), its mechanisms and biological function remains unclear although it may serve to regulate neuronal responses that involve persistent synaptic activity. In this regard, Huganir et al. (52) have shown in vitro that phosphorylation of the nicotinic acetylcholine receptor by the cAMP-dependent protein kinase increases the rate of receptor desensitization. Studies performed in intact muscle preparations have also shown that forskolin, an activator of adenylate cyclase, augments the desensitization of the nicotinic receptor (53) although other authors argue that such modulation is independent of cAMP (54). Calcium ion gene-related peptide has been shown to enhance the rate of desensitization of the nicotinic acetylcholine receptor in cultured rat muscle cells by a mechanism that involves cAMP-dependent phosphorylation of the receptor (55). On the other hand, it has been reported recently that agents that activate protein kinase C regulate the rate of desensitization of nicotinic acetylcholine receptor in cultured sympathetic ganglion neurons (56).

Regarding chloride channel-coupled receptors, it has been shown recently that GABA receptors function in hippocampal cells is maintained by phosphorylation factors (23). In experiments conducted in Xenopus oocytes, protein kinase C activators decrease the amplitude of the GABA, gated current (24), thus suggesting that the activity of these receptors is under the control of this kinase and that neurotransmitters that activate protein kinase C might profoundly affect neuronal signaling. In addition to or instead of regulating receptor activity, glycine receptor phosphorylation by protein kinase C might regulate its rate of internalization and metabolic disposition, as suggested for other types of receptors (15).

In conclusion, our results suggest that phosphorylation of the glycine receptor by protein kinase C may contribute to cross-communication between transmission pathways. Further studies will be necessary to provide information concerning the molecular mechanisms of functional regulation of the glycine receptor in vivo.

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### References