Modulation of Inhibitory Glycine Receptors by Phosphorylation by Protein Kinase C and cAMP-dependent Protein Kinase*

(Received for publication, July 6, 1993, and in revised form, September 20, 1993)

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Recent evidence has suggested a role for phosphorylation in the regulation of ligand-gated ion channels. We have recently shown (Ruiz-Gómez, A., Vaello, M., Valdivieso, F., and Mayor, F., Jr. (1991) J. Biol. Chem. 266, 559-566) that the inhibitory glycine receptor (GlyR) α subunit is phosphorylated in vitro by protein kinase C (PKC). In this report we further show that α subunits of the GlyR can also be phosphorylated by cAMP-dependent protein kinase (PKA) in an in vitro assay. Moreover, incubation of intact rat spinal cord neurons with specific PKC or PKA activators leads to increased phosphorylation of the GlyR α subunits, strongly suggesting a physiological role in its functional modulation. The role of protein phosphorylation in modulating GlyR channels was explored in Xenopus oocytes injected with poly (A)* mRNA isolated from nervous tissue. The treatment of oocytes with phorbol esters or dibutyryl cAMP resulted in a decrease or an enhancement, respectively, of glycine-evoked currents. Our results show that the GlyR can be phosphorylated in vivo in response to activation of either PKC or PKA with opposite functional consequences, suggesting that neurotransmitters affecting the activity of such kinases could profoundly alter glycine-mediated neuronal signaling and modulate synaptic efficacy.

The glycine receptors (GlyRs) are ligand-gated ion channels that mediate inhibitory transmission in the spinal cord and other regions of the central nervous system. The binding of glycine to its receptors promotes a large increase in chloride conductance, which causes membrane hyperpolarization; such actions are selectively antagonized by the competitive antagonist strychnine (Aprison and Daly, 1978). Purified GlyR preparations from mammalian spinal cord contain α (48 kDa) and β (58 kDa) subunits, which form the receptor channel. In some instances, peripheral membrane proteins associated to cytoplasmic domains of the receptor copurify with the GlyR (Pfeiffer et al., 1992; Langosch et al., 1988; Garcia-Calvo et al., 1989; Prior et al., 1992). Molecular cloning studies have revealed the existence of several α subunit isoforms (α1-3) with differing developmental and regional expression (Malosio et al., 1991a, and references therein). Such diversity has been increased by the demonstration of alternative splicing of the α1 subunit mRNA, leading to the expression of a variant (α1al) that contains an 8-amino acid insert in a putative cytoplasmic domain (Malosio et al., 1991b). Biochemical and pharmacological studies have demonstrated that α subunits carry the binding sites for agonists and for strychnine (Ruiz-Gómez et al., 1989, 1990; Schimieden et al., 1989; Kuhse et al., 1990a, 1990b). This indicates that the inhibitory glycine receptor (GlyR) can be phosphorylated and modulated by at least three different protein kinases: cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and an endogenous tyrosine kinase (reviewed in Wagner et al., 1991). PKA and PKC have also been shown to phosphorylate β and γ subunits of the GABA_2 receptor in vitro (Swpe et al., 1992; Moss et al., 1992a). Our laboratory has recently reported that the α subunit of the GlyR is phosphorylated in vitro by PKC in a serine residue close to the fourth transmembrane domain (Ruiz-Gómez et al., 1991) and that glycineric ligands modulate the rate of phosphorylation by this kinase (Vaello et al., 1992).

To clarify the possible physiological role of the phosphorylation of ligand-gated ion channels, two key questions have to be addressed: whether receptor phosphorylation occurs in intact nervous cells in response to specific activators of protein kinases, and which (if any) are the effects of such a covalent modification in receptor function. It is known that phosphorylation of nAChR regulates the rate of desensitization of the

*This work was supported by Comisión Interministerial de Ciencia y Tecnología Grant PB970216, PB92-0435 (to F. M.) and PB90006 (to J. L.), Boehringer Ingelheim and an institutional grant from Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GlyR, glycine receptor; nAChR, nicotinic acetylcholine receptor; PAG, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TPA, the phorbol ester 12-0-tetradecanoylphorbol-13-acetate; GABA, γ-aminobutyric acid.

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Glycine receptor (reviewed in Wagner et al., 1991; Swope et al., 1992; Raymond et al., 1989a, and recent reports using reconstituent GABA<sub>a</sub> receptor subunits or GluR6 receptors expressed in human embryonic kidney cells have demonstrated direct phosphorylation of receptor subunits by PKA leading to either decreased or increased responses to receptor agonists (Moss et al., 1992b; Raymond et al., 1993b; Wang et al., 1993). In line with these reports, we report herein that the subunit of the GlyR is phosphorylated in intact spinal cord cells in response to the presence of either PKC or PKA activators. Moreover, glycine-induced currents in Xenopus oocytes injected with nervous tissue poly(A<sup>+</sup>) mRNA were decreased after phorbol ester treatment of the oocytes and increased after incubation with dibutyryl-cAMP. These results strongly indicate a role for protein phosphorylation in modulating GlyR function in neuronal cells.

**EXPERIMENTAL PROCEDURES**

**Glycine Receptor Phosphorylation in Spinal Cord Cells—**Spinal cord cells were isolated from adult male Wistar rats as previously described (Johnston and Geiger, 1989). Briefly, the spinal cord and brain stem were dissected and rinsed in 10 volumes of ice-cold buffer A (25 mm glucose, 2.7 mm KCl, 1.3 mm MgSO<sub>4</sub>, 25 mm sodium pyrophosphate, 0.2 mm EDTA, 2.5 mg/ml bovine serum albumin, and 50 pg/ml soybean trypsin inhibitor). After 15 min at 37 °C with gentle shaking, the suspensions were centrifuged at 9500 × g for 5 min, pelleted again, and the supernatant subjected to SDS-PAGE (10% gels). In order to dissociate the cells, the medium was supplemented with 0.6 μg/ml collagenase (Boehringer Mannheim), 1% bovine serum albumin, and 50 μg/ml soybean trypsin inhibitor. After 10 min at 37 °C with gentle shaking, the suspensions were centrifuged (9500 × g for 5 min) and the supernatant subjected to SDS-PAGE (10% gels) and autoradiography. A control with preimmune serum was performed in all the experiments.

**In Vitro Phosphorylation Studies—**GlyR was affinity-purified from adult spinal cord and brain by normalizing (1–1 pmol of [H]strychnine-binding sites/assay) by PKC (5 × 10<sup>-5</sup> units) exactly as previously reported by our laboratory (Ruiz-Gómez et al., 1990, 1991). The reaction was stopped by addition of SDS sample buffer followed by SDS-PAGE (10% gels). In order to detect GlyR phosphorylation by PKA, an alternative experimental strategy previously reported by Riosse and Catterall (1987) and Lai et al. (1990) was used. Such strategy has been termed "back-phosphorylation" and is based on the incubation of receptor immunoprecipitates with purified protein kinases. In our experiments, GlyR was solubilized from adult rat spinal cord membranes with Triton X-100 as described (Pfeiffer et al., 1982; Ruiz-Gómez et al., 1989) and immunoprecipitated with the specific anti-peptide antibody Ab384 as described above.

**In other experiments, forskolin-treated or control isolated spinal cord cells were used as a source of GlyR in order to estimate the occurrence of PKA-mediated phosphorylation in vitro.** The immunoprecipitated complexes attached to protein A-agarose are first washed with solubilization buffer and then washed three more times at 4 °C with PKA phosphorylation buffer (50 mm Tris-HCl, pH 7.4, 4.5 mm MgCl<sub>2</sub>, 0.1 mm EDTA, 2 mm dithiothreitol, 0.05% Triton X-100, 12 mm NaF, 30 μg/ml soybean trypsin inhibitor, 600 μg/ml bacitracin, 200 μM ATP). The phosphorylation assay is performed by resuspending the washed pellet in 100 μl of PKA phosphorylation buffer containing 10<sup>-4</sup> units of purified PKA catalytic subunit (generous gift of Dr. Lissi Arceo, University of Granada, Madrid) and [γ-<sup>32</sup>P]ATP (3–5 counts/min/μmol). After incubation for 20 min at 30 °C, the reaction was terminated by rapid sedimentation of the protein A-agarose-attached proteins in a microfuge. The resulting precipitates were washed twice with resuspension/sedimentation in stop buffer (50 mm Tris-HCl, pH 7.4, 10 mm EDTA, 2 mm NaF, 0.2 mg/ml bacitracin, 1% Triton X-100, 10 mm sodium pyrophosphate, 600 μg/ml bacitracin, 10 μg/ml soybean trypsin inhibitor, 1 mm benzanilide, 17 milliunits/ml aprotinin, 2.5 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mm benzotriazol chloride, 2.5 mM iodoacetamide, 16 milliunits/ml aprotinin, 0.24 mg/ml bacitracin, 12 μg/ml soybean trypsin inhibitor (protease inhibitors mixture)). The homogenate was centrifuged at 20,000 g for 1 h in order to obtain a membrane pellet free of myelin (García-Calvo et al., 1989). Membrane proteins were subjected to SDS-PAGE (10% gels) and autoradiography.

**Expression in Xenopus Oocytes and Electrophysiology—**Poly(A<sup>+</sup>)-mRNA was isolated from the brain of 14-day-old rats using a established procedure (Fast Track, Invitrogen). Injection of RNA into Xenopus oocytes and voltage-clamp recordings were performed as previously described (Lerman et al., 1986). Briefly, mature oocytes were dissociated and injected with 50 ng of poly(A<sup>+</sup>)-mRNA dissolved in water. Oocytes were transferred to Leibovitz L-15 culture medium (0.7 strength, Sigma) supplemented with 5 mM HEPES, pH 7.6, 100 units/ml penicillin and 1 mg/ml streptomycin. After 5–9 days, oocytes were transferred to a new 100 μl recording chamber and perfused at a rate of 5 ml/min with control solution (10 mM HEPES, pH 7.2, 116 mM NaCl, 2 mM KCl, 0.5 mM CaCl<sub>2</sub>). The compounds to be assayed (glycine, kainate, GABA, strychnine, phorbol esters, or dibutylryl-CAMP) were dissolved in this buffer medium, bath-applied for the time desired, and then washed out. All experiments were done at room temperature (20°C). Whole-cell current measurements were performed under two-electrode voltage-clamp, at a holding potential of −60 mV. 

M.-L. Vaello and F. Mayor, Jr., manuscript in preparation.
PKC activators promote phosphorylation of the α subunit of the glycine receptor in adult rat spinal cord cells. Spinal cord cells isolated from adult rats were metabolically labeled with [32P]orthophosphate as detailed under "Experimental Procedures" and incubated for 15 min with different concentrations of TPA or vehicle alone. The membrane proteins were solubilized with 1% sodium cholate (A) or 1.5% Triton X-100 (B), and GlyR complexes were subsequently immunoprecipitated using a specific polyclonal antibody generated against a synthetic peptide corresponding to residues 384–392 of the rat α1 sequence (Ab384) or the appropriate preimmune controls. In panel A, spinal cord cells were incubated with vehicle alone (lane 1) or 0.5 μM TPA (lane 2) and solubilized extracts immunoprecipitated with Ab384. In panel B, cells were incubated with 0.5 μM TPA and extracts immunoprecipitated with preimmune serum (lane P) or Ab384 (lane 1). The immunoprecipitated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. The position of molecular weight standards is shown. The arrows indicate the position of the 48 kDa band specifically immunoprecipitated with Ab384 antisemur.

RESULTS

PKC Activators Promote GlyR Phosphorylation in Intact Spinal Cord Cells—Previous data obtained in our laboratory indicated that the α subunit of the GlyR purified from adult rat spinal cord was rapidly and stoichiometrically phosphorylated by PKC (Ruiz-Gómez et al., 1991). In order to test whether the receptor was phosphorylated in situ in response to the presence of messengers leading to PKC activation, spinal cord cells isolated from adult rat tissue were labeled with [32P]orthophosphate and treated for 15 min with 0.5 μM of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, TPA (Fig. IA, lane 2) or vehicle alone (Fig. IA, lane 1). Membrane proteins were solubilized with cholate and immunoprecipitated with a specific polyclonal antipeptide antibody raised against the GlyR (see “Experimental Procedures” for details). In the TPA-treated cells, the GlyR antibody immunoprecipitates a phosphorylated band of ~48 kDa, which corresponds to the reported molecular mass of the α subunit of the GlyR. In a different set of experiments, extracts of TPA-treated cells (obtained in this case with 1.5% Triton X-100 as detergent) were immunoprecipitated either with preimmune serum (Fig. IB, P) or AB-384 GlyR antibody (Fig. IB, I). This result indicates that immunoprecipitation of the labeled 48 kDa band is specific, since it is not observed with the preimmune serum. As will be discussed later, additional, higher M, phosphorylated proteins seem to be immunoprecipitated with the GlyR antibody, specially when membrane proteins are extracted with Triton X-100. Phosphorylation of the 48-kDa polypeptide is promoted by TPA concentrations ranging from 0.1 to 10 μM and is not observed in the presence of inactive TPA analogs (data not shown). Maximal phosphorylation in response to TPA is attained around 15 min of treatment and decreases thereafter (data not shown). A variable, small level of basal phosphorylation was also observed in different experiments (see Fig. 3).

Fig. 2A shows the immunoprecipitated 48-kDa phosphoprotein comigrates in SDS-PAGE gels with the α subunit of purified GlyR phosphorylated in vitro by PKC. Further, a phosphorylated 48-kD protein can be purified from TPA-treated cells by using Ni2+-nitruxynine affinity columns (data not shown). In addition to its specific recognition by GlyR antibodies, these results indicate that the 48 kDa band corresponds to the α subunit of the GlyR. Taken together, our results indicate that the α subunit of the GlyR is phosphorylated in intact spinal cord neurons upon activation of endogenous PKC.

The low abundance of GlyR makes direct sequencing and identification of phosphorylation sites not possible. Therefore, we have used sequence-specific proteolytic agents to cleave the 48-kDa protein and generate phosphopeptide maps. Such phosphopeptide maps were then compared with those previously obtained in our laboratory with the α subunit of the GlyR phosphorylated in vitro by PKC and from the theoretical products of specific cleavage in the rat α1 sequence (Ruiz-Gómez et al., 1991). When the 48-kDa phosphopeptide was subjected to digestion with N-chlorosuccinimide (cleaving at tryptophan residues), a lower molecular mass phosphopeptide of ~14 kDa was produced together with other labeled bands generated as a result of partial cleavage (Fig. 2B). The size of the lower molecular weight phosphopeptide and the overall pattern of cleavage are similar to those obtained with the in vitro phosphorylated α subunit (Ruiz-Gómez et al., 1991). Complete digestion of the immunoprecipitated GlyR subunit with endoproteinase lysine C generated a single labeled peptide of ~2.9 kDa (Fig. 2C).

Cyclic AMP-dependent Phosphorylation of the Glycine Receptor—In parallel experiments we investigated whether activation of endogenous PKA could also lead to GlyR phosphorylation. Fig. 3A shows the phosphorylation level of the immunoprecipitated GlyR α subunit when isolated adult rat spinal cells are incubated for 10 min with vehicle alone (lane 1), the adenyl cyclase activator forskolin, which is known to raise intracellular cAMP levels leading to PKA activation (lane 2) and TPA (lane 3). In addition to the above described effect of
brane proteins were solubilized with receptor. A, 0.6 mCi/ml [32P]orthophosphate and incubated for 10 min with 5 μM TPA (lane 3), 10 μM forskolin (lane 2), or vehicle alone (lane 1). Membrane proteins were solubilized with 1.5% Triton X-100 and extracts subsequently immunoprecipitated using Ab384 as detailed under “Experimental Procedures.” The samples were subjected to 10% SDS-PAGE and autoradiography. B, primary neuronal spinal cord cultures were labeled with 0.4 mCi/ml [32P]orthophosphate as detailed under “Experimental Procedures.” The samples were then subjected to 10% SDS-PAGE and autoradiography. C, in vitro phosphorylation of the α subunit of the GlyR by PKA catalytic subunit. Adult rat spinal cord membranes were solubilized with 1.5% Triton X-100 and immunoprecipitated using Ab384 (lane 2) or preimmune serum (lane 1), washed, and subsequently incubated with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-32P]ATP for 30 min at 30°C. For details see “Experimental Procedures.” The samples were then subjected to 10% SDS-PAGE followed by autoradiography. The arrows point to the 48-kDa phosphoprotein corresponding to the α subunit of the GlyR. D, aliquots of spinal cord cells isolated from adult rats were incubated as described under “Experimental Procedures” for 15 min at 37°C in the presence of 10 μM forskolin (lane 2) or vehicle alone (lane 1). Membrane proteins were solubilized as in A. The GlyR was immunoprecipitated with Ab384, the immunoprecipitates washed as in C and incubated with the catalytic subunit of PKA in the presence of [γ-32P]ATP for 10 min at 30°C as detailed under “Experimental Procedures.” The samples were then subjected to 10% SDS-PAGE and autoradiography. The position of molecular weight standards is shown on the left in all the panels.

PKC activators, an increase over basal phosphorylation is also noted in response to PKA activators. This effect was unexpected, since α subunits of the GlyR do not show consensus phosphorylation sites for PKA (Grenningloh et al., 1987). However, a recent report by Betz and colleagues had shown that an alternative splicing form of the α1 subunit, α1ins, contained an 8-amino acid insert in the intracellular domain including a serine residue that might serve as a potential phosphorylation site for cyclic nucleotide-dependent protein kinases (Malosio et al., 1991b). Northern analysis has indicated that the α1ins form represents ~30% of the total α1 subunit mRNA, which is expressed predominantly in the adult rat spinal cord (Malosio et al., 1991a, 1991b). In this regard, most of our in vitro phosphorylation experiments have been performed in spinal cord neuronal cultures isolated from fresh adult rat tissue instead of in primary neuronal cultures from embryonic rat spinal cord. The rationale for this choice (besides the higher amounts of GlyR-binding sites available) was the fact that different α subunit isoforms are expressed in the adult animal (α1) and the primary cultures, which preferentially express the neonatal α2 isoform (Malosio et al., 1991a; Hoch et al., 1989), and the fact that our previous in vitro PKC phosphorylation studies were performed with adult tissue. In this regard, it is worth noting that when experiment analogous to that shown in Fig. 3A were done in primary neuronal spinal cord cultures (Fig. 3B) the forskolin effect is not apparent (lane 2 versus control in lane 1), whereas a stimulation of GlyR α subunit phosphorylation similar to that found in adult tissue is noted in cultivated cells treated with phorbol esters (lane 3). Fig 3B also illustrates the previously mentioned finding that some GlyR-associated phosphorylated proteins are also immunoprecipitated with the antisera. The faint band below the 48-kDa phosphopeptide probably represents a proteolytic product of the α subunit, whereas the heavily phosphorylated protein of ~95 kDa may represent gephyrin, a tubulin-binding protein which is peripherally associated to cytoplasmic domains of the GlyR (Prior et al., 1992). Gephyrin has been shown to be phosphorylated by endogenous protein kinases (Langosh et al., 1992) and copurifies with the GlyR specially when solubilized with Triton X-100 (Pfeiffer et al., 1982; García-Calvo et al., 1989; Prior et al., 1992) as is the case in the immunoprecipitations were this band is more clearly observed.

To evaluate whether the observed effect of forskolin on the in vivo phosphorylation of the α subunit of the GlyR was a direct or an indirect effect, we tried to demonstrate that purified PKA can directly phosphorylate the GlyR. Since previous attempts with the purified GlyR had proven unsuccessful (Ruiz-Gómez et al., 1991; Langosh et al., 1992), we used an alternative experimental design known as back-phosphorylation. In such experiments, adult rat spinal cord membranes were solubilized and the extracts immunoprecipitated with preimmune serum (Fig. 3C, lane 1) or specific anti-GlyR antibodies (Fig. 3C, lane 2), washed, and incubated in the presence of [γ-32P]ATP and purified PKA catalytic subunit. Fig. 3C shows that, under such experimental conditions, the α subunit of the GlyR is clearly phosphorylated by PKA in a dose-dependent way (not shown) to an estimated stoichiometry of ~0.2 pmol of phosphate/pmol of [3H]strychnine-binding sites, thus suggesting that only a subpopulation of GlyR α subunits is being phosphorylated. It is worth noting that the extent of PKA back-phosphorylation attained in glycine receptors immunoprecipitated from forskolin-treated spinal cord cells is diminished with respect to non-treated cells (compare lane 2 versus 1 in Fig. 3D), thus suggesting that a fraction of glycine receptors are phosphorylated by PKA in vivo, so subsequent in vitro phosphorylation by this kinase is reduced.

Functional Consequences of GlyR Phosphorylation in Response to PKA and PKC Activators—To examine the possible role of the observed GlyR phosphorylation in receptor function, we have used the Xenopus oocytes heterologous expression system. After injection with rat brain poly(A)+ mRNA, oocytes responded to the agonists GABA, kainate, and glycine under voltage-clamp conditions (Fig. 4, A and B, data not shown). Fig. 4, A and B, show that a brief (1–2 min) perfusion of the oocytes with TPA (0.1–1 μM) promotes a time-dependent decrease in the glycine and GABA elicited currents, whereas the response to kainate is not significantly affected. Similar incubations with the inactive TPA analog 4-α-phorbol 12,13-didecanoate (2.5 μM) did not induce any change in response to the different agonists (n = 2, data not shown). A similar effect of phorbol esters, which would lead to the activation of the oocyte PKC, either endogenous or directed by the injected poly(A)+ mRNA, on GABA-A channels has been previously described in Xenopus oocytes injected with chicken brain mRNA (Sigel and Baur, 1988). The lack of effect of PKC
activators on kainate currents has also been reported in the Xenopus oocyte system (Lotan et al., 1990). Thus, our results indicate that GlyR function is negatively modulated by PKC activators. On the contrary, a brief incubation of the oocytes with dibutyryl cAMP, which leads to endogenous PKA activation, results in a moderate but significant increase in glycine-induced currents without affecting the response to kainate (Fig. 5, A and B). These results tie in nicely with recent reports showing that similar concentrations of dibutyryl cAMP promote an enhancement of glycine-activated currents in isolated spinal trigeminal neurons (Song and Huang, 1990). It is worth noting that the phosphatase inhibitor okadaic acid (1 μM) applied in the bath for 2 min led to a selective increase (27% after 60 min) in glycine-induced currents, with no change in kainate or GABA-induced responses (not shown). This result would suggest that a certain level of PKA-mediated GlyR phosphorylation is taken place in the oocyte under basal conditions. Interestingly, the subsequent incubation of a dibutyryl cAMP-treated oocyte (Fig. 5B) with TPA produced a decrease on glycine-induced responses (Fig. 5C) which was similar to that found in untreated oocytes (Fig. 4), thus suggesting independent sites for PKA and PKC phosphorylation and modulation of GlyR. Overall, our results strongly suggest that a dual, opposite modulation of GlyR function can be observed as a consequence of PKA- or PKC-mediated protein phosphorylation.

**DISCUSSION**

The phosphorylation of ligand-gated receptor channels by second messenger-regulated protein kinases such as PKA and PKC seems to emerge as a major mechanism for the modulation of cross-communication between transduction pathways (Huganir and Greengard, 1990; Swope et al., 1992; Raymond et al., 1993a). A better understanding of such mechanisms requires the identification of phosphorylation targets in these receptors both in vitro and in vivo and the characterization of the functional consequences of receptor phosphorylation. In this study, we show that the α subunit of the glycine receptor (GlyR) can be phosphorylated in intact spinal cord neurons in response to the presence of either PKC or PKA activators. Moreover, using the Xenopus oocyte heterologous expression system, we demonstrate that activators of these two different kinases promote opposite functional effects on the GlyR.

**In situ** phosphorylation of the α subunit of the GlyR by PKC is consistent with our previous report showing that this kinase phosphorylates the α subunit of the receptor in vitro, in a cytoplasmic serine residue (Ser-391) close to the putative fourth transmembrane domain (Ruiz-Gómez et al., 1991). The time course of phosphorylation and in vitro experimental conditions are similar to those reported for PKC-mediated phosphorylation of the nAChR and other membrane proteins (Ross et al., 1988; Piller et al., 1989; Clark et al., 1991), thus supporting a direct effect. Phosphopeptide maps generated from the immunoprecipitated 48-kDa phosphoprotein are similar to those obtained after in vitro phosphorylation by PKC of the GlyR α subunit (Ruiz-Gómez et al., 1991). This result suggests that the same serine residue of the α subunit phosphorylated in vitro (Ser-391) is also phosphorylated in vivo upon activation of endogenous PKC, although this has to be confirmed by using recombinant α subunits and site-directed mutagenesis. Phorbol esters clearly increase GlyR phosphorylation in both spinal cord cells isolated form adult rat spinal cord, where α1 is the predominantly expressed subunit isoform (Malosio et al., 1991a) and in embryonic primary neuronal cultures, which express the neonatal form of the α subunits, α2 (Hoch et al., 1989). It is worth noting that these isoforms show high sequence homology in the proposed domain of phosphorylation.

Our data also indicate that the α subunit of the GlyR is phosphorylated in spinal cord neurons isolated form adult rat tissue in response to the presence of 10 μM forskolin. This adenyl cyclase activator has been used at the same concentration and in similar experimental conditions to assess PKA-mediated phosphorylation of nAChR and GABAA receptors in both primary cultures and heterologous expression systems (Miles et al., 1987; Moss et al., 1992b; Swope et al., 1992).
Although modulation of GlyR function by PKA activators has been recently described in spinal trigeminal neurons (Song and Huang, 1990), the target of forskolin stimulation was somewhat surprising, since α subunits do not display a clear consensus sequence for PKA phosphorylation (Grenningloh et al., 1987). However, as mentioned under “Results,” Betz and colleagues have reported the occurrence of an alternative splicing form of the α1 subunit which bears a potential PKA phosphorylation site (Malosio et al., 1991b). Such variant may represent ~30% of the total α subunits in the adult spinal cord (Malosio et al., 1991a). In line with this observation, we show in this paper that the catalytic subunit of PKA can phosphorylate in vitro α subunits of the GlyR immunoprecipitated from adult rat spinal cord extracts (Fig. 3C). On such basis, it is tempting to speculate that PKA would phosphorylate a specific subset of α1 subunits both in vitro and in situ. This situation would be reminiscent to that observed in the case of the homologous GABA_A receptor, where two alternative splicing forms of the γ2 subunit have been shown to differ by the presence of an 8-amino acid insert (as the α_{7,3} subunit which bears a potential PKA phosphorylation site in Xenopus oocytes. This experimental system has been previously employed for assessing the modulation of receptors and channels by protein kinases (Sigel and Baur, 1988; Lerma et al., 1989; Letan et al., 1990; Swope et al., 1990). Our results indicate that agents that activate PKC decrease glycine-induced currents, while PKA modulators increase the response to glycine. Although such data do not provide evidence for the actual phosphorylation of GlyR channels in the oocyte, the results obtained in intact spinal cord cells are consistent with a direct effect of phosphorylation in receptor function. Since GlyR α subunits have been shown to be critical for both ligand binding and channel function (Betz, 1990), it is reasonable to assume that the observed functional effects are related to a subunit phosphorylation. However, a role for phosphorylation of β subunits or other GlyR-associated proteins in regulating GlyR function cannot completely be ruled out. The observed enhancement of glycine responses upon treatment with PKA activators is in agreement with a recent report showing positive modulation of GlyR by PKA in isolated spinal trigeminal neurons, by a mechanism involving increased probability of channel opening (Song and Huang, 1990). We are not aware of reports describing functional effects of PKC activators on GlyR function. Phosphorylation of muscle and neuronal nAChR by PKC has been shown to increase the rate of desensitization to agonists of these receptors. Activation of PKA in muscle cells also leads to an increase in the rate of the rapid phase of desensitization. On the contrary, cAMP analogs seem to enhance the intensity of neuronal nAChR-mediated responses (Wagner et al., 1991; Swope et al., 1992). Very recent evidence indicates that PKA increases the amplitude of responses to glutamate of GluR6 homomeric receptors (Raymond et al., 1993b; Wang et al., 1993). Regarding the GABA_A receptor, recent experiments in 293 cells expressing defined GABA_A receptor subunits have demonstrated that cAMP-dependent phosphorylation decreases the amplitude of GABA response and shows the rate of desensitization of some receptor subtypes (Moss et al., 1992b). PKC phosphorylation has been suggested to decrease GABA_A receptor function (Sigel and Bour, 1988). However, results on the physiological effects of phosphorylation of GABA_A receptors have been complex and sometimes contradictory (discussed in Raymond et al., 1993a; Moss et al., 1992b). This fact may be due, at least in part, to the potentially different regulation by phosphorylation of the different subunits isoforms (Moss et al., 1992a, 1992b). In the case of the GlyR, if our suggestion regarding α_{7,3} as the specific target for PKA phosphorylation is confirmed, only receptor subtypes containing this α subunit variant would have the ability to be modulated by cAMP-dependent protein phosphorylation. Thus, changes in the developmental and/or cellular expression of particular subunit isoforms would lead to the assembly of receptor subtypes with different subunit composition and with variable patterns of phosphorylation and modulation, further contributing to generate receptor diversity and functional heterogeneity within the nervous tissue.

In conclusion, our results indicate that the GlyR can be phosphorylated in intact spinal cord neurons in response to the presence of either PKC or PKA activators and that the stimulation of these transduction pathways leads to a decrease or an enhancement of GlyR functionality, respectively (see Fig. 6). Such dual mechanism of regulation would provide a very sensitive instrument for the integrated modulation of the GlyR by other extracellular messengers acting in the same neuron. The previous or simultaneous activation of a variety of G protein-coupled receptors modulating cAMP or diacylglycerol levels would lead to changes in the subsequent neuronal responses to glycine and in the transmission at medulla and spinal levels (Song and Huang, 1990). Such cross-talking mechanisms would help to integrate different extracellular signals at the neuronal membrane level, and account for transient changes in the efficacy of synaptic transmission, thus contributing to synaptic plasticity.
Acknowledgments—We thank Prof. F. Mayor for continuous encouragement, A. García-Ballesteros for participating in some experiments, and M. Sanz for skillful secretarial assistance.

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