Endogenous Phosphorylation of Distinct γ-Aminobutyric Acid Type A Receptor Polypeptides by Ser/Thr and Tyr Kinase Activities Associated with the Purified Receptor*

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We have investigated the phosphorylation of γ-aminobutyric acid type A (GABAA) receptors purified from bovine cerebral cortex in the absence of added kinases. Incubation of the affinity-purified receptor with [γ-32P]ATP and 500 µM MnCl2 yielded incorporation of 0.45 mol of 32P/mol of muscimol binding sites within 2 h at 30 °C. Mn2+ was much more effective than Mg2+ as activator. Phosphorylation of the receptor was observed on at least three different polypeptides of 51, 53, and 55 kDa. It was predominant on 51- and 53-kDa polypeptides that co-migrate with the [3H]flunitrazepam photoaffinity-labeled bands, suggesting that 32P incorporation mainly occurs on α-subunits. A monoclonal antibody specific for α-subunit adsorbed the endogenously phosphorylated GABAA receptor with a stoichiometry close to 1 mol of phosphate/mol of muscimol. The phosphorylation of the 51-kDa polypeptide, corresponding to α1-subunit, exhibited a micromolar affinity for ATP and sigmoid kinetics (nH = 2). Major incorporation of phosphate occurred on serine and threonine residues in roughly equimolar ratio. By enzyme-linked immunosorbent assay and immunoblotting studies we also detected a minor incorporation on tyrosine residues; this was specific for a 55-kDa polypeptide. Comparison with molecular data suggests that at least α1- and α2-subunits (Ser and Thr residues) and possibly γ-γ-subunits (Tyr residues) are endogenously phosphorylated by multiple kinases, with a clear preference for α1-subunit. The β-subunits were not phosphorylated in our experimental conditions. The corresponding kinase activities are closely associated to the receptor protein, indicating a new complexity in the regulation of the GABAA receptor.

γ-Aminobutyric acid type A (GABAA) receptors are ligand-gated anion channels that mediate most inhibitory synaptic transmission in the central nervous system. Molecular studies have identified five distinct subunits with, for most of them, multiple subtypes; 17 genes have been characterized so far (1, 2). Functional studies have used heterologous expression of several subunits to produce functional GABAA receptors (3, 4). At the native protein level, ligand binding heterogeneity of the receptor was demonstrated by photoaffinity labeling and autoradiography (5, 6).

Phosphorylation is a common mechanism for the regulation of receptor function. All subunit subtypes of the GABAA receptors contain some consensus substrate sequences for kinases such as cAMP-dependent protein kinase, protein-tyrosine kinase, and/or Ca2+-phospholipid-dependent protein kinase C (7). Moreover, type 2 calcium/calmodulin-dependent protein kinase and GMP-dependent protein kinase phosphorylate the intracellular domains of γL and γS fusion proteins (8).

Using purified GABAA receptor, Browning et al. (9) reported that β-subunits could be phosphorylated in the presence of exogenously added cAMP-dependent protein kinase and Ca2+-phospholipid-dependent protein kinase C, Ca2+-phospholipid-dependent protein kinase C and cAMP-dependent protein kinase phosphorylation have been reported to modify the amplitude of GABA-activated currents (4, 10–13).

In acutely dissociated neurons, it has been reported that favorable conditions for phosphorylation were required to prevent “run-down” of GABAA currents (14–16). The authors excluded the possibility that Ca2+-phospholipid-dependent protein kinase C, cAMP-dependent protein kinase, or type 2 calcium/calmodulin-dependent protein kinase could be operative; they proposed that the maintenance of normal GABA currents required the activity of a unique kinase specific for the GABAA receptor.

Although many molecular and biochemical analyses have shown that β1- (4), β3- (17), and γ2-subunits (18) of GABAA receptors are good substrates for several exogenously added kinases, there is little information on possible endogenous phosphorylation by kinase activities associated to the receptor. In GABAA receptor purified from rat cerebellum, it has been reported that an α-subunit could be phosphorylated by a receptor-associated protein kinase (19). In the present study, we describe multiple endogenous kinase activities that co-purify with the GABAA receptor protein purified from cerebral cortex.

EXPERIMENTAL PROCEDURES

Materials—Radioligands were purchased from DuPont NEN Chemicals GmbH (Dreieich, Germany): [3H]muscimol, 16 Ci/mmol; [3H]flunitrazepam, 87 and 103.1 Ci/mmol; and [γ-32P]ATP, 30 Ci/mmol. Hionic Fluor scintillation mixture, toluene, and soluene were from Packard (Groningen, The Netherlands); acrylamide, SDS, glycerine, and the molecular weight electrophoresis calibration kit were from Pharmacia LKB (Uppsala, Sweden); N,N,N',N'-tetramethylethylenediamine was from Serva (Heidelberg, Germany); Comassie Brilliant Blue R-250 was from Fluka Chemie (Buchs, Switzerland); hydrogen peroxide was from Carlo Erba (Milan, Italy); anti-GABA receptor, α-chain (clone bd 24) monoclonal antibody, anti-mouse Ig polyclonal antibody (fluorescent labeled), and the nonradioactive tyrosine kinase assay kit were from
Boehringer Mannheim. Flurazepam, diazepam, and GABA were obtained from Sigma. Other chemicals and solvents of analytical grade were obtained from E. Merck (Darmstadt, Germany).

Preparation of Membranes—Fresh cow brains were obtained from a local slaughter house. White matter was dissected out of brain samples, and the membrane fraction was prepared as described previously (6).

Phosphorylation of the GABA\textsubscript{A} Receptor—Membranes were solubilized in a phosphate buffer (10 mM) containing a final concentration of 1% Triton X-100. The solubilized receptor was applied on an affinity column as described previously (20). For elution, phosphate buffer was replaced by Tris buffer containing Flurazepam (10 mM) with urea at concentrations ranging from 2 to 6 M. Protein was estimated according to Lowry et al. (22).

Binding Studies—The methods used have been previously described (6). Briefly, the GABA\textsubscript{A} site was assayed by measuring the binding of 0.5–40 nM \textsuperscript{3}H Jnusmcimol. The benzodiazepine site was assayed by measuring the binding of \textsuperscript{3}H Flunitrazepam for 60 min at 4°C, with 10 \mu M diazepam for the blank. Activity for both radioactive ligands was determined after polyethylene glycol/bovine \gamma-globulin precipitation and centrifugation. The pellets were then dissolved overnight, and radioactivity was counted.

Phosphorylation—Receptor preparations (5–10 \mu g prot/ml) were incubated in the presence of 0.33 \mu M [\gamma\textsuperscript{32}P]ATP for 30 min at 30°C. The medium contained Heps-Tris buffer at pH 7.3 and different amounts of divalent cations (see figure legends). In some cases the phosphorylated receptor preparation was precipitated and washed with trichloroacetic acid at a final concentration of 10% before counting. In the other cases, after incubation under the same conditions, the preparations were subjected to SDS-polyacrylamide gel electrophoresis. The resolving gel was a linear gradient of 5–15% acrylamide. Strips of gel were cut in 1-mm slices, dissolved at 60°C in H\textsubscript{2}O (35% [m/v]) overnight, and counted (5).

Photoaffinity Labeling—Photoaffinity labeling was performed as described previously (6). Purified receptor was incubated with \textsuperscript{3}H Jflunitrazepam at 4°C for 60 min. After incubation, samples were submitted to UV (360 nm) irradiation for 20 min, precipitated, and then submitted to SDS-polyacrylamide gel electrophoresis, sliced, and counted.

Immuno precipitation—The purified receptor (50 \mu l) was incubated for 2 h at 4°C with varying dilutions of the monoclonal antibody bd 24 in a final volume of 200 \mu l in phosphate buffer containing 0.1% Triton X-100. After addition of 200 \mu l of goat anti-mouse I\gamma G conjugated to agarose with 1% bovine serum albumin, the samples were carefully shaken for 16 h at 4°C (22).

Phosphoamin oacid Analysis—Aliquots of receptor were phosphorylated by [\gamma\textsuperscript{32}P]ATP (3\textsuperscript{rd} mol) in the presence of MnCl\textsubscript{2} (2 mM) and/or MgCl\textsubscript{2} (5 mM) and precipitated by the chloroform-methanol method (23). Chemical digestion was performed in 6 M HCl at 110°C for 1 h (24). The phosphoamino acids were concentrated on Dowex 50W-X8 mini column in a minimum volume of buffer made of formic acid (88%), acetic acid, and deionized water (50:156:1794) at pH 1.9 (25). Samples containing 0.3–0.5 \mu g of phosphoamino acid standards (Tyr(P), Thr(P), and Ser(P)) were subjected to two-dimensional thin layer electrophoresis and chromatography (26). After the run, unlabeled markers were located by spraying with 0.2% ninyhydrin in acetone and developed for 15 min at 65°C. Plates were then autoradiographed.

Tyr osine Kinase Assay Kit—We used the enzyme-linked immunosorbent assay kit developed by Boehringer Mannheim. This includes specific peptide substrates (called protein kinase substrates 1 and 2) that are biotinylated at the amino terminus. After the reaction, phosphorylated and dephosphorylated substrates were immobilized by binding to a streptavidin-coated microtiter plate (96 wells). The fraction of phosphorylated substrate was determined using an anti-phosphotyrosine antibody directly conjugated to peroxidase.

Western Blotting—Purified GABA\textsubscript{A} receptor preparation was transferred from polyacrylamide gels to polyvinylidene difluoride membranes as described by Towbin et al. (27). After incubation with blocking reagent, the preparation was incubated overnight with 0.4 \mu g anti-phosphotyrosine antibody/mliliter of Tris buffer, pH 7.2, at 4°C. Immunostaining was performed using 0.25 units of anti-mouse IgAP/ml buffer at room temperature. Color reaction was obtained by incubating the samples for 20 min with 0.3 mM 4-nitroblue tetrazolium chloride, 0.7 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.1 mM Tris chloride buffer, pH 7.2.

RESULTS

Purification of the GABA\textsubscript{A} Receptor—The technique previously described (20, 22) was modified in order to optimize the yield of endogenous phosphorylation. Phosphate buffer was found more suitable than Tris buffer. Even though the solubilization yield was the same as with Tris buffer, lower concentrations of the detergent Triton X-100 could be used with phosphate buffer (28). Higher concentrations of Triton X-100 (5%) decreased the incorporation of phosphate compared with lower concentrations (1%) (data not shown). The optimal buffer conditions for solubilization were found to be phosphate (10 mM, pH 7.4), containing 1% Triton X-100. During the affinity chromatography, the duration of exposure of the solubilized receptor to urea had to be minimized. Moreover, the highest yield of phosphate incorporation was obtained after elution with 4 M rather than 6 M urea. Extensive dialysis (48 h) was required to eliminate the urea before the assay. Finally, it was found that freezing and thawing of the purified receptor also strongly and irreversibly inhibited the endogenous kinase activities.

Stoichiometry of Endogenous Phosphorylation—The purified receptor was incubated at 30°C in the presence of [\gamma\textsuperscript{32}P]ATP and divalent cations Mg\textsuperscript{2+} or Mn\textsuperscript{2+} (Fig. 1). No significant level of incorporation was obtained in the absence of divalent cations. In a typical experiment with an incubation time of 120 min, we found an incorporation of 0.12 mol of PO\textsubscript{4}/mol of muscimol in the presence of Mg\textsuperscript{2+} (5 mM) and of 0.45 mol of PO\textsubscript{4}/mol of muscimol in the presence of Mn\textsuperscript{2+} (500 \mu M). With higher concentrations of Mn\textsuperscript{2+} (2–5 mM), the stoichiometry rose to 3 mol of PO\textsubscript{4}/mol of muscimol (data not shown).

Electrophoretic Pattern—In the presence of 5 mM Mg\textsuperscript{2+}, we detected a major incorporation in the molecular mass range 51–53 kDa and a minor incorporation at 43–45 kDa (Fig. 2). The doublet at 51–53 kDa corresponds to \omega-subunits; the 51-kDa band has been identified as the \alpha\textsubscript{1}-subunit (29). The 43–45-kDa polypeptides have been shown to be proteolytic products of major GABA\textsubscript{A} receptor subunits (6, 29). We photolabeled the 51–53-kDa bands with \textsuperscript{3}H Jflunitrazepam (2 nm). Both \textsuperscript{32}P- and \textsuperscript{3}H Jflunitrazepam-labeled samples were subjected to the same run of SDS-polyacrylamide gel electrophoresis. We observed a co-migration of both \textsuperscript{32}P- and \textsuperscript{3}H Jflunitrazepam-labeled polypeptides at 51 and 53 kDa. This suggests that the endogenous phosphorylation predominates on 51–53 kDa \omega-subunits and particularly on the \alpha\textsubscript{1}-subunit. There is no direct correlation between the amount of incorporated \textsuperscript{32}P and the relative protein abundance, confirming a

![FIG. 3. Effect of incubation time on endogenous phosphorylation of the GABA\textsubscript{A} receptor. Stoichiometry was calculated as number of moles of phosphate incorporated per mole of muscimol bound in the presence of 5 mM Mg\textsuperscript{2+} (squares) or 500 \mu M Mn\textsuperscript{2+} (circles). The maximal incorporation (120 min) of phosphate in the presence of Mg\textsuperscript{2+} and Mn\textsuperscript{2+} was 0.12 and 0.45 mol of \textsuperscript{32}P/mol of \textsuperscript{3}H Jmuscimol respectively.](http://www.jbc.org/)

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clear preference of the endogenous phosphorylation for the 51-kDa polypeptide (Fig. 2, upper part). We also observed both \([^{3}H]flunitrazepam\) labeling and \(^{32}P\)-incorporation on the 43–45 kDa proteolytic products of \(\alpha\)-subunits. Minor incorporation was detected at molecular masses ranging from 55 to 58 and from 38 to 40 kDa. No incorporation of \(^{32}P\) was observed in other major subunits. Practically no radioactivity was associated with \(\beta\)-subunits. Other proteins that co-purified with the receptor, notably the heavily stained 36-kDa voltage-dependent anion channel protein (22), were not phosphorylated.

A Monoclonal Antibody against \(\alpha\)-Subunit Adsorbs the \(^{32}P\)-labeled GABA<sub>A</sub> Receptor Protein—In order to make sure that the \(^{32}P\) labeling did occur on GABA<sub>A</sub> receptor protein rather than on possible contaminants, we attempted to immunoprecipitate the purified receptor protein using an \(\alpha\)-specific monoclonal antibody named bd 24 (30). Dilutions of 1:50 to 1:50,000 of the antibody were incubated with the \(^{32}P\)-labeled purified receptor. As shown in Fig. 4C, the activity dropped by 50% between 30 and 37°C. This suggests that only half of the activity is thermolabile below 45°C.

Phosphoamino Acid Analyses—In the data shown in Fig. 5, the entire purified preparation was labeled by \(\gamma[^{32}P]ATP\) in the presence of Mg<sup>2+</sup> alone (Fig. 5A) or of both Mg<sup>2+</sup> and Mn<sup>2+</sup> and subjected to two-dimensional phosphoamino acid thin layer electrophoresis (Fig. 5B). The presence of Mn<sup>2+</sup> ions strongly increased the amount of \(^{32}P\)-incorporation for both serine and threonine. Roughly the same amounts of radioactivity were visualized in autoradiograms for serine and threonine residues in the presence or absence of Mn<sup>2+</sup>. It has to be kept in mind that very small amounts of phosphoamino acids could not be detected by this procedure.

There is a single consensus sequence for tyrosine kinase in the \(\gamma\)-subunit (31) that contrasts with the numerous consensus sequences for serine and threonine phosphorylation sites (4). We therefore used more sensitive and specific immunological methods that can detect very low levels of phosphorylated tyrosine residues. For these experiments we used a highly specific anti-phototyrosine antibody. The results are presented in Fig. 6. Aliquots of purified receptor were phosphorylated with 50 \(\mu\)M unlabeled ATP. By enzyme-linked immunosorbent assay (Fig. 6A), we observed a positive reaction only when Mn<sup>2+</sup> (2 mM) was present in the incubation medium. In the presence of 5 mM Mg<sup>2+</sup> alone (i.e. without Mn<sup>2+</sup>), no phototyrosine could be detected by this method. In order to estimate the molecular mass of the polypeptide that is phosphorylated on tyrosine, we used the same antibody for Western blot analysis. Under the same conditions as for enzyme-linked immunosorbent assay, a single labeled band was recognized at an apparent molecular mass of 55 kDa (Fig. 6B). The intensity of labeling increased with the concentration of the antibody. No
significant cross-labeling was observed, even at the highest antibody concentration. This apparent molecular mass is close to the mass of 55.2 kDa deduced from the bovine γ2-subunit sequence (31). Interestingly, the antibody stained neither the 51–53-kDa polypeptides nor any other part of the blot.

DISCUSSION

GABA\(_A\) receptor function is regulated by phosphorylation (4, 13–16, 32). The receptor protein contains consensus sequences for phosphorylation by protein kinases (7). In the present work, we describe kinase activities associated to the affinity-purified GABA\(_A\) receptor from cerebral cortex. In order to characterize endogenous activities, the purification procedure had to be improved. Indeed, we observed that the use of high detergent and urea concentrations during the purification procedure drastically decreased the rate of phosphorylation. Moreover, freezing the purified protein nearly completely abolished this activity. In contrast, phosphate buffer seems to have a protective effect during the solubilization of the protein. These technical differences might explain why a significant level of endogenous phosphorylation was not previously detected (9).

We observed a major phosphorylation in the 51–53-kDa bands. Maximum incorporation of 32P was in the 51-kDa polypeptide. We also detected a minor incorporation of 32P in a band of higher molecular mass (55 kDa). When we studied the effect of ATP concentration on 51-kDa polypeptide phosphorylation, we obtained a sigmoid curve. This suggests that the endogenous protein kinase has allosteric properties with at least two cooperative binding sites for ATP. Similar effects were reported for the insulin-stimulated autophosphorylation of the insulin receptor (33).

Immunoprecipitation of the phosphorylated receptor was obtained with a monoclonal antibody specific for α-subunits, confirming that phosphorylation occurs on these subunits. The 51- and 53-kDa bands co-migrated with two bands photoaffinity-labeled by [\(^{3}H\)]flunitrazepam. These two bands appear identical to those previously identified by partial sequencing and Western blotting as corresponding to the α\(_1\) and α\(_2\) gene products (29). However, data currently available are not yet sufficient to provide definitive proof of their identity. In an earlier study, Sweetnam et al. (19), using a partially purified GABA\(_A\) receptor preparation from rat cerebellum, suggested that an α-subunit was the preferred substrate for endogenous phosphorylation (which was observed only on serine). These results did not rule out the possibility that endogenous phosphorylation might also occur on other types of subunits. Indeed, it was shown later (34, 35) that α\(_1\), α\(_5\), β\(_2\), β\(_3\), γ\(_2\), and δ-subunits are expressed in cerebellum but the other subunit subtypes are not. The absence of expression of α\(_2\) is in agreement with the
lack of 53-kDa polypeptide in cerebellum (5, 6). Our preparation,
which is purified from bovine cortex, contains δ2- and β2-subunits,
and endogenous phosphorylation was observed in δ2- but not in
β-type subunits. In contrast, phosphorylation by cAMP-de-
pendent protein kinase and Ca2+-phospholipid-dependent protein
kinase C never occurs on α-subunits but only on β-subunits (9, 36).
The nature of α-subunit subtypes affects both the sen-
sitivity of the receptor to GABA and the affinity of different
ligands for the benzodiazepine binding site (5, 6). It is thus rea-
sonable to propose that the α-subunits can regulate the
channel conductance in two different ways: the binding of ag-

lant ligands to the NH2-terminal extracellular domain (37)
and the endogenous phosphorylation in the long intracellular
loop. The physiological consequences of phosphorylation on
β-subunits by exogenous kinases are different from those pro-
posed for endogenous phosphorylation (4).

We measured a roughly equimolar 32P-incorporation in Thr
and Ser residues in α-subunits, whereas a previous study (19)
suggested an endogenous phosphorylation of α-subunit only on
serine. A possible explanation for this apparent discrepancy is
that the latter preparation (19) was from cerebellum. The addi-
tional phosphorylation on Thr residue might reflect phosphi-
rylation on other α-subunits (α2,3) expressed in the neocortex
(38).

When our preparation was incubated with ATP + Mn2+, we
also detected the presence of phosphotyrosine on a single 55-
kDa polypeptide. The identification of the band(s) labeled at 55
kDa is made difficult by the presence of several α-, γ-, and
β-subunit subtypes sharing similar molecular mass (1). This
incorporation may occur at least partially on the γ2-subunit as
in the bovine brain, this subunit is the only one containing a
unique consensus sequence for tyrosine phosphorylation (locat-
ed on Tyr403 (31)). The apparent molecular mass has been
estimated at 55 kDa with a highly specific phosphotyrosine
antibody. This corresponds to the deduced mass from the
γ2 gene. However, the apparent molecular mass of the γ2 gene
product is still uncertain due to post-transcriptional events;
specific antibodies also recognized a broad band at 41–47 kDa
(39–41). Thus, the identification of the 55-kDa polypeptide
with the γ2-subunit of the GABA_A receptor is possible but not
duly demonstrated.

The parallels between the conditions reported previously and
the present study are worth noticing. It has been suggested
that GABA_A receptor current is maintained by phosphorylation
involving an unknown kinase and an unknown substrate (14).
Our results bring new insights on the complexity of the α- and
possibly γ-subunit pharmacology and indicate that the nature
of the subunit subtypes composing the GABA_A receptor may
also directly affect the properties of the GABA-activated chlo-
ride channel. The present work suggests an additional hetero-
genicity of receptor function due to modulation by multiple
dergogenous phosphorylations.

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