A Novel Serine Kinase with Specificity for β3-Subunits Is Tightly Associated with GABA<sub>A</sub> Receptors*  

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Tuning of γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor function via phosphorylation of the receptor potentially allows neurons to modulate their inhibitory input. Several kinases, both of the serine-threonine kinase and the tyrosine kinase families, have been proposed as candidates for such a modulatory role in vivo. However, no GABA<sub>A</sub> receptor-phosphorylating kinase physically associated with the receptor has been identified so far on a molecular level. In this study, we demonstrate a GABA<sub>A</sub> receptor-associated protein serine kinase phosphorylating specifically β3-subunits of native GABA<sub>A</sub> receptors. The characteristics of this novel kinase clearly distinguish it from enzymatic activities that have been shown so far to phosphorylate the GABA<sub>A</sub> receptor. We putatively identify this protein kinase as the previously described GTAP34 (GABA<sub>A</sub> receptor-tubulin complex-associated protein of molecular mass 34 kDa). Using expressed recombinant fusion proteins, we identify serine 408 as a major target of the phosphorylation reaction, whereas serine 407 is not phosphorylated. This demonstrates the high specificity of the kinase. Phosphorylation of serine 408 is known to result in a decreased receptor function. The direct association of this kinase with the receptor indicates an important physiological role.

The ionotropic GABA<sub>A</sub> receptor is abundantly expressed in the mammalian brain, and its major function is to mediate fast synaptic inhibition (for reviews, see Refs. 1–3). Binding of GABA to the extracellular domain of the receptor leads to the opening of an intrinsic ion channel followed by chloride influx, countering depolarization of the neuronal resting potential. Also, excitatory actions of the receptor depending on an altered chloride equilibrium potential have been reported (4, 5). A hallmark of the GABA<sub>A</sub> receptor is the many clinically important ways of modulation (3, 6).

Biochemical purification of the GABA<sub>A</sub> receptor (7) and cloning of the first two subunits (8) were followed by the description of so far 14 different mammalian subunits named α1–6, β1–3, γ1–3, δ, and ε. From these subunits, diverse heterodimeric receptor subtypes are generated neurally (9), bearing most likely a pentameric structure (10, 11) equivalent to that of the related nicotinic acetylcholine receptor (12).

As also demonstrated for other ion channels (13), GABA<sub>A</sub> receptor function can be regulated by phosphorylation. This provides a mean for fine tuning inhibitory neuronal inputs. A variety of studies has given evidence for the phosphorylation of the GABA<sub>A</sub> receptor by serine/threonine kinases such as protein kinase C (14–19, 23), cAMP-dependent protein kinase (17, 20–25), cGMP-dependent protein kinase (26, 27), Ca<sup>2+</sup>/calmodulin-dependent kinase II (27), and also by the tyrosine kinase Src (28, 29). These reports point to the intracellular loops of GABA<sub>A</sub> receptor β- and γ-subunits as major phosphorylation targets. The functional consequences of phosphorylation are either inhibition or enhancement of GABA<sub>A</sub> receptor-mediated chloride currents. The results obtained for the effect of a given kinase are sometimes conflicting, probably reflecting the complex nature of GABA<sub>A</sub> receptor regulation by phosphorylation in vivo.

Kinase activities copurifying with native GABA<sub>A</sub> receptors have previously been reported (30, 31). A kinase physically associated with native GABA<sub>A</sub> receptors can be expected to be of major functional relevance in vivo, but the molecular identity of such a protein has not been described so far. This study was conducted to answer the question whether one of the recently identified GABA<sub>A</sub> receptor-associated proteins named GTAPs (32) is a receptor-associated kinase. Our results indeed imply the identity of GTAP34 with a serine kinase that phosphorylates GABA<sub>A</sub> receptor β3-subunits.

MATERIALS AND METHODS

Immunoprecipitation of GABA<sub>A</sub> Receptors—GABA<sub>A</sub> receptors were immunoprecipitated from Triton X-100- or Zwittergent 3-14-solubilized calf brain membranes as described previously (32). For precipitation, the monoclonal antibody bd24 (33, 34) covalently coupled to protein A-Sepharose (bd24-beads) was used. With 10 μl of packed bd24-beads, GABA<sub>A</sub> receptor containing approximately 1 μg of α1-subunit was precipitated. This value was determined by comparing the staining intensity of the α1-subunit, migrating in SDS-PAGE (35) as a sharp 50-kDa band and identified by Western blotting, with known amounts of marker proteins in silver-stained gels. Since around 55 kDa several GABA<sub>A</sub> receptor β-subunits and also receptor-associated tubulin comigrated, the amount of β-subunits present could not be determined.

Phosphorylation of Immunoprecipitated GABA<sub>A</sub> Receptor—For standard assays, 5 μl of bd24-beads obtained after immunoprecipitation were washed five times with 1 ml of ice-cold buffer 1 (25 mM Tris, pH 7.2, 10 mM MgCl<sub>2</sub>, 0.1 mM Na-EGTA, 1% (w/v) β-octyl glucoside). 35 μl of buffer 1 were added, and the reaction was started by adding radio-labeled ATP to concentrations ranging from 50 to 400 μM. The specific activity was between 1000 and 10,000 cpm/pmol using [γ-<sup>32</sup>P]ATP (3000 Ci/mmol; Hartmann Analytic, Braunschweig, Germany). Incubation was done under gentle agitation at 24 °C in a water bath for 30 min unless indicated otherwise. The reaction was stopped by adding SDS-
PAGE sample buffer and heating to 95 °C for 10 min. After 8 or 10% SDS-PAGE,1 silver staining (36), and drying of the gel, phosphorylated proteins were detected by autoradiography using a Kodak BioMax HE enhancer screen. To quantify 32P incorporation, phosphorylated protein bands were excised from gels and homogenized, and the radioactivity was determined by scintillation counting.

To test for the presence of associated kinases already known to phosphorylate GABA<sub>ε</sub> receptor subunits in vitro, the following assay conditions were employed: (a) protein kinase C: 20 mM HEPES-NaOH, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 50 mM phosphatidylserine; (b) cAMP-dependent protein kinase: 40 mM HEPES-NaOH, pH 7.0, 10 mM MgCl<sub>2</sub>, 10 mM EGTA, 10 mM cAMP (cyclic adenosine monophosphate-dependent kinase: 35 mM HEPES-NaOH, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1 mM dithiothreitol, 1 mM calmodulin; (c) GMP-dependent protein kinase: 40 mM Tris-HCl, pH 7.4, 20 mM MgCl<sub>2</sub>, 2 mM cGMP. All buffers additionally contained 1% (w/v) β-oxycetylglucose, and all assays were done with an ATP concentration of 100 μM.

**Deglycosylation of Phosphorylated GABA<sub>ε</sub> Receptors and Western Blotting**—To deglycosylate phosphorylated GABA<sub>ε</sub> receptor probes, 5 μl of bd24-beads obtained from immunoprecipitation were first subjected to a phosphorylation assay (as described above, 50 μM ATP, 60 min). The assay was terminated by washing the bd24-bead precipitate two times with 400 μl of buffer 2 (25 mM Tris-HCl, pH 7.2, 10 mM EDTA, 1% (w/v) β-oxycetylglucose, pepstatin, antipain, and leupeptin each at a concentration of 1 μg/ml) followed by addition of 0.1 mg/ml of neuraminidase, 0.5 μl of buffer 2 and 5 μl (1 unit) of PNGase F (N-glycosidase F, Roche Molecular Biochemicals) were added, followed by 45 min of incubation at 37 °C. For controls, the enzyme was omitted. Deglycosylation was terminated by adding SDS-PAGE sample buffer and heating the probe. After 8% SDS-PAGE, proteins were transferred to nitrocellulose sheets (Hybond-ECL; Amersham Pharmacia Biotech) and immunodecorated in a manner similar to that described previously (32). The following primary antibodies were used: bd24 (2 μg/ml), GABA<sub>ε</sub> receptor β2-subunit-specific monoclonal antibody bd17 (33, 34), GABA<sub>ε</sub> receptor β1-subunit-specific antibody β1-(350–404) (5 μg/ml; Refs. 37 and 38), GABA<sub>ε</sub> receptor β2-subunit-specific antibody β2-(351–405) (2 μg/ml; Refs. 37 and 38), GABA<sub>ε</sub> receptor β3-subunit-specific antibody β3-(345–408) (5 μg/ml; Refs. 37–40), GABA<sub>ε</sub> receptor γ2-subunit-specific antibody γ2-(1–33) (5 μg/ml) and α-tubulin-specific monoclonal antibody B-5-1-2 (Sigma; 1:5000). After incubation with appropriate secondary antibodies, positive bands were detected with the ECL system (Amer sham Pharmacia Biotech). For consecutive decoration of the same blot strip with different antibodies, bound antibodies were stripped off from the blot strip according to the protocol provided by the manufacturer (Amersham Pharmacia Biotech).

**Expression and Purification of Intracellular Loop Fusion Proteins**—The nomenclature of the fusion proteins was based on the published sequences for the GABA<sub>ε</sub> receptor α1-subunit (8), β1–3-subunits (41), and γ2-subunit (42), beginning with the first amino acid (position 1) of the mature protein after removal of the signal peptide. The cDNAs coding for cytoplasmic loop regions of the mature protein after removal of the signal peptide. The cDNAs coding for cytoplasmic loop regions of β1-(351–405) construct by using the QuickChange<sup>TM</sup> mutagenesis kit (Stratagene). The resulting MBP-β2-(351–405) fusion protein and 400 μM ATP. Assays were performed by eluting the bd24-beads with the bound kinase activity by a brief centrifugation and removing the supernatants containing the intracellular loop fusion proteins. The supernatants were either analyzed by SDS-PAGE and autoradiography or by spotting aliquots onto Whatman P82 filters, extensive washing of the filters with 0.85% ortho-phosphoric acid, and scintillation counting.

**Phosphorylation Assays after High Salt Extraction of GABA<sub>ε</sub> Receptor Immunoprecipitates**—Immunoprecipitates bound to bd24-beads were extracted with buffer 1 containing 0.6 M NaCl (high salt extraction) essentially as described previously (32). To maximally maintain kinase activity for phosphorylation assays, the primary incubation of the immunoprecipitate in high salt buffer was shortened to 1 min, and the extracts were diluted immediately after extraction 3-fold with buffer 1, resulting in a NaCl concentration of 0.2 M. Buffer 1 containing 0.2 M NaCl was also used for the phosphorylation assays of the other probes to be compared with the high salt extract. The assay volume was 120 μl for each probe: the nonextracted bd24-beads, the extracted beads (both 5 μl of beads) and the high salt extract (extracted from 5 μl of beads). 5 μg of MBP-β3-(345–408) fusion protein or 0.78 μg of histone was used as kinase substrate. The probes were incubated for 120 min at 24 °C in the presence of 50 μM radiolabeled ATP. Analysis of the assay supernatants was performed as described above.

Gel filtration of high salt-extracted proteins was done in buffer 1 containing 0.6 M NaCl with a fast protein liquid chromatography system on a Superox 12 HR10/30 column (Amersham Pharmacia Biotech). Undiluted extract obtained from 140 μl of bd24-beads was separated at a flow rate of 0.1 ml/min. Starting at the void volume, 2.5 ml of eluate fractions were collected. For phosphorylation assays, 25 μl of each fraction were diluted with 50 μl of buffer 1 containing 4 μg of the MBP-β3-(345–408) fusion protein. To monitor autophosphorylation of separated proteins, identical probes lacking the fusion protein were prepared. Assays were performed for 120 min in the presence of 50 μM ATP, followed by 8% SDS-PAGE and autoradiography.

**Phosphorylation Assays of Immunoprecipitated β2- and β3-Subunits—**Calb brain membranes were solubilized in 10 mM HEPES, 5 mM EDTA, 0.5% β-mercaptoethanol, and 1% Triton X-100, resulting in a SDS concentration of 0.1%. After centrifugation at 165,000 × g for 30 min, the supernatant was precleared with protein A-beads for 90 min at 4 °C. 6 ml of the precleared solution containing 6 mg of protein were incubated with a 5 μg/ml concentration of either the rabbit antibody β2-(351–405) or antibody β3-(1–13) that recognizes β2- and β3-subunits (11) overnight at 4 °C. The antibodies were recaptured with 15 μl of packed protein A-beads and washed five times with buffer A containing 1 μl of 1:1 slurry packed protein A-beads were mixed with 90 μl of high salt-extracted kinase (prepared in 2 μl Tris-glycerol (1:3, extracted and diluted as described above) and incubated for 120 min at 24 °C in the presence of 50 μM radiolabeled ATP. Phosphorylated β2- and β3-subunits were analyzed by SDS-PAGE and autoradiography.

**Phosphoamino Acid Analysis**—One- and two-dimensional analysis of radiolabeled phospha amino acids were performed essentially as described (44). In brief, GABA<sub>ε</sub> receptor immunoprecipitate was incubated in buffer 1 with 50 μM ATP for 60 min, either alone or in the presence of MBP-β3-(345–408) fusion protein. After SDS-PAGE of the whole immunoprecipitate or only the probe supernatant, silver staining, and autoradiography, protein was eluted from gel pieces containing labeled GABA<sub>ε</sub> receptor subunits or labeled MBP-β3-(345–408) fusion protein. Following acid hydrolysis, amino acids were separated by two-dimensional thin layer chromatography and compared with phosphoamino acid standards.

**Two-dimensional Phosphopeptide Maps—**Phosphorylation of immunoprecipitated GABA<sub>ε</sub> receptors and MBP-β3-(345–415) fusion proteins was done as described above. bd24-beads containing the bound phosphorylated receptor were washed with Lys-C digestion buffer (25 mM Tris HCl, pH 8.5, 1 mM EDTA). The supernatant of the phosphorylation assays containing the phosphorylation protein was subjected to a chloroform-methanol extraction (45). Both the phosphorylated receptors and the loop constructs were digested in 200 μl of Lys-C digestion buffer containing 0.1 μg of Lys-C (Roche Molecular Biochemicals) for 19 h at 37 °C. Peptides were lyophilized twice in H<sub>2</sub>O and separated on cellulose thin layer plates in formic acid, glacial acetic acid, and H<sub>2</sub>O at a ratio of 50:156:1794, pH 1.9, 1.8 kV, for 15 min. By ascending chromatography in isobutyric acid, pyridine, glacial acetic acid, l-butanol, and H<sub>2</sub>O at a ratio of 65:53:2:29 in the second dimension (46).
RESULTS

Phosphorylation of Isolated GABA_A Receptor by an Endogenous Kinase Activity—GABA_A receptors were immunoprecipitated from Triton X-100-solubilized calf brain membranes with bd24-beads. The immunoprecipitate was assessed for the presence of an associated kinase activity by incubation with [γ-32P]ATP and subsequent SDS-PAGE analysis. In order to allow different kinases to exert optimal activity during the assay, various buffer compositions were used. In the presence of Mg²⁺, a strong phosphorylation of a 55-kDa band was obtained (Fig. 1, lanes 1 and 3–5). Replacement of Mg²⁺ with Mn²⁺ largely abolished this activity (Fig. 1, lane 2). Under conditions designed to stimulate protein kinase C (Fig. 1, lane 3), cAMP-dependent protein kinase (Fig. 1, lane 4), and calcium/calmodulin-dependent kinase (lane 5) did not promote phosphorylation of the predominant 55-kDa band (see "Materials and Methods").

Fig. 1. Phosphorylation of immunoprecipitated GABA_A receptors by an endogenous kinase activity. Shown is an autoradiogram of phosphorylated GABA_A receptor probes separated on a 10% SDS-PAGE. GABA_A receptors were immunoprecipitated with antibody bd24 covalently bound to protein A-Sepharose (bd24-beads) from Triton X-100-solubilized calf brain membranes. Immunoprecipitate containing 500 ng of α1-subunit was incubated for 30 min at 24 °C in different buffers containing γ-32P-labeled ATP at a concentration of 100 μM and either Mg²⁺ (lanes 1 and 3–5) or Mn²⁺ (lane 2) at a concentration of 10 mM. The standard buffer (lane 1) included 25 mM Tris, pH 7.2, 10 mM MgCl₂, 0.1 mM EGTA, and 1% β-octyl glucoside. Optimal conditions for the activity of protein kinase C (lane 3), cAMP-dependent protein kinase (lane 4), and calcium/calmodulin-dependent kinase (lane 5) did not promote phosphorylation of the predominant 55-kDa band (see "Materials and Methods").

The Endogenous Kinase Activity Preferentially Phosphorylates GABA_A Receptor β-Subunits—Following phosphorylation, GABA_A receptor immunoprecipitates were enzymatically deglycosylated, subjected to SDS-PAGE, and blotted to nitrocellulose membranes, parallel to nondeglycosylated probes. Deglycosylation was done in order to unequivocally differentiate between receptor subunits and subunit isoforms and tubulin. It was indeed found that some proteins comigrating around 55 kDa (i.e., the GABA_A receptor β2-subunit (Fig. 3, lane 7), the GABA_A receptor β3-subunit (Fig. 3, lane 11), and α-tubulin (Fig. 3, lane 27)) showed a differential migration after deglycosylation (Fig. 3, lanes 8, 12, and 28). Radioactivity on the blot was detected by autoradiography, and the resulting pattern of labeled bands was compared with the signals obtained after immunodecoration with specific antibodies recognizing GABA_A receptor α1-, β1-, β2-, β3-, β2/3-, and γ2-subunits and α-tubulin (Fig. 3). After deglycosylation, the number of radioactive bands increased. This might be due to incomplete deglycosylation or to a partial protein degradation during deglycosylation. The predominant radioactive signals at 55 kDa in the nontreated probe (Fig. 3, lanes 1, 5, 9, 13, 17, 21, and 25, upper band) and at 52 kDa in the deglycosylated probe (Fig. 3, lanes 2, 6, 10, 14, 18, 22, and 26, upper band) colocalized best with the signals obtained with an antibody specific for GABA_A receptor β3-subunits (Fig. 3, lanes 9–12). Also, a weak radioactive band at 36 kDa, possibly representing a degradation product of β3-subunits generated during deglycosylation by contaminating proteases, comigrated with a β3-subunit positive signal (Fig. 3, lanes 14 and 16). Only one other antibody, recognizing GABA_A receptor β2- and β3-subunits, also reacted with the dominant radioactive bands of glycosylated and deglycosylated probes (Fig. 3, lanes 13–16), confirming the result with the β3-subunit-specific antibody. The additional bands recognized by the β2/3-subunit-specific antibody were due to the detection of β2-subunits, as evident from the comparison with a β2-subunit-specific antibody (Fig. 3, lanes 7–8). With this antibody, as well as with the antibodies directed against GABA_A receptor α1-, β1-, and γ2-subunits and for α-tubulin, a match with each of the two predominant radioactive bands of the receptor probes (55 kDa for the glycosylated receptor, 52 kDa for the deglycosylated probe) was not obtained. These results indicated a preferential phosphorylation of GABA_A receptor β3-subunits by the GABA_A receptor-associated kinase activity. If it is assumed that β3-subunits are present in the receptor in a stoichiometry of 1:1 with α1-subunits, phosphorylation of β3-subunits almost occurs stoichiometrically (see Fig. 2). However, it should be pointed out that this probably represents an underestimate of the stoichiometry, since α1-subunits presumably assemble preferentially with β2- and not with β3-subunits (9).

In 8 of 19 experiments, in addition to the dominant 55-kDa band also a minor 50-kDa band was observed to be phosphorylated (see Fig. 3, lane 1). The minor radioactive bands at 50 kDa in the nondeglycosylated and at 45 kDa in the deglycosylated probe could be matched with immunopositive signals for GABA_A receptor α1-subunits (Fig. 3, lanes 17–20), β1-subunits (Fig. 3, lanes 1–4), and also β3-subunits (Fig. 3, lanes 9–12). The immunostaining for the β1-subunit revealed a molecular mass of roughly 50 kDa for the glycosylated protein. This value is clearly lower than expected from the sequence similarity between the β1-subunit and the β2- and β3-subunits, both migrating at 55 kDa. Most likely, this discrepancy can be
explained by partial degradation of the β1-subunit during receptor purification, resulting in the loss of a terminal portion of the protein.

A Fusion Protein Containing an Intracellular Portion of the GABA_A Receptor β3-Subunit Can Serve as Kinase Substrate—The GABA_A receptor immunoprecipitate was incubated, under conditions promoting high enzymatic turnover, with a fusion protein consisting of amino acid residues 345–408 of the intracellular loop of the GABA_A receptor β3-subunit fused to MBP (MBP-β3-(345–408)). A stoichiometry of phosphorylation of 0.79 mol of bound phosphate/mol of MBP-β3-(345–408) was obtained (Fig. 4). This result indicated the usefulness of fusion proteins of intracellular loops of GABA_A receptor subunits for further experiments. In another experiment under comparable conditions, a similar enzymatic activity was found.

The GABA_A Receptor-associated Kinase Phosphorylates Serine Residues—The side chain specificity of the GABA_A receptor-associated kinase activity was analyzed by two-dimensional thin layer chromatography of phosphoamino acids after acidolysis of the phosphorylated substrate proteins. A strict specificity of the kinase activity for serine residues was found both in vitro and for the MBP-β1-(328–382) (Fig. 6B) and for the MBP-β1-(350–404) (lanes 3 and 4), β2-(351–405) (lanes 7 and 8), β3-(345–408) (lanes 11 and 12), bd17 (recognizing β2- and β3-subunits; lanes 15 and 16), bd24 (recognizing α1-subunits; lanes 19 and 20), and γ2-(1–32) (lanes 23 and 24). In addition, α-tubulin was detected with antibody B-5-1-2 (lanes 27 and 28). The immunopositive signals were compared with the corresponding autoradiogram (lanes 1 and 2, 5 and 6, 9 and 10, 13 and 14, 17 and 18, 21 and 22, and 25 and 26).

Phosphorylation of Different Fusion Proteins Representing Parts of Intracellular Loops of GABA_A Receptor Subunits—In addition to MBP-β3-(345–408), a C-terminal elongated fusion protein of the β3-subunit, MBP-β3-(345–415), and fusion proteins of parts of the intracellular loops of the GABA_A receptor α1-, β1-, β2-, and γ2-subunits were compared in phosphorylation assays. No radioactive signals were obtained in the case of GST-α1-(328–382) (Fig. 6B, lane 1) and MBP-γ2-(319–366) (Fig. 6B, lane 3), while a stronger signal was found for MBP-β1-(350–417) (Fig. 7B, lane 1). MBP alone (Fig. 7B, lane 9) was not phosphorylated. In Fig. 7C, a quantitative evaluation of the experiment shown in Fig. 7B is given. An additional experiment gave comparable results.

GABA_A Receptor-associated Protein Kinase

Phosphorylation of the fusion protein MBP-β3-(345–408). GABA_A receptor immunoprecipitates were incubated with the fusion protein MBP-β3-(345–408) for various time periods. Probes supernatants containing the phosphorylated fusion protein were spotted onto Whatman P81 filter paper, and the radioactivity was quantified and related to the amount of fusion protein.

Phosphoamino acid and phosphopeptide analysis of phosphorylated kinase substrates. Phosphorylated GABA_A receptor-associated kinase substrates were subjected to phosphoamino acid analysis via two-dimensional thin layer electrophoresis (A and B) or to electrophoresis in the first dimension and to chromatography in the second dimension (C and D). Shown are autoradiograms of the thin layer plates. The positions of the phosphoamino acid standards are outlined by dashed lines (A and B).
The fusion proteins GST-β1-(350–382) and MBP-γ2-(319–366), (A and B, lane 2) were compared with MBP-β3-(345–408) (A and B, lane 3) in phosphorylation assays. Silver staining of the fusion proteins (A) and an autoradiogram of the phosphorylated probes (B) are shown.

These results demonstrated the phosphorylation of residues in the expressed portion of the intracellular loop of the GABA<sub>A</sub> receptor β3-subunit. The mutations S381A, S395A, and S407A did not reduce phosphate incorporation (Fig. 7B, lanes 4–6). The mutation S408A decreased the extent of phosphorylation strongly by 49 ± 8% (n = 3 experiments; see Fig. 7B, lane 7) and identified serine 408 as a major phosphorylation target. The fact that the neighboring serine residue 407 is not used as a substrate points to a highly selective phosphorylation reaction. This experiment was performed three times with comparable results.

The Kinase Activity Is Dissociated from the Receptor by Media of High Ionic Strength—In addition to the standard preparation of GABA<sub>A</sub> receptors derived from Triton X-100-solubilized membranes (Fig. 8A, lanes 1–3), an immunoprecipitate obtained from Zwittergent 3-14-solubilized membranes (32) was also investigated. This preparation results in a different GTAP composition (Fig. 8A, lanes 4–6). With this preparation, MBP-β3-(345–408) phosphorylation comparable with that of the standard receptor preparation was found (Fig. 8B, lanes 1 and 4). In addition, the phosphorylation of the MBP-β3-(345–415; S408A) mutant was reduced to a similar degree (by 54%; mean of two experiments) as with the preparation from Triton X-100-solubilized membranes. Furthermore, the receptor-associated kinase phosphorylated a 55-kDa band of the GABA<sub>A</sub> receptor preparations obtained from Zwittergent 3-14-solubilized membranes (32). A silver stain of the high salt-extracted receptor preparation from TX-100 is shown in Fig. 8B, lane 5. The protein composition of the respective extracts is evident from Fig. 8A, lanes 3 and 6. We compared the phosphorylation of MBP-β3-(345–408) by nonextracted (Fig. 8B, lanes 1 and 4) and high salt-extracted immunoprecipitates (Fig. 8B, lanes 2 and 5) and salt extracts (Fig. 8B, lanes 3 and 6). In both detergents, high salt extraction strongly decreased kinase activities. Nevertheless, it was evident that the kinase activities remaining after high salt extraction were found to distribute almost entirely preference for that from the β1-subunit.

Ser<sup>408</sup> of the β3-Subunit Is a Major Target for Phosphorylation—Several mutant MBP-β3-(345–415) fusion proteins were prepared to identify the position of phosphorylated serine residues in the expressed portion of the intracellular loop of the GABA<sub>A</sub> receptor β3-subunit. The mutations S381A, S395A, and S407A did not reduce phosphate incorporation (Fig. 7B, lanes 4–6). The mutation S408A decreased the extent of phosphorylation strongly by 49 ± 8% (n = 3 experiments; see Fig. 7B, lane 7) and identified serine 408 as a major phosphorylation target. The fact that the neighboring serine residue 407 is not used as a substrate points to a highly selective phosphorylation reaction. This experiment was performed three times with comparable results.

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The kinase was prepared as high salt extract of the immunoprecipitate obtained in Zwittergent 3-14. A shows the phosphorylation of the immunoprecipitate using anti-β2 antibodies and protein A beads (β2), anti-β2/β3 antibodies and protein A beads (β3), or protein A beads only as a control (c). B compares the phosphorylation signals obtained from equimolar amounts of the fusion protein MBP-β3-(345–415) and of histone phosphorylated in solution. C compares the signals of native receptor β3-subunit and histone with the protein kinase and the receptor bound to the immunoprecipitation beads.

into the salt extracts (Fig. 8B, lanes 3 and 6), with no activity phosphorylating MBP-β3-(345–408) remaining in the immunobeads after treatment with high ionic strength (Fig. 8B, lanes 2 and 5). Comparable results were obtained in three similar experiments. The silver stain of the different preparations showed (Fig. 8A) that the only major protein present in the Zwittergent 3-14 extract, GTAP34, was also present in the Triton X-100 extract. This finding indicated that GTAP34 may be identical to the GABA_A receptor-associated kinase. More evidence for this identity was provided by additional experiments described below (see Figs. 9 and 10).

The Kinase Activity Accepts Immunoprecipitated β2- and β3-Subunits and to a Limited Extent Histone as a Substrate—The kinase activity was purified by immunoprecipitation in Zwittergent 3-14 and subsequent high salt treatment of the immunobeads to release the kinase activity into the supernatant (see Fig. 8). Bovine membranes were solubilized under denaturing conditions using SDS; subsequently, the SDS was quenched in Triton X-100, and GABA_A receptor β2- and β3-subunits were immunoprecipitated using subunit-specific antibodies. In both cases, immunoprecipitated subunits were accepted as a substrate by the kinase (Fig. 9A). If the kinase was offered MBP-β3-(345–415) fusion protein or histone as a substrate (Fig. 9B), histone was also used as a substrate, albeit to a smaller extent as compared with MBP-β3-(345–415) fusion protein, which amounted to about 60% on a molar basis (two experiments). When histone was added directly to the immunoprecipitate and the combination was subjected to phosphorylation, histone was also accepted as a substrate, but it was phosphorylated to a much smaller extent than the native receptor subunit β3 (Fig. 9C). As stated above, β3 could only be estimated as a maximum amount. On a molar basis, it may be calculated that phosphorylation of histone amounts to less than 14% of phosphorylation of β3.

GTAP34 Comigrates with the MBP-β3-(345–408)-phosphorylating Kinase Activity in Gel Filtration of High Salt Extract—High salt-extracted GTAPs were separated by gel filtration, and aliquots of collected fractions were silver-stained (Fig. 10A) or assessed for kinase activity using MBP-β3-(345–408) as substrate (Fig. 10B). The kinase activity comigrated with GTAP34. This strongly indicates identity of GTAP34 with the GABA_A receptor-associated serine kinase phosphorylating GABA_A receptor β-subunits. Repetition of the experiment confirmed this result.

The prolonged exposure to high salt concentrations during gel filtration resulted in a strong decrease in kinase activity. The long exposure time needed for autoradiography (5 days compared with 1 h in Fig. 8) revealed the autophosphorylation of a protein with a molecular mass of approximately 57 kDa (Fig. 10C). Due to its low signal strength and because of overlap with the strong signals from GABA_A receptor β-subunits and from the MBP-β3-(345–408) fusion protein, this signal was not detected in the previous experiments. Although this signal may be due to the phosphorylation of a 57-kDa protein by trace amounts of GTAP34, it could also represent an autophosphorylating activity of another GABA_A receptor-associated kinase.

DISCUSSION

We describe here a GABA_A receptor-associated protein kinase with specificity for serine residues. Regulation of GABA_A receptor function by direct phosphorylation through serine/threonine kinases has been comprehensively studied (see Introduction). Biochemical features of the serine kinase described here are (a) the independence on cAMP and cGMP, (b) the independence on Ca²⁺ and phospholipids, (c) the independence on Ca²⁺/calmodulin, and (d) the likely molecular mass of 34 kDa. These biochemical properties, including the molecular weight, distinguishes it from cAMP-dependent protein kinase (59–41 kDa for the catalytic subunit), cGMP-dependent protein kinase (75–86 kDa), protein kinase C (>74 kDa for all the different isoforms), and Ca²⁺/calmodulin-dependent kinases (>37 kDa for all the different isoforms, all described in Ref. 47).

All of these types of kinase have been shown to affect GABA_A receptor function in different expression systems and neuronal preparations. Phosphorylation of the GABA_A receptor by the present serine kinase may be regarded as a potential novel mechanism to exert cellular control on GABA_A receptor func-
tion. This kinase merits special interest because of its physical association with the receptor.

The GAB\textsubscript{\textalpha} receptor-associated kinase has a clear preference for \(\beta_3\)-subunits in the intact receptor. \(\alpha_1\) and \(\gamma_2\)-subunits were not used as a kinase substrate either in the intact receptor or in overexpressed and purified loop constructs. Phosphorylation of \(\beta_2\)-subunits was not evident, and that of \(\beta_1\)-subunits, if occurring at all in native receptors, was quantitatively negligible. The results of previous studies on GAB\textsubscript{\textalpha} receptor-phosphorylating activities associated with the receptor are substantially different from those of the study presented here. The serine kinase activities investigated by Sweetnam et al. (30) and Bureau and Laschet (31) showed strong phosphorylation of \(\alpha\)-subunits, while phosphorylation of \(\beta\)-subunits was found to be either unlikely (30) or even absent (31). In addition, preferential kinase activation by Mn\(^{2+}\) as compared with Mg\(^{2+}\) was found (31), in contrary to the kinase described here.

MBP-loop fusion proteins of parts of the intracellular loops of all \(\beta\)-subunits isoforms could be phosphorylated, with a preference for \(\beta_1\). This contrasts with the observations made with native receptors and indicates, that the recombinant substrate proteins may not be not fully representative for the native kinase substrates. The bacterially overexpressed parts of the intracellular loops may have assumed a conformation different from that found in the native proteins. That this is indeed the case is made likely by the observations on the phosphorylation of different GAB\textsubscript{\textalpha} receptor \(\beta\)-subunits by cAMP-dependent protein kinase (25), where a similar apparent discrepancy was described. Alternative explanations seem to be less likely. For example, the weak or even absent phosphorylation of the native \(\beta_1\)-subunit could possibly be due to a preferential degradation of \(\beta_1\)-subunits as compared with \(\beta_3\)-subunits during receptor purification, but the removal of a short N- or C-terminal portion from the \(\beta_1\)-subunit would not be expected to have an effect on the ability of the \(\beta_1\)-subunit to serve as a substrate. Also, an overrepresentation of the \(\beta_3\)-subunit compared with \(\beta_1\) and \(\beta_2\)-subunits in the receptor preparation seems unlikely, since the \(\alpha_1\)-subunit, the subunit recognized and precipitated by the antibody bd24, is expected to be preferentially coassembled with \(\beta_2\) - but not with \(\beta_3\)-subunits (9). The fact that the \(\beta_3\)-subunit is probably a quantitatively minor protein of the receptor preparation underlines the specificity of the present serine kinase.

Roughly half of phosphate incorporation into a fusion protein containing intracellular sequences of the GAB\textsubscript{\textalpha} receptor \(\beta_3\)-subunit occurred at Ser\(^{408}\). That Ser\(^{407}\) evidently was not used as a substrate pointed to a highly discriminating phosphorylation reaction. It is interesting to note that the homologous serine residue to \(\beta_1\) Ser\(^{406}\) in the \(\beta_1\)-subunit is a substrate for protein kinase C (18), for cAMP-dependent protein kinase and Ca\(^{2+}\)/calmodulin-dependent kinase (27); and in the \(\beta_2\)-subunit it is a substrate for protein kinase C (16). Phosphorylation of the corresponding residues resulted in alteration of GAB\textsubscript{\textalpha} receptor function (16–19). From these findings, it can be predicted that GABA-evoked responses of \(\beta_3\)-subunit-containing GAB\textsubscript{\textalpha} receptors can be modulated by the present serine kinase. In this respect, also the dual mode of GAB\textsubscript{\textalpha} receptor regulation by cAMP-dependent protein kinase-mediated phosphorylation recently described by McDonald et al. (25) is of interest. Phosphorylation of the \(\beta_3\)-subunit at both serine residues Ser\(^{406}\) and Ser\(^{409}\) enhanced GAB\textsubscript{\textalpha} receptor function, while Ser\(^{409}\) phosphorylation alone of a mutated \(\beta_3\)-subunit was shown to inhibit GAB\textsubscript{\textalpha} receptor-mediated chloride currents. It is important to point out here that McDonald et al. (25) use a different numbering system of amino acid residues, our Ser\(^{407}\) and Ser\(^{408}\) corresponding to their Ser\(^{408}\) and Ser\(^{409}\) in \(\beta_3\)-subunits. Future studies on the action of the kinase on functionally expressed GAB\textsubscript{\textalpha} receptors containing different \(\beta\)-subunits will be pivotal to precisely determine its functional consequences.

It is interesting to note that the protein kinase was obtained by immunoprecipitation using an antibody with specificity for the \(\alpha_1\)-subunit. Most probably, the kinase is associated directly with GAB\textsubscript{\textalpha} receptors containing an \(\alpha_1\)-subunit. However, the possibility cannot be excluded that the kinase is associated with another protein or with a GAB\textsubscript{\textalpha} receptor is associated itself directly or indirectly with an \(\alpha_1\)-subunit-containing GAB\textsubscript{\textalpha} receptor. In any case, the link between \(\alpha_1\)-subunit-containing GAB\textsubscript{\textalpha} receptors and the kinase has to be resistant to overnight exposure to TX-100 and Zwittergent 3-14.

The strict specificity of the described protein kinase for \(\beta\)-subunits of the GAB\textsubscript{\textalpha} receptor seems to be lost once the kinase is released from the receptor into solution. At least the \(\beta_2\)-subunit and, to a limited extent, histone (Fig. 9, A and B) can then additionally be used as a substrate. Histone is a very weak substrate for the bound kinase (Fig. 9C). It appears that the apposition of the protein kinase to the substrate plays an important role in its substrate specificity.

Several experiments indicate an identity of the present GAB\textsubscript{\textalpha} receptor-associated serine kinase with GTAP34, a protein that has recently been shown to be associated with GAB\textsubscript{\textalpha} receptors under the present conditions (32). First, the kinase and GTAP34 are present in the immunoprecipitates obtained from Triton X-100 and Zwittergent 3-14; second, extraction of the immunobeads with a medium containing high ionic strength eluted GTAP34 as well as the serine kinase; third, in gel filtration experiments of high salt extract, GTAP34 and the kinase activity co-eluted in the same fractions. This identification on the protein level should lead to a description at the DNA level.

In conclusion, the data presented here point to GTAP34 as being a GAB\textsubscript{\textalpha} receptor-associated serine kinase with preference for \(\beta_3\)-subunits as a substrate. Study of this kinase may contribute to the further understanding of the physiological significance of GAB\textsubscript{\textalpha} receptor subunit diversity (25, 48–50). In addition, GTAP34 could serve as a target for drugs specifically modulating the function of \(\beta_3\)-subunit-containing GAB\textsubscript{\textalpha} receptors.

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**REFERENCES**

1. Macdonald, R. L., and Olsen R. W. (1994) *Annu. Rev. Neurosci.* 17, 569–602
2. Rabow, L. E., Russek, S. J., and Farb, D. H. (1995) *Synapse* 21, 189–274
3. Sieghart, W. (1995) *Pharmacol. Rev.* 47, 181–234
4. Mchenkel, H. B., and Wong, R. K. S. (1991) *Science* 253, 1420–1423
5. McLean, H. A., Cailllard, O., Ben-Ari, Y., and Giaiisra, J.-L. (1996) *J. Physiol.* 496, 471–477
6. Sigel, E., and Buhr, A. (1997) *Trends Pharmacol. Sci.* 18, 425–429
7. Sigel, E., Stephenson, F. A., Mamalaki, C. and Barnard, E. A. (1983) *J. Biol. Chem.* 258, 6965–6971
8. Schofield, P. R., Dallison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Rele, V., Glocenske, T. A., Seeburg, P. H., and Barnard, E. A. (1987) *Nature* 328, 221–227
9. McKernan, R. M., and Whiting, P. J. (1996) *Trends Neurosci.* 19, 139–143
10. Nguyen, N., Green, T. P., Martin, I. L., and Barnard, E. A. (1994) *J. Neurochem.* 62, 815–818
11. Tretter, V., Ebya, N., Fuchs, K., and Sieghart, W. (1997) *J. Neurosci.* 17, 2728–2737
12. Uwein, N. (1993) *J. Mol. Biol.* 229, 1101–1124
13. Smarr, T. G. (1997) *Curr. Opin. Neurobiol.* 7, 358–367
14. Sigel, E., and Baur, R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 6192–6196
15. Sigel, E., Baur, R., and Malherbe, P. (1991) *FEBS Lett.* 291, 150–152
16. Kellenberger, S., Malherbe, P., and Sigel, E. (1992) *J. Biol. Chem.* 267, 25660–25663
17. Moss, S. J., Smart, T. G., Blackstone, C. D., and Huganir, R. L. (1992) *Science*
18. Krishek, B. J., Xie, X., Blackstone, C., Huganir, R. L., Moss, S. J., and Smart, T. G. (1994) Neuron 12, 1081–1095
19. Lin, Y.-F., Angelotti, T. P., Dudek, E. M., Browning, M. D., and MacDonald, R. L. (1996) Mol. Pharmacol. 50, 185–195
20. Heuschneider, G., and Schwarz, R. D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2938–2942
21. Tehrani, M. H. J., Hablitz, J. J., and Barnes, E. M. (1989) Synapse 4, 126–131
22. Porter, N. M., Twyman, R. E., Uhler, M. D., and Macdonald, R. L. (1990) Neuron 5, 789–799
23. Moss, S. J., Doherty, C. A., and Huganir, R. L. (1992) J. Biol. Chem. 267, 14470–14476
24. Kapur, J., and Macdonald, R. L. (1996) J. Neurophys. 76, 2634
25. McDonald, B. J., Amato, A., Connolly, C. N., Benke, D., Moss, S. J., and Smart, T. G. (1998) Nat. Neurosci. 1, 23–28
26. Valenzuela, C. F., Machu, T. K., McKernan, R. M., Whiting, P., Van-Renterghem, B. B., McManaman, J. L., Smith, G. B., Olsen, R. W., and Harris, R. A. (1995) Mol. Brain Res. 31, 165–172
30. Sweetnam, P. M., Lloyd, J., Gallambrodo, P., Malison, R. T., Gallager, D. W., Tallman, J. F., and Nestler, E. J. (1988) J. Neurochem. 51, 1274–1284
31. Bureau, M. H., and Laschet, J. J. (1995) J. Biol. Chem. 270, 26482–26487
32. Kannenberg, K., Baur, R., and Sigel, E. (1997) J. Neurochem. 68, 1352–1360
33. Haring, P., Stahl, C., Schoch, P., Takacs, B., Staelin, T., and Möhler, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4837–4841
34. Ewert, M., Shivers, B. D., Lüddens, H., Möhler, H., and Seeburg, P. H. (1990) J. Cell Biol. 110, 2045–2048
35. Laemmli, U. K. (1970) Nature 227, 680–685
36. Morrissey, J. H. (1981) Anal. Biochem. 117, 307–310
37. Sperr, G., Schwarz, C., Tsunashima, K., Fuchs, K., and Sieghart, W. (1997) Neuroscience 80, 987–1000
38. Jechlinger, M., Pelz, R., Tretter, V., Klausberger, T., and Sieghart, W. (1998) J. Neurosci. 18, 2449–2457
39. Slany, A., Zenula, J., Tretter, V., and Sieghart, W. (1995) Mol. Pharmacol. 48, 385–391
40. Todd, A. J., Watt, C., Spike, R. C., and Sieghart, W. (1996) J. Neurosci. 16, 974–982
41. Ymer, S., Schofield, P. R., Draguhn, A., Werner, P., Kohler, M., and Seeburg, P. H. (1989) EMBO J. 8, 1655–1670
42. Shivers, B. D., Killisch, I., Sprengel, R., Sontheimer, H., Kohler, M., Schofield, P. R. and Seeburg, P. H. (1989) Neuron 3, 327–337
43. Mattes, B., Togel, M., Fuchs, K., and Sieghart, W. (1994) J. Biol. Chem. 269, 25777–25782
44. Hunter, T., and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
45. Wessel, D., and Flugge, U. I. (1983) Anal. Biochem. 138, 141–143
46. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
47. Hardie, G., and Hanks, S. (eds) (1995) The Protein Kinase Factsbook: Protein-Serine Kinases, Academic Press, Inc., London
48. Connolly, C. N., Woolerton, J. R. A., Smart, T. G., and Moss, S. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 91, 9899–9904
49. Bussard, A. B., Kits, K. S., Baker, R. E., Williams, W. P. A., Leyting, Vermeulen, J. W., Voorn, P., Saut, A. B., Bicknell, R. J., and Herbison, A. E. (1997) Neuron 19, 1103–1114
50. Essrich, C., Lorez, M., Benson, J. A., Fritschy, J.-M., and Lüscher, B (1998) Nat. Neurosci. 1, 569–571