Cellular/Molecular

Glyceraldehyde-3-Phosphate Dehydrogenase Is a $GABA_A$ Receptor Kinase Linking Glycolysis to Neuronal Inhibition

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Protein phosphorylation is crucial for regulating synaptic transmission. We describe a novel mechanism for the phosphorylation of the $GABA_A$ receptor, which mediates fast inhibition in the brain. A protein copurified and coimmunoprecipitated with the phosphorylated receptor α1 subunit; this receptor-associated protein was identified by purification and microsequencing as the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Molecular constructs demonstrated that GAPDH directly phosphorylates the long intracellular loop of $GABA_A$ receptor α1 subunit at identified serine and threonine residues. GAPDH and the α1 subunit were found to be colocalized at the neuronal plasma membrane. In keeping with the GAPDH/$GABA_A$ receptor molecular association, glycolytic ATP produced locally at plasma membranes was consumed for this α1 subunit phosphorylation, possibly within a single macrocomplex. The membrane-attached GAPDH is thus a dual-purpose enzyme, a glycolytic dehydrogenase, and a receptor-associated kinase. In acutely dissociated cortical neurons, the rundown of the $GABA_A$ responses was essentially attributable to a Mg^{2+}-dependent phosphatase activity, which was sensitive to vanadate but insensitive to okadaic acid or fluoride. Rundown was significantly reduced by the addition of GAPDH or its reduced cofactor NADH and nearly abolished by the addition of its substrate glyceraldehyde-3-phosphate (G3P). The prevention of rundown by G3P was abolished by iodoacetamide, an inhibitor of the dehydrogenase activity of GAPDH, indicating that the $GABA_A$ responses are maintained by a glycolysis-dependent phosphorylation. Our results provide a molecular mechanism for the direct involvement of glycolysis in neurotransmission.

Key words: **$GABA_A$ receptor phosphorylation; response rundown; receptor-associated kinase; GAPDH; glycolysis; ATP**

Introduction

The control of neuronal excitability is essential for brain function and depends on a continuous supply of glucose (Sokoloff, 1977). *In vivo* brain imaging shows tissue uptake of glucose in excess of that consumed by oxidative metabolism (Fox et al., 1988), indicating that the anaerobic glycolytic-produced ATP may preferentially serve specific nerve cell functions. Indeed, it has been suggested that glycolysis supports excitatory neurotransmission in the retina (Ames et al., 1992) and that it ensures maximal glutamate accumulation into presynaptic vesicles (Ikemoto et al., 2003).

Protein phosphorylation is a major mechanism for regulating ligand-gated ion channels (Levitan, 1999). In the mammalian brain, fast synaptic inhibition is mainly mediated by the GABA type A receptor ($GABA_AR$), which is constituted of hetero-oligomeric assemblies of five subunits, mostly of the α, β, and γ type, each including four transmembrane-spanning domains (TM1–4) (Unwin, 1993). A large intracellular domain (I2) located between TM3 and TM4 contains consensus phosphorylation sites for both Ser/Thr and Tyr protein kinases in several $GABA_AR$ subunits (Moss and Smart, 1996). The β and γ subunits are phosporylated by type 2 calcium–calmodulin-dependent protein kinase (Machu et al., 1993; McDonald and Moss, 1994), cAMP-dependent protein kinase (PKA) (Kirkness et al., 1989; Browning et al., 1990; Moss et al., 1992a), Ca^{2+}-phospholipid-dependent protein kinase C (PKC) (Browning et al., 1990; Moss et al., 1992a; Krishek et al., 1994), cGMP-dependent protein kinase (McDonald and Moss, 1994; Robello et al., 1998), or protein-tyrosine kinase (Bureau and Laschet, 1995; Brandon et al., 2001). The effect of $GABA_AR$ phosphorylation in cellular expression systems or in neuronal preparations are variable: phosphorylation of Ser/Thr residues by PKA or PKC results, in most cases, in a decrease of $GABA_AR$ function (Moss et al., 1992b; Moss and Smart, 1996), although an increase in $GABA_A$-induced currents has also been reported (Lin et al., 1994; McDonald et al., 1998). Phosphorylation by PKA increases or decreases $GABA_A$-mediated currents depending on the type of neuron and on the combination of β isoform expressions (Nusser et al., 1999; Poisbeau et al., 1999). Phosphorylation of the Tyr residues either potentiates or maintains $GABA_A$-mediated inhibition (Moss et al., 1995; Wan et al., 1997; Huang and Dillon, 1998).

The state of phosphorylation of the receptor also depends on

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the rate of dephosphorylation, which has an equally dynamic and critical role in the modulation of synaptic transmission (Jones and Westbrook, 1997; Huang and Dillon, 1998).

In acutely dissociated neurons or in slices, maintenance of $GABA_AR$ function requires favorable conditions for phosphorylation, by an unknown endogenous kinase requiring only the addition of ATP in the intracellular milieu (Stelzer et al., 1988; Chen et al., 1990; Krupp and Feltz, 1993; Gyenes et al., 1994; Kaneda et al., 1995). Using purified $GABA_AR$ from bovine cerebral cortex, we and others have previously shown that this receptor-associated kinase activity is α1 specific (Sweetnam et al., 1988; Bureau and Laschet, 1995). Here, we report that, unexpectedly, the $GABA_AR$ endogenous kinase is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), providing the first example of a direct functional link between glycolysis and neurotransmission.

Materials and Methods

Receptor endogenous phosphorylation. Benzodiazepine affinity chromatography-purified $GABA_ARs$ from bovine cerebral cortex (Stauber et al., 1987; Bureau and Laschet, 1995) (0.5–1 mg protein/ml) were incubated with 0.33 μM [γ-^{33}P]ATP, Mg^{2+}, NADH, and ADP in HEPES-Tris buffer (50 mM at pH 7.3) for 0–100 min at 30°C. GAPDH (rabbit muscle; Boehringer Mannheim, Mannheim, Germany) was added in some experiments. Receptor phosphorylation was also studied in washed brain cell membranes (Stauber et al., 1987; Bureau and Laschet, 1995), with $^{32}P_i$ added at micromolar concentrations to restrict labeling to $GABA_AR$ α1 subunit (Minier et al., 2000). The incubation time was 10 min at 30°C, in the presence or absence of the required glycolytic intermediates [glyceraldehyde-3-phosphate (G3P), NAD^+, P_i, ADP, Mg^{2+}], without addition of the glycolytic enzymes. Proteins were precipitated in a methanol–chloroform mixture and subjected to SDS-PAGE. Radioactivity was counted using a PhosphorImager scanner (Storm 840; Molecular Dynamics, Sunnyvale, CA) after gel exposure on a storage P-screen or by direct scanning with a β-Imager (Biospace Mesures, Paris, France).

Receptor immunoprecipitation. Purified $GABA_AR$ (50 μl), labeled by endogenous phosphorylation, was incubated for 2 hr at 4°C with varying dilutions of an anti-α1 antibody (0.1 ml/ml; clone bd24; Boehringer Mannheim) or an anti-GAPDH antibody (4.9 mg/ml; Chemicon, Temecula, CA) in a final volume of 200 μl with phosphate buffer containing 0.1% Triton X-100. After the addition of 200 μl of goat anti-mouse IgG conjugated to agarose with 1% bovine serum albumin, the samples were carefully shaken for 16 hr at 4°C. The immunosorbent was removed by centrifugation, and the supernatant and pellets were assayed individually. To further isolate the phosphorylated α1 receptor subunit from the autophosphorylated GAPDH, both immunoprecipitated by the anti-GAPDH antibody, the proteins were separated by SDS-PAGE and analyzed by autoradiography as described above. A monoclonal antibody of the same isotype (mouse IgG2a toward *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues; Dako, Trappes, France) was used as a negative control.

Western blotting. Purified $GABA_ARs$ were transferred from polyacrylamide gels to polyvinylidene difluoride membranes. After incubation with a blocking reagent, the preparation was incubated overnight with the anti-GAPDH, the anti-α1 (the same antibodies used for immunoprecipitation), or the negative control antibody in Tris buffer, pH 7.2, at 4°C. Immunostaining was performed using 0.25 U of anti-mouse Ig-AP/ml buffer for 1 hr at room temperature, followed by incubation of the samples for 20 min with 0.3 mM 4-nitroblue tetrazolium chloride/0.7 mM 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris-chloride buffer, pH 7.2.

Fusion protein constructs. Plasmid constructs were obtained as follows. The second intracellular loop region I2α1 (aa 334–420) of the $GABA_AR$ α1 subunit was amplified by reverse transcription-PCR on mRNA extract from rat brain cortex with the primers 5′-CGGAATTCAACTATTTCACCAAGAGAGG-3′ and 5′-CGCTCGAGTCATCGGTCGATTTTGCTGACGCTG-3′, then in-frame inserted in the plasmid pcDNA3.1HisC (Invitrogen, San Diego, CA), between the polylinker *Eco*R1 and *Xho*I sites. Mutagenesis of I2α1 (T337A and S416G double mutant) was performed by PCR on the I2α1 recombinant plasmid with the primers 5′-CGGAATTCAACTATTTCGCCAAGAGAGG-3′ and 5′-CGCTCGAGTCATCGGTCGATTTTGCCGACGCTG-3′, then reinserted in the same vector. Both wild-type (WTI2α1) and mutant (mutI2α1) constructs were separately PCR amplified by the primers 5′-CCGCTCGAGCGGAAGCTTACCATGGGG-3′ and 5′-GGAATTCCGAGTCCTCGGTCGATTTTGC-3′, and the resulting 400 bp cDNAs were in-frame subcloned in a second vector, pEYFP-N1 (BD Biosciences Clontech, Erembodegem, Belgium), between *Xho*I and *Eco*R1. For the control construct [yellow fluorescent protein (YFP)], the WTI2α1 recombinant pEYFP-N1 was digested by *Eco*R1 to release the I2α1 sequence and ligated after Klenow filling, such that the I2α1 insert is replaced by a single Leu codon. All constructions were checked by nucleotide sequencing.

COS7 cells were cultured in DMEM (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum and 100 μg/ml penicillin–streptomycin in a humidified atmosphere of 5% CO_2/95% air and transiently transfected by using Superfect (Qiagen, Courtaboeuf, France) and 10 μg of pEYFP-N1-WTI2α1, pEYFP-N1-mutI2α1, and pEYFP-N1-control, respectively. Fusion protein expressions were assessed by Western blotting with either Xpress (diluted 1:5000; Invitrogen, Leek, The Netherlands) or poly-histidine (diluted 1:3000; clone HIS-1; Sigma, Saint Quentin Fallavier, France) primary antibodies and goat (heavy and light chain) anti-mouse IgG-HRP secondary antibody (diluted 1:1000; Bio-Rad, Ivry-sur-Seine, France) and detected by ECL+*Plus* luminescence (Amersham Biosciences, Buckinghamshire, UK). Recombinant proteins were purified from the cytosolic extracts on a nickel-chelating HisTrap column (Amersham Biosciences, Uppsala, Sweden) and followed-up by fluorescence.

The direct phosphorylation assay with the fusion proteins was the same procedure as for the receptor endogenous phosphorylation, except for the addition of GAPDH. To allow protein–protein interaction, each construct was codialyzed with GAPDH (100 μg/ml). The amounts of the three constructs were equalized according to their fluorescence.

Double fluorescence immunocytochemistry. Primary cultures of hippocampal neurons were prepared from embryonic day 18 rat embryos (Fuhrmann et al., 2002). At 12 d *in vitro*, cells were fixed with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) containing 4% sucrose at room temperature for 15 min, rinsed in PBS, and permeabilized with 0.25% Triton X-100 in 0.02 M PBS containing 0.2% gelatin and 10% normal goat serum for 30 min. Primary antibodies were simultaneously incubated overnight at +4°C. GAPDH mouse monoclonal antibody (Chemicon) was used at 10 μg/ml. The antibody against the α1 subunit of the rat $GABA_AR$ (a gift from Prof. W. Sieghart, Brain Research Institute, Vienna, Austria) (Sperk et al., 1997) was used at 0.5 μg/ml. After rinsing, cells were incubated with biotinylated anti-rabbit Ig (concentrated rabbit link; BioGenex, San Ramon, CA) at a 1:100 dilution for 30 min at room temperature, rinsed again, and incubated in a mixture of Cy3-conjugated streptavidin (1 μg/ml; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 488 goat anti-mouse (1:100; Molecular Probes Europe, Leiden, The Netherlands) for 1 hr at room temperature. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). In the controls in which the primary antibodies were omitted, no immunostaining could be observed. Immunostaining was analyzed using a Leica TCS-SP2 confocal laser-scanning microscope. All pictures are single confocal sections averaged four to six times to reduce noise.

Electrophysiology. Adult Sprague Dawley rats (35–40 d of age) were anesthetized with ether and killed by decapitation. The brains were removed quickly and placed in cold artificial CSF. Cortical neurons were acutely dissociated from 400 μm slices by incubation in protease-XXIII (3 mg/ml; Sigma) at 32°C, followed by mechanical dissociation. After washing, the cells were transferred in a solution containing (in mM) 135 NaCl, 3 KCl, 2 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, 7 TEA-Cl, 10 D-glucose, and 1 μM TTX, pH 7.4. Pyramidal neurons were recorded using borosilicate pipettes (4–5 MΩ) filled with a solution containing (in mM) 130 CsF, 10 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA, and 7 Mg-ATP. Additions were made to this standard pipette milieu for specific experiments: GAPDH (1 μg/ml), NAD^+ (5 or 50 μM), NADH (5 μM), G3P (500 μM),

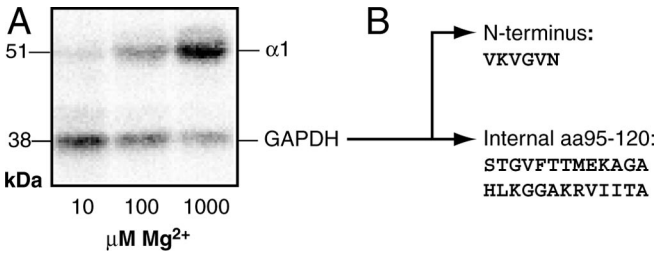

Figure 1. Endogenous phosphorylation of the α1 subunit of purified $GABA_AR$ and identification of the kinase candidate. *A*, Gel autoradiography showing a phosphorylation cascade of copurified 38 kDa protein to the α1 subunit (51 kDa) after a 30 min incubation in the presence of ^{33}P-ATP (0.33 μM) and increasing [Mg^{2+}]. *B*, Microsequencing of the 38 kDa protein revealing two sequences of bovine GAPDH.

ortho-vanadate (100 μM), or okadaic acid (10 μM). The seals were made with these compounds in the pipette. In other experiments, iodoacetamide (500 μM) was added to the bath between recording times $t = 6$ min and $t = 9$ min and maintained until the end of the experiment. Whole-cell peak currents induced by GABA (100 μM; pressure ejections or fast applications, 1 sec pulse every 3 min) were measured for each application and normalized to the maximal response ($t = 0$ min). This maximal response was observed within 0–6 min after patching because, in some cases, there was an initial run-up. Neurons were held at −80 mV such that GABA evoked inward currents (Cl^- equilibrium potential close to −40 mV). In control experiments, CsF was replaced by CsCl: the holding potential was then −40 mV (Cl^- equilibrium potential close to 0 mV). The statistical significance was assessed using a one-way ANOVA and a two-way ANOVA for repeated measures, followed by Dunnett's *post hoc* analysis (one-tailed distribution).

Results

GAPDH is the $GABA_AR$ endogenous kinase

Preparations of $GABA_AR$ purified from bovine cerebral cortex consistently included an additional copurified 38 kDa polypeptide (P38) (Figs. 1*A*, 2*A*). At physiological [Mg^{2+}] (1 mM) and with [^{33}P]ATP (without the addition of any protein kinase or of their activators), $GABA_AR$ phosphorylation occurred at the receptor α1 subunit only (51 kDa), as shown by Western blotting (Fig. 2*A*). These data confirms previous studies (Sweetnam et al., 1988; Bureau and Laschet, 1995; Minier et al., 2000). At 10 mM [Mg^{2+}] or greater, phosphorylation appears also in subunits of higher molecular weight (~53–55 kDa; data not shown). At 10 μM [Mg^{2+}], a marked labeling of P38 was still observed, whereas that of the α1 subunit was very faint; at 100–1000 μM [Mg^{2+}], the ^{33}P-labeling of the α1 subunit increased, whereas that of P38 decreased (Fig. 1*A*). Kinetic studies at 1 mM [Mg^{2+}] suggested that phosphorylation of P38 occurred before any significant incorporation of ^{33}P-phosphate at the α1 subunit (data not shown). We made the assumption that P38 is the receptor α1 subunit endogenous protein kinase.

The P38 copurifying with $GABA_AR$ was separated and microsequenced (Fig. 1*B*). The first six N-terminal amino acids were identical to the N terminus amino acids of bovine (aa 1–6) and human (aa 3–8) GAPDH (Kulbe et al., 1975; Ercolani et al., 1988), a key glycolytic enzyme. To rule out any possible comigration of another N-blocked protein, an internal fragment of P38 was purified after controlled proteolysis by endoproteinase Lys-C and sequenced. The first 26 amino acids obtained corresponded to the aa 95–120 of bovine GAPDH (except for a S119T substitution) and were highly homologous to the aa 97–122 of the human GAPDH. Moreover, an anti-GAPDH monoclonal antibody recognized the copurified P38 band without cross-reacting with any other protein of the purified receptor preparation (Fig. 2*A*).

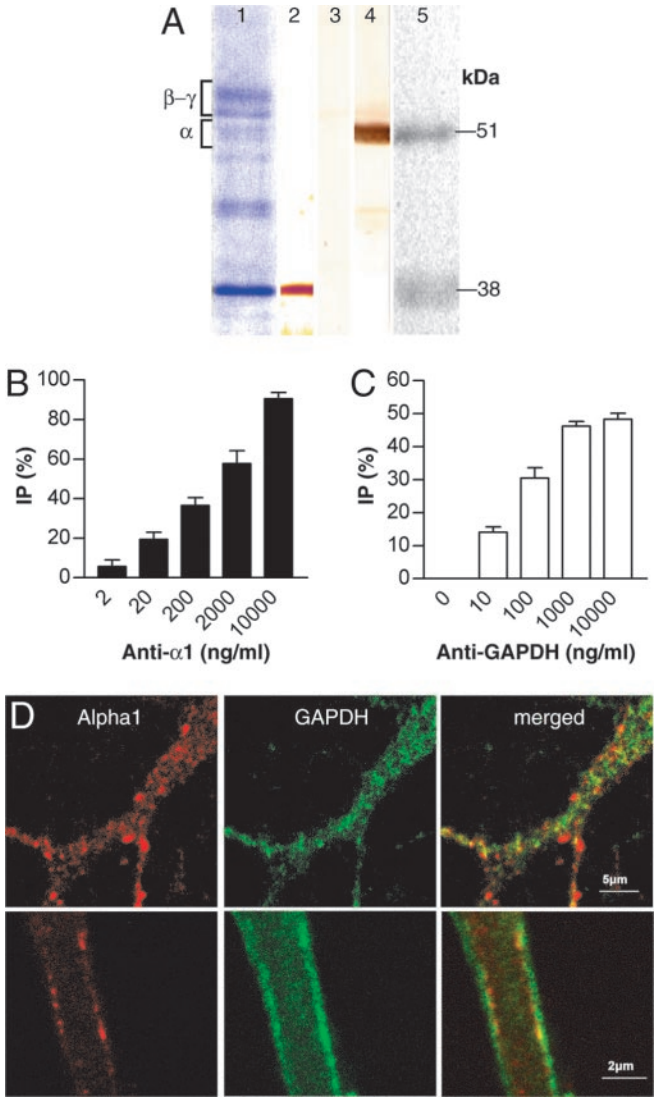

Figure 2. Molecular association between GAPDH and the $GABA_AR$ α1 subunit. *A*, $GABA_AR$ purified preparations from bovine brain cortex were separated and stained showing the main subunits (lane 1), probed with anti-GAPDH (lane 2), negative control (lane3), or anti-α1 (lane 4) monoclonal antibodies at 10 μg/ml, or ^{33}P-autoradiographed after endogenous phosphorylation (lane 5). *B*, Immunoprecipitation of ^{33}P-labeled purified $GABA_AR$ receptor by an anti-α1 antibody at varying concentrations. Error bars are SEM ($n = 3$). *C*, Immunoprecipitation of ^{33}P-labeled purified $GABA_AR$ α1 subunits by an anti-GAPDH antibody at varying concentrations; measurements on isolated α1 subunit were performed by autoradiography after protein gel electrophoresis. Error bars are SEM ($n = 3$). *D*, Subcellular colocalization of the $GABA_AR$ α1 subunit (red) with GAPDH (green) at the cell membrane of rat hippocampal neurons in primary cultures at day 12 *in vitro*, observed by confocal microscopy. The top view shows a neuron, and the bottom view shows a dendrite of another neuron at higher magnification.

Thus, the P38 receptor-associated protein corresponded to GAPDH.

It is known that GAPDH is autophosphorylated in the sole presence of Mg-ATP and transfers its incorporated phosphate to unknown target proteins (Kawamoto and Caswell, 1986). Thus, it is likely that the ^{33}P-labeling of the 38 kDa protein now identified as being GAPDH is attributable to autophosphorylation. The observation that increasing [Mg^{2+}] produced opposite effects on the phosphorylation of GAPDH (decreased) and on that of α1 subunit (increased) (Fig. 1*A*) indicates a phospho-transfer from the autophosphorylated GAPDH to the α1 subunit.

$GABA_AR$–GAPDH association

Nearly 90% of the ^{33}P-labeled $GABA_AR$ was immunoprecipitated by the $\alpha1$-specific bd24 monoclonal antibody at 10 μg/ml (Fig. 2*B*); in control experiments performed with an irrelevant monoclonal antibody (see Materials and Methods) of the same isotype (IgG2a) at the same dilutions, no radioactivity was immunoprecipitated (data not shown). The anti-GAPDH monoclonal antibody immunoprecipitated purified $GABA_AR$, phosphorylated with [^{33}P]ATP and 1 mM Mg^{2+}. To exclude from the total ^{33}P immunoprecipitated by the anti-GAPDH antibody the part attributable to GAPDH autophosphorylation, the $\alpha1$ subunit was further isolated by protein gel electrophoresis, and autoradiographic counting was restricted to the 51 kDa band that is recognized by the anti-$\alpha1$ antibody. At anti-GAPDH antibody concentrations of 1–10 μg/ml, half of the ^{33}P-labeled $\alpha1$ subunit was adsorbed (Fig. 2*C*); in control experiments with the irrelevant monoclonal antidody, no immunoprecipitation was achieved (data not shown). These results indicate that at least 50% of the $\alpha1$ subunits were bound either directly or indirectly to GAPDH. This interaction between $\alpha1$ and GAPDH can be considered as specific because GAPDH remained in high amounts even after stringent washing during receptor preparation (Fig. 2*A*, lane 1). Nevertheless, a partial loss of GAPDH during the procedure cannot be ruled out.

A close association between GAPDH and the $GABA_AR$ $\alpha1$ subunit implies their colocalization at the neuronal plasma membrane. Double fluorescence immunocytochemistry with confocal microscopy was performed on primary cultures of rat hippocampal neurons. On day 12 *in vitro*, $\alpha1$ subunit immunoreactivity formed bright puncta (red) along the surface membrane of somata and dendrites (Fig. 2*D*) of most neurons (16 of 28). Whereas a light and sparse cytoplasmic GAPDH immunostaining (green) was visible in the cell bodies and dendrites of all neurons ($n = 75$), conspicuous labeling was concentrated at the cell membrane, giving a somewhat discontinuous appearance (Fig. 2*D*). On superimposed images, all immunoreactive dots reveal the receptor $\alpha1$ subunit colocalized with the GAPDH-immunoreactive fluorescence at or near the neuronal membrane (Fig. 2*D*). In washed cortical membranes, we measured a GAPDH activity of ~10 μkatal per milligram of protein.

Direct $\alpha1$ ^{337}Thr/^{416}Ser-phosphorylation by GAPDH

The phosphorylation cascade between GAPDH and the $\alpha1$ subunit may be indirect and require one or several intermediates. To rule out this possibility, we tested the direct phosphorylation by GAPDH on purified fusion proteins. The long intracellular domain I2 (also called M3-M4) of the rat $\alpha1$ subunit (I2$\alpha1$), a candidate site for phosphorylation, was expressed in COS7 cells. The constructs include I2$\alpha1$, an N-leader tagged for immunodetection and purification, and a YFP at the C terminus for rapid expression detection. No phosphorylation occurred after incubation of the purified fusion protein with [^{33}P]Mg-ATP (data not shown). When highly purified GAPDH was added, it autophosphorylated (GAPDH labeling) (Fig. 3*C*, 38 kDa band), and the fusion protein containing I2$\alpha1$ was also phosphorylated (Fig. 3*C*, 36 kDa band). A control construct (25 kDa) containing only the tags and YFP was not phosphorylated (Fig. 3*C–E*). This demonstrates that GAPDH directly phosphorylates I2$\alpha1$.

Numerous Thr, Ser, and Tyr phosphorylable residues are present in I2$\alpha1$ (Fig. 3*B*), but it is known that endogenous phosphorylation involves only Ser and Thr residues on $\alpha1$ and possibly $\alpha2$ subunits but not Tyr on these subunits (Bureau and Laschet, 1995). To determine any possible consensus sites for this phosphorylation, we compared the rat I2$\alpha1$ sequence (which is identical to the human, bovine, and mouse sequences) with different $GABA_AR$ subunit sequences. We found at both ends of I2$\alpha1$, two α(1–3, 5) subunit-specific highly conserved regions centered on ^{337}Thr and ^{416}Ser, with a minimum consensus sequence [NXX(T/S)K] (Fig. 3*A*,*B*). Site-directed mutagenesis was performed on the recombinant protein such that the double mutant (T337A and S416G) presented nonphosphorylable residues at both presumed sites. The GAPDH phosphorylation assayed at the plateau value (incubation time longer than 20 min) on the modified protein (36 kDa) showed that 78% of the ^{33}P incorporation observed on the wild type disappeared (Fig. 3*C–E*). The

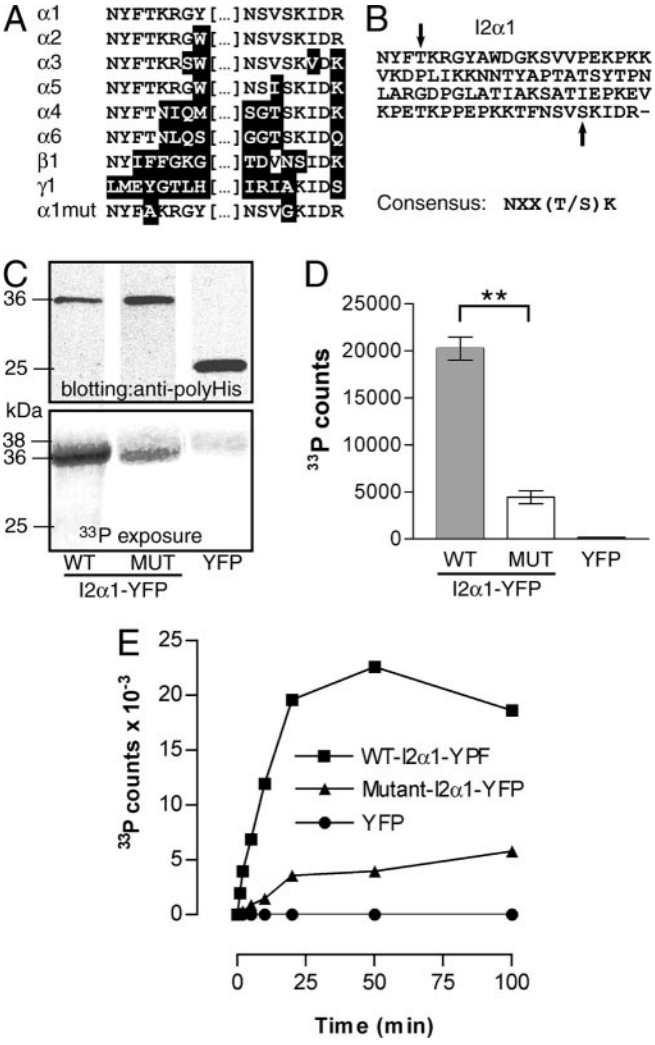

Figure 3. Direct phosphorylation of the recombinant long intracellular domain of the $GABA_A$ α subunit at identified Ser and Thr residues. *A*, Conserved end sequences of the long intracellular domain of mammalian $GABA_AR$ in several α subunits, but not in β-γ subunits. Site-directed mutations of ^{337}Thr and ^{416}Ser in the mutated $\alpha1$ subunit construct (α1mut). Amino acids diverging from the $\alpha1$ subunit are boxed. *B*, The sequence of the long intracellular domain of the $\alpha1$ subunit contains numerous Ser and Thr residues. The arrows indicate mutation sites. The consensus sequence of the presumed phosphorylation sites is shown. *C*, Purified wild-type I2$\alpha1$-YFP (WT), Ser/Thr double mutant I2$\alpha1$-YFP (MUT), or control (YFP) constructs were phosphorylated directly by GAPDH in the presence of Mg^{2+} and ^{33}P-ATP, separated, and probed with an anti-polyHis antibody (top) or ^{33}P-autoradiographed (bottom). *D*, Counting of the ^{33}P incorporation by GAPDH of wild-type I2$\alpha1$-YFP (WT-I2$\alpha1$-YFP), Ser/Thr double mutant (MUT-I2$\alpha1$-YFP), or control (YFP). Error bars are SEM ($n = 3$). The mutation effect on phosphorylation (22% of the wild type) is significant ($p < 0.01$; paired Student's *t* test). *E*, Representative kinetics of the phosphorylation by GAPDH of the constructs mentioned in *C* and *D*.

remaining labeling (22%) may be attributable to less specific phosphorylable residues that we did not attempt to identify. Therefore, ^{337}Thr and ^{416}Ser sites account for most of the I2α1 phosphorylation by GAPDH *in vitro*.

Factors influencing endogenous kinase

At 100 μM [Mg^{2+}], phosphorylation of the $GABA_AR$ α1 subunit was maximally activated twofold by 1–10 μg/ml of added exogenous GAPDH, with an EC_{50} value of ~50 ng/ml (1.3 nM; data not shown; $n = 3$). The phospho-GAPDH was stable at pH 4 but hydrolyzed after a long exposure to neutral and alkaline pH (Kawamoto and Caswell, 1986). For this reason, autophosphorylation of GAPDH was not always apparent in our experiments after gel electrophoresis: labeling was constantly observed only when GAPDH was added at high concentrations.

The reduced GAPDH coenzyme NADH facilitates phosphate transfer from phospho-GAPDH to target proteins (Kawamoto and Caswell, 1986). The transfer of a phosphate group from the phospho-enzyme to the α1 substrate did not require a coenzyme (other than Mg^{2+}), even if it was enhanced by NADH (+33% at 1 μM; data not shown; $n = 3$), most likely through its action on endogenous GAPDH. This effect was not related to the glycolytic activity of GAPDH because no glycolytic substrate was added. Furthermore, NADH is the reduced cofactor of GAPDH and thus opposes glycolytic ATP synthesis. Our results therefore suggest an allosteric interaction between the coenzyme and the kinase catalytic sites. In contrast, the addition of ADP decreased α1 endogenous phosphorylation (IC_{50} value, 2 μM; data not shown; $n = 3$), perhaps as a result of a thermodynamic effect because ADP is produced during GAPDH autophosphorylation. Autophosphorylation of GAPDH does not require a coenzyme; it requires only Mg^{2+}-ATP. The catalytic Cys residue related to the dehydrogenase action of GAPDH does not play a role in its phosphotransferase activity because the sulfhydryl alkylating agent iodoacetamide, a GAPDH inhibitor, did not inhibit phosphorylation of the $GABA_AR$ α1 subunit (data not shown; $n = 3$). These results show that, in the presence of Mg-ATP, phosphorylation of the $GABA_AR$ α1 subunit by GAPDH did not directly require the reactions involved in the dehydrogenase activity.

Glycolysis-dependent membrane receptor phosphorylation

We have previously shown that $GABA_AR$ endogenous phosphorylation in washed membrane preparations is more efficient than that of the purified receptors, and that ^{33}P incorporation from ATP at micromolar concentrations is highly specific for the α1 subunit (Minier et al., 2000). It may be asked whether the membrane preparation retains the capacity to generate ATP from the glycolytic precursors when no exogenous enzyme is added. When the membranes were incubated with the specific GAPDH substrate G3P, together with the other ingredients necessary for glycolysis (NAD^+, ADP, Mg^{2+}, $^{32}P_i$), the α1 subunits were indeed labeled (Fig. 4*A*). In this condition, the only labeled precursor was the [^{32}P]-orthophosphate (no added ATP). Without G3P, the α1 subunit was not phosphorylated. Similarly, α1 phosphorylation did not occur when one of the other intermediates (NAD^+, ADP, Mg^{2+}, $^{32}P_i$) was omitted. Decreasing ADP concentration from 100 to 10 μM enhanced labeling, thus reducing its inhibitory effect. The addition of exogenous cold ATP decreased labeling by isotopic dilution, thus demonstrating a production of [^{32}P]ATP at the membrane and its direct use for $GABA_AR$ endogenous phosphorylation (Fig. 4*B*). From these data, it is also clear that ambient cold ATP can compete for α1 phosphorylation and exchange with the glycolytic [^{32}P]ATP produced at the membrane.

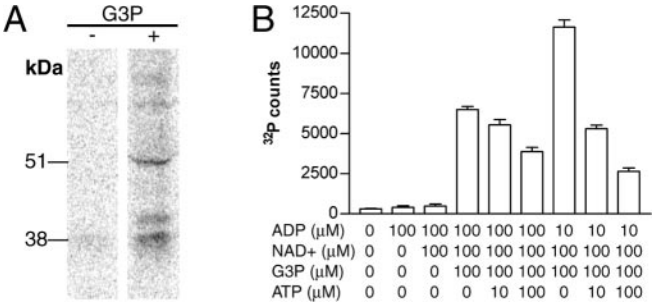

Figure 4. Involvement of membrane-associated glycolysis in the $GABA_AR$ phosphorylation. Local glycolytic ATP production was used for endogenous α1 phosphorylation in bovine cortical washed membranes. *A*, Autoradiograph of washed membranes incubated without any exogenous enzyme or activator, in the presence of ADP (10 μM), NAD^+ (100 μM), Mg^{2+} (1 mM), and $^{32}P_i$ (0.33 μM), without or with the specific GAPDH substrate G3P (100 μM). Bands at 51 and 38 kDa are ^{32}P-labeled α1 subunit and GAPDH, respectively. *B*, Effects of exogenous factors on phosphorylation of the α1 subunit, as expressed as counts in the 51 kDa band, in the presence of Mg^{2+} (1 mM) and $^{32}P_i$ (0.33 μM) of washed membranes. Error bars are SEM ($n = 3$).

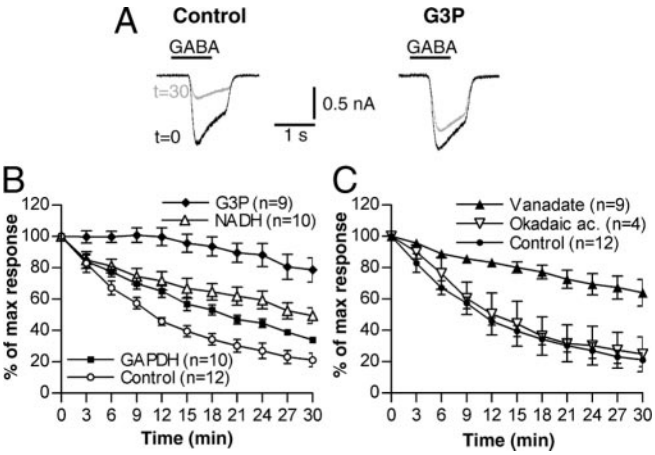

Figure 5. Glycolysis dependence of the functional maintenance of $GABA_A$ currents. *A*, Superimposed whole-cell current traces induced by GABA (100 μM) applied to acutely dissociated rat neocortical neurons at $t = 0$ min (black) and at $t = 30$ min (gray). Rundown of the responses was markedly decreased when G3P was added to the pipette milieu. *B*, Time course of normalized peak currents to GABA applied every 3 min during 30 min (holding potential, −80 mV). The addition of GAPDH (1 μg/ml), NADH (5 μM), or G3P (500 μM) to the pipette milieu reduced the rundown of responses ($p = 0.012$, $p < 0.001$, and $p < 0.001$ for GAPDH, NADH, and G3P, respectively; 2-way ANOVA with Dunnett's *post hoc* test). Error bars are SEM, and *n* is the number of cells recorded. *C*, The phosphatase inhibitor orthovanadate (100 μM) added in the pipette mostly prevented rundown ($p < 0.005$), whereas okadaic acid (10 μM) had no effect.

Function of GAPDH-dependent phosphorylation

In whole-cell recordings from dissociated neurons, the amplitude of $GABA_AR$-mediated responses decreases with successive applications of GABA. This rundown involves a phosphorylation–dephosphorylation process (Stelzer et al., 1988; Chen et al., 1990; Gyenes et al., 1994; Kaneda et al., 1995). Using whole-cell recordings, the currents elicited by GABA (100 μM) were measured in neurons acutely dissociated from rat cortex (Fig. 5*A*). Magnesium ions activate both phosphorylation and dephosphorylation of the receptor (see below), and a concentration of 7 mM Mg-ATP was chosen to provide a robust and reproducible rundown of $GABA_A$ responses. Lowering the [Mg-ATP] led to a more rapid rundown, and changing the [Mg^{2+}]/[ATP] molar ratio also influenced rundown (data not shown). When Mg-ATP was omit-

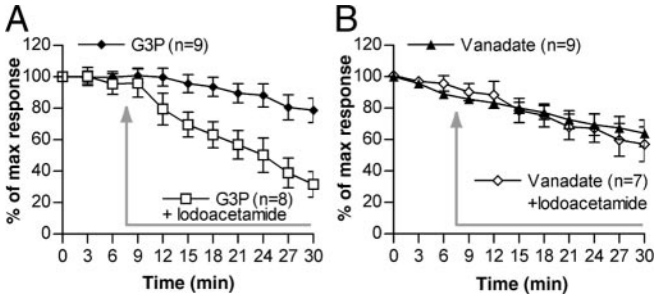

Figure 6. Functional effect of GAPDH inactivation. Iodoacetamide, an inhibitor of the dehydrogenase activity of GAPDH (500 μM), was added to the bath between $t = 6$ min and $t = 9$ min and maintained during the rest of the recordings (arrows). *A*, Iodoacetamide abolished the preventive effect of G3P (500 μM) on rundown (7 mM ATP in the pipette; $p < 0.001$; 2-way ANOVA with Dunnett's *post hoc* test). Error bars are SEM, and *n* is the number of cells recorded. *B*, In the presence of phosphatase inibitor vanadate (100 μM), iodoacetamide could no longer accelerate the rundown of $GABA_A$ responses.

ted from the pipette milieu, GABA responses declined rapidly and were nearly abolished within 9–12 min after patching (data not shown).

With 7 mM Mg-ATP in the intracellular medium, the response declined steadily to 21% of the maximal response after 30 min (Fig. 5*B*). With the addition of GAPDH in the pipette milieu (1 μg/ml), the $GABA_A$ response was reduced to only 34% of the maximum at 30 min (Fig. 5*B*). The response was even more preserved with the addition of G3P (500 μM), the glycolytic substrate of GAPDH, at 79% of the maximum at 30 min. Because this effect was obtained with 7 mM Mg-ATP in the pipette, it shows that prevention of rundown is mainly dependent on glycolytic, but not ambient, ATP. NADH, which increased the kinase activity of GAPDH, also markedly preserved the responses (53% at 30 min) when added at 5 μM to the intracellular solution (Fig. 5*B*). NAD^+ (5 or 50 μM) was much less effective (25% at 30 min; data not shown). From the one-way ANOVA with Dunnett's *post hoc* tests, the difference of response amplitudes compared with the control was significant ($p < 0.01$) at and after $t = 6$ min for NADH and G3P and at and after $t = 12$ min for GAPDH.

We had previously shown that the phosphatase inhibitor vanadate, but not okadaic acid, prevented Mg^{2+}-dependent α1 dephosphorylation in washed cortical membranes (Minier et al., 2000). Therefore, we tested whether these inhibitors were able to modify the time course of the $GABA_A$ responses: indeed, vanadate (100 μM), but not okadaic acid (10 μM), prevented rundown (Fig. 5*C*). These data confirm that rundown depends on a dephosphorylation process and suggest that the same membrane-bound Mg^{2+}-dependent phosphatase is involved in the rundown.

When the glycolytic activity of GAPDH was inactivated by adding iodoacetamide (500 μM) to the bath, the preventive effect of G3P was annihilated and the rundown of the GABA response became again apparent, with a delay of a few minutes (Fig. 6*A*). In contrast, rundown prevention by phosphatase inhibition (vanadate) maintaining the phosphorylated state of the αl subunit was not sensitive to iodoacetamide addition (Fig. 6*B*). Thus, iodoacetamide does not affect by itself the $GABA_A$ responses, and the glycolytic function of GAPDH is required for the maintenance of these responses, even in the presence of 7 mM Mg-ATP. These results show that $GABA_A$R function depends on a phosphorylation process fueled by glycolytic substrates and support a role for GAPDH in this mechanism.

A number of phosphatases can be inhibited by fluoride ions present in the pipette milieu. Control experiments in which fluoride was replaced by chloride did not show any substantial change of the $GABA_A$ current rundown (data not shown).

Discussion

The endogenous kinase responsible for the maintenance of $GABA_A$R-mediated responses was unknown until now. Here, we identify the receptor kinase candidate P38 associated with the purified $GABA_A$R as being the glycolytic enzyme GAPDH and demonstrate that GAPDH directly phosphorylates identified residues of molecular constructs of the $GABA_A$R α1 long intracellular loop. This unexpected identification implies that this dehydrogenase has additionally a protein kinase activity as already shown in the rabbit muscle (Kawamoto and Caswell, 1986). Indeed, if the phospho-GAPDH formed from the added enzyme did not transfer the phosphate moiety to the α1 subunit, this GAPDH would be expected to compete for ATP with the endogenous kinase and to inhibit the α1 phosphorylation, which is contrary to our observations. Similar to some other kinases, GAPDH autophosphorylates before transferring the phosphate to its target, in agreement with previous findings (Kawamoto and Caswell, 1986); such transfer was favored by the coenzyme NADH, as we have presently observed.

This $GABA_A$R endogenous phosphorylation appears α1 specific. We and others have previously reported that an α1-specific endogenous kinase activity is closely associated with purified $GABA_A$R from bovine cerebral cortex (Sweetnam et al., 1988; Bureau and Laschet, 1995). In addition, a selective anti-bovine α1 monoclonal antibody (Ewert et al., 1990; Sperk et al., 1997) immunoprecipitated 90% of the [^{3}H]muscimol binding sites (Bureau and Laschet, 1995), and a similar proportion of the receptor protein phosphorylated in the presence of ^{32}P-labeled ATP (Fig. 2*B*), suggesting that phosphate incorporation occurs essentially at the α1 subunit. We also confirm this α1 specificity by showing that the substrate phosphorylated at physiological [Mg^{2+}] has an apparent molecular weight corresponding to α1 subunits. We also show that 50% of radiolabeled species at 51 kDa were immunoprecipitated by a GADPH antibody (Fig. 2*C*). Taking into account that part of the α1 subunit–GAPDH association could have been disrupted during receptor purification, these data indicate that the anti-GAPDH antibody specifically and quantitatively immunoprecipitates a phosphorylated α1 subunit–GAPDH complex. This is consistent with a high-affinity interaction between GAPDH and the α1 subunit because the addition of exogenous GAPDH enhanced phosphorylation with an estimated EC_{50} value in the nanomolar range. We do not exclude, however, that other endogenous protein kinases may phosphorylate other $GABA_A$R subunits especially at [Mg^{2+}] higher than 10 mM (Kannenberg et al., 1999) with possibly different functional roles. Phosphorylation on β and γ subunits by the classical kinases has been reported, but there is no consistent evidence for phosphorylation of α subunits by these kinases. We have previously demonstrated that the endogenous $GABA_A$R phosphorylation occurred on Ser/Thr residues of α1 in an equimolar proportion (Bureau and Laschet, 1995). Accordingly, we identify phosphorylation sites on single Ser and Thr residues within the long intracytoplasmic loop of the α1 subunit.

We show that GAPDH is a receptor-associated protein: GAPDH and $GABA_A$R α1 subunit are in close molecular association because they were copurified, coimmunoprecipitated, and colocalized at the neuronal plasma membrane. This is consistent with observations showing localization of GAPDH in postsynaptic densities (Wu et al., 1997; Moon et al., 1998; Walikonis et al., 2000) and its association with the inner surface of the plasma

membrane (Daum et al., 1988; Sirover, 1999). However, it is clear from our immunoflurescent imaging that not all the GAPDH located at the membrane is associated with the $GABA_AR$ α1 subunit, even if the reverse is true. The fraction of membrane-bound GAPDH that did not colocalize with the α1 subunit could be accounted for by associations with other membrane proteins such as (Na^+,K^+)-ATPase. This ionic pump is fueled at least partially by glycolytic ATP in both neurons and glial cells (Silver et al., 1997). A functional association at the membrane between GAPDH and (Na^+,K^+)-ATPase and a preference for glycolytic ATP were clearly demonstrated in erythrocytes, in cardiac Purkinje cells, and in fast-twitch skeletal muscle (Mercer and Dunham, 1981; Glitsch and Tappe, 1993; Okamoto et al., 2001).

The phosphorylation of α1 subunits in washed brain membranes with P_i as the sole precursor cannot be explained without the intervention of at least two enzymes of the glycolytic pathway, GAPDH and phosphoglycerate kinase (PGK), working together at the membrane to locally produce the needed ATP. Accordingly, GAPDH and PGK are both present in postsynaptic densities (Wu et al., 1997; Walikonis et al., 2000). Thus, our findings suggest that the GAPDH–PGK association induces a local synthesis of glycolytic ATP to fuel receptor phosphorylation at the postsynaptic density. Interestingly, aldolase and lactate dehydrogenase activities are also found in postsynaptic densities (Wu et al., 1997), allowing the coenzyme NAD^+ to be regenerated, the glycolysis rate to be increased, and thus the ATP production to be accelerated by the Pasteur effect.

The involvement of GAPDH-dependent phosphorylation of the α1 subunit in the prevention of rundown of $GABA_A$ responses is supported by the fact that both processes are favored by the addition of GAPDH, its coenzyme NADH, or its substrate G3P. The limited but significant protection against $GABA_A$ current rundown by GAPDH added to the pipette may be attributable to an increase in the glycolysis turnover or to an increased kinase activity for α1 phosphorylation. Nevertheless, new experimental tools would be helpful to provide more direct evidence that the $GABA_AR$ is phosphorylated by GAPDH on the α1 subunit in isolated intact cells or *in vivo*.

We have previously found a membrane-associated Mg^{2+}-dependent phosphatase that dephosphorylates the α1 subunit and is inhibited by orthovanadate but not by okadaic acid (Minier et al., 2000). This phosphatase is lost during receptor purification. Thus, even in the presence of Mg^{2+} in sufficient amounts, α1 did not dephosphorylate in purified receptors, whereas this phosphatase is present and active in washed brain membrane preparations. We show that vanadate, but not okadaic acid, mostly prevents rundown, suggesting that the phosphatase active in whole-cell recorded neurons has the same pharmacological profile. Rundown thus reflects a time-dependent shift from the phosphorylated to the nonphosphorylated state because of the higher rate of phosphatase compared with kinase activity. Vanadate is often considered as being a specific inhibitor of phosphotyrosine phosphatases in the micromolar range, whereas α1 is phosphorylated on Ser/Thr residues. However, at higher concentrations, vanadate is no longer residue specific (Shenolikar and Nairn, 1991; Morinville et al., 1998). Moreover, some phosphatases expressed in the brain dephosphorylating Tyr residues, but also Ser/Thr residues, are indeed inhibited by vanadate (Zhao et al., 2001). Finally, we cannot exclude that the α1 Ser/Thr-phosphatase(s) activity may depend indirectly on that of a Tyr-phosphatase.

In membranes or cells, the $GABA_A$ receptor must be phosphorylated to function, and this phosphorylation requires the Mg–ATP complex; whereas free Mg^{2+} ions are sufficient to activate the membrane-bound phosphatase. Therefore, varying $[Mg^{2+}]$ and [ATP] and their ratio may be sufficient to modulate the phosphorylation state of $GABA_AR$ and thus its response to the agonist. Yet, it is difficult to know how free intracellular Mg^{2+} is regulated, especially near the inner side of the plasma membrane. We do not rule out the possibility that $[Mg^{2+}]_i$ remains constant; but even in such case, varying the glycolysis rate (and thus the level of ATP available) may be sufficient for $GABA_A$ response modulation.

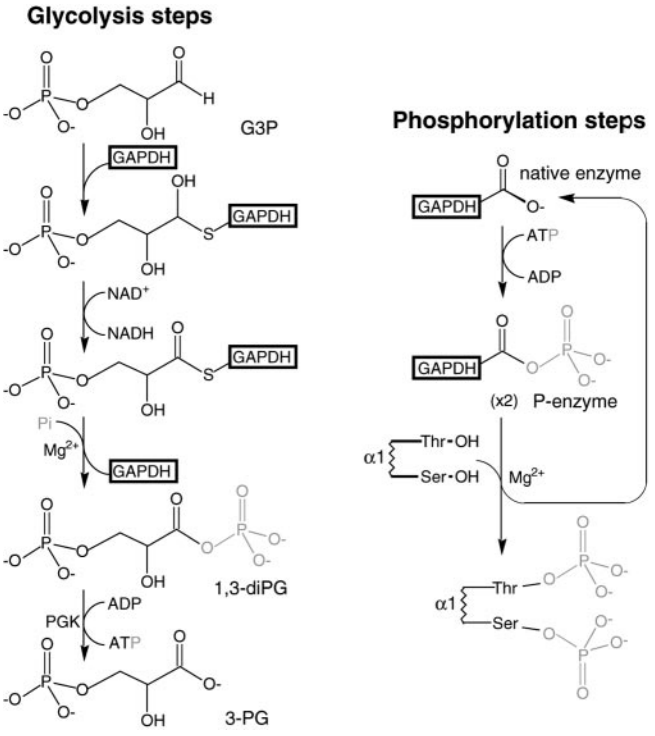

Figure 7. Diagram of the metabolic integration of glycolysis in the $GABA_A$ receptor function. In the present study, we have shown the use of glycolytic ATP for endogenous $GABA_AR$ α1 phosphorylation. The glycolytic substrate G3P is oxidized by the coenzyme NAD^+, then separates from the enzyme by phosphorolysis in the presence of Mg^{2+}, with incorporation of a phosphate (P_i) forming 1,3-diphosphoglycerate (1,3-diPG). This energy-rich bond is finally used to produce the glycolytic ATP. For the phosphorylation steps, GAPDH autophosphorylates with a preference for this glycolytic ATP. The phospho-GAPDH can then transfer the phosphomoeity in the presence of Mg^{2+} to the α1 subunit residues Ser and Thr. All these reactions occur on the inner side of the plasma membrane, in the vicinity of the $GABA_AR$. 3-PG, 3-Phosphoglycerate.

The dehydrogenase inhibitor iodoacetamide did not prevent the kinase activity of GAPDH and did not directly alter the $GABA_AR$ function. Therefore, the rapid and marked effect of iodoacetamide on the rundown of GABA responses observed when the intracellular [ATP] was 7 mM shows that the ATP required for the maintenance of the GABA responses is essentially the glycolytic ATP, probably produced locally at the membrane. Such feature presents the advantage of a local and fast ATP production, and a regulation independent of oxidative energy production. Furthermore, the association of glycolytic enzymes with the receptor complex avoids or limits ATP diffusion. Indeed, it was shown that glycolytic ATP is not in equilibrium with the bulk ATP in the skeletal muscle triads (Han et al., 1992). Our data suggest a similar property for the $GABA_A$ receptor function.

Considering the preference for glycolytic instead of oxidative ATP for $GABA_AR$ phosphorylation, we propose the following

concept of integrated membrane machinery for sustaining fast inhibitory transmission (Fig. 7). The steps of glycolysis required for ATP production are those catalyzed by GAPDH and PGK. GAPDH itself is not phosphorylated in glycolytic reactions, and ATP is not a substrate in this oxidation–reduction cycle. For receptor phosphorylation, there is no need for ATP to diffuse because it can be channeled back, allowing GAPDH to autophosphorylate with a preference for this locally produced glycolytic ATP. The phospho-GAPDH has the capacity to transfer the phospho-moiety in the presence of Mg^{2+} to the $GABA_A$R α1 subunit residues Ser and Thr that are substrates for the kinase activity of receptor-associated GAPDH, this transfer being enhanced by NADH produced by the dehydrogenase activity. All these reactions occur at the inner side of the plasma membrane, in the vicinity of the $GABA_A$R within a macrocomplex.

Finally, because the GABAergic function is glycolysis dependent, it must be modulated by the NAD^+/NADH and ATP/$(ADP+P_i)$ ratios in conjunction with the appropriate GAPDH substrate concentrations. Thus, any pathophysiological state interfering with the energetic metabolism (epilepsy, anoxia, etc.) should alter the efficiency of synaptic GABAergic inhibition. Indeed, it has been shown using positron emission tomography that the administration of a $GABA_A$ agonist increases brain glucose metabolism in normal subjects (Peyron et al., 1994a), even more in temporal lobe epilepsy patients, in which a diffuse hypometabolism is usually present (Peyron et al., 1994b). Therefore, the mechanisms regulating such glycolysis-dependent phosphorylation may be the targets for the development of new drugs in various brain pathologies.

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