THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 43, Issue of October 24, pp. 42006–42011, 2003

Printed in U.S.A.

γ-Hydroxybutyrate Reduces Mitogen-activated Protein Kinase Phosphorylation via $GABA_B$ Receptor Activation in Mouse Frontal Cortex and Hippocampus*

Received for publication, April 22, 2003, and in revised form, August 13, 2003
Published, JBC Papers in Press, August 15, 2003, DOI 10.1074/jbc.M304238200

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γ-Hydroxybutyrate (GHB) naturally occurs in the brain, but its exogenous administration induces profound effects on the central nervous system in animals and humans. The intracellular signaling mechanisms underlying its actions remain unclear. In the present study, the effects of GHB on the activation (phosphorylation) of mitogen-activated protein kinases (MAP kinases), extracellular signal-regulated kinase 1 and 2 (ERK1/2), were investigated. Acute administration of GHB (500 mg/kg, intraperitoneal) induced a fast and long lasting inhibition of MAP kinase phosphorylation in both frontal cortex and hippocampus. The reduced MAP kinase phosphorylation was observed in the CA1 and CA3 areas but not in the dentate gyrus. Pretreatment with the specific γ-aminobutyric acid, type B ($GABA_B$), receptor antagonist CGP56999A (20 mg/kg, intraperitoneal) prevented the action of GHB, and the effect of GHB was mimicked by baclofen, a selective $GABA_B$ receptor agonist, whereas the high affinity GHB receptor antagonist NCS-382 (200 mg/kg, intraperitoneal) had no effect on GHB-inhibited MAP kinase phosphorylation. Moreover, the GHB dehydrogenase inhibitor valproate (500 mg/kg, intraperitoneal), which inhibits the conversion of GHB into GABA, failed to block the effect of GHB on MAP kinase phosphorylation. Altogether, these data suggest that GHB, administered *in vivo*, reduces MAP kinase phosphorylation via a direct activation of $GABA_B$ receptors by GHB. In contrast, GHB (10 mM for 15 min) was found ineffective on MAP kinase phosphorylation in brain slices, indicating important differences in the conditions required for the second messenger activating action of GHB.

γ-Hydroxybutyrate (GHB)[1] is a natural constituent of the mammalian brain derived from the metabolism of γ-aminobutyric acid (GABA) (1). Peripherally administered GHB can readily cross the blood-brain barrier and produces significant behavioral, electrophysiological, and biochemical effects. Clinically, GHB has been used as an anesthetic agent (2), in the treatment of alcohol withdrawal and dependence (3), opiate withdrawal (4), and in sleep disorders (5). Recently GHB has emerged as a major recreational drug of abuse, and its abuse or intoxication has been associated with a mild euphoria, respiratory depression, headache, vomiting, agitation, seizures, and even death (6–8).

GHB has been suggested to play a role as a neurotransmitter or neuromodulator in brain (9–11) and was shown to modulate neuronal excitability and the release of some neurotransmitters in the different brain regions (12–16). Although the precise mechanisms underlying GHB actions have not been established, accumulating evidence supports that GHB acts via $GABA_B$ receptors. For instance, the sedative/hypnotic effect of GHB is entirely mediated by the stimulation of $GABA_B$ receptors (17), and GHB can increase the concentrations of neurosteroids in the brain via a $GABA_B$ receptor-mediated mechanism (18). Despite these findings, little is known about intracellular signaling pathways possibly regulated by GHB in the central nervous system.

The extracellular signal-regulated protein kinase (ERK, also known as mitogen-activated protein kinase, MAP kinase) pathway converts extracellular stimuli at many cell surface receptors including G-protein-coupled receptors into intracellular signals controlling nuclear events and thus plays a crucial role in various physiological and pathological processes (19, 20). Recent studies have implicated the ERK pathway in the mechanisms underlying the actions of many types of substances of abuse (21, 22) including opioid (23), Δ9-tetrahydrocannabinol (24), amphetamine (25, 26), cocaine (26), phencyclidine (27), nicotine (28), and ethanol (29). Interestingly, $GABA_B$ receptor agonists such as baclofen have been shown to promote abstinence and to reduce the use of cocaine, heroin, alcohol, and nicotine (7, 30). These findings, together with the use of GHB in treating alcohol dependence and opioid withdrawal (3, 4, 9), suggest a possible interaction between the intracellular signaling cascades induced by $GABA_B$ receptor activation and some psychoactive drugs. In a previous study, we have demonstrated that GHB reduces neuronal excitability and synaptic activity in neocortical and hippocampal neurons via $GABA_B$ receptor activation (31). In the present study, we have examined the actions of GHB on ERK1 and -2 (ERK1/2) following acute GHB administration to mice at a high dose (500 mg/kg). We present the first evidence for GHB significantly reducing MAP kinase phosphorylation in mouse cortex and hippocampus specifically via the activation of $GABA_B$ receptors.

* This work was supported by National Institutes of Health Grant DA14947 (to I. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GHB, γ-hydroxybutyrate; GABA, γ-aminobutyric acid; $GABA_B$ receptors, GABA type B receptors; MAP kinase, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; aCSF, artificial cerebrospinal fluid; DG, dentate gyrus; HP, hydrogen peroxide; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MKP, MAP kinase phosphatase.

EXPERIMENTAL PROCEDURES

In Vivo Treatment and Slice Preparation—C57Black6 mice, purchased from Harlan Sprague-Dawley (Indianapolis, IN), were maintained in the UCLA Division of Laboratory Animal Medicine (DLAM) vivarium facilities on a 12-h light/12-h dark cycle. The mice had free access to food and water. Six- to 10-week-old mice were used in the present experiments. All our experiments with mice were approved by the UCLA Chancellor's Animal Research Committee in accordance with National Institutes of Health guidelines.

The dose of GHB (purchased from Sigma), 500 mg/kg (intraperitoneally), was chosen based on previous reports (11, 17, 18) and data from GHB abuse in humans. For abuse purposes, subjects can consume 10s of grams of GHB, which would give rise to millimolar concentrations in the volume of distribution for GHB (32). The doses of (±)-baclofen (purchased from Sigma), 20 mg/kg (intraperitoneal), the highly specific $GABA_B$ receptor antagonist CGP56999A (kindly provided by Dr. Wolfgang Froestl, Novartis, Basel, Switzerland), 20 mg/kg (intraperitoneal), the GHB receptor antagonist NCS-382 (purchased from Sigma), 200 mg/kg (intraperitoneal), and GABA dehydrogenase inhibitor valproate (purchased from Sigma), 500 mg/kg (intraperitoneal), were chosen based on previous reports (9, 11, 17, 18) and on our preliminary experiments. CGP56999A, NCS-382, and valproate were administered (intraperitoneal) 10 min prior to GHB administration. All drugs used for *in vivo* injections were dissolved in sterile saline. At various times following the injections, the mice were anesthetized by halothane and subsequently killed by decapitation. The brains were rapidly dissected, frozen on dry ice, and kept at −80 °C until use for Western blot analysis.

For slice preparation, the brain was prepared as above and placed into a cold artificial cerebrospinal fluid (aCSF) containing (mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 D-glucose, pH 7.3, while bubbled with 95% O_2 and 5% CO_2. The brain was glued to a platform, and 600-μm-thick (for hippocampal subfield dissections after *in vivo* drug administrations) or 350-μm-thick coronal slices (for the *in vitro* drug experiments) were cut with a Leica VT1000S vibratome. The slices were incubated for 1 h at 32 °C in bubbled aCSF before further treatment with 10 mM GHB for 15 min (31) or 10 mM hydrogen peroxide (H_2O_2) for 10 min, which was used as a positive control (33). The slices were then microdissected into the respective regions under a microscope and/or directly frozen by putting on dry ice and finally kept at −80 °C for protein analysis.

Western Blot Analysis—The frontal cortex and hippocampus, or the subregions of the hippocampus (CA1, CA3, and dentate gyrus), were homogenized at 4 °C in a lysis buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml pepstatin A. The homogenate was centrifuged at 12,000 × *g* for 25 min, and the supernatant was used for the measurement of protein concentration and phosphorylation analysis. The protein concentration was determined with a DC Protein Assay kit (Bio-Rad).

For phosphorylation analysis of MAP kinases, 5–10 μg of protein was boiled in the 2× sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 12% β-mercaptoethanol, 0.004% bromphenol blue), applied onto a 10% polyacrylamide gel, subsequently transferred to a nitrocellulose membrane (Osmonics Inc.), and blocked with 5% milk in TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). Membrane was incubated with anti-phospho-ERK1/2 (1:1000 dilution; Cell Signaling Technology, New England Biolabs) overnight at 4 °C. After incubation with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Vector Laboratories), the immune complex was detected by ECL (Amersham Biosciences) and exposed to x-ray film (Eastman Kodak Co.). The film was scanned, and the band intensity was analyzed by the NIH Image J software. For the visualization of total MAP kinases, the membrane was stripped with a stripping buffer (0.2 M glycine, pH 2.2, 0.1% SDS, 0.1% Tween 20) at 37 °C for 30 min, re-labeled with the primary antibody against total ERK1/2 (1:1000 dilution; Cell Signaling Technology, New England Biolabs), and detected as described above. In some cases, the membrane was stripped again and labeled with the primary anti-β-actin antibody (Sigma).

Data Analysis—Paired and unpaired *t* tests were performed. Data are expressed as means ± S.E., with *n* indicating the number of animals or slices. Significance level was set at $p < 0.05$ (two-tailed test).

RESULTS

GHB Reduces MAP Kinase Phosphorylation in Frontal Cortex and Hippocampus—Acute administration of a high dose of GHB in rodents has been shown to induce marked decreases in locomotor activity and body temperature (34–36). We have observed similar behavioral effects of GHB in the mice used in our current experiments (data not shown), but our study focused on the effects of GHB (500 mg/kg) on the regulation of MAP kinase activation by using Western blot analysis. All of our measurements are expressed as the ratio (*p*/*t*) of phospho-MAP kinase (*p*) to total MAP kinase (*t*). No difference in the total MAP kinase levels was detected in mouse cortex or hippocampus at different times following GHB administration (as compared with β-actin, data not shown).

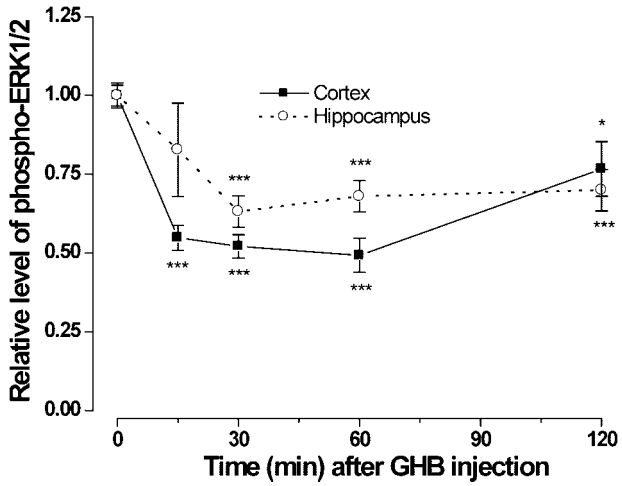

FIG. 1. **Acute GHB administration significantly reduces MAP kinase phosphorylation in mouse frontal cortex and hippocampus.** Measurements of normalized phospho-ERK1/2 levels were done at the indicated times (15, 30, 60, and 120 min) following an acute injection of GHB (500 mg/kg, intraperitoneal). Control mice were injected with saline, and tissue was collected at 30, 60, and 120 min, respectively, after the injection. Because there was no difference between these time points in controls, all control data were combined (designated as 0 min after GHB injection). * and ***, $p < 0.05$ or 0.001 *versus* control, respectively ($n = 5$–7 for each GHB treatment group and $n = 16$ for control).

First, we determined the time course of ERK1/2 activation (phosphorylation) after an acute intraperitoneal injection of GHB (500 mg/kg body weight). Control mice were injected with saline, and their brains were prepared at 30, 60, and 120 min, respectively, following the injection. As there was no difference in the level of ERK1/2 phosphorylation between the control mice at the different time points, all control data were pooled. As shown in Fig. 1, a significantly reduced MAP kinase phosphorylation was observed in both frontal cortex and hippocampus ($n = 5$–7 for each time point of GHB treatment, and a total $n = 16$ for control). A significant effect of GHB on the MAP kinase phosphorylation in frontal cortex was detected as early as 15 min following GHB injection. Thirty minutes following GHB administration, the phosphorylation levels of MAP kinases were reduced to 52 ± 4% of control in frontal cortex ($n = 7$) and to 65 ± 6% of control in hippocampus ($n = 7$). We were able to observe a significant down-regulation of MAP kinase phosphorylation in frontal cortex and hippocampus (to 77 ± 9 and 67 ± 6% of control, respectively, $n = 5$–7) lasting for at least 2 h following GHB injection (Fig. 1).

Possible regional differences in the GHB-dependent regulation of MAP kinase phosphorylation specific to various cortical and hippocampal subfields were identified by microdissecting the 600-μm-thick slices in ice-cold solution into the respective brain regions. The brain regions, including somatosensory cortex and CA1, CA3, and dentate gyrus (DG) of hippocampus, were then subjected to Western blot analysis. As shown in Fig. 2, GHB-induced down-regulation of MAP kinase phosphoryla-

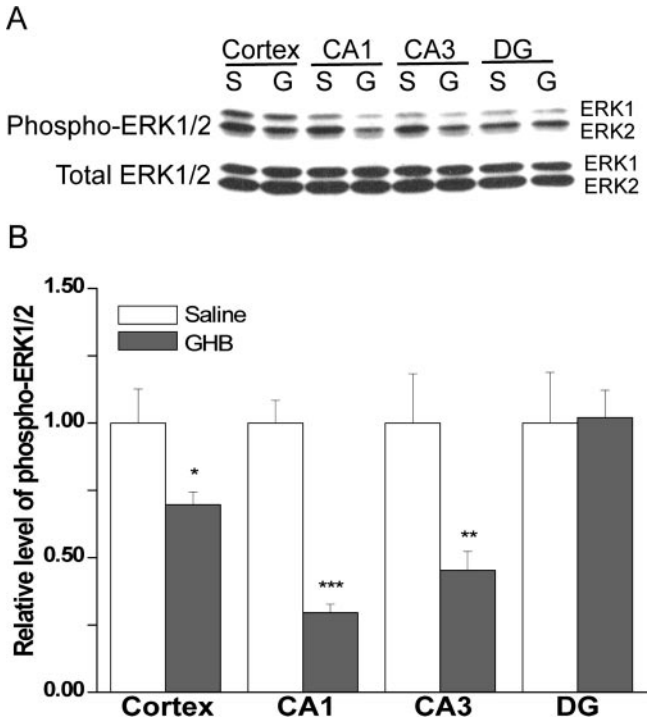

FIG. 2. **GHB-induced decrease of MAP kinase phosphorylation in areas CA1 and CA3 of hippocampus.** *A*, representative Western blot detected with anti-phospho-ERK1/2 or anti-total ERK1/2 antibody. *B*, normalized phospho-ERK1/2 levels were measured in tissue obtained 30 min following the injection of GHB (500 mg/kg, intraperitoneal). A clear reduction of MAP kinase phosphorylation was observed in somatosensory cortex and in CA1 or CA3 but not in DG of hippocampus. *, **, and ***, $p < 0.05$, 0.01, or 0.001 *versus* saline, respectively ($n = 4–8$; *S*, saline; *G*, GHB).

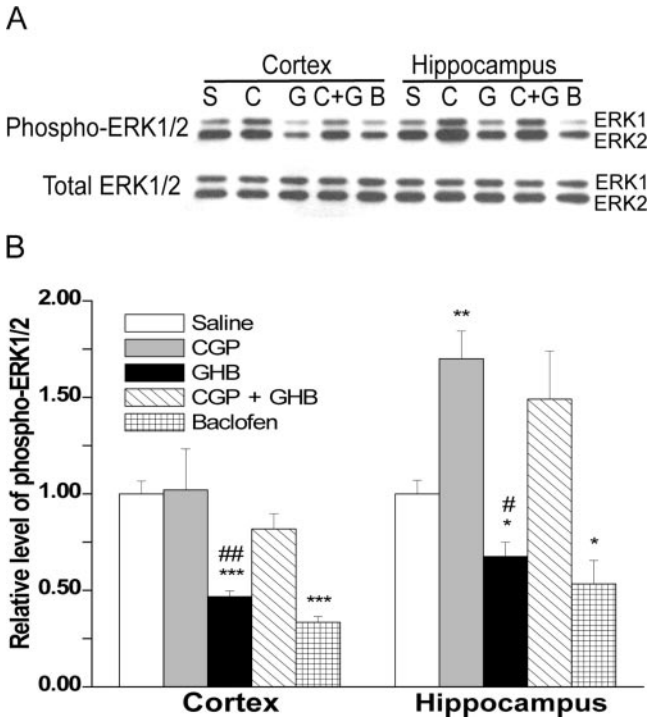

FIG. 3. **The specific $GABA_B$ receptor antagonist CGP56999A reverses and the $GABA_B$ receptor agonist baclofen mimics the effect of GHB on MAP kinase phosphorylation.** *A*, representative Western blot detected with anti-phospho-ERK1/2 or anti-total ERK1/2 antibody. *B*, normalized phospho-ERK1/2 levels. CGP56999A (20 mg/kg, intraperitoneal) was given 10 min prior to GHB injection. The tissue was collected 30 min following one injection of GHB (500 mg/kg, intraperitoneal) or baclofen (20 mg/kg, intraperitoneal). The cortices and hippocampi of brains from the mice subjected to various treatments were then dissected out for Western blot analysis. *, **, and ***, $p < 0.05$, 0.01, or 0.001 *versus* saline; # and ##, $p < 0.05$ or 0.01 *versus* C+G, group, respectively ($n = 5$; *S*, saline; *C*, CGP56999A; *G*, GHB; *C+G*, CGP56999A plus GHB; *B*, baclofen).

tion was detected only in CA1 (to 30 ± 3% of control, $n = 6–8$) and CA3 (to 45 ± 7% of control, $n = 6–8$) but not in DG (to 102 ± 10% of the control, $n = 4–6$). A clear GHB-induced decrease (to 70 ± 5% of control, $n = 4–7$) in MAP kinase phosphorylation was also detected in the somatosensory cortex.

The Effect of GHB on MAP Kinase Phosphorylation Is Not Mediated through the High Affinity GHB Receptor—Because certain effects of GHB have been proposed to occur via high affinity GHB receptors (9, 37, 38), we addressed this point by using the specific GHB receptor antagonist NCS-382 in a separate set of experiments. We found that the phosphorylation levels of MAP kinases in frontal cortex and hippocampus of animals treated with GHB (500 mg/kg for 30 min) were 57 ± 8% of control and 60 ± 21% of control, respectively, whereas the phosphorylation levels of MAP kinases in frontal cortex and hippocampus of animals pre-treated with NCS-382 (200 mg/kg) were 50 ± 8% of control and 50 ± 13% of control ($n = 5–7$). There is no statistically significant difference between the two groups. By itself, NCS-382 (200 mg/kg) did not significantly affect the phosphorylation of MAP kinase in the frontal cortex (85 ± 15% of control) or hippocampus (82 ± 10% of control; $n = 5$). Because pretreatment with NCS-382 (200 mg/kg) failed to reverse GHB-induced inhibition of MAP kinase phosphorylation, the proposed high affinity GHB receptors appear to play no role in mediating the GHB-induced inhibition of MAP kinase phosphorylation.

GHB Affects MAP Kinase Phosphorylation by Acting $GABA_B$ Receptors—We next examined the role of $GABA_B$ receptors in the GHB-induced inhibition of MAP kinase phosphorylation. As shown in Fig. 3, a pretreatment with the specific $GABA_B$ receptor antagonist CGP56999A (20 mg/kg) administered 10 min before GHB prevented the inhibitory effect of GHB (500 mg/kg) on MAP kinase phosphorylation both in cortex and hippocampus, demonstrating that $GABA_B$ receptors are involved in diminishing MAP kinase phosphorylation by GHB. In addition, as shown in Fig. 3, we also found that CGP56999A alone, at the dose used (20 mg/kg, intraperitoneal), significantly up-regulated the phosphorylation of MAP kinases in the hippocampus (170 ± 14.4% of control, $n = 5$) but not in the cortex (102 ± 17% of control, $n = 5$).

We also used baclofen, a well known selective $GABA_B$ receptor agonist, as a positive control for $GABA_B$ receptor activation. Similar to GHB, baclofen (20 mg/kg, intraperitoneal) significantly decreased the phosphorylation of MAP kinases in the cortex (to 33 ± 3% of control, $n = 5$) and hippocampus (to 53 ± 12% of control, $n = 5$), further confirming the role of activation of $GABA_B$ receptors in MAP kinase signaling (Fig. 3).

GABA Is Not an Intermediate in the GHB-induced Inhibition of MAP Kinase Phosphorylation—We sought to determine whether the inhibitory effect of GHB on MAP kinase phosphorylation was due to the activation of $GABA_B$ receptors by GABA that was converted from the exogenously administered GHB. We used valproate, a compound known to inhibit GHB dehydrogenase, hence preventing the conversion of GHB into GABA (9, 39). The phosphorylation levels in GHB-treated animals were 57 ± 8% of control and 60 ± 21% of control in frontal cortex and hippocampus, respectively ($n = 5–7$), whereas the phosphorylation levels in valproate plus GHB-treated animals were 40 ± 9% of control and 48 ± 29% of control in frontal cortex and hippocampus, respectively ($n = 5–7$), indicating that blocking the conversion of GHB to GABA by valproate failed to

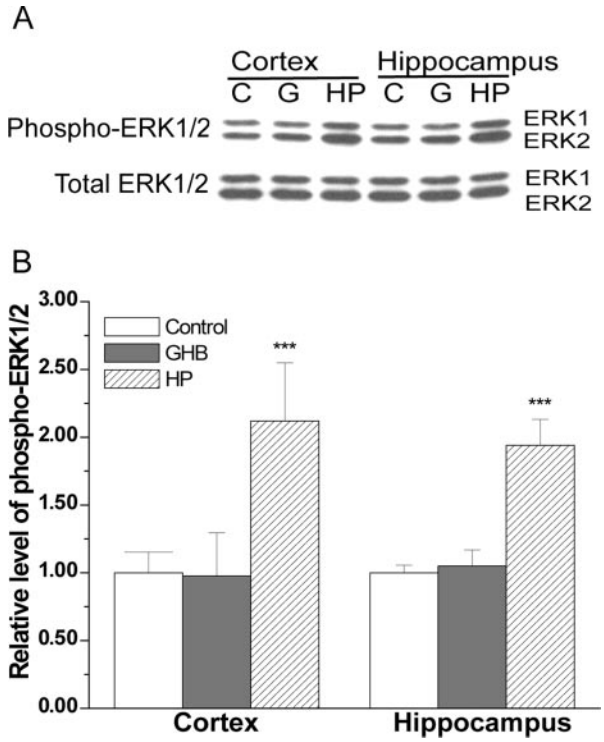

FIG. 4. **GHB has no effect on the MAP kinase phosphorylation in brain slices.** *A*, representative Western blot detected with anti-phospho-ERK1/2 or anti-total ERK1/2 antibody. *B*, normalized phospho-ERK1/2 levels. 350-μm-thick coronal brain slices were removed after decapitation and placed into an ice-cold aCSF bubbled with 95% O_2 and 5% CO_2. The slices were incubated for 1 h at 32 °C in oxygenated aCSF before further treatment with 10 mM GHB for 15 min or 10 mM hydrogen peroxide (H_2O_2) for 10 min (33). ***, $p < 0.001$ *versus* control (n = 5–7 for cortex and 8–19 for hippocampus. *S*, saline; *G*, GHB; *HP*, hydrogen peroxide).

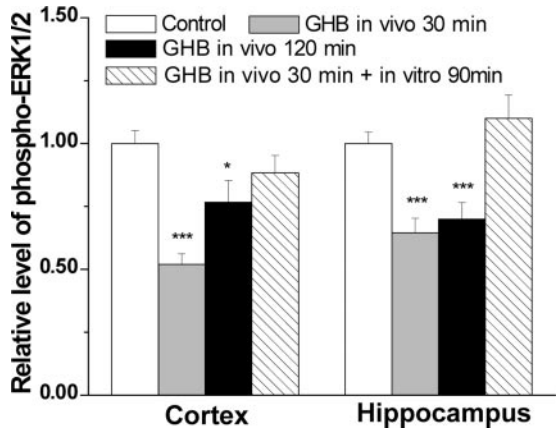

FIG. 5. **A comparison of GHB effects on MAP kinase phosphorylations between *in vivo* and *in vitro* conditions.** MAP kinase phosphorylation was significantly inhibited in frontal cortex and hippocampus *in vivo* 30 min following an acute administration of GHB, and this effect of GHB was still observed *in vivo* 120 min after GHB injection. A 90-min *in vitro* incubation of the slices from the mice acutely administrated with GHB (500 mg/kg for 30 min) in aCSF led to the recovery of phospho-MAP kinase to control levels. The brains were directly used for Western blot analysis or cut into 350-μm-thick coronal slices, which were further incubated for 90 min at 32 °C in aCSF before they were used for Western blot analysis. The third group of mice were injected GHB (500 mg/kg, intraperitoneal), and 120 min later, the brains were removed and dissected out for Western blot analysis. * and ***, $p < 0.05$ or 0.001 *versus* control, respectively (n = 5–7).

prevent GHB-reduced MAP kinase phosphorylation. Thus, GHB-induced inhibition of MAP kinase phosphorylation most likely results from the direct activation of $GABA_B$ receptors by GHB. In addition, a significant inhibitory effect of valproate (n = 5) on MAP kinase phosphorylation was observed in the frontal cortex (52 ± 7% of control) and hippocampus (62 ± 13% of control).

GHB Is Ineffective in Brain Slices—We finally compared the effects of GHB administration *in vivo* and *in vitro* (brain slices), because we have shown previously in brain slices that GHB activates $GABA_B$ receptors in neocortical and hippocampal neurons (31). Although the inhibitory effect of GHB on MAP kinase phosphorylation was remarkable *in vivo* (Figs. 1–3), GHB (10 mM for 15 min) caused no obvious change in MAP kinase phosphorylation in cortical and hippocampal slices (Fig. 4). To address whether MAP kinases could be phosphorylated in brain slices, we also included hydrogen peroxide as a positive control. We confirmed a previous report (33) that this compound induced a significant increase in MAP kinase phosphorylation in brain slices.

We also wanted to find out whether the altered phosphorylation of MAP kinase could be maintained *in vitro*. The effect of GHB on MAP kinase phosphorylation *in vivo* was long lasting, as we were able to detect a significant down-regulation of MAP kinase phosphorylation in cortex and hippocampus (77 ± 9 and 67 ± 6% of control, respectively, n = 5–7) even 2 h following GHB injection (Fig. 1 and Fig. 5). In contrast to our *in vivo* findings, *in vitro* incubation in aCSF of brain slices prepared 30 min after animals were acutely treated with GHB (500 mg/kg) resulted in the recovery of the MAP kinase phosphorylation to control levels (Fig. 5). In addition, we also examined whether slice cutting procedure itself would affect MAP kinase phosphorylation. One whole brain was cut into two equal halves; half was immediately put on dry ice, and the other half was cut into slices in cold aCSF. No differences were observed in the level of MAP kinase phosphorylation between the intact and sliced brain (data not shown).

DISCUSSION

Acute GHB administration in humans or animals induces profound physiological effects. Previously we have demonstrated that GHB, at millimolar concentration *in vitro*, reduces neuronal excitability and synaptic activity in both neocortical and hippocampal neurons mediated via $GABA_B$ receptors (31). In our current experiments, we chose a high of dose of GHB (500 mg/kg), because the concentration of GHB in cerebrospinal fluid or brain in animals and in humans, after an exogenous administration at this dose, reached millimolar levels (14, 40–42), compatible with the affinity of $GABA_B$ receptors for GHB (43). Acute administration of GHB (500 mg/kg) *in vivo* caused a rapid and long lasting inhibition on MAP kinase phosphorylation in both frontal cortex and hippocampus (Fig. 1).

The mechanisms whereby GHB exerts its effects in the central nervous system are still poorly understood. In general, two modes of GHB action have been proposed: through $GABA_B$ receptors or via high affinity GHB receptors that have been suggested to exist in the brain (9, 37, 38). According to binding studies, some actions of GHB may be mediated via highly specific membrane-binding sites (9) that appear to have a different regional distribution than $GABA_B$-binding sites in rodent brain (37). More recently the high affinity GHB receptor has been suggested to be a member of the G-protein-coupled receptor family (38). The overwhelming evidence favors $GABA_B$-receptor-mediated actions of GHB, as many effects of GHB on the central nervous system show striking similarities to the effects produced by the selective $GABA_B$-receptor agonist baclofen and can be attenuated by specific $GABA_B$-receptor antagonists (11–13, 15, 17, 18, 44–47). Clearly, the binding characteristics, reported by Bernasconi *et al.* (1) and Mathivet

et al. (47), also strongly support GHB as a selective $GABA_B$ receptor agonist.

To address whether the high affinity GHB receptors or $GABA_B$ receptors mediate the GHB-induced inhibition of MAP kinase phosphorylation in our experiments, we tested the effects of NCS-382, a specific GHB receptor antagonist shown to reverse some of the effects of GHB (9, 38). Even at the extremely high dose of NCS-382 (200 mg/kg) used in our experiments, NCS-382 was ineffective in reversing the suppressant effect of GHB on MAP kinase phosphorylation. Therefore, the high affinity GHB receptors do not appear to mediate this action of GHB. In contrast, CGP56999A, a specific $GABA_B$ receptor antagonist, completely antagonized the inhibitory effect of GHB on MAP kinase phosphorylation. The antagonism of GHB action by CGP56999A strongly implicates a role of $GABA_B$ receptors in mediating the GHB-induced inhibition of MAP kinase phosphorylation. This was further confirmed by the selective $GABA_B$ receptor agonist baclofen mimicking the effect of GHB on MAP kinase phosphorylation (Fig. 3). Interestingly, the treatment with CGP56999A alone increased MAP kinase phosphorylation in the hippocampus. As $GABA_B$ receptor activation by spontaneous GABA release has been detected in the hippocampus (48), the activation of $GABA_B$ receptors by ambient GABA levels appears to be sufficient to produce an inhibitory effect on the phosphorylation of MAP kinases in the hippocampus. The $GABA_B$ receptor antagonist CGP56999A may have blocked the tonic activation of $GABA_B$ receptors by GABA, consequently increasing MAP kinase phosphorylation. Interestingly, CGP56999A alone caused no up-regulation of MAP kinase phosphorylation in the frontal cortex. This could be due to differences in the two brain regions regarding the levels of ambient GABA or nature of the second messenger systems linked to the tonically activated $GABA_B$ receptors.

We then asked whether the GHB-induced decrease in MAP kinase phosphorylation was due to an indirect activation of $GABA_B$ receptors by GABA converted from the high concentration of exogenously administered GHB through the combined actions of GHB dehydrogenase and GABA transaminase. We used the short chain fatty acid valproate known to inhibit the cytosolic GHB dehydrogenase, thus preventing GHB degradation into GABA (9, 39). As valproate failed to block the GHB-induced decrease in MAP kinase phosphorylation, this effect of GHB is due to the direct activation of $GABA_B$ receptors by GHB and not by GHB-derived GABA acting at $GABA_B$ receptors. In addition, valproate *per se* also inhibited MAP kinase phosphorylation in both frontal cortex and hippocampus. Considering that acute valproate administration (200 mg/kg-600 mg/kg) induces significant increases in brain GHB levels (9, 49), it is very likely that the elevated GHB concentration following valproate administration is responsible for suppressing MAP kinase phosphorylation. However, the possible effects of valproate on other signaling mechanisms cannot be ruled out. Our findings with valproate are inconsistent with the recent report by Yuan *et al.* (50), in which valproate was found to increase MAP kinase phosphorylation in a cultured cell line (human neuroblastoma SH-SY5Y).

Our experiments strongly support an acute reduction in MAP kinase phosphorylation by GHB acting via direct activation of $GABA_B$ receptors in mouse frontal cortex and hippocampus. This finding has important implications for the role of $GABA_B$ receptors and of MAP kinase signaling pathways in the physiological function and regulation. GHB levels in the cerebrospinal fluid remain consistently high (millimolar range) for more than 4 h in mammals after one acute GHB (500 mg/kg) administration (40–42); consequently, even one administration of a high dose of GHB, as often is the case during the abuse in humans, might cause lasting influences in the central nervous system. The GHB-induced down-regulation of MAP kinase phosphorylation, mediated via $GABA_B$ receptors, may represent an important cellular mechanism underlying the effects of GHB on the central nervous system.

The specific mechanism of MAP kinase phosphorylation suppression by an acute GHB administration remains an open question. The most parsimonious explanation is a decrease in cAMP levels and, consequently, in cAMP-dependent protein kinase A (PKA) activity. The stimulation of $GABA_B$ receptors has been linked to the inhibition of adenylyl cyclase, thus decreasing cAMP levels (51), and it is well documented that the cAMP level, through its effect on the PKA, correlates with the activation (phosphorylation) of MAP kinase (52). Another possibility may be a change in protein kinase C (PKC) activity, resulting from the activation of $GABA_B$ receptors leading to the inhibition of voltage-gated Ca^{2+} channels (51). In support of a role of PKA and/or PKC in the GHB-induced inhibition of MAP kinase phosphorylation, the $GABA_B$ receptor agonist baclofen has been shown to reduce a forskolin-stimulated increase in cAMP both *in vivo* and *in vitro* (51), whereas baclofen and GABA have been shown to modulate PKC activity (53). In addition, GHB has been also reported to modulate adenylyl cyclase activity and intracellular Ca^{2+} concentrations (38, 54). The involvement of other protein kinase pathways in the negative regulation of MAP kinase signaling cascade cannot be excluded. For example, several recent studies have identified Akt (also termed protein kinase B), a main downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway, as a potent negative regulator of MAP kinase pathway (55, 56), and PI3K has been shown to mediate certain G-protein-coupled receptor activations of the MAP kinase signaling pathway (57), probably through the regulation of PI3K catalytic activity by the α and/or $\beta\gamma$ subunits of heterotrimeric G-proteins, Ras and calmodulin (58, 59). Calmodulin can also directly modulate the ERK pathway (52, 60).

Recently, much attention has been devoted to the role of the family of dual specificity MAP kinase phosphatases (MKPs), of which MKP-3 is selective for the inactivation of ERKs (61). A significant up-regulation in its expression level in rat hippocampus has been detected after acute amphetamine (62). However, by using a specific antibody against MKP-3 (C-20) (sc-8599, Santa Cruz Biotechnology), we found that the expression level of MKP-3 not to be significantly affected in frontal cortex and hippocampus of mice subjected to acute GHB injection (the expression levels of MKP-3 in frontal cortex and hippocampus of mice 30 min following GHB injection were 91 ± 12 and 109 ± 10%, respectively, $n = 3$). Therefore, it appears unlikely that the reduced MAP kinase phosphorylation is due to increased activation of MAP kinase phosphatases like MKP-3. The GHB-induced inhibition in MAP kinase phosphorylation more likely represents the $GABA_B$ receptor-mediated modulation of MAP kinase (ERK1/2) by different signaling cascades. The exact interactions between the different signaling pathways will require further clarification.

In contrast to the GHB-induced inhibition of MAP kinases after *in vivo* GHB administration, our experiments with brain slices showed that GHB (10 mM for 15 min) did not alter the phosphorylation level of MAP kinases in slices. Although injury itself was reported to strongly activate MAP kinases (63), our careful dissections (hippocampi were kept intact) and the immediate freezing of brain tissues on dry ice make injury less likely a factor affecting the MAP kinase phosphorylation in these tissues, especially hippocampus. Another intriguing observation of our study is that after a 90-min *in vitro* incubation, the phosphorylation level of MAP kinases in slices from the

brains of animals acutely treated with GHB (500 mg/kg for 30 min) recovered to control levels (Fig. 5), whereas a significantly reduced MAP kinase phosphorylation was still observed even after 120 min following one GHB injection (500 mg/kg) *in vivo* (Fig. 1 and Fig. 5). One explanation for this could be that GHB levels *in vivo* remained consistently elevated causing a persistent inactivation of MAP kinases, whereas the *in vitro* recovery of MAP kinase phosphorylation level was due to the incubation of brain slices without GHB stimulation. However, this seems unlikely because GHB (10 mM for 15 min) did not alter the phosphorylation level of MAP kinases in cortical and hippocampal slices. The exact reasons for the discrepancy between the *in vivo* and *in vitro* data remain to be further investigated. In contrast to our findings, a recent report (64) showed that the $GABA_B$ receptor agonist baclofen increased MAP kinase (ERK2) phosphorylation in the CA1 area of hippocampal slices. The inconsistencies between these results may probably be due to the experimental situations such as incubation temperature (these authors used an incubation temperature of 26–28 °C). However, it is also possible that GHB may exert some effects distinct from those induced by baclofen.

In summary, our findings represent the first demonstration that GHB markedly inhibits MAP kinase activation (phosphorylation) via a $GABA_B$ receptor-mediated mechanism. These findings have revealed a novel action of GHB and of $GABA_B$ receptors through ERK MAP kinases in intracellular signaling cascades and thus provide new insights into the intracellular mechanisms underlying the various effects of GHB on the central nervous system (31, 65). Because MAP kinases have been found to play a crucial role in mediating a number of physiological and pathological changes in cell function (19, 20), the down-regulation of this pathway may be relevant to the pathological changes during acute GHB intoxication. Given the role of MAP kinases in the induction of long term functional changes, the neuroadaptative changes (tolerance and dependence) following repeated uses of GHB in rodents and humans (6–8) may involve this mechanism of action. Our present findings may also open novel means for the treatment of acute GHB intoxication by modulating the ERK-signaling pathway. It will be important to elucidate the precise mechanisms whereby GHB inhibits MAP kinase phosphorylation and the possible correlation between the deactivation of MAP kinases specific to certain brain regions and the behavioral effects associated with acute and repeated GHB administrations.

Acknowledgments—We thank Dr. W. Froestl for kindly providing CGP56999A. We are also grateful to Yijun Cui, Nils Ole Dalby, Brian Oyama, Mahsan Rafizadeh, and Weizheng Wei for assistance and help during experiments.

REFERENCES

1. Bernasconi, R., Mathivet, P., Bischoff, S., and Marescaux, C. (1999) *Trends Pharmacol. Sci.* **20,** 135–141
2. Kleinschmidt, S., Schellhase, C., and Mertzlufft, F. (1999) *Eur. J. Anaesthesiol.* **16,** 23–30
3. Gallimberti, L., Canton, G., Gentile, N., Ferri, M., Cibin, M., Ferrara, S. D., Fadda, F., and Gessa, G. L. (1989) *Lancet* **2,** 787–789
4. Rosen, M. I., Pearsall, H. R., Woods, S. W., and Kosten, T. R. (1996) *Neuropsychopharmacology* **14,** 187–193
5. Mamelak, M., Scharf, M. B., and Woods, M. (1986) *Sleep* (*Stuttg.*) **9,** 285–289
6. Timby, N., Eriksson, A., and Bostrom, K. (2000) *Am. J. Med.* **108,** 518–519
7. Okun, M. S., Boothby, L. A., Bartfield, R. B., and Doering, P. L. (2001) *J. Pharmacol. Sci.* **4,** 167–175
8. Nicholson, K. L., and Balster, R. L. (2001) *Drug Alcohol Depend.* **63,** 1–22
9. Maitre, M. (1997) *Prog. Neurobiol.* **51,** 337–361
10. Cash, C. D., Gobaille, S., Kemmel, V., Andriamampandry, C., and Maitre, M. (1999) *Biochem. Pharmacol.* **58,** 1815–1819
11. Nava, F., Carta, G., Bortolato, M., and Gessa, G. L. (2001) *Eur. J. Pharmacol.* **430,** 261–263
12. Xie, X., and Smart, T. G. (1992) *Eur. J. Pharmacol.* **223,** 193–196
13. Xie, X., and Smart, T. G. (1992) *Eur. J. Pharmacol.* **212,** 291–294
14. Gobaille, S., Hechler, V., Andriamampandry, C., Kemmel, V., and Maitre, M. (1999) *J. Pharmacol. Exp. Ther.* **290,** 303–309
15. Hu, R. Q., Banerjee, P. K., and Snead, O. C., III (2000) *Neuropharmacology* **39,** 427–439
16. Ferraro, L., Tanganelli, S., O'Connor, W. T., Francesconi, W., Loche, A., Gessa, G. L., and Antonelli, T. (2001) *J. Neurochem.* **78,** 929–939
17. Carai, M. A., Colombo, G., Brunetti, G., Melis, S., Serra, S., Vacca, G., Mastinu, S., Pistuddi, A. M., Solinas, C., Cignarella, G., Minardi, G., and Gessa, G. L. (2001) *Eur. J. Pharmacol.* **428,** 315–321
18. Barbaccia, M. L., Colombo, G., Affricano, D., Carai, M. A., Vacca, G., Melis, S., Purdy, R. H., and Gessa, G. L. (2002) *Neuropharmacology* **42,** 782–791
19. Sweatt, J. D. (2001) *J. Neurochem.* **76,** 1–10
20. Johnson, G. L., and Lapadat, R. (2002) *Science* **298,** 1911–1912
21. Nestler, E. J. (2002) *Nat. Neurosci.* **5,** (suppl.) 1076–1079
22. Koob, G. F., Sanna, P. P., and Bloom, F. E. (1998) *Neuron* **21,** 467–476
23. Schulz, S., and Höllt, V. (1998) *Eur. J. Neurosci.* **10,** 1196–1201
24. Derkinderen, P., Valjent, E., Toutant, M., Corvol, J. C., Enslen, H., Ledent, C., Trzaskos, J., Caboche, J., and Girault, J. A. (2003) *J. Neurosci.* **23,** 2371–2382
25. Choe, E. S., Chung, K. T., Mao, L., and Wang, J. Q. (2002) *Neuropsychopharmacology* **27,** 565–575
26. Licata, S. C., and Pierce, R. C. (2003) *J. Neurochem.* **85,** 14–22
27. Kyosseva, S. V., Owens, S. M., Elbein, A. D., and Karson, C. N. (2001) *Neuropsychopharmacology* **24,** 267–277
28. Brunzell, D. H., Russell, D. S., and Picciotto, M. R. (2003) *J. Neurochem.* **84,** 1431–1441
29. Kalluri, H. S., and Ticku, M. K. (2002) *Eur. J. Pharmacol.* **439,** 53–58
30. Cousins, M. S., Roberts, D. C., and de Wit, H. (2002) *Drug Alcohol Depend.* **65,** 209–220
31. Jensen, K., and Mody, I. (2001) *Cereb. Cortex* **11,** 424–429
32. Galloway, G. P., Frederick, S. L., Staggers, F. E., Jr., Gonzales, M., Stalcup, S. A., and Smith, D. E. (1997) *Addiction* **92,** 89–96
33. Kanterewicz, B. I., Knapp, L. T., and Klann, E. (1998) *J. Neurochem.* **70,** 1009–1016
34. Kaufman, E. E., Porrino, L. J., and Nelson, T. (1990) *Biochem. Pharmacol.* **40,** 2637–2640
35. Nissbrandt, H., and Engberg, G. (1996) *J. Neural. Transm.* **103,** 1255–1263
36. Cook, C. D., Aceto, M. D., Coop, A., and Beardsley, P. M. (2002) *Psychopharmacology* **160,** 99–106
37. Snead, O. C., III (1994) *Brain Res.* **659,** 147–156
38. Snead, O. C., III (2000) *J. Neurochem.* **75,** 1986–1996
39. Hechler, V., Ratomponirina, C., and Maitre, M. (1997) *J. Pharmacol. Exp. Ther.* **281,** 753–760
40. Snead, O. C., III, Yu, P. K., and Huttenlocher, P. R. (1976) *Neurology* **26,** 51–56
41. Snead, O. C., III (1978) *Neurology* **28,** 636–642
42. Shumate, J. S., and Snead, O. C., III (1979) *Res. Commun. Chem. Pathol. Pharmacol.* **25,** 241–256
43. Berton, F., Brancucci, A., Beghe, F., Cammalleri, M., Demuro, A., Francesconi, W., and Gessa, G. L. (1999) *Eur. J. Pharmacol.* **380,** 109–116
44. Engberg, G., and Nissbrandt, H. (1993) *Naunyn-Schmiedebergs Arch. Pharmacol.* **348,** 491–497
45. Madden, T. E., and Johnson, S. W. (1998) *J. Pharmacol. Exp. Ther.* **287,** 261–265
46. Erhardt, S., Andersson, B., Nissbrandt, H., and Engberg, G. (1998) *Naunyn-Schmiedebergs Arch. Pharmacol.* **357,** 611–619
47. Mathivet, P., Bernasconi, R., De Barry, J., Marescaux, C., and Bittiger, H. (1997) *Eur. J. Pharmacol.* **321,** 67–75
48. McLean, H. A., Caillard, O., Khazipov, R., Ben-Ari, Y., and Gaiarsa, J. L. (1996) *J. Neurophysiol.* **76,** 1036–1046
49. Snead, O. C., III, Bearden, L. J., and Pegram, V. (1980) *Neuropharmacology* **19,** 47–52
50. Yuan, P. X., Huang, L. D., Jiang, Y. M., Gutkind, J. S., Manji, H. K., and Chen, G. (2001) *J. Biol. Chem.* **276,** 31674–31683
51. Bowery, N. G., Bettler, B., Froestl, W., Gallagher, J. P., Marshall, F., Raiteri, M., Bonner, T. I., and Enna, S. J. (2002) *Pharmacol. Rev.* **54,** 247–264
52. Grewal, S. S., York, R. D., and Stork, P. J. (1999) *Curr. Opin. Neurobiol.* **9,** 544–553
53. Tremblay, E., Ben-Ari, Y., and Roisin, M. P. (1995) *J. Neurochem.* **65,** 863–870
54. Ito, Y., Ishige, K., Zaitsu, E., Anzai, K., and Fukuda, H. (1995) *J. Neurochem.* **65,** 75–83
55. Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D., and Glass, D. J. (1999) *Science* **286,** 1738–1741
56. Guan, K. L., Figueroa, C., Brtva, T. R., Zhu, T., Taylor, J., Barber, T. D., and Vojtek, A. B. (2000) *J. Biol. Chem.* **275,** 27354–27359
57. Lopez-Ilasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997) *Science* **275,** 394–397
58. Joyal, J. L., Burks, D. J., Pons, S., Matter, W. F., Vlahos, C. J., White, M. F., and Sacks, D. B. (1997) *J. Biol. Chem.* **272,** 28183–28186
59. Wymann, M. P., and Pirola, L. (1998) *Biochim. Biophys. Acta* **1436,** 127–150
60. Agell, N., Bachs, O., Rocamora, N., and Villalonga, P. (2002) *Cell. Signal.* **14,** 649–654
61. Hafen, E. (1998) *Science* **280,** 1212–1213
62. Takaki, M., Ujike, H., Kodama, M., Takehisa, Y., Nakata, K., and Kuroda, S. (2001) *J. Neurochem.* **79,** 679–688
63. Martin, K. C., Michael, D., Rose, J. C., Barad, M., Casadio, A., Zhu, H., and Kandel, E. R. (1997) *Neuron* **18,** 899–912
64. Vanhoose, A. M., Emery, M., Jimenez, L., and Winder, D. G. (2002) *J. Biol. Chem.* **277,** 9049–9053
65. Banerjee, P. K., Hirsch, E., and Snead, O. C., III (1993) *Neuroscience* **56,** 11–21