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► **To cite this version:**

Haijun Tu, Philippe Rondard, Chanjuan Xu, Federica Bertaso, Fangli Cao, et al.. Dominant role of GABAB2 and Gbetagamma for GABAB receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons.. Cellular Signalling, 2007, 19 (9), pp.1996-2002. 10.1016/j.cellsig.2007.05.004 . hal-00319022

HAL Id: hal-00319022

<https://hal.science/hal-00319022>

Submitted on 5 Sep 2008

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Dominant role of GABA_{B2} and Gβγ for GABA_B receptor mediated-ERK_{1/2}/CREB pathway in cerebellar neurons

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Running title: GABA_{B2}- and Gβγ-mediated ERK1/2 activation by GABA_B.

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Keywords: GABA_B Receptor - ERK_{1/2}- CREB- G_{v0} protein - Gβγ subunit - neurons

Abstract:

γ -aminobutyric acid type B (GABA_B) receptor is an allosteric complex made of two subunits, GABA_{B1} and GABA_{B2}. GABA_{B2} plays a major role in the coupling to G-protein whereas GABA_{B1} binds GABA. It has been shown that GABA_B receptor activates ERK_{1/2} in neurons of the central nervous system, but the molecular mechanisms underlying this event are poorly characterized. Here, we demonstrate that activation of GABA_B receptor by either GABA or the selective agonist baclofen induces ERK_{1/2} phosphorylation in cultured cerebellar granule neurons. We also show that CGP7930, a positive allosteric regulator specific of GABA_{B2}, alone can induce the phosphorylation of ERK_{1/2}. PTX, a G_{i/o} inhibitor, abolishes both baclofen and CGP7930-mediated ERK_{1/2} phosphorylation. Moreover, both baclofen and CGP7930 induce ERK-dependent CREB phosphorylation. Furthermore, by using LY294002, a PI-3 kinase inhibitor, and a C-term of GRK-2 that has been reported to sequester G $\beta\gamma$ subunits, we demonstrate the role of G $\beta\gamma$ in GABA_B receptor mediated-ERK_{1/2} phosphorylation. In conclusion, the activation of GABA_B receptor leads to ERK_{1/2} phosphorylation via the coupling of GABA_{B2} to G_{i/o} and by releasing G $\beta\gamma$ subunits which in turn induce the activation of CREB. These findings suggest a role of GABA_B receptor in long-term change in the central nervous system.

1. Introduction

γ -aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system [1, 2]. It mediates fast synaptic inhibition through GABA_A and GABA_C ionotropic receptors as well as slow and prolonged synaptic inhibition through the metabotropic GABA_B receptor [3]. GABA_B receptor mediates both presynaptic inhibition of neurotransmitter release and post-synaptic inhibition of neuronal excitability [4, 5]. Accordingly, GABA_B receptor is involved in various types of epilepsy, nociception and drug addiction. Activation of the receptor also has myorelaxant activity that are commonly used to treat spasticity associated with multiple sclerosis [2, 6]

GABA_B receptor belongs to the class C of G-protein coupled receptors (GPCRs), together with metabotropic glutamate (mGlu), extracellular Ca²⁺-sensing, and some pheromone and taste receptors [7]. Each of these receptors is composed of an extracellular domain called the Venus flytrap domain (VFT), to which agonists bind, and a heptahelical domain (HD), which is responsible for the recognition and activation of heterotrimeric G-proteins. Whereas mGluRs and Ca²⁺-sensing receptors exist as homodimers, GABA_B receptor is a heterodimer composed of two homologous subunits, GABA_{B1} and GABA_{B2} [8-11].

Heterodimerization of GABA_B receptor is a prerequisite for GABA_B receptor function. The VFT domain of GABA_{B1} is sufficient for ligand binding [7], but its assembly with GABA_{B2} increases the affinity of GABA_{B1} for agonists [12]. Although GABA_{B2} does not appear to bind any natural ligand [13], GABA_{B1} needs to be associated with GABA_{B2} to reach the cell surface [14]. Agonist binding to the GABA_{B1} VFT domain results in its closure and this is likely responsible for a change in the relative position of the two VFT and HD domains [15]. This movement then allows activation of G proteins mediated by the HD domains of GABA_{B2} [16, 17]. Recently, a novel class of compounds called GABA_B positive allosteric modulators (PAM) such as CGP7930, appeared to represent a better therapeutic alternative than

the classical agonist [18, 19]. Although CGP7930 alone was reported to be inactive in several assays, direct activation of the receptor by this positive allosteric modulator acting in the HD domain of GABA_{B2} have also been observed [20].

Functional GABA_B receptor is predominantly coupled to heterotrimeric G_{i/o}-type protein since most of GABA_B receptor-mediated effects are inhibited by pertussis toxin (PTX) [21, 22]. Upon activation of the G protein, the Gβγ complex represses Ca²⁺ influx by inhibiting Ca²⁺ channels [23] and triggers K⁺ channels opening [24, 25]. In the mean time, the Gα_{i/o} subunits modulate the level of cyclic adenosine monophosphate (cAMP) by regulating adenylate cyclase activities [26].

Protein phosphorylation plays a critical role in synaptic plasticity, learning and memory in vertebrates [27]. The Extracellular signal-Regulated protein Kinases 1/2 (ERK_{1/2}), also known as p42/44 mitogen-activated protein kinase (MAPK), signaling cascade plays important roles in the modulation of long-term potentiation in area CA1 of the hippocampus and is required for several forms of learning and memory [28]. Recently, it has been reported that GABA_B receptor induces ERK_{1/2} phosphorylation in the CA1 area of mouse hippocampus [29]. Regulation of ERK_{1/2} signaling cascade by GPCRs is highly complex and cell type-specific [30], and the mechanism of GABA_B receptor-mediated ERK_{1/2} phosphorylation is still poorly understood. Furthermore, GABA_B receptor is reported to bind to the transcription factor CREB2(cAMP responsive element binding protein-2)/ATF4(activating transcription factor 4) through coiled-coil interactions [31-33]. Nuclear translocation of CREB2 is also observed following GABA_B receptor activation [31, 33]. The physiological significance for GABA_B receptor activation-induced CREB2 translocation awaits elucidation.

In the current study, we examine the role of GABA_B receptor in the regulation of ERK_{1/2} and CREB phosphorylation in cultured cerebellar granule neurons. We find that selective GABA_B receptor activation induces ERK_{1/2} phosphorylation that in turn mediates CREB phosphorylation. We also show that this effect occurs via the

GABA_{B2} coupling to G_{i/o} proteins by releasing Gβγ subunits. Interestingly, we show for the first time that selective activation of GABA_{B2} is sufficient to induce CREB phosphorylation.

2. Materials and Methods

2.1 Materials

GABA was obtained from Sigma (Shanghai, China). Baclofen was purchased from Tocris (Fisher-Bioblock, Illkrich, France). GP54626, CGP7930, Pertussis toxin (PTX) were purchased from Calbiochem (US and Canada). Fetal bovine serum, culture medium, and other solutions used for cell culture were from Invitrogen (Shanghai, China). LY294002 and U0126 were purchased from Cell Signaling Technology (Beverly, MA). pRK5 plasmids encoding wild-type GABA_{B1} and GABA_{B2} with an epitope tag at their N-terminal ends under the control of a cytomegalovirus promoter were described previously [14]. The pcDNA3-c-myc-CD8-βARK plasmid, which was composed of the CD8 antigen membrane receptor and a domain containing the G-protein βγ subunit-binding site of GRK2, was a generous gift from Dr. M. De Waard.

2.2 Primary cerebellar granule neuronal cultures

Primary cultures of cerebellar cells were prepared as previously described [34]. Briefly, 1-week-old newborn mice were decapitated and cerebellum-dissected. The tissue was then gently triturated using fire-polished Pasteur pipettes, and the homogenate was centrifuged at 500rpm. The pellet was resuspended and plated in tissue culture dishes previously coated with poly-L-ornithine. Cells were maintained in a 1:1 mixture of DMEM and F-12 nutrient (Life Technologies, Gaithersburg, MD), supplemented with glucose (30mM), glutamine (2mM), sodium bicarbonate (3mM) and HEPES buffer (5mM), deplemented fetal calf serum (10%), and 25mM KCl to improve neuronal survival. Three-days-old cultures contained 1.25×10^5 cells/cm².

2.3 Cell culture and transfection

Human embryonic kidney HEK293 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected by

electroporation as described previously [35]. Cells (10×10^6) were transfected with plasmid DNA containing the cDNAs encoding GABA_{B1} (4 μ g) or GABA_{B2} (4 μ g), and completed to a total amount of 10 μ g of plasmid DNA with the pRK5 empty vector.

2.4 Western blot analysis

Cell lysates from cultures were sonicated, and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories LTD, Hertfordshire, UK). Equal amounts of protein (20 μ g) were resolved by SDS-PAGE on 12% gels. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in TBS and 0.1% Tween 20) for 1 hr. The blots were then incubated with the primary rabbit polyclonal antibodies against phospho-ERK_{1/2} (1:1000; Cell Signaling Technology, Beverly, MA), or with a rabbit polyclonal antibodies against the total ERK_{1/2} (1:1000; Cell Signaling Technology), overnight at 4°C. This was followed by 1 hr incubation with goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies (1:20000; Cell Signaling Technology). Immunoblots were revealed using the enhanced chemiluminescence reagents (Pierce, USA) and visualized using the X-Ray film. The density of immunoreactive bands was measured using NIH image software, and all bands were normalized to percentages of control values.

2.5 Drug treatment

Cultures were washed once with HBS (Ca²⁺ free) and preincubated at 37°C with HBS for 60 min. Cells were treated by adding freshly made drugs to the HBS. At the end of the treatment, cells were washed quickly with ice-cold PBS, pH 7.4 (Ca²⁺ free), lysed with 200 μ L lysis buffer and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. Drugs were dissolved in HBS with or without dimethyl sulfoxide (DMSO) or/and alcohol. Whenever DMSO or/and alcohol were used, HBS containing the same concentration of DMSO or/and alcohol were used as the control vehicle.

3. Results

3.1. Activation of GABA_B receptor increases ERK_{1/2} phosphorylation in neurons

We first studied the effect of GABA and the GABA_B-selective agonist baclofen, on ERK_{1/2} phosphorylation in cultured mouse cerebellar granule neurons (CGNs). We found that GABA at 100μM caused a rapid and transient increase in ERK_{1/2} phosphorylation with no changes in ERK_{1/2} expression levels (Fig. 1A, upper panel). ERK_{1/2} phosphorylation peaked at 10min and then decreased. Similar results were obtained with baclofen at 100μM (Fig. 1A, lower panel). These data showed that the activation of GABA_B receptor leads to increased ERK_{1/2} phosphorylation in CGNs.

To demonstrate that ERK_{1/2} phosphorylation occurs via the specific activation of GABA_B receptor, we evaluated the effect of the GABA_B receptor selective antagonist on baclofen-induced ERK_{1/2} phosphorylation in CGNs. Cells were pretreated for 20min with GABA_B receptors antagonist, CGP54626 (10μM) and then stimulated with baclofen (100μM) for 10min. We found that this antagonist blocked baclofen-induced ERK_{1/2} phosphorylation without to alter ERK_{1/2} expression levels (Fig. 1B), thus demonstrating that GABA_B receptor activation contributes to the baclofen-induced ERK_{1/2} phosphorylation in CGNs.

3.2 GABA_B receptor-mediated ERK_{1/2} phosphorylation occurs through the coupling of GABA_{B2} to G_{v0} protein

GABA_B receptor, reported as a G_{v0} protein-coupled receptor activates its downstream effectors through coupling of GABA_{B2} subunits towards G proteins [22, 23]. Recently, it was demonstrated that CGP7930, a positive allosteric modulator (PAM) of the GABA_B receptor not only modulates GABA_B receptor activity by directly acting on GABA_{B2} HD domain but also activates GABA_{B2} when expressed alone [36]. To evaluate whether GABA_B receptor-mediated ERK_{1/2} effect occurs through GABA_{B2} coupling to G protein, we then studied the effect of CGP7930 on ERK_{1/2} phosphorylation in CGNs. CGP7930 at a concentration of 50μM caused a

rapid, transient and strong increase in ERK_{1/2} phosphorylation in CGNs (Fig. 2A). ERK_{1/2} phosphorylation levels induced upon CGP7930 treatment peaked at 10 min prior to decreasing gradually to the basal level. Pretreatment of CGNs with CGP54626 even at the concentration of 100 μ M did not abolish CGP7930-induced ERK_{1/2} phosphorylation (Fig. 2A, inset panel). These results demonstrate that GABA_{B2} directly activates ERK_{1/2} pathway. In addition, the required association of GABA_{B1} with GABA_{B2} to reach the cell surface [14] indicates that GABA_B receptor mediated- ERK_{1/2} phosphorylation in CGNs occurs through GABA_{B2} coupling to G protein.

We further verified that GABA_B receptor mediated-ERK_{1/2} phosphorylation is also regulated through coupling of G_{i/o} protein by using pertussis toxin (PTX). Neurons were pretreated with PTX at 200ng/ml for 14-18hrs, and then stimulated for 10 min with 100 μ M baclofen or 50 μ M CGP7930 (Fig. 2B). Under those circumstances, PTX inhibited baclofen or CGP7930 -induced ERK_{1/2} phosphorylation in CGNs (Fig. 2B), demonstrating that baclofen or CGP7930 induced ERK_{1/2} activation via G_{i/o} proteins.

We confirmed the observed GABA_B receptor-mediated ERK_{1/2} phosphorylation in HEK293 cells transfected with both GABA_{B1} and GABA_{B2}, or with GABA_{B2} alone. GABA at 100 μ M caused a rapid and transient ERK_{1/2} phosphorylation without change in ERK_{1/2} levels in cells expressing both GABA_{B1} and GABA_{B2} whereas it had no effect on the mock-transfected cells (cells transfected with pRK5 empty vector) (Fig. 3A). CGP7930 alone also induced ERK_{1/2} phosphorylation (Fig. 3B), and this effect was not antagonized by a CGP54626 pre-treatment of the cells (Fig. 3B, inset panel). Pretreatment of the cells with PTX blocked both GABA and CGP7930-induced ERK_{1/2} phosphorylation (Fig. 3B). These results demonstrate that GABA_B receptor-mediated ERK_{1/2} phosphorylation occurs via GABA_{B2} coupling to G_{i/o} protein. In HEK293 cells expressing GABA_{B2} alone, CGP7930 induced an acute phosphorylation of ERK_{1/2} whereas it had no effect on mock-transfected cells (Fig.

4A). Pretreatment of the cells with PTX efficiently reduced CGP7930-mediated ERK_{1/2} phosphorylation with no change in ERK_{1/2} expression levels (Fig. 4B), thus suggesting that the activation of GABA_{B2} by CGP7930 is sufficient to mediate ERK_{1/2} phosphorylation via G_{i/o} protein.

Our results in CGNs and in HEK293 cells transfected with GABA_{B2} alone or with both GABA_{B1} and GABA_{B2}, showed that GABA_B receptor induced ERK_{1/2} phosphorylation via the coupling of GABA_{B2} to G_{i/o} heterotrimeric proteins.

3.3 Gβγ subunits mediate GABA_B receptor-induced ERK_{1/2} phosphorylation

To test whether the coupling of GABA_{B2} to G_{i/o} protein occurs via Gβγ/PI-3 kinase to induce ERK_{1/2} phosphorylation, we treated CGNs with the selective PI-3 kinase inhibitor, LY294002. Interestingly, the inhibition of the PI-3 kinase pathway led to a strong inhibition of both baclofen and CGP7930- induced ERK_{1/2} phosphorylation in neurons (Fig. 5A), thus suggesting an important role for Gβγ derived from G_{i/o} proteins.

We then investigated the role of Gβγ subunits in GABA_B receptor-mediated ERK activation in HEK293 cells expressing the GABA_B receptor. To this end, we used the previously characterized Gβγ-scavenger consisting of the C-terminal region of GRK2 (βARK) fused to the extracellular and transmembrane domains of CD8 which then provides a membrane anchor for βARK's C-tail (CD8-βARK)[37] [38]. The overexpression of CD8-βARK inhibited GABA-induced ERK_{1/2} phosphorylation in HEK293 cells co-expressing both GABA_{B1} and GABA_{B2} and CGP7930-induced ERK_{1/2} phosphorylation in HEK293 cells expressing GABA_{B2} alone (Fig. 5B). Taken together, these results show that GABA_B receptor mediates ERK_{1/2} phosphorylation through Gβγ subunits.

3.4 GABA_B receptor-mediated ERK_{1/2} phosphorylation induces CREB phosphorylation

Phosphorylation of ERK_{1/2} plays an important role in the regulation of gene expression via phosphorylation of nuclear transcription factors [39]. We therefore tested the response of CREB to the GABA_B receptor mediated-ERK_{1/2} pathway. We found that baclofen at 100μM induced a rapid and transient increase in CREB phosphorylation in CGNs (Fig. 6A). Baclofen-induced CREB phosphorylation was abolished by the pretreatment of CGNs with either CGP54626 (Fig. 6B) or MEK_{1/2} inhibitor U0126 (Fig. 6C). Furthermore, the pretreatment of CGNs with U0126 also inhibited CGP7930-induced CREB phosphorylation (Fig. 6C). These results show that GABA_B receptor-mediated CREB phosphorylation occurs through the ERK_{1/2} pathway.

4. Discussion

The main findings of the present study concern the mechanism of GABA_B receptor-mediated ERK_{1/2} phosphorylation. We show that: 1) GABA_B receptor activation leads to increased ERK_{1/2} phosphorylation in cultured cerebellar granule neurons which in turn induces CREB phosphorylation; 2) selective activation of GABA_{B2} by CGP7930 is sufficient for ERK_{1/2} activation in both cultured cerebellar neurons and HEK293 cells transfected with GABA_{B2} alone or in the presence of GABA_{B1}; 3) all these effects rely on a PTX-sensitive G_{i/o} heterotrimeric protein-dependent pathway by releasing Gβγ and by implicating PI-3 kinase pathway, and 4) both baclofen and CGP7930-mediated CREB phosphorylation is ERK_{1/2} dependent.

Several reports have recently shown that G_{i/o}-coupled GABA_B receptor induced ERK_{1/2} phosphorylation [29, 40-43]. However, the signaling cascades transmitting GABA_B receptor signals towards ERK_{1/2} remained unclear. Indeed, the biochemical routes linking GPCRs to ERK_{1/2} are highly complex and cell type-specific [44]. Our results demonstrate the role of Gα_{i/o} protein in selective activation of GABA_B receptor to ERK_{1/2} signaling. Furthermore, it is well established that PI3 kinase acts downstream of Gβγ subunits to mediate GPCR-controlled MAPK activation [45-47]. The inhibition of the PI-3 kinase leading to a strong inhibition of baclofen-induced ERK_{1/2} phosphorylation in neurons suggests an important role for Gβγ derived from G_{i/o} proteins for GABA_B receptor-mediated ERK_{1/2} activation.

We showed that GABA_B receptor-mediated ERK_{1/2} activation requires the presence of GABA_{B2}. These results are compatible with a recent study where GABA_{B1} has been reported to reach the cell surface by co-expression with GABA_A receptors γ2S subunits in heterologous cellular systems but failed to stimulate ERK_{1/2} phosphorylation in the absence of GABA_{B2} [43]. Furthermore, CGP7930, a positive modulator of GABA_B receptor, was reported to potentiate GABA_B receptor activity

by interacting with HD domain of GABA_{B2} [36]. However, the effect of CGP7930 alone on GABA_B receptor activity is controversial. Meanwhile, CGP7930 produced little or no stimulation of the GABA_B receptor activity in some studies [18], others show that CGP7930 alone can stimulate inositol phosphate accumulation in HEK293 cells co-expressing the GABA_B receptor and the chimeric G-protein Gqi9 [36]. Here, we observed CGP7930 alone displays an intrinsic agonist activity on GABA_B receptor mediated-ERK_{1/2} activation in both CGNs and HEK293 cells expressing GABA_B receptor. Competitive antagonist of GABA_B receptors such as CGP54626 that binds on GABA_{B1} did not block the CGP7930-mediated ERK_{1/2} phosphorylation, suggesting that GABA_{B2} activation is sufficient to induce ERK_{1/2} activation. CGP7930-mediated ERK_{1/2} phosphorylation in HEK-293 cells expressing GABA_{B2} alone is compatible with the effect of CGP7930 on inositol phosphate accumulation in HEK-293 expressing GABA_{B2} alone [36].

Several studies suggested that GABA_B receptor plays an important role in memory processing. For example, mice lacking the GABA_{B1} [25] or the GABA_{B2} subunit [48] suffer from severe memory impairment. Increasing evidence has shown that ERK_{1/2} plays an important role in long-term synaptic plasticity and memory through regulating protein synthesis and gene expression [49, 50]. Stimulation of CREB is also critical for long term potentiation [51] through either ERK_{1/2} or p38 pathway [52, 53]. In our study, we demonstrate that CREB phosphorylation is induced by selective activation of GABA_B receptor via an ERK_{1/2}-dependent pathway. These data suggest a role of the ERK/CREB pathway in GABA_B receptor-mediated long-term synaptic plasticity and memory.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grants No. 30530820, No.30470368) and Hi-Tech Research and Development Program of China (863 project) (Grants No. 2006AA02Z326). H. T was supported by the fund of outstanding thesis from Huazhong University of Science and Technology (D0611). We would like to thank Dr Eric Chevet and Dr Jian Zhu for helpful discussions.

Figure Legends:

Figure 1. Activation of $GABA_B$ receptor increases $ERK_{1/2}$ phosphorylation in cultured mice cerebellar granule neurons. *A*, Upper panel, time course of the endogenous $ERK_{1/2}$ phosphorylation after incubation of GABA (100 μ M). Data represent the mean \pm SEM from at least five independent experiments. Lower panel, typical immunoblots used to quantify the phosphorylated $ERK_{1/2}$ (p $ERK_{1/2}$). *B*, Effects of the antagonist CGP54626 on baclofen-induced $ERK_{1/2}$ phosphorylation. CGP54626 (10 μ M) was incubated for 30 min before and during treatment with baclofen (10min). p $ERK_{1/2}$ was quantified as previously in Fig. 1A, and data are the mean \pm SEM from three independent experiments.

Figure 2. $GABA_B$ receptor-mediated $ERK_{1/2}$ phosphorylation occurs through the coupling of $GABA_{B2}$ to $G_{i/o}$ protein. *A*, Effect of the, CGP7930 (50 μ M), a PAM of $GABA_{B2}$ in the increase of $ERK_{1/2}$ phosphorylation in cultured mice CGNs. Data are the mean \pm SEM from three independent experiments. *Inset*, effect of CGP54626 (100 μ M) on CGP7930-induced $ERK_{1/2}$ phosphorylation. *B*, Inhibitory effect of PTX on baclofen- and CGP7930-stimulated $ERK_{1/2}$ phosphorylation. Data are the mean \pm SEM from three independent experiments

Figure 3. $GABA_B$ receptor-mediated $ERK_{1/2}$ phosphorylation in HEK293 cells co-expressing both $GABA_{B1}$ and $GABA_{B2}$. *A*, Effect of GABA (100 μ M) in the increase of transient $ERK_{1/2}$ phosphorylation. Data are the mean \pm SEM from five independent experiments. *B*, Inhibitory effect of PTX on GABA- and CGP7930-stimulated $ERK_{1/2}$ phosphorylation. *Inset*, Effect of CGP54626 (100 μ M) on CGP7930-induced $ERK_{1/2}$ phosphorylation. Data represent the mean \pm SEM from at least three independent experiments.

Figure 4. Activation of $GABA_{B2}$ increases $ERK_{1/2}$ phosphorylation in HEK293 cells expressing $GABA_{B2}$ alone. *A*, Effect of CGP7930 (50 μ M) in the increase of transient $ERK_{1/2}$ phosphorylation. *B*, Inhibitory effect of PTX on GABA- and CGP7930-

stimulated ERK_{1/2} phosphorylation. Data are the mean \pm SEM from at least four independent experiments.

Figure 5. *G β subunit mediates GABA_B receptor-induced ERK_{1/2} phosphorylation.*

A, Effect of LY294002 (20 μ M) on GABA_B receptor-mediated ERK_{1/2} phosphorylation induced by baclofen (100 μ M) or CGP7930 (50 μ M) in CGNs. *B*, Effect of over-expression of c-myc-tagged CD8- β ARK, G β subunits inhibiting peptide, on GABA and CGP7930-induced ERK_{1/2} phosphorylation in HEK293 cells expressing the heterodimer GABA_B and GABA_{B2} alone, respectively. Data are the mean \pm SEM from at least four independent experiments.

Figure 6. *GABA_B dependent CREB phosphorylation is mediated by ERK_{1/2} phosphorylation.*

A, The effect of baclofen (100 μ M) on CREB phosphorylation (pCREB) was determined by immunoblotting, and quantified as previously for pERK_{1/2} in Figure 1A. *B*, Effect of CGP54626 (10 μ M) on baclofen-induced CREB phosphorylation. *C*, Effects of U0126 (10 μ M) on baclofen- or CGP7930- mediated ERK_{1/2} and CREB phosphorylation in CGNs. Data are the mean \pm SEM from at least four independent experiments.

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Figure 1

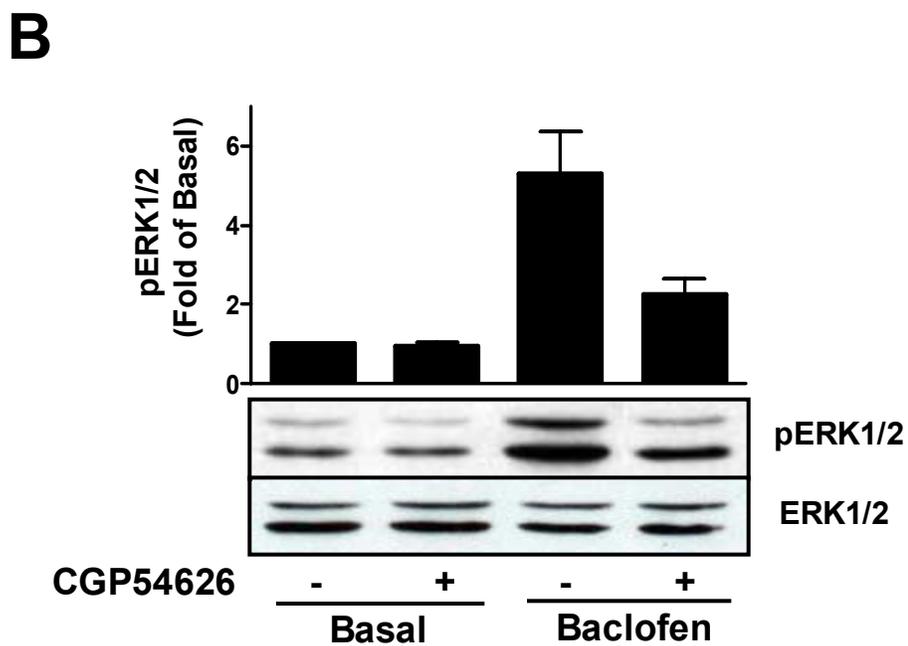
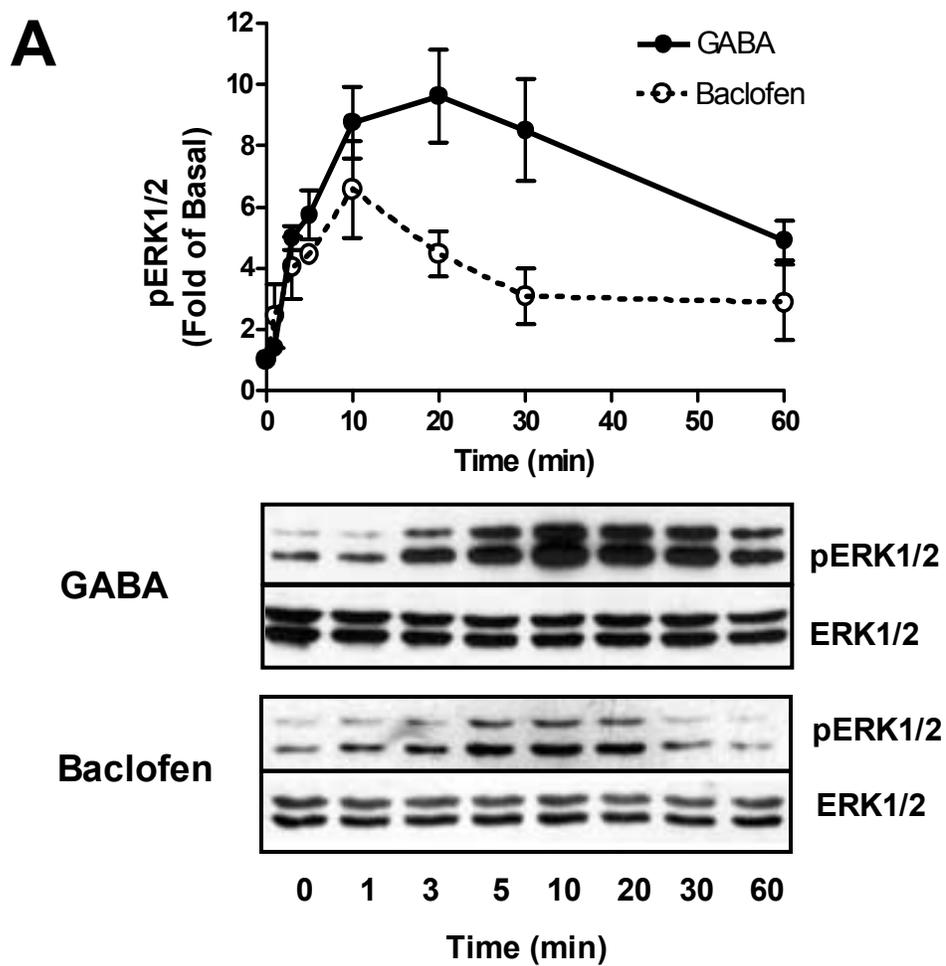


Figure 2

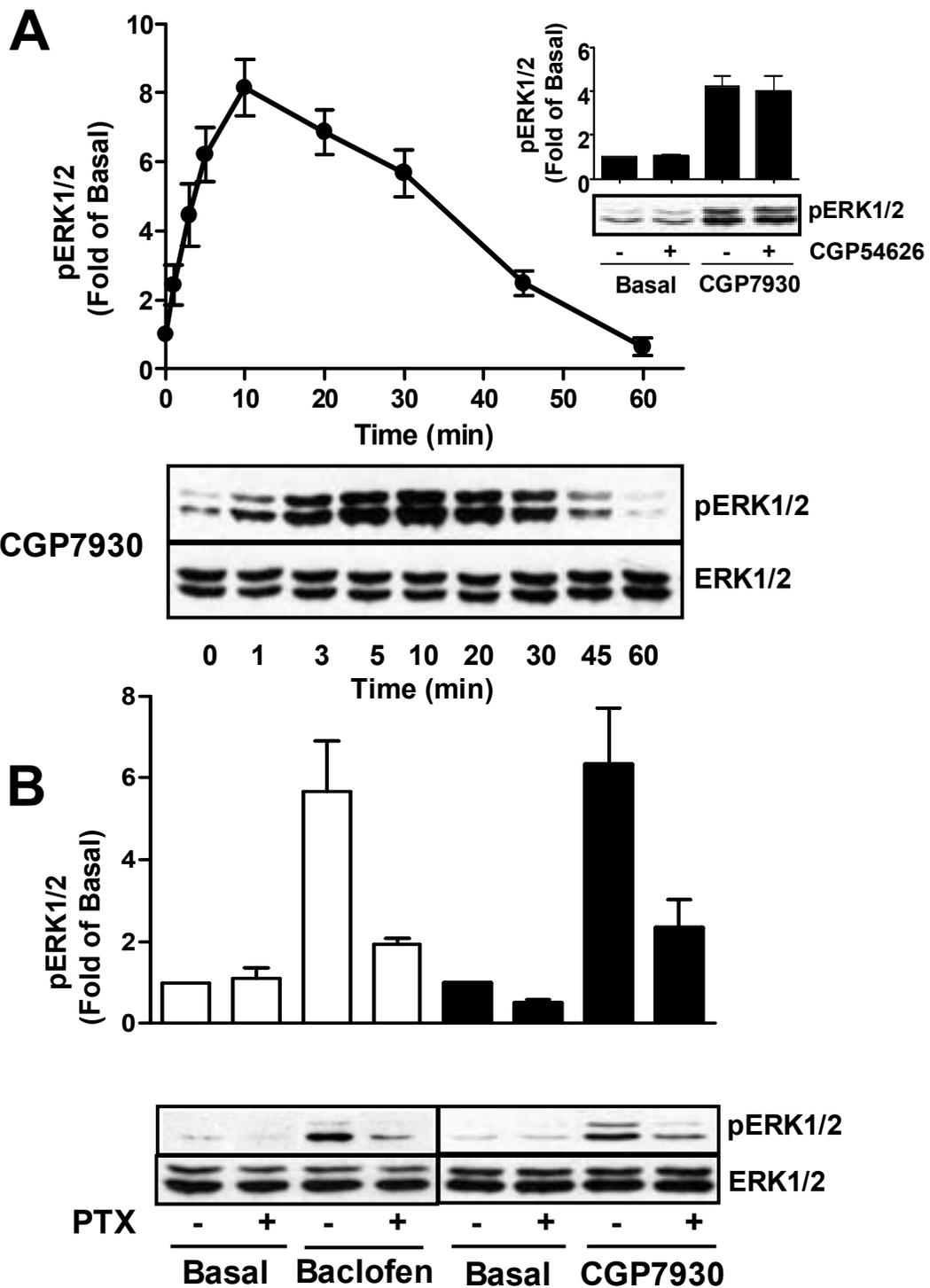


Figure 3

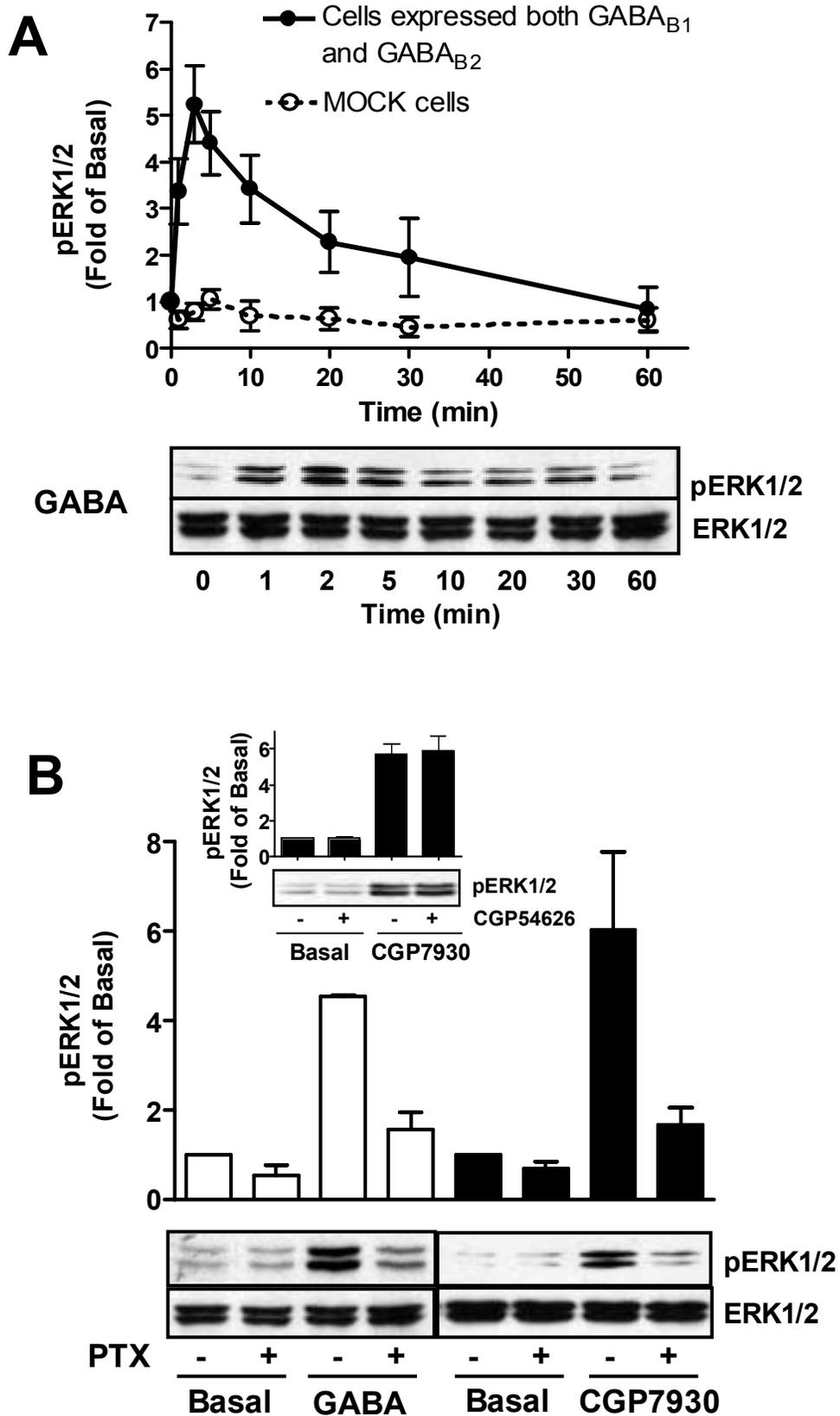


Figure 4

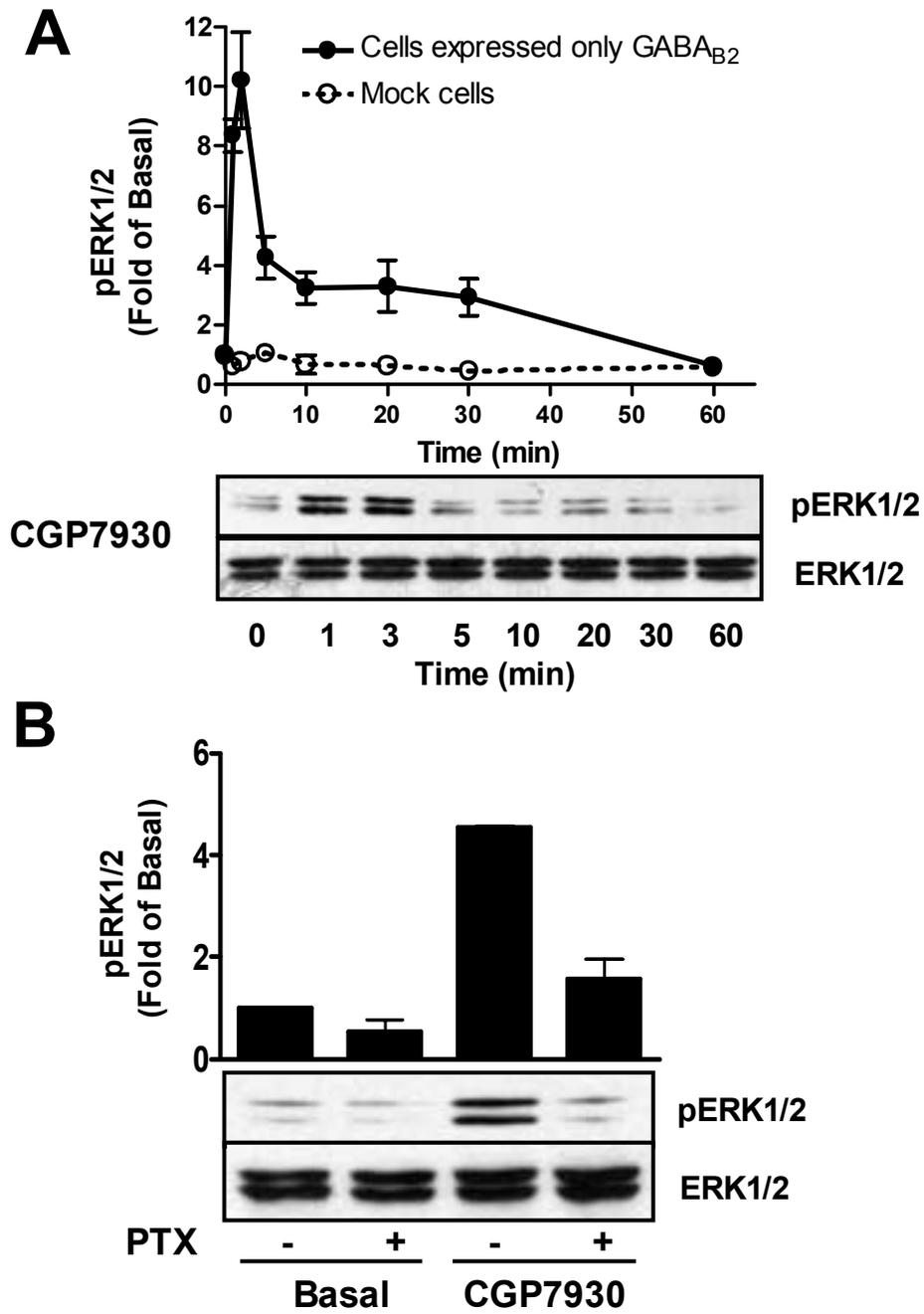


Figure 5

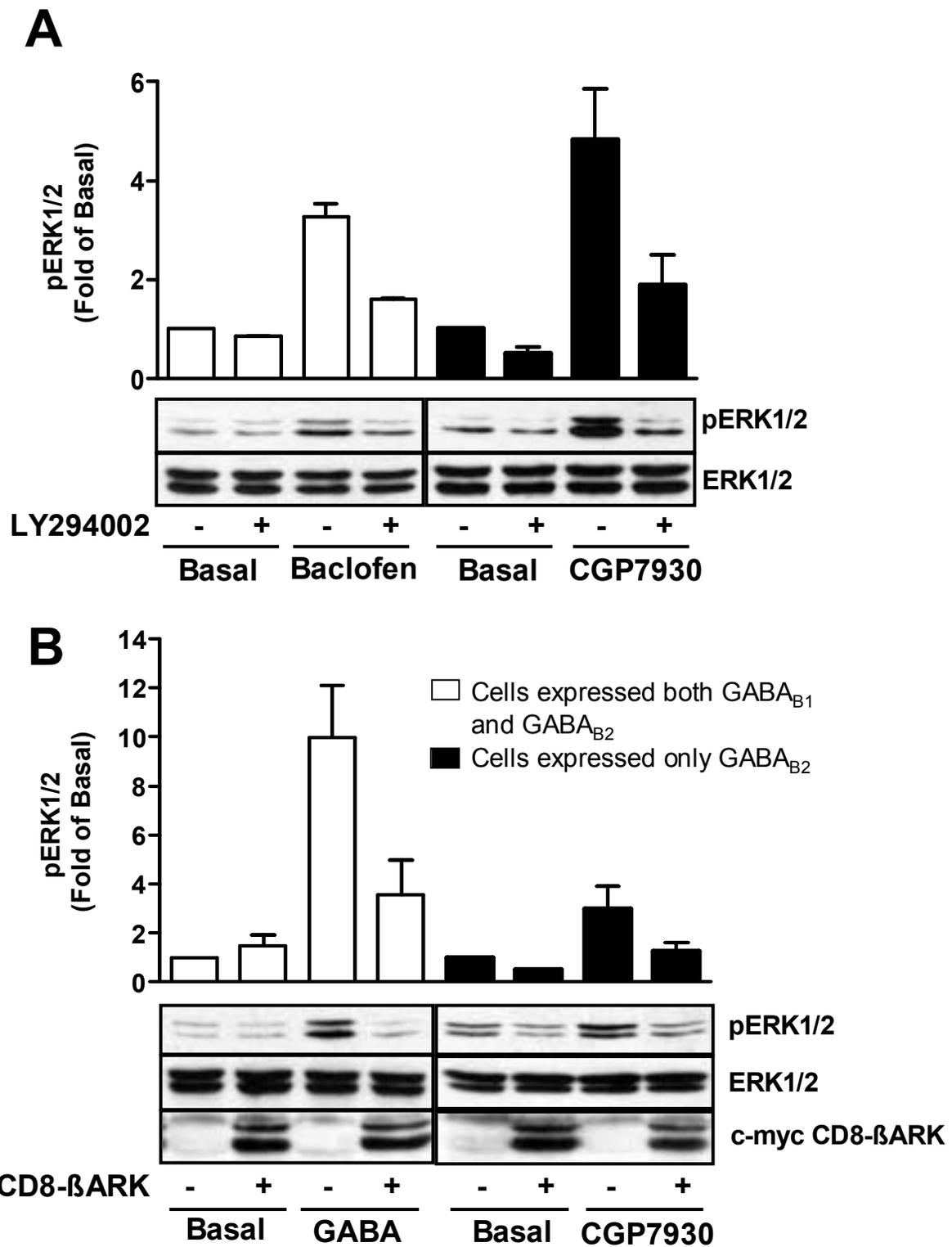
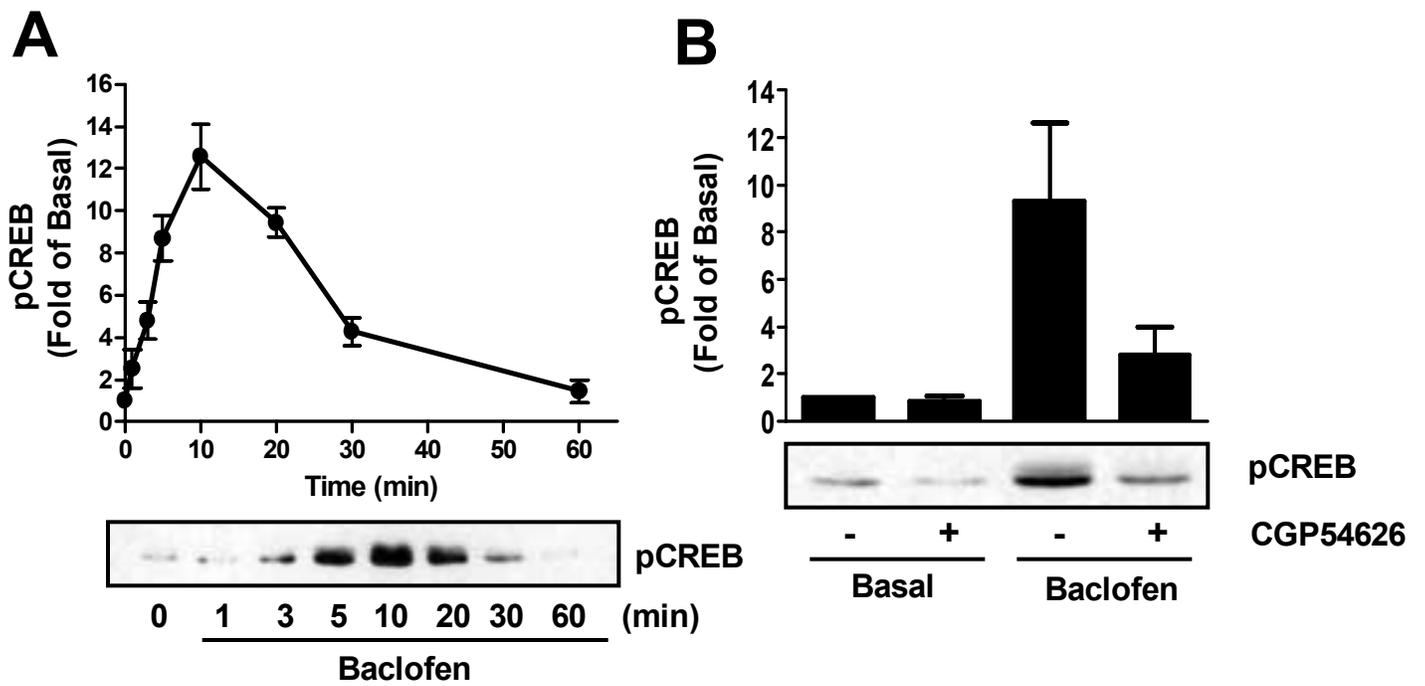


Figure 6



C

