PGE$_2$ Modulates GABA$_A$ Receptors via an EP1 Receptor-Mediated Signaling Pathway

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Key Words
PGE$_2$ • GABA$_A$ receptors • Cerebellar granule cells • EP receptor • PKC

Abstract

Aims: PGE$_2$ is one of the most abundant prostanoids in mammalian tissues, but its effect on neuronal receptors has not been well investigated. This study examines the effect of PGE$_2$ on GABA$_A$ receptor currents in rat cerebellar granule neurons. Methods: GABA$_A$ currents were recorded using a patch-clamp technique. Cell surface and total protein of GABA$_A$ $\beta_{1/2/3}$ subunits was carried out by Western blot analysis. Results: Upon incubation of neurons with PGE$_2$ (1 $\mu$M) for 60 minutes, GABA$_A$ currents were significantly potentiated. This PGE$_2$-driven effect could be blocked by PKC or CaMKII inhibitors as well as EP1 receptor antagonist, and mimicked by PMA or EP1 receptor agonist. Furthermore, Western blot data showed that PGE$_2$ did not increase the total expression level of GABA$_A$ receptors, but significantly increased surface levels of GABA$_A$ $\beta_{1/2/3}$ subunits after 1 h of treatment. Consistently, both PKC and CaMKII inhibitors were able to reduce PGE$_2$-induced increases in cell surface expression of GABA$_A$ receptors. Conclusion: Activation of either the PKC or CaMKII pathways by EP1 receptors mediates the PGE$_2$-induced increase in GABA$_A$ currents. This suggests that upregulation of postsynaptic GABA$_A$ receptors by PGE$_2$ may have profound effects on cerebellar functioning under physiological and pathological conditions.

Introduction

Prostaglandin E$_2$ (PGE$_2$) is one of the most abundant prostanoids in a wide variety of mammalian tissue [1] and it regulates multiple biological processes under both normal and pathological conditions, including cell proliferation, apoptosis, angiogenesis, inflammation, and immune surveillance [2, 3]. In addition, PGE$_2$ has a variety of central
actions such as wakefulness, anorexia and impulsive behavior under both physiological and pathophysiological conditions [4-6]. In recent years, that PGE$_2$ has been found to be important for hippocampal long-term synaptic plasticity and cognitive function [7], and can increase AMPA/Kainate receptor surface expression in the developing preoptic area neurons and glia to cause dendritic spine formation [8]. Moreover, centrally administered PGE$_2$ exhibited anxiolytic-like activity in the elevated plus-maze and open field test in mice [9]. Thus, the effect of PGE$_2$, associated neuronal excitability is gaining attention.

The physiological activity of PGE$_2$ has been shown to be mediated by the EP family of receptors, consisting of four isoforms, EP1-EP4 [10, 11]. EP receptors are G-protein-coupled receptors (GPCRs) that are coupled to Gα proteins, containing either a stimulatory (GαS) or inhibitory (Gαi) subunit, which can subsequently modulate the levels of Ca$^{2+}$, cyclic AMP (cAMP), and inositol phosphate [12]. When taken in combination with the ubiquitous production of PGE$_2$, it is clear that EP receptor signaling accounts for the pleiotropic ability of PGE$_2$ to potently activate diverse biological effects. Past genetic deletion and selective inhibition studies have examined each type of EP receptor, attempting to clarify the various roles PGE$_2$ receptors play in neuronal death induced by excitotoxicity and ischaemic stroke. Inhibition of EP1 receptors has been shown to reduce neuronal damage caused by both excitotoxicity and ischaemic stroke [13]. Contrarily, deletion of the EP2 receptor increases infarct volume in mice after ischaemic stroke [14]. Thus, it is clear that there is a wide variability in the response of target cells to PGE$_2$, which is based on the particular subtype of receptors that are activated as well as their distribution.

Inhibitory neurotransmission in the brain is largely mediated by γ-aminobutyric acid (GABA) acting through GABA type A receptors (GABA$_A$Rs). These receptors are heteropentameric, GABA-gated chloride channels that belong to the Cys-loop ligand-gated ion channel superfamily [15]. GABA$_A$Rs are expressed ubiquitously in neurons along the entire neuraxis. Their activity is important for animal development and neural differentiation [16], but also for the structural and functional maturation of neurons [17, 18]. Deficits in GABA$_A$R-mediated neurotransmission have been implicated in pathophysiological disorders, ranging from anxiety disorders to the etiology of epilepsy and schizophrenia [19-21]. Therefore, studying the mechanisms that regulate the function of GABA$_A$Rs is required for an understanding of the underlying causes of their physiological and pathological functioning. Previous studies indicated GABA$_A$Rs activity was regulated by both endogenous molecules, Zn$^{2+}$ [22], and exogenous drugs, including the widely prescribed anxiolytics, benzodiazepines [23]. Recently, investigation by Suzuki et al’s indicated that PGE$_2$, through the EP1 and EP4 receptors exhibited anxiolytic-like activity, which was inhibited by antagonists for serotonin 5-HT1A, dopamine D1 and GABA$_A$ receptors [9]. Whether and how PGE$_2$ regulates GABA$_A$ receptors through the EP receptors are thus worth further study.

Cultured cerebellar granule neurons (CGNs) have long been a model for studying GABA$_A$ receptors [24]. Importantly, all four types of EP receptors are transcribed in this neuronal type [25]. Therefore, the objective of this study was to determine whether PGE$_2$ was able to modulate GABA$_A$ receptor by specific EP receptor.

Materials and Methods

Ethics statement

All experimental procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC). The protocol was approved by the Committee on the Ethics of Animal Experiments of Fudan University (Permit Number: 20090614-001). All surgeries were performed under sodium pentobarbital anesthesia and all efforts were made to minimize animal suffering.

Cell culture

Cells were derived from the cerebellum of 7- to 8-day-old Sprague-Dawley rat pups as previously described [26]. Isolated cells were plated onto 35 mm diameter Petri dishes coated with poly-L-lysine.
(10 μg/ml) at a density of 10⁴ cells/ml. Cultured cells were incubated at 37°C under 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine (5 mM), insulin (5 μg/ml), KCl (25 mM), and 1% antibiotic-antimycotic solution. All experiments were carried out using primary CGNs after 5–7 days in culture.

**GABA current recordings**

Whole-cell currents from granule neurons were recorded using a patch-clamp technique. For recording GABA currents, the culture medium was replaced with a bath solution containing the following (in mM): NaCl 145, KCl 2.5, HEPES 10, MgCl₂ 1 and glucose 10 (pH adjusted to 7.4 with NaOH). Soft-glass pipettes were filled with an internal solution containing the following (in mM): KCl 145, HEPES 10, CaCl₂ 1, MgCl₂ 1, EGTA 10, and ATP 1 (pH adjusted to 7.2 with KOH). The recordings were performed at 23–25°C. While recording, 100 μM GABA was added to the cells via a rapid perfusion system, which ensured constant perfusion with the bath solution. The GABA receptor current was recorded for 3 s every 40 s. All currents were recorded using an Axopatch 700B amplifier (Axon Instrument, Foster City, CA, USA) operated in voltage-clamp mode and a computer connected to the recording equipment via a Digidata 1440A analog-to-digital (A/D) interface (Axon Instrument, Foster City, CA, USA). Current was digitally sampled at 100 µs (10 kHz). Current signals were filtered by a 1 kHz three-pole Bessel filter. Data acquisition and analysis were performed with pClamp 10.2 software (Axon Instruments, Foster City, CA, USA) and/or Origin8.0 (Microcal Analysis Software, Northampton, MA, USA).

**Biotinylation assay**

Cell surface proteins were biotinylated according to the manufacturer’s protocol as previously described [27]. Briefly, the neurons were incubated with 0.25 mg/mL sulfo-NHS-SS-biotin (Thermo Scientific, Rockford, IL, USA) for 45 min at 4°C and subsequently blocked with 50 mM Tris (pH 8.0) for 20 min at 4°C. The cells were lysed in HEPES-NP40 lysis buffer (see Western blotting section). Biotinylated proteins were pulled down using streptavidin-agarose beads (Thermo Scientific) overnight at 4°C and subsequently washed four times with lysis buffer. The bound proteins were eluted with the sample buffer and used for Western blotting.

**Western blotting**

Rat CGNs were lysed in NP40 lysis buffer (HEPES 20, NaCl 150, EDTA 2, Na₃VO₄ 0.1, NaF 50, in mM, NP-40 0.5%, glycerol 10%) with both a protease inhibitor cocktail and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, USA). Lysates were mixed with an equal volume of Laemmli sample buffer and boiled for 5 min. Samples were electrophoresed by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline Tween (TBST; Tris-base 10mM, NaCl 0.15mM, Tween-20 0.05%) containing 10% nonfat milk, blots were probed with rabbit polyclonal antibodies against GABA₂ α/β 1/2/3 (1:500) (sc-28796 H-50, Santa Cruz Biotechnology, CA, USA), Na⁺/K⁺-ATPase α (1:1000), Phospho-PKC (pan) (1:1000) (#3010, #9371, Cell Signaling Technology, MA, USA), and mouse monoclonal antibodies against GAPDH (1:10000) (KC-5G4, Kangchen Bio-tech, Shanghai China) at 4°C overnight. After washing with TBST, membranes were treated with anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (HPR) at room temperature. Immunoreactivity was detected using a chemiluminescence substrate (Pierce Protein Research Products, Rockford, IL, USA) with the ChemiDoc XRS System and analyzed by Quantity One software (Bio-Rad Laboratories, USA).

**Chemicals**

AH23848, AH6809, Bisindolylmaleimide (Bis), dibutyryl cyclic AMP (db-cAMP), Insulin, KN93, Phorbol 12-myristate 13-acetate (PMA), poly-L-lysine, Prostaglandin E₂ (PGE₂) and SC 19220 were all purchased from Sigma (St. Louis, MO, USA). L-798, 106 was acquired from Tocris Bioscience (Bristol, UK). DMEM, Fetal calf serum and the antibiotic-antimycotic solution were purchased from Gibco Life Technologies (Grand Island, NY, USA). 17-Phenyl Trinor PGE₂ was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

**Statistical analyses**

Statistical analyses were performed with a Student’s t-test with non-paired or paired comparisons, as relevant. Values are given as the means ± SEM, with n representing the number of cells tested. A value of p<0.05 was considered a significant difference between groups. When multiple comparisons were made, the
data were analyzed by one-way ANOVAs followed by the Tukey and Fisher LSD test for samples of more than two. All statistical tests were performed using OriginPro software (OriginLab Corporation, Northampton, MA, USA).

Results

In order to investigate whether PGE\(_2\) modified GABA\(_A\) receptor current amplitudes in CGNs, GABA\(_A\) currents were recorded while the membrane potential was held at −70 mV. 100 µM GABA was given for 3 s with a gravity perfusion system to induce an inward Cl\(-\) current. There was a 40 s interval between each GABA perfusion. Incubation of CGNs with PGE\(_2\) for 1 h significantly augmented GABA\(_A\) currents (Fig. 1A) by 42.6 ± 18.3% (\(n = 8, p < 0.05\)). A PGE\(_2\) concentration of less than 1 µM did not increase GABA\(_A\) current amplitude. In the presence of either 100 nM or 500 nM PGE\(_2\), GABA\(_A\) current amplitude was 99.9 ± 15.5% (\(n = 7, p > 0.05\)) and 93.4 ± 13.5% (\(n = 4, p > 0.05\)) of control (without PGE\(_2\)), respectively. We noted that incubating CGNs with a higher concentration of PGE\(_2\) did not lead to an increasing effect on GABA\(_A\) currents (Fig. 1B). Pre-treatment with 5 µM or 10 µM PGE\(_2\) resulted in a small increase in current amplitude (11.2 ± 9.2% (\(n = 9\)) or 25.0 ± 15.5% (\(n = 6\)), respectively (Fig. 1A). The effect of PGE\(_2\) on GABA\(_A\) currents was time dependent, as incubating CGNs with 1 µM PGE\(_2\) for 15 min, 30 min, and 1 h increased GABA\(_A\) current amplitude by 20.0 ± 9.4%, 42.7 ± 13.7% (\(p < 0.05\)) and 42.6 ± 18.3% (\(p < 0.05\)), respectively (Fig. 1C and 1D, \(n = 8\) to 9, compared to controls).

Since previous studies have indicated that GABA\(_A\) receptor activity can be modulated by activation of protein kinase A [28], we incubated CGNs with a cAMP analog (dibutyryl cAMP, db-cAMP) for 1 h in the bath solution to ascertain whether a PKA-dependent pathway was involved in the effects seen with PGE\(_2\). However, 20 µM db-cAMP was unable to mimic PGE\(_2\) induced increases in GABA\(_A\) current. On the contrary, it reduced current amplitude by 28.6 ± 6.1% (\(n = 8\)) (Fig. 2A).

Since PGE\(_2\) has been shown to increase intracellular calcium and activate PKC in chondrocytes [29], we next sought to determine whether a PKC-dependent pathway was involved in the PGE\(_2\)-induced increases of GABA\(_A\) currents. Incubating CGNs with 100 nM PMA,
Fig. 2. Effect of activation of PKC pathway on PG_{2}-induced increases of GABA_{A} currents. A, Control currents and currents following the application of 20 µM db-cAMP for 1 h (Upper Panel), and statistical analysis of the effect of chronic application of db-cAMP on GABA_{A} currents (Lower Panel). B, Control currents and currents following the application of 100 nM PMA for 1 h (Upper Panel), and statistical analysis of the effect of PKC activation on GABA_{A} currents (Lower Panel). C, Current trace in the present of 10 µM Bisindolylmaleimide (Bis) with or without 1 µM PG_{2} for 1 h (Upper Panel), and statistical analysis of the effect of Bis in chronic PG_{2}-induced increases of GABA_{A} currents (Lower Panel). D, Current trace in the present of 10 µM KN93 with or without 1 µM PG_{2} for 1 h (Upper Panel), and statistical analysis of the effect of KN93 on chronic PG_{2}-induced increases of GABA_{A} currents (Lower Panel). Data are means ± SEM. *, p < 0.05 for two groups connected with a straight line.

Fig. 3. Time-dependent effects of PG_{2}-induced increases on the phosphorylation level of PKC (pPKC). A, Representative Western blot analysis showing the levels of activated PKC (pPKC) after incubation with 1 µM PG_{2} for 5 min to 75 min. B, Quantitative analysis of the levels of pPKC normalized to levels of GAPDH. *, p < 0.05 compared with control (without PG_{2}) by one-way ANOVA.
Moreover, we found that inhibitors of both PKC (Bisindolylmaleimide (Bis)) and CaMKII (KN93) attenuated the effects of PGE$_2$-induced increases in GABA$_A$ currents (Fig. 2C and 2D). In the present of Bis, PGE$_2$ did not increase GABA$_A$ receptor currents, but rather resulted in a slight decrease of GABA$_A$ receptor current amplitude (13.0 ± 7.8%, $p > 0.05$, compared with Bis alone). Application of KN93 resulted in an even smaller PGE$_2$ effect on GABA$_A$ receptor currents, with an increase of only 0.2 ± 18.6% ($p > 0.05$, compared with KN93 alone, $n = 8$). Take together these data suggest that both calcium- and PKC-dependent pathways are involved in PGE$_2$-induced increases in GABA$_A$ currents.

Given our data supporting a role for PKC, we then used Western blotting with phospho-specific antibodies to measure the levels of phosphorylated PKC isoform (pPKC) in response to application of PGE$_2$. PGE$_2$ significantly increased pPKC levels and this effect was time dependent (Fig. 3A and 3B). After incubating CGNs with PGE$_2$ for 5 min, 15 min, 30 min, or 45 min, we found that pPKC levels were increased by 25.0 ± 3.2%, 30.0 ± 8.3%, 32.0 ± 7.1%, and 45.0 ± 9.6%, respectively ($n = 5$, $p < 0.05$ compared with control by one-way ANOVA). Thus, the effect of PGE$_2$ on intracellular pPKC levels in CGNs paralleled its effects on GABA$_A$ receptor currents (Fig. 1C and 1D).

The effects of PGE$_2$ are mediated through a family of G-protein-coupled EP receptors, including EP1-EP4 [10, 11]. Among the four type of EP receptor, EP2 and EP4 couple to increase the amount of intracellular cAMP concentration and subsequently activate PKA
We next sought to determine whether the two remaining receptors, EP1 and EP3, mediated the PGE$_2$-induced increases of GABA$_A$ currents and pPKC levels. This was accomplished pharmacologically by using specific antagonists and a general agonist of EP1 and EP3 receptors.

The present of 20 µM SC19220, an EP1 receptor antagonist, resulted in a reduction of PGE$_2$-induced increases of GABA$_A$ receptor currents (2.2 ± 16.4%, Fig 4A and 4C, n = 11, p>0.05 compared with SC19220 alone). However, an EP3 receptor antagonist, L-798,106, was unable to significantly reduce the effect of PGE$_2$ on GABA$_A$ receptor currents (Fig.4B and 4C). When 20 µM L-798,106 was applied, the effect of PGE$_2$ on GABA$_A$ receptor currents remained at 49.7 ± 27.2% (n = 8, p<0.05 comparing with L-798,106 alone).

Biochemical analysis revealed a similar effect of EP1 and EP3 antagonists on the phosphorylation levels of PKC. Application of SC19220 significantly reduced the effects of PGE$_2$ on pPKC levels. In the presence of SC19220, PGE$_2$ only increased pPKC levels by 12.0 ± 7.8% (n = 5, p>0.05 compared with control). To further verify that only EP1 was responsible for the intracellular effects of PGE$_2$, we administered either an EP2 or EP4 receptor antagonist, AH6809 or AH23848, at concentrations that we have previously demonstrated to be effective [25, 32]. Either antagonist was unable to eliminate the effects of PGE$_2$ on pPKC levels (Fig. 4D). Contrastingly, 1 µM 17-Phenyl Trinor PGE$_2$, which is an agonist of the EP1/EP3 receptor, successfully mimicked the effect of PGE$_2$, leading to an increase of GABA$_A$ currents by 67.7 ± 20.3% (Fig. 4E, n = 10, p<0.05 compared with control). Taken together, these results suggest that the PKC pathway is activated by the EP1 receptor, which mediates the PGE$_2$-induced increases in GABA$_A$ currents.

The increase in GABA$_A$ receptor current could also be due to an increase in the sensitivity to GABA. To investigate this idea, we conducted a dose-response study by applying different concentrations of GABA to CGNs, without or with PGE$_2$ treatment. Currents were recorded while holding the membrane potential at −70 mV. We chose a range of different GABA concentrations, from 100 nM to 1 mM, to induce GABA receptor current in CGNs with 20 s intervals between the applications of each concentration (Fig.5A). The resulting data were fitted with the Hill equation: $I = I_{max}/(1 + (EC_{50}/[GABA])^n)$, where the GABA-induced current I is a function of the GABA concentration, EC$_{50}$ is the GABA concentration required for inducing a half-maximal current, $n_H$ is the Hill coefficient, and $I_{max}$ is the maximum current. The maximum current was then used to normalize the dose-response curve for each individual cell.
trace. The average of the normalized currents for each GABA concentration was used to plot the data. Results indicated that upon pre-treatment with PGE$_2$, the GABA$_A$ receptor current was significantly increased (Fig. 5B). However, there was no significant dose-dependent relationship between GABA concentration, GABA$_A$ receptors, and application of PGE$_2$ (Fig. 5C, compared to control without PGE$_2$). The average EC$_{50}$ was 11.8 ± 2.0 μM for control and 15.0 ± 3.1 μM for PGE$_2$ ($n=7$, $p>0.05$ compared with control). These results indicate that the effect of PGE$_2$ increases on GABA$_A$ receptor current is not due to an increase in sensitivity to GABA.

It is possible that the increase in GABA$_A$ receptor current is the result of either an upregulation of receptor expression levels or an increase in its trafficking to the cell surface. To determine whether either of these responses was underlying the change in current, we used an anti-GABA$_A$β 1/2/3 antibody to measure both total and surface GABA$_A$ receptor levels. This subunit was chosen due to previous immunocytochemical work [33] showing the expression of all three types (β1, β2 and β3) in the granule cell layer. Western blotting data obtained from three independent experiments indicated that there was no significant increase in total protein levels of the GABA$_A$β1/2/3 subunit following 1 h of treatment with 1μM PGE$_2$ (Fig. 6A, $n=3$, $p>0.05$ compared with control). To determine surface levels, both control and PGE$_2$-treated neurons were labeled using a biotinylation assay, and biotinylated GABA$_A$β subunit levels were then detected by immunoblotting with an anti-GABA$_A$β 1/2/3 specific antibody. The membrane bound Na-K ATPase was used as a control for the membrane fraction. As shown in Fig. 6B, β 1/2/3 subunit levels were significantly increased (39.2 ± 8.3%, $n=11$) in the membrane fractions after 1 h of PGE$_2$ treatment. Levels of Na-K ATPase in the membrane fractions were unaffected. Moreover, PGE$_2$ was unable to increase surface level expression of the β 1/2/3 subunit when either PKC or CaMKII was blocked with Bis or KN93 (Fig. 6C and 6D). In the presence of Bis or KN93, the surface levels of β 1/2/3 subunit were 108.1 ± 11.9% ($n=5$, $p>0.05$ compared with Bis alone) and 98.0 ± 17.1% ($n=5$, $p>0.05$ compared with
KN93 alone) of its corresponding control. Also the effects of Bis or KN93 on surface levels of 1/2/3 subunit were limited. Collectively, these findings suggest that PGE₂ increases to GABA_A receptor currents are due to (i) an upregulation of GABA_A receptor surface levels and (ii) an association with either a PKC- or CaMKII-dependent pathway.

Discussion

In the present study, we have demonstrated that incubation of CGNs with PGE₂ increases GABA_A currents through an increase of cell surface GABA_A receptors protein expression. This effect was induced by activation of EP1 receptor-coupled PKC and calmodulin/CaMKII pathways.

Numerous studies have indicated that the physiological activity of PGE₂ is mediated by the EP family of receptors, which consists of four isoforms, EP1-EP4 [10, 11]. EP2 and EP4 receptors couple to increase the concentration of intracellular cAMP [30, 31], but EP3 couples with a resulting decrease in intracellular cAMP levels [34]. The EP1 receptor activates phospholipase C (PLC), which mediates activation of Ca²⁺/phospholipid-dependent protein kinase (PKC) and elevation of cytosolic free calcium [35]. Although our previous study revealed that all the four types of EP receptors are expressed in CGNs [25], pharmacological experiments with both EP receptor-specific antagonists and agonists indicated that PGE₂-induced increase of GABA_A currents is mediated mainly by activation of the EP1 receptor. This is partially consistent with Suzuki et al’s report and explain their findings of which the centrally administered PGE₂ exhibited anxiolytic-like activity through EP1 and EP4 receptors and the GABA_A receptor [9].

In this study, we discovered that the PGE₂ concentrations required to increase the GABA_A currents falls into a narrow range. Applying 1 µM PGE₂ increased GABA_A currents by 42.6 ± 18.3%, while 10 µM of PGE₂ led to a current increase of only 25.0 ± 15.5%. Comparing to our previous report in which 10 µM of PGE₂ applying by perfusion increased voltage-gated Na⁺ current (I_Na) through EP2 receptor coupled PKA signal pathway [25], it is likely that concentrations of PGE₂ greater than 1 µM might activate EP2 or other types of EP receptors CGNs simultaneously, which offsets the EP1 receptor-driven effect on GABA_A currents. Very small changes in PGE₂ concentration cause different effects, implying that it may be a fine tuning mechanism by which neurons adjust their response to PGE₂ through the multiple EP receptors expressed in the same cells. Due to the fact that the exogenous PGE₂ was given at concentrations higher than the physiological range in our studies [25], the affinity of EP1-4 to exogenous PGE₂ in CGNs, and the physiological or pathological relevance of our report need further exploration.

It is generally accepted that the EP1 receptor activates phospholipase C (PLC), which mediates activation of Ca²⁺/phospholipid-dependent protein kinase (PKC) and elevation of cytosolic free calcium [35]. Studies in granule cells of the dentate gyrus have provided evidence that PKC enhances mIPSC amplitudes [36]. Furthermore, it has been shown that PKC causes an enhancement of receptor function in α1β1γ2L expressing cell lines [37] and an increase in mIPSC amplitudes mediated by αβ3γx receptors [38]. In addition to PKC, calcium/calmodulin-dependent activation of CaMKII was reported to increase the number of cell surface GABA_A receptors by phosphorylation of S383 within the β3 subunit of the GABA_A receptor [39]. It is interesting to note that PGE₂/EP1 enhanced intracellular PKC levels in our study. Furthermore, that either a PKC or calmodulin/CaMKII inhibitor reduced the PGE₂-induced increases of GABA_A currents. We thereby suggest that as they are downstream of EP1/PLC/Ca²⁺, both PKC- and calmodulin/CaMKII-dependent pathways are associated with the effects of PGE₂ on increases of GABA_A currents.

PKA is also known to be a modulator of GABA_A receptors, as early studies have indicated that GABA_A receptor activity can be reduced by acute activation of protein kinase A [40], and its expression can be modulated by chronic treatment of intracellular cyclic AMP (cAMP) in granule cells [41]. In this study, PGE₂-induced increases of GABA_A currents was not shown.
to be associated with the activation of the PKA pathway, as our results demonstrated db-cAMP-driven PKA activation induced only a small reduction of GABA$_A$ current.

The effect of PKC activation on GABA$_A$ receptors is diverse, and appears to be dependent on subunit composition [42, 43]. There is some evidence suggesting that PKC upregulates both GABA$_A$ receptor cell-surface expression and their stability at the membrane in subtypes that mediate tonic inhibition [44]. However, in vitro systems expressing both transfected $\alpha$2$\beta$2y2 and native receptors in cultured cortical neurons have shown that PKC activity leads to a decrease in cell-surface GABA$_A$ receptors and associated currents [45, 46]. Interestingly, this effect appears to be independent of direct phosphorylation of the GABA$_A$ receptor, and might involve phosphorylation of some other protein in the endocytic cascade [47]. The diversity of effects reported may reflect variations in experimental protocols, experimental systems, or the distinct mechanism(s) of kinase activation which underlying the effect of PKC activation [43].

Our results show that activation of PKC by PGE$_2$/EP1 did not increase total GABA$_A$ receptor expression levels in CGNs, but significantly increased cell surface levels. This effect was then reduced by a PKC blocker (Fig. 6). Thus, it is highly likely that GABA$_A$ receptors can be upregulated by PKC by increasing GABA$_A$ receptor cell surface expression. We noted that this result seemed to be in conflict with a report by Balduzzi et al. [45] in which phorbol esters decreased cell surface expression of GABA$_A$ receptors in cultured rat cerebellar granule cells. However, since the effect of PKC inhibitors was not tested, it is unknown if this response was due to activation of PKC, and also direct effects of phorbol esters on membrane trafficking cannot exclude. Additionally, we relied on measuring membrane protein levels of all three GABA$_A$ receptor $\beta$ subunits (1-3) with Western blot analysis. This stand in contrast to the method of Balduzzi et al., which relied on immunocytochemical experiments. Considering that the CGN subunit composition of GABA$_A$ receptors is specific and developmentally dependent, a closer analysis of the effects of the PGE$_2$/EP1/PKC pathway on the specific subunit composition of different GABA$_A$ receptors will be needed in the future. Finally, it also remains to be determined whether PKC phosphorylated additional protein(s) in the endocytic or exocytic cascade.

When compared to PKC, the report about the effects of calmodulin/CaMKII on GABA$_A$ receptors was less in previous studies. A study by Marsden et al. [48] in hippocampal neurons indicated that an NMDA-dependent increase in surface GABA$_A$ receptors required Ca$^{2+}$ and CaMKII. Additionally, that the GABA receptor-associated protein (GABARAP) was involved. Recent work has demonstrated that CaMKII is able to increase levels of GABA$_A$ receptors at the cell surface numbers by phosphorylation of S383 within the $\beta$3 subunit of the GABA$_A$ receptor [39]. Similarly, in our study, inhibition of CaMKII activity reduced the PEG2/EP1-driven increase of surface GABA$_A$ receptor levels. This result indicates that the CaMKII increases to GABA$_A$ currents are also mediated by the increase in levels of GABA$_A$ receptors cell surface expression. However, whether the phosphorylation of S383 within the $\beta$3 subunit or where GABARAP are associated with CaMKII-induced increases of GABA$_A$ receptors in CGNs remains to be seen.

Taken together, our study demonstrated a function for PGE$_2$ on modulation of neuronal GABA$_A$ receptor currents. Our results also provide mechanistic evidence for a role of EP1 receptor-mediated PKC and CaMKII activation, resulting in an increase in GABA$_A$ receptor membrane expression. However, we have yet to determine the involvement of either direct phosphorylation of GABA$_A$ receptor by activation of PKC or CaMKII or the role of GABA$_A$ receptor trafficking to the membrane surface. Despite these limitations the intersection of GABA$_A$ receptors and PGE$_2$ should not be underestimated. GABA$_A$ receptor activity is crucial to neuronal excitability and underlies many neurological disorders [19, 30]. Furthermore the importance of PGE$_2$ to fever, inflammation [49], and neurological processes such as hippocampal long-term potentiation [50] and synaptogenesis in the developing preoptic area [51, 52] has been revealed. Given that, the experimental evidence presented here offers a foundation to understand how changes in neuronal activity change are associated with enhanced levels of PGE$_2$ in physiological and pathological conditions.
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Disclosure Statement

The authors declare that they have no conflicts of interest related to this work.

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