Original Paper

PGE₂ Modulates GABA_A Receptors via an EP1 Receptor-Mediated Signaling Pathway

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Key Words

PGE₂ • GABA_A receptors • Cerebellar granule cells • EP receptor • PKC

Abstract

Aims: PGE₂ 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 PGE₂ 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 β1/2/3 subunits was carried out by Western blot analysis. **Results:** Upon incubation of neurons with PGE₂ (1 μM) for 60 minutes, GABA_A currents were significantly potentiated. This PGE₂-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 PGE₂ did not increase the total expression level of GABA_A receptors, but significantly increased surface levels of GABA_A β1/2/3 subunits after 1 h of treatment. Consistently, both PKC and CaMKII inhibitors were able to reduce PGE₂-induced increases in cell surface expression of GABA_A receptors. **Conclusion:** Activation of either the PKC or CaMKII pathways by EP1 receptors mediates the PGE₂-induced increase in GABA_A currents. This suggests that upregulation of postsynaptic GABA_A receptors by PGE₂ may have profound effects on cerebellar functioning under physiological and pathological conditions.

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Introduction

Prostaglandin E₂ (PGE₂) 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, PGE₂ 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 PGE₂ 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 PGE₂ exhibited anxiolytic-like activity in the elevated plus-maze and open field test in mice [9]. Thus, the effect of PGE₂ associated neuronal excitability is gaining attention.

The physiological activity of PGE₂ 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 (Gα_s) or inhibitory (Gα_i) subunit, which can subsequently modulate the levels of Ca²⁺, cyclic AMP (cAMP), and inositol phosphate [12]. When taken in combination with the ubiquitous production of PGE₂, it is clear that EP receptor signaling accounts for the pleiotropic ability of PGE₂ to potentially activate diverse biological effects. Past genetic deletion and selective inhibition studies have examined each type of EP receptor, attempting to clarify the various roles PGE₂ 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]. Contrastingly, 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 PGE₂, 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_ARs). These receptors are heteropentameric, GABA-gated chloride channels that belong to the Cys-loop ligand-gated ion channel superfamily [15]. GABA_ARs 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_AR-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_ARs is required for an understanding of the underlying causes of their physiological and pathological functioning. Previous studies indicated GABA_ARs activity was regulated by both endogenous molecules, Zn²⁺ [22], and exogenous drugs, including the widely prescribed anxiolytics, benzodiazepines [23]. Recently, investigation by Suzuki et al's indicated that PGE₂ 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 PGE₂ 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 PGE₂ 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 10⁶ 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 GABA_A 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 (HRP) 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 PGE₂ modified GABA_A receptor current amplitudes in CGNs, GABA_A currents were recorded while the membrane potential was held at -70 mV. 100 μ M 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 PGE₂ for 1 h significantly augmented GABA_A currents (Fig. 1A) by $42.6 \pm 18.3\%$ ($n = 8$, $p < 0.05$). A PGE₂ concentration of less than 1 μ M did not increase GABA_A current amplitude. In the presence of either 100 nM or 500 nM PGE₂, GABA_A current amplitude was $99.9 \pm 15.5\%$ ($n = 7$, $p > 0.05$) and $93.4 \pm 13.5\%$ ($n = 4$, $p > 0.05$) of control (without PGE₂), respectively. We noted that incubating CGNs with a higher concentration of PGE₂ did not lead to an increasing effect on GABA_A currents (Fig. 1B). Pre-treatment with 5 μ M or 10 μ M PGE₂ resulted in a small increase in current amplitude ($11.2 \pm 9.2\%$ ($n = 9$) or $25.0 \pm 15.5\%$ ($n = 6$)). The effect of PGE₂ on GABA_A currents was time dependent, as incubating CGNs with 1 μ M PGE₂ for 15 min, 30 min, and 1 h increased GABA_A current amplitude by $20.0 \pm 9.4\%$, $42.7 \pm 13.7\%$ ($p < 0.05$) and $42.6 \pm 18.3\%$ ($p < 0.05$), 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 PGE₂. However, 20 μ M db-cAMP was unable to mimic PGE₂-induced increases in GABA_A current. On the contrary, it reduced current amplitude by $28.6 \pm 6.1\%$ ($n = 8$) (Fig. 2A).

Since PGE₂ 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 PGE₂-induced increases of GABA_A currents. Incubating CGNs with 100 nM PMA,

Fig. 1. Effect of PGE₂ on CGN GABA_A receptor currents. A, Current trace for the effect of a 1 h incubation of 1 μ M PGE₂ on GABA_A receptor currents. B, Statistical analysis of the effect of different PGE₂ concentrations on GABA_A receptor current amplitudes. Data are means $\pm$ SEM. *, $p < 0.05$ for two groups connected with a straight line. C, Representative current trace in the absent or presence of 1 μ M PGE₂ for 15 min and 60 min. D, Statistical analysis of the time-dependent effect of 1 μ M PGE₂ on GABA_A currents. Data are means $\pm$ SEM. *, $p < 0.05$ compared with control (without PGE₂).

Fig. 2. Effect of activation of PKC pathway on PGE₂-induced increases of GABA_A currents. A, Control currents and currents following the application of 20 μ M 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 presence of 10 μ M Bisindolylmaleimide (Bis) with or without 1 μ M PGE₂ for 1 h (Upper Panel), and statistical analysis of the effect of Bis in chronic PGE₂-induced increases of GABA_A currents (Lower Panel). D, Current trace in the presence of 10 μ M KN93 with or without 1 μ M PGE₂ for 1 h (Upper Panel), and statistical analysis of the effect of KN93 on chronic PGE₂-induced increases of GABA_A currents (Lower Panel). Data are means $\pm$ SEM. *, $p < 0.05$ for two groups connected with a straight line.

Fig. 3. Time-dependent effects of PGE₂-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 μ M PGE₂ 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 PGE₂) by one-way ANOVA.

a PKC activator, in the bath solution for 1 hour was able to partially mimic PGE₂ enhancement of GABA_A currents (Fig. 2B) and increase GABA_A current amplitude by $20.4 \pm 7.2\%$ ($n = 28$,

Fig. 4. Effects of EP1 and EP3 antagonists and EP1/EP3 agonist on PGE₂-induced augmentation of GABA_A currents. A, Current trace in the presence of 20 μ M EP1 receptor antagonist SC19220 with or without 1 μ M PGE₂ for 1 h. B, Current trace in the presence of 20 μ M EP3 receptor antagonist L-798,106 with or without 1 μ M PGE₂ for 1 h. C, Statistical analysis of the effects of SC19220 and L-798,106 (L-798) on PGE₂-induced increases of GABA_A currents. D, Western blot analysis of the effect of SC19220 (SC19), AH23848 (AH23), L-798,106, and AH6809 (AH68) on pPKC levels in the presence and absence of 1 μ M PGE₂. E, Current traces and statistical analysis of the effect of EP1/EP3 receptor agonist 20 μ M 17-Phenyl Trinor PGE₂ (17-pt PGE₂) on chronic PGE₂-induced increases of GABA_A currents. Data are means $\pm$ SEM. *, $p < 0.05$ for two groups connected with a straight line.

$p < 0.05$). Moreover, we found that inhibitors of both PKC (Bisindolylmaleimide (Bis)) and CaMKII (KN93) attenuated the effects of PGE₂-induced increases in GABA_A currents (Fig. 2C and 2D). In the presence of Bis, PGE₂ did not increase GABA_A receptor currents, but rather resulted in a slight decrease of GABA_A receptor current amplitude ($13.0 \pm 7.8\%$, $p > 0.05$, compared with Bis alone). Application of KN93 resulted in an even smaller PGE₂ effect on GABA_A receptor currents, with an increase of only $0.2 \pm 18.6\%$ ($p > 0.05$, compared with KN93 alone, $n = 8$). Taken together these data suggest that both calcium- and PKC-dependent pathways are involved in PGE₂-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 PGE₂. PGE₂ significantly increased pPKC levels and this effect was time dependent (Fig. 3A and 3B). After incubating CGNs with PGE₂ for 5 min, 15 min, 30 min, or 45 min, we found that pPKC levels were increased by $25.0 \pm 3.2\%$, $30.0 \pm 8.3\%$, $32.0 \pm 7.1\%$, and $45.0 \pm 9.6\%$, respectively ($n = 5$, $p < 0.05$ compared with control by one-way ANOVA). Thus, the effect of PGE₂ on intracellular pPKC levels in CGNs paralleled its effects on GABA_A receptor currents (Fig. 1C and 1D).

The effects of PGE₂ are mediated through a family of G-protein-coupled EP receptors, including EP1-EP4 [10, 11]. Among the four types of EP receptor, EP2 and EP4 couple to increase the amount of intracellular cAMP concentration and subsequently activate PKA

Fig. 5. Effect of incubation of CGNs with PGE₂ on the GABA sensitivity. A, GABA currents elicited by different concentrations of GABA applied to cell with a gravity perfusion system in the presence or absence of 1 μ M PGE₂. B, Curve of GABA_A receptor current amplitude as a function of GABA dose in the presence or absence of 1 μ M PGE₂. C, Plot of the normalized current amplitude as a function of GABA dose in the presence or absence of 1 μ M PGE₂ treatment; Data points were fitted using the Hill function.

Data are means $\pm$ SEM. *, $p < 0.05$ compared with control (without PGE₂).

[30, 31]. We next sought to determine whether the two remaining receptors, EP1 and EP3, mediated the PGE₂-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 μ M SC19220, an EP1 receptor antagonist, resulted in a reduction of PGE₂-induced increases of GABA_A receptor currents ($2.2 \pm 16.4\%$, 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 PGE₂ on GABA_A receptor currents (Fig. 4B and 4C). When 20 μ M L-798,106 was applied, the effect of PGE₂ on GABA_A receptor currents remained at $49.7 \pm 27.2\%$ ($n = 8$, $p < 0.05$ 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 PGE₂ on pPKC levels. In the present of SC19220, PGE₂ only increased pPKC levels by $12.0 \pm 7.8\%$ ($n = 5$, $p > 0.05$ compared with control). To further verify that only EP1 was responsible for the intracellular effects of PGE₂, 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 PGE₂ on pPKC levels (Fig. 4D). Contrastingly, 1 μ M 17-Phenyl Trinor PGE₂, which is an agonist of the EP1/EP3 receptor, successfully mimicked the effect of PGE₂, leading to an increase of GABA_A currents by $67.7 \pm 20.3\%$ (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 PGE₂-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 difference concentrations of GABA to CGNs, without or with PGE₂ 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: $I = I_{\max} / (1 + (EC_{50} / [GABA])^{n_H})$, 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

Fig. 6. Effect of PGE₂ on total and surface levels of GABA_A β subunit expression level in CGNs as measured by Western blot analysis. A, Representative Western blot and statistical analysis of the effect of 1 μ M PGE₂ on total GABA_A β subunit expression levels. The expression level of GAPDH was used as the loading control. B, The effect of 1 μ M PGE₂ on surface levels of GABA_A β subunit as determined by biotinylation. The expression level of Na-K ATPase was used as the loading control. C and D, Effect of PKC and CaMKII inhibitors on PGE₂-induced increases in surface GABA_A β subunit levels. Data are means $\pm$ SEM. *, $p < 0.05$ for two groups connected with a straight line.

trace. The average of the normalized currents for each GABA concentration was used to plot the data. Results indicated that upon pre-treatment with PGE₂, 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 PGE₂ (Fig. 5C, compared to control without PGE₂). The average EC₅₀ was $11.8 \pm 2.0 \mu\text{M}$ for control and $15.0 \pm 3.1 \mu\text{M}$ for PGE₂ ($n = 7$, $p > 0.05$ compared with control). These results indicate that the effect of PGE₂ 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 (β 1, β 2 and β 3) 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 β 1/2/3 subunit following 1 h of treatment with 1 μM PGE₂ (Fig. 6A, $n = 3$, $p > 0.05$ compared with control). To determine surface levels, both control and PGE₂-treated neurons were labeled using a biotinylation assay, and biotinylated GABA_A β 1/2/3 proteins 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 ($39.2 \pm 8.3\%$, $n = 11$) in the membrane fractions after 1 h of PGE₂ treatment. Levels of Na-K ATPase in the membrane fractions were unaffected. Moreover, PGE₂ 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 $108.1 \pm 11.9\%$ ($n = 5$, $p > 0.05$ compared with Bis alone) and $98.0 \pm 17.1\%$ ($n = 5$, $p > 0.05$ 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 αxβ3γx 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 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 $\alpha 1\beta 2\gamma 2$ 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 PGE₂/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 β 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 PGE₂/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 endocytotic or exocytotic 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 Ca²⁺ and CaMKII. Additionally, that the GABA receptor-associated protein (GABARAP) was involved [48]. 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 PGE₂ 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 PGE₂ should not be underestimated. GABA_A receptor activity is crucial to neuronal excitability and underlies many neurological disorders [19, 30]. Furthermore the importance of PGE₂ 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 PGE₂ 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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