Pharmacological Characterization of the Prostanoid Receptor EP3 in Locus Coeruleus Neurons by Single-Unit Extracellular Recordings in the Rat Brain in Vitro

Amaia Nazabal¹, Aitziber Mendiguren¹ and Joseba Pineda¹*

¹ Department of Pharmacology, Faculty of Medicine and Odontology, University of the Basque Country (UPV/EHU), E-48940 Leioa, Bizkaia, Spain
* Author to whom correspondence should be addressed; e-mail: joseba.pineda@ehu.es

Published: 4 December 2015

Abstract: Prostanoids are known to regulate several physiological functions and to play an important role in certain pathophysiological situations such as inflammation. Prostaglandin E₂ receptors (EP) are members of the G protein-coupled receptor superfamily. Four subtypes have been described: EP2 and EP4 (coupled to Gₛ proteins) and EP1 and EP3 (coupled to Gᵥ/o proteins). To date, the function of the prostanoid system in the brain has not been well characterized. The locus coeruleus (LC), the main noradrenergic nucleus in the brain, has been described to express the EP3 receptor. The aim of this study was to characterize the functional relevance of EP3 receptors in the LC by single-unit extracellular recordings in rat brain slices. We performed concentration-effect curves for different endogenous derivatives and selective agonists of EP3 receptors. Thus, increasing concentrations of the EP3/EP1 agonist sulprostone (0.3-80 nM) fully inhibited the neuronal activity of LC cells, with an EC₅₀ value of 15 nM (n = 9). The EP3 receptor antagonist L-798,106 (10 µM) caused a rightward shift (> 8 fold) in the concentration-effect curve for sulprostone, but the EP2 receptor antagonist PF04418948 (10 µM) or the EP4 receptor antagonist L-161,982 (10 µM) failed to cause any rightward shift of sulprostone effect. On the other hand, perfusion with the endogenous PGE₂ (0.3 nM-1.28 µM) or the PGE₁ analogue misoprostol (0.3-320 nM) induced a concentration-dependent inhibition of the firing rate of LC cells, with EC₅₀ values being 51 nM and 112 nM, respectively. Likewise, only the EP3 antagonist L-798,106 (10 µM) caused a rightward shift (> 8 fold) in the concentration-effect curves for these prostanoid agonists (n = 5). In conclusion, LC neurons are regulated in an inhibitory manner by the prostanoid system likely through the EP3 receptor.

Keywords: in vitro; locus coeruleus; electrophysiology; EP3 receptor; prostanoid; PGE2; sulprostone; slice
1. Introduction

Prostanoids system is involved in the regulation of pain, fever and inflammation processes. During inflammation, membrane phospholipids are transformed into arachidonic acid (AA) by the phospholipase A2 enzyme. In turn, AA is metabolized by cyclooxygenase (COX) into PGH₂, which is the common prostanoid precursor of other prostaglandins including prostacyclins and thromboxanes (Yagami et al. 2015). COX-blockers or NSAIDs -non-steroidal anti-inflammatory drug- have been widely used as analgesic, antipyretic and antiinflammatory drugs due to their ability to suppress the production of prostanoids.

Two isoforms of COX (-1 and -2) have been characterized, but the isoform that has been shown to be constitutively expressed in the body is the isoform 1. However, recent studies have suggested that COX-2 could also be constitutively expressed in human and animal brain (Hétu & Riendeau 2005; Martin et al. 2007; Yaksh et al. 2001). This supports the idea of a possible important role of prostaglandins in the central nervous system. Nevertheless, the function of prostaglandins remains unclear since dual effects have been described in the brain. On one hand, PGE₂ seems to promote neuroprotection in injured brain by induction of either BDNF release or reducing the expression of inducible oxide nitric synthase (iNOS) during inflammation (Hutchinson et al. 2009; Levi et al. 1998). On the other hand, EP3 antagonists exert protective effects in the brain after ischemia (Ikeda-Matsuo et al. 2011).

PGE₂ is the most abundant prostaglandin produced in the body and it acts through activation of EP1-4 receptors, which are expressed in neurons and glia (Ito et al. 2001). Classically, EP1 has been described to be coupled to Gi/o protein (Ji et al. 2010), although there is recent evidence that supports its coupling to Gq protein (Liu et al. 2010). EP3 is coupled to G<sub>i/o</sub> protein (Negishi et al. 1995), whereas EP2 and EP4 are coupled to G<sub>i</sub> protein (Fujino & Regan 2006; Sugimoto & Narumiya 2007).

The locus coeruleus (LC) is the main source of noradrenergic innervation in the brain. It is involved in the regulation of numerous physiological functions such as sleep-wake cycle, arousal, cognition/memory, pain, cardiovascular control and rewarding behavior. A role of this nucleus in the production of fever has also been proposed (Almeida et al. 2004).

Several findings have suggested an interaction between the noradrenergic/LC system and prostanoid system. First, in situ hybridization experiments have demonstrated the presence of mRNA for the EP2 and EP4 receptors in this nucleus (Zhang & Rivest 1999). Furthermore, EP3 expression has also been shown by immunoreactivity and double hybridization techniques (Ek et al. 2000; Nakagawa et al. 2000; Nakamura et al. 2001). Second, the LC has been identified as a pivotal nucleus in PGE2-induced thermogenesis (Almeida et al. 2004). Third, administration of PGE2 inhibits, via EP3 receptors the release of noradrenaline in the brain (Exner & Schlicker 1995).

Despite several evidences that suggest a possible role of the prostanoid system in the LC, the functional role of EP receptors in this nucleus remained to be studied. Therefore, the aim of our research was to characterize, by single-unit extracellular recordings in vitro, the functional role of the prostanoid receptor EP3 in LC neurons from the rat brain. For this purpose, we performed concentration-effect curves for several EP3 receptor agonists in the absence and in the presence of different EP receptor antagonists.
2. Results and Discussion

Effect of the EP3/EP1 receptor agonist sulprostone on the firing rate of LC neurons

To evaluate the effect of prostaglandins on the LC neurons, we applied increasing concentrations of the EP3/EP1 agonist sulprostone (0.3-80 nM) and we found that it fully inhibited the neuronal activity of LC cells, with an EC\textsubscript{50} value of 15 nM (n = 9). To identify the EP receptor involved in the inhibitory effect induced by sulprostone, we performed the concentration-effect curves for the EP3/EP1 agonist in the presence of specific antagonists of the EP receptors expressed in the LC: EP2 (PF04418948), EP3 (L-798,106) and EP4 (L-161,982) at 3 and 10 µM. The EP3 receptor antagonist L-798,106 (10 µM) caused a rightward shift (> 8 fold) in the concentration effect curve for sulprostone (Fig. 1). Neither the EP2 receptor antagonist PF04418948 (10 µM) nor the EP4 receptor antagonist L-161,982 (10 µM) caused any rightward shift of sulprostone effect.

These results indicate that the inhibitory effect of sulprostone is mediated by the EP3 receptor, which is known to be coupled to G\textsubscript{i/o} proteins. The inhibitory effect mediated by the EP3 receptor has been shown to occur in other nucleus at the same concentrations used in our study (Ito et al. 2000). Therefore, our results show that EP3 receptor is functionally active in the LC. Future experiments are required to characterize the relevance of other EP receptors to the modulation of the firing activity of LC cells.

Figure 1. Effect of the EP3/EP1 receptor agonist sulprostone on the firing rate of locus coeruleus cells in the absence and in the presence of the EP3 receptor antagonist L-798,106. Representative examples of firing-rate recordings of two LC cells showing the effect of increasing concentrations of sulprostone in the
absence (A) and in the presence (B) of the EP3 antagonist L-798,106. Each horizontal bar represents the period of application of each sulprostone concentration and the vertical lines show the number of spikes recorded every 10 s. The inhibitory effect induced by each application was calculated as a percentage from the basal firing rate. Note that sulprostone inhibits the firing rate of LC cells and that its inhibitory effect is diminished in the presence of L-798,106.

Effect of the endogenous PGE\(_2\) and the PGE\(_1\) analogue misoprostol on the firing rate of LC neurons

In order to study whether endogenous prostanoid compounds mimicked the effect observed with sulprostone on the LC, we applied the endogenous PGE\(_2\) and the PGE\(_1\) analogue misoprostol. Perfusion with the endogenous PGE\(_2\) (0.3 nM-1.28 \(\mu\)M) or the PGE\(_1\) analogue misoprostol (0.3-320 nM) induced a concentration-dependent inhibition of the firing rate of LC cells, with EC\(_{50}\) values being 51 nM and 112 nM respectively. Likewise, only the EP3 antagonist L-798,106 (10 \(\mu\)M) caused a rightward shift (> 8 fold) in the concentration-effect curves for these prostanoid agonists (Fig. 2; n = 5).

These results indicate that sulprostone and the endogenous derivatives act through activation of the same EP receptor; the EP3 receptor. However, sulprostone shows higher potency than the endogenous prostanoid derivatives to inhibit LC cells.

![Figure 2](image-url)

**Figure 2. Effect of the PGE\(_1\) analogue misoprostol on the firing rate of locus coeruleus cells in the absence and in the presence the EP3 receptor antagonist L-798,106.** Representative examples of firing-rate recordings of two LC cells showing the effect of increasing concentrations of misoprostol in the absence (A) and in the presence (B) of the EP3 antagonist L-798,106. Each horizontal bar represents the period of application of each misoprostol concentration and the vertical lines show the number of spikes recorded every 10 s. The inhibitory effect induced by each application was calculated as a percentage from the basal firing rate. Note that misoprostol inhibits the firing rate of LC cells and that its inhibitory effect is diminished in the presence of L-798,106.
3. Materials and Methods

3.1. Animals

Adult male Sprague-Dawley rats weighing 200-300 g were housed under controlled laboratory conditions (22 °C and 12-h light/dark cycles) with free access to food and water. The animals were obtained from the animal house of the University of the Basque Country (Leioa, Spain). All experimental procedures reported in this manuscript were conducted in accordance with U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, and with the European Community Council Directive on “Protection of Animals Used in Experimental and Other Scientific Purposes” of 24 November 1986 (86/609/EEC). The procedures were approved by the Animal Care and Use Committee of the University of the Basque Country. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2. In vitro electrophysiology

3.2.1. Brain slice preparation

In vitro experiments were performed as previously described (Mendiguren & Pineda 2007). Briefly, animals were first anaesthetized with chloral hydrate (400 mg/kg, i.p.) and sacrificed by decapitation. The brain was removed and a block of tissue containing the brainstem was placed in ice-cold modified artificial cerebrospinal fluid (aCSF) where NaCl was equiosmotically substituted with sucrose to improve neuronal viability. Coronal slices of 600 µm thickness containing the LC were cut by an oscillating vibratome and then allowed to recover from the slicing for 90 min in oxygenated aCSF. Next, slices were placed on a nylon mesh and maintained at 33 ± 0.5 C in a modified Haas-type interface chamber continuously perfused with oxygenated aCSF (95% O₂/5% CO₂, pH=7.38) at a flow rate of 1-1.5 ml/min. The aCSF contained (in mM): NaCl 130, KCl 3, NaH₂PO₄ 1.25, D-glucose 10, NaHCO₃ 21, CaCl₂ 2, and MgSO₄ 2.

3.2.2. Recording procedures

Extracellular recordings of single neurons were performed as previously described (Mendiguren & Pineda 2004). The recording electrode consisted of an Omegadot glass micropipette that was pulled and filled with a solution of 50 mM NaCl (tip size of 2-5 µm, 3-5 MΩ). The microelectrode was placed in the LC, which was visually identified in the rostral pons as a dark oval area on the lateral borders of the central gray and the 4th ventricle, just anterior to the genu of the facial nerve. The extracellular signal recorded by the microelectrode was passed through a high-input impedance amplifier system and monitored on an oscilloscope and by an audioanalyzer. Individual (single-unit) neuronal spikes were isolated from the background noise with a window discriminator and counted. The firing rate was represented and analyzed by a PC-based custom-made program, which generated histogram bars representing the cumulative number of spikes in consecutive 10 s bins. Noradrenergic neurons in the LC were identified by the following electrophysiological criteria: a spontaneous and regular discharge, a slow firing rate and a positive-negative biphasic waveform of 3-4 ms duration (Andrade & Aghajanian 1984). We only recorded cells that showed stable firing rates between 0.4 and 1.5 Hz for at least 3-5 min and strong inhibitory effects induced by ME (0.8 mM, 1 min) (higher than 80%). Only one neuron was recorded per slice and only one slice was obtained from each animal.
3.3. Pharmacological procedures

To characterize the effect of prostanoids in the LC neurons, we perfused increasing concentrations of the EP3/EP1 receptor agonist sulprostone (0.3-80 nM) and the endogenous derivatives of prostanoid system (PGE2 and misoprostol, 0.3 nM–1.28 µM). Each concentration of the EP receptor agonists was perfused for at least 1 min. We performed concentration/effect curves for the inhibitory effect of the agonists. In order to study the involvement of EP receptors in the effect observed with the EP receptor agonists, we used specific antagonist for the EP2 receptor (PF04418948), EP3 (L-798,106), and EP4 (L-161,982) at 3 and 10 µM. All the antagonists were perfused for at least 20-30 min before performing the concentration-effect curves for EP receptor agonists.

3.4. Drugs and reagents

Chloral hydrate was obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). Met\(^5\)-enkephalin (ME) was obtained from Bachem (Weil am Rhein, Germany). Sulprostone was purchased from Cayman Chemical (Michigan, USA). PF04418948, L-798,106 and L-161,982 were obtained from Tocris Bioscience (Bristol, UK). Drugs were dissolved in the final volume of aCSF just before each assay and applied by turning a threeway valve that switched from aCSF to the test solution. Stock solutions of the EP antagonist were made in DMSO stored at -25 °C and, on the day of the experiment, diluted in aCSF to their final volume. The maximal final concentration of DMSO was lower than 0.1%.

3.5. Data analysis and statistics

Values are expressed as the mean ± standard error of the mean (S.E.M) of n experiments. The firing rate of LC cells was recorded before (baseline), during and after drug applications. The inhibitory effect of EP receptor agonists was calculated as follows:

\[
E(\%) = \frac{FR_{pre} - FR_{post}}{FR_{basal}} \cdot 100
\]

where FR\(_{pre}\) is the average firing rate for 60 s before application of each concentration, FR\(_{post}\) is the average firing rate after the perfusion of each concentration, and FR\(_{basal}\) is the firing rate for 60 s of each cell at the beginning of the recording. Changes induced by drugs in the firing rate were evaluated by a paired Student's t test when compared within the same cell or by a two-sample Student's t test when compared between different cells. Additionally, to evaluate the possible differences between groups the one-way ANOVA test was used. The level of significance was considered as p=0.05. Curve fitting analysis was performed by the computer program GraphPad Prism (version 5.0 for Windows, San Diego, CA, USA) to obtain the best simple nonlinear fit to the following three-parameter logistic equation:

\[
E = E_{max} / [1 + (EC_{50} / A)^n]
\]

where E is the effect induced by each concentration of the EP3 agonist (A), E\(_{max}\) is the maximal effect, EC\(_{50}\) is the concentration of EP3 agonist needed to elicit a 50% of the maximal effect, and n is the slope factor of the concentration-effect curve.

4. Conclusions
In conclusion, LC neurons are regulated in an inhibitory manner by the prostanoid system likely through the EP3 receptor. Future experiments are required to characterize the relevance of other EP receptors to the modulation of the firing activity of LC cells.

Acknowledgments
This work was supported by the Ministerio de Ciencia e Innovación [Grant SAF2008-03612] and the University of the Basque Country (UPV/EHU) [Grant GIU11/27 and Grant GIU14/29]. Pineda’s research group takes part in a network unit supported by the University of the Basque Country [UFI 11/35]. A. Nazabal was supported by a predoctoral fellowship from the Basque Government. The experiments comply with the current laws of Spain.

Author Contributions
A. Nazabal performed the research and analysed the data. A. Mendiguren and J. Pineda designed the research study. All mentioned authors wrote the manuscript.

Conflicts of Interest
The authors declare no conflict of interest.

References and Notes


