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Signaling pathways leading to prostaglandin E_2 production by rat cerebral frontal cortex ☆

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Abstract

In this paper, we have determined the effect of both muscarinic acetylcholine receptor (mAChR) and exogenous prostaglandin E_2 (PGE_2) on PGE_2 production and cyclooxygenases (COX) mRNA gene expression on rat cerebral frontal cortex. Carbachol and PGE_2 increase endogenous PGE_2 production and the COX-1 mRNA levels by activation of PLA_2s. The COX-1 and COX-2 activity participated in the production of PGE_2 triggered by exogenous PGE_2. While in carbachol-PGE_2 only COX-1 activity is affected. The specific inhibition of PGE_2 receptor was able to impair the increase of endogenous PGE_2 production triggered by both carbachol and exogenous PGE_2. These results suggest that carbachol-activation mAChR increased PGE_2 production that in turn interacting with its own receptor triggers an additional production of PGE_2. Both mechanisms appear to occur by using PLA_2 signaling system. This data should be able to contribute to understand the involvement of PGE_2 in normal brain function and its participation in neuroinflammatory processes.

1. Introduction

Phospholipase A_2 (PLA_2) from mammalian tissues play a role in physiological functions such as defense mechanisms and the production of bioactive lipids [1–3]. In the last years, purification and molecular cloning of PLA_2 has allowed the characterization of several enzymes displaying significant differences in both structural and functional properties. There is significant confusion in the field of PLA_2 because many of the identified PLA_2s were not associated with the specific cellular activities and function [4]. There is an older classification system that still remains based on whether the PLA_2 is secreted from the cell ($sPLA_2$), calcium-dependent and cytosolic ($cPLA_2$) or calcium-independent ($iPLA_2$) [5]. These isoforms have been partially purified and characterized from brain tissue [6,7]. The $sPLA_2$ isoform require millimolar amounts of calcium for activity and behaves as an acute phase protein whose production is induced in a variety of immunoinflammatory conditions [8,9], although its causal role in these conditions has not been ascertained, and there is no clear evidence about its involvement in the release of arachidonic acid (AA) elicited by agonists. Recent studies have shown the ability of $sPLA_2$ to promote mitogenesis by acting on a cell surface receptor [10,11]. It is known that $sPLA_2$ interact with two types of surface receptors, namely the *N* type identified in neurons and the *M* type identified in skeletal muscle [12].

The $cPLA_2$ isoform require micromolar amounts of calcium for translocation to membrane and plays a central role in the release of AA triggered by neurotransmitters [13,14]. The $iPLA_2$ are located in both cytosol [15,16] and membrane fractions [17]. It should

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be mentioned that cross-talk between $sPLA_2$ and $cPLA_2$ has been suggested in signal transduction events in leucocytes and macrophages [18,19] and Kolko et al. [20] observed in neural cells a complex interplay between neurotransmitter-activated $cPLA_2$ and $sPLA_2$.

COX-1 is distributed in neurons throughout the brain, but it is most prevalent in forebrain, were prostaglandins (PGs) may be involved in complex integrative functions, such as modulation of the autonomic nervous system [21,22]. COX-2 is the predominant isoform in the brains of neonate pigs [23] and in the spinal cord of the rat [24], while human brain tissues contain equal amounts of mRNA for COX-1 and COX-2 [25].

Carbachol-triggered PLA_2-catalyzed AA release with subsequent increase in cyclooxygenase (COX) activity with increased prostaglandin E_2 (PGE_2) production has been shown to be coupled to calcium mobilization playing a central role in the release of AA triggered by neurotransmitters [26,27]. This AA can be used by COX enzyme and exert physiological functions on its own. Carbachol is also able to activate selected population of mAChRs, increasing PGE_2 production in cat [28] and rat [29] brain. Previously, we have demonstrated that the muscarinic acetylcholine receptor (mAChR) activation acts on rat cerebral frontal cortex as an early positive regulator of COX-1 mRNA gene expression, closely correlating with phospholipase C activation using common enzymatic pathways associated with the activation of M_3 mAChR [29].

Release of AA in response to mAChR agonist has been reported in astrocytoma cells that possess M_3 mAChR subtypes [30] and in cells transfected with cDNAs that code for human M_1, M_3 and M_5 mAChRs [31]. Although responses to agonist that engage G-protein-coupled receptor show some overlap, there are several distinct responses for each agonist, attributable to efficient coupling of G-protein α subunits to receptors [32] or to triggering of additional signaling pathways [33].

The aim of this work was to determine whether carbachol, mAChR agonist and exogenous PGE_2 are able to induce an increment in PGE_2 production via their own receptors. The participation of phospholipases and cyclooxygenases isoforms signal transduction underlying carbachol and exogenous PGE_2-induced PGE_2 production was studied.

In the present work, we show that the activation of rat cerebral frontal cortex mAChR by carbachol, leads to augmented production of PGE_2 preceded by an activation of PLA_2 and COX-1. This event is associated with the selective activation of PGE_2 receptor that in turn triggers an additional production of PGE_2. Also, exogenous PGE_2 through the activation of its own receptor increased the PGE_2 production employing common signaling pathways that those used by the muscarinic cholinergic agonist.

2. Materials and methods

2.1. Rat cerebral frontal cortex preparations

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires) were housed in our colony in small groups and kept in automatically controlled lighting (lights on 08:00–19:00) and uniform temperature (25 °C) conditions. All animals were used at 3–4 months of age and were cared for in accordance with the principles and guidelines of the National Institutes of Health (NIH No. 8023, revised 1978). Efforts were made to minimize animal suffering such as: killing under anesthesia and reducing the number of animals, as well as using the same animal for all enzymatic assays.

2.2. PGE_2 assay

Rat cerebral frontal cortex slices (10 mg) were incubated for 60 min in 0.50 ml of Krebs Ringer bicarbonate (KRB) gassed with 5% CO_2 in oxygen at 37 °C. Carbachol or exogenous PGE_2 were added 30 min before the end of incubation period and blockers 30 min before the addition of different carbachol or exogenous PGE_2 concentrations. Tissues were then homogenized into a 1.5 ml polypropylene microcentrifuge tube. Thereafter, all procedures employed were those indicated in the protocol of Prostaglandin E_2 Biotrak Enzyme Immuno Assay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). The PGE_2 results were expressed as picogram/milligram of tissue wet weight (pg/mg tissue wet wt).

2.3. mRNA isolation and cDNA synthesis

Total RNA was extracted from rat cerebral frontal cortex slices by homogenization using guanidinium isothiocyanate method [34]. A 20 μl reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mM dNTP and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First strand cDNA was synthesized by incubating rat cerebral frontal cortex in KRB gassed with 5% CO_2 in O_2 pH 7.4 at 37 °C for 60 min. In a selected tube, the reverse transcriptase was omitted to control for amplification from contaminating cDNA or genomic DNA.

2.4. Quantitative PCR

Quantitation of COX isoforms (COX-1, COX-2) mRNA levels was performed by a method that involves simultaneous co-amplification of both the target cDNA and a reference template (MIMIC) with a single set of primers. MIMIC for COX-1 and COX-2 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were

constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). Each PCR MIMIC consists of a heterologous DNA fragment with 5′ and 3′-end sequences that was recognized by a pair of gene-specific primers. The sizes of PCR MIMIC were distinct from those of the native targets. The sequences of oligonucleotide primer pairs used for construction of MIMIC and amplification of COX isoforms and G3PDH mRNAs are listed in Table 1. Aliquots were taken from pooled first-strand cDNA from the same group and constituted one sample for PCR. A series of 10-fold dilutions of known concentrations of the MIMIC were added to PCR amplification reactions containing the first-strand cDNA. PCR MIMIC amplification was performed in 100 μl of a solution containing 1.5 mM $MgCl_2$, 0.4 μM primer, dNTPs, 2.5U Taq DNA polymerase and 0.056 μM Taq Start antibody (Clontech Laboratories). After initial denaturation at 94 °C for 2 min, the cycle condition was 30 s of denaturation at 94 °C, 35 s of extension at 58 °C and 35 s for enzymatic primer extension at 72 °C for 45 cycles for COX isoforms. The internal control was the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). PCR amplification was performed with initial denaturation at 94 °C for 2 min followed by 30 cycles of amplification. Each cycle consisted of 35 s at 94 °C, 35 s at 58 °C and 45 s at 72 °C. Samples were incubated for an additional 8 min at 72 °C before completion. PCR products were subjected to electrophoresis on ethidium bromide-stained gels. Band intensity was quantitated by densitometry using NIH Image software. COX mRNA levels were normalized with the levels of G3PDH mRNA present in each sample, which served to check for variations in RNA purification and cDNA synthesis. The relative mRNA expression of COX was compared with those from the respective control group.

2.5. Drugs

Carbachol, atropine, trifluroperazine (TFP) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma Chemical Company, Saint Louis, MO, USA; U-73122, [1,2,4]oxadiazol[4,3-a]quinoxalin-1 one (ODQ), 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA), arachidonyl trifluoromethyl ketone (AACOCF3), 1-[4,5-bis(4-methoxyphenyl)-2thiazolyl]carbonyl-4-methylpiperazine hydrochloride (FR 122047) and 5-bromo-2-(4-flurophenyl)-3-[4-(methylsulfonyl)phenyl]-tiophene (DuP 697) were from Tocris Cookson Inc., Baldwin, MO, USA.

2.6. Statistical analysis

Student's *t*-test for unpaired values was used to determine significance levels. Analysis of variance (ANOVA) and post hoc test (Dunnett's Method and Student–Newman–Keuls test) were employed when a pairwise multiple comparison procedure was necessary. Differences between means were considered significant if $P < 0.05$.

3. Results

Fig. 1 shows the ability of carbachol to trigger PGE_2 production in a concentration-dependent manner with 1×10^{-7} M proving the maximal response and decreasing thereafter, raising values significantly higher than basal ones. Atropine (1×10^{-7} M) specifically blocked the stimulatory action of carbachol upon PGE_2 production. Histogram of Fig. 1 shows a reduction in carbachol-induced PGE_2 production in the presence of OBAA (5×10^{-6} M) and FR 122047 (5×10^{-8} M). The AACOCF3 (5×10^{-6} M) attenuated the production of PGE_2 while DuP 697 (5×10^{-8} M) was without effect. The stimulatory effect of carbachol on PGE_2 production was inhibited about 35% and 58% with OBAA at 1×10^{-6} and 2.5×10^{-6} M, respectively; while AACOCF3 at 1×10^{-6} and 2.5×10^{-6} M inhibited it about 28% and 49%, respectively.

Of particular interest is the result showed in Fig. 2, in which, a reduction in carbachol-induced PGE_2 production was observed in the presence of PGE_2 receptor blocker (SC 19220, 5×10^{-7} M). In order to discern if exogenous PGE_2 is able to trigger endogenous PGE_2 production, dose–response curves of exogenous PGE_2 were constructed in absence or in presence of the specific PGE_2 receptor antagonist.

Fig. 3 shows the ability of exogenous PGE_2 to increase endogenous PGE_2 production in a concentration-dependent manner, with 1×10^{-9} M proving the maximal

Table 1
Oligonucleotides of primers for PCR

Gene product	Sense	Antisense	Predicted size, bp
COX-1	5′ TAAGT ACCAG TGCTG GATGG 3′	5′ AGATC GTCGA GAAGA GCATCA 3′	160
COX-2	5′ TCCAA TCGCT GTACA AGCAG 3′	5′ TCCCC AAAGA TAGCA TCTGG 3′	242
G3PDH	5′ ACCAC AGTCCA TGCCAT CAC 3′	5′ TCCAC CACCC TGTTG CTGTA 3′	452

Cyclo-oxygenase 1 (COX-1), cyclo-oxygenase 2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

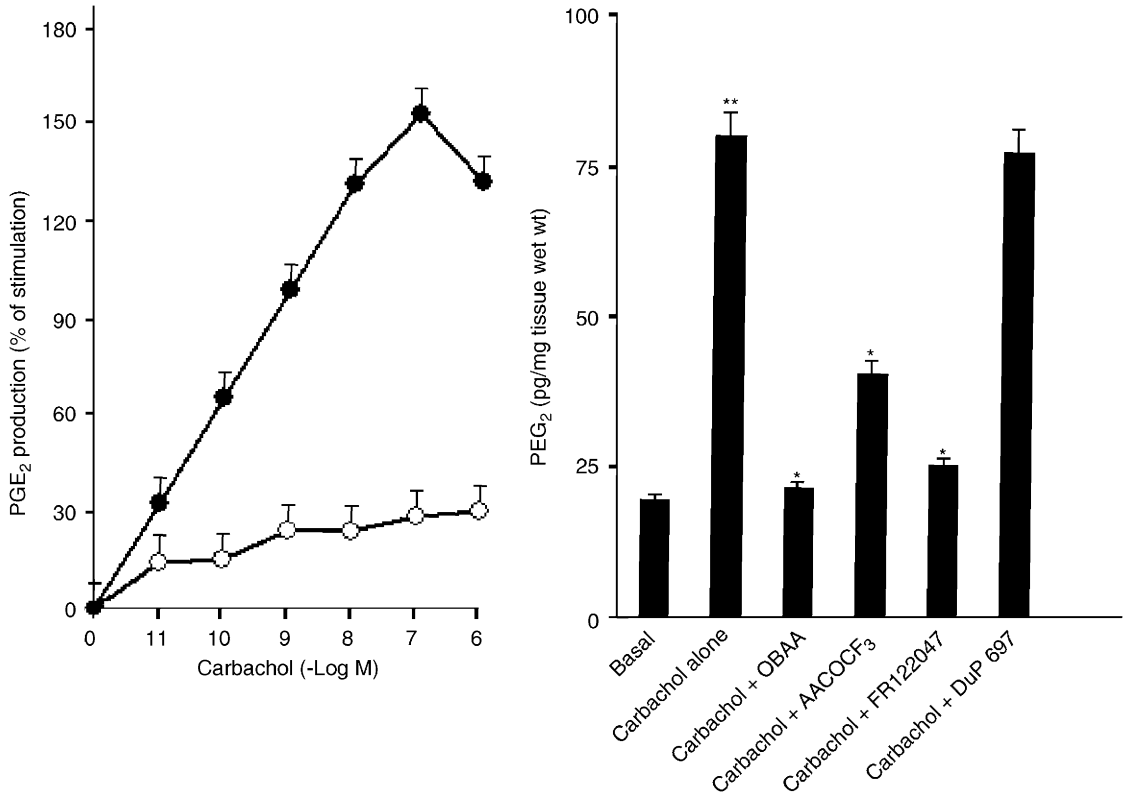

Fig. 1. Concentration–response curves of carbachol in the absence (●) or in the presence of 1×10^{-7} M atropine (○) upon rat cerebral frontal cortex preparations. Results are means ± s.e. mean of seven experiments performed by duplicate. Histogram shows basal, carbachol alone and carbachol in the presence of phospholipases and cyclooxygenases inhibitors. * Differ significantly from basal with $P < 0.001$. ** Differ significantly from carbachol alone with $P < 0.001$.

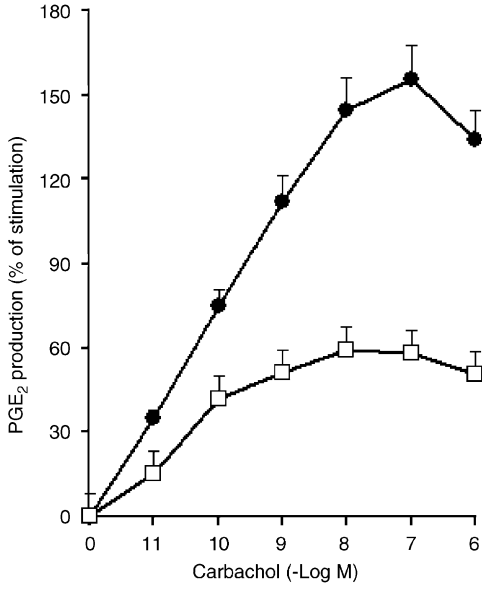

Fig. 2. Concentration–response curves of carbachol in the absence (●) or in the presence of 5×10^{-7} M SC 19220 (□) upon rat cerebral frontal cortex preparations. Results are means ± s.e. mean of eight experiments performed by duplicate.

response and decrease thereafter. An inhibition in exogenous PGE_2-induced endogenous PGE_2 production was observed in the presence of 5×10^{-7} M SC 19220 (a PGE_2 receptor blocker), indicating the specificity of the reaction. Moreover, histogram of Fig. 3 shows a reduction in PGE_2-induced PGE_2 production in the presence of OBAA and AACOCF3 at different concentrations.

As can be seen in Table 2, PGE_2-induced PGE_2 production was inhibited by the presence of COX-1 and COX-2 inhibitors. Moreover, to determine whether the activation of enzymatic pathways commonly associated to PGE_2 production is depending on calcium mobilization, the action of calcium blocker agent (verapamil, 5×10^{-6} M) was studied. Table 2 also shows that verapamil inhibited PGE_2-dependent PGE_2 increase production and the ionophore (A 23187, 5×10^{-6} M) mimicked exogenous PGE_2 action.

With the use of specific oligonucleotide primers for COX-1 and COX-2 mRNA gene expression, RT-PCR amplified products showed single clear bands of the predicted size (Fig. 4). Semi-quantitative reversed transcription polymerase chain-reaction analysis demonstrated that stimulation with carbachol (1×10^{-7} M) or

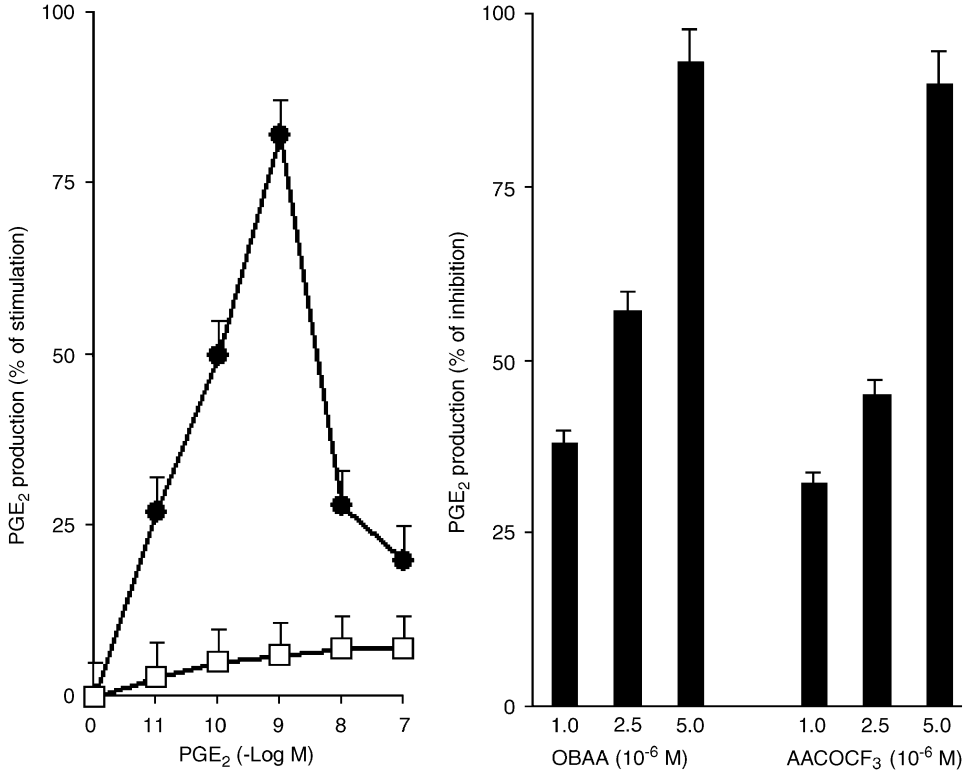

Fig. 3. Concentration–response curves of exogenous PGE_2 in the absence (●) or in the presence of 5×10^{-7} M SC 19220 (□) upon rat cerebral frontal cortex preparations. Results are means ± s.e. mean of seven experiments performed by duplicate. Histogram shows the percentage (%) of inhibition of PGE_2-induced PGE_2 production by different concentrations of OBAA and AACOCF3.

Table 2
Influence of different drugs on endogenous PGE_2 production by exogenous PGE_2

Additions	PGE_2 production (pg/mg tissue wet wt)	*n*
Basal	20 ± 1.6	7
PGE_2 (1×10^{-10} M)	42 ± 3.8	7
PGE_2 (1×10^{-10} M) + FR 122047 (5×10^{-8} M)	18 ± 1.8	5
PGE_2 (1×10^{-10} M) + DuP 697 (5×10^{-8} M)	21 ± 1.9	5
PGE_2 (1×10^{-10} M) + Verapamil (5×10^{-6} M)	25 ± 1.6	4
A 23187 (5×10^{-6} M)	58 ± 3.6	4

Values are the means ± s.e. mean. n = number of experiments.

exogenous PGE_2 (1×10^{-9} M) triggered increase in COX-1 mRNA levels (Fig. 4 upper and lower panels). A reduction in carbachol or PGE_2-induced elevation of COX-1 mRNA levels was observed in the presence of OBAA (5×10^{-6} M) and AACOCF3 (5×10^{-6} M).

4. Discussion

In this study, we show that activation of mAChR of rat cerebral frontal cortex preparations triggers increased production of PGE_2 and this is preceded by PLA_2s activation, which in turn catalyzes PLA_2-AA release and induces immediate early COX-1 mRNA gene expression without affecting COX-2 mRNA levels.

Positive PGE_2 regulation by carbachol has been described in different tissues [35–39] including rat cerebral frontal cortex tissues [40]. We observed that such stimulation was due to PLA_2s and COX-1 through mAChR activation; as it was prevented by specific blockade of these enzymes and by cholinoceptor antagonist agent. Thus, FR 122047 known to selectively inhibit COX-1 [41], OBAA and AACOCF3 known to selectively inhibit PLA_2s [42], have proven to be effective in preventing carbachol-stimulated PGE_2 production, suggesting that this carbachol action is under control of COX-1 and PLA_2s. The lack of the COX-2 specific inhibitor confirmed this statement. However, the rat frontal cortex expressed constitutively both COX-1 and COX-2 enzymes as shown in this paper and reported by others [21,22]. In most cells, COX-1 mediates physiological responses such as modulation of the autonomic nervous system, whereas COX-2 mainly plays a role in inflammation, infection and cellular proliferation [43]. Such functional dichotomy helps to explain the preferential COX-1-carbachol effect.

Recent advances in molecular and cellular biology of PLA_2 have let to the identification of more than 14

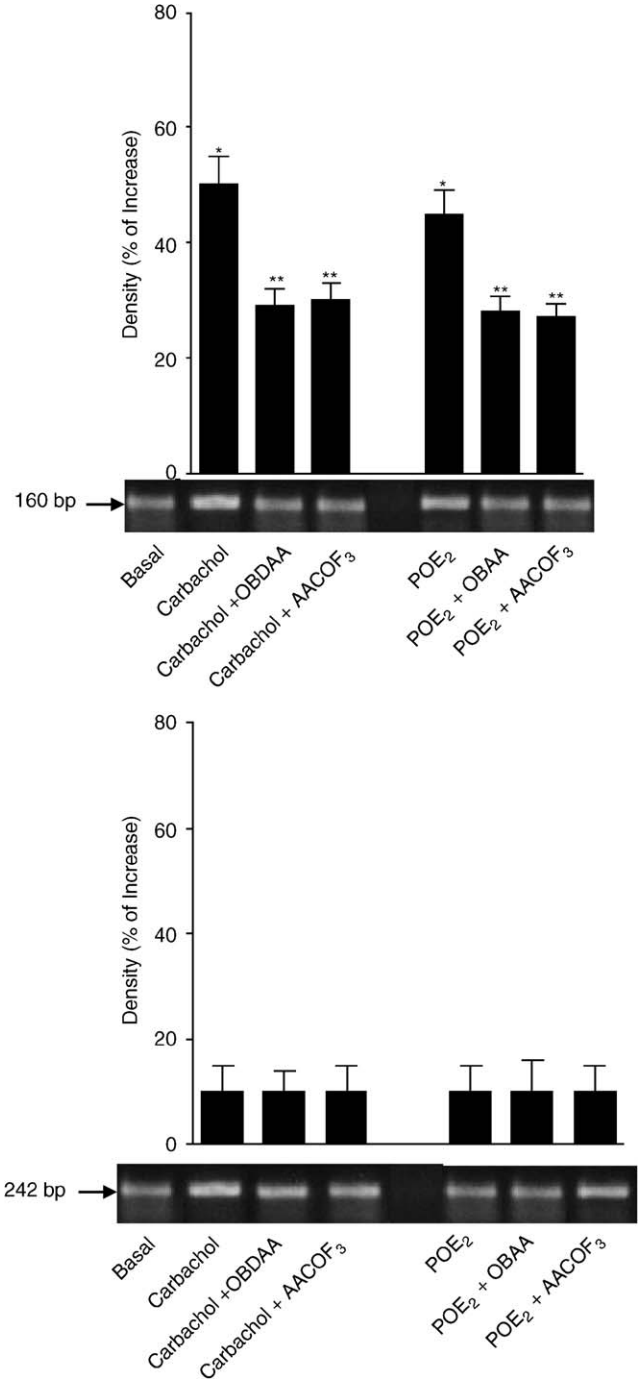

Fig. 4. Effect of carbachol and exogenous PGE_2 on COX-1 mRNA (upper panel) and COX-2 mRNA (lower panel) levels on rat cerebral frontal cortex. Tissue were incubated for 1 h with 1×10^{-9} M carbachol or exogenous PGE_2 in absence or in presence of 5×10^{-6} M OBAA or AACOCF3. Values are means ± s.e. mean of five experiments in each group. RT-PCR products obtained from this analysis are shown. $^{*}P < 0.001$ between basal; $^{**}P < 0.001$ between carbachol or exogenous PGE_2 alone.

isoforms with PLA_2 activity. These isoforms include $sPLA_2$ and $cPLA_2$. Both isoforms have been shown to be present in cerebral frontal cortex of rat brain and in astrocytes and have not only neurochemical importance, but also, are regulatory element in brain tissue function [7,43,44]. In this context, neural cells has shown that neurotransmitter-activated $cPLA_2$ and $sPLA_2$ [20]. Moreover, $sPLA_2$ is released from rat brain synaptosomes or neuronally differentiated PC12 cells upon stimulation via acetylcholine receptor [44].

On the other hand, exogenous PGE_2 itself has a capacity to stimulate endogenous PGE_2 production. Also, PGE_2 acted as an inducer's of early COX-1 mRNA levels. Both effects appear to be preceded by PLA_2s activation as the inhibitors of PLA_2s prevented these effects. The PGE_2 stimuli was able to activate PGE_2 receptor, since a receptor antagonist agent, inhibited the capacity of exogenous PGE_2 to generated endogenous PGE_2.

Carbachol-receptor-triggered PLA_2 catalyzed AA release with COX-1 activation, has been shown to be related to increasing the concentration of intracellular calcium leading to raised cerebral PGE_2 production [21]. As regards calcium mobilization, PGE_2 induces calcium release from intracellular calcium stores, producing an early intracellular calcium peak which is responsible for the sustained plateau of intracellular calcium increase. This seems to be mediated by COX-1, COX-2 and PLA_2s activations as they were blocked by verapamil and mimicked by A23187, an ionophore agent. This mechanism was described for some of the physiological actions of PGE_2 induced by carbachol, i.e. production of platelet-activating factor and AA release in human polimorphonuclear leucocytes [44] and may be required for exogenous PGE_2-release endogenous PGE_2. It is possible that phospholipases and cyclooxygenases activated by PGE_2 with the increase amount of endogenous PGE_2, maintain the chronic state of inflammatory processes.

This pharmacological study describes the involvement of PLA_2s in carbachol and exogenous PGE_2-induced PGE_2 production is the result of their ability to mobilize AA from phospholipids with subsequent activation of COX-1 and COX-2. However, since the enzymatic inhibitory agents used, may be involved in the regulation of other enzymes [1,2,8], the mechanism described here, perhaps quite complex.

In conclusion, this data show the triggering via the PGE_2 of a signal-transduction pathway that includes activation of PLA_2s, COX-1 and COX-2. This work provides the precursors of PGE_2 involved in normal brain function and neuroinflammatory pathophysiological processes. PGE_2 not only acts as inflammatory lipids but also directly modulate neural cell function mAChR activation in rat cerebral frontal cortex.

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