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Signal transduction underlying carbachol-induced PGE_2 generation and cox-1 mRNA expression of rat brain

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Abstract

In this paper we have determined the different signal pathways involved in M_1 and M_3 muscarinic acetylcholine receptor (mAChR) dependent stimulation of cyclo-oxygenase 1 (cox-1) mRNA gene expression and PGE_2 production on rat cerebral frontal cortex. Carbachol stimulation of M_1 and M_3 mAChR exerts an increase in cox-1 mRNA gene expression without affecting cox-2 mRNA expression and increased PGE_2 generation. Besides, increased phosphoinositide (PI) turnover and stimulation of nitric oxide synthase (NOS) and cyclic GMP (cGMP) production. Inhibitors of phospholipase A_2 (PLA_2), COX and phospholipase C (PLC), calcium/calmodulin (CaM), NOS and soluble guanylate cyclase prevent the carbachol effect. These results suggest that carbachol-activation of M_1 and M_3 mAChR increased PGE_2 release associated with an increased expression of cox-1 and NO–cGMP production. The mechanism appears to occur directly to PLC stimulation and indirectly to PLA_2 activation. These results may contribute to understand the effects and side effect of non-steroidal anti-inflammatory drugs in patients with cerebral degenerative diseases.

Keywords: Brain; Phospholipase C; Phospholipase A_2; PGE_2; Calcium/calmodulin; cox-2 mRNA gene; cox-1 mRNA gene; Carbachol; mAChR

1. Introduction

Cyclo-oxygenase (COX) is the enzyme that catalyzes the first steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (AA).

COX activity has long been studied in preparations from sheep seminal vesicles and this enzyme was cloned by three separate groups (Merlie et al., 1988; Yokoyama et al., 1988). The discovery of a second form of COX in the early 1990s was a landmark event in prostanoid biology (Herschman, 1996; Bakhle and Bottin, 1996; Jouzeau et al., 1997).

The inducible enzyme COX-2 is very similar in structure and catalytic activity to the constitutive COX-1. The biological activity of both isoforms can be inhibited by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (Vane, 1971).

The main reason for labeling COX-1 and COX-2 as physiological and pathological, respectively, is that most of the stimuli known to induce COX-2 are those associated with inflammation, i.e. bacterial lipopolysaccharide (LPS) and cytokines such as interleukin (IL)-1, IL-2 and tumor necrosis factor (TNF)-α. Corticosteroids decrease induction of COX-2 as well as the

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anti-inflammatory cytokines (IL-4, IL-10, IL-13) (Bakhle and Bottin, 1996; Onoe et al., 1996).

COX-1, as a constitutive form, is distributed in neurons throughout the brain, but is most prevalent in forebrain, where prostaglandin (PG) of E series (PGE_2) may be involved in complex integrative functions, such as modulation of the autonomic nervous system (Yamagata et al., 1993; Breder et al., 1995). COX-2 is expressed constitutively in only a few tissues including the brain and this expression is restricted to certain parts of the central nervous system (CNS), mainly the cortex, hippocampus, hypothalamus and spinal cord (Breder and Saper, 1996). It is the predominant isoform in the brains of neonate pigs (Jones et al., 1993) and in the spinal cord of the rat (Beiche et al., 1996), while human brain tissues contain equal amounts of cox-1 and cox-2 mRNAs (O'Neil and Fort-Hutchinson, 1993).

The correlation between COX isoforms, PGE_2 and CNS diseases, such as Alzheimer's disease, has been analyzed but the true significance and the normal risk for these diseases taking NSAIDs as anti-inflammatory therapy are still controversial (Cochran and Vitek, 1996; McGeer and McGeer, 1995). The powerful techniques of molecular biology have provided an extensive description of cox-1 and cox-2 gene expression and their mRNA, supporting the involvement of one or both isoforms in physiological or/and pathological processes. All such evidence, nowadays, is subject to debate and further studies are essential to reach definitive conclusions (Beiche et al., 1996; O'Neil and Fort-Hutchinson, 1993).

Release of AA in response to muscarinic acetylcholine receptor (mAChR) agonists has been reported in astrocytoma cells that possess M_3 mAChR subtypes (Wall et al., 1991) and in cells transfected with cDNAs that code for human M_1, M_3 and M_5 mAChRs (Felder et al., 1989). Although responses to agonists that engage G-protein-coupled receptors show some overlap, there are several distinct responses for each agonist, attributable to efficient coupling of G-protein α subunits to receptors (Aragay et al., 1995; Post et al., 1996) or to triggering of additional signaling pathways (Grabham and Cunningham, 1995; Collins et al., 1997).

Muscarinic AChR-triggered phospholipase A_2 (PLA_2)-catalyzed AA release with subsequent increase in COX activity with augmented PGE_2 generation, has been shown to be coupled to the activation of calcium entry from the extracellular medium and intracellular calcium signals (Shuttleworth and Thompson, 1998), playing a central role in the release of AA triggered by neurotransmitters (Bayon et al., 1997). This AA can be used by COX enzyme and exert physiological functions on its own. Like angiotensin, carbachol has been shown to stimulate PGE_2 release through PLA_2 activation (Schlondorff et al., 1997). Indeed, carbachol is also able to activate selected populations of mAChRs, thus increasing PGE_2 production in cat brain (Navarro et al., 1998).

Previously we have demonstrated that carbachol acts on rat frontal cortex as an early positive regulator of M_1 mAChR-mRNA expression, closely correlating with NOS-mRNA using common enzymatic pathways (Sterin-Borda et al., 2003). The aim of this work was to determine whether the mAChR agonist carbachol is able to induce early cox mRNA gene expression and whether such induction correlates with PGE_2 production. The implication of PLC and PLA_2 signal transduction underlying carbachol-induced PGE_2 generation was also studied.

In the present study, we show that the activation of rat cerebral frontal cortex M_1 and M_3 mAChR preparations leads to increased generation of PGE_2 preceded by an activation of PLA_2 and PLC; and increased induction of cox-1 without affecting cox-2 mRNA levels. These events are associated with selective activation of several mAChR subtypes, which was prevented by mAChR antagonist agents. In addition, these findings suggest the participation of cerebral mAChR subtypes in a cross-talk mechanism that involves carbachol-mediated coupling of PLA_2 and PLC and cox-1 gene expression.

2. Materials and methods

2.1. Rat cerebral frontal cortex membrane 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 N° 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. Membranes from cerebral frontal cortex were prepared as previously described (Borda et al., 1998). Briefly, tissues were homogenized in an Ultraturrax at 4 °C in 5 volumes of 10 mM potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose pH 7.5 (buffer A), supplemented with 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 $\mu g\,ml^{-1}$ leupeptin and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3000 g, then at 10,000 g and 40,000 g at 4 °C, for 15 and 90 min, respectively. Resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitor pH 7.5 (buffer B).

2.2. mRNA isolation and cDNA synthesis

Total RNA was extracted from rat cerebral frontal cortex slices by homogenization using guanidinium isothiocyanate method (Chomozynski and Saachi, 1987). 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 Krebs Ringer bicarbonate (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.

2.3. 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, 2.5 U 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.

2.4. Measurement of total labeled phosphoinositide (PI)

Rat cerebral frontal cortex slices were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 mCi [myo-^{3}H]-inositol ([^{3}H]-MI) (Sp.Act. 15 Ci/mmol) from Dupont/New England Nuclear and LiCl (10 mM) was added to determine inositol monophosphate accumulation according to the technique previously described (Borda et al., 1998). Carbachol was added 30 min before the end of the incubation period and blockers 30 min before the addition of different carbachol concentrations. Water-soluble PI was extracted after 120 min incubation. Tissues were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Chloroform (0.62 ml) and water (1 ml) were then added. Samples were centrifuged at 3000 g for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7-ml column of Bio-Rad AG (Formate Form) 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid previously washed with 10 mM Tris–formic pH 7.4. The resin was then washed with 20 volumes of 5 mM myo-inositol followed by 6 volumes of water and PI were eluted with 1 M ammonium formate in 0.1 M formic acid. Fractions (1 ml) were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation and results corresponding to the second peak were expressed following previous criteria (Borda et al., 1998).

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 (Bishop-Bailey et al., 1997).

2.5. Determination of nitric oxide synthase (NOS) activity

NOS activity was measured in cerebral frontal cortex slices by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine according to the procedure previously described for brain slices (Borda et al., 1998). Briefly, after 20 min in preincubation KRB solution, tissues were transferred to 500 ml of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]-arginine (0.5 mCi). Drugs were added and incubated for 20 min under 5% CO_2 in O_2 at 37 °C. Tissues were then homogenized with an Ultraturrax in 1 ml of medium containing 20 mM HEPES pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM leupeptin and 0.2 mM phenyl methane sulfonyl fluoride at 4 °C. After centrifugation at 20,000 g for 10 min at 4 °C, supernatants were applied to 2 ml columns of Dowex AG 50 WX-8 (sodium form); [^{14}C]-citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting.

2.6. Cyclic GMP (cGMP) assay

Rat cerebral frontal cortex slices were incubated in 1 ml of KRB for 30 min and different concentrations of carbachol were added in the last 15 min. When blockers were used, they were added 20 min before the addition of reagents. The residue was dissolved in different buffers for cGMP (Sterin-Borda et al., 1997). Aliquots of 100 ml were taken for the nucleotide determination using a RIA procedure with ^{125}I-cGMP RIA kit from Dupont/New England Nuclear, USA.

2.7. Prostaglandin E_2 (PGE_2) assay

Rat cerebral frontal cortex slices (10 mg) were incubated in the assay buffer, after all reagents were allowed to equilibrate at room temperature prior to use. 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).

2.8. Drugs

Carbachol, pirenzepine, atropine, 4-DAMP, AF-DX 116, trifluoperazine (TFP), acetyl salicylic acid (ASA) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma Chemical Company, Saint Louis, MO, USA; U-73122, [1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA) were from Tocris Cookson Inc., Baldwin, MO, USA; rofecoxib was from Merck, Rahway, NJ, USA.

2.9. 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

3.1. Carbachol effect on cox-1 and cox-2 mRNA levels

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. 1). Semi-quantitative reversed transcription polymerase chain-reaction analysis demonstrated that stimulation with carbachol (1×10^{-7} M) for 1 h increased cox-1 mRNA levels (1A) without modifying cox-2 mRNA levels (1B). Furthermore, a reduction in carbachol-induced elevation of cox-1 mRNA levels was observed in the presence of 5×10^{-5} M L-NMMA, pirenzepine and 4-DAMP. On the contrary, none of the mAChR antagonists had any effect on cox-2 mRNA levels. No effect on carbachol-induced elevation of cox-1 mRNA levels was observed in the presence of AF-DX 116 (data not shown).

3.2. Carbachol effect on PGE_2 generation

Fig. 2 shows the ability of carbachol to trigger PGE_2 release in a concentration-dependent manner, with 1×10^{-7} M proving the maximal response and decreasing thereafter, though to values significantly higher than basal ones. A reduction in carbachol-induced PGE_2 production was observed in the presence of COX-1 inhibitor (ASA, 5×10^{-5} M) and phospholipase A_2 (PLA_2) (OBAA, 5×10^{-6} M) but not by rofecoxib (4×10^{-8} M) at concentration that inhibited COX-2, indicating the specificity of the reaction.

In order to discern which mAChR subtypes were involved in carbachol-induced PGE_2 release, several cholinoceptor antagonist subtype agents were used. Fig. 3 shows that atropine, pirenzepine and 4-DAMP at 1×10^{-7} M attenuated the stimulatory action of carbachol upon PGE_2 production while AF-DX 116 had no effect.

To determine whether the activation of enzymatic pathways commonly associated to M_1 and M_3 mAChR signaling were implicated in carbachol-increased PGE_2

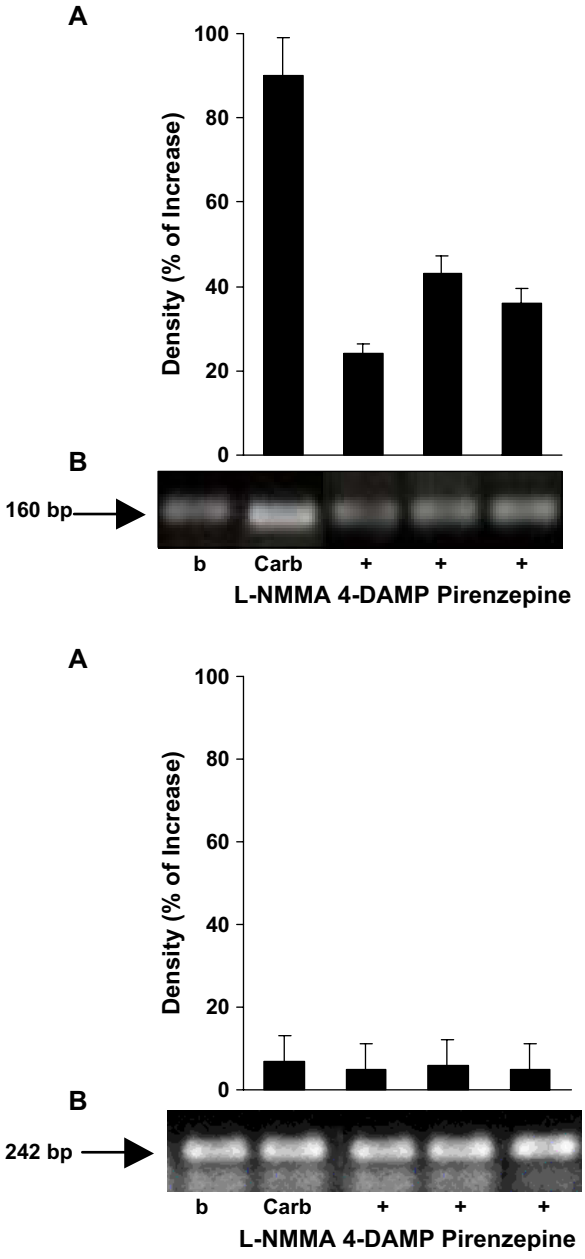

Fig. 1. (A) Carbachol (1×10^{-7} M) action on semi-quantitative RT-PCR analysis for cox-1 (upper panel) and cox-2 (lower panel) mRNA levels from rat cerebral frontal cortex preparations incubated for 1 h in the absence (carb) or in the presence of 5×10^{-5} M L-NMMA or 1×10^{-7} M 4-DAMP and pirenzepine. Basal values (b) correspond to cox-1 and cox-2 mRNA levels after 1 h of incubation. Values are means ± SEM of seven experiments in each group. (B) RT-PCR products obtained from this analysis are shown. $P < 0.001$ between carbachol alone or carbachol in the presence of blockers (upper panel A).

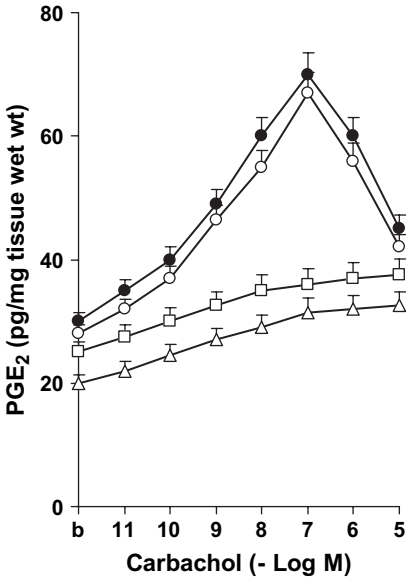

Fig. 2. Concentration–response curves of carbachol in the absence (●) or in the presence of 5×10^{-5} M ASA (□) or 5×10^{-6} M OABB (△) or 4×10^{-8} M rofecoxib (○). Results are expressed as means ± SEM of six experiments performed by duplicate.

generation, several enzymatic inhibitors were used. As depicted in Table 2, inhibition of PLC by 5×10^{-6} M U-73122 (Smallridge et al., 1992), of calcium–calmodulin (CaM) by 5×10^{-6} M TFP (Scharff and Foder, 1984), of NOS by 5×10^{-5} M L-NMMA (Lowenstein and Synder, 1992) and of soluble guanylate cyclase by 5×10^{-5} M ODQ (Garthwaite, 1995) blunted the carbachol-induced increase of PGE_2 levels. The inhibition of protein kinase C (PKC) by 1×10^{-9} M staurosporine (Scharff and Foder, 1984) failed to affect the release of PGE_2. Table 2 also shows that carbachol stimulation resulted in raised PI production, nitric oxide formation and cGMP accumulation.

4. Discussion

In this study we show that activation of M_1 and M_3 mAChR of rat cerebral frontal cortex preparations triggers increased generation of PGE_2. This is preceded by PLC activation, which in turn catalyzes PLA_2-AA release and induces immediate early cox-1 mRNA gene expression without affecting cox-2 mRNA levels.

Our data indicate that the endogenous NO signaling system is the putative transcriptor factor able to increase the rate of transcription in response to mAChR activation, pointing out the role of calcium mobilization in rapid NOS activation. Positive prostaglandin (PGs) regulation by carbachol has been described in diverse tissues (Yokoyama et al., 1988; Herschman, 1996; Schlondorff et al., 1997) including rat cerebral frontal cortex preparations (Navarro et al., 1998). We observed that such stimulation was mostly due to PLA_2 and COX as it was prevented by specific blockade of these two enzymes.

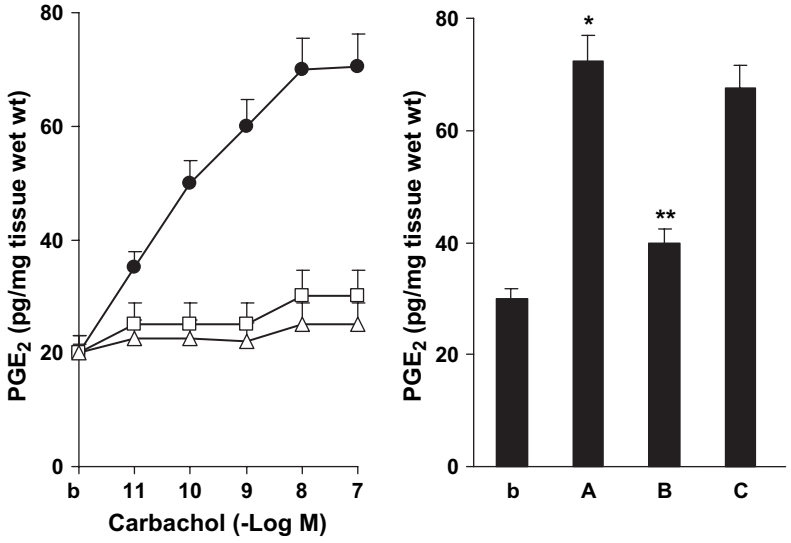

Fig. 3. Concentration–response curves of carbachol in the absence (●) or in the presence of 1×10^{-7} M 4-DAMP (□) or pirenzepine (△) upon rat cerebral frontal cortex preparations. Basal values (b) are shown. Results are means ± SEM of seven experiments performed by duplicate. Histogram shows: b, basal values; A, effect of 1×10^{-8} M carbachol; B, effect of 1×10^{-7} M atropine + 1×10^{-8} M carbachol; C, effect of 1×10^{-6} M AF-DX 116 + 1×10^{-8} M carbachol. *Differ significantly from b with $P < 0.001$. **Differ significantly from A with $P < 0.001$.

The finding that low ASA concentration, known to selectively inhibit COX-1 (Bakhle and Bottin, 1996), has proven to be effective in preventing carbachol-stimulated PGE_2 generation, suggests that this carbachol action is under control of cox-1 mRNA, in agreement with the positive regulation of cox-1 mRNA without changes in cox-2 mRNA expression. 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 reported (Yamagata et al., 1993; Breder et al., 1995). In most cell types, 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 (Jouzeau et al., 1997). Such functional dichotomy helps to explain the preferential cox-1 mRNA carbachol effect. M_1 and M_3 mAChR subtypes appear to be implicated, since M_1 and M_3 antagonistic drugs diminished the carbachol effect on cox-1 expression.

The mechanism by which carbachol stimulates PGE_2 release and cox-1 mRNA levels may be explained by efficient coupling of Gq-protein to M_1 and M_3 cerebral mAChR. Thus, we observed that the activation of M_1 and M_3 mAChR by carbachol seems to involve a stimulation in PI hydrolysis through PLC activation, because agents known to interfere with PLC activity inhibited carbachol action. However, PI hydrolysis intermediates (IP_3–DAG) play a different role whereby the inhibition of CaM blocked PGE_2 generation but PKC inhibition failed to do so.

In addition, in the observed carbachol effect, we demonstrated simultaneous up-regulation on NOS activity and cyclic GMP (cGMP) accumulation. Experiments with inhibitor agents directed to NOS and soluble

Table 2
Effect of carbachol on several enzymatic pathways

Addition	PGE_2(pg/mg tissue wet wt)	PI (area/mg)	NOS (pmol/g tissue wet wt)	cGMP (pmol/mg tissue wet wt)
Basal	29 ± 2	29 ± 3	81 ± 7	350 ± 15
Carbachol	71.5 ± 4*	62 ± 5*	169 ± 12*	986 ± 32*
Carbachol + U-73122	28 ± 2**	28 ± 2**	85 ± 8**	448 ± 24**
Carbachol + TFP	22.5 ± 2**	58 ± 4	79 ± 7**	404 ± 21**
Carbachol + L-NMMA	37.5 ± 3**	60 ± 5	66 ± 7**	382 ± 18**
Carbachol + ODQ	21.5 ± 2**	64 ± 6	172 ± 14	349 ± 19**
Carbachol + staurosporine	67.5 ± 4	62 ± 6	180 ± 16	996 ± 29

Values are means ± SEM of five experiments in each group performed in duplicate. Enzyme activities were measured after incubating for 1 h cerebral frontal cortex preparations (10 mg) in the presence of 1×10^{-7} M carbachol with or without enzymatic inhibitors used at the following concentrations: 5×10^{-6} M U-73122, 5×10^{-6} M TFP, 1×10^{-5} M L-NMMA; 1×10^{-5} M ODQ; and 1×10^{-9} M staurosporine. *$P < 0.001$ compared with basal values (no additions); **$P < 0.001$ compared with carbachol alone.

guanylate cyclase activities confirm the participation of the NO–cGMP system. Moreover, on the basis of our results, we suggest that carbachol can act as an inducer of cox-1 mRNA gene expression through the activation of NOS activity. Concomitant inhibition of cox-1 mRNA gene expression confirms this finding.

Recent work has shown that NO is a powerful stimulant of gene expression associated with carbachol action on cerebral frontal cortex (Sterin-Borda et al., 2003). Reciprocal stimulation of immediate early gene expression between mAChR and direct enzymatic activation of NOS or an increase in their transcription expression was demonstrated (Sterin-Borda et al., 2003).

Carbachol-receptor-triggered PLC catalyzed PLA_2-AA release with cox-1 gene expression and activation, has been shown to be coupled to CaM and NOS activation by increasing intracellular calcium concentration, leading to raised cerebral PGE_2 production. Not unlikely, PLC activation by carbachol is directly regulated while indirect regulation occurs at PLA_2 level through CaM and NOS stimulation, resulting in increased PGE_2 release in rat cerebral frontal cortex. On the basis of our results, carbachol-receptor triggered NO accumulation that in turn co-stimulates both PGE_2 release and the induction of cox-1 mRNA expression. The reduction of both carbachol-induced elevation of cox-1 mRNA levels and PGE_2 release by L-NMMA confirm this statement. It has been reported that exogenous AA in adult human microglia in vitro stimulated PGE via COX-1 but this effect was independent from induction of cox mRNA expression (Hoozemans et al., 2002). It is therefore likely that in the observed carbachol effects the endogenous NO signaling system through the NO accumulation can act as an inducer of cox-1 mRNA levels. The mechanism might imply the stimulation of the same enzymatic machinery that regulates the mAChR-mRNA levels in cerebral frontal cortex using NO as a modulator (Sterin-Borda et al., 2003).

A diagram to tie together the various systems studied and proposing a model for the positive feed-back mechanism whereby carbachol up-regulates cox-1 mRNA levels and PGE_2 generation, is shown in Fig. 4.

Finally, cholinergic abnormalities are the major characteristic in Alzheimer's disease (Kihara and Shimohana, 2004). Therefore, acetylcholinesterase inhibitors or the mAChR activation by the agonist are shown to be effective in the improvement of cognitive

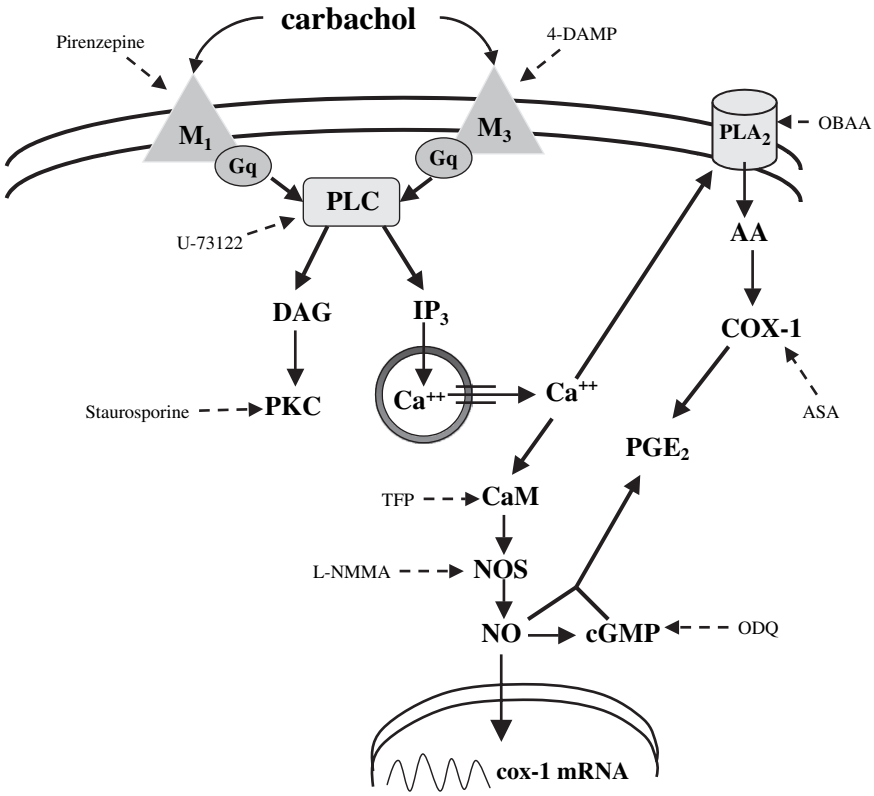

Fig. 4. Proposed model to explain the mechanism whereby carbachol up-regulates cox-1 mRNA gene expression and PGE_2 generation in cerebral frontal cortex preparations. Carbachol acting on Gq-protein M_1 and M_3 mAChR activates PLC mediating production of inositol triphosphate (IP_3) and 1-2-diacylglycerol (DAG). IP_3 triggers intracellular release of calcium (Ca^{2+}) stores. On one hand, free cytosolic Ca^{2+} activates phospholipase A_2 (PLA_2) with PGE_2 generation and, on the other hand, it activates NOS with NO and cGMP production. In turn, NO regulates transcription factors that increase cox-1 mRNA levels. Dotted line indicates the site where inhibitory agents act.

functions and social behaviors (Grutzendler and Morris, 2001). Of particular interest may be the signal transduction pathways mediated through cholinergic receptors activation modulating brain inflammation. Prospective studies provided evidence that chronic use of NSAIDs significantly reduced incidence and slower progression of Alzheimer's disease. Although, the role of inhibition of COX-1 and COX-2 in the beneficial effect of NSAIDs on brain inflammation in Alzheimer's disease is still controversial (Lane et al., 2004). In the light of the complex role of inflammation in neurodegenerative processes as a potential therapeutical target in Alzheimer's disease, the data presented in this study suggest that selective COX-2 inhibitors or classical NSAIDs may prevent not only neuroinflammation but also the cholinergic hypofunction (Giovannini et al., 2003).

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