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Experimental Eye Research 80 (2005) 391–399

EXPERIMENTAL EYE RESEARCH

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Correlations between neuronal nitric oxide synthase and muscarinic M_3/M_1 receptors in the rat retina

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Received 25 November 2003; in revised form 2 September 2004; accepted in revised form 10 September 2004
Available online 10 December 2004

Abstract

This study determined the different signal pathways involved in M_1/M_3 muscarinic acetylcholine receptor (mAChR) dependent stimulation of nitric oxide synthase (NOS) activity/cyclic GMP (cGMP) production and nNOS mRNA expression in rat retina. Exposure of the retina to different concentrations of carbachol caused an increase in NOS activity, cGMP production and phosphoinositol (PI) accumulation. The increase in NOS activity and cGMP content was blocked by L-NMMA and ODQ, respectively. Also, phospholipase C (PLC) and calcium/calmodulin (CaM) inhibition prevented the carbachol activation on NOS/cGMP pathways. Both, 4-DAMP and pirenzepine but not AF-DX 116 blocked the increase in NOS and cGMP induced by carbachol. Carbachol-stimulation of M_1/M_3 mAChR increased nNOS-mRNA levels associated with an increase of endogenous NO and cGMP production. The mechanism appears to occur secondarily to stimulation of PIs turnover via PLC. This triggers a cascade reaction involving CaM and soluble guanylate cyclase leading to NO and cGMP accumulation, that in turn, up regulates nNOS-mRNA gene expression. These results give novel insight into the mechanism involved in the regulation of nNOS-mRNA levels by mAChR activation of retina.
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Keywords: NOS; NO; mAChR; M_3 mAChR; M_1 mAChR; cGMP; nNOS mRNA

1. Introduction

The photoreceptor cells, which are located in the outer nuclear layer and make up about 70% of all retinal cells in rodents, are thought to be the area rich in putative muscarinic acetylcholine receptors (mAChRs). Many authors infer possible functional roles for cholinergic neurotransmission (Hutchins and Hollyfield, 1985; Won-Kyu et al., 2000).

The mAChRs are members of a large family of receptors that mediate signal transduction by coupling with G-protein, which modulate the activity of a number of effector enzymes in addition to regulating ion channels directly, leading to increases or decreases in second messengers or changes in ion concentrations (Nathanson, 1987). Muscarinic AChR subtypes are generally grouped according to their functional coupling, either to the mobilization of intracellular calcium (M_1–M_3–M_5) through the activation of phospholipase C (PLC) or by the inhibition of adenylyl cyclase (M_2–M_4) (Hulme et al., 1990; Hosey, 1992). Five human subtypes of mAChR have been indentified by molecular cloning (Allard et al., 1987). Moreover, recent studies using selective muscarinic antagonists (4-DAMP, pirenzepine, AF-DX 116) indicate that the mAChRs exist most notably as M_3 and M_1 subtypes in retina (Hutchins and Hollyfield, 1985; Gupta et al., 1994; Cheon et al., 2001). M_3 and M_1 receptors are very similar in sequence (Bonner, 1989). Stimulation of mAChR subtypes leads to activation of endogenous NO signaling pathways in different tissues (Sterin-Borda, 1995; Borda et al., 1998). NO and cGMP

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doi:10.1016/j.exer.2004.09.016

have implicated in many neuronal functions including regulation of gene expression (Gudi et al., 1999).

The expression of isoforms of NOS, enzymes responsible for NO production, have been detected (Park et al., 1996; Fujii et al., 1998; Vercelli et al, 2000; Tsumamoto et al., 2002). NO in retina is involved in diverse processes, such as neurotransmission, vasodilatation, host defense and inflammation (Christopherson and Bredt, 1997; MacMicking et al., 1997; Fang, 1997). One of these isoforms is continuously expressed in retina; neuronal NOS (nNOS), is predominantly found in neurons of the central nervous system (Knowles and Moncada, 1994; Park et al., 1996). NO is known to influence neovascularisation in a variety of models of angiogenesis (Papapetropoulos et al., 1997). The real role of nNOS in angiogenesis remains undetermined.

To investigate which mAChR subtypes in retina are activated by the cholinergic agonist carbachol to trigger nNOS activation and its expression in retina, we studied carbachol action on NOS/cGMP pathway activation and nNOS mRNA expression.

2. Materials and methods

2.1. Animals and retina preparations

The animals used, male Wistar rats obtained from our colony were handled in accordance with the Guidelines for Animal Experiments in the Faculty of Dentistry, Buenos Aires University (according with NHI No. 8023 revised 1978). They were housed individually in plastic cages with free access to foot and water, under room conditions of 23 °C and 50% humidity with 10 h at room light alternating with 14 h darkness. The rats were sacrificed by decapitation. After enucleation, an incision was made at the margin of the ora serrata to remove the lens, vitreous and ciliary body. Then, the retina was removed from the choroid and placed in a Petri dish containing Krebs-Ringer bicarbonate (KRB) solution gassed with 95% oxygen with 5% CO_2 at room temperature pH 7·4.

2.2. Nitric oxide synthase (NOS) activity assay

NOS activity was measured in retinas of male Wistar rats using L-[U-^{14}C] arginine as a substrate (Bredt and Snyder, 1989). Briefly, retinas were carefully dissected and incubated in KRB solution containing 18·5 kBq of L-[U-^{14}C] arginine for 20 min before the addition of the agonist. When inhibitors were used they were added from the beginning of the incubation time at the final concentrations indicated in the text. The incubations were carried out under a 50% CO_2 in oxygen atmosphere at 37 °C and stopped by homogenisation of retinas in 1 ml 20 mM HEPES pH 7·4, 0·5 mM EGTA, 0·5 mM EDTA and 1 mM dithiothreitol at 4°C. The supernatants were passed through 2 ml Dowex AG-50 WX-8 columns and L-[U-^{14}C] citrulline was eluted with water and quantified as described before (Bacman, 1998). Nitric oxide synthase activity was inhibited more than 90% by 5×10^{-4} M L-*N*-monomethyl arginine (L-NMMA). In the experiments with NOS activation by carbachol lower concentrations of this arginine analogue were chosen to inhibit the effects in order not to modify basal nitric oxide synthase activity.

2.3. Cyclic GMP (cGMP) assay

Retina preparations were incubated in 1 ml KRB containing 0·1 mM isobutyl methyl xanthine for 30 min gassed with 5% CO_2 in oxygen. Carbachol was added in the last 10 min while inhibitors were included in the incubation volume from the beginning. Reactions were stopped by homogenization. Samples were assayed by RIA using [^{125}I] cGMP from Dupont New England Nuclear (81400 kBq mmol^{-1}) and anti cGMP antisera from Sigma Chemical Co. (St Louis, MO, USA).

2.4. Measurement of total labelled phosphoinositides (PIs)

Retinas were incubated for 120 min in 0·5 ml of KRB gassed with CO_2 in oxygen with 37 kBq [myo-^{3}H] inositol ([^{3}H] MI) (specific activity 533 kBq mmol^{-1}) from Dupont New England Nuclear, LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique of Berridge et al. (1982). Carbachol was added 30 min before the end of the incubation period and the blockers 30 min before the addition of agonist. Water-soluble inositol phosphates were extracted after 120 min incubation following the method of Berridge et al. (1982). Tissues were quickly washed with KRB and homogenized in 0·3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v v^{-1}) to stop the reaction. Then, chloroform (0·62 ml) and water (1 ml) were 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 which had been 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 six volumes of water and inositol phosphates were eluted with 1 M ammonium formate in 0·1 M formic acid. One ml fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation. Results corresponding to the second peak, were expressed as absolute values of area units under the curve per milligram of wet weight tissue (area mg^{-1} tissue wet weight) following the criteria of Simpson's equation (Borda et al., 2002).

2.5. mRNA isolation and cDNA synthesis

Total RNA was extracted from rat retina by homogenisation using the 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 retina in KRB gassed with 5% CO_2 in O_2 pH 7·4 at 37°C for 60 min.

2.6. Quantitative PCR

Quantitation of neuronal NOS (nNOS) isoform 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 nNOS and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was 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 of gene-specific primers. The sizes of PCR MIMIC were distinct from those of the native targets. The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of the nNOS and G3PDH mRNA 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 an initial denaturation at 94°C for 2 min, the cycle condition was 30 sec of denaturalisation at 94°C, 30 sec of extension at 60°C and 45 sec for enzymatic primer extension at 72°C for 45 cycles for nNOS isoform each cycle consisted of 30 sec at 94°C, 30 sec at 55°C and 45 sec at 72°C for 45 cycles. The internal control was the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). PCR amplification was performed with initial denaturalisation at 94°C for 2 min followed by 30 cycles of amplification. Each cycle consisted of 35 sec at 94°C, 35 sec at 58°C and 45 sec0 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. Levels of mRNA were calculated from the point of equal density of the sample and MIMIC PCR products (Fig. 1). nNOS mRNA levels was normalized with the levels of G3PDH mRNA present in each sample, which served to control for variations in RNA purification and cDNA synthesis. The relative mRNA expression of nNOS in each group was compared with those from the respective normal group and reported as a percentage of normal.

Table 1
Oligonucleotides of primers for PCR

Gene product sense	Antisense	Predicted size (bp)	Accession number
nNOS 5′-GCGGA GCAGA GCGGC CTTAT-3′	5′-TTTGGT GGGAG GACCG AGGG-3′	240	NM-017008
G3PDH 5′-ACCA-CAGTCCA TGCCAT CAC-3′	5′-TCCAC CACCC TGTTG CTGTA-3′	452	X-59949

Neuronal nitric oxide synthase (nNOS); glyceraldehydes-3-phosphate dehydrogenase (G3PDH).

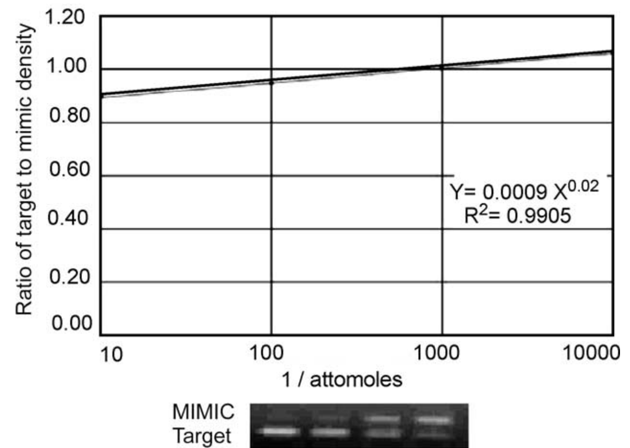

Fig. 1. Analysis of target mRNA levels by competitive PCR. *Top*: quantitative analysis of the competitive PCR experiment is shown. Ordinate, ratio of the target to MIMIC products as determined by band densitometry; abscissa, inverse amount of MIMIC added to the amplification. When the resultant target and MIMIC densitometric values are equal ($y=1$), extrapolation can be performed to yield the target amount. *Bottom*: Neuronal nitric oxide synthase (nNOS) of digoxigemin-labeled competitive PCR autoradiogram.

2.7. Drugs

Carbachol, N^G-monomethyl-L-arginine (L-NMMA), L-arginine, pirenzepine [Sigma Chemical Company, St Louis, MO, USA]; 4-DAMP, AF-DX 116 [ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA]; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), *Nz*-Propyl-L-arginine (NZ) [Tocris Cookson, Inc., Baldwin, MO, USA]. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text.

2.8. Statistical analysis

Student's *t*-test for unpaired values and when multiple comparisons were necessary the Student-Newman–Keuls test was applied. Differences between means were considered significant if $P<0·05$.

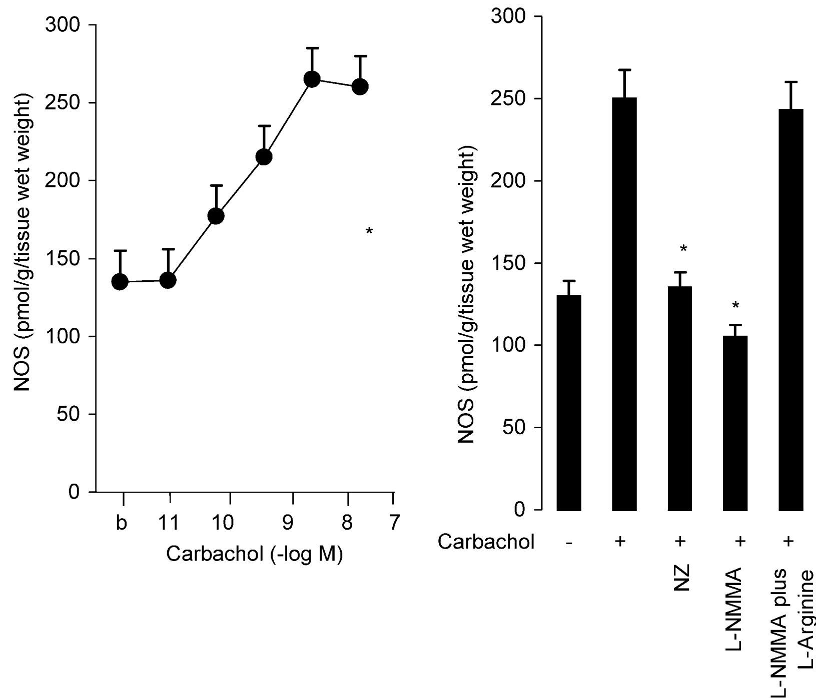

Fig. 2. Carbachol action upon NOS activity. Retina preparations were incubated with increasing concentrations of carbachol (●). Basal value (b) is shown. *Histogram* shows the effect of 1×10^{-8} M carbachol above basal values and the effect of the same concentration of carbachol in the presence of nNOS blocker 1×10^{-5} M NZ, NOS blocker 1×10^{-5} M L-NMMA or L-NMMA $+5\times10^{-4}$ M L-arginine. Results are means ± SEM of seven experiments in each group performed in duplicate. *$P<0{\cdot}001$ compared with carbachol alone.

3. Results

As can be seen in Fig. 2, carbachol increased NOS activity in a concentration-dependent manner, with the maximal effect at 1×10^{-8} M. The carbachol effect on retinal-NOS activity, was blunted by a reversible NOS inhibitor L-NMMA (1×10^{-5} M) (Lowenstein and Synder, 1992) and the natural substrate L-arginine (5×10^{-5} M) reversed the inhibitory action of L-NMMA on the carbachol effect. Moreover, a specific nNOS inhibitor NZ (1×10^{-5} M) (Zhang et al., 1997) attenuated carbachol stimulation (Histogram Fig. 2).

It is well known that NO operates as a second messenger that stimulates soluble guanylate cyclase, increasing cGMP concentrations in target cells (Bredt and Snyder, 1989). We measured cGMP production by retina after carbachol-stimulation. Fig. 3 shows the dose–response curves of

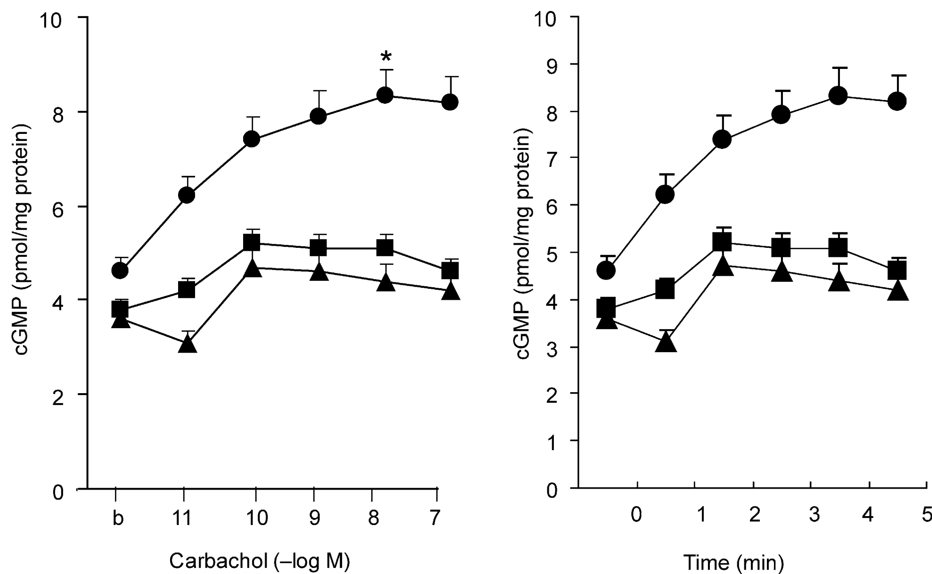

Fig. 3. Dose–response curve of carbachol on cyclic GMP (cGMP) (●). The inhibitory effects of 1×10^{-5} M L-NMMA (■) or 1×10^{-6} M ODQ (▲) on carbachol action are also shown. Data are means ± SEM of six separate determinations in each groups performed in duplicate. *$P<0{\cdot}001$ versus L-NMMA or ODQ.

Table 2
Influence of mAChR blockers on carbachol-effects on NOS activity and cGMP accumulation

Additions	NOS (pmol g^{-1} tissue wet $weight^{-1}$)	CGMP (pmol mg^{-1} protein)
None	138±10	4.82±0.15
Carbachol (1×10^{-8} M)	253±18	8.43±0.17
Atropine (1×10^{-7} M)	165±14*	5.03±0.13*
4-DAMP (1×10^{-7} M)	155±13*	5.18±0.12*
Pirenzepine (1×10^{-7} M)	176±15*	5.67±0.14*
AF-DX 116 (1×10^{-6} M)	240±16	7.92±0.16

Nitric oxide synthase (NOS) activity and cyclic GMP (cGMP) levels were measured after incubating retina with or without mAChR antagonists for 30 min and then for an additional 5 min with carbachol at the indicated concentations. Results are means±SEM of seven experiments in each group performed in duplicate. *Significantly different from carbachol alone with $P<0\cdot001$.

carbachol upon cGMP levels in rat retina. It can be seen that carbachol caused an increase in cGMP content in a concentration dependent manner. Carbachol stimulation also reached the maximal increase at 1×10^{-8} M. Moreover, a reduction in carbachol-induced elevation in cGMP was observed in the presence of both L-NMMA (1×10^{-5} M) and the specific guanylate cyclase inhibitor ODQ (1×10^{-6} M) (Garthwaite et al., 1995), indicating that NO-dependent synthesis of cGMP is involved in the study system.

To determine which mAChR subtypes are implicated in endogenous signalling system triggered by carbachol (1×10^{-8} M) stimulation, different mAChR antagonists were used. Table 2 shows that atropine (mAChR antagonist), 4-DAMP (M_3 mAChR antagonist) and pirenzepine (M_1 mAChR antagonist) (1×10^{-7} M) but not AF-DX 116 (M_2 mAChR antagonist) (1×10^{-6} M) inhibited carbachol induced NOS activation and cGMP production.

In order to investigate the mechanisms by which the stimulation of mAChRs modified NOS/cGMP pathways, retinas were incubated with different enzymatic inhibitors. Table 3 shows that the inhibition of PLC by U-73122 (5×10^{-6} M) decreased the stimulatory action of carbachol on both NOS activity and cGMP production.

Table 3
Influence of enzymatic inhibitors on carbachol-effects on NOS activity and cGMP accumulation

Additions	NOS (pmol g^{-1} tissue wet $weight^{-1}$)	CGMP (pmol $^{-1}$ mg $protein^{-1}$)
None	145±10	3.98±0.12
Carbachol (1×10^{-8} M)	262±18	8.69±0.20
U-73122 (5×10^{-6} M)	148±12*	4.22±0.15*
TFP (5×10^{-6} M)	152±13*	4.56±0.18*
Staurosporine (1×10^{-9} M)	278±19	8.72±0.21

Retina preparations were incubated for 30 min in the absence or in the presence of PLC inhibitor (U-73122), CaM blocker (TFP) and PKC inhibitor (staurosporine) and then carbachol was added at the last 5 min. Values are means±SEM of five determinations performed in duplicate. *Differ significantly from carbachol alone with $P<0\cdot001$.

Moreover, the inhibition of calcium/calmodulin (CaM) by TFP (5×10^{-6} M) significantly inhibited the stimulatory action of carbachol. However, the positive carbachol effect on NOS activity and cGMP production was not altered by the inhibition of protein kinase C (PKC) with staurosporine (1×10^{-9} M).

To analyse if carbachol enhances phosphoinositide (PIs) hydrolysis, retina preparations were incubated with 1×10^{-8} M carbachol in the presence or absence of cholinoceptor antagonists. Fig. 4 shows that carbachol leads to increased PIs production and atropine, 4-DAMP and pirenzepine (1×10^{-7} M), but not AF-DX 116 (1×10^{-6} M) blocked the stimulatory action of the cholinergic agonist, pointing to the participation of M_3 and M_1 mAChRs. As control, U-73122 (5×10^{-6} M) abrogated this effect, indicating that PLC activation was implicated in carbachol-stimulated PIs hydrolysis.

Using RT-PCR in vitro technique, we examined the retina tissues expressing nNOS mRNA levels. Semiquantitative reverse transcription polymerase chain-reaction analysis demonstrated that stimulation with carbachol (1×10^{-8} M) for 1 h increased nNOS-mRNA levels (Fig. 5). A reduction in carbachol-induced elevation of nNOS-mRNA levels could be detected in the presence of 4-DAMP or pirenzepine (1×10^{-7} M) but not AF-DX 116 (1×10^{-6} M) (Fig. 5). The fact that both M_3 and M_1 antagonists blocked the expression of nNOS mRNA, indicate that the action of carbachol on retina nNOS mRNA levels, occurs by the activation of M_3 and M_1 mAChR subtypes, respectively.

In order to determine if the endogenous NO/cGMP signaling system participates in carbachol-induced increases in nNOS-mRNA levels, retinas were incubated with enzymatic inhibitor agents at the same concentration used above. Fig. 6 shows that the inhibition of PLC, CaM, nNOS and soluble guanylate cyclase activities, but not the inhibiton of PKC activity, attenuated the carbachol-induced increase in nNOS-mRNA levels. These results could indicate that carbachol-activation of M_1/M_3 mAChR results in an increased endogenous NO and cGMP production, that in turn, up regulate nNOS early gene expression.

4. Discussion

In the present study, we showed that carbachol can stimulate NOS activity and increase nNOS mRNA gene expression in retina. These carbachol effects appear to be related with activation of M_3/M_1 cholinoceptor subtypes as their effects were blocked by M_3 and M_1 cholinoceptor antagonists. We observed that both receptors efficiently mediate stimulation of PIs accumulation and cGMP production. However, in rat retina M_3 activity is predominant with less activity of M_1, because 4-DAMP was more effective to inhibit carbachol-biological effects than pirenzepine used at the same concentration. This is in

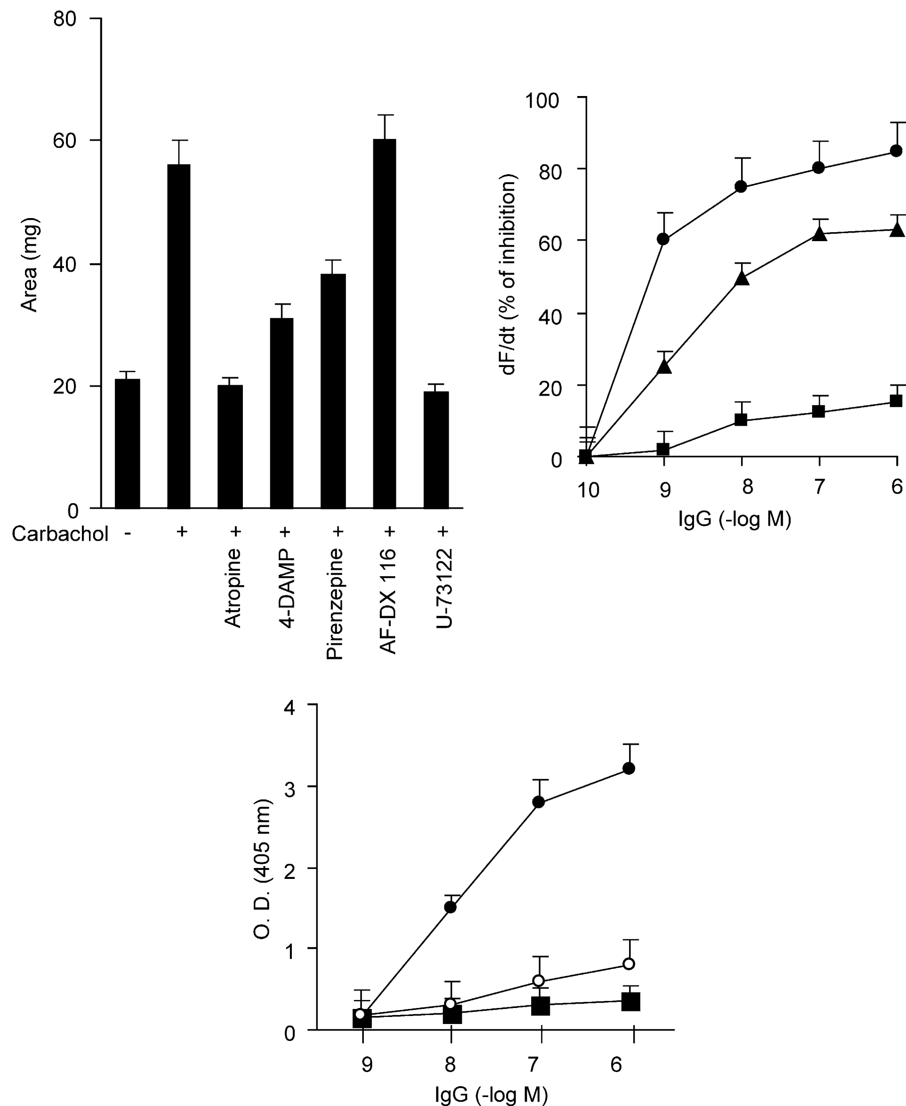

Fig. 4. Increased phosphoinositide hydrolysis by 1×10^{-8} M carbachol. Inhibition of the carbachol effect by treatment with 1×10^{-7} M atropine, 1×10^{-7} M 4-DAMP, 1×10^{-7} M pirenzepine and 1×10^{-6} M AF-DX 116. The action of PLC inhibitor 1×10^{-6} M U-73122 is shown. Values are means±SEM of six experiments performed in duplicate in each group. *Differ significantly from basal values with $P<0{\cdot}001$.

agreement with the findings of Shepard and Rae (1998), who reported that M_3 expression is the predominant subtype with minor contributions of M_1 in retinal cell cultures. The same distribution was reported in human eye using a number of techniques (Zhang et al., 1995; Wiederholt et al., 1996; Gil et al., 1997) that agree with the M_3 subtype being the most abundant.

The mechanism by which carbachol increased NOS activity seems to involve the activation of PIs hydrolysis through PLC activation, since, agents known to interfere with PLC activity inhibited the stimulatory effect of carbachol. However, PIs hydrolysis intermediates (IP_3 and DAG) played a different role on carbachol stimulated NOS activity. While the inhibition of CaM blunted the carbachol stimulatory action, the inhibition of PKC activity was ineffective, indicating that PIs up-regulates retina NOS activity. A constitutive nNOS, which is calcium dependent was described in rat retina, giving further evidence for the possible role of NO in retinal function and neuronal diseases (Bonner, 1989; Blute et al., 2003). In addition, our results showed that carbachol-stimulation of M_3/M_1 mAChRs leads to soluble guanylate cyclase activation, that occurs secondarily to stimulation of NOS activity (Sterin-Borda et al., 2003). The existence of NO/cGMP pathways in rat retina may be linked to the activation of M_3/M_1 mAChRs by carbachol, suggesting that the increment of NO and cGMP modulates the visual signal transmission and/or vision function (Blute et al., 2003).

Nitric oxide (NO) and cGMP have been implicated in many neuronal functions including regulation of gene expression (Gudi et al., 1999). A number of transcription factors that have the capacity to alter the rate of transcription of specific genes in response to receptor-mediated signaling events at the cell membranes have been identified

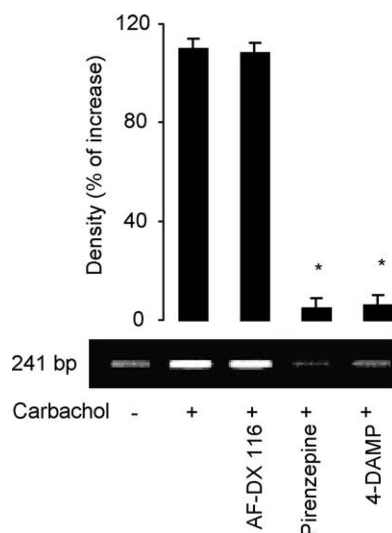

Fig. 5. Carbachol (1×10^{-8} M) action on semiquantitative RT-PCR analysis of nNOS mRNA levels in rat retina. Retinal preparations were incubated for 1 h with carbachol in the absence or in the presence of 1×10^{-6} M AF-DX 116, 1×10^{-7} M pirenzepine, 1×10^{-7} M 4-DAMP. Basal value corresponding to nNOS-mRNA levels after 1 h of incubation is also shown. Values are means ± SEM of five separated retina in each group. *Differ significantly from carbachol alone with $P<0{\cdot}001$. A representative RT-PCR band products obtained from this analysis are shown.

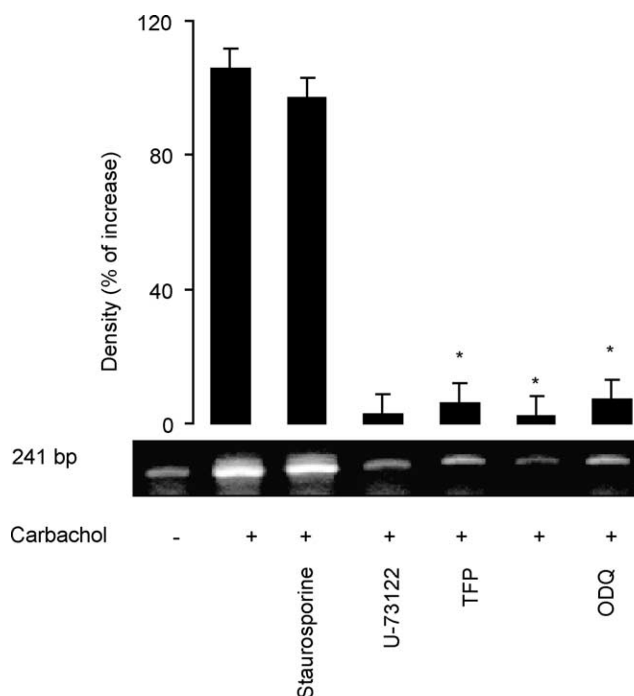

Fig. 6. Carbachol induced increase in nNOS-mRNA levels in rat retina. Retina preparations were incubated 1 h with carbachol (1×10^{-8} Mm) in the absence or in the presence of staurosporine (1×10^{-9} M); U-73122 (5×10^{-6} M); TFP (5×10^{-6} M); NZ (1×10^{-5} M) or ODQ (1×10^{-6} M). Basal values correspond to nNOS-mRNA levels after 1 h of incubation is also shown. Values are means ± SEM of five saparate retina in each group. *$P<0{\cdot}001$ comparing with carbachol alone. A representative RT-PCR band products obtained from this analysis are shown.

(Sheng and Greenberg, 1990). Some of these factors are rapidly and transiently induced following receptor activation and can be identified as early genes. Therefore, we wanted to determine whether the mAChR agonist carbachol acts as an early positive regulator of nNOS gene expression. Our results demonstrate that during one hour of carbachol activation, the expression of nNOS-mRNA levels is increased. This is in accordance with results obtained in carbachol effect on rat cerebral frontal cortex (Sterin-Borda et al., 2003). The fact that the carbachol stimulated increases in nNOS-mRNA levels in rat retina were inhibited by specific M_3 and M_1 cholinoceptor antagonists, points to a mixed responses due to the stimulation of multiple mAChR subtypes. It is likely that the same mAChR subtypes are used by carbachol to activate NOS enzyme and to increase nNOS transcription expression. It has also been reported that NO/cGMP is a powerfull modulator of gene expression (Eigenthaler et al., 1998). Also it has been reported that NO is able to dramatically alter the pattern of early gene expression in hippocampal granule cells (Morris, 1995). However, little is known about the simultaneous stimulation of nNOS early expression and the direct enzymatic activation of NOS. In retina we observed that the nNOS-mRNA gene expression promoted by M_1/M_3 mAChR activation was under the control of an endogenous NO and cGMP signaling system. The mechanism through which carbachol increased nNOS-mRNA levels involved calcium mobilization (Park et al., 1996), that in turn facilitated the stimulation of the common enzymatic machinery whose intermediates (NO/cGMP) can act as an inducer of nNOS expression. Harumi et al. (2003) demonstrated that NO itself and/or the cGMP generated by soluble guanylate cyclase plays a critical roles in early retina embryogenesis. The exact mechanism by which cGMP can lead to nNOS-mRNA gene expression still has to be clarified.

Activation of mAChRs increases the expression of the acetylcholinesterase immediate early gene. This enzyme accelerates the breakdown of acetylcholine to limit increases in cholinergic transmission (von der Kammer et al., 2001). Also, the immediate early gene expression induced by mAChR activation could play an important role in coupling receptor stimulation to long term neuronal response (Albrecht et al., 2000). In this way, the nNOS mRNA levels increased by carbachol may be involved in the regulation of microcirculatory haemodynamics in the rat retina. Development of the retinal vasculature can occur normally without eNOS. Similar levels of NO production, perivascular redistribution of nNOS and increased number of neurons in the eNOS-/-retinas, suggested that the increases in vascular-associated nNOS activity, compensate for the eNOS deficiency in the developing mutant retina (Al-Shabrawey et al., 2003).

NO can be considered as a double-edged sword. At low levels it can exert beneficial effects, but high levels of NO, if they persist uncontrolled, can be detrimental (Colasanti and Suzuki, 2000) due to the generation of highly toxic

compounds, which cause neuronal degeneration and nerve cell death (Przedborsky et al., 2000). Therefore, the hyperactivation of cholinergic system may activate genes that encode nNOS, leading to a subsequent rise in NO, which could contribute to the ethiopathogenesis of retinal diseases. Moreover, in the course of infection, inflammation and hypoxia, both nNOS/mAChRs could act as a modulator and/or as a regulator of other effectors.

Acknowledgements

This work was carried out with the aid of grants from University of Buenos Aires (UBACYT) and Argentine Research National Agency (BID 1202/OC/AR/PICT 5-8250). We also thank Mrs Elvita Vannucchi and Fabiana Solari for expert technical assistance.

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