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Involvement of endogenous nitric oxide signalling system in brain muscarinic acetylcholine receptor activation

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Summary. Biochemical signalling events coupled to muscarinic cholinergic receptors (mAChR), specifically those related to nitric oxide (NO) production, were studied on rat cerebral frontal cortex. The mAChR agonist carbachol was found to exert a specific biphasic action on NO synthase (NOS) activity: low doses ranging between 10^{-9}M to 10^{-7}M lead to NOS activation while higher doses ($>10^{-6}$M) inhibited enzymatic activity. Carbachol stimulatory action was blunted by agents that interfere with calcium-calmodulin while a protein kinase (PKC) inhibitor, staurosporine was able to abrogate the inhibitory effect. Moreover, PKC activity showed maximum translocation to cerebral frontal cortex membranes with carbachol concentrations that inhibited NO production. Products from phosphoinosite (PI) hydrolysis are involved in these actions as carbachol was found to increase PI turnover in a dose dependent manner. These results would serve as an example of cross-talk between both enzymatic pathways.

Keywords: Muscarinic acetylcholine receptor, cerebral frontal cortex, nitric oxide, protein kinase C, phosphoinositide turnover.

Introduction

Nitric oxide (NO) is a stable, free radical gas. Several disciplines has converged to establish NO as a major messenger molecule regulating neuronal transmittion in the brain and peripheral nervous system (Lowenstein et al., 1992; Marletta, 1993). NO is synthetized from the semi-essential amino acid L-arginine by NO synthase (NOS). This process can be inhibited by certain guanidino-substituted arginine analogues, including N^G-monomethyl-L-arginine (L-NMMA) (Lowenstein et al., 1992).

There appear to be at least three distinct forms of NOS: neuronal, endothelial and platelet. The neuronal NOS, which is of the constitutive type, has now been cloned and its cofactor binding sites identified (Bredt, 1992); no

major enzyme induction has yet been demostrated (Lowenstein et al., 1992). Instead, the enzyme is activated by calcium with binds calmodulin as an enzyme cofactor (Bredt and Snyder, 1990).

In the brain, glutamate stimulation of NMDA receptors opens calcium channels that are part of the receptor protein, triggering calcium influx to elicit increases of NOS (Lowenstein et al., 1992). Also, a muscarinic receptor-mediated NO generation has been described in the mouse neuroblastoma clone, NIE-11S (Shintani et al., 1994).

Muscarinic acetylcholine receptor (mAChR) subtypes in the rat brain are intrinsically heterogeneous. Regional distribution studies and binding characterization on mAChR solubilized from rat cerebral cortex, showed that most of the mAChRs in this cerebral region are of the M_1 subtype (Wang et al., 1987; Adem et al., 1988).

It is known that the activation of mAChRs by the agonist carbachol, triggers many different signal transduction pathways including: stimulation of guanosine 3′,5′-cyclic monophosphate (cGMP) production; a decrease in adenosine 3′,5′-cyclic monophosphate (cAMP) through either attenuation of cAMP synthesis or stimulation of cAMP degradation; regulation of several ion channels and stimulation of phospholipase (PL) C, A_2 and D (Feigenbaum and El-Fakahany, 1985; Hosey, 1992; Lai and El-Fakahany, 1987; Liles et al., 1986). Thus, stimulation of brain M_1 mAChR through activation of PLC initiates hydrolysis of inositol phospholipids (PIs) which produces inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 regulates the intracellular calcium concentrations by modifying calcium release from non-mitochondrial internal stores, whereas DAG activates protein kinase C (PKC) (Berridge and Iversen, 1989; Nishizuka, 1986; Lee and Fain, 1991). Studies of signalling events with cloned mAChR subtypes, have revealed differences in their abilities to modulate many different signal transduction pathways that involve PKC and NOS activities (Sandmann and Wurtman, 1991; Sterin-Borda et al., 1995).

Therefore, taking into account the above data, the aim of this paper is to determine the different signalling events involved in M_1 mAChR-dependent activation of NOS on the brain. Results show that the mechanism by which carbachol activation of M_1 mAChR increases NOS activity, appears to occur secondarily to a stimulation of phosphoinositide turnover via PLC activation. This in turn, triggers a cascade reaction involving calcium/calmodulin and PKC activation. Calcium/calmodulin acts as an enzyme cofactor leading to activation of NOS, while PKC down-regulates NOS, decreasing the carbachol effect.

Material and methods

Determination of nitric oxide synthase activity

NOS activity was measured in cerebral frontal cortex by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine according to the procedure described by Bredt and Snyder (1989) for brain slices. Briefly, after 20 min preincubation Krebs-Ringer Bicarbonate (KRB) solution, cerebral frontal cortices were transferred to 500 µl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]-arginine (0.5 µCi).

Appropriate concentrations of drugs were added and the cerebral frontal cortices were incubated for 20 min under 5% CO_2 in O_2 at 37°C. Cerebral frontal cortices 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 μM leupeptin and 0.2 mM phenylmethanesulphonyl fluoride at 4°C. After centrifugation at 20,000 g for 10 min at 4°C, supernants 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.

Protein kinase C (PKC) activity assay

PKC activity was assayed by measuring the incorporation of ^{32}P from gamma-^{32}P-ATP into histone H_1. Incubations were conducted for 30 min at 30°C in a final volume of 85 μl. In final concentrations, the assay mixture contained 25 μM ATP (0.4 μCi), 10 mM Mg_2 acetate, 5 mM β-mercaptoethanol, 50 μg of histone H_1, 20 mM HEPES, pH 7.4 and unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 μg/ml of phosphatidylserine vesicles. The incorporation of ^{32}P phosphate into histone H_1 was linear for at least 30 min. The reaction was stopped by the addition of 2 ml ice-cold 5% trichloroacetic acid, 10 mM H_3PO_4. The radioactivity retained on GF/C glass-fiber filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of calcium and phospholipids. PKM was assayed in the absence of Ca^{++} and phospholipids and in the presence of 10 μM quercetin as specific inhibitor (Junco et al., 1990). The data were expressed in picomol of phosphate incorporated into the substrate per minute and per milligram of protein (pmol/min/mg prot.).

Measurement of total labelled inositol phosphates (IPs)

Rat strips from cerebral frontal cortex were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 μCi [myo-^{3}H]-inositol ([^{3}H]-Ml) (Sp.Act. 15 Ci/mmol) 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 carbachol. Water-soluble IPs were extracted after 120 min incubation following the method of Berridge et al. (1982). Cerebral cortical strips 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) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3,000 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 6 volumes of water and IPs 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 miligram of wet weight tissue (area/mg) following the criteria of Simpson's equation (Camusso et al., 1995). In order to confirm the absence of [^{3}H]- MI in the eluted peaks of IPs, chromatography in silica gel 60 F254 sheets (Merck) was performed using propan-2-ol/6N NH_4OH(14:5) as the developing solvent following the procedure of Hokin-Neaverson and Sadeghian (1976).

Radioligand binding assay

Membranes from cerebral frontal cortex were prepared as described previously (Vanderheyden et al., 1986). In brief, cerebral frontal cortex was homogenized in an

Ultraturrax at 4°C in 5 volumes of potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose (buffer A), pH 7.5, supplemented with 0.1 mM phenylmethylsulphonyfluoride (PMSF), 2 μg ml^{-1} leupeptin and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3,000 g, then at 10,000 g and 40,000 g at 4°C, for 15 and 90 min respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors pH 7.5 (buffer B). Receptor ligand binding was performed as described previously (Vanderheyden et al., 1986). Aliquots of the membrane suspension (30–50 μg protein) were incubated with different concentrations of 1-[benzilic-4.4′-^{3}H(N)]-quinuclidinyl benzilate ([^{3}H]-QNB) (New England Nuclear, Sp. Act. 44.8 Ci/mmol) for 60 min at 25°C in a total volume of 100 μl of buffer B. Binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/C). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Non-specific binding was determined in the presence of 10^{-5} M atropine and never exceded 10% of total binding. Radioactivity bound was lower than 10% of total counts.

Drugs

Carbachol, N^G-monomethyl-L-arginine (L-NMMA), atropine, pirenzepine, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), staurosporine, trifluoperazine (TFP), L-arginine and AFDX 116 were purchase from Sigma Chemical Company, Saint Louis, 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.

Statistical analysis

Student's t-test for unpaired values was used to determine the levels of significance. When multiple comparison were necessary, after analysis of variance, the Student-Newman-Keuls test was applied. Differences between means were considered significant if $p \leq 0.05$.

Results

Figure 1 shows that carbachol modified NOS activity of rat cerebral frontal cortex in a concentration dependent manner. A biphasic effect was observed: at low concentrations (10^{-9} M to 10^{-7} M) the agonist induce a stimulatory action, while at higher ones (10^{-6} M to 10^{-5} M) an inhibitory action was obtained. A reversible NOS inhibitor (L-NMMA 1×10^{-5} M) blunted the action of carbachol, and the natural substrate L-arginine (5×10^{-5} M) completely reversed the inhibitory action of L-NMMA on the carbachol effect. As control, 1×10^{-6} M atropine and pirenzepine also abrogated both effects indicating that mAChR of the M_1 subtype is involved (Table 1).

In order to determine the mechanism by which the stimulation of mAChR modified NOS activity, cerebral frontal cortex preparations were incubated with different inhibitors of the enzymatic pathways known to be involved in the mAChR activation. Figure 2 shows, that the inhibition of PLC by NCDC (5×10^{-6} M), attenuated both the stimulatory and the inhibitory effect of carbachol observed at 1×10^{-7} M and 1×10^{-5} M respectively. To elucidate which pathways gated by polyphosphoinositide turnover (PI), could be involved in these effects, brain preparations were incubated in the presence of an inhibitor of PKC (staurosporine 1×10^{-9} M) or / and an inhibitor of calcium-calmodulin (TFP 5×10^{-6} M). In Fig. 2 it is also shown that TFP

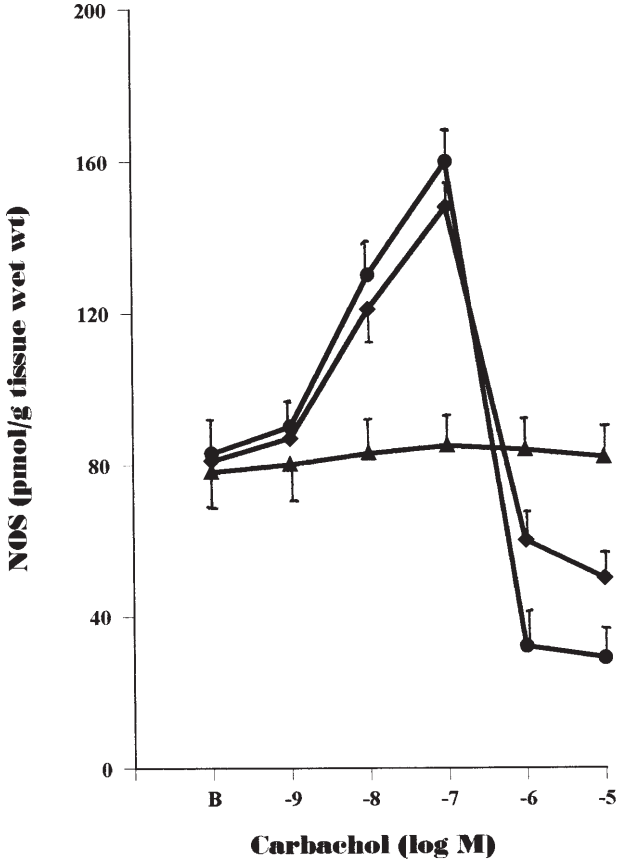

Fig. 1. Biphasic carbachol action upon NOS activity. Cerebral frontal cortex preparations were incubated for 30 minutes as indicated in Materials and Methods: alone (basal, **B**) or in the presence of increasing concentrations of carbachol (•). Inhibition of carbachol effects by L-NMMA (10^{-5}M, ▲) and its reversal with L-arginine (5×10^{-5}M, ■) is also shown. Data are the mean values of five experiments performed in duplicate ± SEM

Table 1. Influence of mAChR blockers on carbachol-effects on NOS activity

Additions	NOS activity (pmol/g/tissue wet wt.)	
	Carbachol 1×10^{-7}M	Carbachol 1×10^{-5}M
None	148 ± 8	54 ± 2
Atropine (1×10^{-6}M)	81 ± 3*	80 ± 5*
Pirenzepine (1×10^{-6}M)	80 ± 2*	84 ± 3*
AFDX 116 (1×10^{-6}M)	139 ± 7	56 ± 2

Nitric oxide synthase (NOS) activity was measured after incubating cerebral frontal cortex preparations with or without mAChR blockers for 20 min and then for an additional 5 min with the indicated carbachol concentrations. Results are the mean ± SEM of five experiments in each group performed in duplicate. *Significantly different from carbachol alone (none) with $P < 0.001$

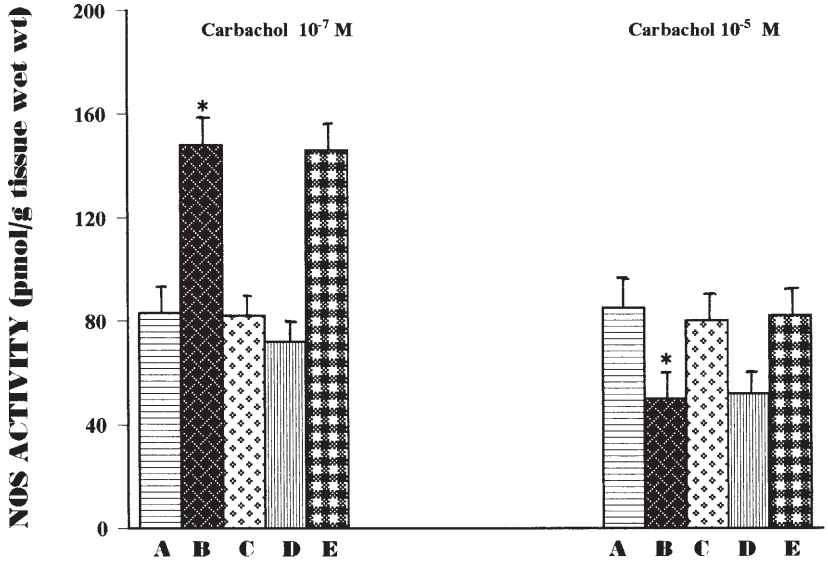

Fig. 2. Stimulatory and inhibitory effect of carbachol (10^{-7}M and 10^{-5}M respectively) on NOS activity. Cerebral frontal cortex preparations were incubated for 30 minutes in the presence of NCDC (5×10^{-6}M, **C**), TFP (5×10^{-6}M, **D**) and staurosporine (1×10^{-9}M, **E**). Tissues were then left for a further 10 minutes in the absence (basal, **A**) or in the presence of the indicated concentrations of carbachol (**B**). Values are mean ± SEM of five determination performed in duplicate. * differ significantly from basal values with $p < 0.05$

significantly inhibited the stimulatory action of 10^{-7}M carbachol, without altering the inhibitory action. Moreover, the negative carbachol effect on NOS activity was prevented by the inhibition of PKC activity by staurosporine.

To analyze if carbachol effects upon brain mAChR lead to a dose – dependent PKC activation, rat cerebral frontal cortex, was incubated with different concentrations of carbachol and PKC enzymatic activity was determined in both cytosolic and membrane fractions. In Fig. 3A carbachol induced PKC traslocation within 30 min of incubation in a dose – dependent manner, increasing the membrane PKC activity significantly. At 10^{-5}M carbachol the PKC activity recovered in membrane decreased, probably due to a proteolytic degradation of PKC. In fact, at this carbachol concentration, the measurement of PKM activity increased by 100% (data non shown). The carbachol effect was blunted by atropine and pirenzepine (Fig. 3B) pointing to the involvement of mAChR of the M_1 subtype in the carbachol induced stimulation of PKC activity. Furthermore, the carbachol effect was abrogated by NCDC (5×10^{-6}M) indicating a phospholipase C mediated activation of PKC.

Figure 4 shows the relationship between carbachol action on both membrane PKC and NOS activity. It can be seen that at low carbachol concentrations (10^{-9}M to 10^{-7}M) the maximal NOS activity is obtained while PKC traslocation is minimum at this point; but higher concentrations of the mus-

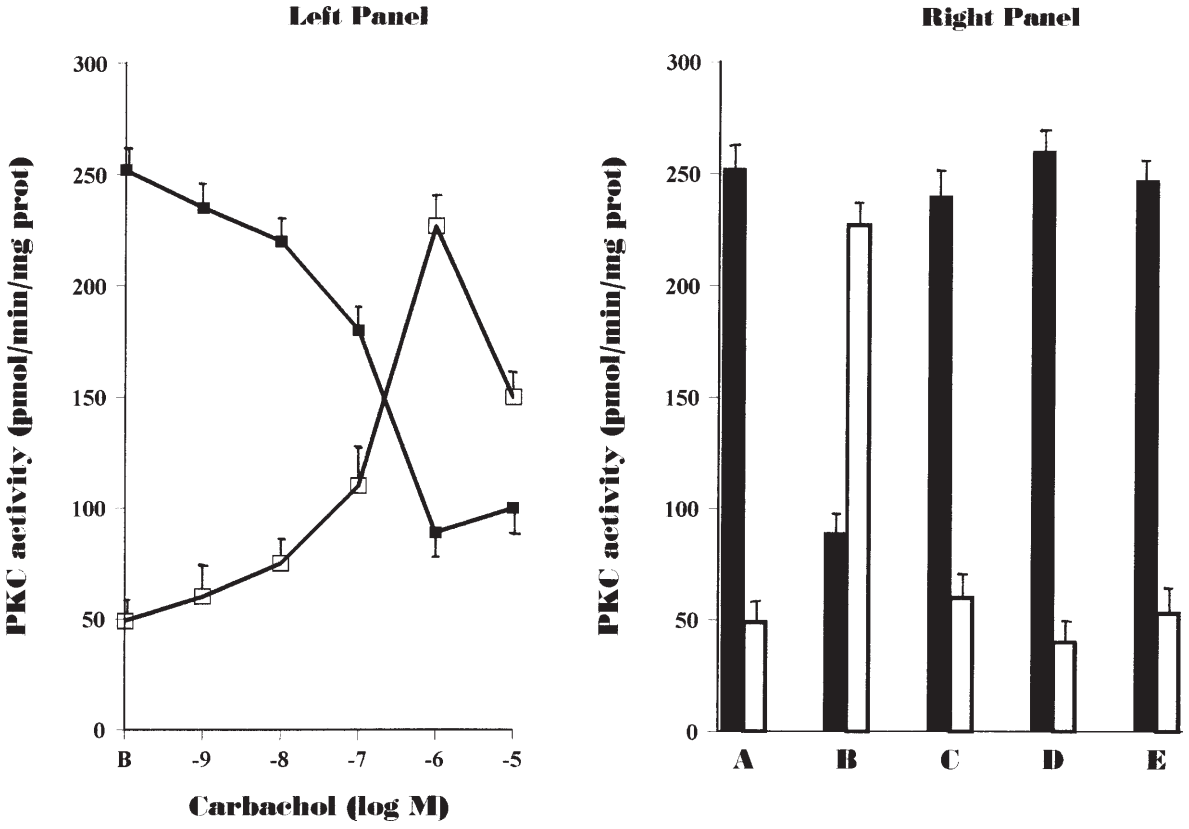

Fig. 3. Activation of cerebral cortex PKC by carbachol. *Left panel* PKC activity was determined on both cytosolic (■) and membrane (□) preparations as indicated in Materials and methods, alone (basal, **B**) or in the presence of increasing concentrations of carbachol. *Right panel* The effect of different blockers on both cytosolic (full bars) and membranes (blank bars) PKC activity upon carbachol stimulation (**B**) above basal values (**A**) was analyzed. Cerebral cortex were preincubated before the addition of the mAChR agonist with the PLC blocker NCDC (5×10^{-6}M, **C**), atropine (1×10^{-5}M, **D**) or pirenzepine (1×10^{-5}M, **E**). Results are the mean ± SEM of four independent experiments performed in duplicate

carinic agonist, induced an important traslocation that, in turn, inhibited the NOS activity.

In order to investigate the possibility that carbachol induces a concentration-dependent activation of phosphoinositide (PI) turnover, brain preparations were incubated with different concentrations of carbachol in the presence or absence of cholinoceptor antagonists. As can be seen in Fig. 5A carbachol increased inositol phosphate (IP) formation. The degree of stimulation induced by carbachol, was directly proportional to its concentration, from 10^{-9}M to 10^{-7}M decreasing thereafter, although to values significantly higher than basal ones. Atropine and pirenzepine (1×10^{-6}M) significantly inhibited the stimulatory action of carbachol, pointing to the participation of M_1 mAChR (Fig. 5B). As control, NCDC also abrogated this

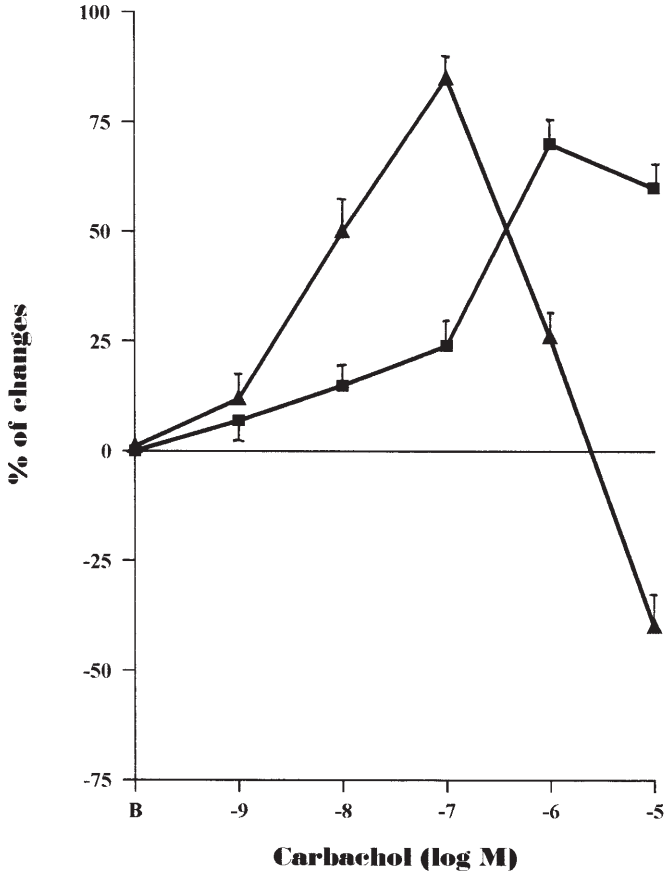

Fig. 4. Comparison of carbachol effect on NOS and PKC activities. Dose-response curves to carbachol upon NOS (▲) and PKC (■) activities are shown in the same coordinates for comparisson. Results are the mean ± SEM of five experiments for NOS and four independent experiments for PKC determination

effect indicating that PLC activation was implicated in carbachol-stimulated PI hydrolysis.

To confirm the M_1 mAChR subtype involvement in the biological effects of carbachol in rat cerebral frontal cortex, radioligand binding assays were performed. Binding of [^{3}H]-QNB to frontal cerebral cortex was a saturable process to a single class of binding sites. The equilibrium parameters calculated from Scatchard plots were Kd (0.53 ± 0.09 nM) and Bmax (620 ± 40 fmol/mg protein). As shown in Table 2, the rank order of potencies of the mAChR antagonist drugs as inhibitors of [^{3}H]-QNB binding was atropine > pirenzepine > AFDX 116, strongly indicating that brain frontal cortex expresses preferentially M_1 mAChR subtype.

Discussion

The present data characterized different signalling events involved in the M_1 mAChR stimulation in rat cerebral frontal cortex. Results show that

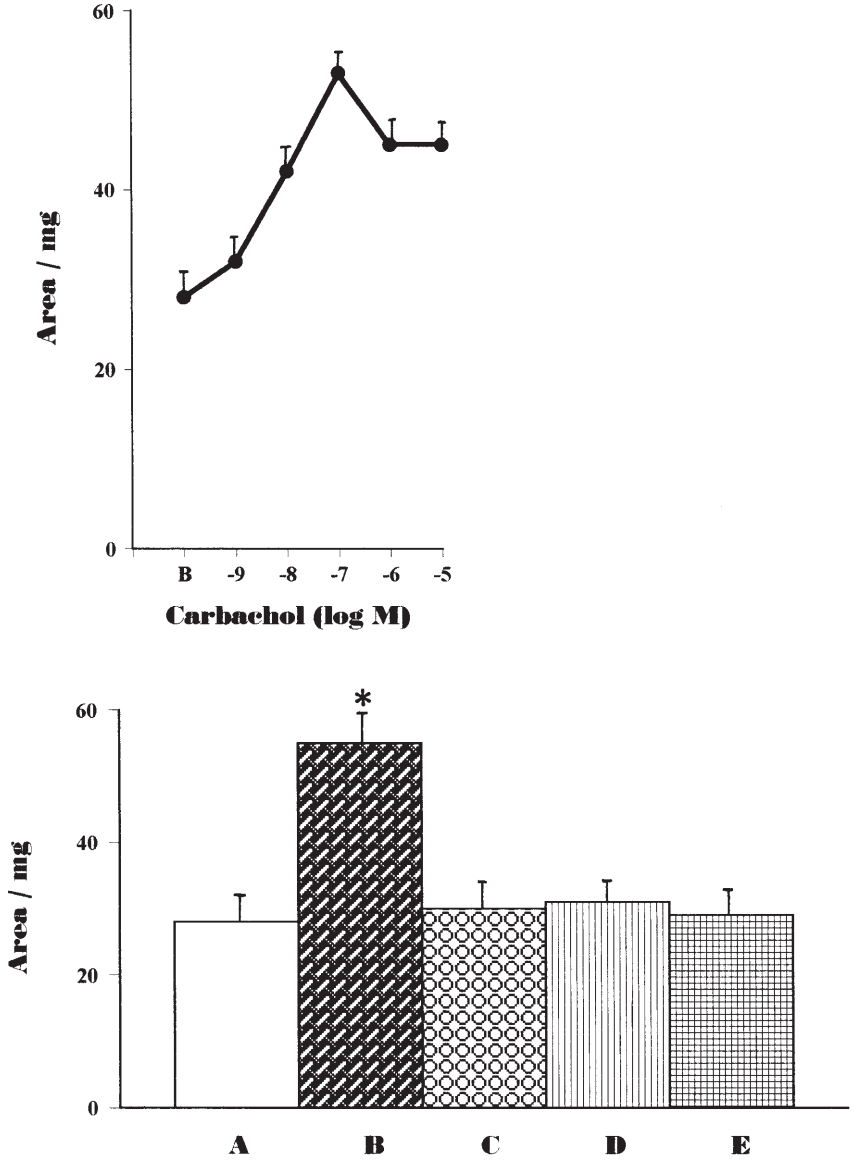

Fig. 5. *Upper panel* Increase of phosphoinositide turnover by different concentrations of carbachol (•). *Lower panel* Inhibition of the effect of carbachol (1×10^{-7}M, **B**) by treatment with staurosporine (1×10^{-6}M, **C**), pirenzepine (1×10^{-6}M, **D**) and NCDC (5×10^{-6}M, **E**). Basal values (**A**) is also shown. Values are mean ± SEM of four experiments performed in duplicate in each group. * differ significantly from basal values with $p < 0.05$

carbachol activation of M_1 mAChR triggers a biphasic NO production, dependent on the concentration of the agonist used. Specifically, at low concentrations carbachol produced an increase in NOS activity, while at high concentrations carbachol decreased it. Both carbachol actions upon NOS activity appeared to be specific, since they were abolished by the stereospecific NOS-inhibitor, L-NMMA (Mulsh and Busse, 1990), and could be

Table 2. Potencies of mAChR antagonists inhibiting ^{3}H-QNB binding on rat cerebral frontal cortex membranes

Cholinoceptor agent	Ki (nM)
Atropine	2.95 ± 0.03
Pirenzepine	23.5 ± 0.17
AFDX 116	93,805 ± 1,825

The inhibition constants (Ki) for the competing agents were calculated from the equation (Cheng and Prussoff, 1973) Ki = $IC_{50}/1([^3H\text{-}QNB])/Kd$ where IC_{50} is the concentration of the competing drug that inhibits 50% of the specific radioligand binding present at a concentration of 0.6 nM. IC_{50} were obtained from competion experiments performed in duplicate at several concentrations of each drug

reversed by the addition of the enzyme substrate L-arginine. Moreover, they seemed to be mediated by M_1 receptor activation as atropine and the M_1 selective antagonist pirenzepine abrogate carbachol effects. In support to this and, as previously demostrated (Wang et al., 1987; Adem et al., 1988), we found that mAChR of the M_1 subtype are preferentially expressed in cerebral frontal cortex as shown by competitive binding assays.

Furthermore, the mechanism of carbachol mAChR stimulation in cerebral cortex seems to involve an increase in PI hydrolysis through PLC activation, since agents known to interfere with PLC activity blunted both (stimulatory and inhibitory) effects of carbachol. PI hydrolysis varied in parallel with carbachol concentrations. However, PI hydrolysis intermediates (IP_3 and DAG) played an oppossite role on carbachol induced NO production. Thus, rapid activation of NOS is related to PI turnover – gated calcium mobilization. On the other hand, inhibition of NOS require high PKC activity.

In fact, low concentrations of carbachol increase NOS activity, whereas poor PKC activation was obtained in this conditions. In contrast, maximal PKC activation was observed at high concentrations of the agonist. It is worth noting that only at the highest concentration of carbachol (10^{-5} M) used, was PKC activity on membranes diminished, but this was in fact due to enzyme degradation, since increased values of PKM were found. These data suggest that at this dose of the agonist an important and significant translocation of the enzyme still ocurred and this was coincident with those that induced inhibition of NOS activity. This statment is based on the fact that the positive effect of carbachol on NOS activity was inhibited by 10^{-5} M of the agonist. In contrast, staurosporine blunted the inhibitory action of carbachol without modifying its stimulatory action.

These results could indicate a cross-talk between both enzymatic pathways: M_1 mAChR coupled intracellular signals appear to be mutually modulated in rat cerebral frontal cortex where different degrees of activation of

PKC and calcium-calmoduline-stimulated NOS were obtained, depending on the concentration of the cholinergic agonist assayed.

The involvement of these pathways in normal or pathological brain functioning or its participation in neuroleptic side effects, would merit future research.

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