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Differential signalling pathways involved in cholinoceptor-dependent stimulation of nitric oxide isoforms in dental pulp

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Abstract

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Aim To investigate the role of muscarinic acetylcholine receptor (mAChR) subtype activity in the regulation of endothelial- (e) and neuronal- (n) nitric oxide synthase (NOS) expression and activity.

Methodology Rat dental pulp tissue was used throughout the study. The e-nos and n-nos mRNA levels were specifically measured using reverse transcriptase polymerase chain reaction procedures that involve simultaneous co-amplification of both target cDNA and a reference template with a single set of primers. NOS activity was measured by the production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine.

Results Stimulation of M_1/M_2 and M_3/M_4 mAChRs with pilocarpine caused an increase in e-nos and n-nos mRNA levels and NOS activity in the dental pulp. The specific mAChR subtype antagonists, L-NMMA, L-NIO and N_2-propyl-L-arginine but not aminoguanidine attenuated all these effects. Inhibitors of phospholipase C (PLC), protein kinase C (PKC) and calcium/calmodulin (CaM) prevented the pilocarpine-dependent increase in n-nos and e-nos mRNA levels and NOS activity.

Conclusions Activation of mAChR subtypes stimulated NOS activity by increasing the production of NO through e-nos and n-nos gene expression and NOS activity. The mechanism appears to occur secondarily to stimulation of CaM and PKC enzymatic activity.

Keywords: dental pulp, e-nos mNRNA, mAChR, n-nos mRNA, nitric oxide synthase, pilocarpine.

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Introduction

The physiological role of parasympathetic system modulation in the dental pulp remains a controversial issue. There is evidence for the presence of cholinergic nerves, muscarinic receptors and acetylcholine-degrading enzymes in pulp tissue (Phohto & Antila 1972). However, a striking feature of dental pulp parasympathetic innervations is its high density relative to other tissues in the body (Zhang *et al.* 1998, Iijima & Zhang 2002). Parasympathomimetic agents cause vasodilatation within the dental pulp tissue of several species (Edwall *et al.* 1973, Okabe *et al.* 1989, Liu *et al.* 1990). Also, triggering of a physiological reflex activation of autonomic nerves in humans evokes a weak pulpal vasodilatation, which is inhibited by atropine (Aars *et al.* 1993). The vasodilator action provoked by acetylcholine has been shown to be dependent on the production of nitric oxide in a number of tissues (Wallace *et al.* 1989, Gardiner *et al.* 1990, White *et al.* 1993).

Thus, muscarinic acetylcholine receptor (mAChR)/nitric oxide (NO) cascade is involved in vasodilation. The development of tenderness to palpation in the buccal sulcus following dental pulp injury has been

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associated with the release of NO via mAChR activation (Yonchara *et al.* 2002, 2003).

Nitric oxide is an intracellular messenger molecule generated by NOS through the oxidation of L-arginine to citrulline. The involvement of NO in dental pulp circulation has been described (Lohinai *et al.* 1995). Three distinct isoforms of NOS have been cloned to date: endothelial (e-NOS), neuronal (n-NOS) and inducible (i-NOS) NOS (Nathan 1992, Liandet *et al.* 2000). The n-NOS is mainly localized on pulpal nerve fibres (Lohinai *et al.* 1997) and e-NOS is detected in endothelial cells of the human dental pulp (Felaco *et al.* 2000). The e-NOS and n-NOS are constitutive isoforms that can rapidly synthesize small amounts of NO, following receptor stimulation (Carmignani *et al.* 2000). The i-NOS is mainly involved in inflammatory processes and produces large amounts of NO for sustained periods of time. NO has a role in nonspecific immune responses, acting as a toxic agent in infections (Moilanen *et al.* 1999, Carmignani *et al.* 2000).

The aim of this paper was to investigate whether the mAChR agonist (pilocarpine) can induce cholinergic NOS activation and nos isoforms – mRNA gene expression in rat dental pulp tissue. The participation of different signal events underlying pilocarpine-induced n-nos and e-nos gene expression and activities was also explored.

Materials and methods

Animals

Male Wistar rats from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires) weighing 220–260 g were used throughout the study. The animal experiments were approved by the local Animal Ethics Committee at the University of Buenos Aires. The animals were subjected to the following environmental conditions 23 °C/25 °C, 12 hours dark/light cycle; they were provided with water and food *ad libitum*. The animals were killed by cervical dislocation.

Exposure of dental pulp

Healthy rat dental pulp tissue of upper incisors (left and right) was extracted following exposure of the pulp chamber using diamond burs. The dental pulp tissue was immediately kept at room temperature in Krebs Ringer bicarbonate (KRB) solution in the presence of CO_2 in oxygen until the various experimental assays were performed.

mRNA isolation and cDNA synthesis

Total RNA was extracted from rat dental pulp tissue by homogenization using the guanidinium isothiocyanate method (Chomozynski & Saachi 1987). As previously described (Sterin-Borda *et al.* 2003), a 20-μL reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mmol L^{-1} dNTPs and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized at 37 °C for 60 min.

PCR procedures

Nitric oxide synthase isoform mRNA levels were determined 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 e-nos and n-nos and glyceraldehyde-3-phosphate dehydrogenase (g3pdh) were constructed using a polymerase chain reaction (PCR) MIMIC construction kit. 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 native targets. The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of NOS isoforms and g3pdh mRNA was as reported previously (Orman *et al.* 2005). 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 was added to PCR amplification reactions containing the first-strand cDNA. Polymerase chain reaction MIMIC amplification was performed in 100 μL of a solution containing 1.5 mmol L^{-1} $MgCl_2$, 0.4 μmol L^{-1} primer, dNTPs, 2.5 U Taq DNA polymerase and 0.056 μmol L^{-1} Taq Start antibody (Clontech Laboratories, Mountain View, CA, USA). After initial denaturation at 94 °C for 2 min, the cycle condition was 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C and 45 s for enzymatic primer extension at 72 °C for 45 cycles for NOS isoforms. The internal control was the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (g3pdh). Polymerase chain reaction 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. Polymerase chain reaction

products were subjected to electrophoresis following procedures described previously (Sterin-Borda *et al.* 2003). Different NOS isoforms, mRNA levels were normalized with the levels of g3pdh mRNA present in each sample, which served to control for variations in RNA purification and cDNA synthesis.

Determination of nitric oxide synthase activity

Nitric oxide synthase activity was measured in rat dental pulp tissue by the 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 pre-incubation KRB solution, tissues were transferred to 500 mL of pre-warmed 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 mmol L^{-1} HEPES pH 7.4, 0.5 mmol L^{-1} EGTA, 0.5 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol, 1 mmol L^{-1} leupeptin and 0.2 mmol L^{-1} phenylmethylsulphonyl fluoride (PMSF) 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.

Measurement of total labelled inositol phosphates

Rat dental pulp tissue was 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]-MI) (Sp. Act. 15 Ci $mmol^{-1}$) from Dupont/New England Nuclear. LiCI (10 mmol L^{-1}) was added for inositol monophosphate accumulation, according to the technique of Berridge *et al.* (1982). Pilocarpine was added 30 min before the end of the incubation period and the blockers 30 min before the addition of the agonist. Water-soluble inositol phosphates (InsP) were extracted after 120 min of incubation as previously described (Sterin-Borda *et al.* 1995).

Protein kinase C assay

Protein kinase C (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 μmol L^{-1} ATP (0.4 μCi), 10 mmol L^{-1} Mg acetate, 5 mmol L^{-1} β-mercaptoethanol, 50 μg of histone H_1, 20 mmol L^{-1} HEPES pH 7.5 and unless otherwise indicated, 0.2 mmol L^{-1} $CaCl_2$ and 10 μg mL^{-1} of phosphatidylserine vesicles. The incorporation of [^{32}P]-phosphate into histone was linear for at least 20 min. The reaction was stopped by the addition of 2 mL of ice-cold 5% trichloroacetic acid, 10 mmol L^{-1} H_3PO_4. The radioactivity restrained on GF/C glass–fibre 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. The data were expressed in picomol of phosphate into the substrate per minute and per milligram of protein (pmol · min^{-1} mg^{-1} $prot^{-1}$).

Drugs

Pilocarpine, AF-DX 116, pirenzepine, 4-DAMP, tropicamide, U-73122, N^G-methyl-L-arginine acetate salt (L-NMMA), trifluoroperazine (TFP) and staurosporine were purchased from Sigma Chemical Company (St Louis, MO, USA); [N^5-1-iminoethyl)-L-ornithine] (L-NIO) and [N_2-propyl-L-arginine] (NZ) were obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Stock solutions were freshly prepared in the corresponding buffers.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. An analysis of variance (ANOVA) and Student–Newman–Keuls test were employed when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant if $P < 0.05$.

Results

As can be seen in Fig. 1a, pilocarpine induced a concentration-dependent increase in the activity of NOS at concentrations ranging from 10^{-10} to 10^{-6} mol L^{-1}. This stimulatory action of pilocarpine was abrogated by the inhibition of NOS activity by 1×10^{-5} mol L^{-1} L-NMMA. As control, L-arginine (5×10^{-5} mol L^{-1}) reversed the L-NMMA effect. To discern which mAChR subtypes were involved in pilocarpine-stimulated NOS activity, several cholinoceptor antagonist subtype agents were used. Figure 1b shows that pirenzepine (1×10^{-7} mol L^{-1}) and AF-DX 116 (5×10^{-7} mol L^{-1}) inhibited the stimulatory action of

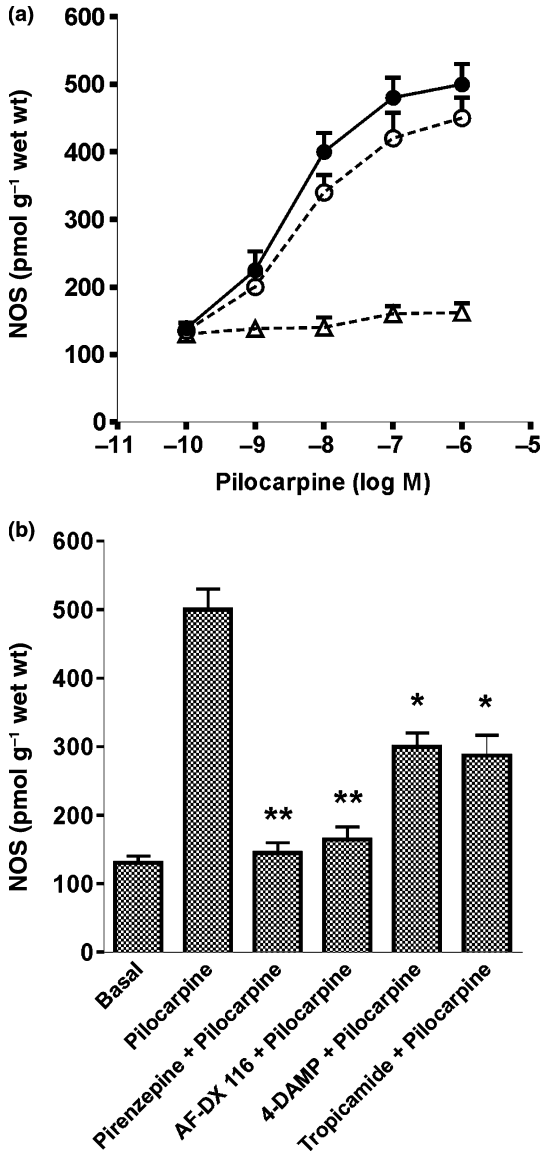

Figure 1 (a) Stimulation in nitric oxide synthase (NOS) activity of rat dental pulp by increasing concentrations of plilocarpine (●). The inhibitory action of 1×10^{-5} mol L^{-1} L-NMMA (△) and reversal of inhibition by 5×10^{-5} mol L^{-1} L-arginine (○) are also shown. (b) Stimulation of NOS activity by 1×10^{-7} mol L^{-1} pilocarpine and inhibition of pilocarpine action by 1×10^{-7} mol L^{-1} pirenzepine and AF-DX 116 and 1×10^{-6} mol L^{-1} 4-DAMP and tropicamide were shown. Basal values are also shown. Values represent the mean ± SEM of eight experiments in each group. *$P < 0.005$ versus pilocarpine; **$P < 0.001$ versus pilocarpine.

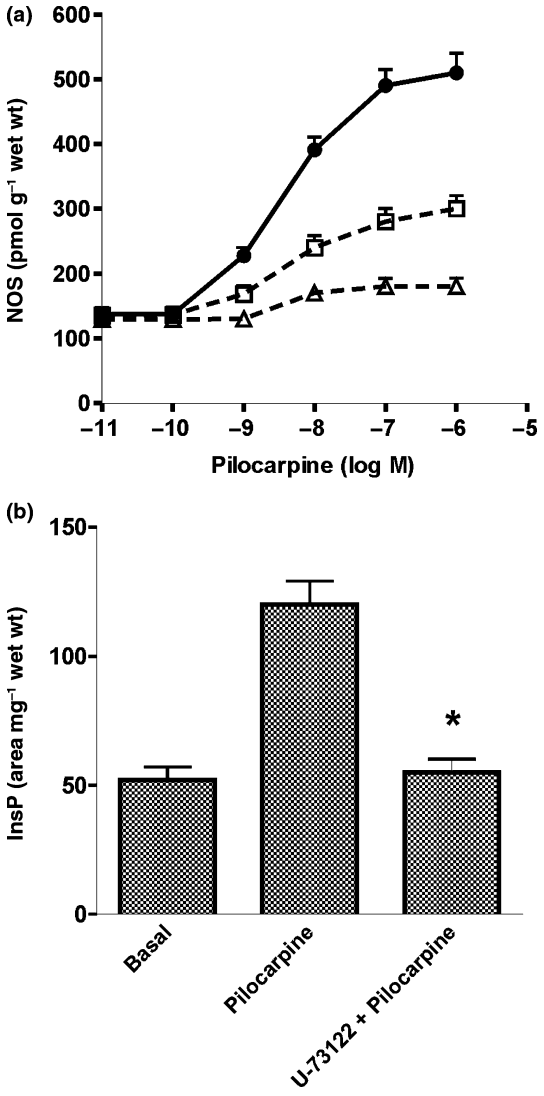

Figure 2 (a) Stimulation in nitric oxide synthase activity of rat dental pulp by increasing concentrations of pilocarpine (●). The inhibitory action of 5×10^{-6} mol L^{-1} U-73122 (△) and 5×10^{-6} mol L^{-1} trifluoroperazine (□) are shown. (b) Stimulation of InsP production by 1×10^{-7} mol L^{-1} pilocarpine and inhibition of pilocarpine action by 5×10^{-6} mol L^{-1} U-73122. Basal value is also shown. Values represent the mean ± SEM of six experiments performed by duplicate in each group. *$P < 0.001$ versus pilocarpine.

pilocarpine by 60% while 4-DAMP (1×10^{-6} mol L^{-1}) and tropicamide (1×10^{-6} mol L^{-1}) inhibited pilocarpine effect only by 40%; indicating the participation of M_1/M_2 and M_3/M_4 mAChR in the action of pilocarpine upon NOS activity.

To determine the nature of the mechanism by which the activation of M_1/M_2 and M_3/M_4 mAChR increase NOS activity, dental pulp tissue was incubated with inhibitors of the enzymatic pathways involved in the mAChR activation. Figure 2a shows that both

5×10^{-6} mol L^{-1} U-73122 (PLC inhibitor) and 5×10^{-6} mol L^{-1} TFP (CaM inhibitor) diminished the stimulatory action of pilocarpine. Additional studies were performed to assess if mAChR subtypes were coupled to InsP turnover and activated PLC, by measuring the production of InsP. Figure 2b shows that 1×10^{-7} mol L^{-1} pilocarpine was able to provoke InsP accumulation in dental pulp tissue and 5×10^{-6} mol L^{-1} U-73122 prevented this pilocarpine action, pointing to the participation of PLC.

To investigate the possibility that PKC might participate in NOS activation through mAChR stimulation, dental pulp was incubated with the PKC inhibitor staurosporine (1×10^{-9} mol L^{-1}). As can be seen in Fig. 3a, staurosporine inhibited the stimulatory action of 1×10^{-7} mol L^{-1} pilocarpine upon NOS activity. Figure 3b demonstrates that under identical experimental condition, the mAChR activation by pilocarpine increased the membrane PKC activity. As control, staurosporine impaired the pilocarpine action.

To demonstrate which isoforms of NOS might be implicated in the pilocarpine-induced NOS stimulation, isolated rat dental pulp tissue was incubated with specific inhibitors of NOS isoforms. As can be seen in Fig. 4, the inhibition of both e-NOS activity by L-NIO (5×10^{-6} mol L^{-1}) and n-NOS activity by NZ (5×10^{-6} mol L^{-1}) inhibited the stimulatory action of pilocarpine on NOS activity. In contrast, the inhibition of i-NOS by aminoguanidine (1×10^{-7} mol L^{-1}) had no effect. The L-NIO was more effective than NZ to inhibit the mAChR agonist effect.

To determine whether the enzyme activities were dependent on each other, dental pulp tissue was incubated with different enzymatic pathway inhibitors involved in mAChR activation. Figure 5 shows that TFP, but not staurosporine, inhibited the remaining available NOS activity when the pulp tissue was pre-incubated with L-NIO. On the other hand, the Fig. 5 shows that staurosporine, but not TFP, was able to inhibit the remaining available NOS activity in the presence of NZ. These results demonstrate that NOS isoforms are independent of each other's mechanism: e-NOS is CaM dependent and PKC independent while n-NOS is PKC dependent and CaM independent.

To determine the role of different enzymes on NOS isoforms activation reverse transcriptase polymerase chain reaction (RT-PCR) was used on e-nos and n-nos mRNA obtained from rat dental pulp tissue. Using specific oligonucleotide primers, RT-PCR-amplified products showed bands of the predicted size for e-nos and n-nos detected in dental pulp (Fig. 6). Figure 6 also

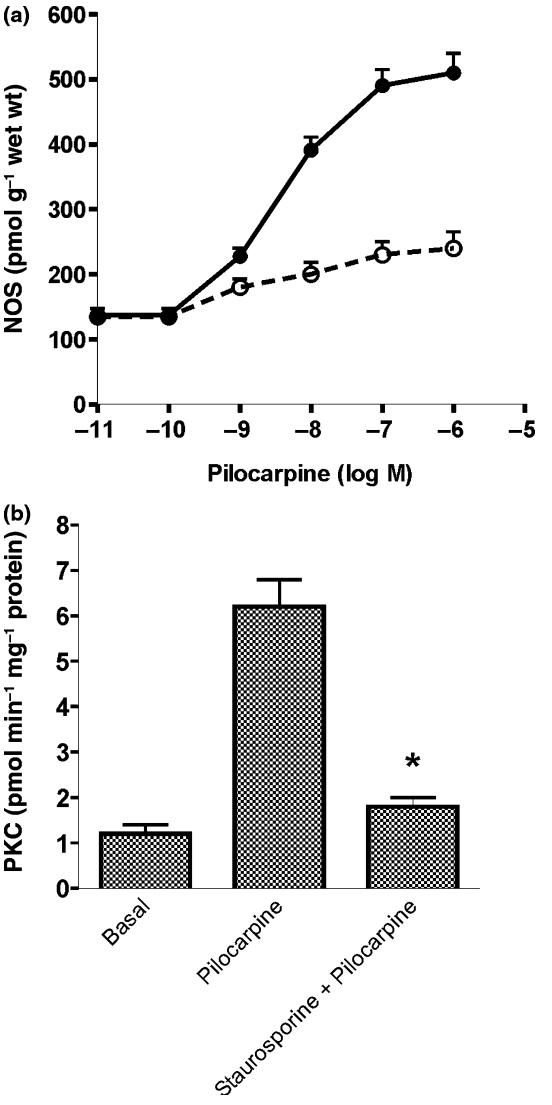

Figure 3 (a) Stimulation in nitric oxide synthase activity of rat dental pulp by increasing concentrations of pilocarpine (●). The inhibitory action of 1×10^{-9} mol L^{-1} staurosporine (○) is shown. (b) Increasing membrane PKC by 1×10^{-7} mol L^{-1} pilocarpine alone or pilocarpine in the presence of 1×10^{-9} mol L^{-1} staurosporine is also shown. Rat dental pulp were incubated for 30 min with 1×10^{-7} mol L^{-1} pilocarpine and cytosol and membrane fractions were obtained as described in the Materials and Methods section. PKC activity was measured in cytosol (c) and membrane (m). Values of PKC expressed as pmol min^{-1} mg^{-1} $prot^{-1}$ were: c = basal 6.9 and pilocarpine 2.2; m = basal 1.9 and pilocarpine 6.3. Values are mean ± SEM of six experiments in each group. *$P < 0.001$ versus pilocarpine.

shows that pilocarpine (1×10^{-7} mol L^{-1}) was able to augment the size of both e-nos and n-nos bands. Staurosporine inhibited pilocarpine-increased e-nos

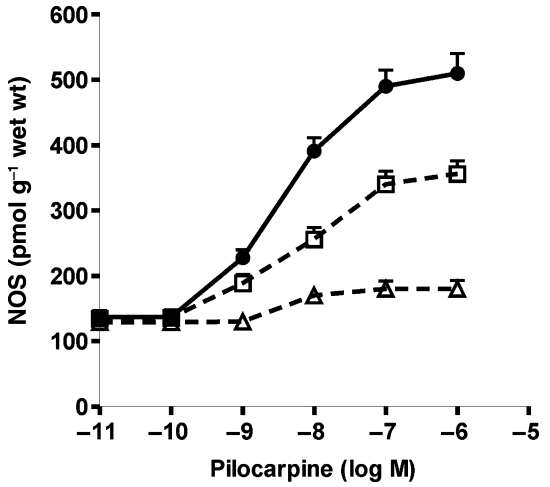

Figure 4 Stimulatory effects triggered by different concentrations of pilocarpine (●) on nitric oxide synthase activity upon rat dental pulp. The actions of 1×10^{-6} mol L^{-1} aminoguanidine (□), 5×10^{-6} mol L^{-1} NZ (○) and 5×10^{-6} mol L^{-1} L-NIO (△) on pilocarpine action are shown. Values represent the mean ± SEM of seven experiments in each group.

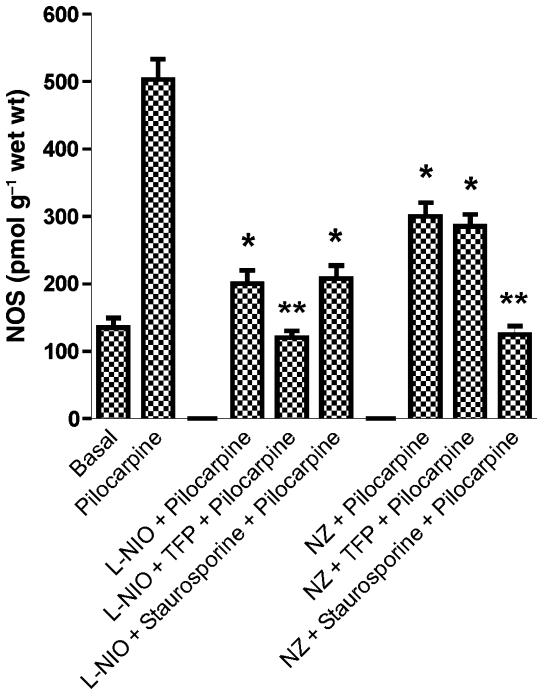

Figure 5 Effect of enzymatic blockers on the pilocarpine-induced increase in nitric oxide synthase (NOS) activity. Rat dental pulp were incubated with 1×10^{-7} mol L^{-1} pilocarpine alone or in the presence of 5×10^{-6} mol L^{-1} L-NIO or L-NIO plus 5×10^{-6} mol L^{-1} trifluoroperazine (TFP) or L-NIO plus 1×10^{-9} mol L^{-1} staurosporine and 5×10^{-6} mol L^{-1} NZ or NZ plus 5×10^{-6} mol L^{-1} TFP or NZ plus 1×10^{-9} mol L^{-1} staurosporine. Basal values correspond to NOS activity without treatment. Values are mean ± SEM of five experiments performed by duplicate in each group. *$P < 0.001$ versus pilocarpine; **$P < 0.005$ versus L-NIO and NZ plus TFP and staurosporine respectively.

mRNA levels while TFP prevented the pilocarpine-increased n-nos mRNA levels.

Discussion

The present data indicate that the activation of M_1/M_2 and M_3/M_4 mAChR of rat dental pulp increased NOS activity and exerted an increase in e-nos and n-nos mRNA gene expression. These results point to a role for PKC and calcium mobilization in rapid activation of NOS and e-nos and n-nos mRNA levels.

Two lines of evidence support these conclusions: on the one hand, the concentration response curves of pilocarpine acting on mAChR increased NOS activity. On the other hand, the pilocarpine action on NOS activity was decreased when NOS and NOS isoforms were inhibited. Both, these effects were receptor-mediated actions demonstrated by virtue of blockade by the selective mAChR subtype antagonists. The rank of potency for the ability of pilocarpine to trigger NOS activity calculated using specific mAChR antagonists showed $M_1 = M_2 > M_3 = M_4$. Although both e-NOS and n-NOS are constitutively expressed in pulp, mAChR agonist activated more effective e-NOS than n-NOS. The fact that, both L-NIO, a specific inhibitor of e-NOS (Rees *et al.* 1990) and NZ, a specific inhibitor of n-NOS (Zhang *et al.* 1997), abrogated pilocarpine increase on NOS activity strongly suggests that e-NOS and n-NOS are constitutive and active in dental pulp tissue.

There are many reports showing that in the denervated rats, acetylcholine or carbachol provoked a vasodilator response on dental pulp tissue. Furthermore, a direct modulation by acetylcholine of sympathetic nerve function has been postulated (Lindmar *et al.* 1968, Kostouros *et al.* 1996). Additional evidence for the presence of mAChR in dental pulp tissue showed that the release of noradrenaline in response to sympathetic nerve stimulation was reduced in the presence of acetylcholine and was antagonized by atropine (Löffelholz & Muscholl 1969). Parker *et al.* (1995) showed that adrenergic nerve terminals in dental pulp were equipped with muscarinic receptors.

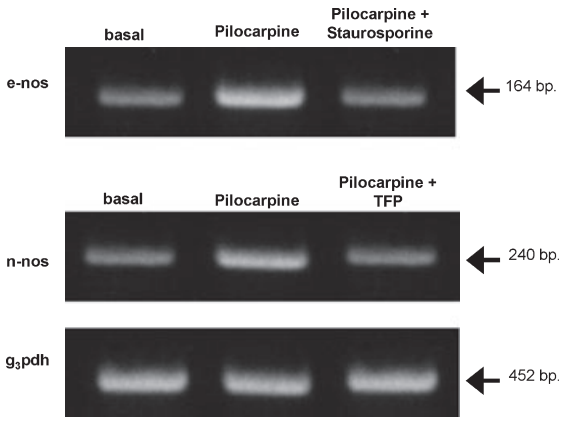

Figure 6 Reverse transcriptase polymerase chain reaction product for endothelial- (upper panel) and neuronal- (lower panel) nitric oxide synthase (NOS) alone or in the presence of 1×10^{-7} mol L^{-1} pilocarpine and pilocarpine in the presence of 1×10^{-9} mol L^{-1} staurosporine or 5×10^{-6} mol L^{-1} trifluoroperazine are shown. Band of $g_3$pdh is also shown. Dental pulp were incubated for 1 h in Krebs Ringer bicarbonate on identical experimental condition that was described for NOS determinations and enzymatic assays. Then, mRNA isolation and DNA synthesis were extracted and determine as described in Materials and Methods section.

The mechanism by which pilocarpine is able to stimulate NOS activity seems to involve an increase in InsP hydrolysis whose intermediates might turn on CaM and PKC. This statement is supported by the fact that, when PLC, CaM, PKC and NOS were inhibited, the action of pilocarpine decreased and the activation of mAChR subtypes by pilocarpine led to an increase in InsP production, PKC translocation and NOS activity.

The nature of pilocarpine stimulation suggests that the NO-mediated pathway might be relevant, as L-NMMA (Mulsh & Busse 1990), L-NIO and NZ but not aminoguanidine (Knowles & Moncada 1994) decreased the pilocarpine effect on NOS activity on dental pulp. Also, in pulp tissues of rats, it was observed that e-NOS and n-NOS were expressed constitutively and i-NOS expression could not be detected in healthy rat pulp tissue. Pilocarpine promotes an increase in e-nos and n-nos gene expression and NOS activity. The mechanism seems to involve differential participation of PKC and CaM activities as TFP (Scharff & Foder 1984) prevented pilocarpine increase of n-nos mRNA levels and NOS activity while staurosporine (Liu *et al.* 1998) prevented pilocarpine increase of e-nos mRNA levels and NOS activity. Thus, PKC is an important inducer of e-nos mRNA gene expression and NOS activity while CaM is required for n-nos mRNA gene expression and NOS activity.

In pulpal stromal cells (endothelium and vascular smooth muscle, nerve fibres and odontoblasts) soluble guanylate cyclase, e-NOS and n-NOS were identified (Korkmaz *et al.* 2005) by immunoreactivity and were able to maintain pulpal homeostasis. The i-NOS was not present in healthy pulp but was induced in pathological processes (pulpitis) (Di Nardo Di Maio *et al.* 2004). NO plays an important physiological role as intercellular and intracellular signal molecule in various organs and cells (Moncada & Higgs 1991, Nathan 1992, Bredt & Snyder 1994). On the other hand, the parasympathetic system via the activation of mAChR is one of the NO-bioregulatory molecules that plays an important role in physiological processes on different tissues (Lohinai *et al.* 1995, Sugiya *et al.* 1998, Berra *et al.* 2005).

A vascular injury or the activation of mAChR promotes the influx of calcium ions (Felaco *et al.* 2000) and the consequent rise of intracellular calcium stimulates both e-NOS- and n-NOS-constitutive NOS isoforms (Kim 1990). Therefore, NO, in healthy dental pulp, most probably can play an important role in the metabolic and trophic activity and can mediate in vasodilation and cell proliferation in this tissue as well (Sase & Michel 1995).

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