# Inflammation Triggers Constitutive Activity and Agonist-induced Negative Responses at $M_3$ Muscarinic Receptor in Dental Pulp

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## Abstract

The purpose of this study was to investigate whether the inflammation of rat dental pulp induces the muscarinic acetylcholine receptor (mAChR) constitutive receptor activity. Pulpitis was induced with bacterial lipolysaccharide in rat incisors dental pulp. Saturation assay with [$^3$H]-quinuclidinyl benzilate ([$^3$H] QNB), competitive binding with different mAChR antagonist subtypes, and nitric oxide synthase (NOS) activity were performed. A drastic change in expression and response to mAChR subtypes was observed in pulpitis. Inflamed pulp expressed high number of $M_3$ mAChR of high affinity, whereas the $M_1$ mAChR is the main subtype displayed in normal pulp. Consistent with the identification of the affinity constant (Ki) of $M_3$ and Ki of $M_1$ in both pulpitis and in normal pulps are the differences in the subtype functionality of these cells. In pulpitis, pilocarpine ($1 \times 10^{-11}$ mol/L to $5 \times 10^{-9}$ mol/L) exerted an inhibitory action on NOS activity that was blocked by J 104129 fumarate (highest selective affinity to $M_3$ mAChR). In normal pulps, pilocarpine ($1 \times 10^{-11}$ mol/L to $5 \times 10^{-9}$ mol/L) has no effect. NOS basal activity was 5.9 times as high in pulpitis as in the normal pulp as a result of the activation of inducible NOS. The irreversible pulpitis could induce a mAChR alteration, increasing the high-affinity receptor density and transduction-coupling efficiency of inducible NOS activity, leading to a spontaneously active conformation of the receptor. Pilocarpine acting as an inverse agonist might be useful therapeutically to prevent necrosis and subsequent loss of dental pulp. *(J Endod 2011;37:185–190)*

## Key Words

Binding assay, constitutive receptor activity, dental pulp, $M_3$ muscarinic receptor, nitric oxide synthase, pilocarpine, pulpitis

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The nature of inflammation in the dental pulp might not be different from that of other organs. Thus, pulpitis is an adaptive immune process characterized by an increase in local blood flow (1), cellular and humoral response activation immune (2), and alteration of neuronal activity (3). The critical role of acetylcholine (ACh) and the vagus nerve has likewise been demonstrated in the immune inflammatory process. Vagotomy blocks behavioral responses and cytokine induction in the brain after a proinflammatory stimulus (4). There is evidence of the presence of cholinergic nerves, muscarinic receptors, and ACh-degrading enzymes in pulp tissue (5,6). Parasympathetic innervations in the dental pulp are distributed around small blood vessels (7), and parasympathomimetic agents control pulpal blood flow, causing vasodilatation (8–10).

Recently we demonstrated a difference in the physiological role of the parasympathetic system in healthy dental pulp and in pulpitis. We have established that in the healthy dental pulp, stimulation of the muscarinic ACh receptor (mAChR) by orthosteric agonist pilocarpine triggered the release of prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO) via the activation of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) (6,11). On the other hand, muscarinic parasympathetic receptor activation might modulate the pulpal inflammatory process, decreasing $PGE_2$ and matrix metalloproteinase-3 (MMP-3) production as a result of inducible nitric oxide synthase (iNOS) overactivity and expression with intracellular accumulation of NO (12,13). The drastic change in tissue response to mAChR activation observed during the inflammation was associated with the high basal NOS activity, $PGE_2$ and MMP-3 production. The down-regulation of NOS activity triggered by pilocarpine in pulpitis was observed when the mAChR agonist was applied even *in vivo* and *in vitro*. Moreover, the drug was more effective at the beginning of pulp inflammation (13), when the expression of proinflammatory enzymes and substances is largely produced by the inflammatory cells (14).

The mAChR belongs to the 7-transmembrane-domain receptor (7-TM) superfamily of G protein–coupled receptors that have the capacity to regulate cellular signaling systems in the absence of occupancy by a ligand (ie, the receptors display constitutive activity) (15). These receptors spontaneously form active states capable of producing elevated basal cellular activity (constitutive activity) in the absence of agonist; this activity can be selectively blocked by ligand (inverse agonist) (16). On the basis of the cooperativeness that links their primary site of interaction with other functional domains of the receptor, inverse agonists have been classified into 3 classes: orthosteric-true inverse agonist, allosteric -true inverse agonist, and allosteric-pseudo inverse agonist, depending on whether they interact with the agonist binding pocket and affect the receptor-signaling domain or bind outside the agonist-binding pocket and still repress the receptor signaling or the ligand also bind ectopically but do not affect the receptor-signaling domain (17).

Constitutive activity and inverse agonist at mAChR have been studied, mainly with receptor mutants (18–21). Evidence for "cross talk" between mAChR subtypes in the regulation of second messengers in Chinese hamster ovary cells has been described (22). Most importantly, the inverse agonist acting in a more physiological setting, such as mAChR in heart membranes (23) and in control of $Ca^{2+}$ and $K^+$ channels in

intact ventricular myocytes (24), was observed. There is evidence that increased cholinergic tone is an important feature of chronic pulmonary disease (25, 26).

In this study, we investigated whether the paradoxical inhibitory effect of pilocarpine observed in pulpitis is associated with inflammation-induced, mAChR-constitutive receptor activity. We provide evidence that irreversible pulpitis can induce a mAChR subtypes alteration, increasing the high-affinity receptor density and the receptor transduction-coupling efficiency of iNOS activity, leading to a spontaneously active conformation of the receptor. Then pilocarpine action on a constitutive mAChR activity might act as an inverse agonist and, consequently, produce an inhibitory effect on NOS activity.

## Materials and Methods

### Animals

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

### Induction of Pulpitis

Under general anesthesia induced with intramuscular ketamine (62.5 mg/kg$^{-1}$) and intramuscular xylocaine, the pulps of the maxillary right and left incisors were exposed by using diamond burs. The entrance of the pulp chamber was enlarged, covering a length of 5 mm with K-files, up to #40, to create sufficient space to apply lipopolysaccharide (LPS). LPS from *Escherichia coli* O111:B4 (Sigma Chemical Co, St Louis, MO) was dissolved in sterile saline at a concentration of 10 mg/mL$^{-1}$, and 4 $\mu$L was applied to the cavities. Sterile saline instead of LPS was applied to evaluate the effects of mechanical stimuli. Entrances to the pulp horns were sealed with temporary filling material (Cavit ESPE, Seefeld, Germany). Animals were killed under ether anesthesia 6 hours after pulp exposure, and the incisors were extracted. LPS caused maximal inflammatory reaction in the coronal area of the pulp at 6 hours, decreasing thereafter, which confirmed a previous report (27). Findings were characterized by disruption of dentinogenesis, blood vessel dilatation, and infiltration of many neutrophils. Fresh dental pulp tissue was kept at room temperature in Krebs Ringer bicarbonate (KRB) solution in the presence of 5% $CO_2$ in oxygen until the various experimental assays were performed. Nontreated, healthy rat dental pulp tissue from the maxillary incisors (right and left) was used as controls.

### Radioligand Binding Assay

Membranes were prepared as previously described (28). In brief, pulps were homogenized in an UltraTurrax homogenizer (IKA Works, Inc, Wilmington, NC) at 4°C in 6 volumes of potassium phosphate buffer and 1 mmol/L $MgCl_2$, 0.25 mol/L sucrose (buffer A; pH 7.5) supplemented with 0.1 mmol/L phenylmethylsulphonylfluoride, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 $\mu$g/mL$^{-1}$ leupeptin, 1 $\mu$mol/L bacitracin, and 1 $\mu$mol/L pepstatin A. The homogenate was centrifuged twice for 10 minutes at 3000*g* and then at 10,000*g* and 40,000*g* at 4°C for 15 and 90 minutes, respectively. The resulting pellets were resuspended in 50 mmol/L phosphate buffer with the same protease inhibitors (pH 7.5; buffer B). Receptor ligand binding was performed as previously described (28). Aliquots of the membrane suspension (30–50 $\mu$g protein) were incubated with different concentrations of [$^3$H]-quinuclidinyl benzilate ([$^3$H] QNB) (Specific Activity 44 Ci/mmol; DuPont/New England Nuclear, Boston, MA) for 60 minutes at 25°C in a total volume of 150 $\mu$L buffer B. The binding was stopped by adding 2 mL ice-cold buffer, followed by rapid filtration (Whatman GF/c; Whatman International Ltd, Maidstone, UK). Filters were rinsed with 12 mL ice-cold buffer, transferred into vials containing 10 mL scintillation cocktail, and counted in a liquid scintillation spectrometer. No specific binding was determined in the presence of $1 \times 10^{-8}$ mol/L atropine, and it never exceeded 10% of total binding. Radioactivity binding was lower than 10% of total counts. For competition binding experiments, membranes were incubated with increasing concentrations of mAChR antagonists pirenzepine and J 104129 fumarate in the presence of 0.40 nmol/L [$^3$H] QNB. Binding data were analyzed with the computer-assisted, curve-fitting program LIGAND.

### Determination of NOS Activity

NOS activity was measured in the rat dental pulp tissue by the production of [U-$^{14}$C] citrulline from [U-$^{14}$C] arginine, as previously described (6). Briefly, the pulp was incubated for 30 minutes in 500 $\mu$L KRB solution containing 18.5 kilobecquerels of L-[U-$^{14}$C] arginine.

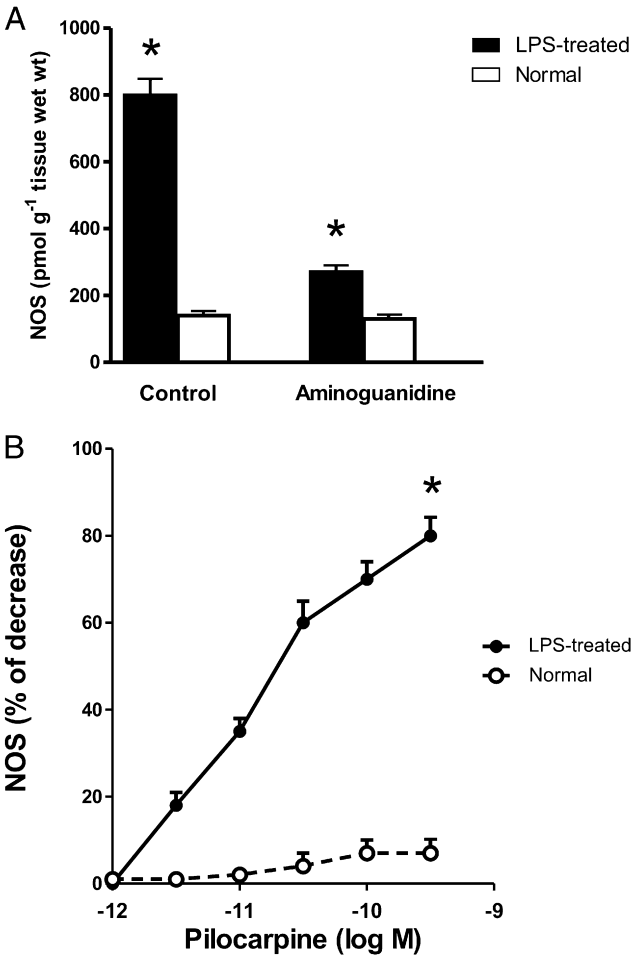


**Figure 1.** (*A*) Basal values of NOS activity in the presence or absence (control) of aminoguanidine ($1 \times 10^{-6}$ mol/L) in LPS-treated pulp and in normal pulp. *$P$ < .0001 vs normal pulp or LPS-treated pulp without aminoguanidine (control). (*B*) Concentration-response curve of pilocarpine on NOS activity in LPS-treated pulps (●) and normal pulps (○). *$P$ < .0001 vs normal. Tissues were incubated for 30 minutes in KRB. Pilocarpine was added 10 minutes before incubation. Values are means ± SEM of $n$ = 6 in each group performed by duplicate.

Inhibitors were added from the beginning of the incubation period at the final concentrations indicated in the text and the agonist pilocarpine (at different concentrations) at 10 minutes before the end of incubation. Incubation was carried out in 5% $CO_2$ in oxygen atmosphere at 37°C. The tissues were then homogenized in an UltraTurrax homogenizer in 500 $\mu$L of medium that contained 20 mmol/$L^{-1}$ HEPES at 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/$L^{-1}$ phenylmethylsulfonyl fluoride at 4°C. Supernatants were applied to 2-mL columns of Dowex AG 50WX-8 (sodium form), and [$^{14}$C] citrulline was eluted with 3 mL water and quantified by liquid scintillation counting (Beckman LS 6500, New York, NY).

## Drugs

Pilocarpine, atropine, pirenzepine, and aminoguanidine were purchased from Sigma Chemical Co (St Louis, MO), and AF-DX 116, tropicamide, and J 104129 fumarate were obtained from Tocris Cookson, Inc (Ellisville, MO). Stock solutions were freshly prepared in the corresponding buffer.

## Statistical Analysis

The Student *t* test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and the Student-Newman-Keuls test were used when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant at $P < .05$.

# Results

Figure 1*A* shows that basal values of NOS activity in LPS-treated pulp at 6 hours (pulpitis) were significantly higher than those of untreated pulp (normal). The inhibition of iNOS by aminoguanidine ($1 \times 10^{-6}$ mol/L) decreased the basal activity of NOS in pulpitis but had no effect on those from normal pulp. These results confirmed previous results (13) indicating that the high activity of NOS in pulpitis could be the result of an elevated activity of iNOS. To assess the influence of mAChR on LPS-treated pulps and normal pulps, we developed concentration-response curves showing the effect of pilocarpine on NOS activity. Figure 1*B* shows that in LPS-treated pulps, pilocarpine triggered an inhibitory action, whereas at the same concentration range the mAChR agonist had no effect in normal pulp.

Figure 2 shows that pilocarpine negative effect was abolished by $1 \times 10^{-7}$ mol/L J 104129 fumarate (an inhibitor with highest affinity for $M_3$ mAChR). Moreover, Figure 2 shows that under identical experimental conditions the inhibition of $M_1$ (by pirenzepine), $M_2$ (by AF-DX 116), and M4 (by tropicamide) showed minimal effect.

Figure 3 shows that in LPS-treated pulps, J 104129 fumarate shifted the pilocarpine inhibitory effect to the right (the Schild plot shows $pA_2$ 11.7 ± 0.3).

Figure 4*A* shows the saturation binding with increasing concentration (0.01–1.5 nmol/L) of [$^3$H] QNB in the membranes of LPS-treated pulps and the rat normal dental pulps. Results revealed the saturable mAChR binding sites that are significantly higher in pulpitis than in normal pulp. Scatchard analysis (Fig. 4*B*) distinguished in LPS-treated pulps the mAChR populations with higher affinity (dissociation constant [$K_d$]) and higher number of sites (maximum binding capacity [$B_{max}$]) than in normal pulp within the concentration range examined: LPS-treated pulp population (medium ± standard error of the mean [SEM], $n = 5$; $B_{max}$, 394.5 ± 18.6 fmol/mg protein; $K_d$, 0.22 ± 0.03 nmol/L); normal pulp population (medium ± SEM, $n = 6$; $B_{max}$, 230.8 ± 14.4 fmol/mg protein; $K_d$, 0.48 ± 0.07 nmol/L).

To determine the subtypes of mAChR, competition binding studies were performed by using pirenzepine and J 104129 fumarate to displace the binding of 0.40 nmol/L [$^3$H] QNB in LPS-treated pulps (Fig. 5*A*) and normal pulps (Fig. 5*B*). As shown, both mAChR antagonists displaced [$^3$H] QNB in a dose-dependent manner. It can be seen in Figure 5*A* that in the LPS-treated pulps, J 104129 fumarate displayed higher affinity binding sites (affinity constant [Ki], $9.2 \times 10^{-11}$ mol/L) than pirenzepine (Ki, $1.1 \times 10^{-8}$ mol/L). On the other hand, in normal pulps (Fig. 5*B*), pirenzepine displayed higher affinity binding sites (Ki, $1.3 \times 10^{-9}$ mol/L) than J 104129 fumarate (Ki, $2.1 \times 10^{-8}$ mol/L).

# Discussion

The major finding of the present study was that progressive inflammation of the pulp caused drastic changes in tissue expression and

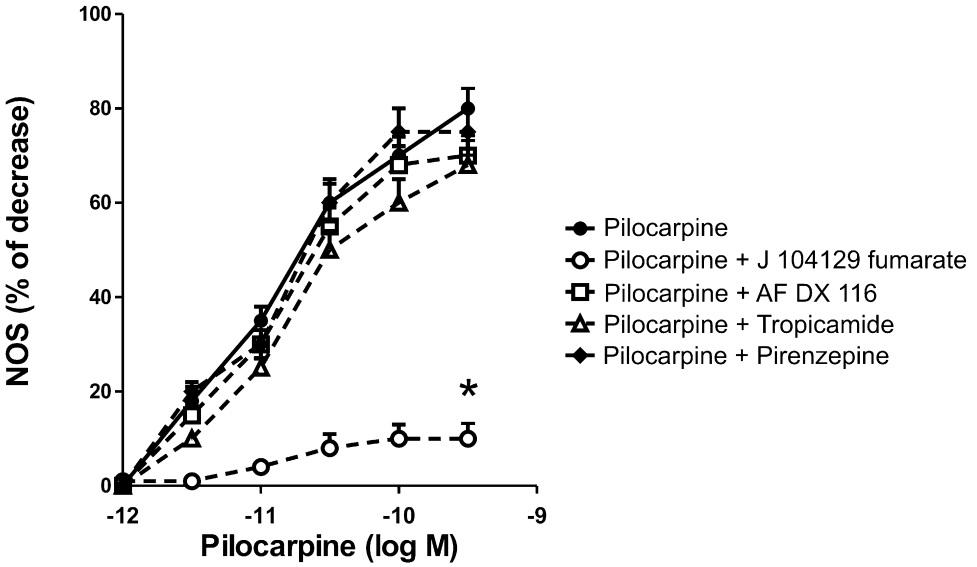


**Figure 2.** Concentration-response curves of pilocarpine in NOS activity of LPS-treated pulp. The pilocarpine negative effect was evaluated alone or in the presence of $1 \times 10^{-7}$ mol/L of different mAChR antagonist subtypes. Values are means ± SEM of $n = 7$ in each group performed by duplicate. *$P < .0001$ vs pilocarpine alone.

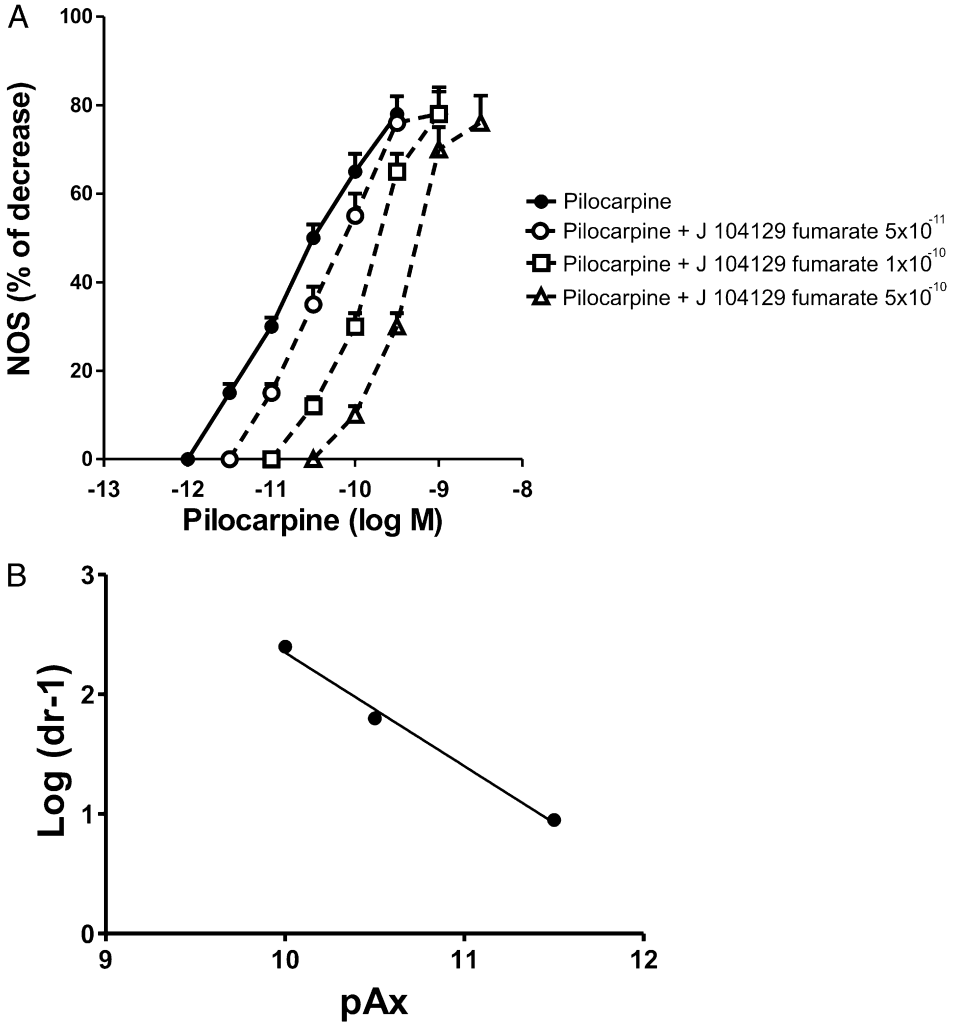


**Figure 3.** (*A*) Effect of $5 \times 10^{-11}$ mol/L, $1 \times 10^{-10}$ mol/L, and $5 \times 10^{-10}$ mol/L of J 104129 fumarate on the inhibitory dose-response curve of pilocarpine on NOS activity of LPS-treated pulps. Each point represents the mean ± SEM of $n = 5$ in each group. (*B*) Schild plots of J 104129 fumarate antagonism of pilocarpine-mediated NOS activity inhibition.

response to mAChR subtypes. We found that $M_1$ and $M_3$ mAChR are expressed in pulpitis differently than in a healthy pulp. Thus, LPS-treated pulps expressed a higher number of mAChRs than did normal pulps. This increment in the number of mAChRs occurred mainly at the expense of $M_3$ mAChR subtype. Thus, competition studies with pirenzepine and J 104129 fumarate indicate a difference in distribution and expression of binding sites for LPS-treated and normal pulps. The fact that J 104129 fumarate showed more affinity than pirenzepine in LPS-treated pulps allowed us to identify higher affinity binding sites for $M_3$ mAChRs than those for $M_1$ mAChRs in pulpitis. On the other hand, experiments with the same mAChR antagonists indicate higher affinity binding sites for $M_1$ mAChRs than those for $M_3$ mAChRs in normal pulps. Consistent with identifying the Ki of $M_1$ mAChRs and $M_3$ mAChRs in both pulpitis and normal pulps are the observed differences in subtype functionality of these cells.

In this study through NOS activity, we determined the specific contributions of $M_1$ and $M_3$ mAChR subtypes in normal and inflamed pulps. We observed that LPS-treated pulps responded to the agonist with negative fashion that depended on the concentrations in which different mAChR subtypes are functionally activated. The $M_3$ antagonist (J 104129 fumarate) blocks the inhibitory effect on NOS, indicating, therefore, that the $M_3$ subtype is linked to the inhibitory response at low concentrations of the agonist. On the contrary, the $M_1$, $M_2$, and $M_4$ antagonists were without effects on pilocarpine effect on NOS activity. Consistent with the identification of the pA2 and Ki for $M_3$ mAChR in pulpitis is the observed difference in subtype functionality of these cells. Our results are in agreement with recent findings made for the identification of orthosteric and allosteric site mutation of $M_2$ mAChR of CHO cells, which demonstrated that alanine substitution of Tyr-80 in orthostatic site TM2 increased the efficacy of pilocarpine, but allosteric site mutation in TM7 and in the second extracellular loop of the receptor had no effect on the functional affinity or efficacy of pilocarpine (29).

The differential effects on ligand efficacy, in addition to evidence of pathway-selective effects, highlight the propensity for ligand and pathway-selective G protein–coupled receptor activation states/conformation likely to be responsible for the phenomenon of ligand-directed signaling of G protein–coupled receptors (29). Thus, previous studies have shown, as we did in this article, that NOS activity in LPS-treated pulps was 5.9 times as high as that of normal pulps. The large basal NOS activity observed in LPS-treated pulps resulted from the induction of iNOS mRNA, with a peak expression at 6 hours, whereas iNOS mRNA expression was not observed in nontreated pulps (12, 27). Moreover, in LPS-treated pulps, the large basal NOS activity resulted from iNOS

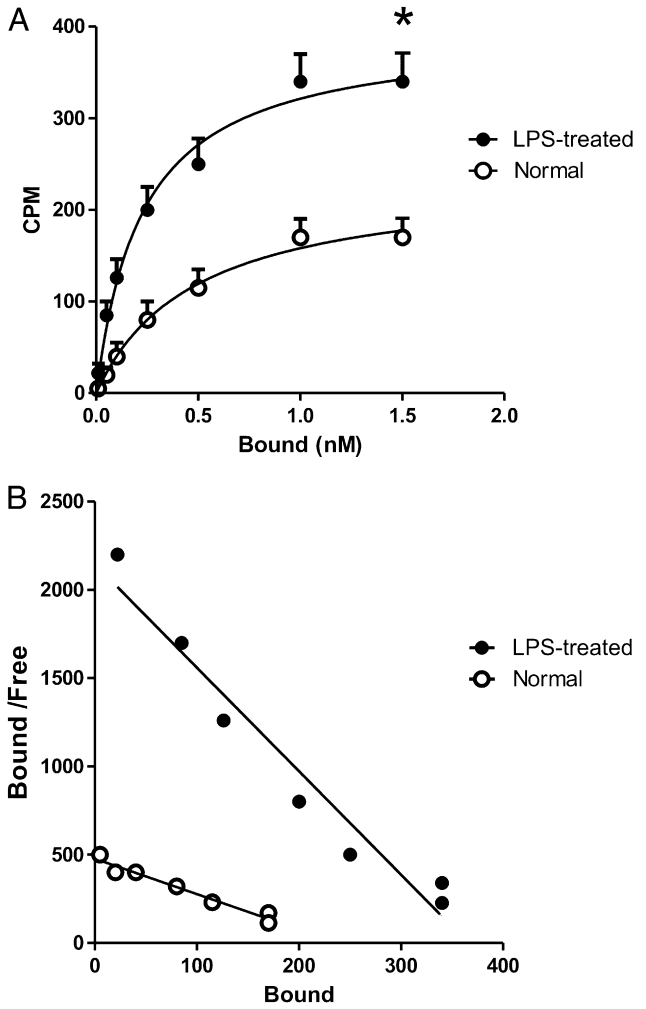


**Figure 4.** (*A*) Saturation of [$^3$H] QNB-binding assays of LPS-treated pulp and normal pulp membranes. Membrane suspensions (30-50 $\mu$g protein) were incubated with different concentrations of [$^3$H] QNB for 60 minutes at 25°C, and receptor ligand binding was performed as indicated in Materials and Methods. *$P$ < .0005 vs normal. (*B*) The corresponding Scatchard plot on the LPS-treated pulp and normal pulp membranes is also shown. Values are mean ± SEM of $n$ = 5 separate experiments in each group performed in duplicate.

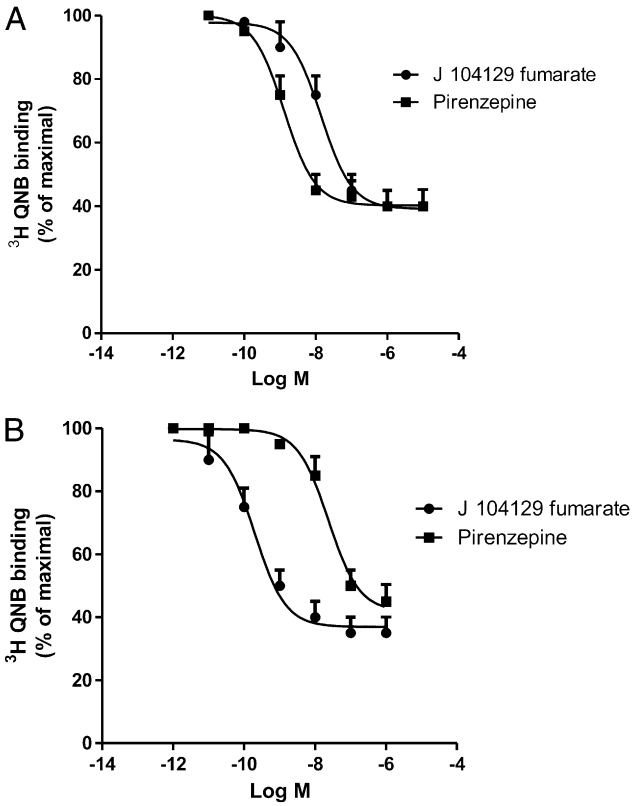


**Figure 5.** Competitive inhibition of [$^3$H] QNB by J 104129 fumarate and pirenzepine specific binding on LPS-treated pulps (*A*) and normal pulps (*B*). Membranes (30-50 $\mu$g protein) were incubated for 30 minutes with increasing concentrations of the antagonists in the presence of 0.40 nmol/L of [$^3$H] QNB. Data are means ± SEM of $n$ = 6 separate experiments performed in duplicate in each group.

overactivity (13). These results agree with the concept that the transition of the inflammatory process into an irreversible pulpitis lowers the eNOS levels and elevates the expression of iNOS (30). Alterations in the functional regulation of mAChR activation observed in irreversible pulpitis might be caused by modifications of the iNOS-mRNA induction levels in response to inflammatory stimuli. Thus, the pilocarpine effect on LPS-treated pulps switched from increase to decrease when iNOS activity was inhibited by aminoguanidine. By contrast, in normal pulp pilocarpine stimulatory effect decreased when the eNOS and nNOS activities were inhibited. Consequently, the nature of pilocarpine inhibition of NOS activity suggests that the iNOS-mediated pathway might be relevant (12). This agrees with a previous report demonstrating that pilocarpine at concentrations that decreased NOS activity also decreased the iNOS mRNA levels. On the other hand, the agonist at concentrations that increased NOS activity increased the eNOS and nNOS mRNA levels (12,13).

The paradoxical inhibitory effect of pilocarpine observed in LPS-treated pulps might be explained through a pharmacologic phenomenon known as constitutive receptor activity, that is, the ability of the receptor to produce signaling without exogenous agonist intervention (15). This spontaneously active state of mAChRs might trigger elevated basal cellular activity that can be inhibited by pilocarpine. The constitutive activity of the receptor observed during inflammation depends on the quantity of mAChRs in an active conformation, which in turn defines the percentage of active receptors in the population. Accordingly, when receptor density is increased, constitutive activity increases as a result of the increase in the absolute number and affinity of receptors. Moreover, during inflammation, the $M_3$ mAChR could adopt a different active conformation. Thus, pilocarpine with a different affinity for active conformation relative to inactive conformation would differentially bind to the active conformation, leading to a ligand-specific response as a result of activation of the $M_3$ mAChR subtype. The constitutive activity of the receptor depends on the efficiency of coupling to transducing molecules (31). Thus, receptor alteration can change the receptor transducer-coupling efficiency. If this is the case in an LPS-treated pulp, irreversible pulpitis might induce mAChR alteration. This increases agonist affinity and potency, increasing the receptor transduction-coupling efficiency to iNOS activity, which leads to a spontaneously active conformation of the receptor. Therefore, pilocarpine action on constitutive mAChR activity might act as an inverse agonist and consequently produce an inhibitory effect on NOS activity. In general, the lack of selectivity at the mAChR is to target allosteric sites, which are topographically distinct and generally consist of regions that show greater sequence divergence among receptor subtype relative to

residues comprising the orthosteric pocket. However, changes in orthostatic site of $M_2$ mAChR of CHO cells increase the efficacy of pilocarpine (29).

Mutations in G-protein–coupled receptors that lead to constitutive activity have been associated with autoimmune diseases (32), hyperthyroidism (33), precocious puberty (34), cell transformation, and cancer (35–37). The association of constitutive activity with some pathologies suggests that inverse agonist might be a useful property in clinical applications (15). The association of constitutive mAChR activity with irreversible pulpitis suggests that pilocarpine acting as an inverse agonist might be useful therapeutically to prevent necrosis and subsequent loss of the dental pulp.

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*The authors deny any conflicts of interest related to this study.*

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