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Activation of nitric oxide synthase through muscarinic receptors in rat parotid gland

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Abstract

Muscarinic receptors play an important role in secretory and vasodilator responses in rat salivary glands. Nitric oxide synthase (NOS) activity was found coupled to muscarinic receptor activation as well as to nitric oxide-mediated amylase secretion elicited by carbachol. Parotid glands presented a predominant M_3 and a minor muscarinic M_1 acetylcholine receptor population, though carbachol stimulated NOS activity only through muscarinic M_3 receptors as revealed in the presence of 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and pirenzepine. Amylase secretion induced by carbachol appeared to be partly mediated by nitric oxide and nitric oxide-induced signaling since *N*-nitro-L-arginine methyl ester (L-NAME) inhibited the effect as well as did methylene blue. A negative regulation of NOS by protein kinase C activation in the presence of a high concentration of carbachol was seen in parotid glands and this inhibition was paralleled by amylase secretion.

Keywords: Nitric oxide (NO) synthase; Parotid gland; Amylase secretion; Nitric oxide (NO) signaling

1. Introduction

Resting and stimulated secretion by salivary glands is under nervous control and each gland contributes differently to the flow and composition of saliva, based on their particular histological and biochemical characteristics. Among the various neurotransmitter and receptors involved in the secretory process, muscarinic receptors expressed on salivary glands are coupled to several signaling pathways, including the L-arginine-nitric oxide signaling pathway (Lohinai et al., 1999; Pérez Leiros et al., 2000). Nitric oxide has proved to play a pivotal role in either physiological or pathological conditions. Certainly, nitric oxide synthesized by constitutive isoforms is involved in neurotransmission through several receptors and it is also synthesized in high amounts during several immune and inflammatory disorders (Moncada et al., 1991; Nathan, 1997). Moreover, its production can be modulated at different levels, namely, by cross-talk with other intracellular pathways simultaneously gated (Bodis and Haregewoin, 1993; Edwards and Garrett, 1993; Förstermann et al., 1994), by expressional control of constitutive isoforms of nitric oxide synthases (NOS) (Förstermann et al., 1998), metabolic–oxidative processes involving other reactive oxygen species (Berlett and Stadtman, 1997) or down-regulation of NOS activity by phosphorylation (Brune and Lapetina, 1991; Bredt et al., 1992; Hagashi et al., 1999). These authors have reported on a regulatory role of NOS phosphorylation by protein kinases A, C and Ca^{2+}-calmodulin-dependent kinase in other tissues and we have reported a strong down-regulation of constitutive NOS by protein kinase C in rat submandibular glands (Pérez Leiros et al., 2000). Moreover, it has been hypothesized that alterations in NOS activity could, in turn, affect receptor signaling thus predisposing the salivary glands to develop functional alterations as we have recently reported for a murine model of sialadenitis (Rosignoli et al., 2001). The aim of the present work was to study further the mechanisms underlying rat parotid secretion with special focus on the activation and regulation of nitric oxide signaling coupled to muscarinic acetylcholine receptors. We present evidence to indicate that both muscarinic M_3 receptors and a minor population of muscarinic M_1 receptors are expressed in parotid glands, being the only muscarinic M_3 receptors coupled to nitric oxide synthase activity and nitric oxide-mediated amylase secretion.

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2. Materials and methods

2.1. Animals

Adult male rats of the Wistar strain (200–250 g) were bred and maintained under specific pathogen-free conditions in the Central Animal Care facility at the University of Buenos Aires (Ciudad Universitaria, Buenos Aires). The studies were conducted in rats fasted overnight, according to standard protocols of the Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires.

2.2. Nitric oxide synthase activity

NOS activity was measured in parotid glands using L-[U-^{14}C]arginine as substrate as previously described (Bredt and Snyder, 1990; Pérez Leiros et al., 2000). Briefly, parotid gland slices (2–4-mm thick) were incubated with 0.2 μCi L-[U-^{14}C]arginine (Amersham Pharmacia Biotech, Buckinghamshire, England, about 300 mCi/mmol) in 500 μl of Krebs–Ringer–bicarbonate (KRB) solution pH 7.4 gassed with 5% CO_2 in O_2 for 30 min at 37 °C. After incubation, the tissues were homogenized, centrifuged at 10,000×*g* for 10 min and [^{14}C]citrulline in the supernatants was separated by ion exchange chromatography on AG 50 W resin (Bio-Rad). Specific NOS activity was assessed in the presence of 10^{-4} M *N*-nitro-L-arginine methyl ester (L-NAME) (Sigma, MO, USA) and constitutive isoforms (Ca^{2+}-dependent) were assessed by incubating the tissues in KRB without Ca^{2+} and with 5 mM EGTA. Nitric oxide production (measured as pmol of [^{14}C]citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal (30 min) periods of time and thus expressed in pmol/g wet weight. When used, inhibitors were included from the beginning of the incubation time and carbachol (Sigma) was added at the final concentrations indicated for the last 20 min.

2.3. Muscarinic acetylcholine receptor binding studies

Parotid gland microsomal membranes were prepared as previously reported (Pérez Leiros et al., 2000). Briefly, the glands were excised and immediately homogenized at 4 °C in 6 to 8 volumes of 50 mM sodium/potassium phosphate buffer pH 7.5, 0.25 M sucrose and supplemented with protease inhibitors (0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM sodium ethylenediaminetetra-acetate, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μM pepstatin A and 1 mM iodoacetamide) as described (Pérez Leirós et al., 1997). The homogenate was centrifuged at 900×*g* twice and the supernatants were collected and spun down at 10,000×*g* for 10 min, both steps almost completely eliminating nucleus, mitochondrial and lisosomal fractions, and the resulting supernatant was centrifuged at 40,000×*g* for 60 min. The pellet was then resuspended in 50 mM phosphate pH 7.5, 5 mM $MgCl_2$ and the same protease inhibitors and stored at −80 °C until used. Radioligand binding assays with the muscarinic receptor antagonist (−)-[^{3}H]quinuclidinyl benzilate were carried out essentially as described (Pérez Leirós et al., 1997; Pérez Leiros et al., 2000). For competition binding assays, membranes (200–300 μg protein) were incubated with about 500 pM (−)-[^{3}H]quinuclidinyl benzilate and increasing concentrations of selective antagonists in a final volume of 500 μl at 37 °C with continuous shaking. Nonspecific binding was assessed with 1 μM atropine. Results were analyzed with the computer-assisted curve fitting program, LIGAND (Munson and Rodbard, 1980).

2.4. Cyclic GMP levels and amylase secretion

We determined guanosine 3′,5′-cyclic monophosphate (cGMP) levels in parotid gland slices by radioimmunoassay with anti 3′,5′-cGMP antisera from Sigma and [^{125}I]cGMP from Dupont/New England Nuclear. Samples were prepared by incubating the tissues for 30 min in 1-ml KRB with 5% CO_2 in O_2 and carbachol was added in the last 20 min. Parotid slices were homogenized and cGMP was extracted in ethanol for subsequent determination and the results were expressed in pmol/g wet weight. For amylase secretion assays, slices were incubated as for cGMP levels but in KRB without glucose. Once incubation was finished, the medium was transferred to other tubes for amylase activity determination (Bernfeld, 1955) and the tissues were homogenized in 50 mM phosphate buffer at 4 °C, spun down at 10,000×*g* for 20 min and the supernatants were used for amylase activity assay. One unit of amylase was defined as the activity of amylase that released 1 mg of maltose per min at 20 °C (Bernfeld, 1955). The results were expressed as percent release of amylase, calculated as the ratio between amylase activity in the incubation medium and total amylase (medium plus homogenate).

2.5. Statistical analysis

Statistical significance of differences was determined by the two-tailed *t*-test for independent populations. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. Differences between means were considered significant at $P<0.05$.

2.6. Drugs

Carbachol, pirenzepine, atropine, phorbol 12-myristate 13-acetate (PMA), methylene blue, L-NAME and anti 3′, 5′-cGMP antisera were from Sigma. The selective muscarinic M_2 receptor antagonist, 11-{[2-(diethylamino) ethyl]-1-piperidinyl} acetyl-5,11-dihydro-6*H*-pirido [2,3-b]-1,4-benzodiazepine-6-one (AF DX 116), was kindly provided by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT) and

the muscarinic M_3 receptor antagonist, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), was from RBI (Natick, MA); all other chemicals used were of analytical grade.

3. Results

3.1. Expression of muscarinic acetylcholine receptor subtypes on parotid glands

We studied the expression of muscarinic acetylcholine receptors on rat parotid gland membranes by means of (−)-[^{3}H]quinuclidinyl benzilate competition binding assays. Fig. 1 shows the order of potency of antagonists to compete for (−)-[^{3}H]quinuclidinyl benzilate binding in these glands. As expected for a predominant muscarinic M_3 receptor subtype population, both atropine and 4-DAMP were the most potent to compete for muscarinic acetylcholine receptors while pirenzepine and AF-DX 116 (muscarinic M_1 and M_2 receptor-selective antagonists) showed lower potency. Interestingly, as we had previously observed in submandibular glands (Pérez Leiros et al., 2000), the shape of the curve for pirenzepine reflected pronounced differences compared with the curves for the other antagonists studied. In fact, computer-assisted curve-fitting showed that pirenzepine curves fitted best ($P<0.05$) to a two-site model with high and low affinity states. The K_i for the high-affinity sites was 26±19 nM, close to K_i values reported for tissues with mostly M_1 expression (Watson et al., 1986). On the other hand, the low-affinity receptor population (K_i=166±89 nM) was similar to that labelled in sublingual glands, fitting a single-site model reported previously (Pérez Leiros et al., 2000) and consistent with the interaction of the antagonist with a population of muscarinic M_3 receptors as previously reported for other tissues with this subtype predominant (Doods et al., 1987).

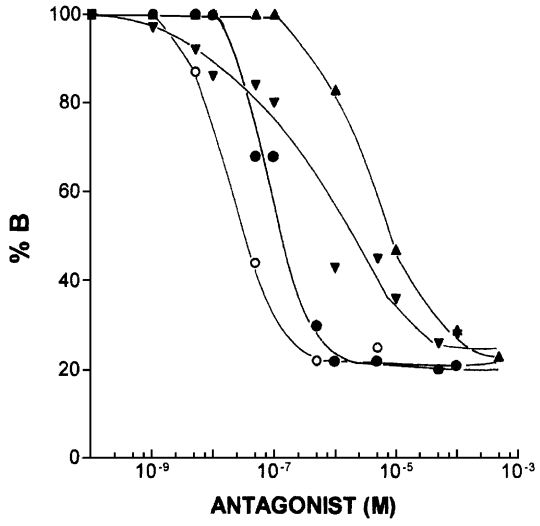

Fig. 1. Competition binding assays of muscarinic antagonists with (−)-[^{3}H]quinuclidinyl benzilate in parotid membranes. Parotid gland membranes were obtained and competition binding assays were performed as indicated in the text with different antagonists such as atropine (○), 4-DAMP (●), pirenzepine (▼) and AFDX-116 (▲). Results shown are representative of four separate experiments performed in duplicate with similar results. %B means percent of (−)-[^{3}H]-quinuclidinyl benzilate ((−)-[^{3}H]-QNB) bound and the 100% B in the absence of cold antagonists was 33.2±4.5 fmol/mg protein.

3.2. Activation of nitric oxide synthase through muscarinic acetylcholine receptors

In order to examine the nitric oxide signaling pathway coupled to muscarinic acetylcholine receptors in parotid glands, we first determined NOS activity in the presence of increasing concentrations of the muscarinic agonist, carbachol. Carbachol stimulated NOS activity in parotid gland slices, displaying a bell-shaped curve with a maximum at 10^{-6} M and decreased thereafter (Fig. 2A). The activation of NOS was specific since it was inhibited by 100 μM L-NAME (Fig. 2A) and the isoforms involved appeared to be constitutive isoforms (Ca^{2+}-dependent) as 5 mM EGTA completely inhibited NOS specific activity (basal: 396±64; EGTA: 184±22 pmol/g, $P<0.01$). The effect of carbachol was blocked by atropine and 4-DAMP but not by pirenzepine, which even reversed the effect of high concentrations of the agonist (Fig. 2B). Based on the fact that protein kinase C activation is one of the downstream steps in the signaling pathway of muscarinic acetylcholine receptors and down-regulated NOS in submandibular glands, we further tested the possibility that protein kinase C could participate in the negative regulation of NOS by high concentrations of carbachol in parotid glands. Table 1 shows that the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), had no significant effect alone but when added together with 10^{-5} M carbachol had an additional inhibitory effect on NOS activity, supporting the possibility of a negative regulation of NOS by protein kinase C in the presence of carbachol in parotid glands.

3.3. Nitric oxide-linked amylase secretion mediated by muscarinic acetylcholine receptors

Evidence of nitric oxide-linked amylase secretion through muscarinic acetylcholine receptors was obtained in parotid glands (Fig. 2C and D). As can be seen in Fig. 2C, carbachol stimulated amylase secretion in a concentration-dependent manner and the effect was inhibited by L-NAME, suggesting a role of nitric oxide in this effect. Regarding the subtype of muscarinic receptors involved in amylase secretion, Fig. 2D shows that 4-DAMP and atropine significantly inhibited the effect on protein secretion induced by carbachol while pirenzepine did not, similarly to the effect observed in NOS activity. To further explore whether the lower activity of NOS at high concentrations of the agonist influenced muscarinic receptor-elicited amylase secretion in parotid

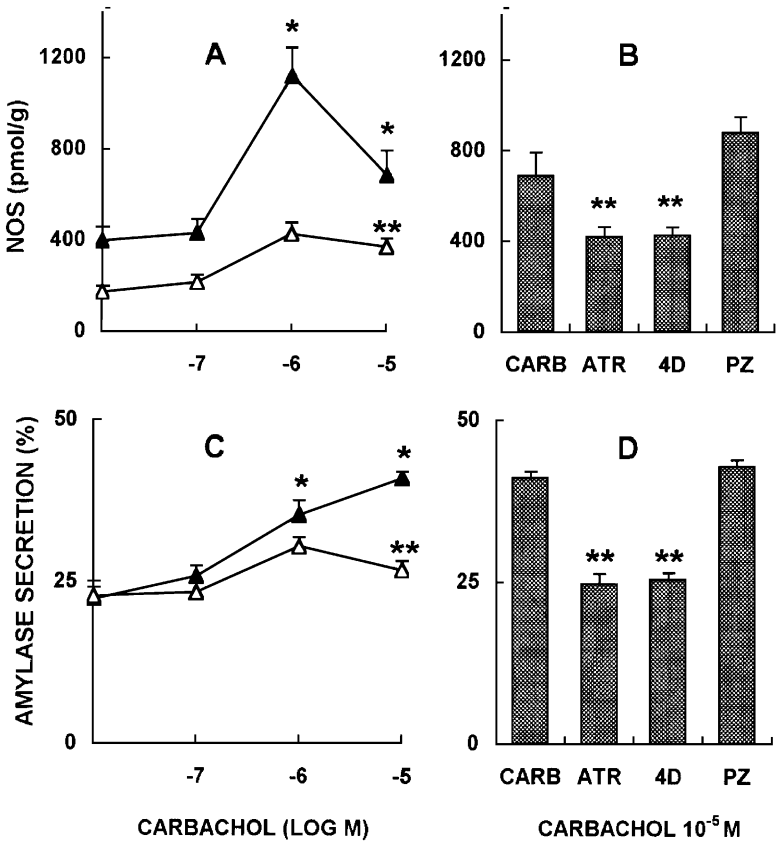

Fig. 2. NOS activity and amylase secretion elicited by carbachol in parotid glands. (A and C) NOS activity and amylase secretion were determined in parotid gland slices in the presence of the indicated concentrations of carbachol as described in Materials and methods, in the absence (▲—▲) or presence (△—△) of 10^{-4} M L-NAME. (B and D) NOS activity or amylase secretion induced by 10^{-5} M carbachol alone (CARB) or in the presence of 10^{-6} M atropine (ATR), 10^{-6} M 4-DAMP (4D) or 10^{-6} M pirenzepine (PZ). Values are means±S.E.M. of at least four separate experiments. (*) indicates significantly different from basal value ($P<0.05$), (**) significantly different from carbachol 10^{-5} M ($P<0.05$).

glands, we repeated the experiments in the presence of PMA. As observed in NOS assays, PMA alone had no effect on amylase while the simultaneous addition of PMA and carbachol induced a decrease of amylase secretion to almost basal levels (Table 1), a result consistent with the fact that nitric oxide favored the secretory effect of carbachol as revealed by L-NAME, and that protein kinase C down-regulated NOS activity as shown above. It is well known that cGMP is a link between nitric oxide and several downstream functional effects in most tissues. In order to assess whether nitric oxide-promoted cGMP levels were involved

Table 1
Effect of PMA on NOS activity and amylase secretion in parotid glands

	NOS (pmol/g)	Amylase secretion (%)
Basal	396 ± 64	22.4 ± 2.7
PMA 10^{-7} M	530 ± 55	23.3 ± 1.5
Carbachol 10^{-5} M	687 ± 108[a]	41.1 ± 1.0[a]
PMA + carbachol	371 ± 36[b]	28.3 ± 3.5[b]

Parotid glands were excised and slices were incubated for 30 min in corresponding medium as indicated for NOS and amylase assays in Materials and methods. Carbachol, PMA or both were added in the last 20 min at the final concentrations stated in the table. Results shown are the means±S.E.M. of at least four different experiments run in duplicate.

[a] Significantly different from basal value ($P<0.05$).

[b] Significantly different from carbachol alone ($P<0.05$).

Table 2
Cyclic GMP production and amylase secretion in parotid glands

	cGMP (pmol/g)	Amylase secretion (%)
Basal	8.5±0.7	22.4±2.7
Carbachol 10^{-6} M	18.1±1.1	35.3±2.3
Carbachol 10^{-5} M	29.4±2.2[a]	41.1±1.0[a]
Carbachol 10^{-5} M+L-NAME	16.4±1.7[b]	26.8±1.2[b]
Carbachol 10^{-5} M+methylene blue	ND	20.9±1.2[b]

Parotid glands were excised and slices were incubated for 30 min in corresponding medium as indicated for cGMP and amylase assays in Materials and methods. Carbachol was added in the last 20 min at the final concentrations stated in the table and L-NAME (10^{-4} M) and methylene blue (10^{-4} M) were included from the beginning of the incubation time. Results shown are the means±S.E.M. of at least four different experiments run in duplicate.

ND: not determined.

[a] Significantly different from basal value in each case ($P<0.05$).

[b] Significantly different from carbachol 10^{-5} M ($P<0.05$).

in amylase secretion following muscarinic receptor activation, we determined cGMP levels and amylase secretion in parotid glands in the presence of carbachol with L-NAME or methylene blue. Table 2 shows that carbachol stimulated cGMP levels at the same concentration that it activated amylase secretion. Moreover, L-NAME inhibited the effect on cGMP as it did that on amylase, and methylene blue, an inhibitor of soluble guanylyl cyclase, inhibited the secretory effect of carbachol (Table 2).

4. Discussion

We present data indicating that muscarinic M_3 acetylcholine receptors and a minor population of muscarinic M_1 subtype are expressed in rat parotid glands with only the former coupled to constitutive nitric oxide synthase activation and nitric oxide-mediated signaling sensitive to protein kinase C regulation, leading to amylase secretion. These conclusions are supported by the following observations: First, competition binding assays with selective antagonists indicated a predominant expression of muscarinic M_3 receptor binding sites and a small population of muscarinic M_1 receptors in parotid membranes. The values calculated for K_i were consistent with a dual site model with a predominant population of muscarinic M_3 acetylcholine binding sites and a low expression of muscarinic M_1 receptors and were comparable to the values of K_i obtained in rat submandibular glands reported previously (Pérez Leiros et al., 2000) as well as for other tissues expressing these subtypes (Watson et al., 1986; Doods et al., 1987). Second, both basal and muscarinic acetylcholine receptor-stimulated activity of NOS in parotid glands was dependent on Ca^{2+}, and thus mediated by constitutive isoforms, as revealed in the presence of EGTA. Also, the effect was specifically inhibited by the substrate analogue, L-NAME, to an extent similar to EGTA, indicating that inducible calcium-independent isoforms, if expressed, have no detectable activity in these glands. Third, only muscarinic M_3 receptors expressed in parotid glands appeared to be coupled to nitric oxide production, as indicated by the ability of 4-DAMP and atropine, but not pirenzepine, to inhibit the effect of the agonist. The combined expression of muscarinic M_3 receptors and a minor population of the M_1 subtype has been found in rat submandibular glands but not in sublingual glands (Pérez Leiros et al., 2000). However, in contrast to the results for parotid glands shown here, both subtypes of receptors expressed in submandibular glands were coupled to nitric oxide production with differential effects, suggesting a fine regulatory tuning operating in those glands that regulated nitric oxide production up or down depending on the concentration of the agonist. Taken together these observations suggest strongly that the internal milieu of different glands may condition the stimulant or inhibitory response to an agonist, depending not only on the relative abundance of receptor subtypes but also on the varying amounts of agonist reaching the receptor. Finally, both cGMP levels and amylase secretion elicited by carbachol were in part mediated by nitric oxide since the inhibition of NOS with L-NAME blocked these effects and the inhibition of guanylyl cyclase with methylene blue blocked amylase secretion. As expected for an effect subsequent to NOS activation and probably not fully dependent on this pathway, the curve for amylase secretion did not exactly parallel that for NOS. Certainly, the curve was right-shifted one order, showing a weakened secretion with 10^{-6} M carbachol compared to the effect on NOS and, at the highest concentration tested, we could not obtain the inhibitory effect on either cGMP production or amylase secretion as it was found for NOS activity. Moreover, the stimulatory effect on amylase seemed to be dependent only on muscarinic M_3 receptors since 4-DAMP but not pirenzepine inhibited secretion. It is interesting to note that, once produced in even minimal amounts, nitric oxide might stimulate soluble guanylyl cyclase for a long time (Bellamy et al., 2000), thus favoring amylase secretion regardless of the lower activity of nitric oxide synthase. To further explore whether the lower response of NOS to carbachol at the highest concentrations tested could be also associated to the activation of protein kinase C as reported for submandibular but not sublingual glands (Pérez Leiros et al., 2000) and because protein kinase C activation is one of the downstream steps in the signaling pathway of muscarinic acetylcholine receptors, we assayed the effect of the protein kinase C activator, PMA, together with carbachol. Actually, PMA not only potentiated the inhibitory effect of carbachol on NOS activation but it also diminished amylase secretion, strongly supporting the existence of a link between nitric oxide and proteinaceous secretion in response to muscarinic stimulation. Interestingly, PMA had no effect alone on either NOS or amylase secretion, suggesting that cross-talk between carbachol-activated signaling and protein kinase C is needed for the inhibitory effect. Moreover, the fact that PMA and carbachol added together could decrease NOS activity to its basal levels and that this, in turn, inhibited amylase secretion, though to a lower extent, suggests that secretion is the result of nitric oxide among other signaling events elicited by carbachol. In this regard, muscarinic and purine receptors have been shown to be the most effective physiological stimuli of Ca^{2+} mobilization in submandibular cells (Liu et al., 2000) and, once released from cellular stores, Ca^{2+} can act through different pathways to trigger secretion. Results shown here clearly suggest that nitric oxide signaling involving Ca^{2+}-dependent isoforms of nitric oxide synthase has a role in amylase secretion in parotid glands and that this pathway is subject to regulation during the last events of the secretory cascade, readily affecting all the nitric oxide production after complete down-regulation of the enzyme by the simultaneous action of protein kinase C and carbachol. The role of nitric oxide in parasympathetic-stimulated saliva and amylase secretion is still controversial. Much evidence has been presented supporting a stimulatory role of this mediator, as nitric oxide-related vasodilation in response to para-

sympathetic stimulation was observed in cat salivary glands (Edwards and Garrett, 1993), and positive modulation of parasympathetic secretion by rat submandibular glands but not sublingual glands was reported (Takai et al., 1999). Regarding the involvement of a nitric oxide signaling pathway in secretion, previous reports have shown an association between nitric oxide and secretory function in vivo (Lomniczi et al., 1998; Lohinai et al., 1999), as well as between nitric oxide and cGMP in parotid acini (Sugiya et al., 1998) and cGMP-related amylase secretion in rabbit parotid glands and acini (Wojcik et al., 1975; Watson et al., 1999). However, to our knowledge, the present report of dual expression of muscarinic M_1 and M_3 receptors in rat parotid glands and of muscarinic M_3 receptor-coupling to nitric oxide signaling and regulation of amylase secretion, extends our previous findings with rat submandibular and sublingual glands (Pérez Leiros et al., 2000). Even though the three major salivary glands share a predominant expression of muscarinic M_3 receptor population, they differ in some of their second messenger responses as can been seen from the results described here and elsewhere (Laniyonu et al., 1990; Iwabuchi and Masuhara, 1992; Pérez Leiros et al., 2000), suggesting that some subtle differences in the regulation of similar signaling pathways may result in a range of responses. The possibility that NOS activity highly regulated by protein kinase C in parotid glands is of neural origin is supported by two main observations. First, it has recently been shown that rat parotid glands highly express neural NOS (Mitsui and Furuyama, 2000) and second, in studies with cultured parotid acinar cells from which neural tissue was removed, PMA plus carbachol did not show inhibition but instead, the cells responded with an increase in amylase secretion in contrast to the results shown here (Möller et al., 1996). Nonetheless, we cannot rule out a contribution of NOS from other sources, such as endothelial or smooth muscle in rat parotid glands, as the three different NOS isoforms have been detected in human salivary glands (Kontinnen et al., 1997), feline submandibular glands (Lohinai et al., 1995) and we have recently reported NOS expression in mouse parotid and submandibular glands (Rosignoli et al., 2001). It has been proposed that nitric oxide may serve a dual purpose in salivary glands as a normal messenger for glandular function and also as a short-lasting inflammatory mediator as well as an antiseptic agent (McCall and Vallance, 1992; Knowles and Moncada, 1994). Thus, in the light of the known multiple functions of nitric oxide and the highly controlled expression of NOS constitutive and inducible isoforms, it seems attractive to look further into the mechanisms mediated by this diffusible molecule in salivary glands to gain more insight into the overall process of secretion.

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