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Differential activation of nitric oxide synthase through muscarinic acetylcholine receptors in rat salivary glands

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Abstract

Muscarinic receptors play an important role in secretory and vasodilator responses in rat salivary glands. Nitric oxide synthase (NOS) appears to be one of the multiple effectors coupled to muscarinic receptors in both submandibular and sublingual glands although some differences have been found depending on the gland studied. First, submandibular glands had a lower basal activity of nitric oxide synthase than sublingual glands and the concentration–response curve for carbachol was bell-shaped in the former but not in sublingual glands. Second, cGMP levels displayed a similar profile to that observed for NOS activity in both glands. Third, protein kinase C also coupled to muscarinic receptor activation in the glands might have a regulatory effect on nitric oxide production since its activity was higher in basal conditions in submandibular than sublingual glands and it also increased in the presence of the agonist at a concentration that inhibited NOS activity in submandibular glands. The effects appear to be partly related to the expression of a minor population of M_1 receptors in submandibular glands absent in sublingual as determined in binding and signaling experiments with the muscarinic receptor antagonist pirenzepine.

Keywords: Submandibular and sublingual glands; Muscarinic acetylcholine receptors; Nitric oxide synthase; Protein kinase C

1. Introduction

Resting and stimulated secretion by salivary glands is under nervous control and each gland contributes differently to flow and composition of saliva based on their particular histological and biochemical characteristics. Salivary cells thus display ranges of responses depending on the subtype of receptors stimulated and probably also on differences in receptor regulation within each gland. Acetylcholine acting on muscarinic receptors of salivary glands mostly activates fluid secretion, while noradrenaline often mediates mobilization of proteinaceous material. These receptor systems act collaboratively and synergistically and probably, this is also the case for various non-adrenergic non-cholinergic (NANC) transmitters in glands (Garrett and Kidd, 1993). There is growing evidence that receptor-mediated secretory effects are susceptible to strong regulation by intermediate intracellular pathways (Quissell et al., 1992). Thus, particular physiologic or metabolic situations can modify or condition intra- and extra-cellular milieu leading to differences in receptor coupling and activation which finally results in varying responses to stimuli. Muscarinic acetylcholine receptors, expressed on both submandibular and sublingual glands, are widely used as targets of a complex network of regulatory inputs. These receptors are coupled to several signaling cascades in these and other tissues with intermediates such as nitric oxide whose production can be in turn modulated by other pathways simultaneously gated (Bodis and Haregewoin, 1993; Edwards and Garrett, 1993; Forstermann et al., 1994). A regulatory role for protein phosphorylation of nitric oxide synthase (NOS) by protein kinase A, C and calcium-calmodulin dependent kinase has been demonstrated in other tissues though little is known about those mechanisms in mammalian salivary glands (Brune and Lapetina, 1991; Nakane et al., 1991; Bredt et al., 1992). The purpose of the present study was to examine the profile of muscarinic acetylcholine receptor

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activation in both salivary glands with special focus on the activation and regulation of NOS activity.

2. Materials and methods

2.1. Muscarinic acetylcholine binding studies

Submandibular or sublingual microsomal membranes were prepared from rats as previously reported (Bacman et al., 1998). Briefly, the glands were excised and immediately homogenized at 4°C in 6–8 volumes of 50 mM sodium/potasium phosphate buffer pH 7.5, 0.25 M sucrose and supplemented with protease inhibitors (0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM sodium ethylenediaminetetraacetate, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μM pepstatin A and 1 mM iodoacetamide) as described (Borda et al., 1997). The homogenate was centrifuged at 900 *g* twice and the supernatants collected and spun down at 10 000 *g* for 10 min and 40 000 *g* for 60 min. The resulting pellet was resuspended in 50 mM phosphates 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 antagonist (−)-[^{3}H]-quinuclidinyl benzilate ((−)-[^{3}H]-QNB) were carried essentially as described elsewhere (Borda et al., 1997; Bacman et al., 1998). For competition binding assays, membranes (200–300 μg protein) were incubated with about 500 pM [^{3}H]-QNB and increasing concentrations of selective antagonists in a final volume of 500 μl at 37°C with continuous shaking. For K_d and B_{max} calculations several concentrations of either [^{3}H]-QNB (50–500 pM) or the selective M_1 antagonist [^{3}H]-pirenzepine (1–15 nM) were used and incubations were carried out as above. Non specific binding was assessed with 1 μM atropine. Results were analyzed with the computer-assisted curve fitting program LIGAND (Mundson and Rodbard, 1980).

2.2. Determination of nitric oxide synthase and protein kinase C activity

Nitric oxide synthase (NOS) activity was measured in submandibular or sublingual glands using L-[U-^{14}C]-arginine as substrate as described earlier (Bacman et al., 1998; Bredt et al., 1992). Whole glands were incubated with 0.5 μCi L-[U-^{14}C]-arginine in 500 μl of Krebs-ringerabicarbonate (KRB) solution pH 7.4 at 37°C. When used, inhibitors were included from the beginning of the incubation time and stimuli were added in the last 15 min. For protein kinase C (PKC) activity, glands were incubated for the indicated times alone or in the presence of stimuli in KRB solution gassed with 5% CO_2 in oxygen and were immediately frozen in liquid nitrogen. PKC was partially purified from subcellular fractions as described (Genaro and Bosca, 1993). Kinase activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into histone H_1 (Genaro and Bosca, 1993). Incubations were conducted for 30 min at 30°C in a final volume of 85 μl containing 25 mM ATP (0.4 mCi), 10 mM magnesium acetate, 5 mM β-mercaptoethanol, 50 mg histone H_1 in 20 mM Hepes pH 7.5 and, unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 mg/ml phosphatidylserine vesicles. The reaction was stopped with 2 ml of ice-cold 5% trichloroacetic acid, 10 mM H_3PO_4 and after filtration on GF/c glass-fiber filters, the radioactivity was quantified. PKC was determined after subtracting ^{32}P incorporation in the absence of Ca^{+2} and phospholipids. Data were expressed in pmol phosphate incorporated per mg protein per minute.

2.3. Inositol phosphates accumulation and cyclic GMP levels

To measure inositol phosphates (IPs) accumulation whole glands were incubated for 120 min at 37°C in 500 μl of KRB with 1 mCi myo-[^{3}H]-inositol ([^{3}H]-MI) (specific activity 46.0 Ci/mmol) from Dupont/New England Nuclear, LiCl (10 mM) according to the technique of Berridge et al. (1982). Carbachol was added in the last 15 min. Glands were quickly washed in KRB, water-soluble inositol phosphates were extracted with chloroform–methanol (1:2, v:v) as described (Berridge et al., 1982) and aqueous phase was applied to a 0.7 ml column of Bio-Rad AG 1×8 anion exchange resin equilibrated in 10 mM Tris-formic acid pH 7.4 and then 0.1 M formic acid. The first peak was eluted with 5 mM myo-inositol and the second peak (IPs) were eluted with 1 M ammonium formate in 0.1 M formic acid. Peak areas were determined by triangulation and results of the second peak (IPs) were expressed as the absolute values of the area under the curve following the criteria of Simpson's Equation. We determined guanosine 3′,5′-cyclic monophosphate (cGMP) levels in whole glands by means of a radioimmunoassay with anti 3′,5′-cGMP antisera from Sigma Chem Co and [^{125}I]-cGMP from Dupont/New England Nuclear. Samples were prepared by incubating whole submandibular or sublingual glands for 30 min in 1 ml KRB with 5% CO_2 in O_2 and carbachol was added in the last 10 min. Glands were homogenized in ethanol and after evaporated, residues were dissolved in 50 mM sodium-acetate buffer pH 6.2 for subsequent cGMP determination.

2.4. 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 $P < 0.05$.

2.5. Drugs

Carbachol, pirenzepine, atropine, anti 3′,5′-cGMP antisera were from Sigma Chem Co (St. Louis, MO). The selective M_2 muscarinic antagonist 11-{[2-(Diethylamino) ethyl]-1-piperidinyl} acetyl-5,11-dihydro-6H-pirido [2,3-b]-1,4-benzodiazepine-6-one (AF DX 116) was kindly provided by Boehringer Ingelheim Pharmaceuticals Inc, Ridgefield, CT and the M_3 muscarinic 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. Activation of nitric oxide synthase signaling pathway mediated by muscarinic acetylcholine receptors

In order to examine the signaling pathways of muscarinic acetylcholine receptors (mAChRs) in salivary glands, we first determined NOS activity in the presence of increasing concentrations of the muscarinic agonist carbachol. We observed significantly higher basal activities of NOS in sublingual than submandibular glands ($P<0.05$) and, while in the former the effect increased gradually with carbachol concentrations, in submandibular glands there was a modest increase at about 1 μM and decreased thereafter (Fig. 1). The activation of NOS was specific since it was inhibited by L-*N*-monomethylarginine (L-NMMA) and dependent on calcium-calmodulin as the addition of trifluoperazine, a calcium-calmodulin inhibitor, significantly inhibited NOS stimulation in both glands ($P<0.05$) (Fig. 1). The possibility that a negative regulation of NOS by protein kinase C (PKC) was responsible for such an altered response was further tested with the protein kinase inhibitor staurosporine at concentrations that inhibit PKC activity. Shown in Fig. 1 is the increase in basal NOS activity in the presence of staurosporine in submandibular glands and the lack of effect of the PKC inhibitor in sublingual glands. If PKC was down-regulating NOS activity in basal conditions, it would be predictable that PKC stimulated through muscarinic acetylcholine receptors would also have a role in inhibiting NOS at high concentrations of the agonist. As expected, the deep inhibition of NOS activity in the presence of the highest

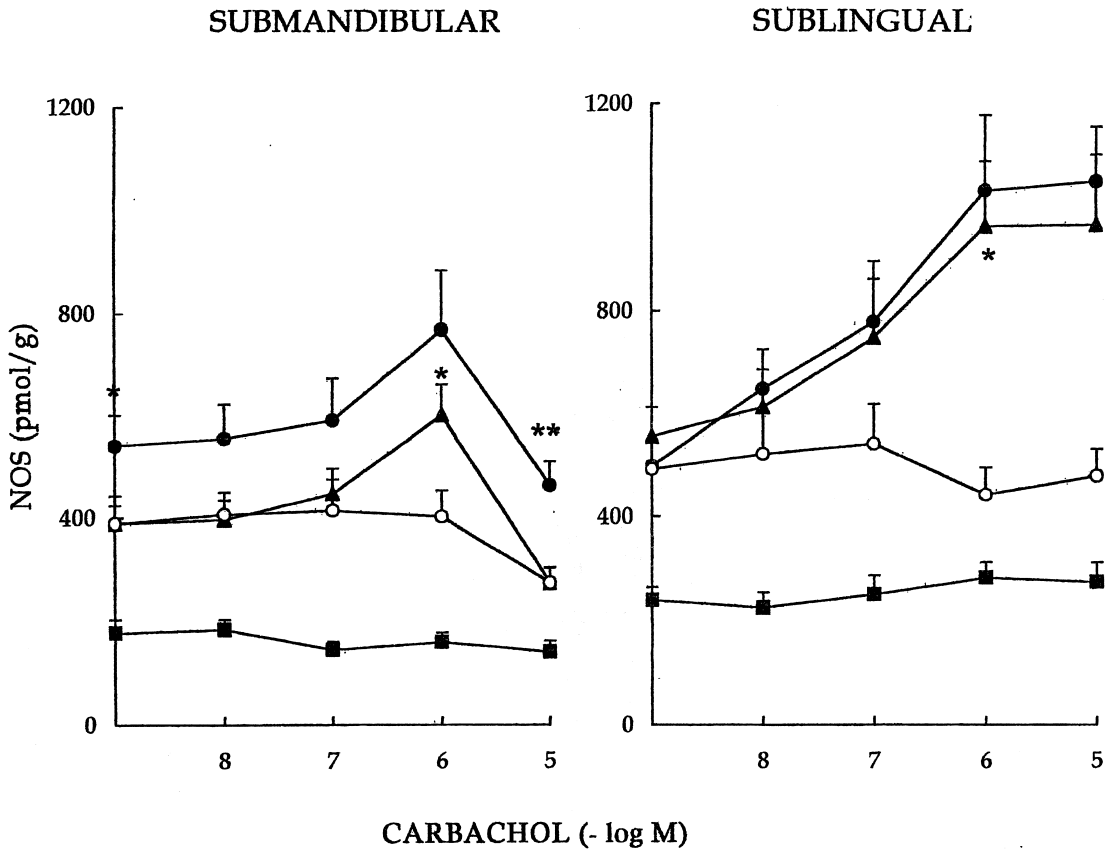

Fig. 1. Effect of carbachol on NOS activity in salivary glands. Submandibular and sublingual glands were incubated with increasing concentrations of carbachol for 15 min alone (▲) or in the presence of 5×10^{-5} M L-NMMA (■), 10^{-6} M trifluoperazine (○) or 2×10^{-9} M staurosporine (●) as indicated in Material and Methods. Values represent the mean±S.E.M. of five experiments. *, significantly different from basal in each gland, $P<0.05$; **, significantly different from 10^{-5} carbachol, $P<0.025$.

concentrations of carbachol tested in submandibular glands was reversed by staurosporine, a phenomenon that was not seen in sublingual glands (Fig. 1).

It is well known that nitric oxide released by constitutive NOS activates soluble guanylil cyclase activity with cyclic GMP production in most tissues. We determined cGMP levels in both glands either in basal or carbachol stimulated conditions. Fig. 2 shows basal submandibular cyclic nucleotide levels lower than sublingual cGMP levels ($P<0.05$) and similar bell-shaped concentration–response curves to carbachol as seen in NOS activity. The effect was inhibited by NOS inhibitor L-NMMA indicating that cGMP stimulation is related to NOS activation in salivary glands. Again, at high concentrations of carbachol the activation of this signaling pathway seemed to be down-regulated in submandibular but not in sublingual glands. The possible role of PKC was then tested by incubating with staurosporine. Fig. 2 shows that staurosporine reversed the declined response to high concentrations of carbachol in submandibular but did not modify carbachol-stimulated cGMP levels in sublingual glands. Moreover, nitric oxide synthase activation and cGMP stimulation were blocked by atropine and by 4-DAMP in both glands while pirenzepine partially blocked NOS activity only in submandibular glands pointing to the participation of M_1 receptor subtype in the effect (Table 1).

3.2. Muscarinic acetylcholine receptor-linked phosphoinositide turnover and PKC activity

Evidence of phosphoinositide turnover linked to mAChR activation via phospholipase C was obtained in both glands (Fig. 3). The effect was dependent on the concentration of carbachol and reached a similar maximal value at 10^{-5} M carbachol in either gland indicating no signs of regulation in contrast to NOS and cGMP responses. Conversely, the glands presented different profiles of protein kinase C activity in basal and carbachol-stimulated situation. Fig. 4 shows a basal translocation of PKC from cytosol to membranes of about 50% in submandibular glands not observed in sublingual glands. This is consistent with levels of PKC activity higher in basal conditions in submandibular glands than ordinarily seen in other tissues and in sublingual glands here. We then tested muscarinic acetylcholine receptor-mediated PKC activation and, as shown in Fig. 4, carbachol (10 μM) significantly stimulated PKC translocation to membranes in both glands while 0.5 μM (effective concentration to activate NOS and

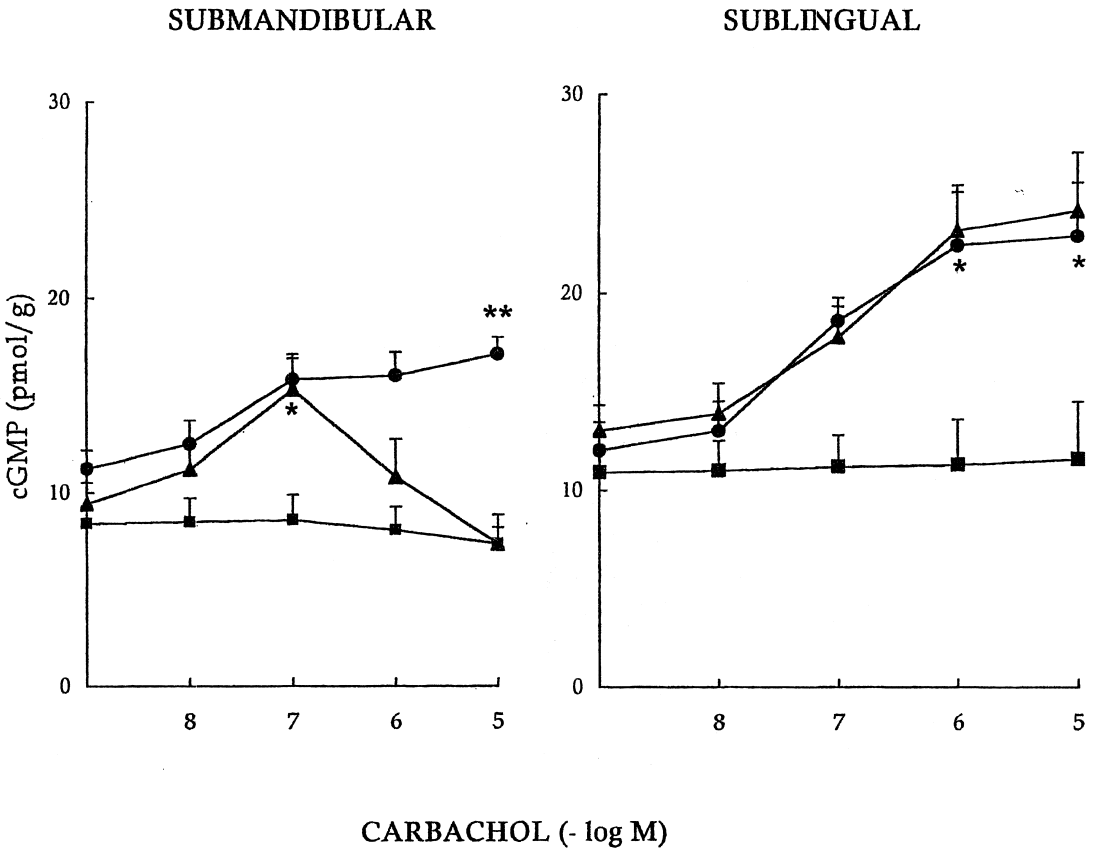

Fig. 2. Effect of carbachol on cGMP levels of salivary glands. Submandibular or sublingual glands were treated with carbachol at different concentrations either alone (▲) or in the presence of 5×10^{-5} M L-NMMA (■) or 2×10^{-9} M staurosporine (●). After 30 min the glands were homogenized and cGMP extracted for evaluation by RIA as indicated in the text. Values are mean±S.E.M. of at least four experiments. *, significantly different from basal in each gland, $P<0.05$; **, significantly different from 10^{-5} M carbachol, $P<0.01$.

Table 1
Effect of pirenzepine on carbachol-stimulated NOS in salivary glands[a]

	Submandibular		Sublingual	
	NOS	cGMP	NOS	cGMP
Basal	389±26	9.2±1.0	557±41	12.3±1.1
Carbachol 5×10^{-7} M	572±52[b]	12.3±0.9[b]	948±106[b]	21.1±1.1[b]
Carbachol+atropine	361±36	8.9±0.8	523±50	11.6±0.9
Carbachol+4-DAMP	376±29	8.9±0.9	544±62	13.3±1.4
Carbachol+pirenzepine	451±31[c]	10.6±0.9	957±74	19.7±2.0

[a] Salivary glands were incubated with carbachol as indicated above for NOS and cGMP determination. The antagonists atropine, 4-DAMP and pirenzepine were included in the incubation volume from the beginning of the incubation time and the final concentrations were 5×10^{-7} M atropine and 10^{-6} M 4-DAMP and pirenzepine. Values correspond to pmol of either NOS or cGMP per gram of tissue wet weight and represent the mean±S.E.M. of four to eight experiments run in duplicates.

[b] Significantly different from basal, atropine and 4-DAMP, $P<0.05$.

[c] Pirenzepine vs. carbachol, $P<0.05$.

cGMP) did not translocate PKC. This observation supports a role for PKC to regulate NOS in submandibular but not in sublingual glands.

3.3. Differential expression of muscarinic acetylcholine receptors on salivary glands

We first studied the expression of muscarinic acetylcholine receptors (mAChR) on rat submandibular and sublingual gland membranes by means of [^{3}H]-QNB competition binding assays. Fig. 5 shows the order of potency of antagonists to compete for [^{3}H]-QNB binding in both glands. As expected for a predominant M_3 receptor subtype population, both atropine and 4-DAMP were the most potent to compete for mAChRs while pirenzepine and AF-DX 116 (M_1 and M_2 muscarinic selective antagonists) showed lower potency in either gland. However, the curves of pirenzepine reflected subtle differences depending on the glands studied. Computer assisted curve-fitting showed that pirenzepine curves fitted best to a single site model in sublingual glands with a Ki value of 115±9 nM consistent with the interaction of the antagonist with a population of M_3 receptors as previously reported for other tissues with this subtype predominant (Doods et al., 1987).

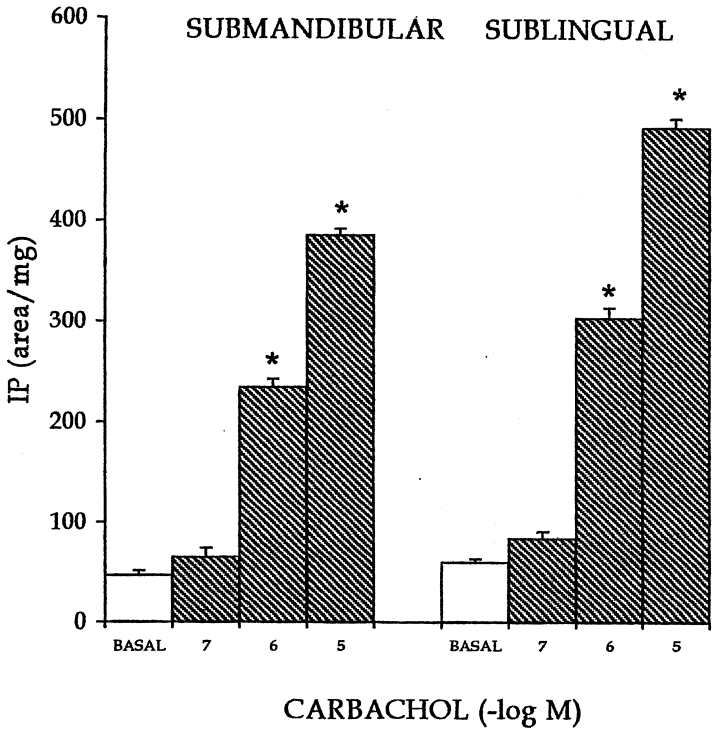

Fig. 3. Inositol phosphates accumulation induced by carbachol in salivary glands. Whole glands were incubated for 120 min with [^{3}H]-myoinositol according to Material and Methods. Carbachol was added in the last 15 min at the concentrations indicated. Values of IPs accumulation were calculated from the areas of the second peak and correspond to the mean±S.E.M. of four experiments. *, significantly different from basal in each gland, $P<0.01$.

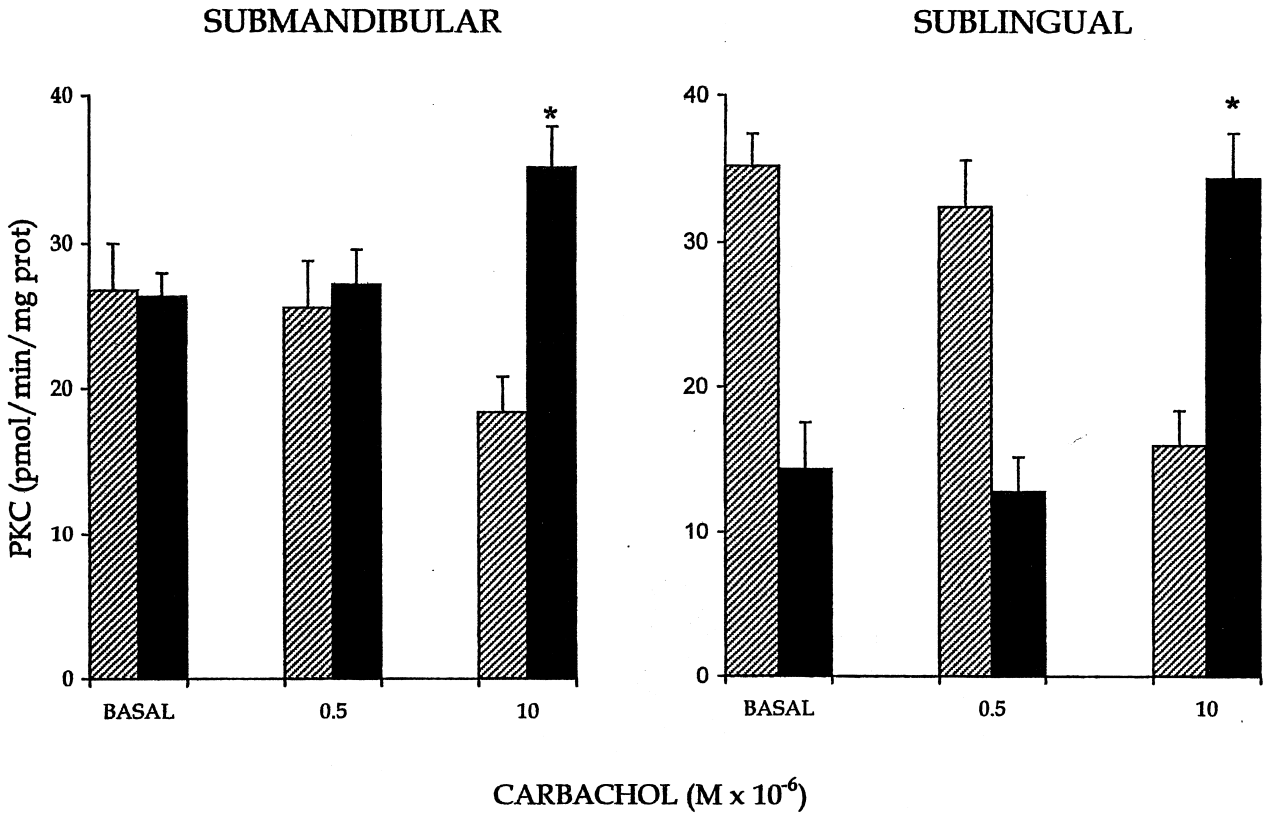

Fig. 4. PKC activity of salivary glands: Effect of carbachol. Submandibular and sublingual glands were incubated for 5 min with 0.5 or 10 μM carbachol as indicated in the chart or without stimulus (basal values), and the activity of PKC was determined both in cytosol (hatched bars) and in the membrane fraction (filled bars) of each gland. Values correspond to the mean±S.E.M. of four experiments run similarly. *, significantly different from basal in submandibular glands, $P<0.05$; **, significantly different from basal in sublingual glands, $P<0.01$.

In contrast, in submandibular glands, the curves fitted well ($P<0.05$) to a two site model with high and low affinity states. The Ki for the high affinity sites was 17±12 nM, close to Ki values reported for tissues with mostly M_1 expression (Watson et al., 1986). On the other hand, low affinity receptor population (Ki=181±94 nM) was similar to that labelled in sublinguals. To explore this different M_1 receptor expression further we carried out [^{3}H]-pirenzepine binding studies in both glands. As shown in Table 2, the maximal number of binding sites (B_{max}) as calculated from saturation curves did not significantly differ between glands indicating a lack of sensitivity of direct [^{3}H]-pirenzepine binding compared to indirect pirenzepine-[^{3}H]-QNB competition assay to measure a relatively small population of M_1 receptors.

4. Discussion

We present data to indicate that both basal and muscarinic acetylcholine receptor-stimulated NOS activity vary depending on the salivary gland studied and the differences observed appear to be related to the activity of protein kinase C as well as to the expression of muscarinic receptors in each gland. These conclusions are based on several observations: First, the activity of NOS stimulated through muscarinic acetylcholine receptors (mAChRs) in both glands was dependent on calcium-calmodulin as it disappeared in the presence of the calcium-calmodulin inhibitor trifluoperazine and specifically it was completely inhibited by the substrate analogue L-NMMA. Second, basal levels of NOS activity and cGMP in submandibular glands were lower than in sublinguals and were substantially increased by the PKC inhibitor staurosporine in the former but not in the latter. Moreover, basal PKC activity was found equally distributed between cytosol and membranes in submandibular glands while it was mostly cytosolic in sublingual glands. This basal translocation of PKC to membranes points to the existence of a different biochemical intracellular environment in submandibular glands that might serve particular functions by conditioning the activity of various pathways. Third, in support of the above observation, not only did cGMP stimulation parallel that of NOS in basal conditions but also in response to the agonist. Hence, submandibular but not sublingual glands presented a bell-shaped curve to carbachol both for NOS activity and cGMP levels. Again, impaired response to high concentrations of the agonist in

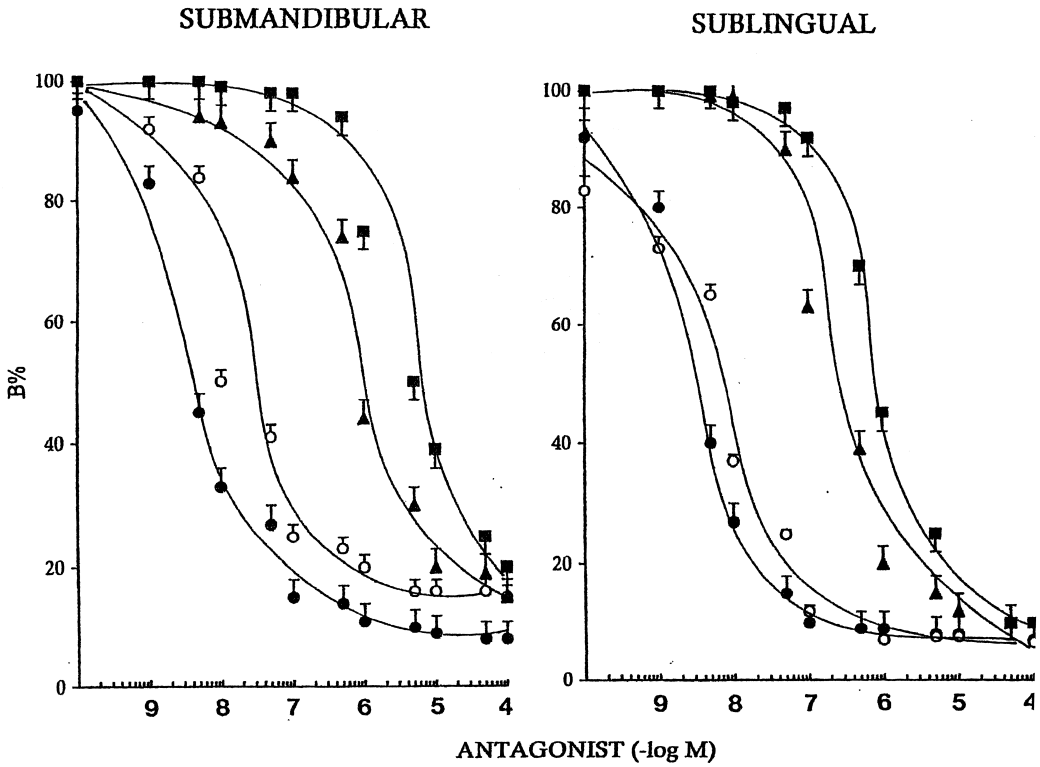

Fig. 5. Competition binding assays of muscarinic antagonists with [3H]-QNB in submandibular and sublingual membranes. Salivary gland membranes were obtained and competition binding assays performed as indicated in the text with different antagonists such as atropine (●), 4-DAMP (○), pirenzepine (▲) and AFDX-116 (■). Values are mean±S.E.M. of four experiments. %B means percent of [3H]-QNB bound and the 100% B in the absence of cold antagonists was 36.9±2.6 fmol/mg protein for submandibular glands and 44.7±3.4 for sublingual glands.

submandibular but not in sublingual glands seemed to be related to the activation of PKC since it was reversed by staurosporine only in submandibular glands. Accordingly, when PKC activity was assayed in response to carbachol, the activation of the enzyme in submandibular glands occurred at relatively high concentrations of the agonist in comparison to those needed to activate NOS in these glands. Conversely, the curves for NOS and PKC were almost parallel in sublingual glands. On the other hand, the differences between glands were not seen when assaying inositol phosphate accumulation, the other pathway simultaneously activated by mAChRs coupled to phospholipase C, supporting the major role of PKC in the system. It is interesting to note that the bell-shaped curves obtained in submandibular glands in response to carbachol seem not to be due to a process of desensitization of receptors but rather to NOS down regulation since the phenomenon was observed in NOS and cGMP but not in inositol phosphate accumulation, a pathway independent of NOS activation that probably participates by providing calcium from intracellular sources. Finally, differences in muscarinic acetylcholine expression were observed in both glands. Though not reflected by the total [^{3}H]-QNB or [^{3}H]-pirenzepine binding sites, the competition curves of selective antagonists indicate a small population of M_1 muscarinic acetylcholine receptors in submandibular glands absent in sublingual glands, a fact that could explain in part the ability of pirenzepine to partially reverse the effect of the agonist on NOS activity. A similar situation was observed in other tissues where the sensitivity of competition curves of pirenzepine-QNB was higher than other methods to unravel a minor population of M_1 receptors (Zhang et al., 1991). The possibility that NOS activity highly regulated by PKC in submandibular glands is from

Table 2
Maximal number of muscarinic receptor binding sites in submandibular and sublingual glands[a]

Radioligand	B_{max} (fmol/mg protein)	
	Submandibular	Sublingual
[^{3}H]-Pirenzepine	20.7±2.2	17.6±2.0
[^{3}H]-QNB	38.2±3.3	47.9±5.6

[a] The membranes from salivary glands were prepared for binding assays as referred to in Material and Methods. B_{max} were calculated from saturation curves performed as indicated in the text with increasing concentrations of either [^{3}H]-pirenzepine (1–15 nM) or [^{3}H]-QNB (50–500 pM) and analyzed with the LIGAND program (Mundson and Rodbard, 1980). Values indicate mean±S.E.M. of at least four experiments with each radioligand in each gland.

neural origins is supported by the observation that M_1 receptors appear to participate in the effects though the major contribution to muscarinic mediated activation appears to be through M_3 receptors. In fact the ability of PKC to inhibit NOS activity previously reported was observed in neural tissue (Bredt et al., 1992). However, we cannot rule out that a contribution of NOS from other sources such as endothelial or smooth muscle may also have a role in submandibular glands. In fact, the presence of NADP-diaphorase activity and three different NOS isoforms has been detected in human salivary glands (Kontinnen et al., 1997) and feline submandibular glands (Lohinai et al., 1995). The role of nitric oxide in glandular secretion is still unclear though nitric oxide metabolites have been found in human saliva after neural stimulation (Bodis and Haregewoin, 1993) and nitric oxide-related vasodilator responses to parasympathetic stimulation have been described in cat salivary glands (Edwards and Garrett, 1993). Certainly, 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, an undoubtedly large variety of physiological roles (McCall and Valiance, 1992; Knowles and Moncada, 1994). Moreover, even though common intracellular messengers can be detected in response to stimuli in each pair of salivary glands, particular characteristics are observed depending on the gland and also on the species studied as it has been shown in rat as opposed to mouse parotid glands regarding the role of cGMP in protein secretion (Harper and Brooker, 1978; Watson et al., 1982). Similarly, different mediators and patterns of exocytotic secretion were seen in parotid and submandibular glands (Butcher and Putney, 1980; Fleming and Bilan, 1987; Castle and Castle, 1998). The importance of protein phosphorylation pathways in the regulation of various systems has been fully discussed (Hosey, 1994). This appears especially interesting in salivary glands where important histological and functional differences between glands are evident and nevertheless each maintain a complex ability to regulate simultaneously internal milieu and metabolic process for secretion (Quissell et al., 1992). The possibility that some of these differences are related to the activation of similar signaling pathways that elicit a range of responses depending on intracellular environment appears reasonable in the light of our results. In fact, glands express mostly M_3 muscarinic receptor population, as it has been shown here and elsewhere (Laniyonu et al., 1990; Iwabuchi and Masuhara, 1992) and strongly differ in some of their second messenger responses as derived from the results presented here. Those differences could be partly associated to protein kinase activity and to subtle variations in the subtypes of receptors minorly expressed. Additional studies are required to understand more fully the particular characteristics of each gland, mainly at the intracellular level, that participate in the modulation of the secretory function.

Acknowledgements

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