Original Article

Cholinergic Autoantibodies from Primary Sjögren's Syndrome Inhibit Mucin Production via Phospholipase C and Cyclooxygenase-2 In the Rat Submandibular Gland

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ABSTRACT

Background: Patients with primary Sjögren's syndrome (pSS) produce functional IgG against cholinoreceptor of exocrine glands modifying their activity. The aim of the present work was to demonstrate pSS IgG antibodies (pSS IgG) interacting with M_3 muscarinic acetylcholine receptors (mAChR) of rats submandibular glands that alter mucin release and production via phospholipase C (PLC) and cyclooxigenase-2 (COX-2) pathways.

Methods: Mucin release and production of prostaglandin E2 (PGE2), and total inositol phosphates (InsP) were measured in rat submandibular gland in the presence of pSS IgG auto antibodies.

Results: The auto antibodies interacting with M3 mAChR decreased mucin release and production through stimulation of PLC and COX-2. This stimulation leads to an incremental increase in InsP production and in PGE2 generation, inducing signalling through the prostaglandin membrane receptors subtype 2 (EP2). Moreover, the decrease in mucin production had negative correlation with PGE_2 generation and InsP accumulation.

Conclusion: IgG in patients with pSS could play an important role in the pathoetiology of dry mouth, decreasing the salivary mucin through the production of proinflammatory substances and leading to the reduction in the protection of the oral tissues.

***Keywords*:** Auto antibodies, InsP, mAChR, Mucin, PGE2, Submandibular gland.

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Introduction

Primary Sjögren's syndrome (pSS) is a T cell-mediated autoimmune disorder characterized by lymphocytic infiltrates, destruction of the salivary glands, and systemic production of autoantibodies against ribonucleoprotein particles (SS-A/Ro and SS-B/La).[1-3] Finding the subtypes M_1, M_3 and M_4 submandibular gland mAChRs-specific auto antibodies in a majority of patiens with pSS is an important advance towards understanding the pathogenesis of pSS.[4-6] However, M_3 mAChR is the major subtype expressed in the membrane of rat submandibular gland and also, M_3 mAChR pSS IgG was more active than M_1 and M_4 mAChRs pSS IgG.[7] It is assumed that autoreactive T cells and M3 mAChR-specific antibodies may recognize auto antigens, triggering autoimmunity in the salivary glands, and leading to clinical symptoms of mouth and eye dryness (sicca syndrome).[8] These data indicate an important role for T and B cell interactions in the pathogenesis of salivary gland autoimmunity. Profound secretary dysfunction may be associated with the capacity of these auto antibodies to impair the acetylcholine action on mAChRs on aqueous salivary secretion.[6] However, the mechanism underlying the mAChR-specific autoantibody-mediated alteration of the secretion of salivary mucins via binding to the M3 mAChR remains to be defined.

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The acinar cells of the submandibular gland receive both adrenergic and cholinergic innervations that regulate secretory responses such as the release of water, electrolytes and proteins. It is accepted that the release of high molecular weight mucin is provoked by the stimulation of the β adrenergic receptors in rat submandibular glands[9,10] and electrical stimulation of chorda tympani and can be induced by injection of bethanechol *in vivo* into a dog submandibular gland.[11] The ability of muscarinic cholinergic stimulation to elicit mucin release is dependent on the increase of intracellular calcium ion in the cells of rat submandibular gland; this highlights the need of calcium function in receptor-mediated mucin release.[12] The increase in intracellular calcium in antral mucous cells results in the stimulation of cyclooxygenase-1 (COX-1) activity, which in turn leads to the prostaglandin E2 (PGE2) synthesis and its subsequent release from cells. The secreted PGE2 may then enhance the calcium-regulated mucin exocytosed by antral mucosal cells.[13] The major goal of the present study was to demonstrate that the serum IgG from pSS patients interacts with M3 mAChR in rat submandibular gland and regulates the release and production of mucin, and to determine if this effect is associated with the activation of phospholipase C (PLC) and cyclooxygenase-2 (COX-2).

Materials and Methods

Subjects and serological tests

Female patients within the range of 35–55 yrs of age, free from receiving treatment for 6 months, and with 7-15 yr from the time of the diagnosis of their illness, were selected from the metropolitan area of Buenos Aires (Argentina). The patients in the present study were 18 women with primary Sjögren's syndrome (pSS) who presented with dry mouth, and 16 healthy women (mean age 45 ± 10 yr) without any systemic diseases (control group). The diagnosis of SS was based on four or more of the criteria published elsewhere.[14] Biopsy results, degree of xerostomia and keratoconjunctivitis sicca, and the results of serological tests on the different groups were the same as previously reported.[15] All participants agreed to participate in the study, according to an approved protocol satisfying the Ethics Committee requirement of Buenos Aires University at the School of Dentistry. The studies were conducted according to the tenets of the Declaration of Helsinki.

Purification of human IgG

Serum IgG fraction from patients with pSS and from normal individuals (as control) were isolated using protein G affinity chromatography as described elsewhere[16] Briefly, serum were loaded onto the protein G affinity columns (Sigma, St Louis, MO, USA). The columns were then equilibrated with 1 M Tris–HCl, pH 8.0, and the columns were then washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine-HCl, pH 3.0, and immediately was neutralized with the corresponding buffer. The concentration and purification of IgG were determined using a radial immunodiffusion assay.

Purification of anti-peptide immunoglobulins by affinity chromatography

The IgG fraction from patients with pSS was independently subjected to affinity chromatography using the M3 mAChR synthesized peptide (25-mer peptide; K-R-T-V-P-D-N-Q-C-FI-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) covalently linked to an affigel 15 (Bio-Rad, Richmond, CA, USA). The IgG fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (PBS) and the non[6] anti-peptide fraction was first eluted using the same buffer. Specific anti-peptide autoantibodies were then eluted using 3 M KSCN/1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentrations of both nonanti- peptide immunoglobulins (Igs) and specific anti-muscarinic receptor peptide Igs (pSS IgG) were determined using radial immunodiffusion assays. Immunological reactivity against the muscarinic receptor peptide was evaluated using an enzyme-linked immunosorbent assay (ELISA).[16]

Measurement of mucin secretion

After removing free connective tissue and fat from submandibular glands, they were cut into small slices that were incubated in 500 μLof Krebs Ringer bicarbonate medium (KRB), pH 7.4, with 5% CO2 in O2 for 30 min at 37 ºC. When inhibitors were used, they were included from the beginning of the incubation time and stimuli were added during the last 15 min. Mucin production and release into the medium were determined using the Alcian Blue methodology as described by Hall et al.[17] and modified by Sarosiek et al.[18] Mucin release and production were expressed as micrograms per milligrams of total protein (μg/mg protein).

PGE2 assay

Rat submandibular gland slices (10 mg) were incubated for 60 min in 500 μL of KRB, gassed with 5% CO2 in O2 at 37ºC. Anti-M3 peptide pSS IgG or pilocarpine were added 30 minutes before the end of the incubation period and blockers were added 30 minutes before the addition of different concentrations of anti-M3 peptide pSS IgG or 1 x 10^{-7} M pilocarpine. The submandibular gland was then homogenized in a 1.5 mL polypropylene microcentrifuge tube. All applied procedures were according to the protocol from the PGE2 Biotrak Enzyme Immuno Assay System (ELISA; Amersham Biosciences, Piscataway, NJ, USA). The PGE2 results were expressed as picograms per milligram of total protein (pg/mg protein).

Measurement of total labelled inositol phosphates (InsP)

Rat submandibular gland slices were incubated for 120 min in 500 μL of KRB gassed with 5% CO2 in O2 with 1 μCi [myo-^{3}H] inositol ([^{3}H]MI) (Sp. Act. 15 Ci mmol-1) from Dupont/New England Nuclear. Litium chloride (LiCI) (10 mM) was added for inositol monophosphate accumulation. Pilocarpine and pSS IgG were added 30 minutes before the end of the incubation period and the blockers were added 30 minutes before the addition of the agonist. Water-soluble InsP were extracted after 120 min and the results were expressed as area units per milligram of tissue wet weight (area/mg wet weight).

Animals

Male Wistar rats weighing within the range of 250-300 g from the Pharmacologic Bioterium (School of Dentistry, University of Buenos Aires) were used throughout the study. The animals were kept in standard environmental conditions were fed with a commercial pellet diet and water. For surgical removal of submandibular glands, the animals were sacrificed using ether. The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

Drugs

Pilocarpine (mAChR agonist), pirenzepine (M_1 mAChR antagonist), 4-DAMP (M_3 mAChR antagonist), U-73122 (PLC inhibitor), and calphostin (PKC inhibitor) were obtained from Sigma Chemical Co.; FR-12204 (COX-1 inhibitor), DuP 697 (COX-2 inhibitor), SC 19220 (EP2 receptor antagonist) and AH 6809 (EP1/EP2 receptor antagonist) were from Tocris Cookson (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted in the bath to achieve the final concentrations as stated in the text.

Statistical analysis

The unpaired Student‘s *t*-test was used to determine statistical significance. Analysis of variance (ANOVA) and a post-hoc test (Dunnett’s method or Student-Newman-Keuls test) were employed when a pair wise multiple comparison procedure was necessary. Differences between means were considered significant if the *P*-value was < 0.05.

Results

At concentrations ranging from 1 x 10-9 M to 1 x 10-6 M, pSS-associated IgG induced a concentration-dependent inhibitory effect on the release and production of mucin by the rat submandibular gland (Figure 1A, B). Normal IgG did not modify either the release or production of mucin (Figure 1A, B). In contrast, pilocarpine increased mucin release (Figure 1A) without modifying its production (Figure 1B).

The M3 mAChR antagonist 4-DAMP, but not the M1 MAChR antagonist, pirenzepine, neutralized the effect of pSS IgG (1 x 10-7 M) on both mucin release and production (Figure 2A). While the stimulatory effect of pilocarpine (1 x 10-7 M) on mucine release was abrogated by pirenzepine; 4-DAMP had no effect (Figure 2B).

To investigate the possibility that PLC and COX-2 activities were involved in the inhibitory effect of pSS IgG on mucin release and production, the effect of selective inhibitors of PLC (U-73122, 5 x 10-6 M) and of COX-2 (DuP 697, 5 x 10-6 M) was determined. U-73122 and DuP 697 prevented the pSS IgG mediated inhibition of mucin release and production. Moreover, SC 19220 (1 x 10-8 M), an EP1 receptor antagonist, also blocked the pSS IgG effects (Figure 3 A, B).

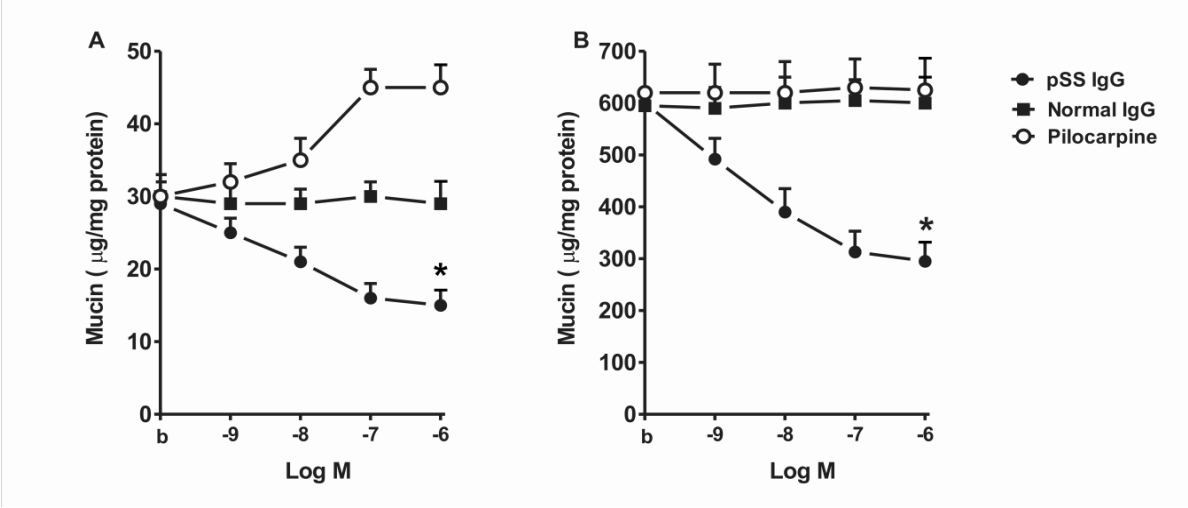


Figure 1. Dose-response-curves of pSS IgG (●), normal IgG (■) and pilocarpine (○) on mucin release (A) and mucin production (B). Rat salivary glands were incubated with each concentration of the reactant for 30 min and the concentration of mucin was assayed as described in the Material and Methods. Values represent the mean ± standard error of the mean (SEM) of 18 patients with pSS and 16 normal subjects. All experiments were performed in duplicate. *P < 0.001 vs. Normal IgG or Pilocarpine.

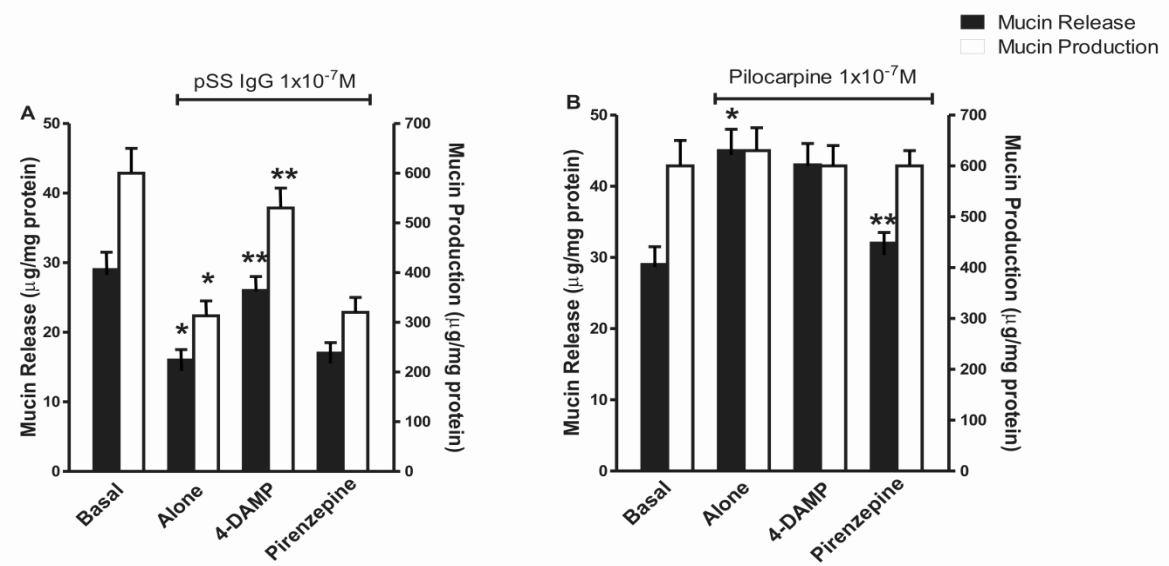


Figure 2. Effect of 1 x 10^{-7} M pSS IgG (A) and 1 x 10^{-7} M pilocarpine (B) on mucin release (black column) and mucin production (white column) by the rat submandibular gland. The salivary glands were incubated for 30 min in the absence (basal) or presence of pSS IgG (alone) or with 1 x 10^{-6} M 4-DAMP or 1 x 10^{-6} M pirenzepine. Values are expressed as the mean ± SEM of 18 pSS patients in each group. Experiments were performed in duplicate. *P < 0.001 vs. Basal. **P < 0.001 vs. pSS IgG alone or pilocarpine alone.

To discern the participation of different second messengers in the signal transduction pathways triggered after pSS IgG binds and activates M3 mAChR, the production of PGE2 and InsP were measured. This clearly demonstrated that pSS IgG (1 x 10-7 M) stimulated the accumulation of InsP and generated PGE2 (Figure 3C, D). The inhibition of PLC by U-73122 and the EP1 receptor antagonist SC 19220, abrogated the stimulatory action of pSS IgG on InsP accumulation (Figure 3C). Moreover, the inhibition of PLC (U-73122) and COX-2 (DuP 697) diminished the stimulatory activity of pSS IgG on PGE2 generation. The production of both InsP and PGE2 was blocked by the M3 mAChR antagonist 4-DAMP (Figure 3C, D).

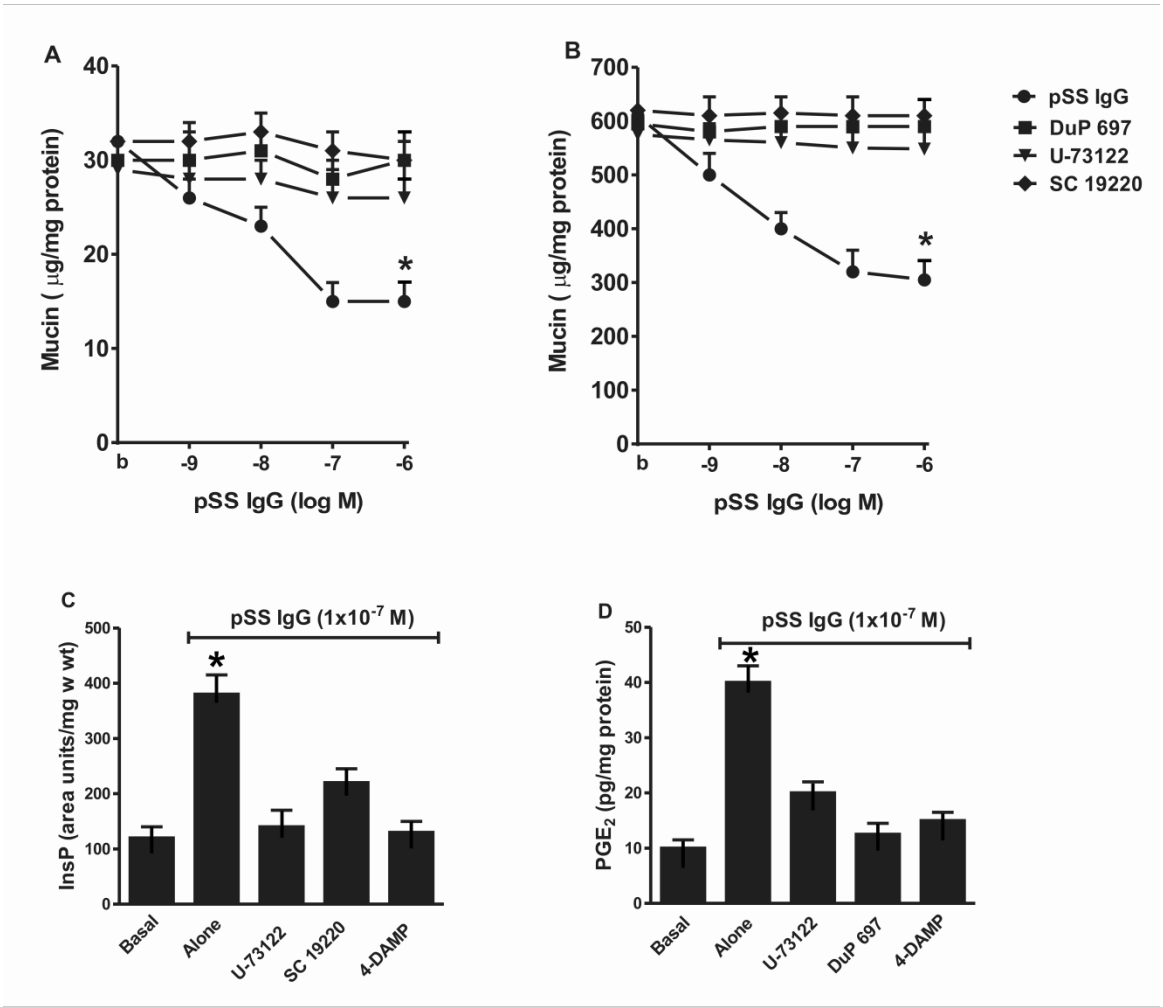
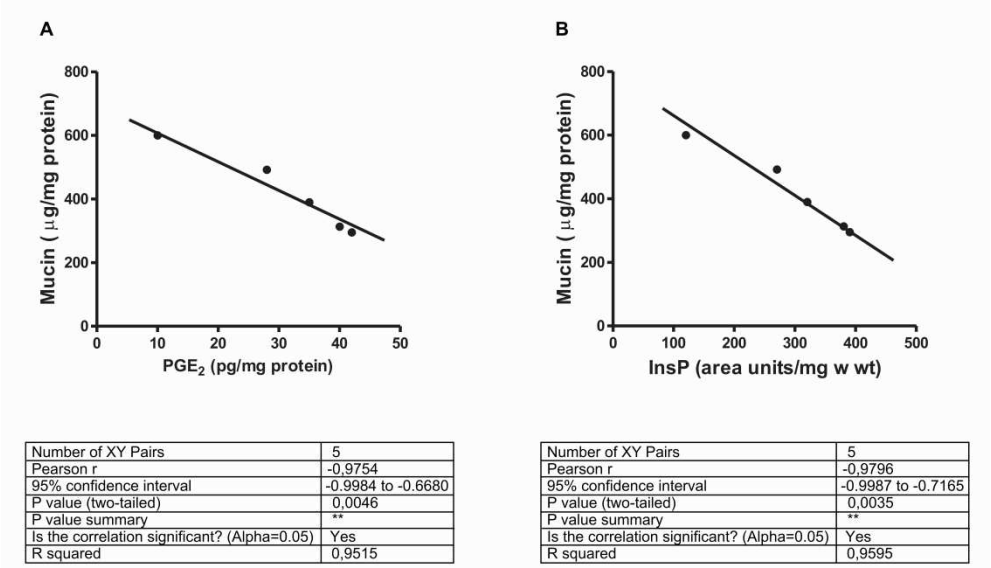


Figure 3. Upper panel: Dose-response curve of pSS IgG alone (●) or in the presence of 5 x 10^{-6} M DuP 697 (■), 5 x 10^{-6} M U-73122 (▼), and 1 x 10^{-8} M SC 19220 (♦) on mucin release (A) and mucin production (B). Values are mean ± SEM of 12 pSS patients in each group. *P < 0.001 vs. pSS IgG + DuP 697, U-73122 or SC 19220. Lower panel: (C) Total InsP accumulation and (D) PGE_2 production in rat submandibular glands in the absence (basal) or presence of 1 x 10^{-7} M pSS IgG (alone), 1 x 10^{-7} M pSS IgG + U-73122 or SC 19220 or 4-DAMP. Each bar represents the mean ± SEM of 12 pSS patients performed in duplicate. *P < 0.001 vs. pSS IgG + U-73122 or SC 19220 or 4-DAMP. C: P < 0.001 vs. pSS IgG + U-73122 or SC 19220 or 4-DAMP. D: *P* < 0.001 vs. pSS IgG + U-73122 or DuP 697 or SC 19220 or 4-DAMP.

There is a significant negative correlation between pSS IgG-stimulated production of PGE_2 and InsP with pSS IgG-mediated inhibition of mucin production (Figure 4A, B). These results demonstrated that the activation of the M_3 mAChR by pSS IgG inhibited mucin production by stimulating InsP accumulation and PGE_2 production.

Table 1 shows comparatively, the pSS IgG inhibitory action with pilocarpine stimulatory activity on mucin release. The pSS IgG-inhibition of mucin release could be prevented by COX-2 (DuP 697) and EP1 receptor antagonist (SC 19220) or by inhibiting the activities of PLC (U-73122) and PKC (calphostin). In contrast, the pilocarpine stimulatory activity was abrogated by COX-1 (FR 1220479) and EP1/EP2 receptor antagonists (AH 6809). PLC (U-73122) and PKC (calphostin) inhibitors had no effect on our system.



Number of XY Pairs	5
Pearson r	-0,9754
95% confidence interval	-0.9984 to -0.6680
P value (two-tailed)	0,0046
P value summary	**
Is the correlation significant? (Alpha=0.05)	Yes
R squared	0,9515

Number of XY Pairs	5
Pearson r	-0,9796
95% confidence interval	-0.9987 to -0.7165
P value (two-tailed)	0,0035
P value summary	**
Is the correlation significant? (Alpha=0.05)	Yes
R squared	0,9595

Figure 4. Negative correlation between the inhibition of mucin production and the stimulation of the production of PGE_2 (A) or accumulation of InsP (B) by the IgG from patients with primary Sjögren's syndrome. Mucin production was plotted as a function of PGE_2 and total InsP.

Table 1. Effect of different inhibitors on mucin release by pSS IgG and pilocarpine on submandibular gland

Additions	pSS IgG ($1x10^{-7}$ M)	Pilocarpine ($1x10^{-7}$ M)
Basal	30 ± 3	29 ± 2
Alone	16 ± 1	45 ± 3
FR 122047 ($5x10^{-8}$ M)	17 ± 1	32 ± 2 *
DuP 697 ($5x10^{-8}$ M)	28 ± 1 *	47 ± 3
SC 19220 ($1x10^{-5}$ M)	31 ± 3 *	46 ± 4
AH 6809 ($3x10^{-5}$ M)	32 ± 3 *	30 ± 3 *
U-73122 ($1x10^{-6}$ M)	26 ± 2 *	44 ± 4
Calphostin ($1x10^{-8}$ M)	29 ± 2 *	43 ± 4

Mucin release by affinity purified M_3 peptide autoantibodies from patients with primary Sjögren's syndrome (pSS IgG) and pilocarpine. Rat submandibular glands were incubated for 30 minutes in the absence (basal) or presence of pSS IgG or pilocarpine alone or with different inhibitors. Pilocarpine or pSS IgG were added in the last 10 minutes. Values are expressed in μg mucin/mg protein and are the mean ± SEM of 6 experiments in each group. * $P < 0.001$ vs. pSS IgG or pilocarpine alone.

Discussion

Autoantibody acting at the level of the post-synaptic M_3 mAChR of rat submandibular gland have been implicated in the autonomic parasympathetic dysfunction in pSS. Mucin secretion from rat submandibular gland is dependent on the parasympathetic stimulation.[19,20] Sjögren's syndrome is an autoimmune disease which is characterized by the hallmark clinical feature of salivary insufficiency associated with focal, periductal and perivenular lymphocytic infiltrates[21]; pSS patients express autoantibodies against M_3 mAChR that, at least in part, are responsible for altering the parasympathetic control of saliva secretion.[22,23] The effectiveness of pilocarpine in stimulating mucin release may suggest that the muscarinic cholinergic system participates in the regulation of mucin release from the rat submandibular gland[24] and the mucin secretory response to the cholinergic agonist was dependent on the M1 mAChR subtype and a calcium dependent mechanism.[25] In contrast to the stimulatory effects of pilocarpine, in this study we demonstrated that patients with pSS produce functional IgG autoantibodies that interact with glandular M_3 mAChR, inhibiting

mucin release and production. The antibody-mediated inhibitory effect correlates with the incremental accumulation of InsP and increase in PGE_2 production. This effect highlighted the possibility that autoantibodies play an important role in the pathogenesis of dry mouth in pSS patients. Furthermore, we even demonstrated the inhibition of mucin or the stimulation of InsP and PGE_2 by pSS IgG, could be abolished by the inhibition of M_3 mAChR, PLC, or COX-2 activities, indicating that pSS IgG functions through M_3 mAChR-mediated signalling in the submandibular gland membranes. Under normal conditions, the constitutive isoform of COX-1 is in virtually all organs and its products help to maintain normal physiological functions such as cytoprotection. This is true for the authentic agonist, pilocarpine, which acts on M_1 mAChR to increase mucin release and provoke an increase in PGE_2 production via the activation of COX-1. In contrast, in an inflammatory setting, the inducible isoform of the enzyme (COX-2) is produced, resulting in the generation of a large amount of pro-inflammatory PGE_2.[26] This pro inflammatory agent mediates acute and chronic inflammation, immunological alterations and

cytotoxic tissue damage.[26] In the present study, we demonstrated that the pro-inflammatory mediator, COX-2, and its product, PGE_2, are induced by pSS-specific autoantibodies. Therefore, the inflammatory process described in the glands of pSS patients might be attributed partly to antibody fixation on membranes that, through interaction with M_3 mAChR, triggers PGE_2 production via COX-2 activation.

An important feature of the differences seen in the effect of pSS IgG and pilocarpine is the ability to decrease or increase mucin release, respectively. This difference could be related to the mAChR and PGE_2 receptor subtypes they interact with. In pilocarpine-stimulated mucin release, the M_1 mAChR and EP2 receptor subtypes are involved; in pSS IgG-decreased mucin release, the M_3 mAChR and EP1 receptor subtypes are involved. Moreover, the activation of M_1 mAChR and EP2 receptors increases cAMP and calcium mobilization,[27] while the activation of M3 mAChR and EP1 receptors decreases cAMP and increases PKC activity.[27] In addition, mucin secretion is stimulated by a number of agents that elevate cAMP concentration[28] and the inhibition of mucin secretion is related to a decrease in cAMP and PKC activation.[24]

Sjögren's syndrome patients present a marked decrease in output per minute of mucin 5B.[29] Mucin 5B is assumed to coat and protect oral tissue.[30-32] The decreased concentration of mucin 5B in the saliva of pSS patients could lead to a reduced protection of oral tissue and increased susceptibility to mucosal damage.[29]

Conclusion

Based on our results, we postulated that the agonist-promoted activation of glandular M3 mAChR initiated by pSS IgG binding and persistent activation results in the production of PGE2 and accumulation of InsP, leading to inhibition of mucin release and production. This inhibition was mediated by IgG autoantibodies in pSS patients and could lead to a reduction in the protection of oral tissues and increased susceptibility to mucosal damage. Moreover, it may also promote the presence of plaque at the level of gingival margin on the tooth leading to caries and periodontal problems, which are frequently seen in SS patients.

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