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Cholinergic autoantibodies from primary Sjögren's syndrome modulate submandibular gland Na^+/K^+-ATPase activity via prostaglandin E_2 and cyclic AMP

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We demonstrate that patients with primary Sjögren's syndrome (pSS) produce functional IgG autoantibodies that interact with the glandular M_3 muscarinic acetylcholine receptors (mAChRs). These autoantibodies act as a partial muscarinic agonist, increasing prostaglandin E_2 (PGE_2) and cyclic AMP production through modifying Na^+/K^+-ATPase activity, but also interfere with parasympathetic neurotransmitter to secretagogue effect. The IgG from patients with pSS has two effects on the submandibular gland. On the one hand, it may act as an inducer of the proinflammatory molecule (PGE_2) that, in turn, inhibits Na^+/K^+-ATPase activity. On the other hand, it plays a role in the pathogenesis of dry mouth, abolishing the Na^+/K^+-ATPase inhibition and the net K^+ efflux stimulation of the salivary gland in response to the authentic agonist pilocarpine, decreasing salivary fluid production.

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Key words: anti-M_3 IgG; cyclic AMP (cAMP); cholinergic peptides; M_3 muscarinic acetylcholine receptor (M_3 mAChR); Na^+/K^+-ATPase activity; prostaglandin E_2 (PGE_2); submandibular gland

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Fluid and electrolyte secretion by the submandibular salivary gland occurs in two steps (1, 2). First, a plasma-like fluid is secreted by the acinar cells in the acinar–intercalated duct region. Second, the electrolyte composition of the acinar fluid in the ductal system is modified. The ducts re-absorb Na^+ and Cl^-, secrete K^+ and HCO_3-, and reduce the osmolality of the fluid, apparently without a significant change in the volume of the secreted fluid (3).

The epithelium of salivary glands, like the epithelium of intestine, is a single layer of columnar cells that regulates the flow of water by creating concentration gradients with vigorous coupled transports of water and NaCl (4). In this epithelium, the electrogenic cationic pump Na^+/K^+-ATPase is essential for the transcellular movement of water and ions, at the apical and basolateral salivary gland levels (5, 6), being involved in the re-absorption of Na^+. The salivary glands produce hypotonic saliva, the volume and composition of which varies with the type of stimulus used. Cholinergic and alpha adrenergic stimulation result in the secretion of saliva, but adrenergic stimulation does not cause as large an output of saliva as cholinergic stimulation (7).

Both cholinergic and alpha adrenergic stimulation inhibit Na^+ re-absorption (8, 9); beta adrenergic stimulation has variable effects, depending on the agonist concentration (10). The neurotransmitter acetylcholine also modulates the ion fluxes occurring through the enterocytes (11, 12) and the acinar salivary cells (13). It is well known that the primary effector mechanism activated by the muscarinic acetylcholine receptor (mAChR) is the hydrolysis of phosphoinositides by phospholipase C (PLC) (14) and the production of free fatty acids by phospholipase A_2 (PLA_2) (15). Acinar cells express a higher number of mAChRs of the M_3 subtype than duct (16). The action of the muscarinic cholinergic agonist on the mAChR of the M_3 subtype generates the second messengers, namely inositol triphosphate ($InsP_3$) and prostaglandin E_2 (PGE_2); each of these metabolites activates salivary secretion, mobilizes calcium from intracellular stores, and regulates salivary ion and water absorption (17).

Sjögren's syndrome (SS) is an autoimmune disease with the hallmark of clinical features of salivary insufficiency associated with focal, periductal, and perivenular lymphocytic infiltrates (15). Profound secretory dysfunction can be associated with the presence of autoantibodies to the M_3 mAChR that have variously been reported to have agonistic and antagonistic effects (18). However, the real mechanism by which the M_3 mAChR autoantibodies, when bound to the M_3

mAChR, evoke an alteration of the secretion of saliva is still to be defined.

Herein, the biological action of autoantibodies that interact with peptide sequences belonging to human M_3 mAChR was evaluated. We considered it especially relevant to investigate whether circulating mAChR autoantibodies not only bind and activate M_3 mAChR subtypes, but also modify the Na^+/K^+-ATPase activity and provoke increased generation of PGE_2 and cyclic AMP (cAMP). Moreover, the antagonistic action of the autoantibodies against the secretogogue effect of parasympathetic intervention was also analyzed.

Material and methods

Subjects and serological tests

Women (35–55 yr of age), free of treatment for 6 months, and with 7–15 yr since diagnosis, were selected from the metropolitan area of Buenos Aires (Argentina). The patients used in this present work were 15 women with primary Sjögren's syndrome (pSS) and dry mouth, and 18 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 from VITALI *et al*. (19). Biopsy results, degree of xerostomia and keratoconjunctivitis sicca, and results of serological tests of the different groups are presented in Table 1. All participants gave their informed consent to participate in the study, according to an approved protocol satisfying the Ethics Committee requirement of Buenos Aires University in the School of Dentistry. The studies were conducted according to the tenets of the Declaration of Helsinki.

Peptides

A 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), corresponding to the amino acid sequence of the second extracellular loop of the human M_3 mAChRs, was synthesized from F-moc-amino acids activated using the 1-hydroxy benzo triazole/dicyclo hexyl carbodimide (HOBt/DCC) strategy in an automatic peptide synthesizer (Applied Biosystems Model 431A. The peptide was desalted, purified by high-performance liquid chromatography (HPLC), and subjected to amino-terminal sequence analysis by automatic Edman degradation in an Applied Biosystems 470 A Sequence. A 25-mer unrelated peptide (S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S) was synthesized as a negative control.

Table 1

Various tests performed on patients with primary Sjögren's syndrome (pSS) and on normal individuals

Tests	pSS patients	Normal individuals (basal)
Biopsy (score focus)	15/15 (100%)	0/15 (0%)
	3.7 ± 0.09	0
Xerostomia (stimulate salivary flow, ml 15 min^{-1})	15/15 (100%)	0/15 (0%)
	1.8 ± 0.5	17.2 ± 1.7
Keratoconjunctivitis sicca*	14/15 (93%)	0/15 (0%)
Anti-Ro (SS-A)	7/15 (47%)	0/15 (0%)
Anti-La (SS-B)	5/15 (34%)	0/15 (0%)
Anti M_3 peptide pSS IgG (absorbance, 405 nm)	15/15 (100%)	0/15 (0%)
	1.29 ± 0.06	0.17 ± 0.03
Anti-non-related peptide IgG (absorbance, 405 nm)	0/15 (0%)	0/15 (0%)
	0.00 ± 0.00	0.00 ± 0.00

Results are expressed as the number of positives per total with percentages in parentheses. Score focus, salivary flow, and absorbance are shown as mean values ± standard error of the mean (SEM).
*Schirmer test < 5 mm (positive); breakup time < 10 s (positive).
pSS patients: 15 women with pSS and dry mouth.
Normal individuals (basal): 15 normal women.

Purification of human IgG

The serum IgG fraction from patients with pSS and from normal individuals (as a control) was isolated by protein G affinity chromatography, as described elsewhere (20). Briefly, sera were loaded onto the protein G (Sigma, St Louis, MO, USA) affinity column 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 neutralized. The concentration and purification of IgG were determined using a radial immune diffusion assay.

Purification of anti-peptide Igs by affinity chromatography

The IgG fraction from patients with pSS was independently subjected to affinity chromatography on the synthesized peptide covalently linked to an AffiGel 15 gel (Bio-Rad, Richmond, CA, USA). The IgG fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (PBS) and the non-peptide fraction was first eluted with the same buffer. Specific anti-peptide autoantibodies were then eluted with 3 M KSCN/1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentrations of both non-anti-peptide Igs and specific anti-muscarinic receptor peptide Igs were determined by radial immunodiffusion assays, and their immunological reactivity against the muscarinic receptor peptide was evaluated by enzyme immunoassay (ELISA) (20).

Total submandibular gland membrane preparations

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, of Buenos Aires) were housed in our colony in small groups and maintained under conditions of automatically controlled lighting and uniform temperature. The animals were cared for in accordance with the principles and guidelines of the NIH.

Acinar and duct submandibular gland membrane preparations

Rat male acinar and duct membranes were prepared as described previously (16). Briefly, salivary glands were minced in ice-cold oxygenated Krebs Ringer bicarbonate (KRB) and incubated in 10 ml of KRB containing collagenase (2–4 mg ml^{-1}) for 40 min in a shaking water bath at 37°C. The glands were dispersed and the cells were washed with Ca^{2+}/Mg^{2+}-free KRB, as reported previously (15). The duct cells in the upper phase and the acinar cells in the lower phase were collected. Submandibular gland acini were homogenized for 10 s, twice, in 50 mM

phosphate buffer, pH 7.4, in an Ultra-Turrax (setting 5). The homogenate was centrifuged for 10 min at 1,000 *g*. The pellets were discarded, and the supernatants were centrifuged (10,000 *g*) at 4°C for 10 min and then at 40,000 *g* for 60 min. The resulting pellets were resuspended in the same buffer (16).

Membrane preparation for ATPase determination

Free connective tissue and fat were gently removed from the submandibular glands, which were cut into small slices, placed in tubes containing 500 μl of KRB solution (pH 7.4) bubbled with 95% O_2 and 5% CO_2, and incubated at 37°C for 30 min. When used, inhibitors were included from the start of the incubation, and the affinity-purified M_3 peptide antibody (anti-M_3 peptide pSS IgG) and pilocarpine were added for the last 20 min. The reaction was stopped by removing the submandibular gland slices and homogenizing them at 4°C in tubes containing 750 μl of 10 mM Tris–HCl, 1 mM EDTA, and 400 mM LiBr (hypotonic shock), supplemented with the protease inhibitor, phenylmethylsulfonyl fluoride, at a concentration of 0.1 mM. The homogenates were centrifuged for 10 min at 1,000 *g* and the supernatants were collected and spun down for 20 min at 9,000 *g*. Both steps almost completely eliminated nuclei, mitochondrial, and lisosomal fractions, and the resultant supernatant was centrifuged for 60 min at 100,000 *g*. The pellet was then resuspended in 10 mM Tris–HCl/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride, and stored at −70°C until used.

Determination of Na^+/K^+-ATPase activity

Membrane aliquots (approximately 10–20 μg of protein) were transferred to the Na^+/K^+-ATPase assay medium [final volume 172 μl, containing: 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 160 mM Tris–HCl (pH 7.4), and 4 mM Na_2ATP] and incubated for 30 min at 37°C in the absence or presence of 5×10^{-4} M ouabain. When ouabain was present, NaCl and KCl were omitted from the incubation medium and replaced with Tris–HCl. The reaction was stopped by the addition of 40 μl of cold 30% trichloroacetic acid. Samples were centrifuged at 3,000 *g* for 10 min and the inorganic phosphate liberated (total ATPase activity) was measured. Na^+/K^+-ATPase activity was calculated as the difference between the means of the total ATPase activity and the ouabain-sensitive ATPase activity.

Net K^+ efflux determination

Rat submandibular gland slices, prepared as described above, were incubated in 1 ml of oxygenated KRB buffer at 37°C in the presence or absence of different concentrations of the anti-M_3 peptide pSS IgG and pilocarpine, ouabain 5×10^{-4} M and the anti-M_3 peptide pSS IgG and pilocarpine plus ouabain 5×10^{-4} M or 4-diphenylacetoxi-*N*-methylpiperidine methiodide (4-DAMP) 10^{-5} M or M_3 synthetic peptide (10 μg ml^{-1}). Two-hundred-microlitre aliquots of the medium were removed at 2, 5, and 10 min. At the end of the incubation period (10 min), the slices were homogenized in the remaining KRB medium and the K^+ concentration in the homogenate and in the aliquots removed in the course of the experiments was measured in triplicate in an Instrumentation Laboratories flame photometer. Net K^+ efflux is presented as the percentage of the K^+ present in the slices.

PGE_2 assay

Rat submandibular gland (10 mg) was incubated for 60 min in 500 μl of KRB, gassed with 5% CO_2 in oxygen, at 37°C. Anti-M_3 peptide pSS IgG and pilocarpine were added 30 min before the end of the incubation period and blockers were added 30 min before the addition of different concentrations of anti-M_3 peptide pSS IgG and 1×10^{-7} M pilocarpine. The submandibular gland was then homogenized into a 1.5 ml polypropylene microcentrifuge tube. Thereafter, all procedures used were those indicated in the protocol of the PGE_2 Biotrak Enzyme Immuno Assay System (ELISA; Amersham Biosciences, Piscataway, NJ, USA). The PGE_2 result was expressed as nanograms per milliliter.

Determination of cAMP

To determine the cAMP levels, tissues were incubated in 1 ml of KRB for 30 min. After incubation, submandibular gland were homogenized in 2 ml of absolute ethanol and centrifuged at 6,000 *g* for 15 min at 4°C. The pellets were then re-homogenized in ethanol/water (2:1) and the supernatants were collected and evaporated to dryness. The residue was dissolved in 400 μl of 50 μM sodium acetate buffer, pH 6.2, and aliquots of 100 μl were taken for nucleotide determination using a radio immunoassay procedure of a [^{3}H]cAMP-RIA kit from Dupont New England Nuclear (Boston, MA, USA).

Drugs

Pilocarpine, pirenzepine, tropicamide, and indomethacin were obtained from Sigma Chemical Co.; FR-12204, DuP697, and OBAA were from Tocris Cookson (Ellisville, MO, USA), and 4-DAMP was kindly provided by Boehringer Ingelheim Pharmaceuticals (Ingelheim, Germany). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text.

Statistical analyses

The Student's *t*-test for unpaired values was used to determine the significance level. Analysis of variance (ANOVA) and the *post hoc* test (Dunnett's method and Student–Newman–Keuls test) were employed when a pairwise multiple-comparison procedure was necessary. Differences between means were considered significant if the *P*-value was < 0.05.

Results

Figure 1 shows the potential of serum IgG from patients with pSS to stimulate the generation of PGE_2 (Fig. 1A) and the production of cAMP (Fig. 1C) in a concentration-dependent manner. Normal IgG was unable to stimulate the production of PGE_2 or cAMP (Fig. 1A,C). To determine the mAChR subtype responsible for the action of pSS IgG, the effects of 4-DAMP, pirenzepine, and tropicamide were studied. Table 2 shows that 4-DAMP (1×10^{-7} M) inhibited the maximal stimulatory effect of the pSS IgG observed at 1×10^{-6} M by

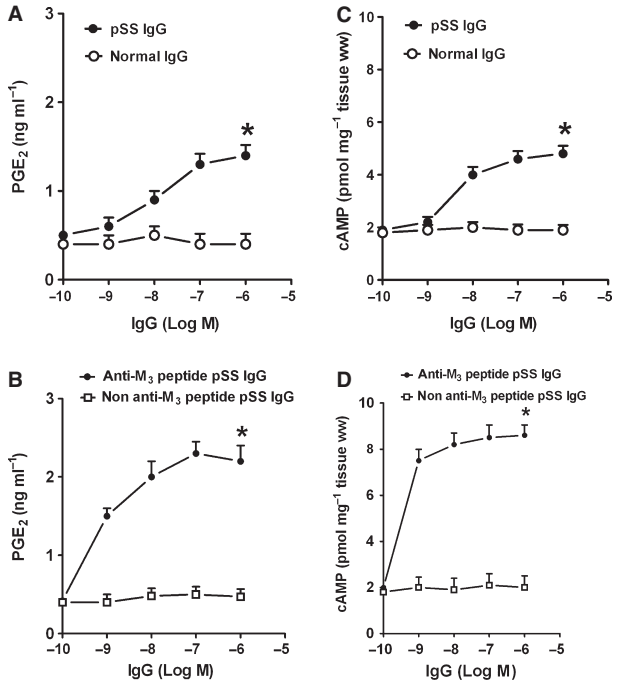

Fig. 1. Dose–response curve of total pSS IgG-induced, anti-M_3 peptide pSS IgG-induced (●), normal IgG-induced (○), or non-anti-M_3 peptide pSS IgG-induced (□) stimulation of PGE_2 (A and B) and cAMP (C and D). Rat salivary glands were incubated with each concentration of the reactant for 1 h, and the concentrations of PGE_2 and cAMP were assayed as described in the Material and methods. Values represent the means ± standard error (SE) of 15 patients with pSS and 15 normal subjects. Experiments were performed in duplicate. *, $P < 0.001$ vs. normal IgG (A and C) or non-anti-M_3 peptide pSS IgG (B and D). anti-M_3 peptide pSS IgG, affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome; cAMP, cyclic AMP; PGE_2, prostaglandin E_2; pSS IgG, IgG fraction from patients with primary Sjögren's syndrome.

89% for PGE_2 and cAMP. When 4-DAMP was used at 1×10^{-8} M, the maximal stimulatory effect of the pSS IgG was inhibited by about 50% (data not shown). Neither 1×10^{-7} M pirenzepine nor 1×10^{-7} M tropicamide affected the stimulatory effect of pSS IgG on PGE_2 and cAMP production. Pirenzepine and tropicamide resulted in 11% of inhibition at the highest dose used (1×10^{-6} M). As 4-DAMP showed a greater inhibitory potency, we investigated if the M_3 mAChR subtype was involved in the pSS IgG-induced increases in PGE_2 and cAMP. Knowing that there is strong homology between the amino acid sequences of the rat and human M_3 synthetic peptide corresponding to the second extracellular loop of the M_3 mAChR (21), we studied the M_3 mAChR-mediated effect of autoantibodies from pSS patients on rat submandibular glands using the affinity-purified M_3 peptide antibodies (anti-M_3 peptide pSS IgG). It can be seen, in Fig. 1B, that the anti-M_3 peptide pSS IgG induced significantly higher amounts of PGE_2 than the non-anti-M_3 peptide pSS IgG. The concentration–response curve produced in response to the presence of the anti-M_3 peptide pSS IgG showed higher potency and affinity than that of the corresponding total pSS IgG. Anti-M_3 peptide pSS IgG increased the production of cAMP in a manner similar to that of pSS IgG (Fig. 1D). The non-affinity-purified, anti-M_3 peptide pSS IgG eluted from the column stimulated the production of PGE_2 (Fig. 1B) and cAMP (Fig. 1D) to levels similar to those induced by normal IgG.

Table 2

Effect of different subtype muscarinic acetylcholine receptor (mAChR) antagonists on the production of prostaglandin E_2 (PGE_2) and cyclic AMP (cAMP) when stimulated with IgG from patients with primary Sjögren's syndrome (pSS IgG)

Additions	PGE_2 (ng ml^{-1})	cAMP (pmol mg^{-1} tissue w/w)
None	0.45 ± 0.02	1.81 ± 0.1
pSS IgG	1.74 ± 0.2*	4.96 ± 0.3*
pSS IgG + 4-DAMP 1×10^{-7} M	0.51 ± 0.04**	2.02 ± 0.1**
pSS IgG + pirenzepine 1×10^{-7} M	1.31 ± 0.8	4.22 ± 0.8
pSS IgG + tropicamide 1×10^{-7} M	1.68 ± 0.1	4.81 ± 0.4

Results are expressed as the mean ± standard error of the mean (SEM) of five experiments in each group. Data show the levels of PGE_2 and cAMP produced by rat submandibular glands incubated for 60 min in Krebs Ringer bicarbonate (KRB). The pSS IgG was added, at 1×10^{-6} M, 30 min before the end of the incubation period and the blockers were added 30 min before the addition of pSS IgG.
*Values differ significantly from control (none) values ($P < 0.001$). **Values differ significantly from pSS IgG alone ($P < 0.001$).

Figure 2 demonstrates that, under identical experimental conditions, there is a significant correlation between anti-M_3 peptide pSS IgG-stimulated production of PGE_2 and cAMP. These results demonstrate that the

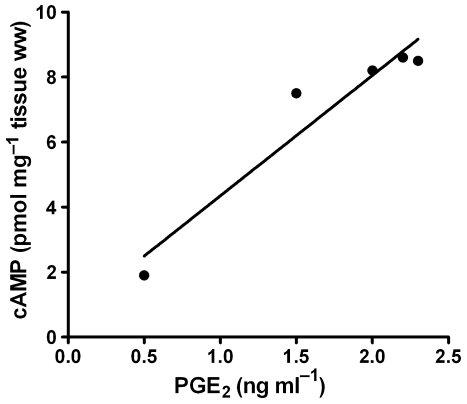

Fig. 2. Correlation of the stimulatory effect of affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG), used at concentrations ranging from 1×10^{-9} to 1×10^{-6} M, on the production of prostaglandin E_2 (PGE_2) and cyclic AMP (cAMP). Cyclic AMP was plotted as a function of PGE_2.

activation of the M_3 mAChR by anti-M_3 peptide pSS IgGs stimulating the accumulation of cAMP may occur as a result of the increased production of PGE_2.

To determine the mAChR subtype specificity of the anti-M_3 peptide pSS IgG on PGE_2 and cAMP, the antibody was assayed in the presence of 4-DAMP and M_3 synthetic peptide. The antagonist 4-DAMP significantly blunted the action of anti-M_3 peptide pSS IgG, as did the M_3 synthetic peptide, on the induction of PGE_2 (A) and cAMP (B). On the contrary, the authentic mAChR agonist, pilocarpine (1×10^{-7} M), had no effect on the system (Fig. 3A,B).

To discern which arachidonic acid (AA) cascade enzymes are implicated in anti-M_3 peptide pSS IgG-generated PGE_2 and cAMP production, several inhibitors of this enzymatic cascade reaction were used. It can be seen in Fig. 4A,B that the inhibition of PLA_2 by OBAA (5×10^{-6} M), the inhibition of cyclooxygenase-1 (COX-1) by FR 122047 (5×10^{-8} M), or the inhibition of COX-2 by DuP697 (5×10^{-8} M), prevented the stimulatory action of anti-M_3 peptide pSS IgG on the induction of PGE_2 and cAMP. Indomethacin (5×10^{-6} M), acting as a non-specific COX-1 and COX-2 blocker, also inhibited the action of the anti-M_3 peptide pSS IgG.

To investigate whether the autoantibody can modify the gland secretory function, we studied the ability of anti-M_3 peptide pSS IgG to induce changes in the activity of Na^+/K^+-ATPase (Fig. 5A). It can be seen that, over the concentration range 1×10^{-10} to 1×10^{-6} M, the autoantibody from pSS patients exerted an inhibitory effect on Na^+/K^+-ATPase in whole submandibular gland membranes. This action was observed only in acini membranes; there was no effect on duct membranes. The inhibitory effect on Na^+/K^+-ATPase of the autoantibody was mimicked by exogenous PGE_2 (Fig. 5B).

To assess whether the M_3 mAChR subtype was involved in the regulatory action of the anti-M_3 peptide pSS IgG on the Na^+/K^+-ATPase pump, a submandibular gland was incubated with the selective M_3 antagonist, 4-DAMP (1×10^{-7} M), and the M_3 synthetic peptide (10 μg ml^{-1}). Figure 6A shows that both 4-DAMP and M_3 synthetic peptide prevented the inhibi-

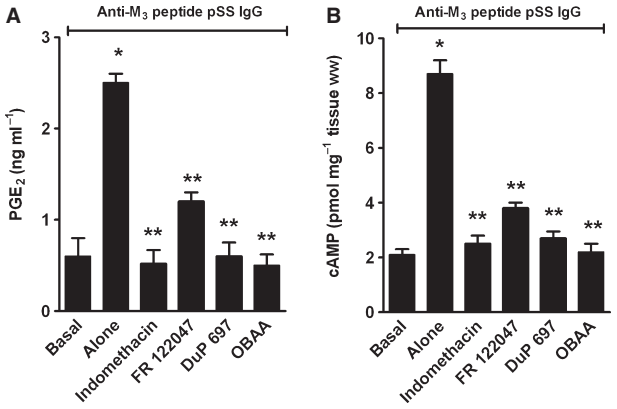

Fig. 4. Effect of affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG; 1×10^{-8} M) on the production of prostaglandin E_2 (PGE_2) (A) and cyclic AMP (cAMP) (B). The salivary glands were incubated for 1 h in the absence (basal) or presence of anti-M_3 peptide pSS IgG, alone, or with 5×10^{-6} M indomethacin, 5×10^{-8} M FR 122047, 5×10^{-8} M DuP697 or 5×10^{-6} M OBAA. Values are expressed as the mean ± standard error of the mean (SEM) of 15 pSS patients in each group. Experiments were performed in duplicate. *, $P < 0.001$ vs. basal. **, $P < 0.001$ vs. anti-M_3 peptide pSS IgG alone.

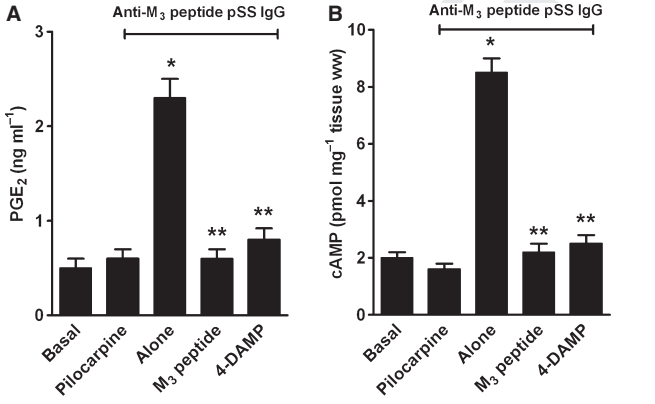

Fig. 3. Effect of pilocarpine (1×10^{-7} M) and affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG; 1×10^{-8} M) on the production of prostaglandin E_2 (PGE_2) and cyclic AMP (cAMP). The salivary glands were incubated for 1 h in the absence (basal) or presence of pilocarpine or of anti-M_3 peptide pSS IgG, alone, or with M_3 peptide (10 μg ml^{-1}) or 4-diphenylacetoxi-*N*-methylpiperidine methiodide (4-DAMP; 1×10^{-7} M). Values are expressed as the mean ± standard error of the mean (SEM) of 15 pSS patients in each group. Experiments were performed in duplicate. *, $P < 0.001$ vs. basal. **, $P < 0.001$ vs. anti-M_3 peptide pSS IgG alone.

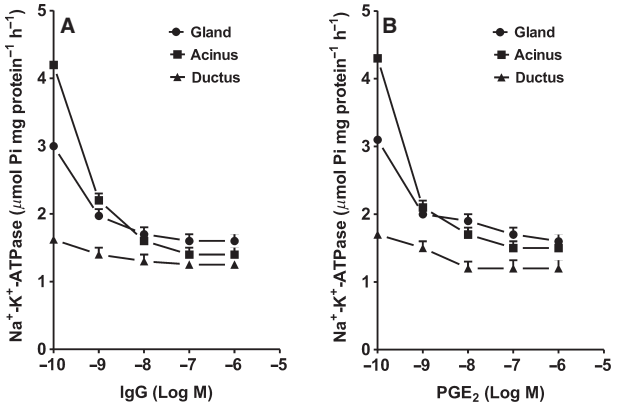

Fig. 5. Dose–response curve of affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG) (A) and prostaglandin E_2 (PGE_2) (B) on Na^+/K^+-ATPase activity in whole submandibular gland, acini, or duct. Values are expressed as the mean ± standard error of the mean (SEM) of 15 pSS patients or of five PGE_2 experiments in each group. Experiments were performed in duplicate.

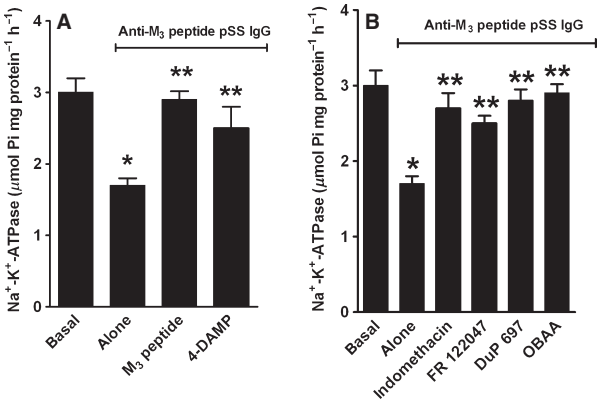

Fig. 6. Effect of the affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG; 1×10^{-8} M) on Na^+/K^+-ATPase activity. The salivary glands were incubated for 1 h in the absence (basal) or presence of anti-M_3 peptide pSS IgG, alone, or with (A) M_3 peptide (10 $\mu g\ ml^{-1}$) or 4-diphenylacetoxi-*N*-methylpiperidine methiodide (4-DAMP; 1×10^{-7} M) and (B) in the presence of 5×10^{-6} M indomethacin, 5×10^{-8} M FR 122047, 5×10^{-8} M DuP697, or 5×10^{-6} M OBAA. Values are means ± standard error of the mean (SEM) of 15 pSS patients in each group. Experiments were performed in duplicate. *, $P < 0.01$ vs. basal. **, $P < 0.05$ vs. anti-M_3 peptide pSS IgG alone.

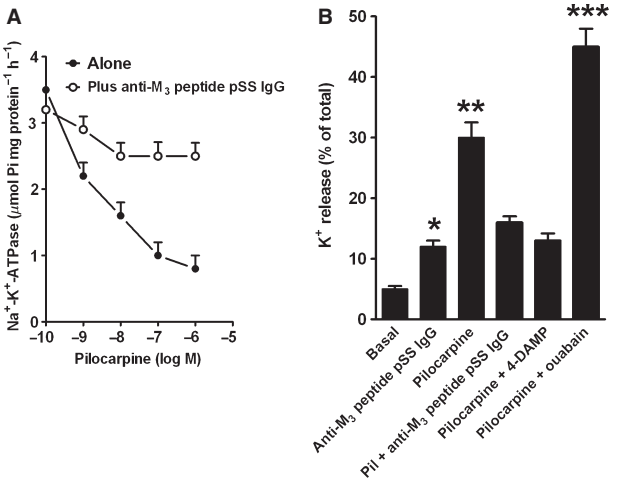

Fig. 7. (A) Dose–response curve of pilocarpine, alone (●), or in the presence of 1×10^{-9} M affinity-purified M_3 peptide antibodies from patients with primary Sjögren's syndrome (anti-M_3 peptide pSS IgG) (○), on Na^+/K^+-ATPase activity. Values are expressed as the mean ± standard error of the mean (SEM) of five experiments in each group performed in duplicate. (B) Extent of net K^+ efflux from rat submandibular glands in the absence (basal) or presence of 1×10^{-9} M pilocarpine, 1×10^{-9} M anti-M_3 peptide pSS IgG, 1×10^{-9} M pilocarpine (pil) + 1×10^{-9} M anti-M_3 peptide pSS IgG, 1×10^{-9} M pilocarpine + 1×10^{-7} M 4-diphenylacetoxi-*N*-methylpiperidine methiodide (4-DAMP), or 1×10^{-9} M pilocarpine + 5×10^{-4} M ouabain. Each bar represents the mean ± standard error of the mean (SEM) of six experiments performed in duplicate.

tory action of anti-M_3 peptide pSS IgG on the Na^+ pump. To determine whether an endogenous AA cascade participates in the pSS autoantibody-induced inhibition of Na^+/K^+-ATPase, a submandibular gland was incubated with selective inhibitors of PLA_2 and COX-1 and COX-2 pathways. Figure 6B shows that the inhibition of PLA_2, COX-1, COX-2, and nonselective COX-s indomethacin resulted in blocking the inhibitory effect of the autoantibody on Na^+/K^+-ATPase activity. These results indicate that anti-M_3 peptide pSS IgG M_3 mAChR stimulation may inhibit glandular Na^+/K^+-ATPase as a result of an increase in PGE_2 generation via PLA_2, COX-1, and COX-2 activation.

To evaluate whether the anti-M_3 peptide pSS IgG binding with the same receptor domain could interfere with the cholinergic agonist, the action of pilocarpine on Na^+/K^+-ATPase in the presence of anti-M_3 peptide pSS IgG was tested. Figure 7A shows that by pre-incubating the salivary gland with the autoantibody (0.5×10^{-9} M) for 30 minutes before completion of the dose–response curve of pilocarpine, the inhibitory action triggered by the cholinergic agonist was blunted.

To assess whether alteration in the modulatory action of autoantibodies and pilocarpine on the Na^+/K^+-ATPase could result in an alteration of ionic fluxes, we studied the net K^+ efflux in the presence of the anti-M_3 peptide pSS IgG and/or pilocarpine. Figure 7B shows that the anti-M_3 peptide pSS IgG (1×10^{-9} M) induced a significant increase in net K^+ efflux, but the increase was less ($P < 0.001$) than that induced by 1×10^{-9} M pilocarpine. However, the same figure shows that pre-incubating 1×10^{-9} M anti-M_3 peptide pSS IgG for 10 minutes before the addition of 1×10^{-9} M pilocarpine inhibited the increment of net K^+ efflux triggered by the cholinergic agonist. This effect mimicked the action of the specific M_3 mAChR antagonist on the pilocarpine effect. The combination of ouabain (5×10^{-4} M), an inhibitor of the Na^+/K^+ pump, and pilocarpine (1×10^{-9} M) resulted in synergism of the net K^+ efflux, indicating the participation of the Na^+/K^+-ATPase in the observed effects.

Discussion

In this work, we provide new evidence for a common pathogenic link between SS and muscarinic acetylcholine system dysfunction. We have demonstrated that patients with pSS produce functional IgG autoantibodies that interact with glandular mAChR. These autoantibodies act as a partial muscarinic cholinergic agonist, increasing PGE_2 generation and cAMP accumulation, thus modifying Na^+/K^+-ATPase activity; but these autoantibodies also interfere with the parasympathetic neurotransmitter modulating the ionic fluxes to acinar cell membranes of the salivary gland. Thus, the IgG from patients with pSS abolished the Na^+/K^+-ATPase inhibition of the salivary gland in response to the authentic agonist pilocarpine.

Here we have proposed two effects of the autoantibodies; on the one hand, the possibility that they play a role in the pathogenesis of dry mouth of pSS patients

and, on the other hand, that pSS IgG may act as an inducer of pro-inflammatory molecules.

The results obtained in this work demonstrate that the Na^+-K^+/ATPase is one of the main targets in the action of anti-M_3 peptide pSS IgG on salivary glands, and that the acini could be the main region of interaction, as the acini M_3 mAChR subtype was the most reactive to pSS IgG (16).

Moreover, it has been demonstrated that pSS autoantibodies, in addition to monoclonal anti-human M_3 mAChR, 'recognize' the apical region of the acinar cell membranes (18). In addition, muscarinic receptors of the M_3 subtype are present in the salivary gland, where they mediate protein secretion, electrolytes, and water (22) on the basolateral surface membranes of the acinar cells (23, 24). Moreover, apical acinar Na^+/K^+-ATPase might participate in series with the basolateral sodium and chloride entry pathways in driving secretory electrolyte flux (25).

Furthermore, we demonstrated that inhibition of the modulatory action of pilocarpine on Na^+/K^+-ATPase activity by the anti-M_3 peptide pSS IgG resulted in alteration of the ionic fluxes that play a key role in fluid secretion (13). The results show that the autoantibodies interfered with the muscarinic agonist-induced increase in the net K^+ efflux from the salivary gland. Moreover, inhibition of the Na^+ pump by ouabain potentiated the increase in net K^+ efflux triggered by pilocarpine, indicating the participation of that enzyme in the pilocarpine-stimulated K^+ efflux. Considering both the role of Na^+/K^+-ATPase in saliva secretion, and the effect of muscarinic stimulation, we propose that patients with pSS produce autoantibodies with the capacity to interact with these receptor subtypes and interfere with the effect of the cholinergic agonist associated with Na^+/K^+ ATPase activity, inhibiting the effect of the secretagogue on the endogenous acetylcholine. In this respect, it is known that salivary glands are target organs for M_3 mAChR autoantibodies of pSS patients and that xerostomia is a main manifestation of the disease. In this sense, an association between the presence of autoantibodies against glandular M_3 mAChR and the presence of xerostomia of pSS patients is well documented (18).

A new finding of this work is the demonstration that the anti-M_3 peptide pSS IgG behaves as a cholinergic agonist, triggering an increased amount of pro-inflammatory PGE_2 in response to the receptor-mediated signalling of events at the salivary cell membrane. The fact that the inhibition of COX-1 and COX-2, as well as that of PLA_2, abrogated the stimulatory action of the autoantibodies on PGE_2 indicates that this action occurs before the activation of PLA_2 and COX-s. It is important to note that the inhibition of COX-2 was more effective than the inhibition of COX-1 in preventing the production of PGE_2 by the autoantibody because COX-1 is the constitutive isoform present under normal conditions and COX-2 acts specifically during the inflammation present in the salivary gland by the action of the anti-M3 peptide pSS IgG. This may explain the lack of an authentic agonist to trigger PGE_2 generation.

Prostaglandin E_2 is known to bind four different types of prostanoid receptors, known as EP 1–4, which activate different signalling pathways. EP2 and EP4 signal through an increase in cAMP (26) via the Gs protein. The results obtained here suggest that the anti-M_3 peptide pSS IgG, acting on the M_3 mAChR of the salivary glands, increases PGE_2 generation and that the prostanoid acting on its own receptor (probably EP2) increases cAMP production, as observed previously (27).

It is now clear that PGE_2 regulates inflammation and apoptosis, which is characterized by a decrease in the activity of Na^+/K^+-ATPase (28), while an increase in the activity of the Na^+/K^+ pump was shown to occur in cell regeneration (29).

Regulation of the Na^+/K^+-ATPase by prostaglandin has been demonstrated; PGE_2 caused a down-regulation of the Na^+/K^+-ATPase activity through an increase of cAMP (30, 31). In accordance with this, the results obtained in this work suggest that the anti-M_3 peptide pSS IgG, by increasing PGE_2 production with subsequent cAMP accumulation, could be a regulatory element that inhibits the salivary Na^+/K^+ pump. The fact that 4-DAMP and the M_3 synthetic peptide blocked the action of the pSS autoantibodies showed that submandibular gland M_3 mAChRs are implicated in both increasing the production of PGE_2 and inhibiting the Na^+/K^+ pump. Furthermore, the involvement of PGE_2 in the inhibition of Na^+/K^+-ATPase activity is demonstrated by the fact that the inhibition of PLA_2, COX-1, and COX-2 (as well as indomethacin) abrogated the action of the anti-M_3 peptide pSS IgG on the Na^+/K^+ pump. It is known that rat submandibular gland expresses M_1, M_2, M_3, M_4, and M_5 mAChR subtypes (32). The effects on inflammation may involve M_1, M_3, and M_5 mAChRs. The fact that neither pirenzepine nor the non-anti M_3 peptide IgG had any effect on the action of pSS IgG, confirm that the M_3 mAChR is the major receptor subtype responsible for the pSS IgG effects currently studied. However, the participation of the M_5 mAChR subtype cannot be disregarded.

On the basis of our results, we postulate that the early agonist-promoting activation of M_3 mAChR initiated by autoantibodies binding to, and persistently activating, the cholinoreceptors, results in the production of large amounts of pro-inflammatory PGE_2, thus contributing to immune inflammation of the submandibular gland. Subsequently, it is possible that the anti-M_3 peptide pSS IgG, continuously interacting with the same receptor domain, could limit the effectiveness to the endogenous muscarinic agonist (acetylcholine), eliciting a loss of secretory response of the glandular acinar cell.

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