

Functional epitope of muscarinic type 3 receptor which interacts with autoantibodies from Sjögren's syndrome patients

N.-Y. Koo¹, J. Li¹, S.-M. Hwang¹, S.-Y. Choi¹, S. J. Lee¹, S.-B. Oh¹, J.-S. Kim¹, E. B. Lee², Y. W. Song² and K. Park¹

Objectives. Recently, autoantibodies directed against muscarinic type 3 receptor (M3R) have been reported in patients with primary SS. However, the precise epitope(s) of the M3R that interacts with SS autoantibodies remains unclear. The aim of this study was to identify the functional epitope of M3R which interacts with SS immunoglobulin G (IgG).

Methods. Purified IgGs were obtained from the sera of seven SS patients (six primary and one secondary SS) and two normal persons. We examined whether SS IgG inhibits M3R function and identified the epitope using six synthetic peptides covering all the extracellular domains of M3R by microspectrofluorimetry and surface plasmon resonance-based optical biosensor system (BIAcore system).

Results. A volume of 0.5 mg/ml SS IgG inhibited carbachol (CCh)-induced $[Ca^{2+}]_i$ transient (CICT) in human submandibular gland (HSG) cells. However, co-incubation of SS IgG with the 6th peptide (514–527 amino acid region) corresponding to the third extracellular loop of M3R, recovered CICT. The result was further confirmed by BIAcore analysis. We found that the 6th peptide interacts with IgGs from three primary SS patients in a concentration-dependent manner. The synthetic peptide which consists of amino acids 228–237 corresponding to the COOH-terminus of the second extracellular loop of M3R also bound to SS IgG. However, normal IgGs did not interact with the 6th peptide.

Conclusions. The results suggest that the third extracellular loop of M3R represents a functional epitope bound by SS IgG, and thereby partly inhibits M3R function.

KEY WORDS: Autoantibodies, Muscarinic type 3 receptor, Sjögren's syndrome, Functional epitope.

Introduction

SS is a systemic autoimmune disorder accompanying sicca symptoms, which is characterized by the lymphocytic infiltration in exocrine glands as well as severe glandular dysfunction, dry eye and dry mouth [1]. The mechanisms of the exocrine secretion are well documented [2, 3]. Stimulus-secretion coupling in salivary glands is initiated by the release of acetylcholine (ACh) from the parasympathetic nerves, which is mediated by an increase of intracellular Ca^{2+} [4–6] that culminates in the activation of the Ca^{2+} -dependent K^+ and Cl^- channels [6]. Efflux of these anions induces primary fluid secretion from the acinar cells. Therefore, any disturbance of ligand binding to the muscarinic receptor or a disturbance in any step of these signal transduction processes will result in secretory dysfunction.

Autoantibodies from the sera of SS patients against muscarinic type 3 receptor (M3R) by various techniques, including SDS-PAGE, immunoblotting, radioligand binding and ELISA has been reported [7–10]. There has been increasing evidence that SS immunoglobulin G (IgG) affects the function of muscarinic receptors. Infusion of serum IgG from human primary SS patients to non-obese diabetic Igmu null mice results in the loss of secretory function [9]. In addition, passive transfer of SS IgG reproduced the pathophysiology of overactive bladder [11]. Acute or chronic SS IgG application inhibited the function of muscarinic receptors at the cellular [12, 13] and tissue levels [14, 15].

In spite of the positive results from functional assays, the existence of the inhibitory SS IgG in patients that interact with M3R and its epitope(s) remains still controversial [16]. Studies for a direct interaction between epitope and SS IgG using peptides [17] or by conventional immunological approaches [18] have been

unsuccessful. Recently, the second extracellular loop of the M3R has been suggested as a possible epitope for SS IgG [19, 20]. Particularly, full inhibitory activity of the antibody was observed with a 10 amino acid epitope located at the COOH-terminus of the second loop [21]. However, the study only examined the role of second extracellular loop. Although the second extracellular loop of M3R has been reported to be an epitope for anti-receptor autoantibodies in certain autoimmune disorders [22–24], autoantibodies could also bind to other extracellular loops; in the mu-opioid receptor, simultaneous binding of IgG to the first and third extracellular loop mimics morphine-induced receptor activation [25]. The precise epitope involved in the interaction with SS IgG remains unknown, although the second extracellular loop of M3R is the most likely candidate.

In this study, we have determined the functional epitope of M3R which interacts with autoantibodies from SS patients using microspectrofluorimetry and plasmon surface resonance-based optical biosensor system (BIAcore system), a newly developed system mainly used for detection of molecule interaction. We tested the direct binding of purified SS IgG with peptides, not only with the second loop but with all the extracellular domains of M3R. Hence, this may be the first line of evidence that the third extracellular loop of M3R may interact with SS IgG.

Materials and methods

Solution and reagents

A normal bath solution used for Ca^{2+} measurement contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 (adjusted with NaOH) giving an osmolarity of 300–310 mOsmol/kg H_2O . The fura-2 acetoxy-methyl (fura-2 AM) was obtained from Molecular Probes (Eugene, OR, USA). Trypsin-EDTA and carbachol (CCh) were obtained from Sigma (Poole, UK). The collagenase was purchased from Worthington (Lakewood, UK). The peptides were synthesized by Pepton (Taejeon, Korea).

¹Department of Physiology, School of Dentistry and ²Internal Medicine, Seoul National University and Dental Research Institute, Seoul, South Korea.

Submitted 22 October 2007; revised version accepted 28 January 2008.

Correspondence to: K. Park, Department of Physiology, School of Dentistry, Seoul National University, Yeongeong Dong 28, Chongno Ku, Seoul 110-749, South Korea. E-mail: kppark@snu.ac.kr

HSG cell culture

The HSG cells were a generous gift from Dr Toshiko Atzumi in Meikai University in Japan. The cells were grown in a suspension in 3 ml tissue culture plates at 37°C in 95% air–5% CO₂, and were maintained in a minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Each plate was refreshed twice a week. Cells were harvested by brief incubation with trypsin–EDTA solution (Gibco BRL, Carlsbad, CA, USA). After the subculture, the cells were incubated with either the control or SS IgG mixture for 24 h prior to CCh stimulation in order to measure the [Ca²⁺]_i.

Serum IgG from SS patients

The purified serum IgGs were obtained from seven SS patients. Patients were enrolled at the Rheumatology Clinic, Seoul National University Hospital, between November 2004 and February 2005. All patients were diagnosed according to the revised international classification criteria for SS [26]. Six patients had primary SS and one patient had secondary SS (RA) (Table 1). The patients were all females with ages ranging from 33 to 63 yrs (53 ± 15 yrs, mean age ± s.e.). The serum IgG was obtained from two healthy controls, who did not show any sign of SS symptoms and were negative to the serological tests. IgG was isolated from the serum using the Affigel 102® (Biorad) affinity purification [27], and the concentration of the purified IgG in the eluted buffer solution was 7.11 ± 3.18 mg/ml (*n* = 2) and 7.15 ± 0.68 mg/ml (*n* = 7) from control and SS group, respectively. The purified IgG diluted in MEM was added to the incubation medium containing the HSG cells with a final concentration of 0.5 mg/ml dissolved in a normal bath solution for Ca²⁺ measurement. The present study was approved by the Medical Ethics Committee of Seoul National University. All patients gave informed consent for participation in this study.

Fluorescent dye loading and [Ca²⁺]_i measurement

After incubation with the serum IgG for 24 h, the HSG cells were harvested by incubation with trypsin–EDTA solution. Cells were loaded with 2 μM fura-2/AM (fura) in 2 ml of HEPES-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose; pH 7.4) for 30 min at room temperature (25°C). Then cells were rinsed with HEPES-buffered solution and incubated for a further 30 min to allow complete de-esterification of intracellular AM esters. Ratiometric fluorescence measurements were performed using an Olympus microscope with a MetaFluor® version 6.1 imaging system (Universal Imaging, West Chester, PA, USA). After cells were attached to cover glass, fura fluorescence was recorded at excitation wavelengths of 340 and 380 nm, with an emission wavelength of 510 nm at 37°C. The results are presented as the measured 340 nm/380 nm ratio (Ca²⁺ fluorescence ratio, F340/F380). All the results are presented as a mean ± s.e. Statistical analysis was performed using Student's unpaired *t*-test.

Design of six peptides constituting M3R

The human M3R (hM3R) is a transmembrane receptor with seven transmembrane domains. Protein sequences exposed to the outside of the cells are predicted according to the hydropathy plot based on the protein sequence, and six peptides were designed to cover all the extracellular portion of the hM3R [28, 29]. Excess amount of all six M3R peptides (10 μM each) were added into the incubation medium containing SS IgG to block the effect of anti-M3R antibody of SS IgG. The sequences of six peptides used in this experiment are shown in Table 2.

TABLE 1. Clinical and serological findings of SS cases

Patient	Age	Sex	Dry mouth	Dry eye	Primary/secondary	RF	ANA	Anti-Ro	Anti-La
SS #6	68	F	Yes	Yes	S	+	+	+	–
SS #7	52	F	Yes	Yes	P	+	+	+	–
SS #8	51	F	Yes	Yes	P	+	+	+	–
SS #9	51	F	Yes	Yes	P	+	+	+	+
SS #33	55	F	Yes	Yes	P	+	+	+	–
SS #43	38	F	Yes	Yes	P	–	+	+	+
SS #53	65	F	Yes	Yes	P	+	+	+	–

S: secondary SS; P: primary SS.

TABLE 2. Amino acid sequences of synthetic peptides corresponding to the extracellular loop of the hM3R

Peptide	Sequences	Amino acid residues
1st	MTLHNNSTTSPLFPNISSSWIHSPSDA	1–27
2nd	HSPSDAGLPPGTVTHTFGSYNVSRAAGN	22–48
3rd	SRAAGNFSSPDGTTDDPLGGHTVWQV	43–78
4th (1st loop)	MNRWALGNLACDLW	131–144
5th (2nd loop)	LFWQYFVGKRTVPPGECFIQFLSEPT	205–230
6th (3rd loop)	NTFCDSCIPKTFWN	514–527
M3R ^{228–237}	EPTITFGTAI	228–237

Surface plasmon resonance analysis

Direct binding of SS IgG with each synthetic peptide was determined using BIAcore 2000 (BIAcore Piscataway, NJ, USA). Each peptide was covalently immobilized on the surface of CM-5 sensor chip using a *N*-hydroxysuccinimide/*N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (NHS/EDC) amine coupling kit (BIAcore) and their immobilizing values were estimated as 3000 resonance units (RU). The component of running buffer was as follows: 150 mM NaCl, 10 mM HEPES, 3 mM EDTA, 0.005% surfactant P20 (pH 7.4). SS IgGs were diluted to the appropriate concentration with running buffer, subsequently injected into the sensor chip immobilized with peptides. The basal level of binding affinity of each peptide with SS IgG was ~20–100 RU. For the kinetic assay, each peptide was modified with biotin and a streptavidin chip was used. The disulphide bond of the 6th peptide was reduced by 1 mM dithiothreitol (DTT) in running buffer. The Surface plasmon resonance (SPR) response curves were analysed with BIAcore evaluation software, version 3.1. The dissociation rate constant (*k*_d) was determined from a plot of ln(*R*₀/*R*) vs time, with *R* being the SPR signal at time *t*; the association rate constant (*k*_a) was determined from a plot of ln(abs(d*R*/d*t*)) vs time. The apparent association and dissociation constants were calculated from the kinetic constants: *K*_D = *k*_d/*k*_a.

Binding reaction analysis

Signal changes on the activated/blocked control channel were subtracted from the peptide–hM3R binding interactions using in-line reference and the subtracted sensorgrams were analysed. The curves generated with serial analyte concentrations were applied globally to the 1:1 Langmuir binding model with or without correction for baseline drifting depending on baseline status. The χ² test was used to evaluate the quality of fit between the experimental data and individual binding models. The χ² value represents the sum of squared differences between the experimental data and reference data at each point.

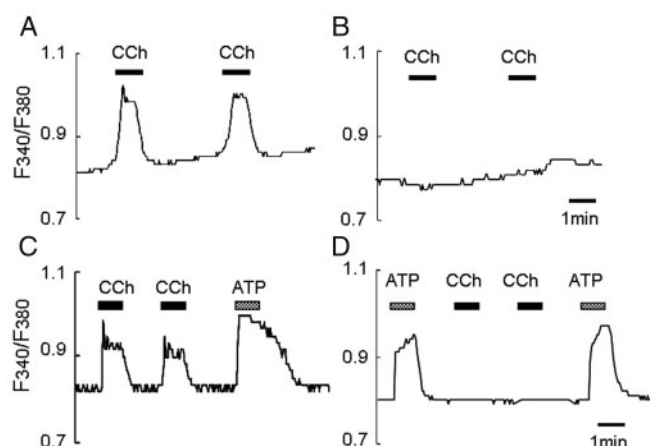


FIG. 1. A typical $[Ca^{2+}]_i$ response to CCh (10^{-4} M) or ATP (10^{-4} M) stimulation in HSG cells. Cells were pre-incubated with 0.5 mg/ml normal (A and C) or SS IgG (B and D) 24 h before stimulation. (A) CCh evokes a reproducible $[Ca^{2+}]_i$ response in the cells incubated with normal IgG, (B) but not in the cells incubated with SS IgG. (C) Both CCh and ATP increase $[Ca^{2+}]_i$ in the cells incubated with normal IgG, (D) but CICT was not observed in the cells incubated with SS IgG. Note that ATP-induced $[Ca^{2+}]_i$ response is still reproducible. The vertical axis represents the Ca^{2+} fluorescence ratio (F340/F380).

Results

The effect of SS IgG on the CCh-induced $[Ca^{2+}]_i$ transient in HSG cells

CCh is a well-known muscarinic cholinergic agonist that increases cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in salivary acinar cells including HSG cells, which express M3R [13]. Figure 1A shows a typical $[Ca^{2+}]_i$ response to 10^{-4} M CCh in HSG cells. We can see the reproducible $[Ca^{2+}]_i$ response by CCh stimulation. The magnitude ($\Delta[Ca^{2+}]_i$) of the CCh-induced $[Ca^{2+}]_i$ transient (CICT) was 0.236 ± 0.018 (mean $\pm$ S.E., $n = 8$). To investigate effects of SS IgG on CICT, cells were incubated with 0.5 mg/ml SS IgG for 24 h before CCh stimulation. Figure 1B shows CICTs after incubating cells with the SS IgGs for 24 h. CICTs were almost completely inhibited by $94.8 \pm 0.65\%$ (0.012 ± 0.001 , $n = 9$, $P < 0.05$) compared with the control groups, which were incubated with the same concentration of normal IgG. Next, we investigated to examine whether the inhibitory effect of SS IgG is specific to M3R. Figure 1C shows typical $[Ca^{2+}]_i$ responses to CCh and ATP (10^{-4} M) after incubation of the cells with normal IgG. Both CCh and ATP increased $[Ca^{2+}]_i$. The magnitude of the ATP-induced $[Ca^{2+}]_i$ increase was 0.154 ± 0.004 ($n = 8$). However, in cells incubated with SS IgG, CICT was abolished (Fig. 1D). ATP-induced $[Ca^{2+}]_i$ response was still reproducible in these cells. Its magnitude was 0.156 ± 0.008 ($n = 8$), which is not significantly different to those in the cells incubated with normal IgG ($P > 0.1$).

The determination of a functional epitope of M3R which interacts with SS IgG

To identify a functional epitope of M3R which interacts with SS IgG, we designed six synthetic peptides covering all the extracellular domains of hM3R. The location of each peptide and its sequence are shown in Table 1. After incubating HSG cells with 0.5 mg/ml SS IgG plus each synthetic peptide for 24 h, we examined the effect of each peptide on CICT. The 1st, 2nd and 3rd peptides were added together, since they together constitute the N-terminal of M3R. The other peptides, including the 4th, 5th and 6th peptides, corresponding to the first, second and third extracellular domains, were added separately. Incubation of HSG cells with synthetic peptides including the 1st, 2nd, 3rd, 4th and 5th peptides had little effect on the CICT (Fig. 2A–C);

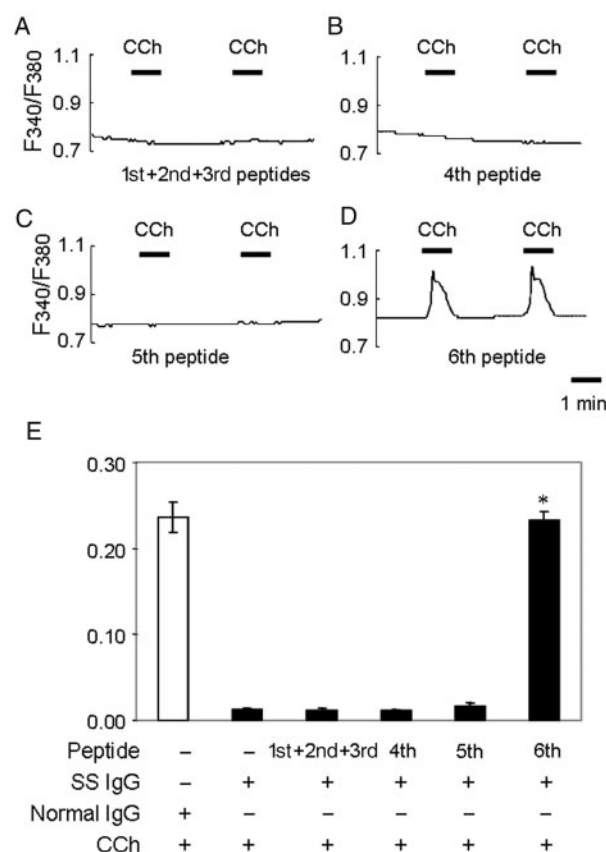


FIG. 2. Effects of co-incubation of synthetic peptides with pooled SS IgG for 24 h on CICT. Effect of mixture of synthetic peptide on CICT with (A) 1st, 2nd and 3rd synthetic peptide, (B) 4th peptide, (C) 5th peptide and (D) 6th peptide. (E) Summarized results. CICT was recovered to $97.07 \pm 5\%$ ($n = 12$, indicated by asterisk) compared with the control group by co-incubation of the 6th peptide with SS IgG.

CICTs were not observed as shown in Fig. 1B. However, the 6th peptide, which corresponds to the third extracellular loop of M3R, recovered CICT (Fig. 2D). We tested the peptides on CICT (in the absence of SS IgG), but they had little effect on the CICT (data not shown). Figure 2E shows a summarized result ($n = 8-19$). The magnitude of CICT in HSG cells that had been incubated with SS IgG plus the 6th peptide was 0.233 ± 0.011 ($n = 11$), which is not significantly different to those of control group (0.236 ± 0.018 , $*P > 0.1$).

Interaction of SS IgG with M3R peptide mimotopes using BIAcore system

Next, we examined whether the 6th peptide which recovered CICT binds to SS IgG using a BIAcore system (BIAcore 2000, BIAcore). After fixing of each peptide on the CM-5 sensor chip by amine coupling, the pooled SS IgG was superfused on the chips. Figure 3A, C, E shows a typical BIAcore analysis with the pooled IgGs from primary SS patients. The binding affinity was tested with 300, 600 and 1200 nM IgG for each peptide. A significant binding was observed in the 4th and the 6th peptide, both in a concentration-dependent manner (Fig. 3A, E). The value of the binding affinity of the 4th and the 6th peptide with SS IgG in 1200 nM peptide was 35.0 ± 0.10 ($n = 3$) and 91.66 ± 0.77 ($n = 3$) RU, respectively. We then compared the binding effects of the 4th and the 6th peptide with the pooled control IgG to rule out non-specific effects. Figure 3B, D, F shows typical BIAcore analysis data with normal IgG. Normal IgG also interacted with the 4th peptide (Fig. 3B) but not with the 6th peptide (Fig. 3F), suggesting

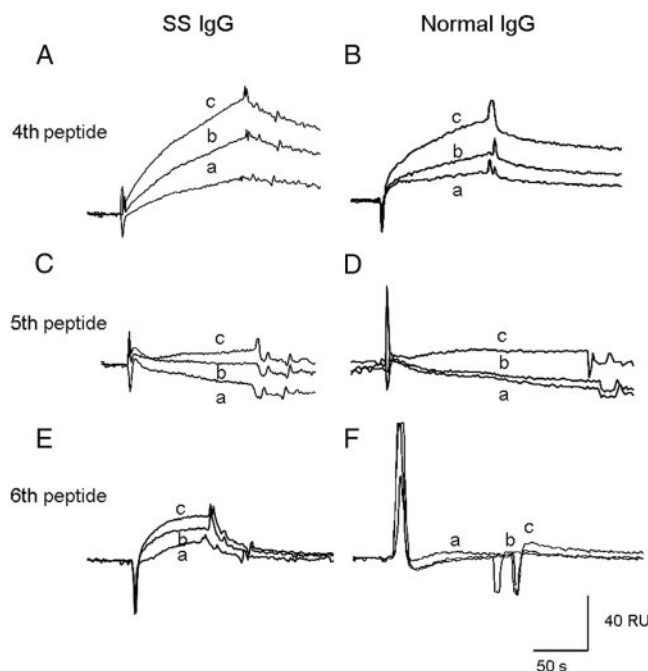


FIG. 3. A representative sensorgram in BIAcore analysis showing interaction between hM3R synthetic peptides and IgGs. Each peptide was immobilized on a CM5 sensor chip. 300 (A), 600 (B) and 1200 nM (C) of the pooled SS IgG (A, C and E) or normal IgG (B, D and F) were loaded into the sensor cells. Sensorgram of the 6th peptide seems to suggest binding to SS IgG, but not to control IgG. Vertical axis indicates RU vs time.

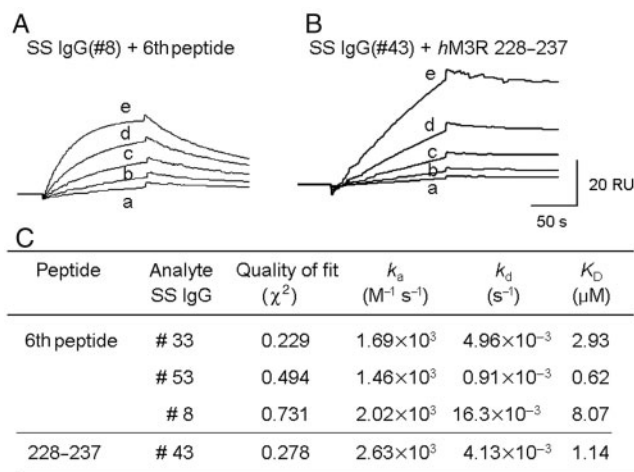


FIG. 4. (A) Representative sensorgrams for the interaction between IgG from one SS patient at various concentrations (a: 250 nM, b: 500 nM, c: 1000 nM, d: 2000 nM, e: 4000 nM) with the 6th peptide. (B) Sensorgrams for the interaction between IgG from another SS patient with hM3R²²⁸⁻²³⁷. (C) Table represents data from experiments. These parameters demonstrate that there are high-quality global fittings to a 1:1 Langmuir binding model from the SS patients (nos. 33, 53, 8 and 43).

that the first extracellular loop is a site for non-specific binding to IgG. We went on further to examine whether such an interaction between SS IgG and 6th peptide has any individual variations. After immobilization of biotin-modified 6th peptide on the streptavidin sensor chip, various concentrations of SS IgG were injected into the sensor chip. Figure 4A shows a representative sensorgram from one SS patient (no. 8). A strong binding of the 6th peptide to IgG was also observed in the other two patients (no. 33, no. 53). The kinetic parameters of k_a , k_d and K_D were

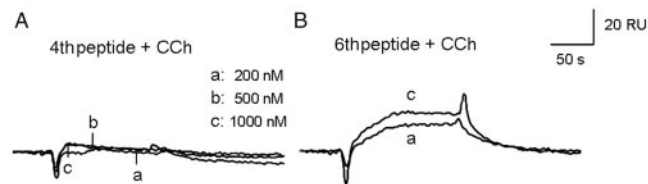


FIG. 5. BIAcore analysis of interaction between CCh and 4th (A) or 6th peptide (B). The 4th and 6th peptide were immobilized on a CM5 sensor chip. Various concentrations of CCh (a: 200 nM, b: 500 nM and c: 1000 nM) were loaded into the sensor cells. From the sensorgram, the binding of 6th peptide to CCh resulted in enhanced RU values compared to 4th peptide.

determined using BIAcore evaluation software. As shown in Fig. 4C, the apparent dissociation constant (K_D) of SS IgG binding to the 6th peptide was $3.87 \pm 2.2 \mu M$ ($n=3$), indicating a strong interaction between two molecules. We also tested the synthetic peptide, which consists of amino acids 228–237 (Table 1), corresponding to the COOH-terminus of the second extracellular loop of M3R, since this peptide showed a strong inhibitory activity on CCh evoked colon contraction in the previous work [21]. Although the 5th peptide did not bind to SS IgG, the synthetic peptide of M3R²²⁸⁻²³⁷ bound to SS IgG in a concentration-dependent manner in our BIAcore analysis (Fig. 4B). We further examined whether the 6th peptide that interacted with SS IgG can also interact with CCh, an M3R agonist in salivary epithelial cells.

Interaction of CCh with M3R peptide mimotopes

We tested the interaction of the 4th peptide with 200, 500 and 1000 nM CCh. We did not find any evidence of CCh binding to the 4th peptide (Fig. 5A). However, a significant binding of CCh with the 6th peptide was observed in 200 and 1000 nM concentration of CCh (Fig. 5B).

Discussion

In our experiments, HSG cells originating from human submandibular duct epithelium were used to see the inhibitory effect of SS IgG on M3R. These cells not only express functional M3R, but also play a key role in salivary secretion [13]. Our result demonstrated that incubation of HSG cells with IgG from sera of SS patients significantly inhibited $[Ca^{2+}]_i$ increase, suggesting that the muscarinic receptor function of HSG cells is inhibited by SS IgG. On the contrary, the control group, in which the same concentration of normal IgGs was used, had little effect on muscarinic receptor function. We demonstrated that Ca^{2+} signalling mechanisms of HSG cells were intact following exposure to SS IgG. Although CICT was almost completely abolished by SS IgG, the ATP-induced $[Ca^{2+}]_i$ increase was still reproducible in the same setting. It is therefore unlikely that inhibition of CICT by SS IgG is caused by the catastrophic process of cells. Our results strongly suggest that SS IgG specifically inhibits M3R function by interacting with muscarinic receptors.

Both our microspectrofluorimetry and BIAcore analysis demonstrated that the 6th peptide corresponding to the third extracellular loop of M3R strongly binds to SS IgG. Co-incubation of HSG cells with SS IgG plus any other peptide had little effect on CICT, which had been inhibited by SS IgG incubation but recovery was possible in the presence of 6th peptide. All kinetic parameters for the 6th peptide in BIAcore analysis, compared with those of the other peptides suggest that there is a strong binding between SS IgG and the 6th peptide corresponding to the third extracellular loop of M3R. However, our result does not exclude the possibility of interaction between the second extracellular loop of M3R with SS IgG. Although we did not find evidence of binding of the 5th peptide corresponding to the second

extracellular loop to SS IgG, we found that the synthetic peptide consisting of amino acids 228–237 corresponding to the COOH-terminus of the second extracellular loop of M3R (M3R^{228–237}), also binds to SS IgG in BIAcore analysis (Fig. 4B). We tested this peptide, since it showed a strong inhibitory activity on CCh evoked colon contraction in previous work [21]. We are not able to understand at the moment why the 5th peptide corresponding to the full sequence of the second loop does not bind to SS IgG. Nonetheless, our results not only support the previous work but also have verified our novel finding, the binding of the third extracellular loop of M3R with SS IgG.

The majority of G protein-coupled receptors including the muscarinic receptors contain a pair of conserved Cys residues in their first and second extracellular loop (Cys 140 and Cys 220). Biochemical and site-directed mutagenesis studies with rhodopsin [30], the β_2 -adrenergic receptor [31] and the M1 muscarinic receptor [32] suggest that these two Cys residues are engaged in a disulphide bond, which may be required for folding of the muscarinic receptor protein [33], but it is not essential for ligand binding and G protein coupling [34]. It has been known that two Cys residues in the third extracellular loop do not form a disulphide bond, suggesting the functional epitope we determined may not be conformational. Furthermore, throughout the experiments, we added 0.01 M DTT in the running buffer during BIAcore analysis to keep the synthetic peptide in reduced form. Binding of the 6th peptide with CCh further supports our conclusion that the third extracellular loop of M3R binds to SS IgG.

In our experiments, only a subset of SS IgGs from primary SS patients interacted with the 6th peptide. Roughly 60% of the tested IgG from SS patients disrupted M3R-mediated contraction in colon (Prof. T. P. Gordon, personal communication). Autoantibodies against SS-A/Ro and SS-B/La [35], anti-salivary gland antibodies, RF [36] and ANA are found in patients with SS. However, these autoantibodies are associated not only with SS, but also with SLE, subacute cutaneous LE, congenital heart block and neonatal lupus. In our study, the three primary SS patients (SS 33, SS 53 and SS 8), whose antibodies interacted with the 6th peptide, belonged to the group that is positive to RF, ANA and anti-Ro (Table 2). The primary SS patient (SS 43) whose IgG interact with the amino acid 228–237 region of M3R was positive to ANA, anti-Ro and anti-La. It is unclear at the moment whether these different antibody profiles directly or indirectly affect binding of SS IgG to M3R epitope. The difference of interaction sites might be due to epitope spreading. Although no attempts have been made to study epitope spreading with anti-muscarinic autoantibodies, humoral immune response targeted towards a single epitope may be followed by spreading to involve several epitopes on the same protein and even to apparently unrelated antigens [37, 38].

In summary, IgGs from the sera of SS patients specifically inhibited M3R function in HSG cells. The third extracellular loop of M3R appears to act as a functional epitope as well as M3R^{228–237}, since the synthetic peptide corresponding to the third extracellular loop of M3R strongly binds to IgGs from half of primary SS patients in our study. If the epitope in the third extracellular loop of M3R is functional, it could be a potential therapeutic target for SS. Further work might be necessary to explain the individual variation in the binding of third extracellular loop of M3R with SS IgG. We also can not rule out the possibilities that this binding depends on ethnicity or IgG subtype.

Acknowledgements

We would like to thank Prof. Tom Gordon for helpful advice.

Funding: This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, KRF-2005-015-E00207).

Disclosure statement: The authors have declared no conflicts of interest.

References

- 1 Fox RI, Stern M. Sjögren's syndrome: mechanisms of pathogenesis involve interaction of immune and neurosecretory systems. *Scand J Rheumatol Suppl* 2002;116:3–13.
- 2 Baum BJ. Principles of saliva secretion. *Ann NY Acad Sci* 1993;694:17–23.
- 3 Nauntofte B. Regulation of electrolyte and fluid secretion in salivary acinar cells. *Am J Physiol* 1992;263:G823–37.
- 4 Ambudkar IS. Regulation of calcium in salivary gland secretion. *Crit Rev Oral Biol Med* 2000;11:4–25.
- 5 Ambudkar IS, Hiramatsu Y, Lockwich T, Baum BJ. Activation and regulation of calcium entry in rat parotid gland acinar cells. *Crit Rev Oral Biol Med* 1993;4:421–5.
- 6 Park K, Case RM, Brown PD. Identification and regulation of K⁺ and Cl[−] channels in human parotid acinar cells. *Arch Oral Biol* 2001;46:801–10.
- 7 Bacman S, Perez Leiros C, Sterin-Borda L, Hubscher O, Arana R, Borda E. Autoantibodies against lacrimal gland M3 muscarinic acetylcholine receptors in patients with primary Sjögren's syndrome. *Invest Ophthalmol Vis Sci* 1998;39:151–6.
- 8 Bacman S, Sterin-Borda L, Camusso JJ, Arana R, Hubscher O, Borda E. Circulating antibodies against rat parotid gland M3 muscarinic receptors in primary Sjögren's syndrome. *Clin Exp Immunol* 1996;104:454–9.
- 9 Robinson CP, Brayer J, Yamachika S *et al.* Transfer of human serum IgG to nonobese diabetic Igmu null mice reveals a role for autoantibodies in the loss of secretory function of exocrine tissues in Sjögren's syndrome. *Proc Natl Acad Sci USA* 1998;95:7538–43.
- 10 Bacman S, Berra A, Sterin-Borda L, Borda E. Muscarinic acetylcholine receptor antibodies as a new marker of dry eye Sjögren syndrome. *Invest Ophthalmol Vis Sci* 2001;42:321–7.
- 11 Wang F, Jackson MW, Maughan V *et al.* Passive transfer of Sjögren's syndrome IgG produces the pathophysiology of overactive bladder. *Arthritis Rheum* 2004;50:3637–45.
- 12 Dawson LJ, Stanbury J, Venn N, Hasdimir B, Rogers SN, Smith PM. Antimuscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. *Arthritis Rheum* 2006;54:1165–73.
- 13 Li J, Ha YM, Ku NY *et al.* Inhibitory effects of autoantibodies on the muscarinic receptors in Sjögren's syndrome. *Lab Invest* 2004;84:1430–8.
- 14 Waterman SA, Gordon TP, Rischmueller M. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. *Arthritis Rheum* 2000;43:1647–54.
- 15 Goldblatt F, Gordon TP, Waterman SA. Antibody-mediated gastrointestinal dysmotility in scleroderma. *Gastroenterology* 2002;123:1144–50.
- 16 Dawson L, Tobin A, Smith P, Gordon T. Antimuscarinic antibodies in Sjögren's syndrome: where are we, and where are we going? *Arthritis Rheum* 2005;52:2984–95.
- 17 Cavill D, Waterman SA, Gordon TP. Failure to detect antibodies to extracellular loop peptides of the muscarinic M3 receptor in primary Sjögren's syndrome. *J Rheumatol* 2002;29:1342–4.
- 18 Dawson LJ, Allison HE, Stanbury J, Fitzgerald D, Smith PM. Putative anti-muscarinic antibodies cannot be detected in patients with primary Sjögren's syndrome using conventional immunological approaches. *Rheumatology* 2004;43:1488–95.
- 19 Kovacs L, Marczinovits I, Gyorgy A *et al.* Clinical associations of autoantibodies to human muscarinic acetylcholine receptor 3(213–228) in primary Sjögren's syndrome. *Rheumatology* 2005;44:1021–5.
- 20 Marczinovits I, Kovacs L, Gyorgy A *et al.* A peptide of human muscarinic acetylcholine receptor 3 is antigenic in primary Sjögren's syndrome. *J Autoimmun* 2005;24:47–54.
- 21 Cavill D, Waterman SA, Gordon TP. Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjögren's syndrome. *Scand J Immunol* 2004;59:261–6.
- 22 Goin JC, Borda E, Leiros CP, Storino R, Sterin-Borda L. Identification of antibodies with muscarinic cholinergic activity in human Chagas' disease: pathological implications. *J Auton Nerv Syst* 1994;47:45–52.
- 23 Magnusson Y, Marullo S, Hoyer S *et al.* Mapping of a functional autoimmune epitope on the beta 1-adrenergic receptor in patients with idiopathic dilated cardiomyopathy. *J Clin Invest* 1990;86:1658–63.
- 24 Eftekhari P, Salle L, Lezoualc'h F *et al.* Anti-SSA/Ro52 autoantibodies blocking the cardiac 5-HT4 serotoninergic receptor could explain neonatal lupus congenital heart block. *Eur J Immunol* 2000;30:2782–90.
- 25 Mace G, Blanpied C, Emorine LJ, Druet P, Dietrich G. Morphine-like activity of natural human IgG autoantibodies is because of binding to the first and third extracellular loops of the mu-opioid receptor. *J Biol Chem* 1999;274:20079–82.

Rheumatology key message

- Purified IgGs from the sera of SS patients significantly inhibited M3R function in HSG cells. The third extracellular loop of M3R appears to act as a functional epitope.

- 26 Vitali C, Bombardieri S, Jonsson R *et al.* Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;61:554–8.
- 27 Fisher JM, Sossin W, Newcomb R, Scheller RH. Multiple neuropeptides derived from a common precursor are differentially packaged and transported. *Cell* 1988;54: 813–22.
- 28 Tusnady GE, Simon I. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. *J Mol Biol* 1998;283:489–506.
- 29 Tusnady GE, Simon I. The HMMTOP transmembrane topology prediction server. *Bioinformatics* 2001;17:849–50.
- 30 Karnik SS, Sakmar TP, Chen HB, Khorana HG. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc Natl Acad Sci USA* 1988;85:8459–63.
- 31 Dixon RA, Sigal IS, Candelore MR *et al.* Structural features required for ligand binding to the beta-adrenergic receptor. *EMBO J* 1987;6:3269–75.
- 32 Kurtenbach E, Curtis CA, Pedder EK, Aitken A, Harris AC, Hulme EC. Muscarinic acetylcholine receptors. Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. *J Biol Chem* 1990;265:13702–8.
- 33 Savarese TM, Wang CD, Fraser CM. Site-directed mutagenesis of the rat m1 muscarinic acetylcholine receptor. Role of conserved cysteines in receptor function. *J Biol Chem* 1992;267:11439–48.
- 34 Hulme EC, Birdsall NJ, Buckley NJ. Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* 1990;30:633–73.
- 35 Wahren M, Solomin L, Pettersson I, Isenberg D. Autoantibody repertoire to Ro/SSA and La/SSB antigens in patients with primary and secondary Sjögren's syndrome. *J Autoimmun* 1996;9:537–44.
- 36 Atkinson JC, Royce LS, Wellner R, Pillemer SR, Bermudez D, Fox PC. Anti-salivary antibodies in primary Sjögren's syndrome. *J Oral Pathol Med* 1995;24:206–12.
- 37 Deshmukh US, Gaskin F, Lewis JE, Kannapell CC, Fu SM. Mechanisms of autoantibody diversification to SLE-related autoantigens. *Ann NY Acad Sci* 2003;987:91–8.
- 38 Monneaux F, Muller S. Epitope spreading in systemic lupus erythematosus: identification of triggering peptide sequences. *Arthritis Rheum* 2002;46:1430–8.