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ORIGINAL ARTICLE

Applying Surface Plasmon Resonance to Monitor the IgE-Mediated Activation of Human Basophils

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ABSTRACT

Background: The histamine releasing test which detects histamine released from basophils *in vitro* is safe, sensitive and widely used for clinical examination in the field of allergy. However, basophils of certain individuals do not release histamine, because of dysfunctions in their intracellular signal transduction (non-responder). To overcome potential shortcomings of the histamine releasing test, we applied surface plasmon resonance (SPR) to detect the activation of basophils.

Methods: Basophils of patients with allergy, and those of non-allergic volunteers were isolated from peripheral blood. A batch of basophils obtained from a healthy volunteer was treated with lactic acid and IgE of a patient with atopic dermatitis in order to replace their endogenous IgE. They were fixed on the sensor chip of the SPR apparatus, pretreated with or without various inhibitors for intracellular signal transduction, and exposed to the antigens or anti-IgE antibody.

Results: When basophils were sensitized with antigen specific IgE, they immediately caused the increase of resonance angle (AR) in response to either anti-IgE antibody or corresponding antigens, even when they did not release histamine. Moreover, the dose dependent reactions of basophils were reflected by the increase of AR as well as the release of histamine. The increase of AR in response to anti-IgE antibody was reduced by pre-treatment of basophils with inhibitors for intracellular signal transduction, but not more than the level for histamine release.

Conclusions: SPR biosensors may be superior to the histamine release test for studying functions of human basophils including those not releasing histamine.

KEY WORDS

basophil, biosensor, histamine release, IgE, surface plasmon resonance

INTRODUCTION

The identification of antigen that provokes mast cell activation is crucial to avoid anaphylactic shock and the aggravation of atopic diseases, such as atopic dermatitis, allergic rhinitis and asthma. The detection of antigen specific IgE in serum implies hypersensitivity against the antigen. Thus, a variety of immunological methods, such as CAP-RAST™, Ala-STAT™ and ADVIA Centaur™, to detect antigen specific IgE have been developed and utilized in clinical practice.[1] However, there are often substantial discrepancies between these serological tests and clinical symptoms.[1]

In vivo tests, such as skin tests and antigen challenge tests, are more reliable in reflecting the clinical scenario. However, these tests may be painful, and could potentially evoke anaphylactic shock when a patient is extremely sensitive to a particular antigen.[2] Moreover, the intradermal injection of an antigen may sensitize subjects who are not sensitive to the antigen. Both mast cells and basophils in subjects sensitized with the same repertoire of IgE release histamine in response to the antigen. Therefore, the *in vitro* histamine release test with basophils of peripheral blood is sensitive, safe and gives reliable information regarding antigen that causes type I hypersensitivity.

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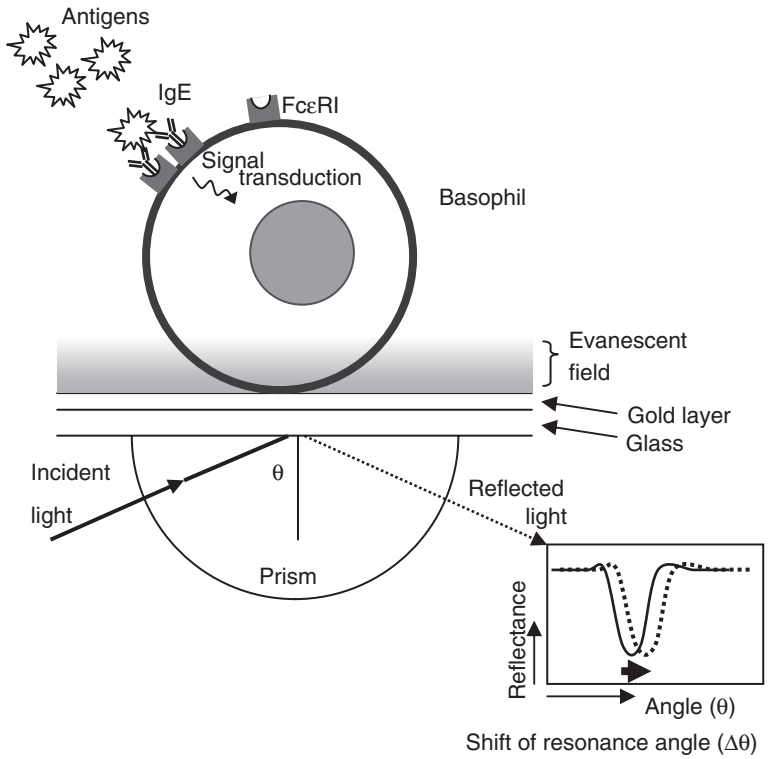

Fig. 1 Principle of the activation of basophils via the high affinity IgE receptor and analysis of SPR signals. Basophils were fixed to the surface of gold layer coated on a glass plate. The laser beam is directed toward the cell via a prism and reflected from there to the monitor which detects the shift of the resonance angle. The angle of resonance is proportional to the reflex index in the evanescent field, a few hundred nanometers from the surface. The reflex index reflects the amount (density) of the molecules in the evanescent field.

Griese *et al*.[3] reported that the histamine release test showed higher sensitivity and specificity than the skin test or RAST analysis based on the comparison with bronchoprovocation of extrinsic asthmatic children. However, basophils of a certain population of individuals, who showed type I hypersensitivity *in vivo*, do not release histamine upon the activation of the IgE receptors *in vitro*.[4] Except for the histamine release from basophils, there is no apparent clinical difference between such patients and ordinary basophil-reactive subjects.

The surface plasmon resonance (SPR) biosensor detects a change of the reflex index on the surface of a sensor chip in a real time fashion with high sensitivity. Therefore, it has been widely utilized for the analysis of binding and dissociation of a ligand to its receptor fixed on a sensor chip.[5] We recently found that degranulation of mast cells on the sensor chip caused unexpectedly large changes of SPR signal.[6,7] In this study we developed a method to detect basophil activation by a SPR biosensor (Fig. 1).

METHODS

REAGENTS

Chemicals were from the following sources: human serum albumin (HSA), 4,4'-dithio dibutyric acid (DDA), N-hydroxysuccinimide (NHS), and 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) from Sigma-Aldrich Japan (Tokyo, Japan); genistein, piceatannol, PP1 and wortmannin from Calbiochem (San Diego, CA, USA); fetal calf serum (FCS) from Biowest (Paris, France); RPMI-1640 medium, DMEM and penicillin streptomycin liquid from Invitrogen (Carlsbad, CA, USA); Goat anti-human IgE antibody from Seikagaku Co. (Tokyo, Japan). BA312 antibody[8] was kindly provided by Shionogi Pharmaceutical, Co., Ltd. (Osaka, Japan) and was used for capturing basophils on sensor chips. A monoclonal antibody (25H3) against human IgE was provided as a kind gift from Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan) and was used for purification of human IgE from a serum of a patient with atopic dermatitis. Mite antigen (300 AU/ml) in 0.04% phenol and 0.05% glycerol

Table 1 Values of total serum IgE and CAP-RAST scores of subjects

Subject	Total IgE (IU/mL)	CAP-RAST score
Atopic dermatitis 1	n.d.	n.d.
Atopic dermatitis 2	8659	mite 4
Allergic rhinitis 1	237	mite 4
Allergic rhinitis 2	273	mite 4
Allergic rhinitis 3	270	mite 4
Psoriasis	726	n.d.
Prurigo	800	n.d.
Soybean allergy	809	soybean 3
Healthy control 1	25	n.d.
Healthy control 2	160	n.d.
Healthy control 3	n.d.	n.d.

n.d.: not done

was purchased from the Bayer Corporation (Elkhart, IN, USA). Semi-purified sweat antigen was prepared as reported previously.[9] Soybean antigen was prepared by gel filtration chromatography from the water-extract of soybean. Ten grains of soybean were soaked in water overnight, homogenized in 30 milliliters of phosphate-buffered saline (PBS), and filtered by means of a strainer (70 μm pore-size; Becton, Dickinson and Company, Bedford, MA, USA), followed by membrane filtration (0.22 μm pore-size; Millipore, Billerica, MA, USA). The resulting extract was gel-filtrated with Superdex 75 PC 3.2/30™ using the SMART™ system (GE Healthcare Bio-sciences KK; Tokyo, Japan), at a flow rate of 50 μl/min. Twenty microliters of eluted fractions were subcutaneously injected into a patient with soybean allergy. The fractions that caused wheal and flare reactions of the patient were pooled and used as a stimulant of the histamine-release test and SPR analysis. All stimulants and their vehicles were appropriately diluted with the buffer for histamine release assays as described previously.[6]

SUBJECTS

The following subjects were included in this study: two patients with atopic dermatitis, (atopic dermatitis 1, 2), three patients with allergic rhinitis, (allergic rhinitis 1–3), a patient with soybean allergy (soybean allergy), a patient with psoriasis vulgaris (psoriasis), a patient with prurigo nodularis (prurigo), and three healthy volunteers without apparent allergic symptoms, (healthy control 1–3). The amounts of antigen-specific IgE in sera of the patients were measured using CAP-RAST™.[1] Values of total and antigen-specific IgE of the subjects (patients and healthy volunteers) are shown in Table 1. The protocol of the study was approved by the ethics committee of the Faculty of Medicine at Hiroshima University. All patients and volunteers were informed of the aim and protocol and agreed to participate in the study.

HISTAMINE RELEASE ASSAY

Histamine release assays were carried out as reported previously.[9] Briefly, peripheral blood leukocytes containing approximately 1% basophils were obtained from patients and from healthy volunteers. Mite antigen, soybean antigen and sweat antigen were used as the stimulants in the histamine release test. A goat anti-human IgE antibody (Seikagaku Co., Tokyo, Japan) was used as a positive control for these assays at a 3,000-fold dilution. To study the effect of inhibitors for intracellular signal transduction, cells were preincubated with indicated concentrations of genistein, piceatannol or PP1 for 30 minutes or wortmannin for 10 minutes at 37℃, and consequently incubated with buffer, antigen or anti-IgE antibody in the presence of the inhibitors. Histamine was extracted and measured by reverse-phase HPLC.

ISOLATION OF BASOPHILS FROM PERIPHERAL BLOOD

Fourteen milliliters of venous blood were collected by venopuncture using EDTA/2Na containing tubes (Vacutainer™; Becton-Dickinson and Company, Franklin Lakes, NJ, USA) and separated by Ficoll-Hypaque™ (GE Healthcare Bio-sciences KK; Tokyo, Japan) density gradient centrifugation to isolate mononuclear cells. Basophils were then semi-purified from the mononuclear cell suspension using a basophil isolation kit and an automated magnetic cell sorter (autoMACS™; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The basophil isolation kit employed a cocktail of antibodies against CD3, CD7, CD14, CD15, CD16, CD36, CD45RA and HLA-DR to deplete non-basophils and isolate untouched basophils from the mononuclear cells. The basophils were then washed and centrifuged twice in the buffer for perfusion (0.8% NaCl, 0.02% KCl, 0.52mM NaH_2PO_4, 10mM HEPES, 0.1% Glucose, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.03% HSA, pH 7.4), which was also used for the histamine release assay. Using Kimura's staining method, the purity of the obtained basophils was found to be greater than 50%.[10]

MONITORING OF BASOPHIL ACTIVATION USING SPR

SPR signals generated in the presence of basophils with or without stimulants were analyzed using an SPR apparatus, SPR-CELLIA™ (Moritex Corporation, Tokyo, Japan). Sensor chips (27 × 20 mm) with 50-nm thick gold films were sterilized with 70% ethanol and chemically activated according to the manufacturer's instructions.[7] Seventy microliters of anti-basophilic antibody (BA312, 100 μg/ml) were placed on two spots on the activated surface of a sensor chip

and incubated for 10 minutes at room temperature. After washing of the surface with PBS, 50–70 µl of basophil suspension (containing approximately 2.3×10^4 to 1.25×10^5 cells) in buffer was placed on the area treated with BA312 antibody. A half to three hours later, the sensor chip with basophils was rinsed with buffer, and preincubated in the presence or absence of 100 µM genistein, 100 µM piceatannol or 10 µM PP1 for 30 minutes or 10 nM wortmannin for 10 minutes in CO_2-incubator at 37℃. The sensor chip was then rinsed with buffer and positioned in a flow-cell unit of the SPR apparatus with two chambers. The cells placed in the chambers were perfused with buffer at a flow rate of 10 µl/min for IgE sensitization or 20 µl/min in all other experiments. Buffer with or without stimulants was injected from the side of the inlet at the same rate of flow as that in the preincubation stage.

PURIFICATION OF IgE FROM THE SERUM OF A PATIENT WITH ATOPIC DERMATITIS

IgE was affinity purified from the sera of a patient with atopic dermatitis, whose peripheral leukocytes released histamine by the mite antigen (described below). Anti-human IgE antibody (25H3) was coupled to NHS-activated Sepharose 4FF (GE Healthcare Bio-Sciences KK, Tokyo, Japan) according to the manufacturer's instructions. The gel was equilibrated in PBS and packed onto a 1 × 5 cm column (econo-column™, Bio Rad, Hercules, CA, USA) (human IgE affinity-column). The serum was diluted with two volumes of PBS and applied to the affinity-column. After washing the column thoroughly with PBS, the IgE antibody was eluted with 0.1M glycine-HCl buffer (pH 2.5). To neutralize the pH immediately, 1 milliliter of each fraction was collected in a tube containing 0.25 milliliters of phosphate buffer (2M, pH 7.2). IgE-containing fractions were pooled, filtered (0.22 µm filter) (Millex-GV™, Millipore Corp., Bedford, MA, USA), and stored at 4℃ until use. The purity of the IgE preparation was confirmed (approximately >95%) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

REMOVAL OF IgE AND PASSIVE SENSITIZATION OF BASOPHILS WITH THE PATIENT'S IgE

To study whether the SPR signals provoked by stimulation of basophils with antigens were mediated by IgE or not, we utilized healthy volunteers' basophils that released histamine in response to anti-IgE antibody, but not to mite antigen. Basophils of healthy volunteers were treated with lactic acid to remove endogenous IgE and passively sensitized with purified IgE of patients with atopic dermatitis (AD-IgE) as described previously.[9,11]

RESULTS

SPR SIGNALS SHOW THE INCREASE OF AR IN RESPONSE TO STIMULI THAT INDUCED HISTAMINE RELEASE.

Basophils were prepared from a patient with atopic dermatitis (atopic dermatitis 1), two patients with allergic rhinitis with mite-specific IgE (allergic rhinitis 1, 2) and an apparently healthy volunteer (healthy control 1). The dermatitis of the patient with atopic dermatitis was aggravated after sweating, whereas the healthy volunteer did not experience any skin problems even after profuse sweating. The basophils were either fixed on a sensor chip for SPR apparatus or suspended in assay tubes for the histamine release test, and exposed to appropriate stimuli or vehicles. When basophils of a patient with allergic rhinitis (allergic rhinitis 1) were exposed to anti-IgE antibody on the sensor chip for SPR apparatus, the angle of resonance (AR) rapidly increased for 5 to 10 minutes and was sustained at a high level or further continued to increase (Fig. 2a). The injection of buffer or goat IgG with the same isotype antibody as anti-IgE antibody caused no, or only transient and minute change of the AR, regardless of the presence of basophils on the sensor chip (Fig. 2a). The change of AR with various concentrations of anti-IgE antibody and that of mite antigen increased in a dose dependent manner (Figs. 2a, c). Anti-IgE antibody and mite antigen also induced histamine release from the basophils in assay tubes in a dose dependent manner (Figs. 2b, d). Similar results were obtained with basophils of another patients with allergic rhinitis (allergic rhinitis 2) and those with atopic dermatitis (atopic dermatitis 1) (data not shown).

Specificity of the reaction detected by SPR was further studied with basophils of allergic and non-allergic donors and various antigens. Basophils of the patient with atopic dermatitis 1 showed the increase of AR and histamine release in response to sweat antigen as those of the patients with allergic rhinitis stimulated with anti-IgE antibody or mite antigen (Figs. 3a, b). However, no change of AR or histamine release was induced when basophils of healthy control 1 were exposed to the sweat, mite or soybean antigens (Figs. 3b, c, d).

SPR SIGNALS OBTAINED FROM BASOPHILS OF A HEALTHY VOLUNTEER THAT WERE PASSIVELY SENSITIZED WITH AD-IgE.

Basophils of a healthy volunteer (healthy control 1) were treated with lactic acid to remove endogenous IgE, and then were immobilized on a sensor chip. After washing with running buffer, the basophils were passively sensitized with AD-IgE in the flow cell during the period indicated by the first horizontal bar. Exposure of the cells to AD-IgE(20 µg/ml, or 50 µg/ml) induced a subtle increase in the SPR signal, but

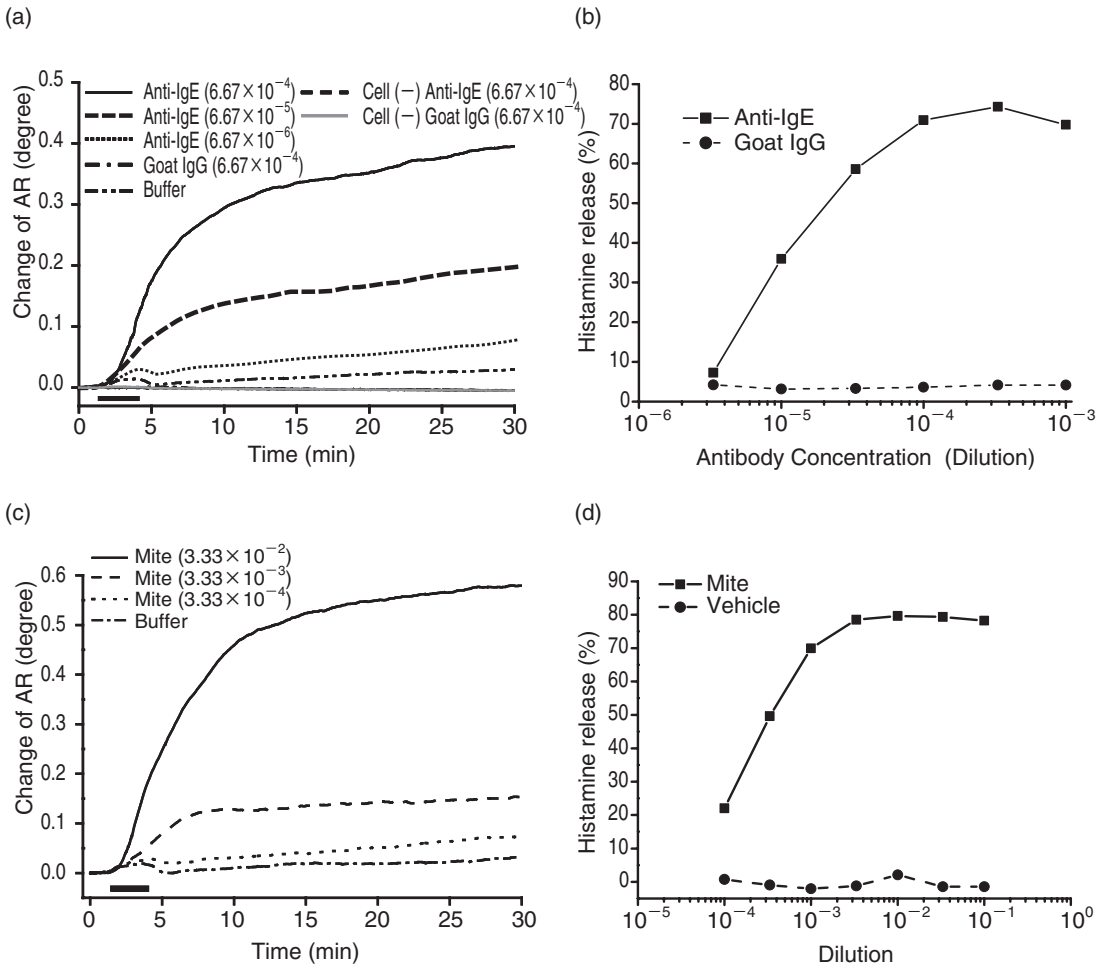

Fig. 2 SPR signals and histamine release by basophils of a patient with allergic rhinitis. Basophils of a patient with allergic rhinitis (allergic rhinitis 1, CAP-RAST™ score 4 for mite antigen) were immobilized on two spots of a sensor chip equipped in a flow-cell unit of the SPR apparatus (**a, c**) or suspended in tubes for histamine release tests (**c, d**). (**a**) The basophils were washed with running buffer, and then exposed to various concentrations of anti-human IgE antibody (anti-IgE), goat IgG as an isotype-control antibody, or a vehicle (running buffer itself) for antibodies, during the periods indicated by a horizontal bar. The control experiments were performed without basophils in the same manner. Angles of resonance (AR) were measured during the perfusion and then changes were plotted against time (Change of AR). (**b**) The leucocytes containing basophils obtained from the donor were stimulated with various concentrations of anti-human IgE antibody or the goat IgG. The amount of histamine was measured by the HPLC system, as described in the Materials and Methods section. (**c**) Basophils were washed, exposed to various concentrations of mite antigen or a vehicle (0.04% phenol and 0.05% glycerol) for mite antigen, during the periods indicated by a horizontal bar. (**d**) The leucocytes were stimulated with various concentrations of mite antigen or vehicle (0.04% phenol and 0.05% glycerol) appropriately diluted with the buffer. Similar results shown in (**a**−**d**) were obtained with the basophils of another patient with allergic rhinitis (allergic rhinitis 2) and those with atopic dermatitis 1 (data not shown).

did not induce any significant increase in the signal by the end of the period. After rinsing the loosely attached IgE with running buffer, mite antigen was perfused into the flow cell during the periods indicated by the second horizontal bar. The mite antigen induced a robust increase of AR, which continued even after the exposure was terminated. Moreover, a larger increase of AR was induced when basophils were sensitized with a higher concentration (50 μg/ml) of AD-IgE (Figs. 4a, b). However, neither significant change of SPR signal nor histamine release was induced by mite antigen, when cells were not exposed to AD-IgE (Figs. 4a, b; broken line). The basophils treated with lactic acid and passively sensitized with AD-IgE released histamine in response to mite antigen (Fig. 4C).

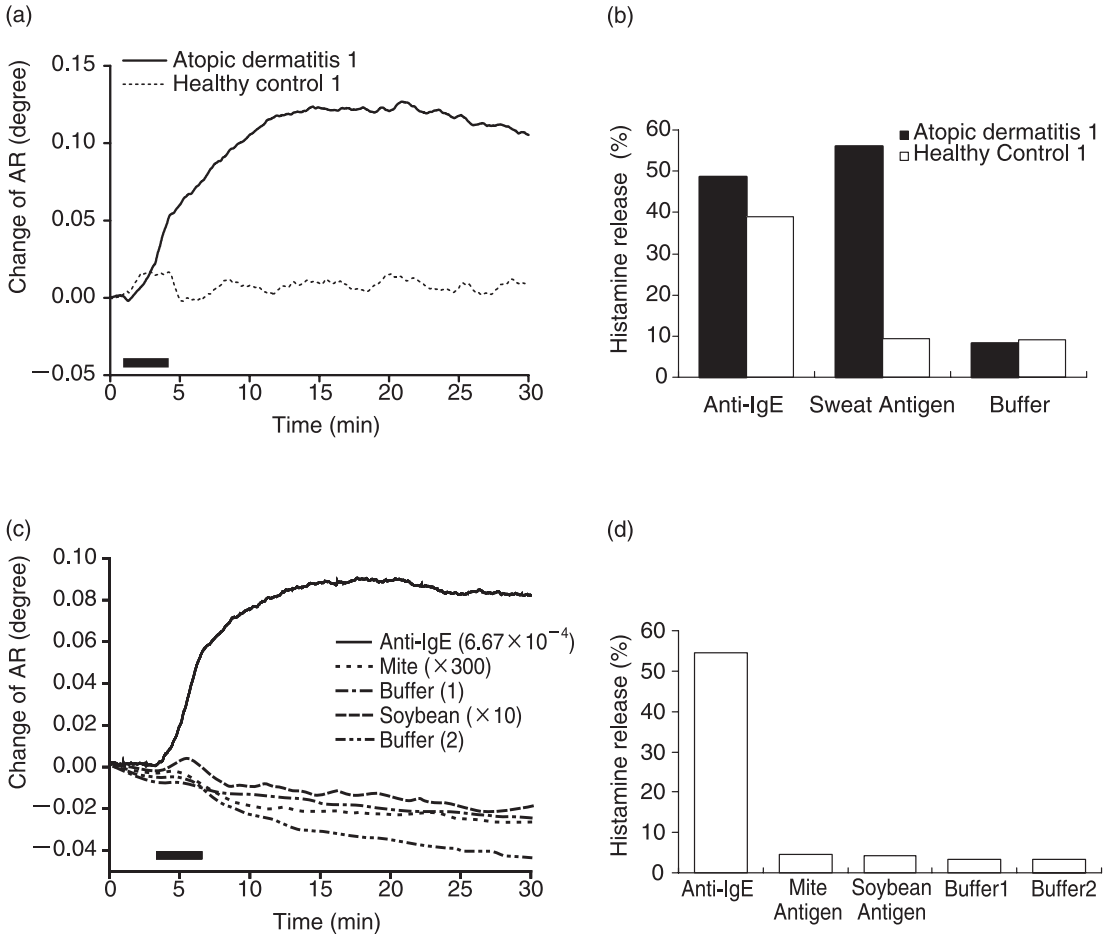

Fig. 3 Specificity of the reaction of basophils obtained from allergic and non-allergic donors against various antigens. (**a**) Basophils of a patient with atopic dermatitis (atopic dermatitis 1) (solid line) and those of a healthy volunteer (healthy control 1) (broken line) were exposed to sweat antigen during the periods indicated by a horizontal bar. (**b**) Leucocytes of the same donors were stimulated with anti-human IgE antibody (anti-IgE), sweat antigen, or buffer. (**c**) Basophils of the healthy volunteer (healthy control 1) were exposed to anti-IgE antibody, mite antigen, soybean antigen during the periods indicated by a horizontal bar. Buffer 1 is the running buffer itself and utilized for dilution of anti-IgE antibody, sweat antigen and soybean antigen. Buffer 2 contained 0.04% phenol and 0.05% glycerol in Buffer 1 and was used for dilution of mite antigen. (**d**) Leucocytes of the healthy volunteer were stimulated with the same stimuli employed in the experiment in (**c**).

SPR SIGNALS DETECT THE CHANGES OF AR IN RESPONSE TO STIMULI ON BASOPHILS THAT DO NOT RELEASE HISTAMINE.

To further investigate whether the SPR sensor may detect any sign of reaction of basophils that do not show histamine release in standard tests, we studied the change of AR with such basophils in response to anti-IgE antibody and antigens. Seven donors were recruited for this study; two healthy volunteers (healthy control 2, 3), two patients with non-allergic skin diseases (psoriasis and prurigo), and three patients with allergic disorders (allergic rhinitis 3, atopic dermatitis 2, and a patient with soybean allergy). The sensitivities of the allergic patients for respective antigens were also diagnosed by clinical episodes. Basophils of all these donors released no or only marginal amounts of histamine in response to anti-IgE antibody or respective antigens (Figs. 5b, d, f and Figs. 6b, d). However, they all caused the prompt increase of AR in response to anti-IgE antibody (Figs. 5a, c, e and Figs. 6a, c). Basophils of the patients with atopic dermatitis and those with soybean allergy also caused apparent increases of AR in response to respective stimuli (Figs. 6a, c). Similar results were obtained with basophils of healthy control 3 for stimulation by anti-IgE antibody or with basophils of the patient with atopic dermatitis 2 for stimulation by mite antigen (data not shown) . The degrees and durations of the increase of AR in response to the stimuli were dependent on the donor of basophils and/or the kind

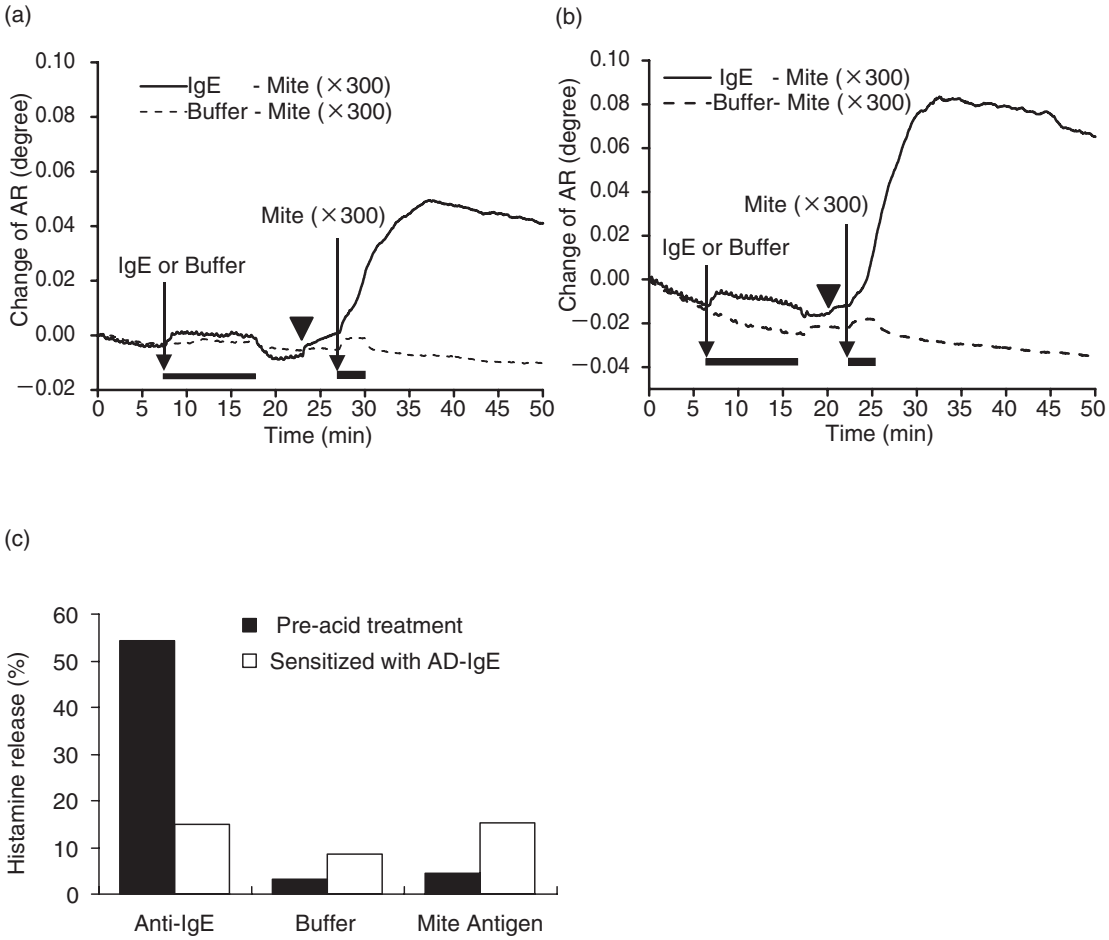

Fig. 4 SPR signals obtained from basophils of a healthy volunteer that were passively sensitized with AD-IgE. Basophils of a healthy volunteer (healthy control 1) were treated with lactic acid to remove endogenous IgE and immobilized on a sensor chip. (**a, b**) The basophils were passively sensitized with or without purified IgE of a patient with atopic dermatitis (AD-IgE) (**a**; 20 μg/ml, **b**; 50 μg/ml) during the periods indicated by the first horizontal bar at flow rate of 10 μl/min. After a rinse by perfusion with running buffer, the cells were then exposed to mite antigen during the periods indicated by the second horizontal bar at flow rate of 20 μl/min. An arrow head indicates the changing point of flow rate. (**c**) The amounts of histamine released from basophils obtained from the same donor and stimulated with anti-human IgE antibody (anti-IgE) or mite antigen. Closed columns indicate the amounts of histamine released from basophils without lactic acid treatment. Open columns show those treated with lactic acid followed by re-sensitization with AD-IgE.

of stimuli. These variations of the SPR signal evoked by basophils may be due to the variations of the cell numbers fixed on a sensor chip, and/or heterogeneity of the signal transductions adjacent to the plasma membrane, including those specifically detected by SPR. In any case, the changes were clear enough to be distinguished from those with basophils exposed to the buffer alone.

EFFECTS OF VARIOUS INHIBITORS FOR INTRACELLULAR SIGNAL TRANSDUCTION ON HISTAMINE RELEASE AND SPR SIGNALS.

To elucidate the molecular mechanism of SPR signals raised by basophil activations, we pre-treated basophils with various inhibitors of intracellular signal transduction; genistein, a tyrosine kinase inhibitor; piceatannol, a Syk inhibitor; PP1, a Src tyrosine kinase inhibitor, and wortmannin, a PI3 kinase inhibitor. The histamine release in response to anti-IgE antibody was inhibited in a dose-dependent manner by all reagents. SPR signals derived from basophils in response to anti-IgE antibody were also impaired by these inhibitors (Fig. 7). Degrees of inhibition in both histamine release and SPR signals were variable either by the type of inhibitors or donors of basophils (n = 3). However, inhibition of SPR signal never exceeded those of histamine release (Fig. 7).

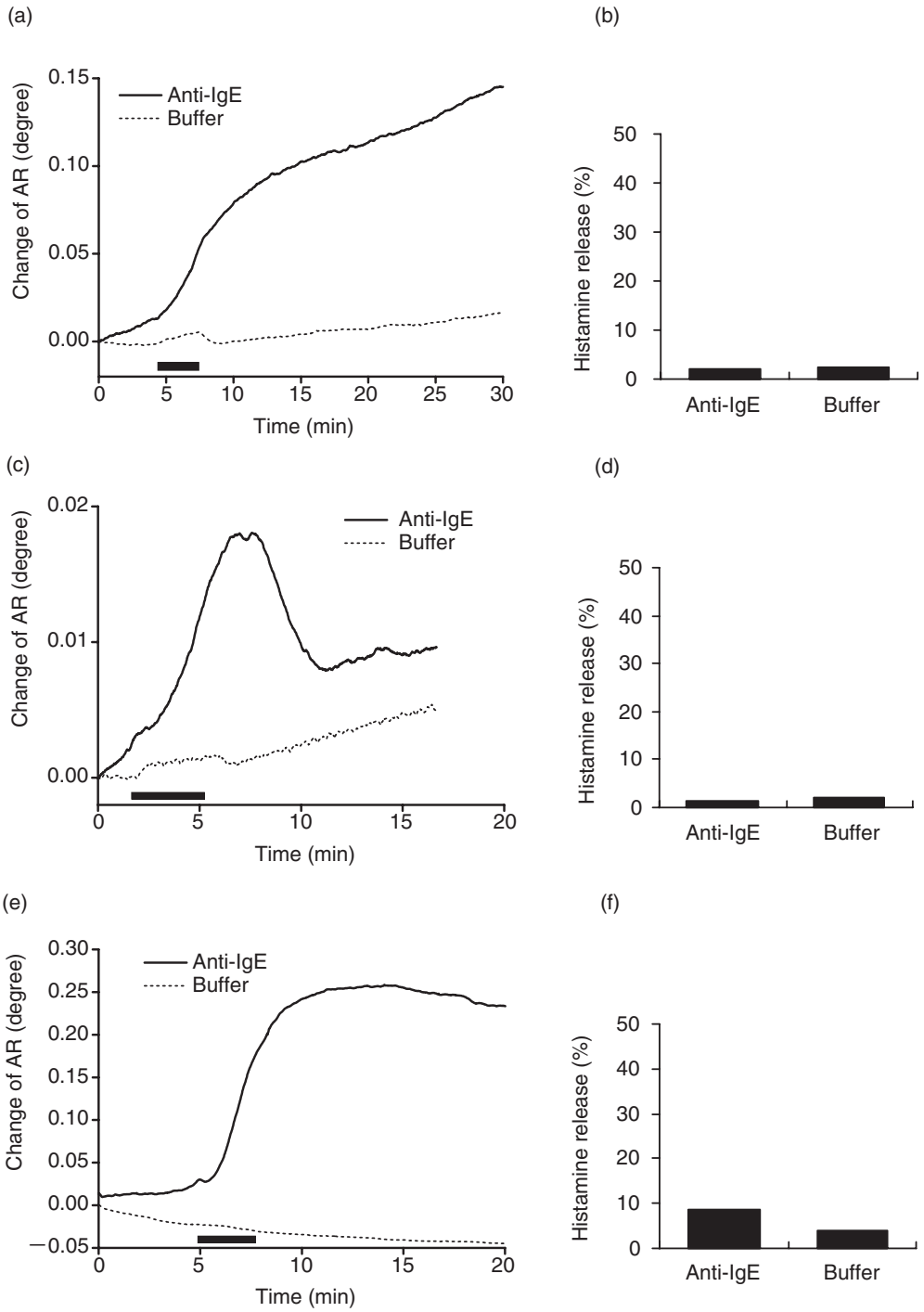

Fig. 5 SPR signals obtained from basophils of a healthy control or non-allergic patients that release no or marginal amounts of histamine. Basophils obtained from a healthy volunteer (healthy control 2) (**a, b**) and those from a patient with psoriasis vulgaris (**c, d**), and pruritus nodularis (**e, f**) were stimulated with anti-human IgE antibody (anti-IgE) as experiments shown in Figures 2−4. No or only marginal amounts of histamine release were detected (**b, d, f**). However, various degrees of increases in AR were observed in response to the stimulation with anti-human IgE antibody (**a, c, e**). Similar results were obtained with basophils of another donor (healthy control 3) (data not shown).

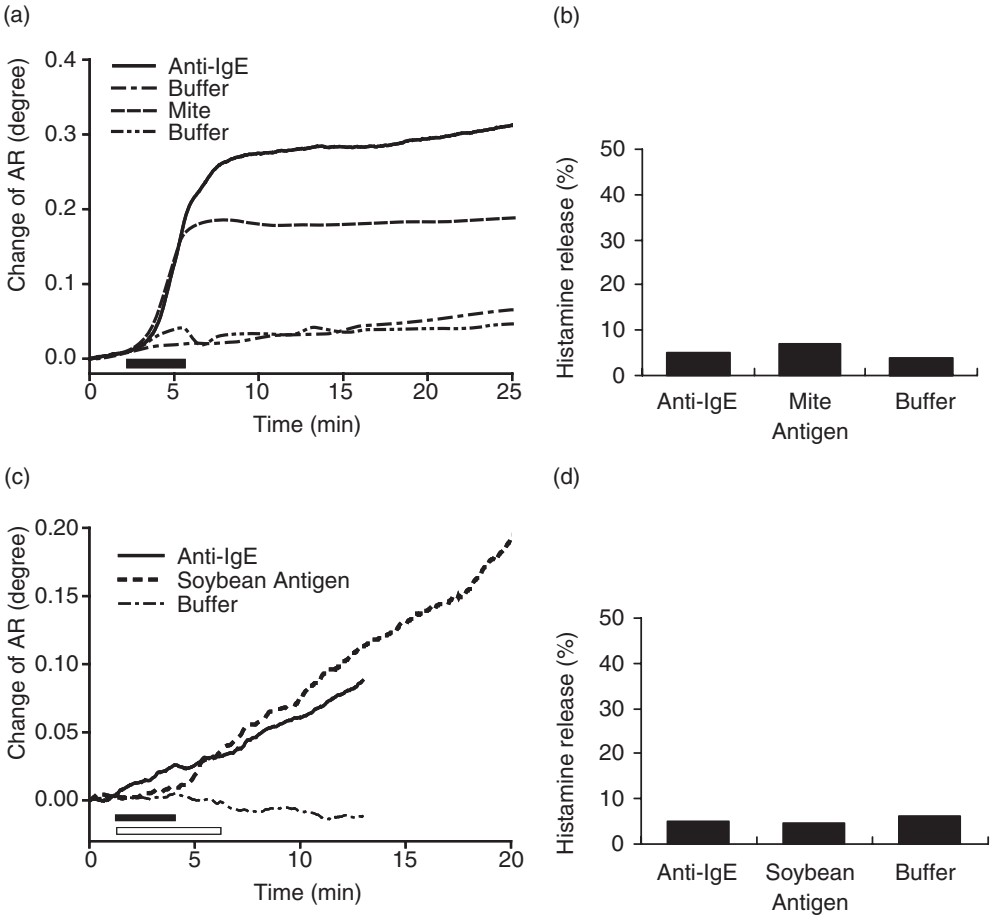

Fig. 6 SPR signal obtained from basophils of patients with allergy that release no or marginal amounts of histamine. Basophils obtained from a patent with allergic rhinitis (allergic rhinitis 3) with mite-specific IgE (CAP-RAST™ score 4) (**a, b**) and a patient with soybean allergy with soybean-specific IgE (CAP-RAST™ score 3) (**c, d**) were stimulated with anti-human IgE antibody (anti-IgE) or respective antigens. After a rinse with running buffer, the stimulants were perfused onto/into the flow cell during the periods indicated by the corresponding horizontal bars (■ ; anti-IgE antibody, □ ; soybean antigen) (**a, c**) . No or only marginal amounts of histamine were released in response to either stimulation (**b, d**) . However, substantial amounts of increase in AR were observed in response to anti-IgE antibody and antigens (**a, c**). Similar results were obtained with basophils of a patient with atopic dermatitis (atopic dermatitis 2) (data not shown).

DISCUSSION

Basophils and mast cells release histamine in response to antigens or anti-IgE antibody and cause anaphylactic symptoms. In this study, we have demonstrated that the SPR biosensor detects the change of AR due to the activation of human basophils in both a dose-dependent and real time manner. The change of AR was initiated in a few minutes and sustained for 10 to 30 minutes or longer. Moreover, such reactions were detected by an SPR sensor, even when no apparent reaction of the cells was detected by the standard histamine release test.

The plasmon in a metal layer surface may be resonated with a laser beam irradiated from the other side of the layer with a certain inward angle (angle of resonance; AR) (Fig. 1). The AR is determined by the reflex index in the field of evanescence (ca 600 nm) on the metal and can be measured via the analysis of reflected light spectrum. The reflex index in the field of evanescence proportionally increases in accordance to the binding of biomolecules, such as protein, lipid and nucleic acid. Therefore, SPR has been widely utilized for sensors to analyze the binding and dissociation of ligands to and from their receptors in real time without labeling.

We recently found that living cells on the sensor chip caused unexpectedly large change of AR when the cells were activated under physiological conditions. The increase of AR was barely induced by the

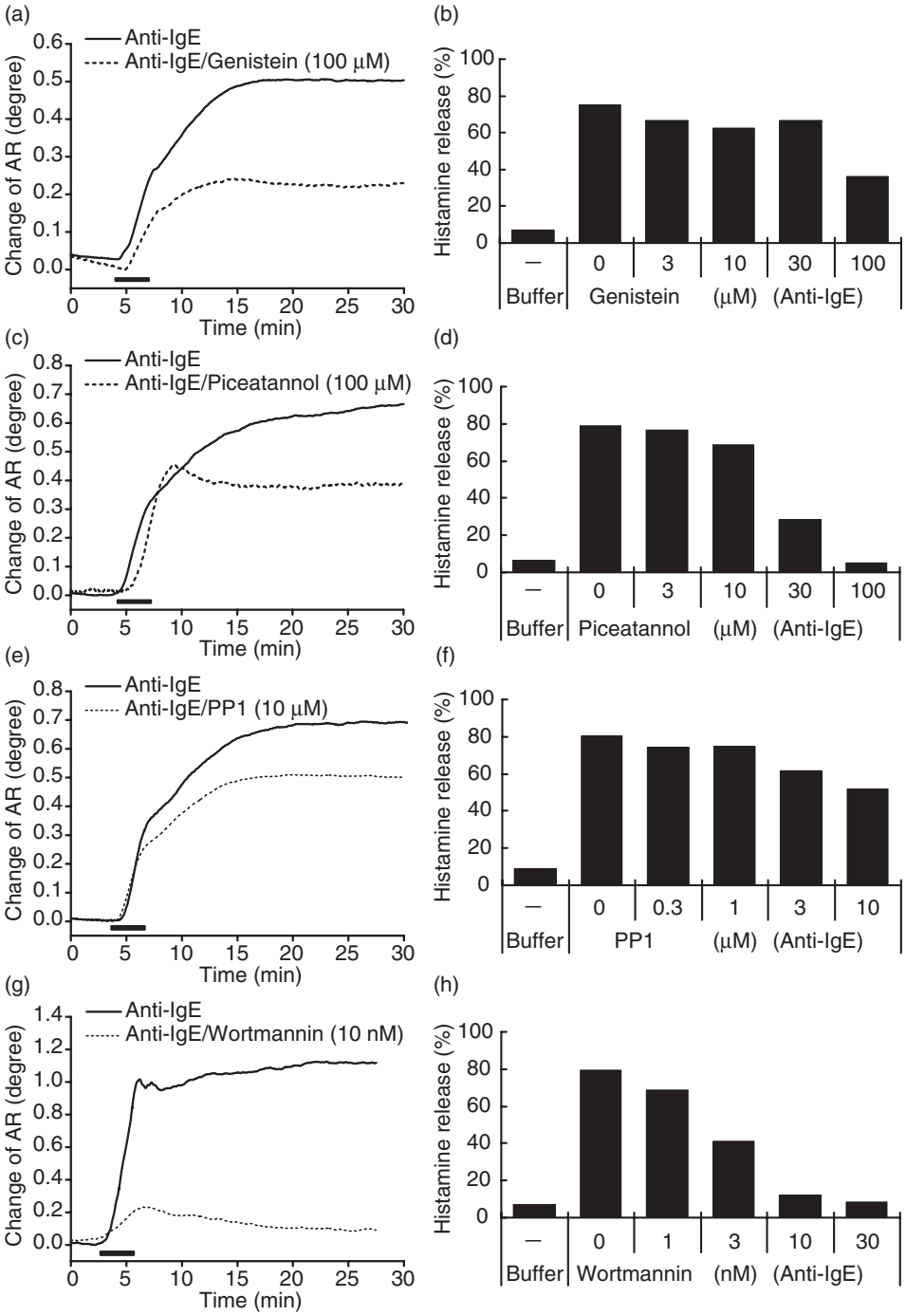

Fig. 7 Effects of various inhibitors for intracellular signal transductions on histamine release and SPR signals. Basophils of patients with allergic rhinitis (allergic rhinitis 1) were immobilized on a sensor chip (**a, c, e, g**) or suspended in assay tubes (**b, d, f, h**) and pretreated in the presence or absence of indicated concentrations of genistein (**a, b**), piceatannol (**c, d**) or PP1 (**e, f**) for 30 minutes or wortmannin (**g, h**) for 10 minutes at 37℃, and then exposed to anti-human IgE antibody (anti-IgE). Both histamine release and SPR signals were impaired by pretreatment with inhibitors. When basophils were prepared from two other donors (healthy control 1 and allergic rhinitis 2), degrees of the impairment were either large or small as compared with results shown in this figure. However, those on SPR signals were always partial and have never been larger than those of histamine release.

binding of monomeric IgE or hapten to RBL-2H3 mast cells, which does not cause exocytosis. However, a consequential binding of antigens that bind to IgE and aggregate FcεRI on RBL-2H3 cells induced a large increase of AR.[6] A similar change of AR in response to the antigen was detected even when the change of cell structure and movement was inhibited by pre-treatment with actin polymerization inhibitors, Toxin B or mycaloride.[7] On the other hand, the changes of AR were abolished when cells were pretreated with genistein, a tyrosine kinase inhibitor that prevents the intracellular signal transduction initiated by the activation of FcεRI. Moreover, activation of the adenosine A3 receptor on RBL-2H3 cells also induced a sustained increase of AR. This stimulation induced only a marginal amount of exocytosis by itself, but showed a large synergistic effect on antigen-induced exocytosis.[6] These results suggest that SPR reflects early events of intracellular signal transduction rather than the exocytosis or morphological changes of mast cells, detectable by light microscopy. In this study, we have demonstrated that human basophils also induce a similar change of AR when stimulated with antigen after proper sensitization with IgE. The reaction was detected even with basophils that did not release histamine in spite of the IgE sensitization for antigen (Figs. 5, 6). Recently, Fang *et al.*[12] also reported the change of local reflex index by the activation of living cells without exocytosis. They employed the technique of resonant waveguide grating to analyze the reactions of epidermoid carcinoma A431 cells in response to epidermal growth factor (EGF) and bradykinin via EGF receptors and bradykinin B2 receptors, respectively.

The assays of serum-IgE titer and allergen-specific IgE titer have been used for the diagnosis of type I hypersensitivity. However, these do not represent the allergic symptoms themselves and do not necessarily reflect the functional hypersensitivity of patients. On the other hand, the histamine released from basophils and/or mast cells is an important mediator that causes allergic symptoms, by directly acting on blood vessels, nerves, glands and trachial smooth muscles. Therefore, the histamine release test with basophils *in vitro* is a reliable and safe examination for the state of allergy.[13] However, it has been known that basophils of some individuals (non-responders) do not release histamine upon the activation of their high affinity IgE receptors (FcεRI).[5] The mechanism of such functional insufficiency is not fully clear. Kepley *et al.* reported that such basophils failed to express syk protein, a tyrosine kinase which binds and phosphorylates the γ-subunit of FcεRI,[14] but they release histamine when activated by non-IgE mediated stimuli such as calcium ionophores, which circumvent the cell surface receptor. Moreover, Lavens-Phillips *et al.*[15] found that basophils of non-responders have at least 10-fold less protein expression of lyn and syk kinases compared with basophils of ordinary individuals. On the other hand, Vonakis *et al.*[16] have shown that basophils of patients with chronic idiopathic urticaria fail to release histamine due to the increased expression of Src-homology 2-containing-5'-inositol phosphatases (SHIPs).

Since SPR detects changes that affect the reflex index in the near field of the sensor chip, it should detect the kinetics of molecules associated with plasma membrane regardless of their enzymatic activities. This may include the aggregation of FcεRI, the association of signaling molecules, including phosphatases and raft formation in the plasma membrane. Pre-treatment of basophils with genistein, a tyrosine kinase inhibitor; piceatannol, a Syk inhibitor; PP1, a Src tyrosine kinase inhibitor; or wortmannin, a PI3 kinase inhibitor, impaired the increase of AR to various degrees. The increase of AR was almost abolished as well as histamine release by wortmannin, especially in a late phase of the reaction, suggesting that the activation of PI3 kinase and/or its downstream signals in basophils were largely reflected by SPR signals (Fig. 7g). Although both degrees of inhibition of histamine release and those of SPR signals by the other inhibitors were variable among donors for basophils, effects of inhibitors on SPR signal never exceeded those of histamine release in all experiments.

A possible explanation for such discrepancies between the inhibitory effects on histamine release and those on SPR signals may be a difference between the threshold for histamine release and that for SPR signals, or the events contribute to histamine release and those to SPR signals. SPR signal should reflect any change of molecular mass distribution in the field of evanescence regardless of their biological activities. On the other hand, histamine release is induced only when positive signaling activities, such as the phosphorylation of tyrosine kinases and the increase of intracellular calcium, become superior to negative signaling activities, such as the activation of phosphatases including SHIP-1 and SHIP-2. Thus, it is feasible that SPR signals reflect cellular reactions more sensitively than whole cell functions, such as mediator release and morphological changes. We recently investigated contributions of intracellular signaling molecules on SPR signals induced by antigen in RBL-2H3 cells. In that study, we identified PKCβ as a key molecule for the increase of AR by techniques of siRNA and overexpression.[17] Further studies are necessary to confirm whether it is applicable for human basophils.

A biosensor with a cell-electrode, based on the semiconductor technology was developed[18] and also applied to the detection of real-time monitoring of IgE-mediated mast cell activation. The impedance of a RBL-2H3 cell-attached electrode correlated with the morphological change of and the release of

β-hexosaminidase from the cells. However, precise relations between molecular events in the cell and the impedance of cell-attached electrode were also unclear. Therefore, the SPR-based cell sensor should be superior to the cell-electrode sensor in that it detects the reactions of non-adhering cells, including basophils that do not complete histamine release.

The limitations of the present method for practical use are the time and the amount of blood needed to obtain basophils for the analysis; namely three hours and 14 milliliters blood for each course of the kinetic studies. However, once basophils are obtained and located in the SPR-biosensor, their reactions to various stimuli are detected rapidly and sensitively, even for those of non-responders. The development of a method for the rapid isolation of basophils and downsizing of the SPR apparatus would be beneficial. This approach will result in the sensor becoming a useful tool for detecting some events of the activation of human basophils, not only for basic scientific research but also for the purpose of clinical examinations.

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