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Surface plasmon resonance biosensor detects the downstream events of active PKCβ in antigen-stimulated mast cells

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Abstract

Surface plasmon resonance (SPR) biosensors detect large changes of angle of resonance (AR) when RBL-2H3 mast cells are cultured on a sensor chip and stimulated with antigen. However, the detail of molecular events that are responsible for such large changes of AR remained unknown. In this study, we investigated the relationship between intracellular signaling events induced by antigen and the change of AR, by genetic manipulation of intracellular signaling molecules; spleen tyrosine kinase (Syk), src-like adaptor protein (SLAP), linker for activation of T cells (LAT), growth-factor-receptor-bound protein 2 (Grb2), Grb2-related adaptor protein (Gads), and isotypes of protein kinase C (PKC). RBL-2H3 mast cells overexpressing dominant-negative Syk or SLAP, which both interfere with active Syk, exhibited only minimal increase of AR in response to antigen stimulation. Likewise, the interference of the activation of LAT and Gads, by expressing dominant-negative LAT and Gads, respectively, resulted in nearly complete suppression of the antigen-induced increase of AR. The cells overexpressing PKCs, apart from PKCβ, showed a reduced extent of increase of AR in response to antigen stimulation. Moreover, the introduction of the small interfering RNA targeted against PKCβ suppressed the antigen-induced increase of AR. These results indicate that the activation of Syk, LAT, Gads, and subsequent PKCβ is indispensable for the antigen-induced increase of AR of mast cells detected by SPR biosensors.

Keywords: Biosensor; Surface plasmon resonance; Mast cells; FcεRI; Intracellular signaling

1. Introduction

Surface plasmon resonance (SPR) biosensors are capable of characterizing the binding of detectants in the field of resonance on a sensor chip in real-time without any labeling (Homola, 2003). They are useful to study the interactions of any biological molecules from proteins, oligonucleotides, and lipids through to small substances phages, viral particles, and cells (Rich and Myszka, 2000). We previously reported that the SPR sensors can detect unexpectedly large changes of the angle of resonance (AR), when the adherent RBL-2H3 mast cells (Hide et al., 2002) or the non-adherent human basophils (Yanase et al., 2007b) were cultured on a biosensor chip and stimulated by antigen, indicating that SPR biosensors could be appropriate for the real-time and non-label detection of the activation of living cells. In addition, recently, we also found that the change of AR reflects intracellular events in living cells rather than changes in the size of the area to which cells adhere (Yanase et al., 2007a). However, the exact intracellular events or signaling molecules responsible for the change of AR have not been identified yet.

Mast cells play a central role in immediate hypersensitivity reactions through the high affinity IgE receptor (FcεRI) on their surface (Rivera and Gilfillan, 2006). The aggregation of FcεRI by multivalent antigen and the antigen-specific IgE results in the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the FcεRI β and γ subunit by Lyn. The phosphorylated ITAM provides the binding sites to the SH2 domain of Syk and activates Syk. The activated Syk phosphorylates the linker for activation of T cells (LAT). The phosphorylation of LAT results in the direct or indirect recruitment of several signaling molecules including growth-factor-receptor-bound protein 2 (Grb2), Grb2-related adaptor

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Table 1
Primers for cloning, mutagenesis, and siRNA

Cloning primers (restriction enzyme sites are underlined)
- LAT
  - Sense: GGGGTACCATGGAAGCAGACGCCTTGAGC
  - Antisense: GCTCTAGACAGTTAAGTTCCTGCAGGTTCTC
- Gads
  - Sense: GGGGTACCCAAGACATGGAAGCCACTGCCAAG
  - Antisense: GCTCTAGATAAGCCTTGCAGTACGACTCATCGC
- Grb2
  - Sense: GGGGTACCGCGCTCAGAATGGAAGCCATCGCC
  - Antisense: GCTCTAGATGCTTCTTAGACGTTCCGGTTCACTG
- dnSyk
  - Sense: GACTAGTGCAGACATGGCGGGCAATGC
  - Antisense: GCTCTAGACTTTTGACATGGTACCGTGAGG
- PKCα
  - Sense: GGGGTACCACCATGGCTGACGTTTTCCCG
  - Antisense: GCTCTAGATCATACTGCACTCTGTAAGATGG
- PKCβ
  - Sense: GACTAGTCGCGCGCAAGATGGCTGACCCGGCTG
  - Antisense: GCTCTAGACGGATCTACTTAGCTCTTGACTTCAGG
- PKCδ
  - Sense: GACTAGTATCATGGCACCCTTCCTGCGCATC
  - Antisense: GCTCTAGAGCTTAATTAAATGTCCAGGAATTGCTC
- PKCε
  - Sense: GACTAGTTTCACCATGGTAGTGTTCAATGGC
  - Antisense: ATAAGAATGCGGCCGCTTCTCAGGGCATCAGGTCTTCACC
- PKCθ
  - Sense: GACTAGTGAACAACCCATGTCACCGTTTCTTCG
  - Antisense: GCTCTAGAAGGTTCAGGAGCAAATGAGAGTCTCC

Mutagenesis primers
- dnLAT-Y171F
  - Sense: GGAGTCATGTGAAGATTTCGTGAATGTCCCTGAGAG
  - Antisense: CTCTCAGGGACATTCACGAAATCTTCACATGACTCC
- dnLAT-Y191F
  - Sense: GATGGGAGCCGGGAGTTTGTGAATGTGTCCCAG
  - Antisense: CTGGGACACATTCACAAACTCCCGGCTCCCATC
- dnGads
  - Sense: GATGGAACCCAAGATCAGGGTCAC
  - Antisense: AGGAAACTCGATATCTATAAAATTCTTAG
- dnGrb2
  - Sense: GACATAGAACAGATGCCACAGCAG
  - Antisense: ATGTGGTTTCATTTCTATGTAATTCTTG

siRNA primers
- PKCβ
  - Sense: AGGCTACACAAACCTTAATGTCTCGTTCCCCCG-GTGTTTCGTCC TTTCCACAAG
  - Antisense: CGGCGAAGCTTTTTCCAAAAAAGGGGAAC-GAGACATTAAG GCTACACAAACCTT
- GFP
  - Sense: ATCCTACACAAAGATATAGACGTTGTGGCTGC-CGGTGTTTCGTC CTTTCCACAAG
  - Antisense: CGGCGAAGCTTTTTCCAAAAAACAGCCA-CAACGTCTATATC CTACACAAAGATA

protein (Gads), SH2-containing leukocyte-specific 76 kDa (SLP-76), phospholipase Cγ1 (PLCγ1), and PLCγ2 (Gilfillan and Tkaczyk, 2006). The activation of PLCγ induces the increase of cytosolic calcium levels and the activation of protein kinase C (PKC), through catalyzing phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), respectively (Rivera and Gilfillan, 2006). These events finally lead to the release of preformed cytosolic granules, namely, degranulation. In another signaling pathway, the phosphorylated LAT recruits Grb2 and a guanine nucleotide-exchange factor, son of sevenless homologue (Sos), and then activates Ras-MAP kinase cascade. The activation of MAP kinases is thought to be essential for the release of arachidonic acid though the activation of cytosolic phospholipase A_2 and the production of cytokines (Zhang et al., 1997).

In this study, by genetically manipulating the function or expression of these molecules critically involved in the antigen-induced signaling pathway, we investigated the relationship between the change of AR detected by SPR biosensors and the antigen-induced signaling events in RBL-2H3 mast cells.

2. Materials and methods

2.1. Reagents and antibodies

The sources of reagents and antibodies were: dinitrophenyl-human serum albumin (DNP-HSA), anti-DNP IgE (SPE-7), and phorbol 12-myristate 13-acetate (PMA), Sigma (St. Louis, MO); polyclonal antibody against Syk (N-19), Santa Cruz (Santa Cruz, CA); polyclonal antibodies against Myc-tag, LAT, and Gads, Upstate (Lake Placid, NY); polyclonal antibodies against Grb2, Erk, and phosphorylated-Erk, Cell Signaling (Danvers, MA); monoclonal antibodies against PKC isoforms, BD Bioscience Pharmingen (San Diego, CA); monoclonal antibody against glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Ambion (Austin, TX).

2.2. Vectors

The expression vector coding mouse src-like adaptor protein (SLAP) was described previously (Hiragun et al., 2006).

The cDNAs of mouse LAT, Gads, Grb2, dominant-negative form of mouse Syk (dnSyk) (Shi et al., 2006), PKCβ, PKCδ, PKCε, PKCθ, and human PKCα were amplified by RT-PCR, digested by the specific restriction enzymes, and then ligated into pEF6-Myc/His vector (Invitrogen, Carlsbad, CA). The myc-tagged dominant-negative form of LAT (dnLAT, Y171/191F) (Zhang et al., 1998) was generated by the site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the wild type LAT. The dominant-negative forms of Gads (dnGads, delSH2) and Grb2 (dnGrb2, delSH2) (Asada et al., 1999) were generated by deletion of SH2 domain from the wild type Gads and Grb2, respectively. The expression vectors for siRNA targeted against PKCβ (target sequence: 1766–1786) and green fluorescent protein (target sequence: 439–459) were generated by the silencer express cassette kit (Ambion), pSEC-neo (Ambion), and their specific oligonucleotides. The primers for generating the expression vectors in this study were shown in Table 1.

2.3. Cell culture and transfection

RBL-2H3 cells were maintained with minimum essential medium (Invitrogen) supplemented with 15% fetal calf serum, L-glutamine, and antibiotics. All plasmids were purified by EndoFree® Plasmid Maxi Kit (Qiagen, Valencia, CA). RBL-2H3 cells were suspended in Dulbecco's modified Eagle's Media containing 25 mM Hepes (Invitrogen) at a concentration of 4×10^7 cells/ml. Cells (100 μl) were mixed with 15 (pEF6 vectors) or 10 (pSEC vectors) μg of plasmid and transfected by electroporation (Gene Pulser, BioRad, Hercules, CA) at 250 V/250 μF.

2.4. SPR analysis

The method measuring the change of AR of living cells in response to antigen stimulation was described previously (Hide et al., 2002; Yanase et al., 2007a). Briefly, on the day before experiments, cells were transfected with the indicated constructs, and then cultured on biosensor chips (1.2×10^4 cells/(60 μl site)) with 50 ng/ml of anti-DNP-IgE overnight. The sensor chip was equipped in a flow-cell unit of the SPR apparatus, SPR-CELLIA (Moritex, Nagoya, Japan). The cells placed in the chambers were preincubated for 20 min with glucose saline/pipes buffer (Hiragun et al., 2006) and stimulated with 50 ng/ml of DNP-HSA for 3 min. The change of AR during cellular activation was monitored by SPR-CELLIA for up to 30 min.

2.5. Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline and lysed in the lysis buffer (25 mM Tris–HCl, pH 7.5,

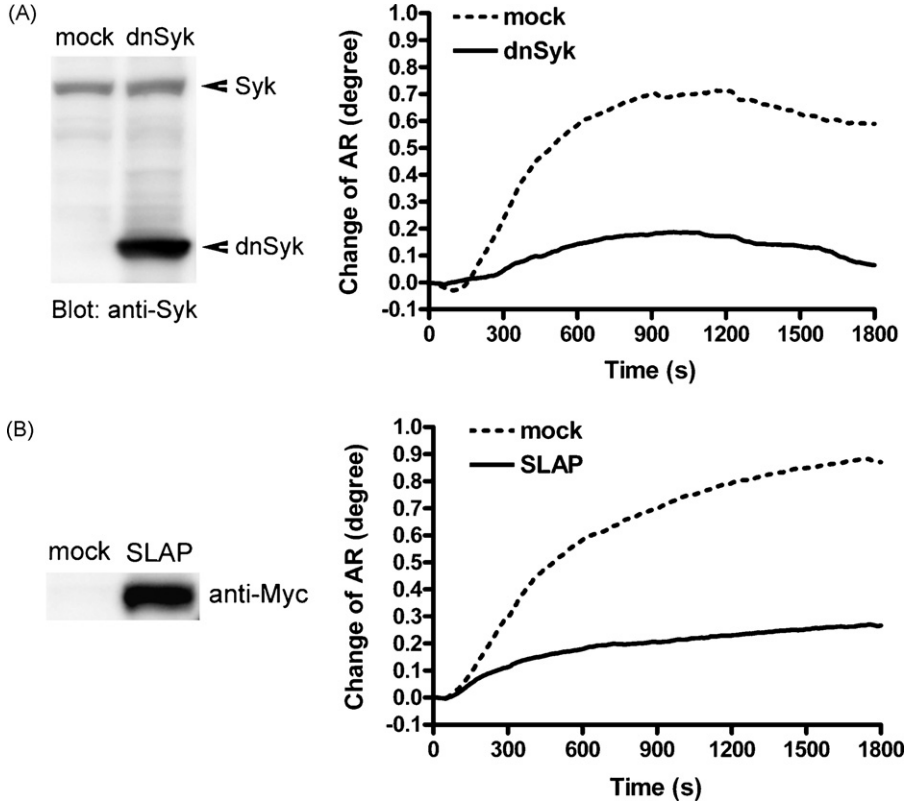

Fig. 1. The antigen-induced increase of AR in RBL-2H3 mast cells is dependent on the activation of Syk. RBL-2H3 mast cells were transfected with vector alone (mock), dnSyk, or SLAP and then cultured overnight. The whole cell lysates were immunoblotted for the detection of dnSyk (A, left panel) or myc-tagged SLAP (B, left panel) by the use of antibodies against Syk or myc-tag, respectively. These transfected cells were cultured on the sensor chips with anti-DNP IgE overnight, stimulated with DNP-HSA for 3 min, and then the change of AR was monitored by SPR-CELLIA for up to 30 min (A and B, right panel). A representative data of three independent experiments is shown for each panel.

150 mM NaCl, 1% nonidet P-40, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 2 μg/ml pepstatin) (Hiragun et al., 2006) and incubated for 30 min on ice. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane blots were probed with the indicated primary antibodies, and the immunoreactive proteins were visualized by the use of horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

3. Results

3.1. The activation of Syk is indispensable for the antigen-induced increase of AR in mast cells

The dominant-negative form of Syk (dnSyk), which contains only the first 261 amino acids (the tandem SH2 domains of Syk), but lacks the catalytic domain of Syk, is known to interfere with the endogenous active Syk kinase (Shi et al., 2006). RBL-2H3 cells overexpressing dnSyk (Fig. 1A, left panel) showed only minimal change of AR, whereas the cells expressing empty vector (mock) exhibited a large change of AR in response to antigen stimulation (Fig. 1A, right panel). The role of Syk on the antigen-induced increase of AR was further validated by transfecting SLAP which has been reported to suppress the antigen-induced activation of Syk by its binding with Syk in RBL-2H3 mast cells (Hiragun et al., 2006). RBL-2H3 cells overexpressing SLAP (Fig. 1B, left panel) showed minimal increase of AR in response to antigen stimulation (Fig. 1B, right panel), as with the cells made to overexpress dnSyk.

3.2. LAT and Gads are indispensable, but Grb2 is dispensable for the antigen-induced increase of AR in mast cells

The linker for activation of T cells, one of the direct substrate of Syk kinase (Siraganian, 2003), has been reported to possess a crucial role on the activation of mast cells, such as calcium signal, degranulation, and cytokine production (Saitoh et al., 2000). The cells made to overexpress dnLAT (Fig. 2A, left panel), which was mutated on tyrosine 171 and 191 to phenylalanine (Zhang et al., 1998), exhibited a much reduced change of AR in response to antigen stimulation as compared with mock-transfected cells (Fig. 2A, right panel). Likewise, the cells overexpressing dnGads (Fig. 2B, left panel), which lacks the internal SH2 domain and interferes with the endogenous active Gads to form a complex with LAT, SLP76, and PLCγ (Kikuchi et al., 2001; Silverman et al., 2006; Houtman et al., 2005), showed a minimal increase of AR in response to antigen stimulation (Fig. 2B, right panel). In contrast to these cells, the cells overexpressing dnGrb2 (Fig. 2C, left panel) showed the same extent of increase of AR with mock-transfected cells (Fig. 2C, right panel), although the activation of Ras-MAP kinase pathway was suppressed (Fig. 2D).

3.3. The PKC isoforms, except PKCβ, possess a negative regulatory role on the antigen-induced increase of AR in mast cells

We next studied the involvement of PLCγ/DAG/PKC pathway in the change of AR induced by antigen. PLCγ is recruited from cytosol to bind to LAT in response to antigen and activates PKC isoforms. As previously reported, phorbol 12-myristate 13-acetate (PMA), a pharmacological mimic of DAG, evoked a small SPR signal without the aggregation of FcεRI (Fig. 3A; Hide et al., 2002). However, the cells made to overexpress PKCα, δ, ε, and θ isoforms showed a reduced extent of increase of AR as compared with the mock-transfected cells in response to antigen stimulation (Fig. 3B, D–F). In contrast to these PKC isoforms, the cells overexpressing PKCβ showed the same extent of increase of AR with mock-transfected cells (Fig. 3C). The kinetics of SPR signals was unchanged by the overexpression of PKC isoforms. Among PKC isoforms, PKCγ and η were omitted according to the finding that these isoforms were not detectable in RBL-2H3 mast cells (Ozawa et al., 1993). These results suggested that among conventional and novel PKC isoforms that are expressed in mast cells, only PKCβ might possibly have a positive regulatory role on the antigen-induced increase of AR.

3.4. The impaired expression of PKCβ causes the reduction of the antigen-induced increase of AR

The role of PKCβ on the antigen-induced SPR signal was validated by the siRNA targeted against PKCβ. The introduction of the siRNA into RBL-2H3 cells impaired the expression of endogenous PKCβ (Fig. 4, left panel) and resulted in the reduction of AR increase (Fig. 4, right panel) in response to antigen stimulation, indicating that PKCβ has a positive regulatory role in the antigen-induced increase of AR.

4. Discussion

The phenomenon of SPR is caused by an evanescent wave, which penetrates into a few 100 nm from the surface of a gold film. Therefore, the SPR biosensors should detect 'something' on and/or just above plasma membrane. In previous studies, we have investigated the mechanism which causes the antigen-induced change of AR by the use of several pharmacologic inhibitors (Hide et al., 2002). An inhibitor of protein tyrosine kinases, genistein, suppressed the entire change of AR in RBL-2H3 cells. In addition, an inhibitor of phosphatidylinositol 3-kinase, wortmanin, substantially suppressed the change of AR in RBL-2H3 cells. These findings indicated that the change of AR, which is monitored by SPR biosensors, could be the reflection of the intracellular signaling events. Recently, Fang et al. monitored the epidermal growth factor (EGF)/EGF receptor-induced activation of living cells by resonant waveguide grating biosensors, and reported that their monitored optical signals were strongly dependent on the Ras/MAPK signaling pathway, and partially dependent on PKC activation (Fang et al., 2005).

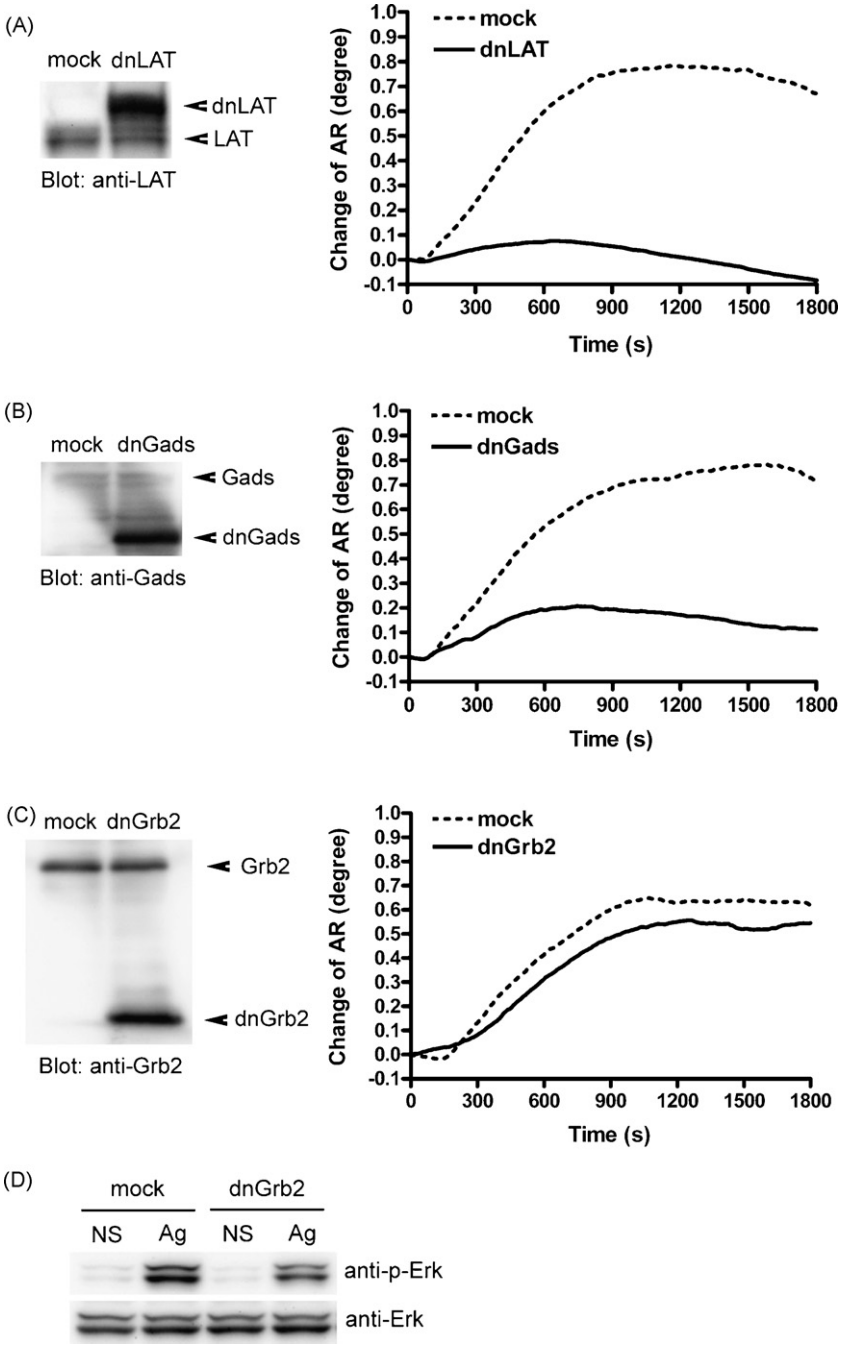

Fig. 2. The antigen-induced increase of AR in mast cells is dependent on the activation of LAT and Gads, but not Grb2. RBL-2H3 mast cells were transfected with mock, dnLAT (LAT-Y171/191F), dnGads (Gads-delSH2), or dnGrb2 (Grb2-delSH2), and then cultured overnight. The whole cell lysates were immunoblotted for the detection of dnLAT (A, left panel), dnGads (B, left panel), or dnGrb2 (C, left panel) by the use of antibodies against LAT, Gads, or Grb2, respectively. These transfected cells were cultured on the sensor chips with anti-DNP IgE overnight, stimulated with DNP-HSA for 3 min, and then the change of AR was monitored by SPR-CELLIA for up to 30 min (A–C, right panel). The dnGrb2-transfected cells were non-stimulated (NS) or stimulated with antigen (Ag) for 15 min, and then lysed. The whole cell lysates were immunoblotted for the detection of phosphorylated Erk or Erk itself (D). A representative data of three independent experiments is shown for each panel.

Syk is an essential proximal tyrosine kinase and initiates the antigen-induced signaling events in mast cells (Fig. 5; Siraganian, 2003). The interfering active Syk kinase by the overexpression of either dominant-negative isoform of Syk or SLAP resulted in a large suppression of change of AR (Fig. 1). Moreover, the interference of the downstream signaling molecule of Syk, LAT and Gads, also caused a suppression of the antigen-induced increase of AR (Fig. 2A and B). However, the interference of Grb2, another signaling molecule that associates with and is activated by LAT did not affect the SPR signals (Fig. 2C). These results definitely indicated that the increase of AR is due to a downstream phenomenon of the activation of Syk, LAT, and Gads. It is well established that the activation of these signaling molecules leads to further signaling events such

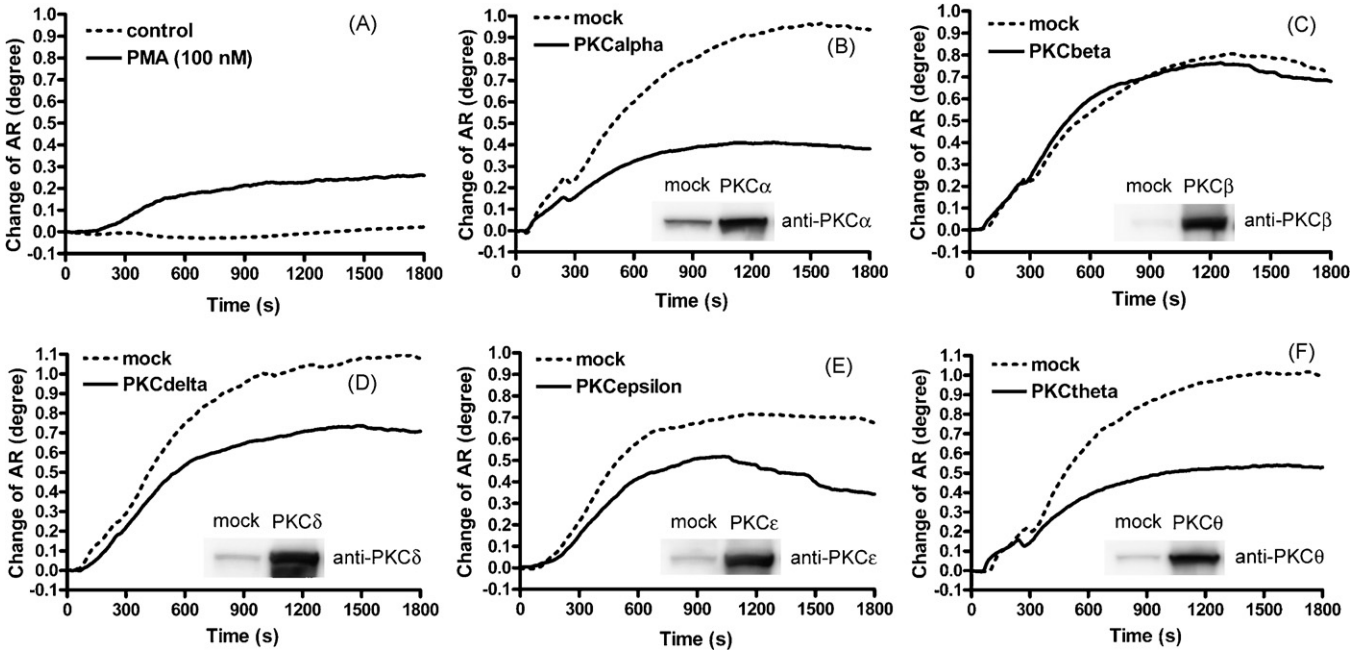

Fig. 3. Overexpression of PKC isoforms, except PKCβ, suppresses the antigen-induced increase of AR in mast cells. The non-transfected RBL-2H3 cells (A), and cells transfected with mock or PKC isoforms (B–F) were cultured on the sensor chips with anti-DNP-IgE overnight, stimulated with 50 nM PMA (A) or DNP-HSA (B–F) for 3 min, respectively, and then the changes of AR were monitored by SPR-CELLIA for up to 30 min. The whole cell lysates were immunoblotted for the detection of the overexpressed PKC isoforms by the use of the indicated antibodies (B–F, inserted figures). A representative data of three independent experiments is shown for each panel.

as the activation of PLCγ, calcium mobilization, activation of PKC, and subsequent degranulation (Fig. 5; Rivera and Gilfillan, 2006; Siraganian, 2003; Gilfillan and Tkaczyk, 2006).

By genetically manipulating the expression of PKC isoforms, we found that PKCβ has a positive role whereas other PKC isoforms have a negative regulatory role in the antigen-induced increase of AR in mast cells (Figs. 3 and 4). Moreover, the result that the excessive PKCβ did not enhance the antigen-induced increase of AR (Fig. 3C) suggests that the change of AR reflects a downstream phenomenon evoked by the active PKCβ rather than the activation of PKCβ itself. Our findings of PKCβ on antigen-induced SPR signals consistent with the previous studies reports the positive regulatory role of PKCβ in antigen-induced histamine release (Ozawa et al., 1993) and cytokine production (Chang et al., 1997) of mast cells. However, among other PKC isoforms, PKCδ (Cho et al., 2004) and PKCθ (Liu et al., 2001) have also been reported to positively regulate degranulation in FcεRI signaling. Since any reaction that increases reflex index in the field of evanescence on a sensor chip causes the increase of AR, intracellular events that inhibit cell activation may also increase AR detected by SPR, or vice versa. Therefore, SPR signals evoked by antigen should reflect the overall PKC activations, regardless of their roles for cell activation. Our observations suggest that PKCβ-induced increase of AR is most dominant among such reactions. The possible explanation of the smaller increase of AR induced by PMA than

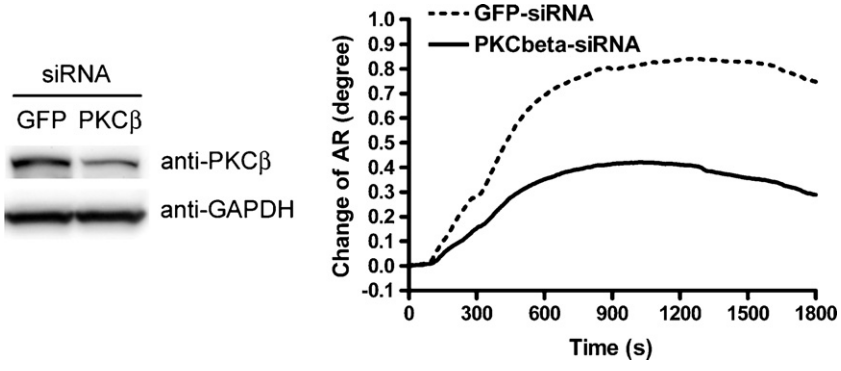

Fig. 4. The siRNA targeted against PKCβ suppresses the antigen-induced increase of AR in mast cells. RBL-2H3 mast cells were transfected with the siRNA against green fluorescent protein (GFP) or PKCβ. The whole cell lysates were immunoblotted for the detection of PKCβ or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by the use of their specific antibodies (left panel). The transfected cells were cultured on a sensor chip with anti-DNP-IgE overnight, stimulated with DNP-HSA for 3 min, and then the change of AR was monitored by SPR-CELLIA for up to 30 min (right panel). A representative data of three independent experiments is shown for each panel.

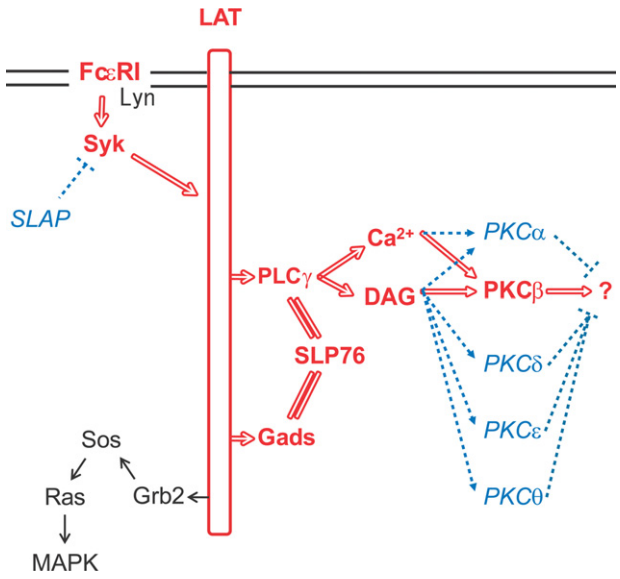

Fig. 5. The intracellular signaling events in antigen-activated mast cells. Following FcεRI aggregation, LAT becomes phosphorylated by Syk. The activated LAT recruits several signaling molecules: Grb2, Gads, PLCγ, and SLP76, and then activates the downstream signaling cascades. SLAP suppresses the activation of Syk by its binding with Syk. The mainstream signaling molecules, which thought to be imperative for the antigen-induced increase of AR, were highlighted in bold. Molecules which inhibit the increase of AR were depicted in italic.

that of antigen stimulation is that the optimal activation of the conventional PKC isoforms, including PKCβ, also needs the increase of intracellular Ca^{2+}, which binds to their C2 domain (Newton, 1995). These cascades are summarized and shown in Fig. 5.

5. Conclusions

The antigen-induced change of AR is dependent on early intracellular signaling events, such as the activation of Syk, LAT, and Gads. The exact molecular events, which are directly expressed as the change of AR, are still unknown, but are demonstrated to be in the downstream events of the activation of PKCβ. The SPR analysis of cells overexpressing and/or having a defect in certain kind of molecules, such as those employed in this study, should be useful not only to identify the event detected as the change of AR, but also to investigate the molecular events specifically on and/or just above the plasma membrane.

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