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FcεRI Signaling of Mast Cells Activates Intracellular Production of Hydrogen Peroxide: Role in the Regulation of Calcium Signals¹

Yoshihiro Suzuki,² Tetsuro Yoshimaru, Takashi Matsui, Toshio Inoue, Osamu Niide, Satoshi Nunomura, and Chisei Ra

Earlier studies, including our own, revealed that activation of mast cells is accompanied by production of reactive oxygen species (ROS) that help to mediate the release of the inflammatory mediators, including histamine and eicosanoids. However, little is known about the mechanisms of ROS production, including the species of oxidants produced. In this study we show that in both the RBL-2H3 mast cell line and bone marrow-derived mast cells, FcεRI cross-linking stimulates intracellular oxidative burst, including hydrogen peroxide (H₂O₂) production, as defined with the oxidant-sensitive dyes dichlorofluorescein and scopoletin and the selective scavenger ebselen (2-phenyl-1,2-benziselenazol-3(2H)-one). The oxidative burst was observed immediately after stimulation and was most likely due to an NAD(P)H oxidase. Experiments using selective pharmacological inhibitors demonstrated that activation of tyrosine kinases and phosphatidylinositol-3-kinase is required for induction of the oxidative burst. Blockade of the oxidative burst by diphenyleneiodonium impaired the release of preformed granular mediators, such as histamine and β-hexosaminidase, and the secretion of newly synthesized leukotriene C₄, whereas selective scavenging H₂O₂ by ebselen impaired leukotriene C₄ secretion, but not degranulation. Sustained elevation of cytosolic calcium through store-operated calcium entry was totally abolished when ROS production was blocked. In contrast, selective depletion of H₂O₂ caused a considerable decrease and delay of the calcium response. Finally, tyrosine phosphorylation of phospholipase Cγ and the linker for activation of T cells, an event required for calcium influx, was suppressed by diphenyleneiodonium and ebselen. These studies demonstrate that activation of the intracellular oxidative burst is an important regulatory mechanism of mast cell responses. *The Journal of Immunology*, 2003, 171: 6119–6127.

Mast cells, basophils, and the rat basophilic leukemia (RBL-2H3) cell line express FcεRI on their cell surface. Aggregation of FcεRI by multivalent IgE-Ag complexes or by anti-FcεRI Abs initiates a cascade of biochemical events that lead to degranulation, secretion of inflammatory mediators, and production of cytokines, contributing to allergic responses (1, 2). Several signal transduction pathways are involved in the process, including activation of Lyn and Syk tyrosine kinases, activation of phospholipase Cγ (PLCγ),³ subsequent activation of a calcium influx pathway that is termed store-operated calcium entry (SOCE), or capacitative calcium entry (3–6).

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radical, are small, diffusible, and

ubiquitous molecules that are produced by virtually every cell type using divergent enzyme systems (7). In addition, activation of neutrophils results in the release of high amounts of superoxide into extracellular spaces, which plays an indispensable role in host defense (8). ROS were originally thought to be an accidental byproduct of respiratory energy production in mitochondria and to be deleterious to biological systems. However, in a growing number of systems, the production of ROS is useful and even required for physiological responses. Recent works have revealed that both nonphagocytic cells and plant cells also release superoxide upon activation, although the production is much smaller than that seen in phagocytic cells (9). It was shown that a variety of tissues and cells, including vascular smooth muscle cells (10, 11), fibroblasts (12–14), B and T cells (15–17), and endothelial cells (18), produce superoxide and/or H₂O₂ upon stimulation. However, little is known about the mechanisms of such production of ROS, although some of them, including smooth muscle cells and endothelial cells, use the phagocytic NADPH oxidase in superoxide production. Increasing evidence has suggested that ROS act as signal intermediates in intracellular signaling to gene activation by cytokines and some growth factors through the modulation of redox-sensitive transcription factors such as NF-κB and AP-1 (19, 20).

Earlier studies have shown that activation of rat peritoneal mast cells is accompanied by increased intracellular levels of ROS. Both pharmacological agents, including gold compounds, compound 48/80, and the calcium ionophore A23187, and physiologically relevant stimulants, including Ag, nerve growth factor, and substance P, stimulate intracellular generation of ROS in rat peritoneal mast cells (21–23), although the nature of the ROS generated has not been defined. However, there are conflicting data regarding

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³ Abbreviations used in this paper: PLC, phospholipase C; BMDC, bone marrow-derived cultured mast cells; [Ca²⁺]_i, cytosolic free calcium concentration; DCF, dichlorofluorescein; DCFH-DA, dichlorodihydrofluorescein-diacetate; DNP, 2,4-dinitrophenol; DPI, diphenyleneiodonium chloride; LAT, linker for activation of T cells; L-NMMA, L-N^G-monomethylarginine; LT, leukotriene; PI-3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride; PY, phosphotyrosine; ROS, reactive oxygen species; SOCE, store-operated calcium entry; TNP, 2,4,6-trinitrophenol.

whether rat peritoneal mast cells actually can produce ROS. Swindle et al. (24) claimed that the production of ROS observed in rat peritoneal mast cells is attributed to contaminating macrophages rather than to mast cells by themselves.

We previously reported that the RBL-2H3 mast cell line produces ROS upon FcεRI cross-linking and releases them into the extracellular spaces (25, 26). The production of ROS is sensitive to diphenyleioidonium (DPI), a broad-spectrum inhibitor of flavoprotein-containing oxidoreductases (25). Similar DPI-inhibitable ROS production occurs in human leukocytes under both physiological (upon Ag stimulation) and pathological (upon allergen challenge) conditions (26). Furthermore, blockade of ROS production by DPI or antioxidants such as (–)-epigallocatechin gallate suppresses IgE-mediated histamine release and leukotriene C₄ (LTC₄) (27). Thus, in these cells ROS may help to mediate the release of the inflammatory mediators, including histamine and eicosanoids. However, previous studies have not addressed the mechanisms of the oxidative burst, including the species of oxidants produced, and those of the regulation of mediator release.

We demonstrate in this study that FcεRI signaling of mast cells activates intracellular oxidative burst, including H₂O₂ production, most likely via an NAD(P)H oxidase and that this is involved in the regulation of calcium influx and mediator release. We also demonstrate that H₂O₂ can regulate tyrosine phosphorylation of PLCγ and the adaptor molecule linker for activation of T cells (LAT), requisite events for the induction of calcium influx.

Materials and Methods

Reagents

Superoxide dismutase, L-N^G-monomethylarginine (L-NMMA), rotenone, scopoletin, ebselen (2-phenyl-1,2-benzisoxazol-3(2H)-one), thapsigargin, and anti-2,4-dinitrophenol (anti-DNP)-IgE mAb (clone SPE-7) were obtained from Sigma-Aldrich (St. Louis, MO). Anti-2,4,6-trinitrophenol (anti-TNP) IgE mAb (clone IgE-3) was purchased from BD Pharmingen Japan (Tokyo, Japan). TNP-BSA conjugate (25 molecules of TNP coupled to one molecule of BSA) was purchased from Cosmo Bio (Tokyo, Japan). DNP-BSA conjugate (33 molecules of DNP coupled to one molecule of BSA) was obtained from Calbiochem (San Diego, CA). DPI, wortmannin, and LY294002 were obtained from Biomol (Plymouth Meeting, PA). Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR). Ab against PLCγ and LAT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-PLCγ 2 (tyrosine 1217) and PLCγ2 mAb were purchased from Cell Signaling (Beverly, MA). Fluo-3AM and lanthanum chloride were obtained from Dojindo (Kumamoto, Japan) and Wako Chemicals (Tokyo, Japan), respectively.

Mice

All experiments were performed according to the guidelines of Nihon University.

Cell culture

The RBL-2H3 cells obtained from the National Institute of Health Sciences (Japan Collection of Research Biosources; cell number JCRB0023) were grown in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Life Technologies, Tokyo, Japan) in a 5% CO₂-containing atmosphere as described previously (25, 26). The RBL-2H3 cells were harvested by incubating them in HBSS-supplemented bicarbonate (pH7.4) containing 1 mM EDTA/0.25% trypsin for 5 min at 37°C. RBL cells were suspended in complete DMEM at a density of 5 × 10⁵ cells/ml and plated on a 24-well plate at the density of 2 × 10⁵ cells/well. Bone marrow-derived cultured mast cells (BMMC) were generated from 4- to 8-wk-old BDF₁ mice as described previously (28). Briefly, cells were incubated in the enriched RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (Nissui Pharmaceutical, Tokyo, Japan), 100 U/ml penicillin and streptomycin (Life Technologies), 10⁻⁴ M 2-ME (Wako, Tokyo, Japan), 110 μg/ml sodium pyruvate (Wako), 1% MEM nonessential amino acid solution (Life Technologies), and 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors. Cells were grown in a 5% CO₂-containing atmosphere at 37°C. After 4–6 wk of

culture, cells were confirmed for cell surface expression of FcεRI and were used for experiments (>95% mast cells).

Cell stimulation

BMMC was sensitized with 1 μg/ml anti-TNP IgE for 60 min at 37°C. IgE-sensitized cells were washed with PBS and suspended in HBSS. The IgE-sensitized cells were stimulated with 30 ng/ml TNP-BSA or 62.5 ng/ml DNP-BSA in HBSS at 37°C for 30 min for measurements of mediators, respectively.

Measurement of intracellular ROS production by fluorometry

A fluorometric assay was used to determine intracellular levels of ROS. DCFH-DA is a nonpolar compound that is converted into a membrane-impermeable nonfluorescent polar derivative (DCFH) by cellular esterase after incorporation into cells. The trapped DCFH is rapidly oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by intracellular peroxides such as hydrogen peroxide. For analyses of RBL-2H3 cells, cells (2 × 10⁵/ml) plated on a 24-well plate were incubated with anti-TNP IgE (0.1 μg/well) overnight at 37°C. For analyses of BMMC, cells grown on a six-well plate were sensitized with anti-TNP IgE (1 μg/well) for 30 min at 37°C. IgE-sensitized cells were incubated with DCFH-DA at a final concentration of 5 μM for 30 min at 37°C, washed twice, and resuspended in HBSS. DCFH-DA-loaded cells were incubated for 8–13 min, and the resultant oxidized DCF was analyzed at 40-s intervals using a microplate fluorometer (Fluoroskan Ascent CF; Labsystems, Helsinki, Finland; excitation and emission at 485 and 527 nm, respectively). In inhibition experiments the agents tested were added to cells just before or 30 min before stimulation.

Measurement of intracellular ROS production by flow cytometry

IgE-sensitized cells (5 × 10⁵/ml) suspended in HBSS were incubated with 5 μM DCFH-DA for 15 min at 37°C in a final volume of 450 μl. Then 50 μl of Ag (10×) was added and incubated at 37°C for the time indicated before harvest. Cells were washed, resuspended in HBSS on ice, and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA; excitation and emission at 488 and 530 nm, respectively). In inhibition experiments the agents tested were added to cells just before or 30 min before stimulation.

Measurement of H₂O₂ production

The production of H₂O₂ was determined by HRP-catalyzed oxidation of fluorescent scopoletin according to the method of Harpe and Nathan (29) with minor modifications. Briefly, IgE-sensitized cells were washed and added with inhibitors in HBSS, pH 7.4, containing 1 mM NaN₃, then stimulated with Ag for 10 min. Cells were pelleted down by centrifugation, and the supernatants were analyzed for H₂O₂ release. To assess intracellular H₂O₂ production, after supernatants were removed, the cells were lysed with 1% Triton X-100, and the cell lysates were analyzed. Samples were added to reaction mixture containing HBSS (pH 7.4), 80 μM scopoletin, and 2 U/ml HRP in a 96-well plate and incubated for 40 min. The decrease in scopoletin fluorescence was measured using a microplate fluorometer (Fluoroskan Ascent CF; Labsystems; excitation and emission at 335 and 460 nm, respectively). The concentration of the oxidant in the samples was calculated using the standard curve, which was made by adding known concentrations of the authentic H₂O₂ instead of the samples.

Degranulation assay

Degranulation was assessed by measuring β-hexosaminidase release. Briefly, cells were activated as described above, and β-hexosaminidase activity in supernatants was determined spectrophotometrically. Briefly, 40 μl of supernatant or cell lysates and 100 μl of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (in 0.4 M citrate and 0.2 M phosphate buffer, pH 4.5) were added to each well of a 96-well plate, and color was developed for 30 min at 37°C. Enzyme reaction was terminated by adding 200 μl of 0.2 M glycine-NaOH, pH 10.7. The absorbance at 405 nm was measured in a microplate reader (Bio-Rad 550; Nippon Bio-Rad Laboratories, Osaka, Japan). Cells were lysed with 0.1% Triton X-100, and the β-hexosaminidase activity of the extracts was measured (total). The β-hexosaminidase activity in nonstimulated cells (the spontaneous release, <5% of the total was subtracted from the enzyme activity in stimulated cells (test). The percentage of β-hexosaminidase released into the supernatant was calculated using the following formula: release (%) = (test – spontaneous)/(total – spontaneous) × 100. In some experiments the supernatant was tested for activity.

LTC₄ secretion assay

LTC₄ release was determined as described previously (25, 26). Briefly, cells were activated as described above, and the LTC₄ content in supernatants was determined by an LTC₄ ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol.

Calcium measurements

Measurement of cytosolic calcium was performed using the calcium-reactive fluorescence probe, Fluo-3, according to the method described by Kunzelmann-Marche et al. (30) with slight modifications as described previously (31). Briefly, RBL-2H3 suspension (10⁶ cells/ml in HBSS) was incubated with 4 μM Fluo-3AM for 30 min at 37°C and then washed twice with HBSS and resuspended in the medium supplemented with 1 mM CaCl₂. To study calcium release and calcium entry separately, aliquots of the Fluo-3-loaded cells were resuspended in the medium supplemented with 1 mM EGTA instead of 1 mM CaCl₂. Fluo-3 fluorescence was monitored at 5-s intervals up to 3 min with a microplate fluorometer (Fluoroskan Ascent CF; Labsystems; excitation and emission at 485 and 527 nm, respectively). The cytosolic free calcium concentration ([Ca²⁺]_i) was calculated using the equation: $[Ca^{2+}]_i = K_d [(F - F_{min}) / (F_{max} - F)]$, where K_d is the dissociation constant of the Ca²⁺-Fluo-3 complex (400 nM), F_{max} represents the maximum fluorescence (obtained by treating cells with 5 μM A23187), F_{min} represents the minimum fluorescence (obtained for A23187-treated cells in the presence of 1 mM EGTA), and F is the actual sample fluorescence.

Immunoblotting

Tyrosine phosphorylation of whole proteins was determined by immunoblotting with the anti-phosphotyrosine (anti-PY) mAb 4G10 as described previously (25). Briefly, samples (cell lysate and the immunoprecipitate) were subjected to SDS-PAGE using a 10% separation gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membrane was incubated with 3% BSA or 0.5% gelatin in PBS at 4°C overnight or for 1 h at room temperature. The PVDF membrane was incubated with 0.2 μg/ml anti-PY mAb 4G10 for 1 h at room temperature and then with HRP-conjugated species-specific anti-mouse Ig (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 1 h at room temperature. To detect PLCγ2 tyrosine phosphorylation, the membranes were probed with anti-phosphor-PLCγ2 (tyrosine 1217)-specific mAb. After extensive washing of the membrane, the immunoreactive proteins were visualized using the ECL kit (Amersham Pharmacia Biotech) according to the recommendations of the manufacturer. The PVDF membrane was exposed to Fuji RX film (Fuji Film, Tokyo, Japan).

Immunoprecipitation

Immunoprecipitation was performed by magnetic bead separation (MACS separation; Miltenyi Biotec, Gladbach, Germany) as recommended by the supplier with minor modifications as described previously (31). Briefly,

10⁷ cells were solubilized with 1 ml of ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM Na₃VO₄, 2 mM EDTA, 0.2 mM *p*-amidinophenylmethanesulfonylfluoride, 20 μM leupeptin, and 0.15 U/ml aprotinin) for 30 min on ice. Cell lysates were centrifuged at 8000 × *g* for 10 min at 4°C. An aliquot (100 μl) of the supernatant was used for analyzing total tyrosine phosphorylation. For analysis of LAT tyrosine phosphorylation, the remainder was incubated with 10 μg of anti-LAT Ab, followed by 50 μl of protein G-conjugated microbeads (MAGmol Protein G Microbeads; Miltenyi Biotec) for 30 min on ice. The samples were applied to microcolumns in the magnetic field of the micro-MACS separator, and the columns were rinsed four times with 200 μl of the lysis buffer and once with 100 μl of low salt wash buffer (50 mM Tris-HCl (pH 8) containing 1% Nonidet P-40). Finally, 50 μl of preheated (95°C) 1× SDS sample buffer was applied to the columns, and eluate containing immunoprecipitate was collected.

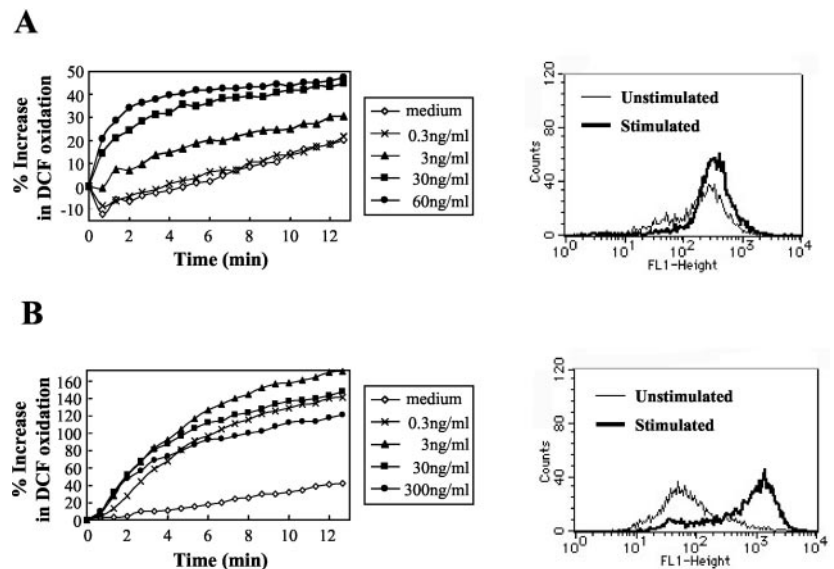
Results

FcεRI cross-linking stimulates intracellular production of ROS

The production of ROS upon FcεRI cross-linking in mast cells was measured using the cell-permeable, oxidation-sensitive dye DCFH-DA. DCFH-DA is nonfluorescent until oxidized by ROS. Cells loaded with the probe were stimulated, and DCF fluorescence was monitored for 13 min. An increase in DCF fluorescence indicates oxidation by peroxides, including hydrogen peroxide, peroxyxynitrite, and/or hydroxyl radical (32).

When DCF fluorescence in RBL-2H3 cells was measured with a microplate fluorometer, a small, but significant, DCF oxidation was observed even in unstimulated (medium-treated) control cells, and this increased with time considerably, indicating ambient ROS production. After addition of Ag (TNP-BSA) to cells sensitized with IgE (clone IgE-3), DCF oxidation was significantly increased above the basal level (Fig. 1A, left). The effect was dose dependent with Ag; a minimal effective dose of 3 ng/ml (1.4-fold increase compared with the level of control cells) and higher concentrations (≥30 ng/ml) of Ag induced maximally an ~2.6-fold increase. The dose (30 ng/ml) was comparable to that required for maximal degranulation, as determined by β-hexosaminidase release. Under these optimal conditions DCF oxidation increased immediately upon FcεRI stimulation, reaching its peak by 5 min and maintaining this level or increasing with time throughout the time periods monitored (at least 13 min). In contrast, when cells were stimulated by lower concentrations (≤3 ng/ml) of Ag, DCF oxidation increased gradually with time without reaching a peak. Basically the same results were obtained with different IgE clone (SPE-7)

FIGURE 1. FcεRI-triggered intracellular ROS production in mast cells. IgE-sensitized RBL-2H3 cells (A) and BMMC (B) were incubated with 5 μM DCFH-DA for 30 min, washed twice, and resuspended in HBSS. DCFH-DA-loaded cells were stimulated with Ag at the indicated concentrations, and ROS-mediated DCF oxidation was measured at 40-s intervals for 13 min using a microplate fluorometer (left). The data are expressed as the percent increase in DCF oxidation above the unstimulated controls and are representative of at least three separate experiments with similar results. IgE-sensitized RBL-2H3 cells (A) and BMMC (B) suspended in HBSS were incubated with 5 μM DCFH-DA for 15 min and then incubated with Ag (30 ng/ml) for 5 min before harvest. Cells were then washed and resuspended in HBSS on ice, and ROS-mediated DCF oxidation was assayed by flow cytometry (right). The data are representative of four separate experiments with similar results.



and Ag (DNP-BSA; data not shown). To verify intracellular production of ROS, we analyzed DCF oxidation by flow cytometry. A substantial increase in DCF oxidation was observed in RBL-2H3 cells after FcεRI cross-linking by IgE-Ag complexes (Fig. 1A, right). A much more prominent increase in DCF oxidation was observed when FcεRI on BMMC was cross-linked (Fig. 1B). The increase was also dose dependent with Ag, and the kinetics were similar to those seen with RBL-2H3 cells. The increase tended to be observed much more clearly with a flow cytometer than with a microplate fluorometer. These results indicate that FcεRI cross-linking stimulates intracellular ROS production in mast cells.

Oxidative burst is mediated by flavoenzymes, most likely NAD(P)H oxidase

We previously showed that DPI, a broad-spectrum inhibitor of flavoprotein-containing oxidoreductases (33), inhibited the release of ROS upon stimulation with IgE-Ag complexes (25, 26). Consistent with previous findings, DPI reduced IgE-mediated ROS production in a dose-dependent manner (Fig. 2A). DPI at concentrations $\geq 11 \mu\text{M}$ was effective, and the agent (100 μM) reduced ROS generation profoundly ($\geq 80\%$ inhibition). DPI at concentrations ranging from 11–100 μM had no inhibitory effect when added to the cells just before stimulation. Because DPI has also been reported to inhibit NO synthase (34) and mitochondrial ROS generation (35), it was possible that the effect of DPI resulted from its actions on them. To test this possibility we also examined the effects of several selective inhibitors on IgE-mediated ROS production. In contrast to DPI, neither L-NMMA, a specific inhibitor of NO synthase (Fig. 2B) nor rotenone, a potent inhibitor of the mitochondrial electron transport chain, had an inhibitory effect on the oxidative burst (Fig. 2C). These results indicate that the IgE-mediated production of ROS is mediated by flavoenzyme, most likely an NAD(P)H oxidase.

Tyrosine kinases and phosphatidylinositol 3-kinase (PI-3K) appear necessary for induction of the oxidative burst

To understand the mechanisms by which FcεRI cross-linking stimulates ROS production, we tested the effects of divergent pharmacological inhibitors of selective signaling pathways on the oxida-

tive burst. When cells were treated with piceatannol, a selective inhibitor of Syk kinase (36), a remarkable reduction in IgE-mediated ROS production was observed (Fig. 3A). PP1, a selective inhibitor for Src-like kinases (37), also inhibited the oxidative burst in a dose-dependent manner (Fig. 3B). In addition, wortmannin, which selectively inhibits PI-3K (38), suppressed the response in a dose-dependent manner (Fig. 3C). Similar effects were observed with another PI-3K inhibitor LY294002 (data not shown). In contrast, U73122, a selective inhibitor for PLC up to 1 μM , had no significant inhibitory effect, although the agent suppressed calcium influx in a dose-dependent manner, with a minimal effective concentration of 0.3 μM (data not shown). Basically the same results were obtained with flow cytometric analyses. These results indicate that tyrosine kinases and PI-3K appear necessary for induction of the oxidative burst.

H₂O₂ is produced upon FcεRI activation in a flavoenzyme-dependent manner

To gain some insight into the species of oxidants produced, we tested the effect of a seleno-organic compound, ebselen, on the oxidative response. This glutathione peroxidase mimetic agent has been shown to possess a unique action. Unlike other antioxidants, the agent selectively scavenges peroxides, including H₂O₂ (39). As we expected, ebselen suppressed IgE-mediated ROS production in a dose-dependent manner. The effect was still observed even when the agent was added to cells just before stimulation (Fig. 4A). Under these conditions, the agent had no effect on ROS produced spontaneously. To ensure H₂O₂ production, we attempted to assess cellular levels of the oxidant directly. HRP-catalyzed oxidation of fluorescent scopoletin has been widely used to assess the occurrence of H₂O₂ in various cell systems. Because the agent became nonfluorescent when binding to the oxidant, the amount of the oxidant can be assessed by measuring the decrease in the fluorescent. Consistent with the idea that ROS are produced spontaneously, a substantial level of H₂O₂ (1–4 μM) was constitutively observed in unstimulated cells, whereas the oxidant released into medium was $< 2 \mu\text{M}$. A higher level (8–15 μM) of H₂O₂ production was observed in cell lysates, but not in supernatants, following

FIGURE 2. Effects of inhibitors for various ROS-producing systems on the oxidative burst. DCFH-DA-loaded cells were incubated for 30 min with DPI (A), L-NMMA (B), or rotenone (C) at the indicated concentrations and then stimulated with Ag (30 ng/ml). ROS-mediated DCF oxidation was measured using a microplate fluorometer as described in Fig. 1. The data are expressed as the percent increase in DCF oxidation above the unstimulated controls and are representative of at least three separate experiments with similar results.

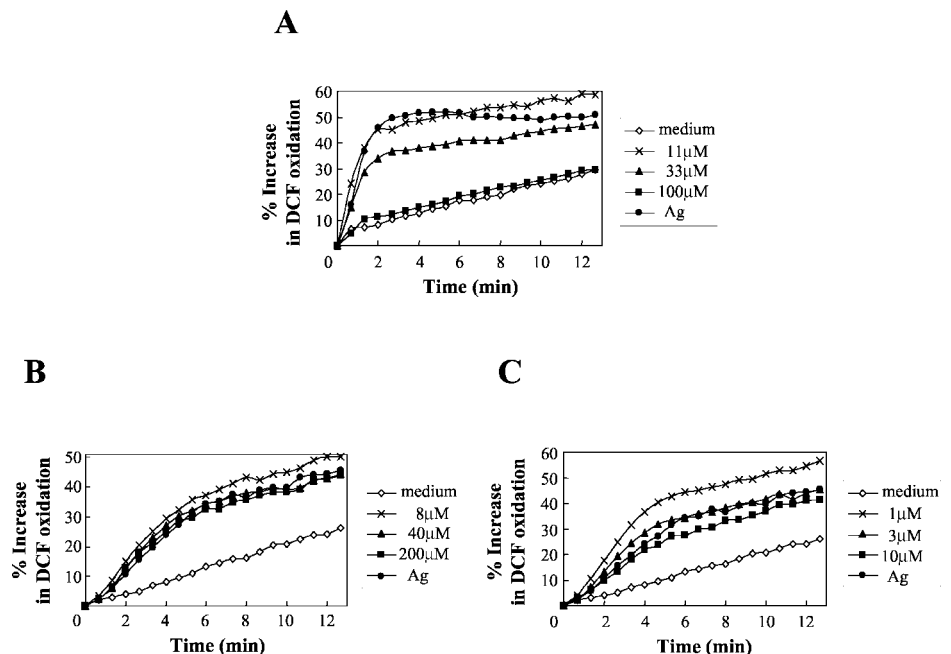
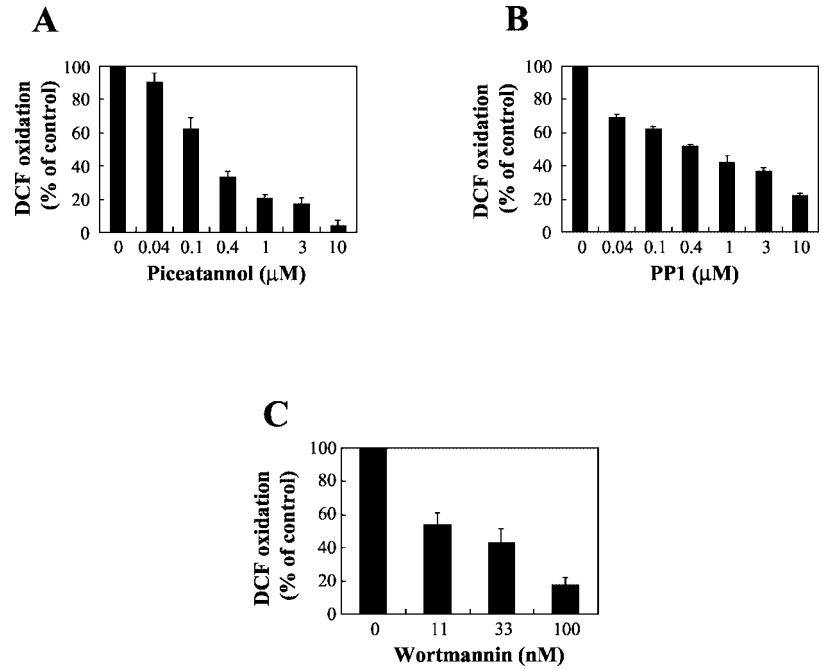


FIGURE 3. Effects of inhibitors of various signaling components on the oxidative burst. DCFH-DA-loaded cells were incubated for 30 min with piceatannol (A), PP1 (B), or wortmannin (C) at the indicated concentrations and then stimulated with Ag (30 ng/ml). ROS-mediated DCF oxidation was measured using a microplate fluorometer as described in Fig. 1. The data are expressed as a percentage of the control value, where DCF oxidation in the cells stimulated with Ag alone is 100%, and represent the mean \pm SE of three separate experiments with similar results.



stimulation with IgE-Ag complexes (Fig. 4B). There was a tendency for a smaller increase to be observed in cells with a higher background H₂O₂ production. The increase in H₂O₂ production was observed by 2 min after stimulation, reaching its peak by 10 min (data not shown). The effect was usually smaller than that of mercuric chloride (100 μ M), which was used as a positive control. Furthermore,

treatment with DPI before stimulation suppressed IgE-mediated H₂O₂ production in a dose-dependent manner, with a minimal effective concentration of 11 μ M (Fig. 4C). The dose was comparable to that at which IgE-mediated DCF oxidation was considerably reduced. In addition, the production of H₂O₂ was suppressed by piceatannol and PP1, like DCF oxidation. Taken together, these results indicate that

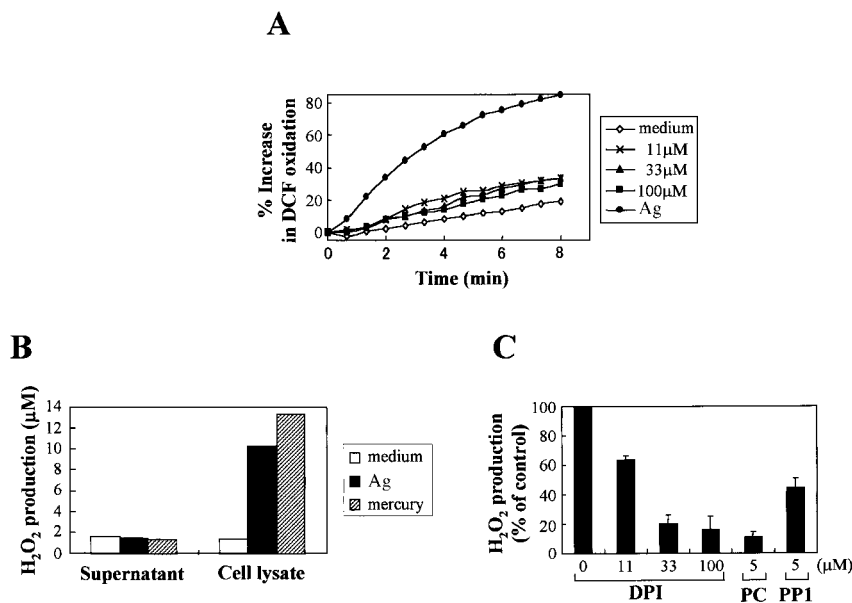


FIGURE 4. Fc ϵ RI-triggered H₂O₂ production. A, DCFH-DA-loaded cells were incubated with ebselen at the indicated concentrations and then stimulated with Ag (30 ng/ml). ROS-mediated DCF oxidation was measured using a microplate fluorometer for 8 min at 40-s intervals. The data are expressed as the percent increase in DCF oxidation above the unstimulated controls and are representative of three separate experiments with similar results. B, IgE-sensitized RBL-2H3 cells were washed, added with inhibitors in HBSS, pH 7.4, containing 1 mM NaN₃, and then stimulated with Ag (30 ng/ml) or mercuric chloride (100 μ M) for 10 min. Cells were pelleted down by centrifugation, and the supernatants were analyzed for H₂O₂ release. After removing supernatants, the cells were lysed with 1% Triton X-100, and cell lysates were analyzed for intracellular H₂O₂ production using a microplate fluorometer as described in *Materials and Methods*. The concentration of H₂O₂ in the samples was calculated using the standard curve, which was made by adding authentic H₂O₂ at a known concentration instead of the samples. The data are representative of three separate experiments with similar results. C, IgE-sensitized RBL-2H3 cells were incubated with DPI, piceatannol (PC), or PP1 at the concentrations indicated and then stimulated with Ag (30 ng/ml), and intracellular H₂O₂ production was assessed as described above. The data are expressed as a percentage of the control, where H₂O₂ production in the cells stimulated with Ag alone was 100%, and represent the mean \pm SE of at least two separate experiments with similar results.

FcεRI cross-linking activates intracellular H₂O₂ production by a flavoenzyme-dependent mechanism.

Blockade of the oxidative burst impairs inflammatory mediator release

If the ROS produced play a role in regulating mast cell activation, then decreasing their levels by blocking their production will be expected to affect mast cell activation. In fact, we previously showed that in RBL-2H3 cells, DPI treatment suppresses IgE-mediated histamine release in a dose-dependent manner with a 50% inhibitory concentration of 50 μM (25). As shown in Fig. 5A, a similar effect was observed with β-hexosaminidase release. High concentrations (≥100 μM) of DPI considerably reduced the release. After a 30-min treatment with DPI up to 200 μM, cell viability was >95% when determined by trypan blue dye exclusion, clearly indicating that the effects were not due to the cytotoxicity of the agent. In contrast, ebselen up to 100 μM had no effect on β-hexosaminidase release (Fig. 5B) and histamine release (data not shown).

Stimulation of RBL-2H3 cells through FcεRI also results in de novo synthesis and the secretion of LTs into medium (1, 2). Therefore, we next examined the effects of DPI and ebselen on LTC₄ secretion. A dose-dependent increase in LTC₄ secretion was observed upon stimulation with IgE-Ag complexes, although the basal LTC₄ contents as well as the amplitude of the increase varied considerably in different experiments. Despite this variability, DPI treatment inhibited IgE-mediated LTC₄ secretion in a dose-dependent manner with a 50% inhibitory concentration of 11 μM (Fig. 5C). DPI at high concentrations (≥33 μM) profoundly inhibited the secretion. Basically the same results were obtained with the anti-FcεRI-stimulated LTC₄ secretion (data not shown). Similarly, ebselen at concentrations ranging from 11–100 μM reduced IgE-mediated LTC₄ secretion in a dose-dependent manner, although at each concentration the agent was less effective than DPI (Fig. 5D). These results indicate that blocking the oxidative burst impairs inflammatory mediator release.

Blocking the oxidative burst impairs calcium influx

The above-mentioned results indicate that blockade of ROS production impairs the release of preformed granular mediators and

newly synthesized LTC₄. Because calcium signals have been implicated to play a role in the induction of these events, there was the possibility that ROS regulate the signals, thereby modulating mediator release. To test this possibility, we determined whether altered ROS production affected calcium signals. Sustained calcium rises were observed after FcεRI cross-linking. Although the calcium response was profoundly impaired in the calcium-free medium, a substantial, but transient, calcium rise up to ~50 nM was still observed (data not shown), indicating that calcium influx occurs by SOCE. This was further confirmed by the fact that the calcium response was totally abolished in the presence of La³⁺ to block calcium influx (Fig. 6A) or by an SOCE antagonist, SK&F96365 (Fig. 6B). DPI treatment suppressed calcium influx in a dose-dependent manner, with a prominent effect at concentrations of ≥33 μM (Fig. 6C). The dose was comparable to that required for strong inhibition of ROS production. Ebselen also dose-dependently suppressed the calcium influx with a minimal effective dose of 11 μM (Fig. 6D). The dose was comparable to that which inhibited the production of ROS, as determined by DCF oxidation. However, unlike DPI, a slower calcium influx was still observed even in the presence of ebselen (100 μM).

Tyrosine phosphorylation of PLCγ and the adaptor molecule LAT is ROS dependent

We next examined the possible roles of ROS in signaling events upstream of calcium influx. Tyrosine phosphorylation-dependent activation of PLCγ occurs upstream and is required for the induction of SOCE. Therefore, we tested whether this event was ROS sensitive. IgE-sensitized cells were treated with 100 μM each of DPI and ebselen and then stimulated with Ag. The stimulated cells were solubilized with detergent, and the lysates were immunoprecipitated using specific Ab against PLCγ, then the immunoprecipitates were analyzed by immunoblotting with the anti-PY mAb 4G10. As shown in Fig. 7A, a substantial increase in tyrosine phosphorylation of PLCγ1 (4.4-fold) was observed upon stimulation with Ag, and DPI and ebselen considerably reduced each increase (for PLCγ1, 71 ± 7 and 47 ± 11% inhibition, respectively; mean ± SD; n = 3). Because we failed to obtain reliable results with PLCγ2 tyrosine phosphorylation in immunoprecipitation experiments, we next performed immunoblotting analysis using anti-

FIGURE 5. Effects of DPI and ebselen on IgE-mediated degranulation and LTC₄ release. IgE-sensitized RBL-2H3 cells were incubated with DPI (A and C) or ebselen (B and D) at the concentrations indicated for 30 min, and then stimulated with Ag (30 ng/ml) for 30 min at 37°C. The β-hexosaminidase activity (A and B) and LTC₄ (C and D) contents in supernatants were determined enzymatically and by ELISA, respectively. The data are expressed as a percentage of the control, where β-hexosaminidase activity or LTC₄ content in the cells stimulated with Ag alone was 100%, and represent the mean ± SE of at least three separate experiments with similar results.

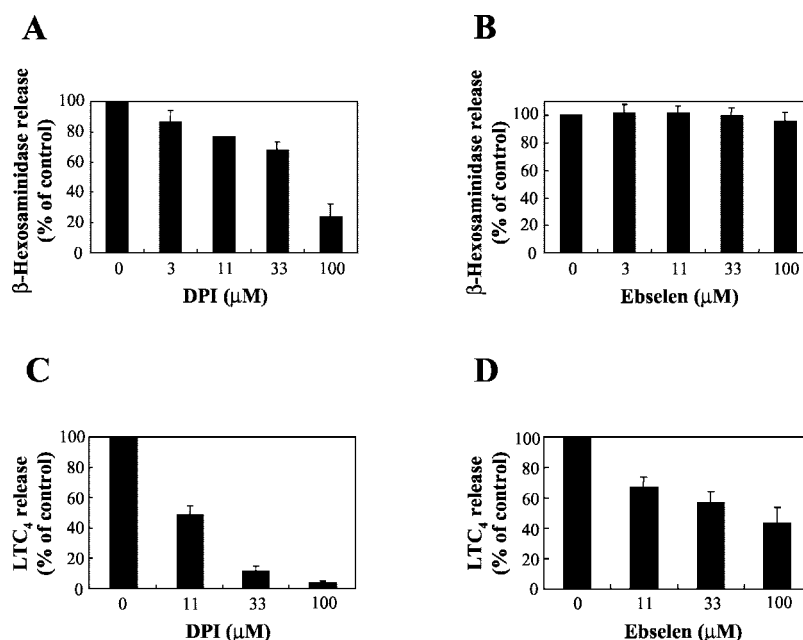
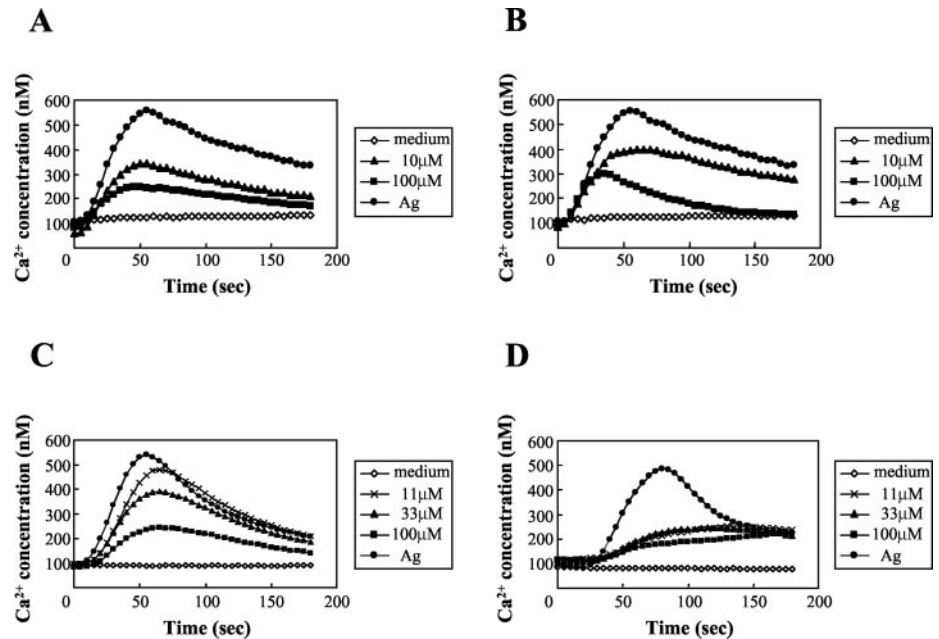


FIGURE 6. ROS regulate calcium influx by SOCE. RBL-2H3 cells were incubated with 4 μ M Fluo-3AM at 37°C for 30 min. The Fluo-3-loaded cells suspended in HBSS supplemented with 1 mM CaCl₂ were stimulated with Ag (30 ng/ml) in the presence or the absence of La³⁺ (A) or SK&F96365 (B) at the concentrations indicated. C and D, The Fluo-3-loaded cells suspended in HBSS supplemented with 1 mM CaCl₂ were incubated for 30 min with DPI at the concentrations indicated and then stimulated with Ag (30 ng/ml; C) or were incubated with ebselen at the concentrations indicated just before stimulation with Ag (30 ng/ml; D). The data shown as the calculated [Ca²⁺]_i are representative of three experiments with similar results.



phospho-PLC γ 2 mAb. The results are shown in Fig. 7B. DPI suppressed PLC γ 2 tyrosine phosphorylation profoundly (90 \pm 12% inhibition; n = 3), whereas ebselen inhibited it moderately (57 \pm 23% inhibition; n = 3). Increased tyrosine phosphorylation of the adaptor molecule LAT also occurs upstream of calcium influx and is shown to play an important role in the induction of capacitative calcium influx. Fc ϵ RI cross-linking remarkably increased LAT tyrosine phosphorylation and DPI, and to a lesser extent, ebselen suppressed the effect (66 \pm 18 and 53 \pm 13% inhibition; n = 3; Fig. 7C). These results indicate that tyrosine phosphorylation of PLC γ and LAT is ROS dependent.

Discussion

Previous studies had suggested that mast cell activation may involve ROS production, but did not address the species of oxidants produced. In this study we provide evidence indicating that Fc ϵ RI signaling of mast cells activates intracellular oxidative burst including H₂O₂ production. Furthermore, the data presented in this report show that the oxidative burst is involved in the regulation of calcium signals, degranulation, and LTC₄ secretion.

It is important to understand the mechanisms by which Fc ϵ RI cross-linking activates the oxidative burst. The present data suggest that the oxidative burst is most likely due to activation of an NAD(P)H oxidase. Because the oxidative burst is abrogated by selective inhibitors of Src-like kinases, Syk kinase, and PI-3K, the activation processes may involve these signaling components. An Src-like kinase, Lyn, and Syk kinases play pivotal roles in Fc ϵ RI signaling leading to calcium signals (1, 2). Furthermore, it was shown that PI-3K is essential for mast cell activation, including degranulation (38). In contrast, all Src-like kinases, Syk kinase, and PI-3K have been shown to be involved in intracellular signaling leading to H₂O₂ production in phagocytic and nonphagocytic cells (40–43). Thus, the oxidative burst appears to locate between these signaling components and calcium signals.

The involvement of PI-3K in the oxidative burst also provides a clue about the enzymes involved. Karlsson et al. (43) have shown that phorbol myristate acetate induces two different types of oxidative responses of neutrophils. Besides the respiratory burst, the agent induces another type of oxidative response that does not result in the release of superoxide but in intracellular production of

the radical. Furthermore, the authors suggested that two pools of NADPH oxidase exist: one localized in the plasma membrane and the other in the granule membranes. In addition, activation of these pools differs regarding involvement of PI-3K; the release of superoxide is independent of PI-3K, whereas intracellular production

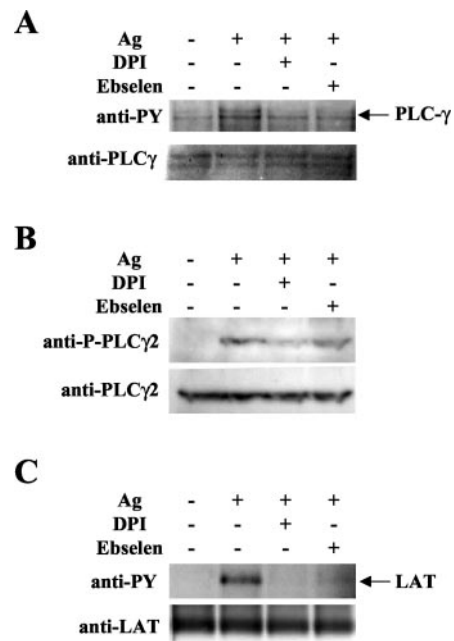


FIGURE 7. ROS-dependent tyrosine phosphorylation of PLC γ and LAT. A and C, IgE-sensitized RBL-2H3 cells were incubated with 100 μ M each of DPI or ebselen for 30 min and then stimulated with Ag (30 ng/ml) for 2 min at room temperature. The stimulated cells were lysed with detergent, PLC γ (A) and LAT (C) were precipitated from the cell lysates using specific Ab, and their tyrosine phosphorylation was analyzed by immunoblotting with the anti-PY mAb. To verify equal loading, the blots were reprobed with mAb against the proteins themselves. The data are representative of three separate experiments with similar results. B, The cells stimulated as described above were subjected to immunoblotting analysis using anti-phospho-PLC γ 2 mAb. To verify equal loading, the blots were reprobed with anti-PLC γ 2 mAb.

of the radical is dependent on the enzyme. We previously showed that FcεRI cross-linking results in the release of ROS, primarily superoxide, into medium (25, 26). Furthermore, our current study reveals a small, but significant, intracellular production of superoxide upon FcεRI cross-linking. Therefore, a similar PI-3K-dependent pathway might play a role in the oxidative burst observed in this study.

Previous studies using divergent antioxidants suggested that FcεRI signals may involve or be sensitive to ROS (22, 25–27, 44, 45). Our findings that DPI, an inhibitor of the oxidative burst, suppresses both degranulation and LTC₄ secretion strongly suggest the importance of ROS in these responses. The fact that ebselen, which shows glutathione peroxidase-like activity, also suppresses LTC₄ secretion supports this idea. Unlike DPI, ebselen has little effect on the release of preformed granular mediators such as histamine and β-hexosaminidase, although also these two responses were shown to be ROS sensitive previously (22, 25, 26, 44, 45) and in the present work. These observations indicate that another species of oxidant whose production is sensitive to DPI treatment might be more important than H₂O₂ in regulating degranulation. DPI is known to be a potent inhibitor of superoxide production, and as mentioned above, FcεRI cross-linking can activate the production. Therefore, superoxide is a likely candidate for such oxidant. We are currently investigating this possibility.

A few processes proximal to FcεRI aggregation, including activation of tyrosine kinases and elevation of cytosolic calcium, play common roles in different cellular responses, whereas processes distal to the receptor activation are involved in the specified responses. Therefore, the wide effects of blocking the oxidative burst strongly suggest the roles of oxidants in a somewhat early signaling event(s) commonly involved in these different responses. We demonstrated in this report that calcium influx by SOCE is sensitive to ROS. It is thought that depletion of calcium stores by either inositol-3,4,5-triphosphate-dependent or -independent pathways results in activation of calcium release-activated Ca²⁺ channels. Thus, activation of PLCγ and the resulting inositol-3,4,5-triphosphate production are critical for the induction of SOCE. Our data demonstrated that the PLCγ tyrosine phosphorylation that is required for their activation is abolished when ROS production is impaired. We also demonstrated that LAT tyrosine phosphorylation is ROS sensitive. In current model the adaptor molecule LAT plays an essential role as scaffold in the formation of macromolecular signaling complex for which tyrosine phosphorylation of LAT is required (46). In fact, it has been shown that LAT deficiency considerably impairs multiple events, including calcium influx, degranulation, and cytokine production in BMDC (46). Interestingly, these effects of LAT deficiency are very similar to those of impaired ROS production observed in the present study. Collectively, endogenous oxidants appear to regulate PLCγ activation and LAT-organized formation of the signaling complex, thereby contributing to the regulation of calcium influx.

In summary, we demonstrated in this study that FcεRI signaling induces intracellular oxidative burst, including H₂O₂ production, and that this event is deeply involved in the regulation of calcium influx and mediator release. The studies suggest that activation of intracellular oxidative burst is an important regulatory mechanism of mast cell activation. Further investigations of the biochemical and biological consequences of increased ROS in mast cells are currently ongoing in our laboratory.

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