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Silver Activates Calcium Signals in Rat Basophilic Leukemia-2H3 Mast Cells by a Mechanism That Differs from the FcεRI-Activated Response¹

Yoshihiro Suzuki,^{2*} Tetsuro Yoshimaru,^{*} Takashi Matsui,^{*} and Chisei Ra[†]

We previously showed that silver stimulates degranulation and leukotriene (LT) C₄ production in rat basophilic leukemia mast cells and now show that silver induces these events by a mechanism that differs from the FcεRI-mediated response. In common with FcεRI cross-linking, silver induced tyrosine phosphorylation of extracellular signal-regulated kinases and furthermore, PD98059, a specific inhibitor of extracellular signal-regulated kinase kinase dose-dependently inhibited the silver-induced LTC₄ production. In contrast to FcεRI cross-linking, silver had no effect on the production of IL-4 and TNF-α, indicating that different mechanisms are involved in the activation by these two stimuli. In line with this, silver had no or only marginal effect on the tyrosine phosphorylation of FcεRIβ, Lyn, Syk, and linker for activation of T cells, the early and crucial events in FcεRI signaling. Silver induced calcium signals that were involved in the metal-induced degranulation, but not LTC₄ production. Unlike Ag, the silver-induced calcium signals were resistant to the depletion of thapsigargin-sensitive calcium stores and the inhibition of tyrosine kinases and phospholipase Cγ. These findings indicate that silver activates mast cells by bypassing the early signaling events required for the induction of calcium influx. Our data strongly suggest the existence of an alternative pathway bypassing the early signaling events in mast cell activation and indicate that silver may be useful for analyses of such alternative mechanisms. *The Journal of Immunology*, 2002, 169: 3954–3962.

There are growing needs to understand effects of environmental heavy metals on the immune system. It has been shown that mercury causes type I and IV allergic reactions, and in vitro sensitizes rat peritoneal mast cells for the FcεRI-mediated mediator release (1–4). Mercury is well known to induce autoimmunity in genetically susceptible humans (5) or experimental animals. Administration of mercuric chloride to the Brown Norway rat induces an autoimmune syndrome characterized by generation of autoantibodies (6–8), tissue injury in the form of necrotizing vasculitis, and marked increase of total serum IgE concentration (9–11). A similar disease was observed in mercury-treated New Zealand rabbits and mice (12–14).

Although less is known about effects of other heavy metals on immune cells, interestingly, Hultman et al. (15) have reported that silver nitrate also highly induces autoimmunity in genetically susceptible mice, causing the production of autoantibodies similar to those observed in mercury-induced autoimmunity. Furthermore, like mercuric chloride, silver nitrate has been shown to enhance the production of superoxide by neutrophils stimulated with chemotactic peptide (16). These observations indicate that mercury and silver share some biological effects on the immune cell system.

In line with this, we have recently demonstrated that similar to mercuric chloride, silver nitrate strongly induces degranulation and leukotriene (LT)³ C₄ production in rat basophilic leukemia (RBL)-2H3 cells (17). Silver nitrate at subtoxic concentrations (as low as 3 μM) is effective enough, whereas other heavy metals including zinc, copper, cadmium, and nickel have little effect on degranulation at concentrations up to 1 mM. The effects of silver can be observed as rapidly as 5 min after administration. Furthermore, silver induces increased tyrosine phosphorylation of the focal adhesion kinase (FAK), an important event in degranulation pathway downstream of the induction of calcium influx and/or the activation of protein kinase C (18). These findings clearly indicate that activation by silver is the result of intracellular signaling rather than that of cytotoxicity or nonspecific binding to sulfhydryl group-containing substances. In the present study, we attempted to understand the molecular mechanisms of the silver-induced cell activation and demonstrate that silver activates mast cells by a mechanism that bypasses the early events in FcεRI signaling.

Materials and Methods

Reagents

Silver nitrate, silver sulfate, mercuric chloride, sodium nitrate, and lanthanum chloride were obtained from Wako Pure Chemicals (Osaka, Japan). All other heavy metal salts used were the components of a skin patch test kit for metal allergy (Kyokuto Pharmaceutical, Tokyo, Japan). A23187, HRP, and anti-DNP-IgE mAb SPE-7 were obtained from Sigma-Aldrich (St. Louis, MO). DNP-bovine albumin conjugate (33 molecules of 2,4-DNP coupled to 1 molecule of BSA) was obtained from Calbiochem (San Diego, CA). PD98059, U-73122, and U-73343 were obtained from Biomol (Plymouth Meeting, PA). Fluo-3-acetoxymethyl ester (fluo-3-AM) was

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³ Abbreviations used in this paper: LT, leukotriene; RBL, rat basophilic leukemia; FAK, focal adhesion kinase; fluo-3-AM, fluo-3-acetoxymethyl ester; PY, phosphotyrosine; LAT, linker for activation of T cells; ERK, extracellular signal-regulated kinase; PVDF, polyvinylidene difluoride; MAPK, mitogen-activated protein kinase; pp, phosphorylated protein; SOCE, store-operated calcium entry; [Ca²⁺]_i, cytosolic free calcium concentration; Tg, thapsigargin; PL, phospholipase.

purchased from Molecular Probes (Eugene, OR). The anti-phosphotyrosine (PY) mAb 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Abs against Lyn, Syk, linker for activation of T cells (LAT), and FAK were all from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated species-specific anti-mouse and anti-rabbit Ig were obtained from Amersham (Bucks, U.K.).

Cell stimulation

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 5% CO₂. The RBL-2H3 cells were harvested by incubating them in HBSS containing 1 mM EDTA, 0.25% trypsin for 5 min at 37°C. RBL cells were suspended in complete DMEM at concentrations of 5 × 10⁵ cells/ml, and plated on a 24-well plate at the density of 2 × 10⁵ cells/well. Then, the cells were sensitized with 1 μg/ml of anti-DNP IgE overnight at 37°C. IgE-sensitized cells were washed with PBS and suspended in DMEM containing 20 mM HEPES, pH 7.4 (HEPES-DMEM). The IgE-sensitized cells were stimulated with 1 μg/ml of DNP-BSA in HEPES-DMEM at 37°C for 30 min for measurements of mediator and cytokine release. When the effect of silver or other metals was tested, RBL-2H3 cells were incubated with the corresponding chemicals for 30 min, and supernatant was analyzed. For the analysis of overall tyrosine phosphorylation, cells were incubated at 37°C for the time indicated.

Histamine release assay

Histamine release was determined as described previously (17, 19). Briefly, after stimulating RBL-2H3 cells with Ag or metal ions tested at 37°C for 30 min, supernatants were collected and histamine content in supernatants was determined using a commercially available ELISA kit (ICN Pharmaceuticals, Costa Mesa, CA) according to the manufacturer's protocol. Cells were lysed with 0.05% Triton X-100, and histamine content of the extracts was measured (total). The amount of histamine in nonstimulated cells (the spontaneous release, around 12% of total) was subtracted from the amount of histamine in stimulated cells (test). The percentage of histamine released into the supernatant was calculated by using the following formula:

$$\text{release \%} = (\text{test} - \text{spontaneous}) / (\text{total} - \text{spontaneous}) \times 100$$

LTC₄ production assay

LTC₄ release was determined as described previously (19). Briefly, after stimulating RBL-2H3 cells with Ag or metals tested at 37°C for 30 min, supernatants were collected. LTC₄ content in supernatants was determined by a LTC₄ ELISA kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol.

Cytokine production assay

IL-4 and TNF-α contents in supernatant were determined by a solid phase sandwich ELISA kit (BioSource International, Camarillo, CA) for rat IL-4 and TNF-α, respectively, according to the manufacturer's protocol. Briefly, the assay plate wells had been coated with specific Ab to rat IL-4 or TNF-α. Samples, including standards of known rat IL-4 or TNF-α contents were added to these wells, followed by the addition of a biotinylated second Ab. After incubating for 2 h (1.5 h for TNF-α) at room temperature, these wells were washed four times with wash buffer and added with streptavidin-HRP conjugate. After incubating for 30 min (45 min for TNF-α), these wells were washed four times to remove the entire unbound enzyme, and added with a substrate solution. These wells were then allowed to stand in the dark for 30 min at room temperature to develop color. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad 550; Nippon Bio-Rad Laboratories, Osaka, Japan).

Immunoprecipitation

Immunoprecipitation was performed by magnetic bead separation (MACS separation; Miltenyi Biotec, Gladbach, Germany) as recommended by the supplier with minor modifications. 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*-amidinophenylmethanesulfonyl fluoride, 20 μM leupeptin, and 0.15 U/ml aprotinin) for 30 min on ice. The cell lysate was centrifuged at 12,000 × *g* for 10 min at 4°C. An aliquot (100 μl) of the supernatant was used for analyzing tyrosine phosphorylation of whole proteins. For analysis of the tyrosine phosphorylation of signaling molecules, the remainder was incubated with 5–10 μg of Ab against each molecule 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 μ columns in the magnetic field of the μ MACS separator and the columns were rinsed four times with 200 μl of 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.

Immunoblotting

Tyrosine phosphorylation of whole proteins, extracellular signal-regulated kinases (ERKs), and signaling molecules was determined by immunoblotting with the anti-PY mAb 4G10. 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 1 h at room temperature. For analysis of tyrosine phosphorylation of whole and signaling molecules, the PVDF membrane was incubated with 0.2 μg/ml of the anti-PY mAb for 1 h at room temperature and then with HRP-conjugated species-specific anti-mouse Ig (Amersham) for 1 h at room temperature. For analysis of ERK tyrosine phosphorylation, the membranes were incubated with 0.5 μg/ml of the phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr²⁰²/Tyr²⁰⁴) mAb (New England Biolabs, Hertfordshire, England) for 1 h at room temperature and then with HRP-conjugated species-specific anti-mouse Ig (Amersham) for 1 h at room temperature. After extensive washing of the membrane, the immunoreactive proteins were visualized using an ECL kit (Amersham) according to the recommendations of the manufacturer. The PVDF membrane was exposed to Fuji RX film (Fuji Film, Tokyo, Japan).

Measurement of cytosolic free calcium concentration ([Ca²⁺]_i)

Measurement of [Ca²⁺]_i was performed using the calcium-reactive fluorescence probe, fluo-3, according to the method described by Kunzelmann-Marche et al. (20) with slight modifications. Briefly, RBL-2H3 suspension (10⁷ cells/ml in 5% HBSS) were incubated with 4 μM fluo-3-AM for 30 min at 37°C and then washed twice with 5% HBSS and resuspended in the medium supplemented with 1 mM CaCl₂. To study Ca²⁺ release and Ca²⁺ 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 by a microplate fluorometer (Fluoroskan Ascent CF; Labsystems, Helsinki, Finland) (excitation and emission at 485 and 527 nm, respectively). [Ca²⁺]_i was calculated using the equation: [Ca²⁺]_i = K_d((F - F_{min})/(F_{max} - F)), where K_d is the dissociation constant of the Ca²⁺-fluo-3 complex (450 nM). F_{max} represents the maximum fluorescence (obtained by treating cells with 5 μM A23187), and F_{min} represents the minimum fluorescence (obtained for A23187-treated cells in the presence of 1 mM EGTA). F is the actual sample fluorescence.

Results

Silver specifically induces degranulation and LTC₄ production in RBL-2H3 mast cells

RBL-2H3 mast cells are mucosal mast cell types that are major models for the study of IgE-mediated degranulation (21, 22). We previously showed that RBL-2H3 cells released histamine in response to the stimulation with silver and mercury, whereas other metals including zinc, copper, cadmium, and nickel were without effect (17). Consistent with the previous data, silver nitrate strongly induced histamine release from RBL-2H3 cells (Fig. 1A). This effect was observed as rapidly as 5 min after the addition of the chemical and was dose-dependent with a minimal effective dose of 3.1 μM (24.1% release). The effect of silver nitrate (10 μM) (41.3 ± 5.2% release, mean ± SE, *n* = 3) was stronger than that of Ag (1 μg/ml) (20.5 ± 1.1% release) and at a high concentration (100 μM) (60 ± 7% release), the chemical was as potent as A23187 (2 μM) (70.3 ± 9.4% release). Silver sulfate, but not sodium nitrate, showed a similar effect, indicating that the effect is attributed to silver but not to nitrate. After a 30-min treatment with silver nitrate at concentrations up to 100 μM, cell viability was >95%, when determined by trypan blue dye exclusion, clearly indicating that the effect was not due to the cytotoxicity of the metal.

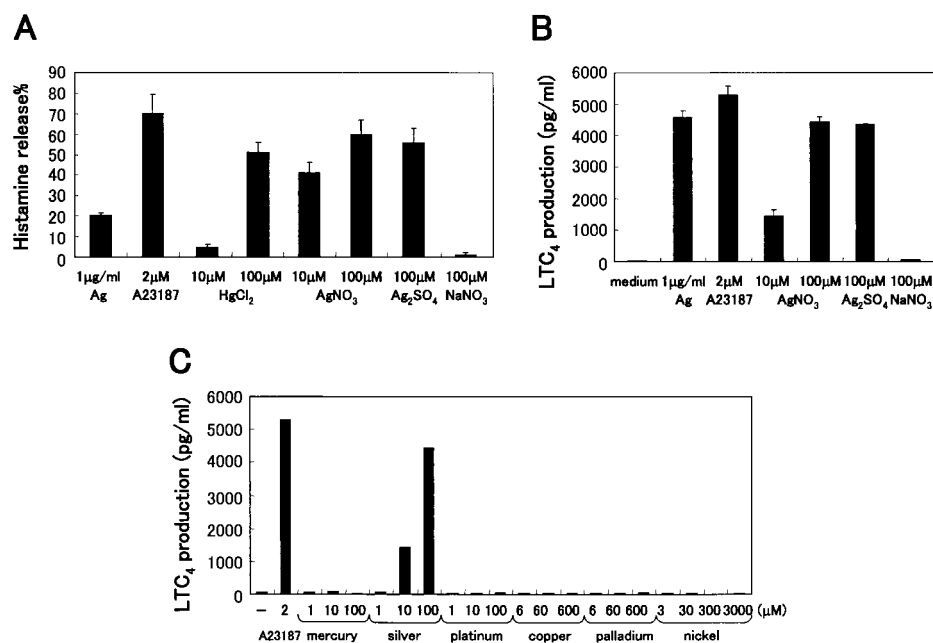


FIGURE 1. Silver-induced degranulation and LTC₄ production. RBL-2H3 cells were suspended in complete DMEM at a concentration of 5×10^5 cells/ml, and plated on a 24-well plate at the density of 2×10^5 cells/well. RBL-2H3 cells were sensitized with anti-DNP IgE (1 $\mu\text{g/ml}$) overnight at 37°C. Cells were washed with PBS and resuspended in DMEM containing 20 mM HEPES, pH 7.4 (HEPES-DMEM). IgE-sensitized cells were incubated with Ag (1 $\mu\text{g/ml}$) for 30 min at 37°C. When the effect of the heavy metals or A23187 was tested, cells not pre-exposed to IgE were incubated with chemicals tested at the concentrations indicated or with A23187 (2 μM) for 30 min at 37°C. C, HgCl₂, AgNO₃, H₂PtCl₆, CuSO₄, PdCl₂, and NiSO₄ were used as compounds of mercury, silver, platinum, copper, palladium, and nickel, respectively. Histamine (A) and LTC₄ contents (B and C) in supernatants were determined by ELISA as described in *Materials and Methods*. Results are mean \pm SE of three separate experiments with similar results.

In our previous study, silver was also found to induce LTC₄ production (17). To test the specificity of the effect, RBL-2H3 cells were incubated with varying concentrations of a range of compounds containing heavy metals such as mercury, silver, zinc, copper, cadmium, nickel, palladium, and platinum, and LTC₄ production was determined by ELISA as described in *Materials and Methods*. The basal level of LTC₄ varied considerably in different experiments. Despite this variability, silver nitrate could reproducibly induce significant LTC₄ production (Fig. 1B). Silver nitrate (100 μM) could induce LTC₄ production as effectively as Ag (1 $\mu\text{g/ml}$). Silver sulfate, but not sodium nitrate, also induced LTC₄ production, indicating that the effect is attributed to silver rather than to nitrate. The induction was observed within 5 min after the addition of silver, reaching a maximal level at 30 min (17). The effect of silver nitrate was dose-dependent with a minimal effective dose of 3.1 μM (11-fold stimulation compared with basal levels) and a maximal effective dose of 100 μM (74-fold stimulation). In contrast, all other metal compounds including mercury had little effect on LTC₄ production at various concentrations ranging from 1 μM to 3 mM (Fig. 1C).

Silver does not induce the production of IL-4 and TNF- α

Mast cells are known to produce various cytokines including IL-4 and TNF- α in response to IgE-Ag challenge. Therefore, we next examined whether silver could also induce cytokine production. The addition of Ag to IgE-sensitized RBL-2H3 cells resulted in an increase in IL-4 production, which could be initially detected at 2 h after the stimulation and a maximal response was observed at 4 h (Fig. 2A). The effect was dose-dependent with an optimal dose of 250 ng/ml (data not shown). In contrast, at longest (4 h), silver nitrate (100 μM) had no effect on IL-4 production (Fig. 2A) and without effect at various concentrations ranging from 3 to 100 μM . Also silver sulfate had no effect. In contrast, consistent with the

previous report (4) mercuric chloride induced a substantial amount of IL-4 production. The production was initially observed at 2 h after the stimulation and increased with time at longest another 2 h (Fig. 2A). The effect was dose-dependent with a minimal effective dose of 25 μM (data not shown). IgE-Ag stimulation also induced TNF- α production and the effect was initially observed at 1 h after stimulation, reaching a maximal level at 2 h and maintaining during another 2 h (Fig. 2B). The effect was dose-dependent with an optimal dose of 250 ng/ml (data not shown). In contrast, neither silver nitrate (100 μM) nor mercuric chloride (100 μM) had effects of at longest 4 h with almost no effect at varying concentrations ranging from 3 to 100 μM (Fig. 2B). Silver sulfate also had no effects. These results indicate that silver has no effects on the production of these two cytokines.

Role of ERKs in silver-induced LTC₄ production but not in degranulation

Cross-linking of Fc ϵ RI induces the tyrosine phosphorylation and activation of MAPK (23–25), resulting in the activation of cytosolic phospholipase (PL) A₂ and release of arachidonic acid (25). Thus, the activation of MAPK seems to play an important role in Fc ϵ RI-dependent LTC₄ production, we next examined whether silver induced LTC₄ production through the MAPK pathway. To address this possibility, we determined whether silver could induce the tyrosine phosphorylation of MAPK. Upon Fc ϵ RI cross-linking or silver exposure, tyrosine phosphorylation of ERKs was analyzed by immunoblotting with specific Ab against the phospho-p44/42 ERKs (Thr²⁰²/Tyr²⁰⁴). In accordance with considerable variation in the level of LTC₄ production in unstimulated cells, the basal level of phosphorylation of ERK1 and ERK2 varied considerably in different experiments. Despite this variability, silver induced an increase in the tyrosine phosphorylation of ERK1 and ERK2, as did Ag (Fig. 3). The effect was dose-dependent with a

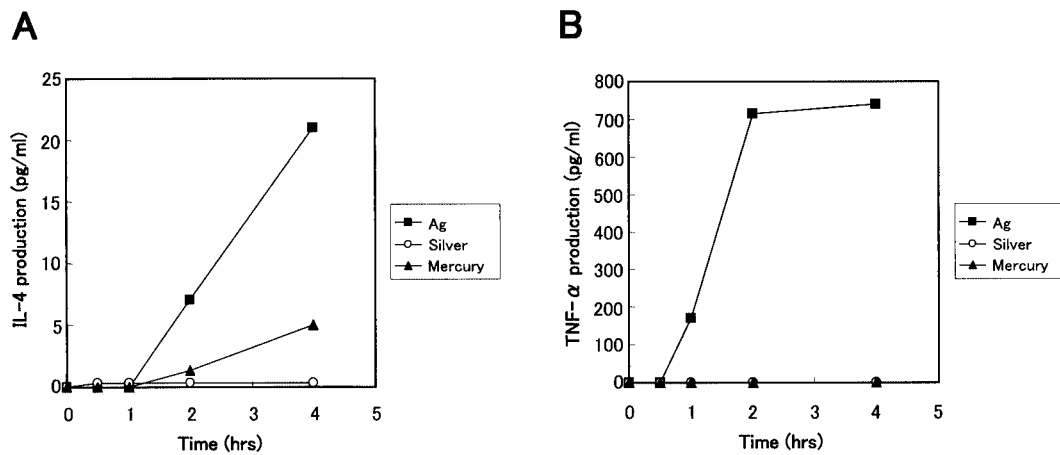


FIGURE 2. Silver has no effect on cytokine production. Cells were incubated with Ag (1 $\mu\text{g/ml}$) or the chemicals indicated for 0.5, 1, 2, and 4 h at 37°C, and IL-4 (A) or TNF- α content (B) in supernatants was determined by ELISA. Results are representative of three different experiments with similar results.

minimal effective concentration of 1 μM . Mercury also dose-dependently increased the tyrosine phosphorylation of ERK1 and ERK2 (Fig. 3), although the metal had no effect on LTC₄ production.

We next examined the effect of the specific inhibitor of ERK kinase, PD98059 (26, 27), on mediator release. PD98059 at concentrations ranging from 6.25 to 50 μM had little effect on histamine release induced by Fc ϵ RI cross-linking (Fig. 4A) or by silver (Fig. 4B). In contrast, the chemical dose-dependently inhibited LTC₄ production induced by Fc ϵ RI cross-linking (Fig. 4C) and by silver (Fig. 4D). The minimal effective dose was 6.25 μM (35.8 \pm 4.1% inhibition for Ag, 41.6 \pm 8.5% inhibition for silver, mean \pm SE) and high concentrations (>12.5 μM) of the chemical inhibited the release induced by Fc ϵ RI cross-linking, as well as by silver, remarkably (>76% inhibition). These results suggest that ERKs play a role in the silver-induced LTC₄ production but not in degranulation, as observed with Fc ϵ RI cross-linking.

Silver has no effect on tyrosine phosphorylation of Fc ϵ RI β , Lyn, Syk, and LAT

In agreement with our previous report (17), exposure of RBL-2H3 cells to silver nitrate resulted in an increase of tyrosine phosphorylation of several proteins (Fig. 5, *left panel*). The increase in most tyrosine-phosphorylated proteins reached a maximal level as rapidly as 2 min after the addition of silver and returned to the basal levels at 10 min. This effect was very transient as compared with

that of Fc ϵ RI cross-linking, which could be observed, at shortest, until 10 min after the stimulation (Fig. 5, *left panel*).

We further assessed the capability of silver to induce tyrosine phosphorylation of several important signaling molecules including Fc ϵ RI β , Lyn, Syk, and LAT. Cells stimulated for 2 min were lysed, immunoprecipitated with Ab against each molecule, and analyzed by immunoblotting with an anti-PY mAb. As shown in Fig. 5, a clear increase in the tyrosine phosphorylation of Fc ϵ RI β , Lyn, Syk, and LAT was observed after Fc ϵ RI cross-linking. In contrast, silver nitrate had no effect on the tyrosine phosphorylation of Fc ϵ RI β , Lyn, and LAT, with a marginal effect on the tyrosine phosphorylation of Syk (Fig. 5, *right panel*) and basically the same results were obtained with silver sulfate (data not shown). These results show that unlike Fc ϵ RI cross-linking, silver has no effect on these signaling responses proximal to Fc ϵ RI activation. In contrast, silver induced the tyrosine phosphorylation of FAK and phosphorylated protein (pp) 77, as reported previously (17).

Silver induces calcium signals that are involved in degranulation but not in LTC₄ production

The increase of $[\text{Ca}^{2+}]_i$ plays a crucial role in the activation of a variety of cell types including mast cells (28–30). Therefore, we next measured changes in $[\text{Ca}^{2+}]_i$ by using the Ca²⁺-reactive fluorescent probe fluo-3. As demonstrated in Fig. 6A, after Fc ϵ RI cross-linking, $[\text{Ca}^{2+}]_i$ increased immediately, reaching its maximum as rapidly as 15 s after the stimulation, and declining rapidly thereafter. Chelation of extracellular calcium by EGTA greatly reduced the magnitude of the calcium signal observed within 1 min. However, a substantial increase in $[\text{Ca}^{2+}]_i$ could be still observed. As shown in Fig. 6B, a calcium channel blocker, lanthanum chloride, also inhibited Ag-induced calcium response. The effect was dose-dependent with a minimal effective dose of 12.5 μM . Higher concentrations (>50 μM) of the compound inhibited the response profoundly. These results indicate that the elevation in $[\text{Ca}^{2+}]_i$ results from both the mobilization of calcium from an intracellular store and the entry of extracellular calcium. Silver induced a slightly delayed increase in $[\text{Ca}^{2+}]_i$ during a 3-min monitoring. Interestingly, $[\text{Ca}^{2+}]_i$ was increased gradually with time and no $[\text{Ca}^{2+}]_i$ spike was observed, although the increase level was usually comparable to that induced by Fc ϵ RI cross-linking. A similar effect was observed with silver sulfate (data not shown), indicating that the effect is attributed to silver. The effect was

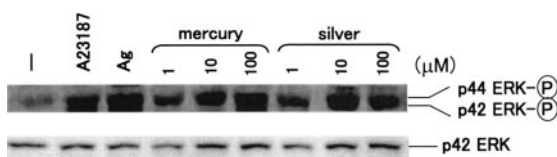


FIGURE 3. Silver-induced ERK tyrosine phosphorylation. Cells were incubated with Ag (1 $\mu\text{g/ml}$), A23187 (2 μM), AgNO₃ (100 μM), and HgCl₂ (100 μM) for 5 min at 37°C, and lysed. Proteins were separated by SDS-PAGE using a 10% separation gel under reducing conditions, and transferred to PVDF membranes. After extensive washing, the membranes were probed with the mAb specific for phospho-ERK (Thr²⁰²/Tyr²⁰⁴) and the immunoreactive proteins on the membranes were visualized using ECL. To verify equal loading, the blots were also probed with Ab against nonphosphorylated forms of ERK (p42^{ERK}). Basically the same results were obtained with Ag₂SO₄.

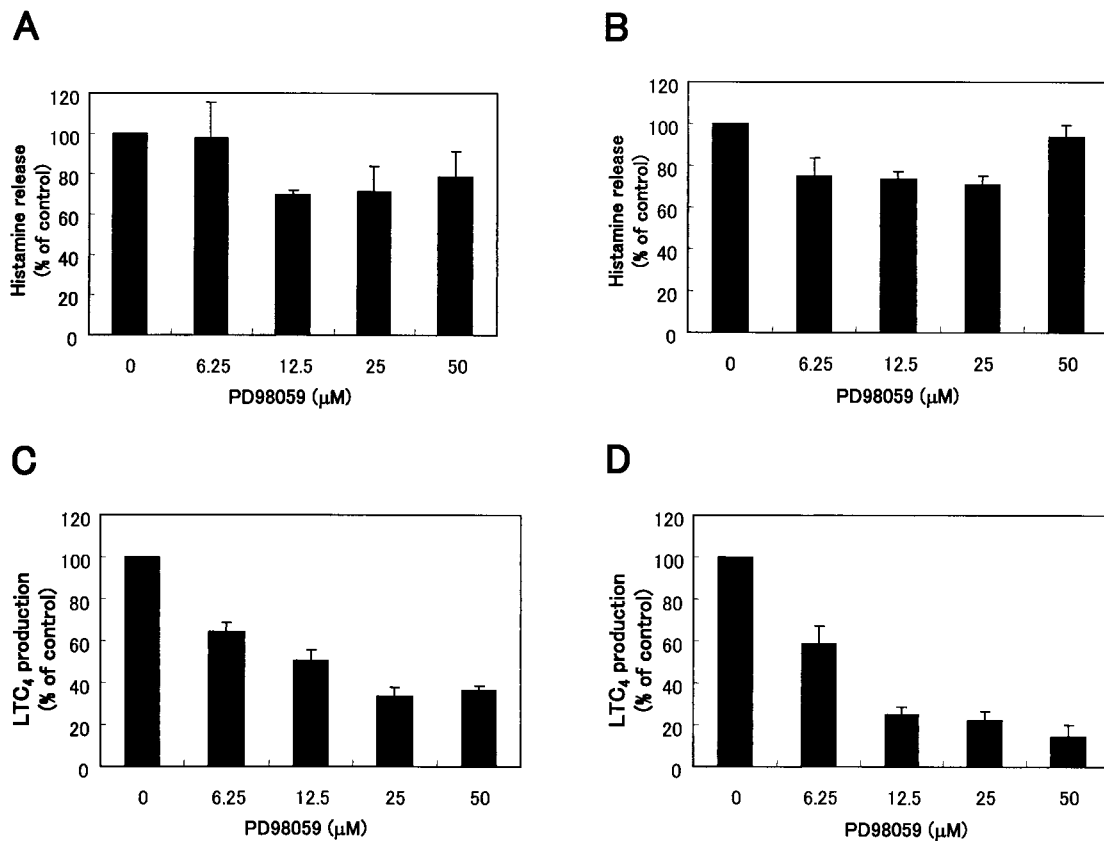


FIGURE 4. Effects of PD98059 on IgE-mediated and silver-induced degranulation and LTC₄ production. Cells were incubated with PD98059 at the concentrations indicated for 30 min, and then stimulated with Ag (1 μg/ml) (A and C) or AgNO₃ (100 μM) (B and D) for 30 min at 37°C. Histamine (A and B) and LTC₄ contents (C and D) in supernatants were determined by ELISA. Results are expressed as the percentage of control where histamine and LTC₄ release from the cells stimulated with Ag or AgNO₃ in the absence of PD98059 is 100%. Results are the mean ± SE of three different experiments with similar results.

dose-dependent with a minimal effective concentration of 10 μM, which was comparable to that effective enough to induce mediator release. In the absence of extracellular calcium, the silver-induced calcium signals were abolished almost completely (Fig. 6C).

Blocking calcium entry by lanthanum also suppressed the silver-induced calcium signals dose-dependently but the effect was moderate (<60% inhibition) even when used at the highest concentration (100 μM). Mercury also increased [Ca²⁺]_i in a dose-

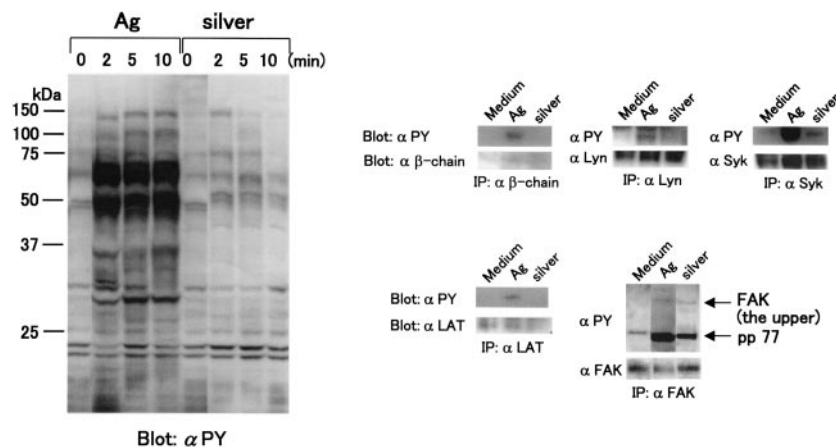
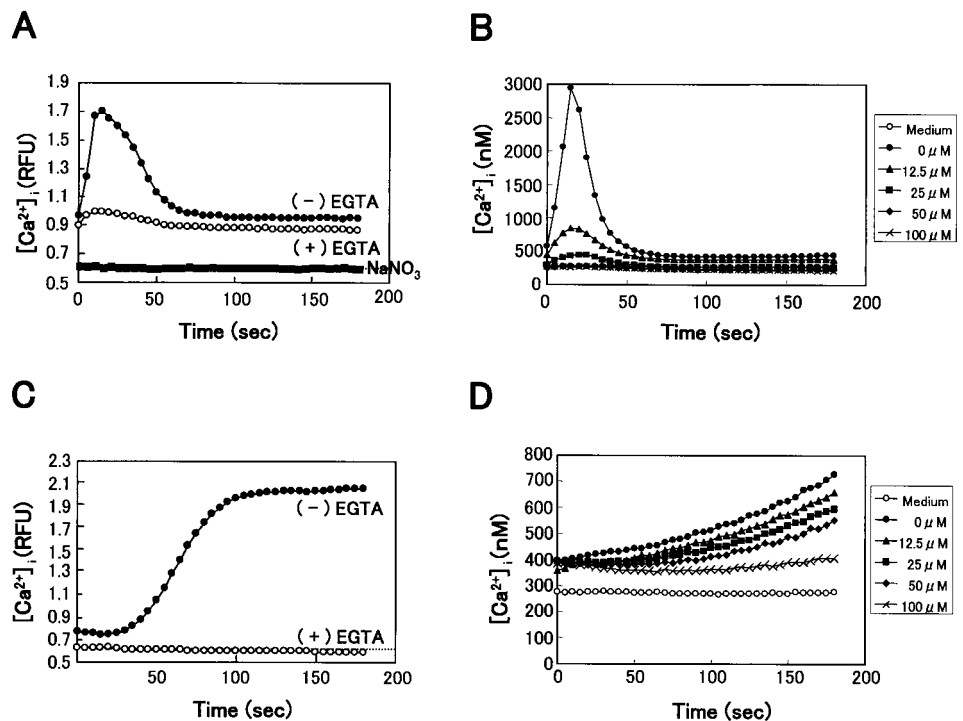


FIGURE 5. Effect of silver on tyrosine phosphorylation. Cells were incubated with Ag (1 μg/ml) or AgNO₃ (100 μM) for the time indicated, and lysed. Proteins were separated by SDS-PAGE using a 10% separation gel under reducing conditions, and transferred to PVDF membranes. After extensive washing, the membranes were probed with the anti-PY mAb, and the immunoreactive proteins on the membranes were visualized using ECL. B. After stimulation with Ag (1 μg/ml) or AgNO₃ (100 μM) for 2 min at room temperature, cells were lysed and immunoprecipitated with Ab against FcεR1β, Lyn, Syk, LAT, or FAK, and tyrosine phosphorylation of each molecule was analyzed by immunoblotting with anti-PY mAb as described above. To verify equal loading, the blots were also probed with Ab against the proteins themselves. Results are representative of three different experiments with similar results. In FAK tyrosine phosphorylation, the blots of Ag- and silver-treated samples were exposed for 15 and 5 min, respectively. Basically the same results were obtained with Ag₂SO₄.

FIGURE 6. Silver induces extracellular calcium influx. Cells were incubated with 4 μM fluo-3-AM at 37°C for 30 min and resuspended in the medium supplemented with 1 mM CaCl_2 (EGTA^-) or in the medium supplemented with 1 mM EGTA instead of 1 mM CaCl_2 (EGTA^+). The fluo-3-loaded cells were stimulated with Ag (1 $\mu\text{g}/\text{ml}$) (A and B) or AgNO_3 (100 μM) (C and D). B and D, The fluo-3-loaded cells were stimulated in the absence or presence of lanthanum chloride at the concentrations indicated. Results shown in relative fluorescence units or calculated $[\text{Ca}^{2+}]_i$ are representative of three experiments with similar results.



dependent manner with a minimal effective dose of 10 μM , but EGTA had only a marginal effect on the increase (data not shown).

To determine the role of calcium signals in the silver-induced responses, we examined the effect of these two calcium modulators. In the presence of EGTA, silver-induced histamine release was suppressed moderately (59% inhibition) (Fig. 7A). Lanthanum also inhibited silver-induced histamine release at concentrations that inhibited the calcium signals. Under the same conditions, Ag-induced histamine release was inhibited more profoundly (>80% inhibition) by EGTA and lanthanum (Fig. 7B). Basically, the same results were obtained with the β -hexosaminidase assay (data not shown). In contrast, neither EGTA treatment nor lanthanum treatment affected LTC_4 production irrespective of the stimulus tested (Fig. 7, C and D). These results show that the calcium signals play a role in silver-induced degranulation but not in LTC_4 production.

Silver-induced calcium signals are resistant to the depletion of thapsigargin (Tg)-sensitive calcium stores, and the inhibition of tyrosine kinases and $\text{PLC}\gamma$

Tg, a cell-permeable sesquiterpene lactone, can elevate $[\text{Ca}^{2+}]_i$ by inhibiting the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (31, 32). Thus, it is possible that under extracellular calcium-free conditions, Tg treatment causes the elevation of $[\text{Ca}^{2+}]_i$, which leads to depletion of the Tg-sensitive calcium stores. Thus, Tg has been widely used as a useful tool for studying the involvement of store-operated calcium entry (SOCE). Indeed, when RBL-2H3 cells were treated with Tg in the presence of EGTA, $[\text{Ca}^{2+}]_i$ was gradually increased with its maximum at 3 min after the addition of Tg and returned to unstimulated levels at 5 min, suggesting that in these cells, intracellular calcium stores seemed to be depleted. In the Tg-treated cells, no elevation in $[\text{Ca}^{2+}]_i$ was observed after Fc ϵ RI cross-linking even when cells were replenished with calcium (Fig. 8A), indicating that cross-linking induces the calcium response through a SOCE mechanism. By contrast, silver could still induce a substantial elevation of $[\text{Ca}^{2+}]_i$ in the Tg-treated cells, although the effect was slightly reduced (Fig. 8B). These

results show that unlike Ag, silver-induced calcium signals are resistant to the depletion of Tg-sensitive calcium stores.

We previously showed that two different tyrosine kinase inhibitors, piceatannol and pp1, dose-dependently inhibited IgE-mediated degranulation, whereas they had little effect on silver-induced degranulation (17). As calcium signals are required for mast cell degranulation, we next examined the effect of these chemicals on the signals. As shown in Fig. 9A, both of these chemicals dose-dependently inhibited the IgE-Ag-mediated calcium influx, although piceatannol was stronger than pp1, which was in accordance with their effects on degranulation (17). By contrast, neither chemical at concentrations up to 40 μM had effects on the silver-induced calcium signals (Fig. 9B).

To determine the role of receptor-mediated PLC activation in calcium signals, we examined the effects of the inhibitor of $\text{PLC}\gamma$ U-73122 on the Ag- and silver-induced calcium signals. As shown in Fig. 10A, the compound dose-dependently inhibited Ag-induced calcium influx at a minimal effective dose of 1 μM (30% inhibition). By sharp contrast, U-73122 at concentrations ranging from 37 to 3000 nM had a slight stimulatory (>120%) rather than inhibitory effect on silver-induced calcium signals (Fig. 10B). In contrast, its inactive analog U-73343 had no inhibitory effect irrespective of the stimulus tested (data not shown). Collectively, these results show that unlike Ag, silver induces calcium signals by a mechanism that is resistant to the inhibition of tyrosine kinases and $\text{PLC}\gamma$.

Discussion

We previously reported that silver induced degranulation and LTC_4 production in RBL-2H3 cells, as did cross-linking of Fc ϵ RI on their surfaces by multivalent IgE-Ag complexes. The data presented in this study clearly demonstrate that the mechanism of silver-induced activation is distinct from that of receptor-mediated activation, although some signaling pathways are commonly involved in them. Cross-linking of Fc ϵ RI induces the tyrosine phosphorylation and activation of ERKs, which in turn results in the activation of cytosolic PLA_2 and release of arachidonic acid (23–

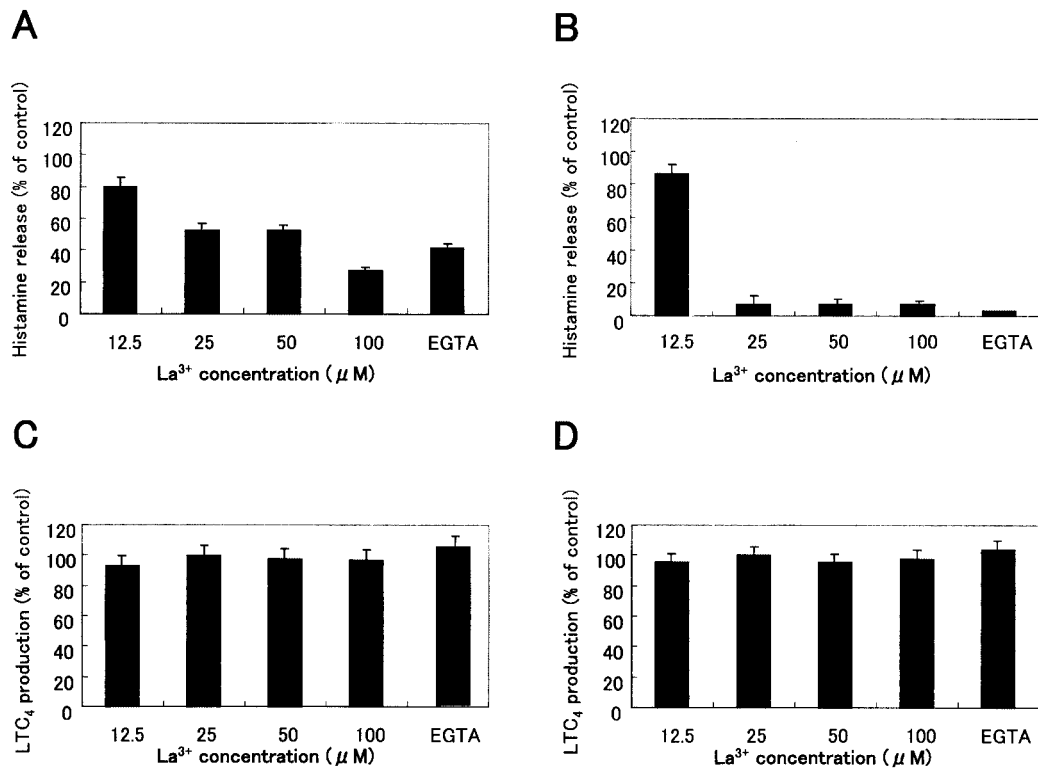


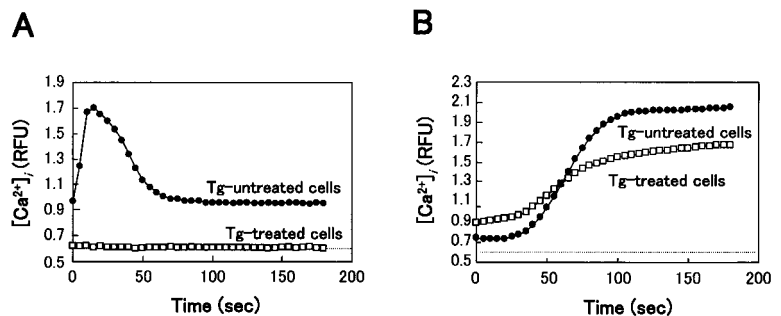
FIGURE 7. Silver-induced calcium influx is involved in degranulation, but not in LTC_4 production. Cells were stimulated with AgNO_3 (100 μM) (A and C) or Ag (1 $\mu\text{g}/\text{ml}$) (B and D) for 30 min at 37°C in the absence or presence of 1 mM EGTA or lanthanum chloride at the concentrations indicated, and then histamine (A and B) and LTC_4 contents (C and D) in supernatants were determined by ELISA. Results are expressed as the percentage of control where histamine release and LTC_4 production from the cells stimulated with Ag or AgNO_3 in the absence of additives is 100%. Results are mean \pm SE of two different experiments with similar results.

25). Consequently, the ERK pathway plays a crucial role in IgE-mediated LTC_4 production. The present data indicate that silver also induces the tyrosine phosphorylation of ERKs and that inhibition of ERK kinase suppresses the silver-induced LTC_4 production. In contrast, the inhibition of ERK kinase has no, or only marginal if any, effect on the silver-induced degranulation. These findings support the notion that silver activates mast cells by an ERK-dependent mechanism that is similar to Fc ϵ RI-mediated activation, although at present the direct molecular target of silver remains to be identified. As demonstrated in this study, mercury can induce ERK tyrosine phosphorylation without effect on LTC_4 production. This strongly suggests the involvement of another pathway in intracellular signaling to LTC_4 production, which is commonly activated by Fc ϵ RI cross-linking and silver.

Several lines of evidence clearly show that Fc ϵ RI does not mediate the effects of silver. First, Fc ϵ RI cross-linking induced the production of IL-4 and TNF- α under our experimental conditions, whereas silver could not. Second, silver could induce none of the

tyrosine phosphorylation of Fc ϵ RI β , Lyn, Syk, and LAT. Cross-linking of Fc ϵ RI causes activation of Lyn that is weakly associated with Fc ϵ RI β and the resulting phosphorylation of the immunoreceptor tyrosine-based activation motif found in Fc ϵ RI β and γ -chains. The phosphorylation in turn leads to recruitment and activation of Lyn and Syk. Very recently, it has been shown that downstream of Syk activation, LAT plays an essential role as a scaffold in the formation of the macromolecular signaling complex involving growth factor receptor-bound protein 2, Src homology 1 domain-containing leukocyte protein of 76 kDa, Vav1, and LAT and that tyrosine phosphorylation of LAT is required for the role (33, 34). In LAT-deficient bone marrow-derived mast cells, multiple events including calcium signals, degranulation and cytokine production are considerably impaired (33). Thus, the tyrosine phosphorylation of Fc ϵ RI β , Lyn, Syk, and LAT is an important signaling response. Therefore, it is possible that the failure of silver in inducing cytokine production results from the absence of the involvement of molecules essential for the production. The

FIGURE 8. Silver-induced calcium signal is resistant to the depletion of Tg-sensitive calcium stores. Cells were incubated with 4 μM fluo-3-AM at 37°C for 30 min and resuspended in the medium supplemented with 1 mM EGTA instead of 1 mM CaCl_2 . The fluo-3-loaded cells were treated with Tg (1 μM) for 5 min. The cells were resuspended in the medium supplemented with 1 mM CaCl_2 and then stimulated with Ag (1 $\mu\text{g}/\text{ml}$) (A) or with AgNO_3 (B). Fluo-3 fluorescence was monitored at 5-s intervals up to 3 min by a microplate fluorometer. Results shown in relative fluorescence units are representative of three experiments with similar results.



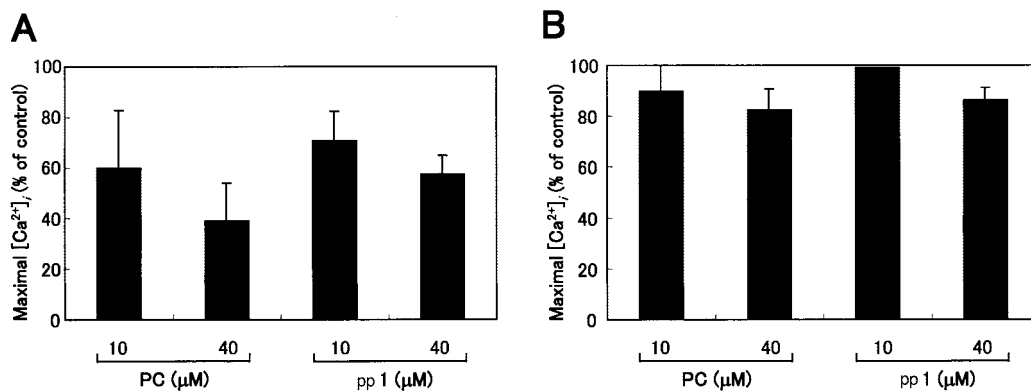


FIGURE 9. Silver-induced calcium influx is resistant to the inhibition of Lyn and Syk kinases. Cells were treated with piceatannol (PC) or pp1 at the concentrations indicated at 37°C for 30 min, and then stimulated with Ag (1 $\mu\text{g/ml}$) (A) or AgNO_3 (100 μM) (B), and $[\text{Ca}^{2+}]_i$ was monitored as described in the Fig. 6 legend. Results are expressed as the percentage of control where $[\text{Ca}^{2+}]_i$ in the cells stimulated with Ag or AgNO_3 in the absence alone is 100%. Results are mean \pm SE of three separate experiments with similar results.

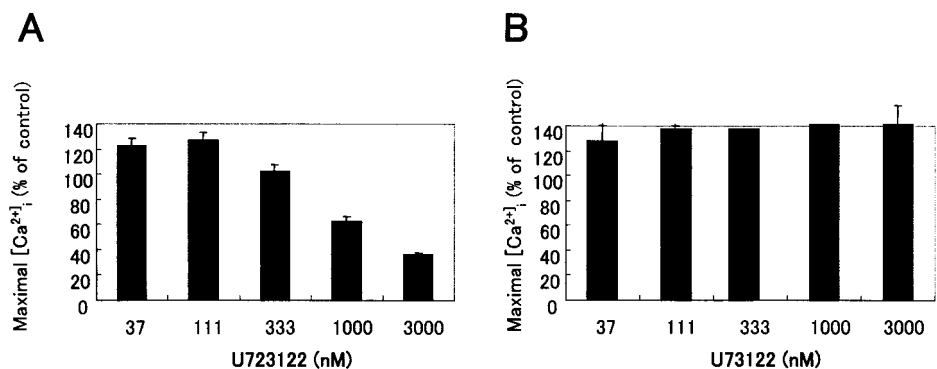
capability of silver to induce calcium signal, degranulation, and LT release indicates that silver can activate an alternative pathway bypassing the usual early signaling responses including LAT tyrosine phosphorylation. With respect to this, it should be noticed that like their normal counterparts LAT-deficient mast cells mobilize calcium and degranulate in response to Tg (33, 34). Similarly, Lyn-deficient mast cells show MAPK activation, degranulation, and cytokine production (35, 36), although they are impaired in some signaling responses including phosphorylation of the $\text{Fc}\epsilon\text{RI}\beta$ and γ . These facts indicate that in the absence of Lyn or LAT, an alternative pathway contributing to these responses must be activated. Taken together, it is likely that silver activates mast cells by using such an alternative mechanism.

The induction of a receptor-mediated cytosolic calcium signal involves two closely coupled events: 1) a rapid and transient release of calcium from the endoplasmic reticulum stores (the mobilization of calcium), 2) followed by slowly developing extracellular calcium entry (30, 37). In many cell types including mast cells, depletion of the intracellular calcium stores induces calcium entry across the plasma membrane through so-called "capacitative entry or SOCE" (38, 39). Analysis of the behavior of intracellular calcium provides another line of evidence that $\text{Fc}\epsilon\text{RI}$ does not mediate the effect of silver. The patterns of the rise in $[\text{Ca}^{2+}]_i$ induced by $\text{Fc}\epsilon\text{RI}$ cross-linking and silver are quite different. Consistent with the SOCE model, $\text{Fc}\epsilon\text{RI}$ -induced elevation in $[\text{Ca}^{2+}]_i$ involves a rapid and transient release of calcium from an intracellular pool, followed by slowly developing extracellular calcium entry. Furthermore, depleting Tg-sensitive calcium stores completely abolished the calcium influx. In contrast, silver induced a

slowly developing rise in $[\text{Ca}^{2+}]_i$, which was accompanied by no apparent rapid and transient release of calcium (calcium spike). In addition, depleting Tg-sensitive calcium stores showed an only marginal effect on the calcium response. As mentioned above, in the SOCE model, calcium influx is absolutely dependent on the calcium release from Tg-sensitive stores, which is generally accepted to be mediated by inositol-1,4,5-triphosphate, a product of phosphoinositide hydrolysis by activated $\text{PLC}\gamma$. It is shown that activation of $\text{PLC}\gamma$ requires LAT-mediated translocation to the cell membrane and tyrosine phosphorylation by activated Syk and Bruton tyrosine kinase. Consequently, Lyn and Syk tyrosine kinases and LAT are all important components to activate calcium influx. Consistent with this paradigm, $\text{Fc}\epsilon\text{RI}$ -mediated calcium influx is quite sensitive to the tyrosine kinase inhibitors, piceatannol and pp1, and the $\text{PLC}\gamma$ inhibitor U73122. By contrast, the silver-induced calcium response is insensitive to all of these inhibitors. Piceatannol and pp1 are shown to preferentially inhibit Syk (40, 41) and Lyn (42, 43), respectively. These data demonstrate that silver-mediated calcium responses do not require $\text{PLC}\gamma$ activity unlike $\text{Fc}\epsilon\text{RI}$ responses. Thus, our finding suggests that silver might induce calcium influx by a mechanism that differs from SOCE. Alternatively, silver might induce the release of calcium from mitochondrial or other sources and not necessarily from the extracellular environment.

In conclusion, our present findings strongly suggest that silver induces biological responses by bypassing the usual signaling events required for the induction of calcium influx. Further investigations on the mechanisms by which silver bypasses these signaling events are ongoing in our laboratory.

FIGURE 10. Silver-induced calcium signal is resistant to the inhibition of $\text{PLC}\gamma$. Cells were treated with U73122 at the concentrations indicated at 37°C for 30 min, and then stimulated with Ag (1 $\mu\text{g/ml}$) (A) or AgNO_3 (100 μM) (B), and $[\text{Ca}^{2+}]_i$ was monitored as described in Fig. 6. Results are expressed as the percentage of control where $[\text{Ca}^{2+}]_i$ in the cells stimulated with Ag or AgNO_3 in the absence alone is 100%. Results are mean \pm SE of two separate experiments with similar results.



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