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Short Communication

The Phenoxazine Derivative Phx-1 Suppresses IgE-Mediated Degranulation in Rat Basophilic Leukemia RBL-2H3 Cells

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Abstract. Antigen-induced aggregation of the high affinity IgE receptor (FcεRI) on mast cells induces degranulation to release chemical mediators, leading to acute allergic inflammation. We have demonstrated that the treatment of rat mast cells, RBL-2H3, with a phenoxazine derivative Phx-1 (2-amino-4,4α-dihydro-4α,7-dimethyl-3*H*-phenoxazine-3-one) suppresses the antigen-induced degranulation. Biochemical analysis reveals that the complementary signaling pathway through Gab2 and Akt is inhibited by this compound in mast cells. These findings suggest that phenoxazine derivatives may have a therapeutic potential for allergic diseases by inhibiting mast cell degranulation.

Keywords: phenoxazine derivative, mast cell, degranulation

Phenoxazine compounds like actinomycin D exert anti-proliferative activity by blocking transcription (1, 2). In addition, phenoxazines inhibit the proliferation of various cell lines including human epidermoid carcinoma cells, human lung carcinoma cell lines, and lymphocytes (3). A new phenoxazine derivative, 2-amino-4,4α-dihydro-4α,7-dimethyl-3*H*-phenoxazine-3-one (Phx-1) was generated by the reaction of 2-amino-5-methylphenol with hemoglobin (4). Administration of Phx-1 into mice transplanted with Meth A carcinoma cells or leukemia cells extensively suppressed the growth of tumor transplants. Phx-1 has a three-ring structure that is similar to that of actinomycin D as well as to that of Emodin (1,3,8-trihydroxy-6-methylanthraquinone), which exhibits immunosuppressive activities. Phx-1 possesses an immunosuppressive effect on lymphocytes and monocytes. Previously, we have shown that treatment of Phx-1 on avian B cells results in a suppression of surface IgM expression and subsequent tyrosine phosphorylation of cellular proteins (5). In the present study, we have tested the effect of two phenoxazine derivatives, Phx-1 and Phx-2 (3-amino-1,4α-dihydro-4α,8-dimethyl-2*H*-phenoxazine-2-one) on IgE-mediated mast cell activation. The structures of these phenoxazine derivatives are shown in Fig. 1.

Engagement of the high affinity IgE receptor (FcεRI) on rat mast cells (RBL-2H3) initiates a number of biochemical events that eventually result in degranulation and cytokine production (6). Using this system, we attempted to analyze the effect of these phenoxazine derivatives on IgE-mediated degranulation (Fig. 2). RBL-2H3 cells were treated without or with different concentrations of phenoxazine derivatives, Phx-1 and

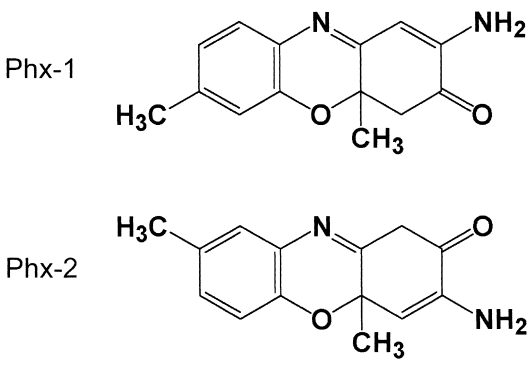

Fig. 1. Chemical structures of new phenoxazine derivatives Phx-1 and Phx-2 used in this study.

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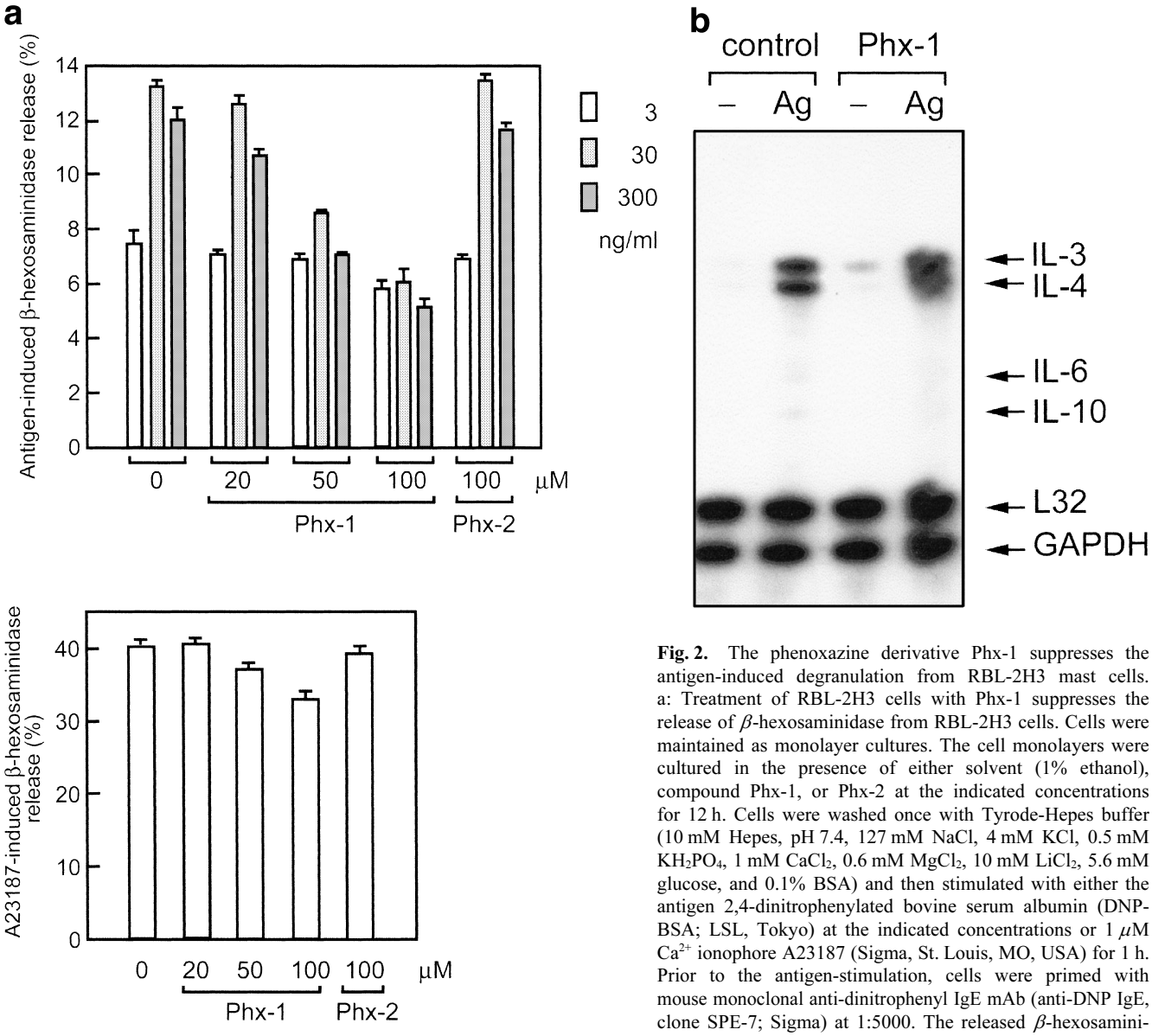

Fig. 2. The phenoxazine derivative Phx-1 suppresses the antigen-induced degranulation from RBL-2H3 mast cells. a: Treatment of RBL-2H3 cells with Phx-1 suppresses the release of β-hexosaminidase from RBL-2H3 cells. Cells were maintained as monolayer cultures. The cell monolayers were cultured in the presence of either solvent (1% ethanol), compound Phx-1, or Phx-2 at the indicated concentrations for 12 h. Cells were washed once with Tyrode-Hepes buffer (10 mM Hepes, pH 7.4, 127 mM NaCl, 4 mM KCl, 0.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM $LiCl_2$, 5.6 mM glucose, and 0.1% BSA) and then stimulated with either the antigen 2,4-dinitrophenylated bovine serum albumin (DNP-BSA; LSL, Tokyo) at the indicated concentrations or 1 μM Ca^{2+} ionophore A23187 (Sigma, St. Louis, MO, USA) for 1 h. Prior to the antigen-stimulation, cells were primed with mouse monoclonal anti-dinitrophenyl IgE mAb (anti-DNP IgE, clone SPE-7; Sigma) at 1:5000. The released β-hexosaminidase was recovered from the cultured medium. Antigen- and A23187-induced releases were subtracted by the basal release and normalized by the total activity. The results were shown as the mean ± S.E.M. from three independent experiments. b: Effect of phenoxazines on multiple cytokine genes translation. RBL-2H3 cells were treated with solvent (1% ethanol, control) or 50 μM Phx-1 for 12 h. For antigen-stimulation, cells were incubated in the presence of anti-DNP IgE. Cells were then stimulated without (–) or with 30 ng/ml antigen DNP-BSA (Ag) for 1 h. Each 20 μg of total RNA was hybridized with the ^{32}P-labeled RNA probes and treated with RNase. The protected double stranded RNA was separated by the urea gel and analyzed by autoradiography. The results are representative of 2 experiments.

Phx-2. Analysis of degranulation was measured by the release of β-hexosaminidase as previously described (7, 8). Treatment of cells with Phx-1, but not Phx-2, showed inhibition of antigen-induced β-hexosaminidase release in a dose-dependent manner (Fig. 2a, upper panel). Rhx-1 at 50 μM did not impair the viability of RBL-2H3 cells (data not shown). A23187-induced β-hexosaminidase release was also suppressed by Phx-1 treatment (Fig. 2a, lower panel). The degree of suppression was comparable to that observed in antigen-induced degranulation, when compared using the absolute values (total amount of β-hexosaminidase). Therefore, this result demonstrates that the phenoxazine derivative Phx-1 suppresses the process(es) of degranulation in mast cells.

Besides degranulation, aggregation of FcεRI induces activation of the signaling pathway to translate the multiple cytokine genes. The effect of phenoxazine derivatives on cytokine gene transcription was quantita-

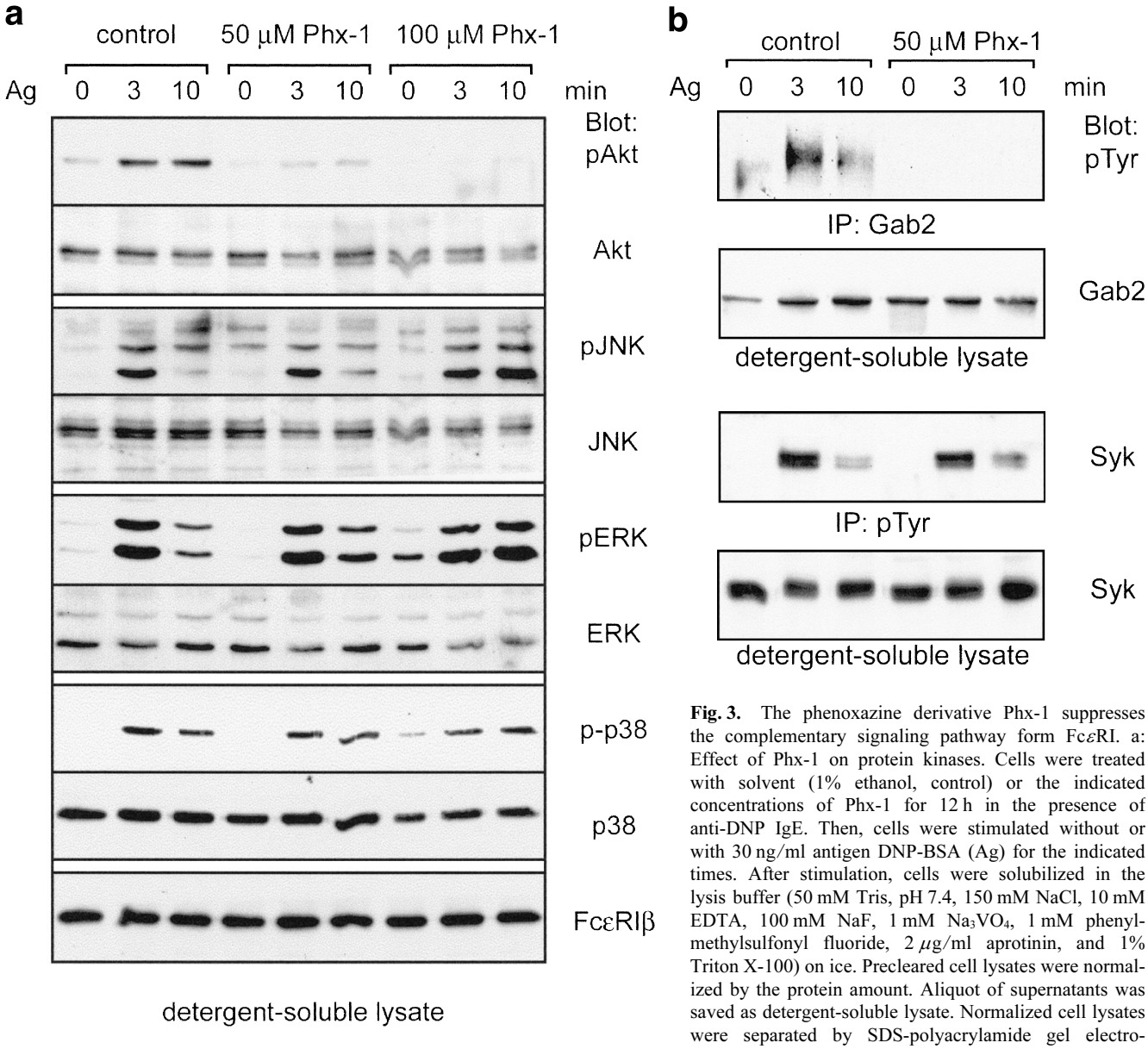

Fig. 3. The phenoxazine derivative Phx-1 suppresses the complementary signaling pathway form FcεRI. a: Effect of Phx-1 on protein kinases. Cells were treated with solvent (1% ethanol, control) or the indicated concentrations of Phx-1 for 12 h in the presence of anti-DNP IgE. Then, cells were stimulated without or with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. After stimulation, cells were solubilized in the lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 1% Triton X-100) on ice. Precleared cell lysates were normalized by the protein amount. Aliquot of supernatants was saved as detergent-soluble lysate. Normalized cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by the immunoblotting using anti-phospho-Akt (pAkt) and anti-Akt, anti-phospho-JNK (pJNK) and anti-JNK, anti-phospho-ERK (pERK) and anti-ERK, anti-phospho-p38 MAPK (p-p38) and anti-p38 MAPK, or anti-FcεRIβ. b: Effect of Phx-1 on antigen-induced tyrosine phosphorylation of Gab2 and Syk. Cells were treated with solvent (1% ethanol, control) or 50 μM Phx-1 for 12 h in the presence of anti-DNP IgE. Then, cells were stimulated without or with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times and solubilized in the lysis buffer. Normalized cell lysates were immunoprecipitated with either anti-Gab2 or anti-phosphotyrosine (pTyr) antibody. The immunoprecipitates and detergent-soluble lysates were analyzed by immunoblotting with anti-pTyr and anti-Gab2 antibodies (upper panels) or with anti-Syk antibody (bottom panels). The results are representative of 2 independent experiments.

tively measured by the RNase protection assay (8). Cells were treated with solvent alone (1% ethanol) or 50 μM Phx-1 and stimulated without or with antigen DNP-BSA (Fig. 2b). Translation of cytokine genes such as interleukin (IL)-3 and IL-4 was not affected by the treatment with Phx-1 at concentrations that inhibited the antigen-induced degranulation. Translation of house keeping genes (L32 and GAPDH) was not affected (Fig. 2b, bottom). These results demonstrate that the phenoxazine derivative Phx-1 has a selective function to suppress the antigen-induced degranulation, but not cytokine synthesis, in RBL-2H3 mast cells.

Next, we examined the effect of Phx-1 on FcεRI-mediated activation of intracellular signal transduction pathways (Fig. 3). Aggregation of FcεRI induces the activation of conventional and complementary path-

ways, both of which contribute to the degranulation and cytokine production. Cells were primed with IgE in the presence of either solvent or Phx-1 and then stimulated with the antigen. The detergent-soluble lysates were prepared and analyzed by the immunoblotting and immunoprecipitation as described previously (8). In control cells, the antigen stimulation induces phosphorylation of Akt, JNK (c-Jun N-terminal kinase), ERK (extracellular signal-regulated kinase), and p38 MAPK (mitogen-activated protein kinase) (Fig. 3a). Treatment of cells with Phx-1 at 50 μM dramatically suppressed the antigen-induced phosphorylation of Akt (Fig. 3a, upper panels). However, neither phosphorylation of JNK, ERK, nor p38 was affected by Phx-1 even at 100 μM. Protein amounts of all these protein kinases, FcεRIβ and tubulin were identical (Fig. 3a and data not shown). These results suggest that the treatment of Phx-1 inhibits the activation of Akt in the complementary signaling pathway (9). Furthermore, treatment with Phx-1 suppresses the expression of surface IgM in B cells (5) but not FcεRI in mast cells (this report), suggesting that Phx-1 may have a cell-type specific function.

Previously, FcεRI-mediated activation of mast cells was thought to be depend solely on a pathway that requires Lyn, Syk, and LAT (conventional pathway). A genetic study on the adaptor molecule Gab2 (Grb2 associated binder-2) suggested that FcεRI-mediated activation involves heterogeneity in signals from the receptor. The adaptor molecule Gab2 is a critical component for regulating antigen-induced mast cell activation via phosphatidylinositol 3-kinase (PI3-kinase) and Akt through the complementary signaling pathway from FcεRI in mast cells (9, 10). The product of PI3-kinase, phosphatidylinositol 3,4,5-triphosphate (PIP_3), recruits the pleckstrin homology domain of cellular proteins such as Akt to the plasma membrane. Although Fyn, but not Lyn, was reported to phosphorylate Gab2 in mast cells, it is still possible that Syk regulates the function of Gab2 because lack of Syk expression abrogates the antigen-induced degranulation and cytokine synthesis in mast cells (9, 11). The molecular mechanism of Gab2-mediated activation of PI-3 kinase is still unclear.

Since treatment of cells with Phx-1 down-regulates the antigen-induced activation of Akt, we focused on the molecule upstream of Akt (Fig. 3b). The treatment of cells with 50 μM Phx-1 completely abrogated the antigen-induced tyrosine phosphorylation and mobility shift of Gab2 (Fig. 3b, upper panel). This result demonstrates that Phx-1 may down-regulate Gab2 and therefore the complementary pathway from FcεRI in RBL-2H3 cells. Besides Gab2, a protein-tyrosine kinase Syk has also been shown to be critical for FcεRI-mediated degranulation and cytokine production in mast cells (11, 12). Thus, we tested the effect of Phx-1 on the activation of Syk as a parameter in the conventional activating pathway from FcεRI. Treatment of cells with 50 μM Phx-1 had no effect on tyrosine phosphorylation of Syk, which correlates with the enzymatic activation of Syk (Fig. 3b, lower panels). These results indicate that treatment with Phx-1 selectively affects the antigen-induced degranulation by inhibiting the Gab2-PI3-kinase-Akt-mediated complementary pathway in mast cells.

Intracellular signaling molecules in mast cells are potential targets for development of anti-allergic drugs. So far, genetic studies have demonstrated that protein-tyrosine kinase Syk, adaptor molecules LAT (linker for activation of T cell), SLP-76 (SH2-containing lymphocyte phosphoprotein of 76 kDa), and Gab2 are essential for the antigen-induced mast cell activation (13). 3BP2 (c-Abl SH3-binding protein-2) and Cbl-b have also been shown to regulate the antigen-induced mast cell activation in RBL-2H3 cells (7, 8). Previously, compound ER-27319 (3,4-dimethyl-10-(3-aminopropyl)-9-acridone oxalate) was reported to inhibit FcεRI-mediated degranulation and tyrosine phosphorylation of Syk, but not FcεRI in mast cells (14). In addition, recent findings demonstrated that protease-activated receptors expressed in peritoneal mast cells are involved in the degranulation and may have a potential to generate anti-allergic drugs (15). Our results reveal that a potential target of Phx-1 is the adaptor protein Gab2 or its regulatory molecule(s).

In this report, we have demonstrated that the new phenoxazine derivative Phx-1 suppresses the antigen-induced degranulation in cultured mast cells. Although there is a structural similarity between Phx-1 and Phx-2, their biological functions seem to be quite different. These findings suggest that new phenoxazine derivatives might lead to development of anti-allergic drugs.

Acknowledgments

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