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Inhibition of IgE-mediated histamine release by myosin light chain kinase inhibitors

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SUMMARY: Wortmannin, a specific inhibitor of myosin light chain kinase(MLCK) blocked IgE mediated histamine release from rat basophilic leukemia cell(RBL-2H3) and human basophils dose-dependently. Its IC_{50} was 20 nM for RBL-2H3 cells and 30 nM for human basophils. There was complete inhibition at the concentration of 1 μM. Wortmannin inhibited partially the A23187 induced histamine release from RBL-2H3 cells(40% inhibition at 1 μM). This inhibition was not accompanied by any significant effect on cytosolic free calcium concentration($[Ca^{2+}]_i$). KT5926, another MLCK inhibitor, inhibited histamine release comparably with wortmannin and blocked to some degree the increase of $[Ca^{2+}]_i$ in RBL-2H3 cells. Thus, the phosphorylation of myosin seems to be involved in signal transduction through $Fc_\epsilon RI$.

Basophils and mast cells release inflammatory mediators such as histamine following the cross-linking of the high affinity IgE receptor($Fc_\epsilon RI$)(1-3). Rat basophilic leukemia cells(RBL-2H3), a cultured analog of rat mucosal mast cells, provide a suitable model for study of Fc_ϵ receptor($Fc_\epsilon RI$) and mechanism of histamine

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Abbreviations
RBL-2H3 cells, rat basophilic leukemia 2H3 clone cell line; IC_{50}, inhibitory concentration at 50%; $Fc_\epsilon RI$, high affinity IgE receptor on basophil/mast cells; DNP_{48}-HSA, 48 molecules of 2,4-dinitrophenyl conjugated to 1 molecule of human serum albumin; MLCK, myosin light chain kinase.

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release(4). Studies of morphological changes following antigen stimulation has shown the extensive ruffling of the plasma membrane(5). Actin-myosin interaction is regulated by phosphorylation of the 20 kDa myosin light chain via myosin light chain kinase (MLCK), and this coupling is involved in not only the contraction of smooth muscle, but also seems to be in secretion process of nonmuscle cells.

Recently, wortmannin, a microbial product, and KT5926, a chemical derivative of K252, were found to be a specific inhibitors of MLCK(6,7). In this article, we studied the effect of wortmannin and KT5926 on histamine release to clarify the role of phosphorylation of myosin in the exocytosis of mast cell(8).

MATERIALS AND METHODS

Materials Wortmannin(MS-54) and KT5926 was prepared as described previously(6,7). Both were used after dissolution with dimethyl sulfoxide. ML-9 was purchased from Seikagaku Kogyo(Tokyo, Japan). Goat anti-human IgE antibody was purchased from Medical & Biological Labs Co.,Ltd.(Nagoya,Japan). Calcium ionophore A23187 was from Sigma Chemical Co.(St.Louis,MO).

Fura-2-AM was obtained from Dojindo(Kumamoto, Japan). Monoclonal anti-dinitrophenol(DNP) monoclonal IgE, DNP_{48}-human serum albumin(HSA), and monoclonal anti-$Fc_{\epsilon}RI$ α-chain antibody(BA3) were gifts from Dr. R.P.Siraganian (NIH,USA) and prepared described previously (9).

Buffers Pipes-buffered saline contained 119 mM NaCl, 5 mM KCl, 025 mM Pipes, 5.6 mM glucose, 0.1% bovine serum albumin, 1mM Ca^{2+}, and 0.4 mM Mg^{2+} adjusted to pH 7.4.

Histamine Release from cells RBL-2H3 cells were cultured as described previously(10). Human leukocytes were prepared by dextran sedimentation described previously(11). Histamine release from cells were done described previously (12). Cells were incubated with PIPES buffered saline in the presence of various concentration of inhibitors at 37°C for 15 min followed by the challenge. After additional incubation for 45 min at 0°C, supernatants were collected and histamine was measured by automated fluorometric technique as described previously(13).

Measurement of cytosolic free calcium concentration ($[Ca2+]_i$) Method in detail was described previously(14-16). Briefly, IgE-sensitized RBL-2H3 cell($6x10^5$/ml) were loaded with fura-2-AM(6 μM). Fluorescence measurements were made in a 1cm quartz cuvette using a Shimazu RF-5000 spectrophotometer at 37°C(excitation, 335nm and 365nm; emission 500nm). During monitoring, cells were preincubated with the inhibitor for 10 min and challenged with DNP_{48}-HSA.

RESULTS AND DISCUSSION

In order to clarify the participation of cytoskeleton, especially, myosin on the molecular mechanism of exocytosis in mast cell and basophils, we utilized MLCK inhibitor, wortmannin and KT5926, for histamine release from RBL-2H3 cells and the human basophils as well-defined in-vitro system. Both materials

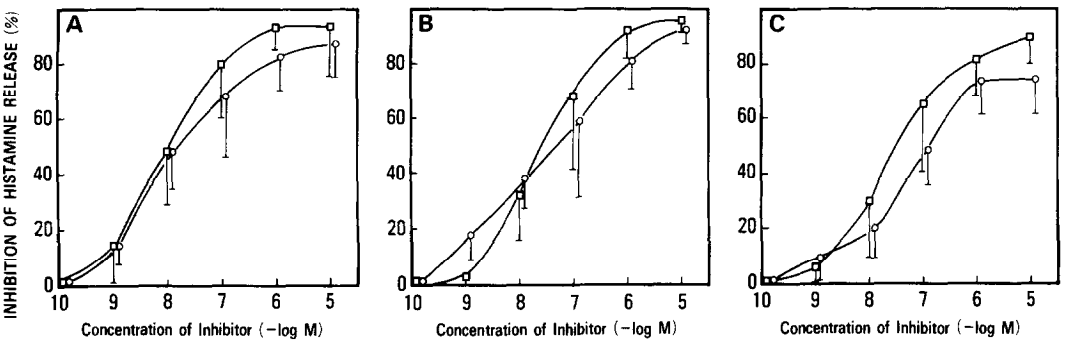

Fig.1. Dose-dependent inhibition of IgE-mediated histamine release from RBL-2H3 cells(A), human basophils(B) and monoclonal anti-receptor antibody-stimulated histamine release(C) with inhibitors. □,wortmannin, o,KT5926.
Cells were preincubated with various concentrations of inhibitor at 37°C for 15 min followed by challenge with DNP-HSA (A) or with goat anti-human IgE(B) or anti-receptor antibody BA3 (C). After additional incubation for 45 min in PIPES buffered saline, supernatant were collected and histamine was measured by automated fluorometric technique. Experiments for A, B and C were repeated 7, 3 and 4 times, and their average and S.E.are shown. Dimethyl sulfoxide dissolved in solution had no effect on histamine release.

did not release histamine directly. As shown in Fig.1A, both materials inhibited IgE mediated histamine release from IgE sensitized RBL-2H3 cells after challenge with antigen (DNP_{48}-HSA) dose-dependently. The potency was almost similar; IC_{50} for wortmannin and KT5926 were 20 nM and 30 nM, respectively. There was complete inhibition at the concentration of 1 μM of both. Another MLCK inhibitor, ML9, did not show such a high potency for inhibition(IC_{50}, 50 μM)(8). The inhibition of [^{3}H] serotonin release was comparable with that of histamine (data not shown). Preincubation time of inhibitors were 10 min in these experiments, whereas the potency of inhibition became half with 3 min of preincubation time with both inhibitors. Wortmannin and KT5926 also inhibited histamine release from freshly prepared human basophils activated with anti-human IgE(Fig.1B). IC_{50} for wortmannin and KT5926 was 30 nM and 100nM, respectively. Similar results were obtained with using anti-$Fc_{\epsilon}RI$ α-chain monoclonal antibody BA3(Fig.1C). The antigen DNP_{48}HSA can crosslink multiple sites of $Fc_{\epsilon}RI$, whereas mAb BA3 can only two sites of receptor with its $F(ab)_2$ fragments(10).

Calcium ionophore A23187 can release histamine without direct crosslinking of $Fc_{\epsilon}RI$ by bypassing the crosslinking $Fc_{\epsilon}RI$. To assess the degree of involvement of myosin phosphorylation at the

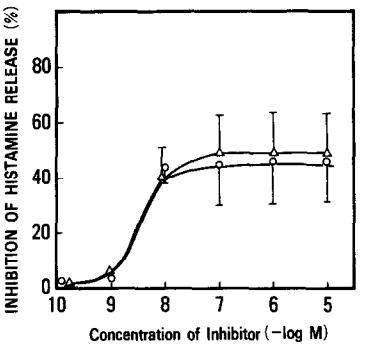

Fig.2. Effect of inhibitors on A23187-activated histamine release from RBL-2H3 cells. o,wortmannin, △,KT5926.
Cells were preincubated with various concentrations of inhibitors at 37°C for 15 min followed by challenge with 0.3 μg/ml of A23187. Experiments were repeated 4 times and their average and S.E. are shown.

crosslinking site and in the movement of secretory vesicles, we studied the effect of wortmannin and KT5926 on the histamine release from RBL-2H3 cells activated with 0.3 μg/ml of A23187. Both materials inhibited histamine release for 40% at the concentration of over 10 nM; both could not inhibit histamine release completely even with the higher concentration (Fig2).

Next, we studied the effect of the inhibitors on increase in $[Ca^{2+}]_i$ during the IgE mediated activation using the fura-2 labeled RBL-2H3 cells.. As shown in Fig.3, wortmannin had no effects on the increase in $[Ca^{2+}]_i$ at the concentration 10^{-6} and 10^{-5} M (A,B,C) where histamine release was suppressed (Fig.1). In contrast, KT5926 blocked the increase in $[Ca^{2+}]_i$ dose-dependently at 10^{-7} and 10^{-8} M(Fig.3 D,E,F). Thus, wortmannin could inhibit IgE-mediated histamine release from RBL-2H3 cells without any significant effects on the increase in $[Ca^{2+}]_i$. This suggests that the phosphorylation of myosin light chain is involved at the downstream of calcium influx initiated with the crosslinking of $Fc_\epsilon RI$ on the mast cell.

Two acting site of MLCK can be speculated: first, myosin light chain phosphorylation might modify the Fc_ϵ receptor-associated cytoskeleton such as actin polymerization; second, MLCK may be involved in the movement of vesicles and fusion of the vesicles to membrane at the final secretory phase. Both inhibitor blocked IgE-mediated histamine release completely, whereas both (over 10 nM) inhibited only 40 % of histamine release activated with A23187 (Fig.2) which can bypass the $Fc_\epsilon RI$ for the signal

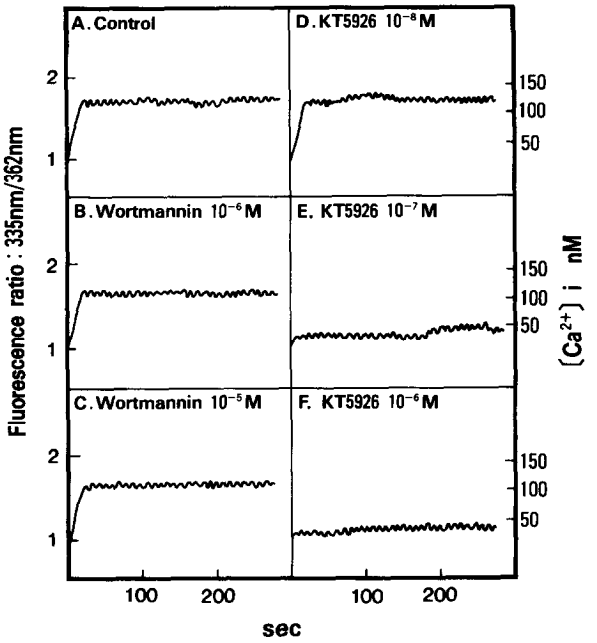

Fig.3. Effect on $[Ca^{2+}]_i$ in RBL-2H3 cells with wortmannin and KT5926.

IgE-sensitized RBL-2H3 cells($6x10^5$) were loaded with 6 μM of fura-2-AM. Cells were preincubated with inhibitor for 10 min and challenged with DNP_{48}-HSA at 37°C. Cytosolic free calcium was monitored during activation with using a Shimazu RF-5000 spectrophotometer(excitation, 335nm and 365nm; emission 500nm). Wortmannin and KT5926 did not have any effect on fluorescence measurement within the range of concentration used in this system, though KT5926 had autofluorescence at more than 30 μM. The ordinate shows the fluorescence ratio of 335nm/365nm and the abscissa shows the time after antigen stimulation (sec). These traces are representatives of three experiments.

transduction. Therefore, these MLCK inhibitor suppress predominantly signalling through $Fc_{\epsilon}RI$, but still contributing partially the block of movement of vesicles. In fact, it was reported that the signalling and morphological change in RBL-2H3 cells were different between IgE- and A23187-mediated histamine release(5,17-19). Recently, Knol et al.(20) reported the inhibition of histamine release induced with anti-IgE from human basophils with using wortmannin, but the exact targeting site of wortmannin and the significance of cytoskeleton in early phase signalling through $Fc_{\epsilon}RI$ have not been discussed by them.

Although the relation of MLCK to other signalling apparatus during histamine release is not clear, at least, protein kinase C was reported to phosphorylate myosin light chain (21) or MLCK was described to phosphorylate myosin light chain(22). High specificity of wortmannin is due to its direct binding to unique

catalytic site for MLCK(7). Since the chemical structure of KT5926 is also similar to the other protein kinase inhibitor, staurosporin(23), its inhibition may be due to the interaction with the ATP-binding site in the catalytic domain. Recently it was reported by kinetic study that KT5926 had an additional effect to inhibit strongly Ca^{2+} calmodulin kinase II(24). Therefore, the difference of effect on Ca^{2+} influx between wortmannin and KT5926 may be explained by the specificity of inhibitory effect of two(6,7).

Taken together, the phosphorylation of myosin, as one of the $Fc_{\epsilon}RI$ associated cytoskeleton, seems to be involved in early phase of signalling through $Fc_{\epsilon}RI$ following Ca^{2+} influx.

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