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MY-1250, a major metabolite of the anti-allergic drug repirinast, induces phosphorylation of a 78-kDa protein in rat mast cells

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Abstract—Repirinast (MY-5116; isoamyl 5,6-dihydro-7,8-dimethyl-4,5-dioxo-4*H*-pyrano[3,2-*c*]quinoline-2-carboxylate) is an anti-allergic drug of demonstrated effectiveness for treating bronchial asthma in humans. MY-1250 (5,6-dihydro-7,8-dimethyl-4,5-dioxo-4*H*-pyrano[3,2-*c*]quinoline-2-carboxylic acid), the major active metabolite of repirinast, inhibits antigen-induced histamine release from sensitized rat peritoneal exudate cells (PEC). When purified rat mast cells were treated with MY-1250 (2.5×10^{-5} M) for 1 min, phosphorylation of a specific mast cell protein of apparent molecular mass of 78 kDa was observed as previously reported for sodium cromoglycate (SCG). Phosphorylation of this protein induced by MY-1250 and SCG occurred in a concentration–dependent manner with IC_{50} values of 2.0×10^{-7} and 1.4×10^{-5} M, respectively. MY-1250 did not inhibit calcium ionophore A23187 (1 μg/mL)-induced histamine release from rat PEC. In the presence of calcium ionophore A23187 (1 μg/mL), phosphorylation of this protein induced by MY-1250 was not evident. In conclusion, MY-1250 induced phosphorylation of a 78-kDa protein in rat mast cells and MY-1250 may inhibit histamine release by regulating phosphorylation of this protein in rat mast cells.

Reprinast (MY-5116, isoamyl 5,6-dihydro-7,8-dimethyl-4,5-dioxo-4*H*-pyrano[3,2-*c*]quinoline-2-carboxylate) is an orally active anti-allergic drug shown to be effective in the treatment of bronchial asthma in humans [1]. When repirinast is absorbed in humans or animals, it is metabolized to MY-1250 (5,6-dihydro-7,8-dimethyl-4,5-dioxo-4*H*-pyrano[3,2-*c*]quinoline-2-carboxylic acid)[2]. MY-1250 inhibits histamine release from rat peritoneal exudate cells (PEC) in a concentration-dependent manner with an IC_{50} value of 1.2×10^{-7} M [3].

Sieghart *et al.* [4] reported that when rat mast cells were treated with compound 48/80 or calcium ionophore A23187, phosphorylation of proteins with apparent molecular masses of 68, 59, and 42 kDa was evident within 10 sec. Phosphorylation of a protein of 78 kDa was not evident until 30–60 sec after the addition of compound 48/80. It was suggested that the early rapid phosphorylation of three proteins might be involved in the initiation of histamine release, while the later phosphorylation of the 78-kDa protein might be associated with the termination of histamine release. Treatment of rat mast cells with sodium cromoglycate (SCG*) also induced phosphorylation of a 78-kDa protein, suggesting that SCG may inhibit histamine release by regulating phosphorylation of a mast cell protein [5, 6]. To investigate the inhibitory mechanism of MY-1250 on histamine release, we examined the effect of MY-1250 on the phosphorylation of proteins in rat mast cells.

Materials and Methods

Drugs and chemicals. MY-1250 and SCG were synthesized at Mitsubishi Kasei Corp. (Yokohama, Japan). [^{32}P]-Orthophosphate, carrier-free in aqueous solution, was obtained from Amersham; calcium ionophore A23187 was purchased from Calbiochem-Behring. Buffer used throughout was the HEPES-buffered salt solution (HBSS) composed of 137 mM NaCl, 27 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.5 mg/mL BSA, 10 mM HEPES; pH 7.4.

Purified rat peritoneal mast cells. Male Wistar rats (200–300 g, Japan SLC Inc., Japan) were exsanguinated and 10 mL of HBSS containing 5 U/mL heparin were injected i.p. After gently massaging the abdominal cavity for 1 min, the fluid was collected from the peritoneal cavity and centrifuged at 110 *g* for 5 min at 4°. The obtained cell pellet was then resuspended in HBSS. Centrifugation was repeated three times and the final pellet was suspended in HBSS. The mast cells were counted following staining with toluidine blue. Mast cells were isolated from rat PEC suspensions using 38% BSA solution [7]. Two millilitres of PEC (2×10^6 cells/mL) were layered on 3 mL of 38% BSA solution and the mixture was centrifuged at 450 *g* for 25 min at 10°. Cells at the interface were discarded and the pellet was resuspended in HBSS and washed by centrifugation to remove residual BSA.

Phosphorylation of mast cell proteins. Purified rat peritoneal mast cells were incubated in 400 μL at a density of 4×10^6 mast cells/mL with 500 μCi [^{32}P]orthophosphate. After 40 min, the incubation mixture was centrifuged at 100 *g* for 5 min at 4° and washed three times in HBSS to remove excess ^{32}P. The final cell density was adjusted to 2×10^6 mast cells/mL. Eighty microlitres of cell suspension (2×10^6 mast cells/mL) were added to the test compound (20 μL) which was then incubated at 37° for 1 min. The reaction was stopped by the addition of 25 μL of SDS solution containing 12.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.125 M Tris–HCl buffer, pH 6.8. The mixture was placed in a boiling water bath for 5 min. Samples were stored at −20° before the analysis of SDS–PAGE.

SDS–PAGE. SDS–PAGE was carried out using a 10% polyacrylamide gel according to the method of Laemmli [8]. After electrophoresis, the slab gels were stained with Coomassie brilliant blue R250 (0.125% in 50% methanol, 10% acetic acid) and destained in 50% methanol and 10% acetic acid. After drying the gels using a gel dryer (Bio-Rad Laboratories), autoradiography was performed at −70° using Kodak X-Omat RP X-ray film (Eastman Kodak). Gels were calibrated using a standard molecular mass kit (Pharmacia). To provide a graphical representation, autoradiographs were scanned with a scanning densitometer (Dual-wavelength TLC scanner, CS-930, Shimadzu, Japan) and the corresponding area was measured. The basal phosphorylation in the absence of drug was set as one arbitrary unit.

*Abbreviations: SCG, sodium cromoglycate; PEC, peritoneal exudate cells; HBSS, HEPES-buffered salt solution; BSA, bovine serum albumin.

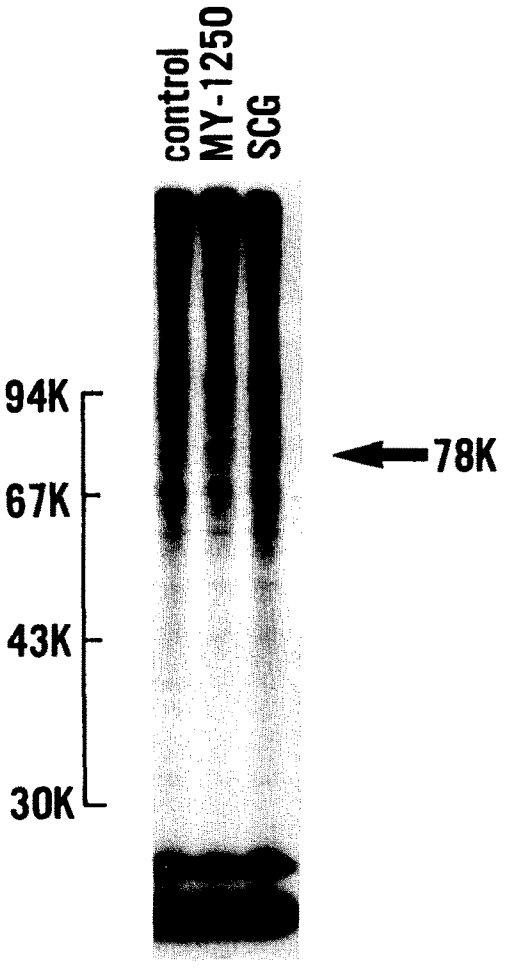

Fig. 1. Autoradiograph showing MY-1250- and SCG-induced phosphorylation of rat mast cell protein. Purified rat mast cells (purity more than 93%) were preincubated with [^{32}P]orthophosphate and then incubated with MY-1250 (2.5×10^{-5} M) or SCG (2.0×10^{-4} M) for 1 min. These cells were then dissolved in a solution containing 10% SDS and analysed for radioactive phosphate incorporation by autoradiography. Molecular mass was estimated using a standard molecular mass kit.

Analysis of data. The IC_{50} value for protein phosphorylation was defined as the concentration of drug required to produce 50% of the maximum phosphorylation. The IC_{50} values were estimated from concentration–response curves by best fit regression.

Results

When purified rat mast cells (purity more than 93%) were treated with MY-1250 (2.5×10^{-5} M) for 1 min, phosphorylation of a 78-kDa protein was induced (Fig. 1). The phosphorylated protein had the same time-related and phosphorylation characteristics as that phosphorylated by treatment with SCG (2.0×10^{-4} M) for 1 min. To examine the degree of phosphorylation, the incorporation of radioactive phosphate into the 78-kDa protein was estimated using a scanning densitometer. The incorporation of radioactive phosphate into the 78-kDa protein induced by MY-1250 and SCG occurred in a concentration-dependent manner with IC_{50} values of 2.0×10^{-7} and 1.4×10^{-5} M, respectively (Fig. 2).

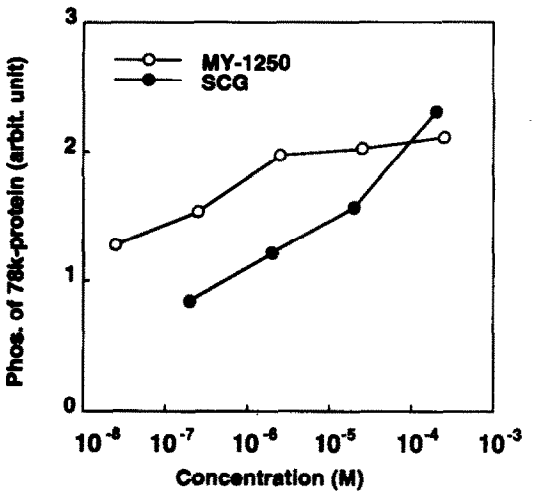

Fig. 2. Graphical representation of the effects of various concentrations of MY-1250 and SCG. Autoradiographs were scanned with a scanning densitometer and the degree of phosphorylation of protein is represented in arbitrary units. One arbitrary unit represents the basal phosphorylation level in the absence of drug. Each value represents the mean of two experiments.

When purified rat peritoneal mast cells were treated with calcium ionophore A23187 (1 μg/mL) for 1 min, phosphorylation of 68- and 59-kDa proteins was observed. When purified mast cells were treated with both MY-1250 (2.5×10^{-5} M) and calcium ionophore A23187 (1 μg/mL) for 1 min, phosphorylation of 68- and 59-kDa proteins, but not the 78-kDa protein, was induced (Fig. 3).

Discussion

Sieghart *et al.* [4] reported that compound 48/80 induced phosphorylation of 68-, 59- and 42-kDa proteins in purified rat peritoneal mast cells. These phosphorylations were very rapid and peaked at 10 sec after the addition of compound 48/80. Phosphorylation of a 78-kDa protein was not evident until 30–60 sec after the addition of compound 48/80. Phosphorylation of this 78-kDa protein occurred after most of the histamine had been released. Also, SCG induced the rapid phosphorylation of the 78-kDa protein in isolation. These results suggested that phosphorylation of the 78-kDa protein represented a termination factor of histamine release [5]. In this study, we reported that MY-1250 also induced phosphorylation of a 78-kDa protein in rat mast cells.

We have reported previously [9] that MY-1250 inhibited antigen-induced histamine release from rat PEC in a concentration-dependent manner with an IC_{50} value of 1.2×10^{-7} M. SCG also showed an inhibitory effect on histamine release from rat PEC with an IC_{50} value of 1.0×10^{-5} M. In the present investigation, we showed that both MY-1250 and SCG induced phosphorylation of a 78-kDa protein in rat mast cells in a concentration-dependent manner with IC_{50} values of 2.0×10^{-7} and 1.4×10^{-5} M, respectively. The concentration range over which phosphorylation of the 78-kDa protein could be induced corresponds with that which inhibited antigen-induced histamine release from rat PEC.

Calcium ionophore A23187 induces histamine secretion in several tissues including rat PEC [10], and at the same concentrations also induces phosphorylation of 68-, 59- and 42-kDa proteins in rat mast cells [4]. In our experiments, A23187 induced phosphorylation of 68- and 59-kDa proteins in rat mast cells. However, we could not detect

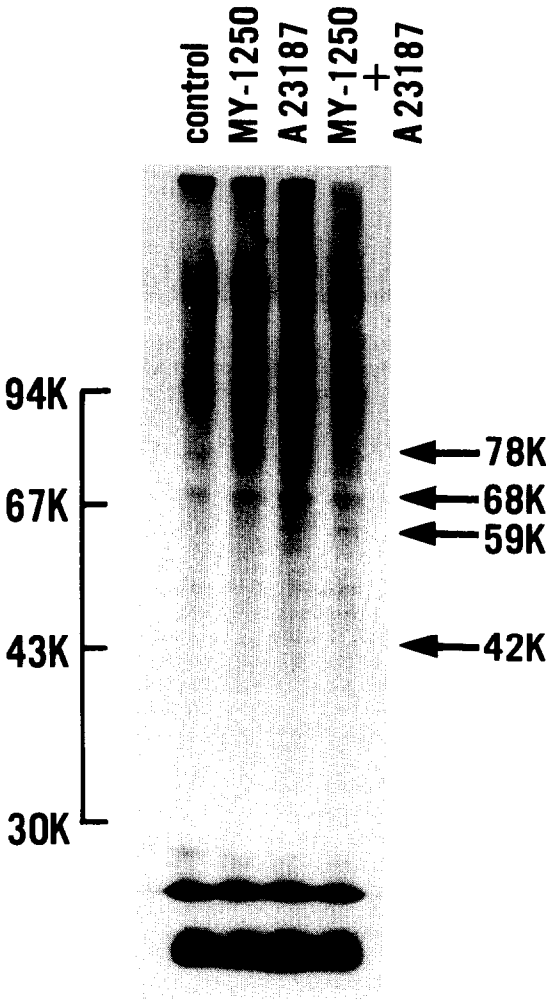

Fig. 3. Autoradiograph showing the effects of MY-1250 and calcium ionophore A23187 on the phosphorylation of mast cell proteins. Purified mast cells were incubated with MY-1250 (2.5×10^{-5} M), calcium ionophore A23187 (1 μg/mL) or both for 1 min. Protein phosphorylation of mast cells was analysed as described in Materials and Methods.

phosphorylation of a 42-kDa protein. It was reported [4] that the phosphorylation level of a 42-kDa protein was lower than those of other proteins, which may explain the lack of detection of phosphorylation of this protein in our experiments. MY-1250 (2.5×10^{-5} M) did not prevent either A23187 (1 μg/mL)-induced histamine release (data not shown) or phosphorylation of the 78-kDa protein in the presence of the calcium ionophore. This result suggests a correlation between inhibition of histamine release and phosphorylation of the 78-kDa protein.

Furthermore, it has been reported previously [11] that REV2871, which has an inhibitory effect on histamine release from rat PEC, also induces phosphorylation of the 78-kDa protein in rat mast cells. These results indicate that phosphorylation of the 78-kDa protein may play an important role in the regulation of the cell secretory system in rat mast cells and that anti-allergic drugs commonly exert their anti-secretory effect by inducing phosphorylation of this protein.

In conclusion, MY-1250 induced phosphorylation of a 78-kDa protein in rat mast cells which we suggest is involved in the termination or limitation of histamine release.

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