Immunopharmacological Studies on TBX, a New Antiallergic Drug
(2) Inhibitory Effects on Histamine Release from Peritoneal Mast Cells and Lung Fragments of Rats

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Accepted May 26, 1988

Abstract—The ability of 9-methyl-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one potassium salt (TBX) to inhibit histamine release from both peritoneal exudate cells (PEC) containing mast cells and lung fragments of rats was investigated in vitro. Low concentrations of TBX dose-dependently inhibited IgE-mediated histamine release from PEC of passively sensitized animals; its IC50 was $5.1 \times 10^{-9}$ g/ml. When TBX was added simultaneously with the antigen challenge, the highest inhibition was obtained. In contrast, extension of preincubation time with the agent resulted in a marked decrease in the inhibition of histamine release. The potent inhibition of histamine release by TBX was observed equally in glucose-free as well as complete Tyrode’s solution, whereas TBX reduced its inhibitory action in Ca$^{2+}$-free or D$_2$O-supplemented medium. In addition, TBX inhibited compound 48/80 but not calcium ionophore A23187-induced histamine release from normal PEC. With regard to the intracellular cyclic AMP level in normal PEC, it was significantly enhanced by a high concentration of TBX ($10^{-3}$ g/ml). TBX also inhibited antigen-induced histamine release from lung fragments of actively immunized animals. Interestingly, TBX displayed non-competitive inhibition of cyclic AMP-dependent phosphodiesterase derived from lung homogenates; its $K_i$ value was $8.70 \times 10^{-4}$ M.

In the preceding companion paper (1), 9-methyl-3-(1H-tetrazol-5-yl)-4H-pyrido[1,2-a]pyrimidin-4-one potassium salt (TBX), which is chemically unrelated to disodium cromoglycate (DSCG) or tranilast, has been reported to be an orally effective drug capable of inhibiting passive cutaneous anaphylaxis (PCA) mediated by homologous IgE or IgG antibody in rats and guinea pigs. In addition, this agent has been shown to have no antagonistic actions on released chemical mediators and to be essentially devoid of $\beta$-adrenergic or hormonal stimulation. Thus, it was suggested that the inhibitory activity of TBX on homologous PCA resulted from the inhibition of chemical mediator release from mast cells.

The present experiments were undertaken to investigate the inhibitory effects of TBX on histamine release from both peritoneal mast cells and lung fragments of rats. The results obtained indicate that TBX is a potent inhibitor of histamine release induced by an immunological or non-immunological stimulus. The possible mode of inhibitory action of TBX on the histamine release were also investigated preliminarily.

Materials and Methods

Animals: Male Wistar rats weighing about 300 g were obtained from Shizuoka Laboratory Animal Center.

Chemicals: The chemicals used were theophylline (Wako), deuterium oxide (D$_2$O, Miles), compound 48/80 (Sigma) and calcium ionophore A23187 (A23187, Cal-
biochem). Theophylline and compound 48/80 were dissolved in physiological saline, whereas A23187 was dissolved in dimethylsulfoxide (Wako) immediately before use. All of the materials employed in this study were exactly as described in the preceding companion paper (1).

Antigen-induced histamine release from peritoneal exudate cells (PEC): Rats were passively sensitized with i.p. injection of 1 ml of homologous IgE serum, taken from the animals which had been immunized with 2,4-dinitrophenyl-conjugated Ascaris extract (DNP-As) mixed with killed Bordetella pertussis and boosted 5 days later by DNP-As alone according to the method of Tada and Okumura (2); the anti-DNP IgE antibody titer of the antiserum obtained 8 days later from the first immunization was 1:256 as determined by 48-hr homologous PCA in rats. After passive sensitization for 48 hr, they were bled and injected i.p. with 10 ml/animal of Tyrode’s solution containing 0.3% bovine serum albumin (BSA) and 5 units/ml of heparin. After gentle massage of the abdomen for 2 to 3 min, PEC were obtained and washed 3 times with Ca²⁺-free Tyrode’s solution. Mast cells in the suspension of PEC were stained with 0.05% toluidine blue, and PEC were adjusted to a concentration of 5 X 10⁴ mast cells/ml using Tyrode’s solution containing 0.3% bovine serum albumin (BSA) and 5 units/ml of heparin. After gentle massage of the abdomen for 2 to 3 min, PEC were obtained and washed 3 times with Ca²⁺-free Tyrode’s solution. Mast cells in the suspension of PEC were stained with 0.05% toluidine blue, and PEC were adjusted to a concentration of 5 X 10⁴ mast cells/ml using Tyrode’s solution containing 0.3% bovine serum albumin (BSA) and 5 units/ml of heparin. After gentle massage of the abdomen for 2 to 3 min, PEC were obtained and washed 3 times with Ca²⁺-free Tyrode’s solution. Mast cells in the suspension of PEC were stained with 0.05% toluidine blue, and PEC were adjusted to a concentration of 5 X 10⁴ mast cells/ml using Tyrode’s solution containing 0.3% bovine serum albumin (BSA) and 5 units/ml of heparin. 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Compound 48/80- or A23187-induced histamine release from PEC of normal rats: PEC from normal rats (5 X 10⁴ mast cells/ml) were prewarmed for 10 min at 37°C, challenged either with compound 48/80 (final concentration of 0.03 μg/ml) or with A23187 (final concentration of 0.05 μg/ml), and further incubated for 10 min. Histamine concentration in the supernatant was determined as described above.

Antigen-induced histamine release from lung fragments of actively immunized rats: Rats were immunized with DNP-As either mixed with Bordetella pertussis or emulsified with complete Freund’s adjuvant (CFA) as described in the preceding companion paper (1). Animals displaying the high titer of anti-DNP IgE or IgG antibody were selected, and their lungs were isolated. Lung fragments were prepared with a tissue sectioner (TC-2 Sorval) and washed thoroughly with Tyrode’s solution. The lung fragments (200 mg) were suspended in 1.6 ml of Tyrode’s solution containing 20 mM HEPES buffer, prewarmed for 15 min at 37°C, challenged with DNP-BSA at a final concentration of 1 mg/ml, and further incubated for 30 min. Histamine concentration in the supernatant was determined by the enzymatic radioassay, and the total content of histamine was also assayed in the intact tissue which had been previously boiled for 10 min.

Measurement of cyclic AMP in rat PEC: PEC taken from normal rats were adjusted to a concentration of 10⁶ mast cells/ml. The cell suspension (0.45 ml) was prewarmed at 37°C for 10 min, and each drug solution (0.05 ml) was added, followed by an additional incubation for 10 min. The reaction was terminated by cooling in ice water, and the cell pellets were obtained by centrifugation. Cyclic AMP was extracted according to the method of Steiner et al. (4). Briefly, extraction of cyclic AMP from PEC was performed 3 times by adding 6% trichloroacetic acid (TCA, Wako). TCA was removed from the extract with water-saturated ether, and cyclic AMP in the water layer was succinylated according to the manual instructions. The succinylated cyclic AMP obtained was measured using a commercially available radioimmunoassay kit (Yamasa).

Measurement of cyclic AMP-dependent phosphodiesterase (cyclic AMP-PDE) from rat lungs: The lung tissue from normal rats was homogenized with 50 mM Tris-HCl buffer containing 1 mM MgCl₂ (pH 8.0). The supernatant was obtained by centrifugation at 1,300 x g for 10 min, further centrifuged at
22,000×g for 30 min, and used as a source of cyclic AMP-PDE (protein content, 1.67 mg/ml). The activity of this enzyme was measured according to the method of Russell et al. (5). Briefly, enzyme solution containing cyclic AMP-PDE was diluted 10-fold with 50 mM Tris-HCl buffer supplemented with 1 mM MgCl₂ and 3.75 mM 2-mercaptoethanol (Sigma), and each drug was added, followed by incubation for 10 min at 30°C. Both ³H-cyclic AMP (New England Nuclear) and non-radioactive cyclic AMP (Sigma) were added and further incubated for 15 min. The reaction was terminated by boiling for 3 min, and the mixture was then incubated in the presence of king cobra venom (Sigma) at a final concentration of 250 μg/ml at 30°C for 10 min. One ml of a 50% suspension of Dowex 1-×2 (Dow Chemical) was added in order to remove unconverted cyclic AMP, followed by standing at room temperature for 15 min. ³H-adenosine as a final product was determined by a liquid scintillation counter (Aloka LSC-635).

Statistical analysis: Results were expressed as the mean±S.E. Statistical significance was determined by Student's t-test. IC₅₀ was obtained by the logit method.

Results

Effect on antigen-induced histamine release in PEC of passively sensitized rats: The effect of preincubation time with TBX at a final concentration of 10⁻⁴ g/ml on antigen-induced histamine release from PEC containing mast cells was first investigated in rats passively sensitized with homologous anti-DNP IgE serum. This anaphylactic histamine release was performed in the presence of 10 μg/ml of PS. As illustrated in Fig. 1, when added simultaneously with the antigen challenge, TBX showed the highest inhibition of IgE-mediated histamine release from PEC. In contrast, extension of the preincubation time (5 and 15 min) with TBX resulted in a marked decrease in the inhibition of histamine release. Second, a dose-response study pertaining to the inhibition of histamine release was done by adding TBX together with antigen, and its ability to inhibit histamine release was compared with that of DSCG or tranilast under identical conditions. As shown in Fig. 2, histamine release was inhibited in a dose-dependent fashion by TBX at low concentrations of 10⁻¹⁰ to 10⁻⁷ g/ml, and its inhibitory activity reached a plateau at a concentration of 10⁻⁷ g/ml, where the inhibition of histamine release was approximately 85%. Similarly, both DSCG and tranilast displayed dose-dependent inhibition of this histamine release. Note that the inhibitory action of TBX upon histamine release was markedly superior to that of DSCG or tranilast; IC₅₀ values of these 3 drugs were 5.1×10⁻⁹ g/ml for TBX, 1.6×10⁻⁶ g/ml for DSCG and 1.3×10⁻⁵ g/ml for tranilast, respectively. Finally, the mechanism by which TBX inhibited IgE-mediated histamine release from mast cells was preliminarily investigated using modified Tyrode's solution. The results are shown in Fig. 3. Both a sufficient level of histamine release (19.4±1.66%) and the clear inhibition of histamine release by TBX (10⁻⁸ and 10⁻⁹ g/ml) were observed in the complete medium. In addition, glucose-free medium also gave almost the same extent of histamine release (20.2±1.31...
Fig. 2. Effect of TBX (○), DSCG (●) and tranilast (△) on antigen-induced histamine release from PEC of rats passively sensitized with homologous anti-DNP IgE serum. Net histamine release in the control was 24.7±1.79%. Each point represents the mean±S.E. of 4 to 12 experiments.

Fig. 3. Effect of TBX on antigen-induced histamine release from PEC of rats passively sensitized with homologous anti-DNP IgE serum. Each point indicates the mean±S.E. of 4 to 6 experiments. **: Statistically significant difference from the control at P<0.01.
% and dose-dependent inhibition of histamine release by TBX. In contrast, a significant decrease in histamine release (9.5±1.89%, P<0.01) was observed in Ca²⁺-free medium containing no chelating agents such as EDTA or EGTA, where only TBX at a concentration of 10⁻⁷ g/ml was able to slightly inhibit the histamine release. On the other hand, the significant enhancement of histamine release (45.1±3.20%, P<0.01) was seen in Tyrode's solution supplemented with 45% D₂O. D₂O-augmented histamine release was significantly inhibited by 10⁻⁷ g/ml of TBX, although the inhibitory activity of TBX was much weaker in the D₂O-supplemented medium than in the complete one.

Effect on compound 48/80 or A23187-induced histamine release from PEC of normal rats: PEC obtained from normal rats were non-immunologically stimulated with compound 48/80 or A23187. Drugs were added simultaneously with either stimulant. Figure 4 illustrates the inhibitory effect of TBX on histamine release from PEC stimulated by 0.03 μg/ml of compound 48/80 which induced 9.8±2.75% histamine release. TBX at concentrations of 10⁻⁹ to 10⁻⁶ g/ml dose-dependently inhibited the histamine release, and its inhibition reached a plateau at a concentration of 10⁻⁶ g/ml. Similarly, the dose-dependent inhibition of this histamine release was obtained with DSCG at concentrations of 10⁻⁶ and 10⁻⁵ g/ml, although DSCG was less potent than TBX. In contrast to TBX and DSCG, tranilast did not show any significant inhibition of compound 48/80-induced histamine release. However, TBX (10⁻⁷ to 10⁻⁴ g/ml) displayed little or no inhibition of histamine release that was caused by 0.1 μg/ml of compound 48/80 and amounted to about 40% (data not shown). On the other hand, none of the drugs (10⁻⁷ to 10⁻⁴ g/ml) showed significant inhibition of histamine release induced by 0.05 μg/ml of A23187, which amounted to the net control release of 44.2±3.40%, as indicated in Fig. 5. Note that the spontaneous histamine release from PEC was unaffected by all the drugs (10⁻⁶ to 10⁻⁴ g/ml) (Fig. 6).

Effect on antigen-induced histamine release from lung fragments of actively immunized rats: Rats were immunized with DNP-As and either Bordetella pertussis or CFA, and the lung fragments obtained were preincubated with TBX at concentrations of 10⁻⁶ to 10⁻⁴ g/ml for 15 min before antigen challenge. As shown in Fig. 7, TBX clearly inhibited antigen-induced histamine release.
from lung fragments of actively immunized rats, regardless of the kind of adjuvant used, although the inhibitory activity of TBX was not compared with that of DSCG or tranilast. Note that there were no significant differences between the two kinds of lung fragments obtained from *Bordetella pertussis* and CFA-treated animals with respect to the inhibition of histamine release by TBX.

**Effect on intracellular cyclic AMP level in PEC of normal rats:** Drugs were incubated with PEC of normal rats at 37°C for 10 min, and cyclic AMP extracted from the cells was succinylated for radioimmunoassay. The results are summarized in Table 1. The intracellular level of cyclic AMP in intact PEC containing 10⁶ mast cells was 10.2±0.47 pmol. Pretreatment of PEC with TBX at a high concentration of 10⁻³ g/ml resulted in a significant increase in cyclic AMP levels (15.5±0.76 pmol, P<0.01). Similar results were obtained with both isoproterenol and theophylline.

**Effect on cyclic AMP-PDE from lung preparation of normal rats:** The results obtained were analyzed according to the method of Lineweaver and Burk. As illustrated in Fig. 8, TBX as well as DSCG showed the non-competitive inhibition of cyclic AMP-PDE. In contrast, theophylline displayed the competitive inhibition of this enzyme. With regard to K<sub>i</sub> values, they were 8.70×10⁻⁴ M (TBX), 3.25×10⁻³ M (DSCG) and 2.50×10⁻⁴ M.

![Fig. 7. Effect of TBX on antigen-induced histamine release from lung fragments of rats actively immunized with DNP-As in combination with either *Bordetella pertussis* (—) or CFA (—). Net histamine release in the control was 12.2±1.46% in animals given *Bordetella pertussis*, and it was 6.5±0.89% in animals given CFA. Each point represents the mean±S.E. of 3 to 7 experiments.

**Table 1.** Effect of TBX, isoproterenol and theophylline on the intracellular cyclic AMP level in PEC of normal rats

| Drug         | Concentration (g/ml) | Cyclic AMP containing 10⁶ mast cells (pmol/PEC) | Ratio |
|--------------|----------------------|-----------------------------------------------|-------|
| Control      | —                    | 10.2±0.47                                     | 1.00  |
| TBX          | 10⁻⁴                 | 9.7±0.39                                      | 0.95  |
|              | 10⁻³                 | 15.5±0.76**                                   | 1.52  |
| Isoproterenol| 2×10⁻⁶               | 12.5±0.75*                                    | 1.23  |
| Theophylline | 10⁻³                 | 26.7±0.95**                                   | 2.62  |

PEC of normal rats were incubated with each drug at 37°C for 10 min. Each value represents the mean±S.E. of 4 experiments. **: Statistically significant difference from the control at P<0.05 and P<0.01, respectively.
In the preceding companion paper (1), the inhibition of chemical mediator release from mast cells was suggested to be responsible for the PCA inhibitory activity of TBX. The present study was designed to investigate its ability to inhibit histamine release from both PEC and lung fragments of rats, and the results obtained clearly indicate that TBX is a potent inhibitor of histamine release. For example, antigen-induced histamine release from peritoneal mast cells of rats passively sensitized with homologous IgE antibody was inhibited in a dose-dependent fashion by much lower concentrations of TBX than those of DSCG and tranilast; IC50 values for these 3 drugs were 5.1 x 10^{-9} g/ml (TBX), 1.6 x 10^{-6} g/ml (DSCG) and 1.3 x 10^{-5} g/ml (tranilast), respectively. Note that in order to obtain the highest inhibition of histamine release, TBX had to be added simultaneously with the antigen challenge. Prolonged pretreatment with TBX, however, resulted in a marked decrease in the inhibition of this histamine release. Similar findings were reported by a number of investigators who demonstrated a decay in the inhibition of histamine release resulting from increasing preincubation times.

Fig. 8. Lineweaver-Burk plots for the effects of TBX, DSCG and theophylline on cyclic AMP-PDE from rat lung preparations.
with DSCG (6-9). Although this phenomenon is not strictly understood, a decrease in the inhibition of histamine release by prolonged preincubation times with TBX or DSCG may be due to the tachyphylaxis often found with some antiallergic drugs (10-12). In addition, pretreatment of mast cells with DSCG, followed by a second exposure to the same drug, is well known to reduce its inhibitory activity on histamine release (6-9). Indeed, there existed in vivo tachyphylaxis to TBX's inhibition of IgE-mediated homologous PCA in rats (1), but in vitro tachyphylaxis to TBX's inhibition of histamine release from mast cells was not directly confirmed in the present study. A few hypotheses concerning the mechanism of tachyphylaxis to DSCG have been proposed; they have been already described in detail in the discussion of the preceding companion paper (1). Nevertheless, TBX's tachyphylaxis observed in the rat system may be of little or no significance in the clinical field, since self-inhibition of antiallergic drug is reported to be demonstrable only in rat preparations (8).

Preliminary experiments were performed to clarify the possible mode of inhibitory action of TBX on the IgE-mediated histamine release from rat mast cells in modified Tyrode's solution. Almost the same inhibition of anaphylactic histamine release by TBX was observed in glucose-free medium in which metabolic inhibitors such as 2-deoxyglucose (13, 14), antimycin A (15) and papaverine (16) were found to display higher degrees of inhibition of histamine release, compared with the use of the complete medium. The results demonstrating that the inhibitory activity of TBX on histamine release was hardly modified in glucose-free medium strongly suggest that this agent exerts little influence on energy metabolism. Indeed, doxantrazole (16), one of the potent antiallergic drugs whose properties differed entirely from the antimetabolites is known to inhibit IgE-mediated histamine release from rat mast cells in glucose-free medium to the same extent as in complete medium, showing no modification of the cellular ATP content. On the other hand, the inhibition of histamine release by \(10^{-7}\) g/ml of TBX was found to be slight in Ca\(^{2+}\)-depleted medium containing no chelating agents such as EDTA or EGTA. The decrease in the inhibition of histamine release was significant, as compared with the effect in complete medium. Both anaphylactic and non-anaphylactic histamine release from mast cells is well-known to be triggered by an increase in the intracellular Ca\(^{2+}\) level; such an increased concentration of Ca\(^{2+}\) in the cytosol results from either uptake of extracellular Ca\(^{2+}\) or Ca\(^{2+}\) release from the intracellular store or both (17-26). Interestingly, TBX at concentrations higher than \(10^{-7}\) g/ml showed clear inhibition of IgE-mediated histamine release from mast cells even in Ca\(^{2+}\)-depleted medium (data not shown). Results consistent with the present findings were reported by White and Pearce (27) who demonstrated that DSCG inhibited anaphylactic histamine release both in the presence and absence of extracellular Ca\(^{2+}\). In addition, DSCG has been shown both to prevent the uptake of extracellular Ca\(^{2+}\) from the channel (17, 28) and to inhibit Ca\(^{2+}\) release from the intracellular source (29). Therefore, TBX may exert its inhibitory influence on the influx of extracellular Ca\(^{2+}\) as well as on the efflux of intracellular cation. With regard to \(D_2O\)-enhanced histamine release, the inhibition by TBX was no more than by about 40% at a concentration of \(10^{-7}\) g/ml, whereas it showed an approximately 85% inhibition of histamine release in the complete medium. Similar findings were obtained with DSCG (30). It could thus be suggested that TBX as well as DSCG might not affect the formation of microtubules due to assembly of tubulin facilitated by \(D_2O\). On the other hand, TBX and DSCG but not tranilast showed the dose-dependent inhibition of compound 48/80-induced histamine release from normal mast cells. The inhibition by the former two drugs was demonstrable only when the cells were stimulated with a low concentration (0.03 \(\mu g/ml\)) of compound 48/80. In contrast, all the drugs displayed little or no inhibition of A23187-induced histamine release. Our results are in agreement with those reported by many investigators (31-35). However, there are a few papers demonstrating that DSCG inhibits A23187-induced histamine release from rat mast cells (36, 37). Drugs capable of increasing the
intracellular cyclic AMP level are generally known to inhibit histamine release caused by antigen or compound 48/80 but not by A23187 (24, 33, 38, 39). Indeed, a high concentration of TBX significantly increased the intracellular cyclic AMP level in PEC containing mast cells, although purified mast cells were not employed in this experiment. Furthermore, it is interesting to note that TBX showed more potent inhibition of cyclic AMP-PDE from rat lung preparations than DSCG. Both TBX and DSCG displayed non-competitive inhibition of this enzyme activity in contrast to theophylline that shows competitive inhibition. Note that antigen-induced histamine release was inhibited equally in two kinds of lung fragments from Bordetella pertussis and CFA-treated rats by TBX.

Taken all together, the results obtained here strongly suggest that the inhibition of histamine release by TBX, which is a more potent inhibitor of histamine release than DSCG and tranilast, may be attributed to a combination of inhibition of Ca²⁺ movement and elevation of intracellular cyclic AMP level; the latter might be triggered by the inhibition of cyclic AMP-PDE. Further studies are needed to clarify the mechanism by which TBX inhibits histamine release induced by both immunological and non-immunological stimuli.

References
1 Yanagihara, Y., Kasai, H., Kawashima, T. and Shida, T.: Immunopharmacological studies on TBX, a new antiallergic drug. (1) Inhibitory effects on passive cutaneous anaphylaxis in rats and guinea pigs. Japan. J. Pharmacol. 48, 91–101 (1988)
2 Tada, T. and Okumura, K.: Regulation of homocytotropic antibody formation in the rat. I. Feedback regulation by passively administered antibody. J. Immunol. 106, 1002–1011 (1971)
3 Yanagihara, Y. and Shida, T.: Immunopharmacological actions of the new antiallergic drug 11-oxo-11H-pyrido[2,1-b]quinazoline-2-carboxylic acid. Effects on type I hypersensitivity reactions in human leukocytes and in human and monkey lungs. Arzneimittelforschung 36, 1627–1631 (1986)
4 Steiner, A.L., Parker, C.W. and Kipnis, D.M.: Radioimmunoassay for cyclic nucleotides I. Preparation of antibodies and iodinated cyclic nucleotides. J. Biol. Chem. 247, 1106–1113 (1972)
5 Russell, T.R., Terasaki, W.L. and Appleman, M.M.: Separate phosphodiesterases for the hydrolysis of cyclic adenosine 3’5’-monophosphate and cyclic guanosine 3’5’-monophosphate in rat liver. J. Biol. Chem. 248, 1334–1340 (1973)
6 Sung, Cheng-po., Saunders, H.L., Krell, R.D. and Chakrin, L.W.: Studies on the mechanism of tachyphylaxis to disodium cromoglycate. Int. Arch. Allergy Appl. Immunol. 55, 374–384 (1977)
7 Sung, Cheng-po., Saunders, H.L., Lenhardt, E. and Chakrin, L.W.: Further studies on the tachyphylaxis to DSCG. The effect of concentration and temperature. Int. Arch. Allergy Appl. Immunol. 55, 385–394 (1977)
8 Thomson, D.S. and Evans, D.P.: Inhibition of immediate hypersensitivity reactions by disodium cromoglycate. Requirements for activity in two laboratory models. Clin. Exp. Immunol. 13, 537–544 (1973)
9 Kusner, E.J., Dubnick, B. and Herzig, D.J.: The inhibition by disodium cromoglycate in vitro of anaphylactically induced histamine release from rat peritoneal mast cells. J. Pharmacol. Exp. Ther. 184, 41–46 (1973)
10 Evans, D.P., Marshall, P.W. and Thomson, D.S.: Inhibition of immediate hypersensitivity reactions in the rat by ICI 74,917 and disodium cromoglycate. Tachyphylaxis and cross-reactivity in vivo and in vitro. Int. Arch. Allergy Appl. Immunol. 49, 417–427 (1975)
11 Welton, A.F., Hope, W.C., O’Donnel, M., Baruth, H., Crowley, H.J., Miller, D.A. and Yaremko, B.: Ro 22-3747: A new antiallergic agent for the treatment of immediate hypersensitivity diseases. J. Pharmacol. Exp. Ther. 228, 57–64 (1984)
12 Khandwala, A., Coutts, S.M. and Weinryb, L.: Antiallergic activity of nylidrin hydrochloride (RHC 3432-A). I. Effect on release of histamine in vitro from rat peritoneal mast cells, guinea pig lung slices and human basophils. Int. Arch. Allergy Appl. Immunol. 70, 295–302 (1983)
13 Lichtenstein, L.M. and Debernard, R.: Immediate allergic response: In vitro action of cyclic AMP-competitive and other drugs on the two stages of histamine release. J. Immunol. 107, 1131–1136 (1971)
14 Kaliner, M. and Austen, K.F.: Cyclic AMP, ATP and reversed anaphylactic histamine release from rat mast cells. J. Immunol. 112, 664–674 (1974)
15 Diamant, B., Norn, S., Fielding, P., Olsen, N., Ziebell, A. and Nisson, J.: ATP level and CO₂
production of mast cells in anaphylaxis. Int. Arch. Allergy Appl. Immunol. 47, 894–908 (1974)

16 Garland, L.G. and Johanson, T.: The relationship between energy metabolism and the action of inhibitors of histamine release. Br. J. Pharmacol. 61, 237–242 (1977)

17 Foreman, J.C., Hallett, M.B. and Mongar, J.L.: The relationship between histamine secretion and calcium uptake by mast cells. J. Physiol. (Lond.) 271, 193–214 (1977)

18 Ishizaka, K., Foreman, J.C., Sterk, A.R. and Ishizaka, T.: Induction of calcium flux across the rat mast cell membrane by bridging IgE receptors. Proc. Natl. Acad. Sci. U.S.A. 76, 5858–5862 (1979)

19 Ennis, M., Trunch, A., White, J.R. and Pearce, F.L.: Calcium pools involved in histamine release from rat mast cells. Int. Arch. Allergy Appl. Immunol. 62, 467–471 (1980)

20 Pearce, F.L., Ennis, M., Trunch, A. and White, J.R.: Role of intracellular and extracellular calcium in histamine release from rat peritoneal mast cells. Agents Actions 11, 51–54 (1981)

21 Ennis, M., Trunch, A., White, J.R. and Pearce, F.L.: Inhibition of histamine secretion from mast cells. Nature 289, 186–187 (1981)

22 Douglas, W.W. and Ueda, Y.: Mast cell secretion (histamine release) induced by 48/80. Calcium-dependent exocytosis inhibited strongly by cytochalasin only when glycolysis is rate limiting. J. Physiol. (Lond.) 234, 97–98 (1973)

23 Ennis, M., Atkinson, G. and Pearce, F.L.: Inhibition of histamine release induced by compound 48/80 and peptide 401 in the presence and absence of calcium, implications for the mode of action of anti-allergic compounds. Agents Actions 10, 222–228 (1980)

24 Tasaka, K., Mio, M. and Okamoto, M.: Changes in intracellular Ca²⁺ distribution of rat peritoneal mast cells before and after histamine release. Agents Actions 18, 61–64 (1986)

25 Johanson, T.: Mechanism of histamine release from rat mast cells induced by the ionophore A23187: Effects of calcium and temperature. Br. J. Pharmacol. 63, 643–649 (1978)

26 Foreman, J.C., Mongar, J.L. and Gomperts, B.D.: Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. Nature 245, 249–251 (1973)

27 White, J.R. and Pearce, F.L.: Effect of anti-allergic compounds on anaphylactic histamine secretion from rat peritoneal mast cells in the presence and absence of exogenous calcium. Immunology 46, 361–367 (1982)

28 Foreman, J.C., Hallett, M.B. and Mongar, J.L.: Site of action of the antiallergic drugs cromoglycate and doxantrazole. Br. J. Pharmacol. 59, 473–474 (1977)

29 White, J.R., Ishizaka, T., Ishizaka, K. and Sha'afi, R.I.: Direct demonstration of increased intracellular concentration of free calcium as measured by quin-2 in stimulated rat peritoneal mast cell Proc. Natl. Acad. Sci. U.S.A. 81, 3978–3982 (1984)

30 Saijo, T., Ashida, Y., Kuriki, H., Kanno, M. and Maki, Y.: Inhibitory action of 6-ethyl-3-(1H-tetrazol-5-yl)chromone (AA-344) on IgE-, IgGα- or chemical agent-induced histamine release from isolated rat mast cells. Japan. J. Pharmacol. 29, 531–540 (1979)

31 Welton, A.F., Hope, W.C., Crowley, H.J. and Salvador, R.A.: In vitro studies on the mechanism of action of a new antiallergic. Ro 21-7634, Agents Actions 11, 345–351 (1981)

32 Garland, L.G. and Mongor, J.L.: Differential histamine release by dextran and the ionophore A23187: The actions of inhibitors. Int. Arch. Allergy Appl. Immunol. 50, 27–42 (1976)

33 Goto, K., Hisadome, M. and Terasawa, M.: Inhibitory effect of Traxanol sodium on IgE mediated histamine release from passively-sensitized mast cells of the rat in vitro. Int. Arch. Allergy Appl. Immunol. 68, 332–337 (1982)

34 Khandwala, A., Coutts, S. and Weinnyb, I.: Antiallergic activity of Tiaramide (RHC 2592-A). Int. Arch. Allergy Appl. Immunol. 65, 159–168 (1982)

35 Khandwala, A., Coutts, S., Dally-Meade, V., Jariwala, N. and Huang, F.: RHC 3024: anti-allergic activity in vitro and comparison with disodium cromoglycate and other antiallergic agents. Int. Arch. Allergy Appl. Immunol. 70, 311–320 (1983)

36 Nagai, H., Kelly, K. and Sehon, A.H.: The inhibition of histamine release by antiallergic drugs. Int. Arch. Allergy Appl. Immunol. 56, 307–315 (1978)

37 Johnson, H.G. and Bach, M.K.: Prevention of calcium ionophore induced release of histamine in rat mast cells by disodium cromoglycate. J. Immunol. 114, 514–516 (1975)

38 Lichtenstein, L.M.: The mechanism of basophil histamine release induced by antigen and by the calcium ionophore A23187. J. Immunol. 114, 1692–1699 (1975)

39 Foreman, J.C., Mongar, J.L., Gomperts, B.D. and Garland, B.D.: A possible role for cyclic AMP in the regulation of histamine secretion and the action of cromoglycate. Bioch. Pharmacol. 24, 538–540 (1975)