Effects of MKS-492 on Antigen-Induced Bronchoconstriction and Allergic Reaction in Guinea Pigs and Rats

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ABSTRACT—Effects of R[+]-8-([1-[3,4-dimethoxyphenyl]-2-hydroxyethyl]amino)-3,7-dihydro-7-[2-methoxyethyl]-1,3-dimethyl-1H-purine-2,6-dione (MKS-492), a reported type III isozyme inhibitor of cyclic nucleotide phosphodiesterase, on antigen- or platelet activating factor (PAF)-induced bronchoconstriction and allergic reactions in guinea pigs and rats were investigated. 1) MKS-492 inhibited antigen-induced bronchoconstriction in guinea pigs. Aminophylline also inhibited the reaction. 2) MKS-492 inhibited PAF-induced bronchoconstriction and inhibited the increase in airway responsiveness to histamine in guinea pigs, although aminophylline failed to affect these reactions. 3) MKS-492 relaxed guinea pig tracheal muscle in vitro more potently than aminophylline. 4) MKS-492 inhibited leukotriene B4 (LTB4)-induced airway eosinophilia in guinea pigs. 5) MKS-492 inhibited passive cutaneous anaphylaxis and mediator-induced skin reactions in rats more potently than aminophylline. Both drugs inhibited antigen- and phospholipase A2-induced histamine release from guinea pig lung tissue. 6) MKS-492 inhibited PAF-induced O2− generation from guinea pig alveolar macrophages. These results indicate that MKS-492 is a more potent inhibitor of allergic bronchoconstriction and PAF- or LTB4-induced inflammatory reactions in guinea pigs and the allergic cutaneous reactions in rats when compared to aminophylline.

Keywords: Phosphodiesterase inhibitor (type III), Aminophylline, Bronchodilator, MKS-492, Bronchial asthma

Theophylline has been a useful drug for the treatment of asthma for over 50 years. Although some pharmacological actions of theophylline, such as phosphodiesterase (PDE) inhibition and adenosine antagonism, have been studied, the precise mechanisms responsible for its activity in the treatment of asthma are not yet fully elucidated (1–5). Among its actions, however, PDE inhibition has been considered to be important for asthma therapy. Recently, PDE isozymes have been characterized, and much attention has been paid to the application of distinct PDE isozyme inhibitors as a remedy for asthma (6–11), because the data obtained may explain the relationship between PDE inhibition and the therapeutic effect of theophylline for asthma and may lead to the development of more effective drugs. The existence of PDE isozymes in airway smooth muscle and airway inflammatory cells has already been demonstrated by many investigators (7–10). In 1991, Torphy and Undem reported that the inhibition of type III or V PDE is related to bronchodilation and that the inhibition of type IV PDE causes an anti-inflammatory effect in airways (8). However, the anti-inflammatory action of type III PDE inhibitors (SK&F 94120 and sigazodan) is mainly exerted through a mechanism not involving the inhibition of inflammatory cell function (6, 12–15). A type IV PDE selective inhibitor, tibenelast, and type III and IV PDE inhibitors, benzafentrine and zardaverine, are reported to be effective anti-asthmatic agents (8, 9). At present, however, only limited information is available about the action of PDE isozyme inhibitors. R[+]-8-([1-[3,4-Dimethoxyphenyl]-2-hydroxyethyl]amino)-3,7-dihydro-7-[2-methoxyethyl]-1,3-dimethyl-1H-purine-2,6-dione (MKS-492) is a selective inhibitor of cyclic GMP-inhibited PDE (type III PDE), and it is reported to inhibit antigen-induced airway inflammation (16–18). Many recent investigations have indicated the contribution of airway inflammation to the development of asthma (19–22). Therefore, in the present study, to elucidate the relationship between type III PDE inhibition and the therapeutic effect for asthma, the effects of...
MKS-492 on antigen- or platelet activating factor (PAF)-induced bronchoconstriction in guinea pigs and allergic inflammatory reactions in guinea pigs and rats were studied in comparison to those of aminophylline, which has a 2,000-times less potent inhibitory activity against type III PDE (16).

MATERIALS AND METHODS

Animals

Female Hartley guinea pigs weighing 250–300 g were used for preparation of antiserum. Male Hartley guinea pigs weighing 300–500 g were used for the other experiments. In some studies, male Wistar rats weighing 180–220 g were also used. All animals were purchased from Japan SLC, Inc. (Hamamatsu).

Antigen and antiserum

Benzylpenicilloyl bovine-γ-globulin (BPO-BGG) and bovine serum albumin (BPO-BSA) were used as antigens for the guinea pigs. Anti-BPO-BGG guinea pig serum was prepared according to the method described by Levine and Redmond (23). The IgE antibody titer was 1 : 211, as estimated by 7-day homologous passive cutaneous anaphylaxis (PCA). Dinitrophenylated Ascaris suum extract (DNP-As) and bovine serum albumin (DNP-BSA) were used as antigens for rats. Anti-DNP-As serum was prepared according to the method described by Tada and Okumura (24), with a slight modification. The IgE antibody titer of the antiserum preparation estimated by homologous PCA was 1 : 24.

Materials

MKS-492 was kindly donated by Sandoz Pharma, Ltd. (Basel, Switzerland). The following were obtained from the indicated commercial sources: Aminophylline and metyrapone (Aldrich Chemical Co., Inc., Milwaukee, WI, USA); histamine dihydrochloride (Histamine) and dimethyl sulfoxide (DMSO) (Nacalai Tesque, Inc., Kyoto); serotonin creatininsulfate (5-HT; Merck, Darmstadt, Germany), sodium phenobarbital (phenobarbital), leukotriene C₄ (LTC₄) and leukotriene B₄ (LTB₄) (Wako Pure Chemical Industries, Ltd., Osaka); PAF (Bachem, Torrance, CA, USA); phorbol myristate acetate (PMA), gallamine triethiodide (gallamine), phospholipase A₂ (PLA₂, cobra venom from Naja naja) (Sigma Chemical Co., St. Louis, MO, USA); and sodium pentobarbital (pentobarbital, Nembutal; Abbott Laboratories, North Chicago, IL, USA). PAF was dissolved in 0.25% BSA-saline. LTC₄ and PMA were dissolved in DMSO and then diluted with saline.

Antigen-induced bronchoconstriction

Guinea pigs were passively sensitized with anti-BPO-BGG guinea pig serum (0.5 ml/kg) by an intravenous injection via the forearm vein. Both 24 hr and 30 min before intravenous injection of antigen, metyrapone (5 mg/kg, i.v.), which inhibits 11β-hydroxylase in glucocorticoid biosynthesis, was given to sensitized guinea pigs in order to decrease glucocorticoid levels, because the serum glucocorticoid levels in guinea pigs are much higher than those in humans, especially in bronchial asthmatic patients (25, 26). The procedure for measuring alterations in respiration has been described previously (25). In brief, at 48 hr after sensitization, the guinea pigs were anesthetized with pentobarbital (50 mg/kg, i.p.). The trachea was cannulated (tracheotomy), and the cannula was connected to both a transducer (MFP-1100, TV-142, TU-241, TP-602T; Nihon Kohden, Tokyo) and a respirometer (RM-25, RPM-6018; Nihon Kohden). Then the guinea pigs were challenged by intravenous injection of BPO-BSA (30 µg/kg) saline solution. Alterations in respiration were measured by counting changes in the respiratory rate, and the volume and ratio of expiration time to inspiration time. MKS-492 and aminophylline were administered intravenously 5 min before the antigen challenge.

PAF-induced bronchoconstriction and increase in airway responsiveness to histamine

To measure PAF-induced bronchoconstriction and increases in airway responsiveness, previously described procedures were employed (27). Guinea pigs were anesthetized with pentobarbital (10 mg/kg, i.p.) and phenobarbital (120 mg/kg, i.p.) and paralyzed with gallamine (10 mg/kg, i.m.). The animals were ventilated with air (8 ml/kg, 60 strokes/min) using a rodent ventilator (New England Medical Instruments, Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method described by Konzett and Rössler (28) using a bronchospasm transducer (model 7020; Ugo Basile, Varese, Italy) connected to the tracheal cannula.

Airway reactivity to intravenous injection of histamine (3 µg/kg) was determined according to the method described by Anderson et al. (29) with a slight modification. Histamine was injected 2 times via the jugular vein to obtain a pre-challenge value. The interval of histamine injections was 7 min. Seven minutes after the latter injection of histamine, the animals were challenged with a single bolus dose of PAF (25 ng/kg, i.v.), and then histamine was again injected 7 min later. For the control, saline containing 0.25% BSA (vehicle) was used instead of PAF. Drugs were injected intravenously 5 min before PAF injection. Data were expressed as the percentage of the post-challenge value against the pre-challenge value.
Studies of guinea pig tracheal muscle relaxation

Guinea pigs were stunned and exsanguinated. The trachea was excised, trimmed of excess tissue, and cut longitudinally in the cartilage tissue area. The open trachea was cut into 16 to 20 segments. Four segments, tied together to form a chain, were placed in an organ bath containing Tyrode's solution. Changes in the tone of the preparation, with 0.5-g initial resting tension, were recorded isotonically (MEC, ME-4013; World Medical Co., Ltd., Nagoya). Drugs to be examined were added cumulatively after the carbachol (10^{-6} M)-caused contraction reached a plateau. Relaxing potency was calculated as a percentage of the relaxation induced by isoproterenol (10^{-9} M).

Measurement of leukocyte count in bronchoalveolar lavage fluid (BALF)

In order to measure the number of leukocytes in the airway, BALF was obtained from guinea pigs by the previously reported method (30). Four hours after the intratracheal injection of LTB4 (10 μg), the animals were sacrificed by means of an intraperitoneal injection of pentobarbital (260 mg/kg). BALF was obtained by the slow injection of 10 ml of sterile saline into the trachea using a cannula, after which the fluid was withdrawn. The number of nucleated cells in lavage fluid was counted in a smear prepared using a cytocentrifuge and stained with May-Grunwald and Giemsa stain.

PCA and cutaneous reactions

PCA and cutaneous reactions caused by allergic chemical mediators were examined at the same time in the same rats (31). As allergic mediators, histamine, 5-HT and LTC4 were used. On the shaved dorsal skin of rats, 6 reaction sites were marked, and 0.1 ml of 40-fold diluted anti-DNP-As serum was injected intradermally into 1 of the 6 reaction sites for passive sensitization. At the same time, 0.1 ml of saline was injected into another site as a control for PCA. Forty-eight hours after the sensitization, 0.1 ml of 10^{-5} g/ml of histamine, 5×10^{-7} g/ml of 5-HT or 3×10^{-6} g/ml of LTC4 was injected into 3 of the remaining sites intradermally. The last site was used as a control for cutaneous reactions and received an injection of an equivalent volume of saline. Immediately after the injections of mediator solutions and saline, 1 mg of DNP-BSA and 5 mg of Evans blue dye dissolved in saline were given intravenously. Thirty minutes later, the rats were sacrificed, and the reaction sites were excised for determination of extravasated dye. Each experimental group consisted of 6 rats, and the reaction sites were rotated within the group. Extravasated dye in each reaction site was extracted and determined colorimetrically according to the method described by Katayama et al. (32).

Histamine release from guinea pig lung tissue

Lungs from guinea pigs passively sensitized with anti-BPO-BGG guinea pig IgE antibody (0.5 ml anti-serum/head) were chopped with a McIlwain tissue chopper and then suspended in 10 times volume of Tyrode's solution. Histamine release was induced by incubation with antigen (BPO-BSA, 10^{-6} g/ml) at 37°C for 30 min. The amount of histamine in the incubation medium was measured according to the fluorescence method of May et al. (33).

PLA2-induced histamine release was obtained by incubation of chopped lung tissue from non-sensitized guinea pigs with PLA2 (0.5 IU/ml) at 37°C for 30 min. The released histamine was assayed as described above.

Measurement of superoxide anion (O_{2}^{-}) production

The generation of superoxide anion (O_{2}^{-}) was measured according to the method of Yamashita et al. (34). Guinea pig alveolar macrophages were prepared according to the previously described method (35). In brief, the animals were killed by intraperitoneal injection of pentobarbital (150 mg/kg). The trachea was cannulated, and the airway lumen was washed with three aliquots (10 ml) of saline containing 0.1% BSA warmed at 37°C. The lavage fluid from each animal was centrifuged (150×g at 4°C for 10 min). The cell pellets were gently washed with Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution (HBSS, pH 7.3) and finally suspended at 2×10^{6} cells/ml in HBSS containing 0.1% BSA. These cells contained more than 92% alveolar macrophages as identified by May-Grunwald and Gimsa staining. These preparations always contained more than 98% viable cells as measured by trypan blue staining. Cells in 1 ml HBSS containing 0.1% BSA were pre-incubated with cytochrome C (1.24 mg/ml) and cytochalasin B (5 μg/ml). After 10 min, cells were stimulated by PAF (10^{-6} g/ml) or PMA (2×10^{-7} g/ml) for 10 min. Drugs to be tested were added 5 min before the stimulation. The reaction was terminated by transferring the test tubes into an ice bath, followed by centrifugation at 1,500×g for 10 min at 4°C. An aliquot of the supernatant was then measured spectrophotometrically at 550 nm. The amount of reduced cytochrome C was calculated from the molar extinction coefficient of 21.1 mM^{-1}cm^{-1}. Results are expressed as the number of nanomoles of cytochrome C reduced by superoxide dismutase.

Statistical analyses

Statistical analyses were performed by Student's t-test and Dunnett's multiple range test (36).
RESULTS

Antigen-induced bronchoconstriction in vivo in guinea pigs

In metyrapone-treated guinea pigs, the antigen-induced bronchoconstriction and prolongation of the ratio of expiration time to inspiration time were clearly potentiated as described before (25). The effects of MKS-492 and aminophylline are indicated in Figs. 1 and 2. MKS-492 at doses of 3 and 10 mg/kg inhibited antigen-induced bronchoconstriction in a dose-related manner (Fig. 1). In contrast, aminophylline at a dose of 3 mg/kg did not affect bronchoconstriction, although at a dose of 10 mg/kg, the drug inhibited it almost completely (Fig. 2).

PAF-induced bronchoconstriction in vivo in guinea pigs

Figure 3 shows the results of PAF-induced bronchoconstriction in guinea pigs. PAF (150 ng/kg administered intravenously) caused bronchoconstriction, causing an increase in air overflow of 75.6±4.30%, compared with the maximum contraction obtained by clamping off the tracheal cannula. MKS-492 at doses of 1 and 3 mg/kg completely inhibited the PAF-induced bronchoconstriction, but the same doses of aminophylline failed to affect it.

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Fig. 1. Effect of MKS-492 on antigen-induced bronchoconstriction in guinea pigs. Guinea pigs were passively sensitized with anti-BPO-BGG IgE antibody and pretreated with metyrapone. The drug was administered intravenously 5 min before antigen challenge. Each point represents the mean of 6 to 8 animals. ∗: P<0.05. Control (○); MKS-492: 3 mg/kg (Δ), 10 mg/kg (▲).

Fig. 2. Effect of aminophylline on antigen-induced bronchoconstriction in guinea pigs. Guinea pigs were passively sensitized with anti-BPO-BGG IgE antibody and pretreated with metyrapone. The drug was administered intravenously 5 min before antigen challenge. Each point represents the mean of 6 or 7 animals. ∗: P<0.05. Control (○); aminophylline: 3 mg/kg (□), 10 mg/kg (■).
PAF-induced increase in airway responsiveness to histamine

Histamine (3 μg/kg) administered intravenously caused transient bronchoconstriction, causing an increase in air overflow of 12.2 ± 2.75% (n = 12), compared with the maximum contraction obtained by clamping off the tracheal cannula. Although intravenous injection of PAF (25 ng/kg) produced a negligible bronchoconstriction by itself, it induced a significant increase (325.7 ± 36.3%) in airway responsiveness to histamine. When MKS-492 at doses of 1 and 3 mg/kg was administered 5 min before PAF injection, the PAF-induced increase in airway responsiveness to histamine was inhibited significantly (Fig. 4). Aminophylline at doses of 1 and 3 mg/kg only showed a tendency of inhibition.

Relaxation of tracheal muscle contracted with carbachol

Figure 5 indicates the effect of MKS-492 and aminophylline on the relaxation of isolated guinea pig tracheal muscle previously contracted with carbachol. Tracheal muscle preparations without any drug treatment (control) relaxed only slightly in a time-dependent manner. Both drugs relaxed the preparations concentration-dependent-
Fig. 6. Effect of MKS-492 and aminophylline on LTB₄-induced cell accumulation in the airway of guinea pigs. Each drug was administered intraperitoneally 30 min prior to treatment with LTB₄ (10 pg/trachea), and bronchoalveolar lavage fluid was recovered 4 hr after the stimulation. Results of the group not treated with PAF are indicated as vehicle. Each column represents the mean±S.E. of 7 to 9 animals. To: total cell, Ma: macrophage, Eo: eosinophil, Ne: neutrophil, Ly: lymphocyte, *P<0.05.

LTB₄-induced increase in leukocyte count in BALF

LTB₄ at a dose of 10 µg increased the number of total cells, eosinophils and neutrophils in BALF (Fig. 6). When MKS-492 was administered intraperitoneally at doses of 30 and 100 mg/kg, 30 min before the injection of LTB₄, the increases in eosinophils and neutrophils were inhibited. Aminophylline at a dose of 30 mg/kg also inhibited the increase of neutrophils.

PCA and mediator-induced cutaneous reactions

Figure 7 indicates the results of PCA and mediator-induced cutaneous reactions. MKS-492 and aminophylline were administered intraperitoneally 30 min prior to induction of reactions. MKS-492 at doses of 10–100 mg/kg significantly inhibited PCA, histamine- and 5-HT-induced cutaneous reactions in a dose-dependent manner. It also showed a tendency to inhibit the LTC₄-induced cutaneous reaction. On the contrary, although aminophylline at doses of 10 and 30 mg/kg inhibited the PCA and histamine-induced cutaneous reaction similarly to MKS-492, it had no effect on the 5-HT- and LTC₄-induced cutaneous reactions.
Histamine release

Figure 8 indicates the results of histamine release from guinea pig lung tissue. Although MKS-492 and aminophylline inhibited the antigen-induced release of histamine only at a concentration of $10^{-4}$ M, they inhibited PLA$_2$-induced release of histamine at concentrations of $10^{-6}-10^{-4}$ M.

$O_2^-$ production

Figure 9 indicates the effect of MKS-492 and aminophylline on $O_2^-$ production from guinea pig alveolar macrophages stimulated by PMA or PAF. MKS-492 at a concentration of 100 pM inhibited the production of $O_2^-$ by PAF, but failed to affect that caused by PMA. Aminophylline had no effect on $O_2^-$ production at concentrations of 100 and 300 pM.

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**Fig. 8.** Effect of MKS-492 and aminophylline on antigen- or PLA$_2$-induced release of histamine from guinea pig lung tissues. Each column represents the mean±S.E. of 4 to 6 experiments. *P<0.05, **P<0.01.

**Fig. 9.** Effect of MKS-492 and aminophylline on PMA- and PAF-induced superoxide anion generation from alveolar macrophages of guinea pigs. Each column represents the mean±S.E. of 4 experiments. *P<0.05.
DISCUSSION

The bronchodilating property of theophylline is considered to be important for asthma treatment. MKS-492 showed about 10 times more potent bronchodilating activity than aminophylline in an in vitro experiment. Antigen-induced bronchoconstriction in guinea pigs in vivo was also inhibited by MKS-492, which was similar to the effect of aminophylline. In addition, MKS-492 showed a potent inhibitory activity on PAF-induced bronchoconstriction. These results suggest that MKS-492 is expected to be effective for asthma in clinical trials through its bronchodilating activity. In addition, MKS-492 inhibited rat cutaneous allergic reactions, LTC4-induced leukocyte accumulation in guinea pig airways, antigen- or PLA2-induced histamine release and PAF-induced O2- production. These effects of MKS-492 seem to be beneficial for the treatment of allergic inflammation in the airway, which plays an important role in the production of bronchial asthma (19 – 22).

In 1989, we studied the anti-allergic mechanisms of intracellular cyclic AMP-elevating agents and indicated that these agents, including theophylline, inhibit not only PCA but also mediator-induced cutaneous reactions elicited at the same time in the same rats (37). In the present study, MKS-492 similarly inhibited not only PCA but also histamine- and 5-HT-induced cutaneous reactions, and it showed a tendency to inhibit LTC4-induced cutaneous reaction. It is suggested, therefore, that MKS-492 inhibits the vascular permeability increase caused by mediators from activated mast cells, and that the action of MKS-492 contributes to the inhibition of PCA. It is interesting that the inhibition of PCA was more potent than those against the mediator-induced cutaneous reactions, suggesting that another mechanism is also involved in the inhibition of PCA. As antigen-induced histamine release from mast cells in lung fragments of guinea pigs was inhibited by MKS-492, the action may also contribute to the inhibition of PCA. Aminophylline inhibited PCA and the histamine-induced cutaneous reaction similarly to MKS-492, although it failed to inhibit 5-HT- and LTC4-induced cutaneous reactions. Higher doses of aminophylline may inhibit these reactions.

In the present results, MKS-492 exhibited potent inhibitory effects on PAF-induced reactions, whereas aminophylline in a similar dose range did not inhibit them. These results support the previous reports by Morley et al. (16, 17). Regarding the role of PAF in bronchial asthma and allergic diseases, however, a definitive conclusion cannot yet be drawn, although the existence of PAF in pathological lesions and the ability to elicit symptoms similar to disease have been confirmed by many investigators (38 – 41). Furthermore, many specific PAF antagonists have been examined, but none of them has been established as a remedy for asthma and allergic diseases. Although the molecular mechanisms of the inhibitory effects of MKS-492 on PAF-induced reactions have not been studied here, it may be a characteristic feature of MKS-492, not observed with aminophylline.

MKS-492 is a specific type III PDE inhibitor, and its potency is reported to be 2,000 times that of aminophylline (16). In the present results, the in vitro bronchodilating effect and inhibitory effects on PAF-induced reactions of MKS-492 were clearly more potent when compared to those of aminophylline. In the other experiments, however, the inhibitory activity of MKS-492 was almost comparable to or slightly more potent than that of aminophylline when compared to the effective dosage. It is difficult, therefore, to discuss the differences observed in these two agents in relation to type III PDE inhibition. Further experiments on absorption, distribution and metabolism of these agents are necessary to clarify the mechanisms of action.

In conclusion, MKS-492, a specific type III PDE inhibitor, exhibits more potent bronchodilating, anti-allergic and anti-inflammatory activities in guinea pigs and rats when compared to aminophylline. Further experiments, however, are necessary to elucidate the relationship between type III PDE inhibition and anti-allergic actions.

REFERENCES

7. H. Nagai et al.


