Effect of Oxitropium Bromide (Ba253) on Isolated Respiratory Smooth Muscle and Release of Chemical Mediators from Passively Sensitized Lung Fragments

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Abstract—The effect of oxtitropium bromide (Ba253), a quaternary scopolamine derivative, on the resting tonus and agonist-induced contraction of isolated guinea pig airway smooth muscle and on the anaphylactic release of histamine and immunoreactive leukotrienes (i-LTs) from lung fragments were investigated and compared with those of Sch1000, atropine and isoproterenol. Ba253 dose-dependently inhibited the acetylcholine (ACh)-induced contraction of the isolated trachea and lung parenchyma. The degree of inhibitory potency was similar to that of Sch1000 and 10 times higher than that of atropine. Ba253 minimally influenced the resting tonus or contractions induced by other agonists including histamine, serotonin and LTD₄. Sch1000 and atropine had similar or slightly stronger inhibitory effects on the tonus and contractions than Ba253. On the other hand, low concentrations of isoproterenol solely relaxed the resting tonus and inhibited the agonist-induced contractions of both preparations. Neither Ba253 nor Sch1000 inhibited the anaphylactic release of histamine and LTs from both guinea pig and human lung fragments, but both mediator releases from either species were slightly inhibited with dose-dependency by atropine and potently inhibited by isoproterenol. From these results, it is suggested that Ba253 is a relatively specific antagonist to cholinergic receptors and might be possibly effective as an inhalant for asthma.

Although mechanisms of onset and development of asthma are still unclear, the state of excitement of parasympathetic nerves (1, 2) or depression of sympathetic nerves (3), abnormality of non-adrenergic and non-cholinergic inhibitory nerves (4) and the like have been proposed as causing a predisposition for asthma. On the other hand, it has been recently re-evaluated that anti-cholinergics are useful as therapeutics for asthma, since atropine is recognized as being effective for patients with the disease by inhalation of the drug (5–9). Therefore, it has been suggested that the abnormality of the parasympathetic nervous system plays an important part in causing asthma or the state of airway hypersensitivity. For this reason and for more clinical effectiveness, ipratropium bromide (Sch1000), which is an N-isopropyl derivative of atropine, has been developed as an antiasthmatic inhalant. It has been reported that the experimental results suggest a significant efficacy of the drug as a clinical prophylactic for asthma (10–12).

More recently, oxtitropium bromide (Ba253), a derivative of scopolamine, has been developed for more effective therapy of asthma by inhalation (13).

In this paper, the effects of Ba253 on the resting tonus and contractions induced by various agonists of the isolated guinea pig airway smooth muscles and on the anaphylactic release of histamine and leukotrienes (LTs) from passively sensitized guinea pig and human lung fragments were investigated.
Materials and Methods

Drugs
Drugs used and their sources were as follows: (8r)-6β,7β-epoxy-8-ethyl-3α[-(-)-tropaxyloxy]-1αH,5αH-tropanium bromide (oxitropium bromide, Ba253) and ipratropium bromide (Sch1000) (Nippon Boehringer Ingelheim, Kawanishi) and atropine sulfate (Merck, Darmstadt) and β-isopropisoterenol D+bitartrate (Nakarai Chem., Kyoto). The structures of Ba253 and Sch1000 are shown in Fig. 1.

Animals
Male Hartley guinea pigs weighing 250–300 g were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. They were used for the experiments after breeding for 3 to 6 weeks under a temperature of 22±1.5°C and humidity of 55±15%.

The macroscopically normal portions of the human lung, which were obtained from the resection for lung carcinoma, were used.

Antigens
1. Benzylpenicilloyl bovine gamma globulin [(BPO)BGG]: (BPO)BGG was prepared from benzylpenicillin potassium (Meiji Confectionery, Tokyo) and BGG (Sigma Chem., St. Louis) according to the method of Levine and Redmond (11). Calculating from the Penamaldate method (15) for the BPO group and the microhiuret method for BGG, the number of BPO groups bound to a BGG molecule was estimated as 29. The lyophilized antigen was dissolved in physiologic saline before use.

2. Mite antigen: Mite extracts (from Dermatophagoides farinae, supplied by Dr. H. Nagai of Gifu Pharmaceutical University) were dissolved in physiologic saline at a concentration of 2×10⁻³ g/ml and stored at -80°C until use.

Anti (BPO)BGG guinea pig serum
According to the method of Levine et al. (16), the guinea pig was sensitized once every two weeks with 5 μg (BPO)₂₉BGG/1 mg Al(OH)₃/ml/time. Two weeks after the eighth sensitization, blood was drawn from the carotid artery. The antiserum titer was 1:4000 when evaluated by 7-day passive cutaneous anaphylaxis, and the antiserum was kept at −80°C until use.

Human atopic serum
Human atopic serum, which possessed a radioallergosorbent test value of more than 30%, was used after 5-fold dilution with Ca²⁺-free Tyrode’s solution.

Preparation of the isolated guinea pig trachea and lung parenchyma
The isolated guinea pig tracheal and lung parenchymal strips were prepared as follows: After the guinea pig was killed by a blow on the head and the lung was perfused through the pulmonary artery with Ca²⁺-free Tyrode’s solution (20 ml/animal), the lung and trachea were isolated. The trachea was cut into two-cartilage-ring-wide segments. Four tracheal chain strip preparations, each of which consisted of four segments ligatured with the cartilage ends, were prepared from one animal. Four lung parenchymal strips were prepared: each consisted of a piece of the lung surface cut into a strip of 2 mm diameter and 2 cm length.

1. Measurement of movement of the isolated airway smooth muscle: Both the guinea pig tracheal and lung parenchymal strips were suspended in the Magnus bath, respectively. Conditions of the experiment were as follows: physiologic solution: Tyrode’s solution, temperature: 31±0.1°C, loading weight: 300 mg, organ bath: 2 or 3 ml. Movement was isotonically recorded (isotonic transducer: TD-112S and Recorder: Fig. 1. Chemical structures of Ba253 and Sch1000.
2. The resting tonus: After almost constant contractions of the isolated guinea pig trachea or lung parenchyma were observed by repeated application of the final concentration of $10^{-7}$ g/ml histamine, the influence of the drugs on the resting tonus was examined by the addition of various doses by the cumulative method, employing a 20-min interval for each dose. The influence of drugs on the resting tonus was expressed as percentage (%) to the maximal relaxation induced by $10^{-6}$ g/ml isoproterenol.

3. The contractions induced by agonists: After almost constant contractions of the isolated guinea pig trachea or lung parenchyma were observed by repeated application of the respective agonists, the influence of the drugs on the contraction was examined by the treatment with various doses of drugs 5 min prior to the addition of agonists. The final concentrations and duration of agonists to the preparation were as follows: trachea [acetylcholine (ACh, Wako Pure Chem., Osaka): $10^{-6}$ g/ml and 5 min, histamine (Wako Pure Chem., Osaka): $10^{-6}$ g/ml and 5 min, serotonin (Wako Pure Chem., Osaka): $10^{-6}$ g/ml and 5 min, LTD4 (Wako Pure Chem., Osaka): $3 \times 10^{-10}$ g/ml and 15 min] and lung parenchyma (ACh: $10^{-6}$ g/ml and 5 min, histamine: $10^{-6}$ g/ml and 5 min, and LTD4: $3 \times 10^{-10}$ g/ml and 10 min).

The influence of the drugs on the contraction induced by agonists was expressed as % of inhibition according to the following formula: % of inhibition = $(A - B/A) \times 100(\%)$, where $A$ is the contraction height (cm) induced by agonist without drug, and $B$ is the contraction height induced by agonist in the presence of the drug.

Release of histamine and LTs from passively sensitized lung fragments

1. Guinea pigs: Guinea pigs were intraperitoneally sensitized with 2 ml/animal of anti(BPO)BGG guinea pig serum. After 48 hr, following killing by a blow on the head and perfusing the lung with physiologic saline through the pulmonary artery, the lung was isolated. Then, the lung parenchyma was cut into fragments of about $1 \times 0.7 \times 0.7$ mm in size with a McIlwain tissue chopper and washed with Tyrode's solution (100 ml/lung) on gauze. Defined weights of 400 to 600 mg of lung fragments were distributed into individual tubes and suspended with 0.98 ml/100 mg wet tissue of Tyrode's solution. Following preincubation at 37°C for 5 min and treatment with drugs for 5 min, the lung fragments were challenged with 0.01 ml/100 mg wet tissue of $10^{-5}$ g/ml antigen and incubated at 37°C for 10 min. After termination of the reaction, the reaction suspension was filtered on gauze to remove lung fragments, and the filtrate was collected into an icerewater chilled tube. The anaphylactic filtrate was centrifuged at $1,700 \times g$ for 15 min at 4°C, and the resultant supernatant was divided for histamine and LT assays, and stored at $-20°C$ and $-80°C$ until assay, respectively.

2. Human: The human lung parenchyma was cut into fragments of $1 \times 0.8 \times 0.8$ mm in size with a McIlwain tissue chopper. Subsequent to washing with Ca2+-free Tyrode's solution (50 ml/g wet tissue) on gauze, the fragments were passively sensitized with a 5-fold dilution of human atopic serum (10 ml/g wet tissue) at 37°C for 2 to 4 hr. Following washing with Ca2+-free Tyrode's solution (50 ml/g wet tissue) on gauze, suspending with Ca2+-free Tyrode's solution (10 ml/g wet tissue) and then allowing them to stand at room temperature for 1.5 hr, the fragments were further washed with Ca2+-free Tyrode's solution. Conditions of distribution, addition of Tyrode's solution, addition of drug, challenging and incubation were the same as described in subsection 1) Guinea pigs.

3. Histamine and LT assays: Histamine in the anaphylactic medium was fluorometrically assayed according to the method of May et al. (17). For the estimation of histamine contents in the tissue, to the lung fragments, which were not challenged with antigen, was added 1% perchloric acid (5 ml/100 mg wet tissue), and the suspension was treated in a boiling water bath for 8 min followed by centrifugation at $1,700 \times g$ for 30 min at 4°C. The resultant supernatant was subsequently treated in the same manner as the anaphylactic medium for fluorometric assay of histamine.
LTs in the anaphylactic medium were measured by radioimmunoassay (RIA). Namely, 50 μl of 1% gelatin (Merck, Darmstadt) and 4 ml of ice-chilled ethanol were added to 1 ml of the sample, and the mixture was allowed to stand at 0°C for 30 min. After centrifugation at 1,700×g for 30 min at 4°C, the supernatant to which 40 μl of 2% gelatin was added was evaporated to dryness under reduced pressure; then the residue was dissolved in 100 μl of 0.3 M HEPES (Nakarai Chem., Kyoto) buffer (pH 7.5) containing 0.1% gelatin, 100 μl of the tracer and 100 μl of anti-LTB4 or anti-LTC4 serum and incubated for 18 hr at 4°C. After completion of the reaction, 500 μl of 0.5% dextran-coated charcoal was added to the reaction mixture and allowed to stand at 0°C for 15 min. Five hundred μl of the supernatant from the centrifugation at 1,700×g for 15 min at 4°C were dissolved in a scintillator (Riaflour, New England Nuclear, Boston) and counted for bound [3H]-LTB4 or LTC4. A tritiated LTB4 assay reagent system (Amer sham, Buckinghamshire) and a [3H]-LTC4 RIA KIT (New England Nuclear, Boston) were purchased for the LT assay. Amounts of LTB4 and LTC4 estimated were expressed as immunoreactive (i-)LTB4 and i-LTC4, respectively.

Results

Effect on the isolated guinea pig airway smooth muscle

1. Effect on the resting tonus: Influences of the drugs on the resting tonus of the isolated guinea pig trachea and lung parenchyma are shown in Fig. 2.

Ba253 at 10⁻⁸ to 10⁻⁵ g/ml modestly but concentration-dependently relaxed the isolated trachea. Sch1000 at the same concentration range also caused concentration-dependent relaxation, which was slightly stronger than that by Ba253. Atropine had no effect at these concentrations. On the other hand, low concentrations (10⁻¹²–10⁻¹⁰ g/ml) of isoproterenol showed a relaxation in a concentration-dependent fashion.

In the isolated lung parenchyma, both Ba253 and atropine at 10⁻⁸ to 10⁻⁵ g/ml produced weak relaxations without concentration-dependency. On the other hand, Sch1000 induced a dose-dependent contraction by 10 to 30% at 10⁻⁷ to 10⁻⁵ g/ml. Isoproterenol had a marked relaxing effect at 10⁻¹⁰ to 10⁻⁸ g/ml.

2. Effect on the contraction induced by ACh: Influences of the drugs on the con-
traction of the isolated guinea pig trachea and lung parenchyma induced by $10^{-6}$ g/ml ACh are shown in Fig. 3.

At the concentrations of $10^{-9}$ to $10^{-7}$ g/ml, Ba253, Sch1000 and atropine all induced concentration-dependent inhibition of the contraction of the trachea. The inhibitory potency was in the following order: Ba253 slightly greater than Sch1000, which was greater than atropine. Isoproterenol did have any effect at the concentration of $10^{-9}$ g/ml, but inhibited it by 55% at $10^{-8}$ g/ml. Isoproterenol at concentrations higher than $10^{-7}$ g/ml, however, did not show any additional inhibition.

The contraction of the lung parenchyma induced by ACh was concentration-dependently inhibited by 25 to 100% by $10^{-9}$ to $10^{-8}$ g/ml of Ba253. In the same concentration range, Sch1000 showed an inhibition curve similar to that of Ba253. Atropine at $10^{-9}$ g/ml had no effect on the contraction, but concentration-dependent inhibition was observed at $10^{-8}$ to $10^{-6}$ g/ml. The inhibitory curve of atropine was shifted more to the right than those of Ba253 and Sch1000, and 10 times higher concentrations of atropine were needed to inhibit the contraction than those of Ba253 or Sch1000. On the other hand, $10^{-11}$ and $10^{-10}$ g/ml of isoproterenol did not inhibit the contraction, but produced concentration-dependent inhibition at $10^{-8}$ to $10^{-7}$ g/ml.

3. Effect on the contraction induced by histamine: Figure 4 shows the results of the drugs on the contraction of the isolated guinea pig trachea and lung parenchyma induced by $10^{-6}$ g/ml histamine. The contraction of the isolated trachea was hardly affected by $10^{-8}$ to $10^{-6}$ g/ml of Ba253, but slightly inhibited by $10^{-5}$ and $10^{-4}$ g/ml. Similarly to Ba253, only $10^{-4}$ g/ml of Sch1000 modestly inhibited the contraction. However, $10^{-6}$ to $10^{-4}$ g/ml of atropine inhibited the contraction in a concentration-dependent fashion. Isoproterenol at $10^{-10}$ to $10^{-8}$ g/ml also concentration-dependently antagonized the contraction.

The contraction of the isolated lung parenchyma was concentration-dependently inhibited by all of the drugs. The inhibition was 3 to 83% at $10^{-8}$ to $10^{-4}$ g/ml of Ba253, 3 to 39% at $10^{-8}$ to $10^{-4}$ g/ml of Sch1000, 12 to 93% at $10^{-8}$ to $10^{-4}$ of atropine and 41 and 89% at $10^{-9}$ and $10^{-8}$ g/ml of isoproterenol, respectively.

4. Effect on the contraction induced by serotonin: The influences of the drugs on the contraction of the isolated guinea pig trachea induced by $10^{-6}$ g/ml serotonin are shown in

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**Fig. 3.** Effect of Ba253 and related compounds on ACh ($10^{-6}$ g/ml)-induced contraction of the isolated guinea pig trachea and lung parenchyma. Each point represents a mean±S.E. of 10 experiments. ○Ba253, ●Sch1000. △Atropine, ▲Isoproterenol.
Fig. 4. Effect of Ba253 and related compounds on histamine (10^{-6} g/ml)-induced contraction of guinea pig trachea and lung parenchyma. Each point represents a mean±S.E. of 10 experiments. ○Ba253, ●Sch1000, △Atropine, ▲Isoproterenol.

Fig. 5. Effect of Ba253 and related compounds on serotonin (10^{-6} g/ml)-induced contraction of the isolated guinea pig trachea. Each point represents a mean±S.E. of 10 experiments. ○Ba253, ●Sch1000, △Atropine, ▲Isoproterenol.

Fig. 5.

Ba253 did not affect the contraction even at high concentrations as 10^{-4} g/ml. Sch1000 and atropine showed weak inhibition at 10^{-4} g/ml. However, isoproterenol showed fair influence on the contraction, with 34 and 99% inhibition at 10^{-9} and 10^{-8} g/ml, respectively.

5. Effect on the contraction induced by LTD₄:

Figure 6 shows the effect of the drugs on the contraction of the isolated guinea pig trachea and lung parenchyma induced by 3×10^{-10} g/ml of LTD₄.

The contraction of the trachea was modestly affected by high concentrations of Ba253, Sch1000 and atropine. On the other hand, isoproterenol produced complete inhibition at 10^{-9} g/ml.

Ba253, Sch1000 and atropine produced approximately 10% inhibition of the lung parenchymal contraction at 10^{-4} g/ml. Similarly to the tracheal results, isoproterenol of 10^{-9} g/ml showed fair inhibition of the contraction.

Effect on the release of chemical mediators from the passively sensitized lung fragments

1. Guinea pigs: Figures 7 and 8 show the effect of the drugs on the release of histamine and i-LTs from the passively sensitized guinea pig lung fragments, respectively.

Ba253, Sch1000 and atropine had no effects on release of histamine, i-LTB₄ and i-LTC₄ at concentrations of 10^{-6} to 10^{-4} g/ml. Either 10^{-6} or 10^{-5} g/ml of isoproterenol showed strong inhibition by 70% of the release of histamine, i-LTB₄ and i-LTC₄.

2. Human: Figures 9 and 10 represent the effect of the drugs on the release of histamine...
Fig. 6. Effect of Ba253 and related compounds on LTD4 (3×10^{-10} g/ml)-induced contraction of the isolated guinea pig trachea and lung parenchyma. Each point represents a mean±S.E. of 10 experiments. ○Ba253, ●Sch1000, △Atropine, ▲Isoproterenol.

Fig. 7. Effect of Ba253 and related compounds on histamine release from passively sensitized guinea pig lung fragments. Each column represents a mean±S.E. of 3 experiments. Anaphylactic histamine release and histamine contents were 4.92±0.72 and 18.6±1.14 μg/g tissue, respectively.

and i-LTs from the passively sensitized human lung fragments.

Ba253 at 10^{-8} and 10^{-5} g/ml did not inhibit the histamine release, but 10^{-4} g/ml slightly decreased the release. Sch1000 at 10^{-5} g/ml and atropine at 10^{-4} g/ml also modestly inhibited the histamine release. Any of these anticholinergics at concentrations of 10^{-6} to 10^{-4} g/ml caused slight enhancement or slight inhibition of the release of i-LTB4 and i-LTC4, which was neither concentration-dependent nor consistent. On the other hand, isoproterenol markedly decreased the release of not only histamine, but also i-LTB4 and i-LTC4.

Discussion

Ba253, a quaternary ammonium derivative of scopolamine, has been developed for the purpose of achieving more potent and longer acting effects with less adverse reactions, due to its quaternary structure and topical application, than atropine (13).

In this paper, pharmacologic effects of Ba253 on the in vitro respiratory organs were investigated.

Ba253 induced slight relaxation of the isolated guinea pig trachea and lung paren-
chyma in which much higher concentrations of this drug were needed, as compared to isoproterenol. On the other hand, although Sch1000 relaxed the guinea pig trachea, the compound induced slight contraction of the lung parenchyma in a concentration-dependent fashion. It is well-known that compounds possessing quaternary ammonium such as d-tubocurarine cause the degranulation of mast cells (18). It was supposed that Sch1000, which has a quaternary ammonium moiety, might induce the resultant contraction of the lung parenchymal smooth muscle by causing the release of histamine.
from mast cells. However, Ba253, which is also a quaternary ammonium-possessing compound in its structure, did not induce a contraction. The cause for the difference in behavior between these compounds in the guinea pig lung parenchyma is not known. Furthermore, the reason for the difference between the contraction of the lung parenchyma and the relaxation of the trachea by Sch1000 was also not identified. One possibility is that it reflects the different susceptibilities of mast cells in these tissues to drugs. Ba253 as well as Sch1000 induced strong inhibition of the contractions of both isolated trachea and lung parenchyma by ACh. On the other hand, Ba253 showed no inhibition or only slight inhibition of the contraction by histamine, LTD₄ and serotonin of the isolated trachea and/or lung parenchyma. In general, a compound possessing a potent anticholinergic effect is known to show non-specific antagonism of the smooth muscle contractions induced by various agonists at its high concentrations. However, as shown in the results, Ba253 is suggested to be a relatively more specific antagonist towards ACh than atropine. Although isoproterenol showed the potent inhibition of the contraction induced by any agonist in both preparations, a relatively low concentration of the drug tended to potentiate the ACh-induced lung parenchymal and ACh-induced tracheal contractions. To elucidate the results or for possible explanation on the mechanism of the enhanced contractions, further experiments are needed.

Ba253 hardly affected the anaphylactic release of histamine, i-LTB₄ or i-LTC₄ from both guinea pig and human lung fragments, although atropine had a modest inhibitory effect on the release of these mediators from both tissues. Sch1000 at concentrations of 10⁻⁵ and 10⁻⁴ g/ml showed a tendency of slightly enhanced release of histamine from the guinea pig lung fragments, which might result in the contraction of the isolated lung parenchyma as described above. In marked contrast to these drugs, isoproterenol potently inhibited the histamine and i-LT release from both species through the resultantly increased cellular levels of cyclic 3',5'-adenosine monophosphate, as reported by several groups (19, 20).

It has been recognized that ACh induces the enhancement of anaphylactic histamine release mediated through the elevation of cyclic 3',5'-guanosine monophosphate in the cell (21). In our separate experiment, the treatment with 10⁻⁷ g/ml ACh enhanced the anaphylactic histamine release from guinea pig lung fragments by 122±8.1% of the control (N=3). The enhancement was abolished to control levels by 10⁻⁶ g/ml of all the anticholinergics: Ba253 (101±17%),
Sch1000 (96±11%) and atropine (100±
8.1%).

From these in vitro experiments, Ba253 was suggested to be possibly effective on cholinergic nervous system-associated asthma because it has been proposed that hypersensitive abnormality of cholinergic muscarinic receptors slightly or largely contributes to this disease as well as airway hypersensitivity.

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