Antiallergic Effects of 4-[2-Oxo-3-(1H-Tetrazol-5-Yl)-2H-Chromen-8-Yloxy]-Butyric Acid

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Accepted February 17, 1989

Abstract—Antiallergic effects of 4-[2-oxo-3-(1H-tetrazol-5-yl)-2H-chromen-8-yloxy]-butyric acid (C4C) were studied. C4C is an active principal metabolite of an orally effective antiallergic agent, KP-136. C4C (0.2–1 mg/kg, i.v.) markedly inhibited the mast cell-mediated homologous PCA of rats and the experimental allergic asthma of rats and guinea pigs, although it had almost no effects on heterologous PCA and compound 48/80-induced cutaneous response in rats. C4C (0.2 mg/kg, i.v.) was scarcely effective on cutaneous responses induced by intradermal injection of histamine and serotonin which are principal chemical mediators of rat homologous PCA, and it blocked the decrease of skin histamine content after the PCA. In addition, C4C (0.01–0.5 µg/ml) inhibited the increase of 45Ca uptake of mast cells, the histamine release and the degranulation induced by the antigen-antibody interaction. These effects of C4C were much the same as those of KP-136. From the above findings, C4C is considered to be an antiallergic agent that inhibits the mast cell activation by blocking the calcium influx, and it shares similar pharmacological properties with KP-136.

In previous reports, it was demonstrated that the new compound 8-hexyloxy-3-(1H-tetrazol-5-yl)-2H-chromen-2-one (KP-136) is an orally effective antiallergic agent (1), and its mechanism of action depends upon the block of calcium influx that might cause mast cell activation (1, 2). The present study describes the antiallergic effects of a principal metabolite of KP-136, 4-[2-oxo-3-(1H-tetrazol-5-yl)-2H-chromen-8-yloxy]-butyric acid (C4C) (Fig. 1), and the results have been compared with those of KP-136 and a classical antiallergic drug, disodium cromoglycate (DSCG).

Materials and Methods

Animals: Wistar rats, Hartley guinea pigs and Japanese white rabbits were purchased from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan) and subjected to investigation after housing for at least 7 days.

Test compounds: C4C, KP-136 and DSCG (Intal®, Fisons) were used. These compounds were dissolved in 0.5% KHCO3 (C4C and KP-136) or saline (DSCG) for intravenous doses (2 ml/kg) and suspended in 0.5% carboxymethyl cellulose sodium for oral doses.

Fig. 1. Chemical structures of C4C and KP-136.
Antiserum: Rat anti-2,4-dinitrophenyl-coupled Ascaris (DNP-As) serum was prepared in female rats weighing about 200 g as described by Tada and Okumura (3). The antibody titer of this antiserum was 1:512, as estimated by PCA in the rat (48-hr latent period). Rat anti-ovalbumin serum was prepared in male rats weighing 180-200 g as described by Orange et al. (4) and heated at 56°C for 240 min. The antibody titer of this heated antiserum was 1:64, as estimated by PCA in the rat (5-hr latent period). Rabbit anti-ovalbumin serum was prepared in male rabbits weighing about 3 kg as described by Koda et al. (5). The antibody titer of this antiserum was 1:16,000, as estimated by PCA in the guinea pig (4-hr latent period). Guinea pig anti-benzylpenicilloyl bovine gamma globulin (BPO-BGG) serum was prepared in female guinea pigs weighing 350-500 g according to the method of Levine et al. (6). The antibody titer of this antiserum was 1:800, as estimated by PCA in the guinea pig (8-day latent period).

Cutaneous responses: Male rats weighing 120-180 g were subjected to these experiments. For PCA tests, antiserum diluted with saline was injected at 0.05 ml/site intradermally onto the shaved back of rats; and after appropriate time intervals, 0.5% Evan's blue solution containing a corresponding antigen (2 mg/ml) was injected at the volume of 2.5 ml/kg, intravenously. On the other hand, histamine dihydrochloride (Nakalai tesque) (100 µg/ml), serotonin creatinine sulfate (Merck) (2 µg/ml) or compound 48/80 (Sigma) (2 µg/ml) in saline was injected at 0.05 ml/site intradermally onto the shaved back of rats intravenously injected with 2.5 ml/kg of 0.5% Evans' blue solution. Animals were sacrificed to remove the skin with the blueing lesion 30 min after elicitation. The leaked-dye was extracted and measured according to the method of Harada et al. (7). The skin histamine content was measured as described previously (1).

Histamine release and mast cell degranulation: The sensitized peritoneal exudate cells were taken from rats weighing 140-180 g with a 24-hr latent period, into which anti-DNP-As serum (0.1 ml) was injected intraperitoneally and suspended at a density of 5×10⁶ mast cells/ml in physiological buffered saline (PBS), pH 7.2 [137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 6H₂O, 5.6 mM glucose and 5% (v/v) of 0.1 M phosphate buffer]. For histamine release assay, 2.5 ml of the cell suspension were mixed with 0.5 ml of PBS containing a test compound and DNP-As. The mixture was incubated at 37°C for 10 min and centrifuged at 700×g for 10 min. The histamine contents of the precipitate and the supernatant were measured by the fluorometric method of Shore et al. (8). The mast cell degranulation assay was carried out as reported previously (9). The sensitized cell suspension (50 µl) was mixed with 50 µl of PBS containing a test compound and DNP-As in a Bellco micro-slide culture chamber. The chamber was incubated at 37°C for 10 min, and the cells on microscope slides were fixed and stained by the addition (100 µl chamber) of 20% neutral formalin solution containing 0.2% toluidine blue. The proportion of mast cells undergoing degranulation was assessed for about 200 mast cells under microscopic observation.

45Ca uptake: The sensitized mast cells were isolated from the previously described peritoneal exudate cells according to the method of Johnson and Moran (10) and suspended in PBS at a density of 2.5×10⁶ cells/ml. To study the 45Ca uptake of the isolated mast cells, the cell suspension (0.2 ml) was incubated at 37°C for 3 min prior to addition of 45CaCl₂ (25 µCi/ml, New England Nuclear) (0.1 ml). At 1 min after 45CaCl₂ addition, a solution of the compound to be tested (0.1 ml) was added and immediately antigen (DNP-As, 100 µg/ml) (0.1 ml) containing phosphatidyl L-serine (5 µg/ml) was challenged to the cells. After incubation for 10 min at 37°C, 2.5 ml of ice-cold PBS was added to terminate incorporation. The cells were collected by centrifugation for 5 min at 300×g and rapidly washed twice with PBS. The final cell pellets were solubilized in 0.1 ml of 1 N NaOH, and radioactivity was determined by a liquid scintillation counter (Aloka, Model LSC-3600).

Experimental asthma: Rat asthmatic response was induced according to the method (11) reported previously. Anti-DNP-As
serum (1 ml) was injected intravenously to each 120–150 g male rat; and 72 hr later, DNP-As (5 mg/kg) in saline was injected intravenously to a sensitized rat to induce allergic asthma. In the rats with asthmatic behaviors, the duration of expiration became markedly longer than that of inspiration. This was specific for the anaphylaxis and easily judged by the movement of the thorax. The prolongation of expiratory time with more extensive movement of the thorax than that of the normal rat was taken as severe respiratory distress. On the other hand, allergic asthma in guinea pigs was induced as follows: Anti-BPO-BGG serum (0.3 ml) was injected intravenously to each 400–500 g male guinea pig. Eight days later, the sensitized animals were anesthetized by subcutaneous injection of 25% urethane (10 ml/kg) on the back. One end of a polyethylene cannula was surgically attached to the trachea and the other end was connected with a ventilator (Shinano, type SN-480-7) and bronchospasm transducer (Ugo Basile). Then, gallamine triethiodide (Sigma) in saline (5 mg/kg) was injected intravenously to eliminate the spontaneous respiration. Benzylpenicilloyl bovine serum albumin, prepared as described by Levine et al. (6), was dissolved in saline and injected at 0.5 mg/kg, intravenously, 120 min after injection of urethane. The degree of airway obstruction was measured according to a modification of the method of Konzett and Rossler (12). Changes in airway resistance was expressed as % of the response seen in the case of complete obstruction of the trachea, and a value of more than 50% was taken as a severe asthmatic response.

Isolated tracheal muscle preparation: The trachea was taken from male guinea pigs, weighing 500–590 g, that were sacrificed by bleeding and then cut into 1.5 mm wide to make tracheal rings. Five specimens were tied at cartilage sites crossing each smooth muscle layer. This preparation was incubated in Tyrode's solution at 37°C, which was bubbled with 95% O₂/5% CO₂. The tension change of the tracheal muscle preparation was recorded through an isotonic transducer (Nihon Kohden, type TD-112S). C4C and KP-136 in 0.5% KHCO₃ or isoproterenol in saline was added cumulatively to the Tyrode's solution at a 1/200 volume. Relaxation percentage was calculated from the maximum relaxation value (100%) produced by replacement of the bathing medium with Tyrode's solution containing no CaCl₂.

Statistical analysis: The significance of difference was determined by Dunnett's method following one-way analysis of variance or the x²-test. The ID₅₀ and IC₅₀ were calculated by linear regression analysis.

Results

Rat 48-h homologous PCA: This PCA was elicited in rats sensitized with 1:32 dilution of anti-DNP-As serum. The amount of leaked-dye was 7.7 to 9.5 μg on the average. On this dye-leakage, C4C given intravenously showed a dose-dependent inhibition with an ID₅₀ of 0.09 mg/kg. In addition, C4C at 0.2 mg/kg produced an equipotent effect to 1 mg/kg KP-136 or 5 mg/kg DSCG. However, C4C was less effective by the oral route because it only produced a maximum inhibition of about 35% even at the high dose of 100 mg/kg, whereas KP-136 showed an overt inhibitory effect of about 66% at the oral dose of 2 mg/kg (Table 1).

Rat 5-h homologous PCA, rat 4-h heterologous PCA and compound 48/80-induced cutaneous response: Rats were intradermally sensitized with 1:16 dilution of rat anti-ovalbumin serum or 1:8 dilution of rabbit anti-ovalbumin serum to elicit each PCA with a 5-hr or 4-hr latent period. The mean amount of leaked-dye was 11.3 μg for 5-h homologous PCA, 7.2 μg for 4-h heterologous PCA and 9.4 μg for compound 48/80 response. C4C (0.2 mg/kg, i.v.) produced about 60% inhibition on 5-h homologous PCA, having an activity similar to that of C4C. On the other hand, KP-136 (1 mg/kg, i.v.) also remarkably inhibited 5-h homologous PCA, having an activity similar to that of C4C.

Histamine- and serotonin-induced cutaneous responses: The dye leakage was 9.2 to 10.5 μg following the injection of histamine or serotonin. C4C (0.2 mg/kg, i.v.) had almost no effects on these cutaneous responses, and KP-136 (1 mg/kg, i.v.) or DSCG (5 mg/kg, i.v.) also did not (Table 3).
### Table 1. Effects of C4C, KP-136 and DSCG on rat 48-h homologous PCA

| Compound       | Dose (mg/kg) | Amount of leaked-dye (μg/site) | Inhibition (%) |
|----------------|--------------|--------------------------------|----------------|
| (Intravenous)  |              |                                |                |
| Control (0.5% KHCO₃) | —            | 9.5±1.3                        |                |
| C4C            | 0.05         | 6.9±1.0                        | 27             |
|                | 0.1          | 3.1±0.8**                      | 67             |
|                | 0.2          | 0.7±0.4**                      | 93             |
| KP-136         | 1            | 1.3±0.5**                      | 86             |
| Control (saline) | —            | 7.7±0.7                        |                |
| DSCG           | 5            | 0.3±0.2**                      | 96             |

Test compounds were given intravenously just before antigen challenge or orally 15 min before antigen. Each value indicates the mean±S.E.M. of 4 to 5 animals. *P<0.05, **P<0.01: Significantly different from the control as examined by Dunnett's method.

### Table 2. Effects of C4C, KP-136 and DSCG on 5-h homologous PCA, 4-h heterologous PCA and compound 48/80-induced cutaneous response in rats

| Compound (i.v.) | Dose (mg/kg) | Amount of leaked-dye (μg/site) | 5-h homologous PCA | 4-h heterologous PCA | compound 48/80 response |
|-----------------|--------------|--------------------------------|---------------------|----------------------|-------------------------|
| Control         | —            |                                | 11.3±1.2            | 7.2±0.6              | 9.4±0.4                 |
| C4C             | 0.2          | 4.9±0.3                        | 7.3±0.7             | 8.9±1.0              |                         |
| KP-136          | 1            | 2.3±0.3                        | 6.6±0.6             | 8.5±1.2              |                         |

Test compounds were given intravenously just before antigen challenge. Each value indicates the mean±S.E.M. of 4 animals.

### Table 3. Effects of C4C, KP-136 and DSCG on histamine- and serotonin-induced cutaneous responses and on decrease of skin histamine content after PCA

| Compound (i.v.) | Dose (mg/kg) | Amount of leaked-dye (μg/site) | Histamine content (μg/g of tissue) |
|-----------------|--------------|--------------------------------|-----------------------------------|
|                 |              |                                | PCA site                           |
| Control (0.5% KHCO₃) | —            | 10.5±0.7                        | 11.7±1.2**                      |
| C4C             | 0.2          | 10.2±1.4                        | 23.0±1.8                         |
| KP-136          | 1            | 11.2±1.3                        | 21.5±1.1                         |
| Control (saline) | —            | 9.3±1.0                         | 14.5±1.1*                        |
| DSCG            | 5            | 8.1±1.2                         | 21.3±1.1                         |

Test compounds were given intravenously just before elicitation. Each value indicates the mean±S.E.M. of 4 to 5 animals. *P<0.05, **P<0.01: Significantly different from the control site as examined by Dunnett's method.
**Skin histamine content**: The skin histamine content apparently decreased after 48-h homologous PCA in rats, suggesting the release of tissue histamine. This decrease of histamine content was almost completely inhibited by either C4C (0.2 mg/kg, i.v.), KP-136 (1 mg/kg, i.v.), or DSCG (5 mg/kg, i.v.) (Table 3).

**Histamine release and degranulation**: The addition of antigen (20 μg/ml) to sensitized peritoneal exudate cells induced the release of intracellular histamine (36.6 to 57.0%) and the degranulation of mast cells (50.2 to 64.9%). C4C (0.002 to 0.05 μg/ml) inhibited this histamine release and degranulation in a dose-dependent manner (Table 4), confirming that it is an inhibitor of mast cell activation, like KP-136 and DSCG. The IC50 was 0.03 μg/ml for histamine release and 0.04 μg/ml for degranulation.

**45Ca uptake**: The incubation (10 min at 37°C) of rat sensitized mast cells in the presence of antigen resulted in an increase of 45Ca uptake from 418.2±27.2 to 2076.9±287.3 cpm. This increase of 45Ca uptake was apparently inhibited by C4C (0.5 μg/ml), KP-136 (0.1 μg/ml) and DSCG (50 μg/ml) (Table 5).

**Experimental asthma**: Table 6 shows the effects on rat experimental asthma. C4C (0.05-0.2 mg/kg, i.v.) produced an obvious inhibitory effect against the antigen-induced asthmatic response, decreasing the proportion of rats with severe respiratory distress. KP-136 (1 mg/kg, i.v.) and DSCG (5 mg/kg, i.v.) had similar inhibitory effects. On the other hand, the antiasthmatic effect of C4C was also confirmed in guinea pigs during the development of asthma induced by antigen (Table 7). C4C (i.v.) at 1 mg/kg showed an obvious inhibitory effect against the asthmatic response of guinea pig, which reached a maximal level 3 to 5 min after antigen challenge, but at a dose of 0.2 mg/kg, it had almost no effects. KP-136 (1 mg/kg, i.v.) also produced a similar activity, although DSCG (i.v.) was less effective even at a high dose of 50 mg/kg.

**Relaxation effect on isolated trachea**: C4C had a relaxation activity on isolated guinea}

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### Table 4. Effects of C4C, KP-136 and DSCG on antigen-induced histamine release and mast cell degranulation

| Compound | Histamine release | Degranulation |
|----------|-------------------|---------------|
|          | Concentration (μg/ml) | Inhibition (%) | Concentration (μg/ml) | Inhibition (%) |
| C4C      | 0.002             | 5.9           | 0.01             | 20.4           |
|          | 0.01              | 36.2          | 0.02             | 43.0           |
|          | 0.05              | 83.6          | 0.05             | 62.8           |
| KP-136   | 0.01              | 76.1          | 0.01             | 63.0           |
| DSCG     | 2                 | 68.7          | 2                | 59.9           |

Each value indicates the mean of 3 to 4 samples. The percentage of histamine release or degranulation in the antigen control was between 36.6 to 57.0% or between 50.2 to 64.9%, respectively.

### Table 5. Effects of C4C, KP-136 and DSCG on antigen-induced increase of 45Ca uptake

| Compound | Concentration (μg/ml) | 45Ca uptake (c.p.m/5×10⁶ mast cells) | Inhibition (%) |
|----------|----------------------|-------------------------------------|---------------|
|          |                      | Antigen (−)                          | Antigen (+)   |               |
| Control  | —                    | 418.2                               | 2076.9        | 69.1          |
| C4C      | 0.5                  | 306.7                               | 805.7         | 69.1          |
| KP-136   | 0.1                  | 416.6                               | 418.5         | 99.9          |
| DSCG     | 50                   | 272.3                               | 769.8         | 70.0          |

Each value indicates the mean of 3 samples.
Table 6. Effects of C4C, KP-136 and DSCG on allergic asthma in rats

| Compound (i.v.) | Dose (mg/kg) | No. of severely asthmatic rats/tested |
|-----------------|--------------|--------------------------------------|
|                 | 0            | 10/11                                |
|                 | 0.02         | 9/10                                 |
| C4C             | 0.05         | 5/10*                                |
|                 | 0.1          | 3/10**                               |
|                 | 0.2          | 0/10**                               |
| KP-136          | 0            | 9/10                                 |
|                 | 1            | 1/10**                               |
| DSCG            | 0            | 9/10                                 |
|                 | 5            | 1/10**                               |

Test compounds were given intravenously just before the antigen challenge. *P<0.05, **P<0.01: Significantly different from the control as examined by the χ²-test.

Table 7. Effects of C4C, KP-136 and DSCG on allergic asthma in guinea pigs

| Compound (i.v.) | Dose (mg/kg) | No. of severely asthmatic guinea pigs/tested 1 min | 3 min | 5 min | 10 min |
|-----------------|--------------|---------------------------------------------------|-------|-------|--------|
| Control (0.5% KHCO₃) | ---          | 3/7                                                | 6/7   | 6/7   | 5/7    |
| C4C             | 0.2          | 3/7                                                | 5/7   | 5/7   | 4/7    |
|                 | 1            | 0/6                                                | 1/6*  | 1/6*  | 1/6*   |
| KP-136          | 1            | 0/6                                                | 2/6   | 1/6*  | 1/6*   |
| Control (saline) | ---          | 2/8                                                | 6/8   | 7/8   | 5/8    |
| DSCG            | 50           | 2/8                                                | 6/8   | 6/8   | 3/8    |

Test compounds were given intravenously just before the antigen challenge. *P<0.05: Significantly different from the control as examined by the χ²-test.

Table 8. Relaxation effects of C4C, KP-136 and isoproterenol on isolated trachea

| Compound     | Dose (mg/kg) | Relaxation (%) |
|--------------|--------------|----------------|
| C4C          | 10           | 12.8±2.4       |
|              | 20           | 24.0±6.2       |
|              | 50           | 37.0±8.9       |
|              | 100          | 51.8±7.9       |
| KP-136       | 2            | 54.8±10.5      |
| Isoproterenol| 0.001        | 67.5±7.4       |

Each value indicates the mean±S.E.M. of 4 to 5 preparations.

pig trachea under normal tone but at high doses (Table 8). The concentration for 50% relaxation was 90 μg/ml. This effect was about fifty times less potent than KP-136 and much less effective than the β-stimulant isoproterenol.

Discussion

The pharmacological properties of C4C, a principal metabolite of KP-136, were studied,
and it was revealed that C4C had potent antiallergic activities, although its oral activity was much less potent.

In rat cutaneous models, C4C taken intravenously produced potent inhibitory effects against IgE-dependent (1) 48-h homologous PCA and IgGa-dependent (1) 5-h homologous PCA, although the compound had almost no effects against 4-h heterologous PCA and compound 48/80 response. Thus, C4C is considered to be an inhibitor of type I allergic homologous PCA mediated by mast cells. In addition, C4C is not an antagonist of mediators and its mode of action is postulated to be the blockage of mediator release from mast cells because C4C had no effects on cutaneous responses induced by intradermal injection of histamine and serotonin, which are principal mediators of rat type I allergy, and blocked the decrease of skin histamine content after the PCA as well as a classical inhibitor of mediator release, DSCG. The inhibitory effects against histamine release from peritoneal exudate cells and mast cell degranulation are consistent with the above postulation. Moreover, C4C was effective on antigen-induced increase of $^{45}$Ca uptake by mast cells. Therefore, as might be implied by previous studies with DSCG (13, 14) and KP-136 (2), the protective effects of C4C during mast cell activation would be a consequence of blocking the increase in calcium permeability that will subsequently cause histamine release and degranulation (15).

The pathogenesis of mast cell mediated type I allergy consists of an increase in vascular permeability and the contraction of the smooth muscle. The former causes urticaria and conjunctivitis, and the latter plays a central role in development of bronchial asthma. Accordingly, C4C which blocked the mediator release would produce an inhibitory effect on allergic asthma. Indeed, it was confirmed that C4C taken intravenously was markedly effective on rat allergic asthma. A further experiment demonstrated that C4C was also active against guinea pig allergic asthma, suggesting that C4C might have a different character from DSCG which was ineffective against this asthmatic response. Although a major antiasthmatic mechanism of C4C is postulated to be the blockage of mediator release as described previously, the possibility that the relaxation of smooth muscle might contribute as an additive mechanism is not excluded, because C4C, but only at high doses, showed a relaxation activity on isolated guinea pig trachea.

As mentioned above, C4C is a potent inhibitor of type I allergy with a blocking activity on mediator release, and it shares very similar pharmacological properties with KP-136. Therefore, C4C detected at high levels in the plasma of rats and humans that were orally given KP-136 is speculated to partly contribute to the antiallergic effects of KP-136.

Acknowledgments: We are grateful to the IAP project team for the gift of Bordetella vaccine and to Mr. Ryoichi Nagatahira, Mr. Junichi Yoshida, Mr. Kiyonoshin Ichikawa, Mr. Kaoru Kubo and Ms. Yumi Nagata for technical assistance.

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