Effects of \( \omega \)-Conotoxin GVIA on the Activation of Capsaicin-Sensitive Afferent Sensory Nerves in Guinea Pig Airway Tissues

Hiroshi Morimoto, Atsushi Matsuda, Makoto Ohori and Takashi Fujii

Department of Pharmacology, New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,
1-6, 2-chome, Kashima, Yodogawa-ku, Osaka 532, Japan

Received November 1995 Accepted March 21, 1996

Abstract—We examined the effects of \( \mathrm{Ca}^{2+} \) channel antagonists on various respiratory reactions induced by the activation of capsaicin-sensitive afferent sensory nerves. Intravenous (i.v.) injection of the N-type \( \mathrm{Ca}^{2+} \) channel antagonist \( \omega \)-conotoxin GVIA (CgTX) (1–20 \( \mu \)g/kg) dose-dependently inhibited capsaicin-induced guinea pig bronchoconstriction, whereas i.v. administration of the L-type antagonist nicardipine (100 \( \mu \)g/kg), the P-type antagonist \( \omega \)-agatoxin IVA (AgaTX) (20 \( \mu \)g/kg) or the OPQ family-type antagonist \( \omega \)-conotoxin MVIIC (CmTX) (20 \( \mu \)g/kg) had no effect. However, CgTX (20 \( \mu \)g/kg) failed to inhibit substance P-induced guinea pig bronchoconstriction. CgTX (20 \( \mu \)g/kg) significantly inhibited cigarette smoke-induced guinea pig tracheal plasma extravasation, but not the substance P-induced reaction. CgTX also reduced electrical field stimulation-induced guinea pig bronchial smooth muscle contraction (0.01–10 \( \mu \)M) and capsaicin-induced substance P-like immunoreactivity release from guinea pig lung (0.14 \( \mu \)M). This evidence suggests that N-type \( \mathrm{Ca}^{2+} \) channels modulate tachykinin release from capsaicin-sensitive afferent sensory nerve endings in guinea pig airway tissue.

Keywords: N-type \( \mathrm{Ca}^{2+} \) channel, Substance P release, Sensory nerve, Airway

Our previous study showed that neurogenic inflammation in the airway plays an important role in the pathogenesis of asthma (1). By using novel tachykinin antagonists (2, 3), we directly demonstrated that airway inflammation is generated by tachykinins released from capsaicin-sensitive afferent sensory nerve endings, which are stimulated by various types of irritants like cigarette smoke (4). These results suggested that the agent, which blocks the excitation of capsaicin-sensitive afferent sensory nerves, may be a valuable tool for the treatment of airway diseases such as asthma.

The entry of \( \mathrm{Ca}^{2+} \) into presynaptic nerve endings through voltage-dependent \( \mathrm{Ca}^{2+} \) channels is essential for the release of neurotransmitters within the nervous system (5). Capsaicin-evoked tachykinin release from sensory nerve endings is also due to an influx of \( \mathrm{Ca}^{2+} \) (6), but its mechanism is not well-known. Biophysical studies have identified several types of voltage-dependent \( \mathrm{Ca}^{2+} \) channels in neurons and other excitable tissues (7). The N-type \( \mathrm{Ca}^{2+} \) channel, which regulates neurotransmitter release from synaptic endings, can be distinguished from the neuronal L-type \( \mathrm{Ca}^{2+} \) channel (8). The neurotoxin \( \omega \)-conotoxin GVIA (CgTX), isolated from the fish-hunting snail Conus geographus, has been shown to be a potent, selective and irreversible inhibitor of N-type voltage-dependent \( \mathrm{Ca}^{2+} \) channels. CgTX has also been shown to inhibit neurotransmission in a number of in vitro preparations. While, in the mammalian brain, other less well-defined subtypes of voltage-dependent \( \mathrm{Ca}^{2+} \) channels also appear to be important in the regulation of neurotransmitter release (9). This latter group of channels, recently referred to as the OPQ family (10), includes the P-type calcium currents initially characterized in Purkinje cells and the Q-type and O-type currents of cerebellar granule cells. \( \omega \)-Conotoxin MVIIC (CmTX), isolated from the fish-hunting snail species Conus magus, inhibits OPQ family-type \( \mathrm{Ca}^{2+} \) channels. \( \omega \)-Agatoxin IVA (AgaTX), isolated from the spider Agelenopsis aperta, specifically blocks P-type \( \mathrm{Ca}^{2+} \) channels.

In this report, we now examined the effects of these \( \mathrm{Ca}^{2+} \) channel antagonists on the respiratory reactions induced by the activation of capsaicin-sensitive afferent sensory nerves and clarified that N-type \( \mathrm{Ca}^{2+} \) channels regulate the release of tachykinins from their endings.
MATERIALS AND METHODS

Bronchoconstriction in guinea pigs in vivo
Male Hartley guinea pigs (290–480 g; Nihon SLCL, Shizuoka) were anesthetized by i.p. injection of sodium pentobarbital (10 mg/animal). The jugular vein and trachea were cannulated, and the animals were ventilated artificially (5 ml of air, 60 strokes/min). The pressure in the respirator system, i.e., the insufflation pressure, was measured constantly using a transducer (TP 200T; Nihon Kohden, Tokyo). Each agonist was injected i.v. every 15 min through the jugular vein cannula to induce bronchoconstriction. Agonists were administered repeatedly (approximately 6 times) until a reproducible constriction (control response) was obtained. Drugs were dissolved in saline and administered i.v. (2 ml/kg) 2 min before the further challenge was repeated. Administration of the vehicle had no effect. The resulting constriction was compared with the control constriction. The following agonists were used at the indicated doses: substance P (10 nmol/kg) and capsaicin (10 nmol/kg).

Tracheal plasma extravasation in guinea pigs in vivo
Male Hartley guinea pigs (300–400 g) were injected i.v. with Evans Blue dye (20 mg/kg, dissolved in saline). Guinea pigs were immediately, either injected with a chemical mediator or exposed to cigarette smoke. Substance P (32 nmol/kg) was dissolved in saline and intravenously injected. The cigarette exposure regime involved intense passive smoke exposure and is a modification. Male Hartley guinea pigs (300–400 g) were killed and then their bronchi were removed rapidly. The main bronchi was mounted in a 5-ml organ baths filled with warmed (37°C) and oxygenated (95% O2, 5% CO2) standard Tyrode’s solution containing 137.0 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1.05 mM MgCl2, 0.4 mM NaH2PO4, 5.6 mM dextrose and 11.9 mM NaHCO3 (pH 7.5) under a resting tension of 0.5 g. After a 60 min equilibration period, bronchi were stimulated by 10 nM neurokinin A or electrical field stimulation (EFS) in the presence of atropine and propranolol (both 1 μM). EFS was delivered using two parallel platinum wires connected to a stimulator, and square-wave pulses of supramaximal voltage (50 V) and 1 msec pulse duration were applied for 30 sec every 30 min at a frequency of 10 Hz. After a reproducible response was obtained (control response), the drug was added 10 min prior to the final neurokinin A or EFS. The response obtained in the presence of drug was compared with the control response.

Substance P release from guinea pig lung
The procedure of Ray et al. (13) was used with certain modifications. Male Hartley guinea pigs (300–400 g) were killed, and then their lungs were perfused (6 ml/min, 37°C) with oxygenated (95% O2, 5% CO2) Krebs-Ringer HEPES buffer containing 138.0 mM NaCl, 5.6 mM KCl, 1.0 mM CaCl2, 1.0 mM MgCl2, 1.0 mM NaH2PO4, 11 mM NaHCO3, 10 mM dextrose, 20 mM HEPES, 30 μM bacitracin and 1 μM phosphoramidon (pH 7.5) via a cannula which was inserted into the pulmonary artery through the right ventricle. The left atrium was opened to collect the outflow. Fifteen minutes after the start, perfusates from one period (15 min; i.e., 90 ml) were collected on ice in beakers containing hydrochloric acid to give a final concentration of 0.1 M. Each fraction was desalted on Sep-Pak C18 cartridges (Waters, Milford, MA, USA) as described for somatostatin (14), and the peptides were concentrated to a final volume of 1 ml. The recovery from the Sep-Pak cartridge was more than 90% for radiolabelled substance P. Chemical irritation of tissues was achieved by perfusion with buffer containing 1 μM capsaicin for 5 min during the 2nd collection period. Substance P-like immunoreactivity was measured by radioimmunoassay. The amount of substance P released by capsaicin was calculated by subtracting the level detected in the 1st period perfusate from that in the 2nd period perfusate. Drugs were added in Krebs-Ringer HEPES buffer throughout the experiment.

Materials
Substance P, α-conotoxin GVIA (CgTX), α-conotoxin MVIIC (CmTX), α-agatoxin IVA (AgaTX) and phosphoramidon were purchased from Peptide Institute, Inc. (Osaka). Nicardipine and bacitracin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Capsaicin was from Nacalai Tesque Chemical Company.
(Kyoto). $^{[25]}$I-Substance P (74 TBq/mmol) and anti-substance P antiserum for the radioimmunoassay were purchased from Amersham Int., Ltd. (Buckinghamshire, UK). The doses of Ca$^{2+}$ channel antagonists used in this study were as previously described (15–17).

Statistical analyses

Results are each given as the mean±S.E.M. of four experiments. Statistical analyses were performed by either analysis of variance followed by Dunnett’s multicomparison test (bronchoconstriction) or by means of the unpaired Student’s t-test (others). P<0.05 or less was considered as indicative of significance.

RESULTS

Effects of Ca$^{2+}$ channel antagonists on bronchoconstriction in guinea pigs

We examined the effects of Ca$^{2+}$ channel antagonists on capsaicin-induced bronchoconstriction in guinea pig airway induced by capsaicin. I.v. injection of the N-type Ca$^{2+}$ channel antagonist CgTX (1–20 pg/kg) dose-dependently inhibited capsaicin-induced constriction (Fig. 1). In contrast, i.v. injection of other type Ca$^{2+}$ channel antagonists, nicardipine (100 pg/kg), CmTX (20 pg/kg) and AgaTX (20 pg/kg) had no effect (Fig. 2). Substance P-induced guinea pig bronchoconstriction was unaffected by CgTX at a dose of 20 pg/kg (Fig. 3). At the doses used, both agonists produced half maximum increases in the bronchoconstriction of the guinea pigs. The increased pressure of bronchoconstriction induced by these agonists were 21.8±3.0 cmH$_2$O (substance P) and 26.8±1.0 cmH$_2$O (capsaicin). None of the Ca$^{2+}$ channel antagonists tested altered baseline airway resistance (% inhibition of baseline: 20 µg/kg CgTX, 1.4±1.4; 100 µg/kg nicardipine, 0.0±0.0; 20 µg/kg AgaTX, 0.5±1.9; 20 µg/kg CmTX, -1.8±1.2).

Effects of CgTX on tracheal plasma extravasation in guinea pigs

The effects of CgTX on guinea pig tracheal plasma extravasation induced by cigarette smoke were examined. I.v. injection of CgTX significantly inhibited cigarette smoke-induced guinea pig tracheal plasma extravasation (Table 1). However, substance P-induced tracheal plasma extravasation was not affected by CgTX at a dose of 20 µg/kg. CgTX (20 µg/kg) per se did not alter Evans Blue dye content in guinea pig trachea (Control, 9.4±0.5 µg/g tissue; 20 µg/kg CgTX, 9.2±0.2 µg/g tissue; N = 4).

Effects of CgTX on isolated guinea pig bronchial smooth muscle contraction

The effects of CgTX on isolated guinea pig bronchial smooth muscle contraction were examined. After electrical field stimulation, isolated guinea pig bronchial smooth muscle evoked tachykinin-dependent prolonged

![Fig. 1: Effects of i.v. injection of 1 µg/kg (○), 5 µg/kg (●), 10 µg/kg (△) or 20 µg/kg (▲) ω-conotoxin GVIA on capsaicin-induced guinea pig bronchoconstriction. Significantly different from the control, *P<0.05, **P<0.01. Values are given as % inhibition of control bronchoconstriction and are the mean (±S.E.M.) of four experiments.](image-url)
Fig. 2. Effects of i.v. injection of 20 μg/kg ω-conotoxin GVIA (○), 100 μg/kg nicardipine (●), 20 μg/kg ω-conotoxin MVIIIC (△) or 20 μg/kg ω-agatoxin IVA (▲) on capsaicin-induced guinea pig bronchoconstriction. Significantly different from the control, **P<0.01. Values are given as % inhibition of control bronchoconstriction and are the mean (±S.E.M.) of four experiments. Values for 20 μg/kg ω-conotoxin GVIA are the same as those shown in Fig. 1.

Fig. 3. Effects of ω-conotoxin GVIA on capsaicin (○)- and substance P (●)-induced guinea pig bronchoconstriction. Significantly different from the control, **P<0.01. Values are given as % inhibition of control bronchoconstriction and are the mean (±S.E.M.) of four experiments. Values for 20 μg/kg ω-conotoxin GVIA on capsaicin-induced guinea pig bronchoconstriction are the same as those shown in Fig. 1.
Table 1. Effect of \(\omega\)-conotoxin GVIA on guinea pig tracheal plasma extravasation induced by cigarette smoke and substance P

| Treatment      | Cigarette smoke | Substance P |
|----------------|-----------------|-------------|
|                | Evans Blue dye (µg/g tissue) | Inhibition (%) | Evans Blue dye (µg/g tissue) | Inhibition (%) |
| Control        | 47.9±5.7        | 61.9±3.7    |
| \(\omega\)-Conotoxin GVIA 20 µg/kg | 18.8±2.3** | 60.7        | 59.2±4.2    | 4.4          |

\(\omega\)-Conotoxin GVIA was applied 2 min before cigarette smoke exposure or substance P administration. Each value indicates the mean (±S.E.M.) of four experiments. **P<0.01, significantly different from the vehicle group.

contraction (5). CgTX inhibited electrical field stimulation-induced tachykinin-dependent contraction in a dose-dependent manner (Table 2). On the other hand, neurokinin A-induced guinea pig bronchial smooth muscle contraction was not affected by CgTX at a dose of 10 µM.

Effects of CgTX on substance P-like immunoreactivity release from guinea pig lung

To clarify the mechanism involved in CgTX inhibition of capsaicin-induced guinea pig airway constriction and cigarette smoke-induced guinea pig bronchial smooth muscle contraction, the effects of CgTX on capsaicin-induced substance P-like immunoreactivity release from guinea pig lung were examined (Table 3). CgTX (0.14 µM) significantly reduced the capsaicin-induced release of substance P-like immunoreactivity from guinea pig lungs.

DISCUSSION

It has been suggested that a various of stimuli act on irritant receptors in respiratory mucosa and produce various respiratory reactions via the release of tachykinins from the endings of afferent sensory nerve endings (18). The influx of \(Ca^{2+}\) into presynaptic nerve endings through voltage-dependent \(Ca^{2+}\) channels is essential for the release of neurotransmitters within the nervous system. \(Ca^{2+}\) influx is a key step in excitation-release coupling in afferent sensory nerves (6). For asthma research, it is important to decide which type of \(Ca^{2+}\) channels are involved in the activation of afferent sensory nerves. In this study, we found that the N-type \(Ca^{2+}\) channel antagonist CgTX inhibited capsaicin-induced guinea pig airway constriction, cigarette smoke-induced guinea pig tracheal plasma extravasation and electrical field stimulation-induced guinea pig bronchial smooth muscle contraction. These reactions are dependent on tachykinins because they were inhibited by tachykinin antagonists (3, 4). In contrast, CgTX did not influence substance P-induced guinea pig airway constriction, substance P-induced guinea pig tracheal plasma extravasation or neurokinin-induced guinea pig bronchial smooth muscle contraction. These results suggested that CgTX blocks the release of tachykinins from afferent sensory nerves, but does not antagonize the affect of tachykinins on their receptors in guinea pig airways. However, CgTX did significantly reduce the capsaicin-induced release of substance P-like immunoreactivity from guinea pig lungs, while the other type of \(Ca^{2+}\) channel antagonists did not affect capsaicin-induced guinea pig airway constriction.

Recently, Hamilton and Lundy (19) reported that...
ruthenium red inhibited $[^{25}]$I-CgTX binding, Ca$^{2+}$ influx and $[^{3}H]$-dopamine release in chicken forebrain and rat striatal synaptosomes. They concluded that ruthenium red reduced the neurotransmitter release by directly blocking N-type Ca$^{2+}$ channels. In airway tissues, ruthenium red also inhibits bronchoconstriction and calcitonin gene-related peptide release induced by the activation of capsaicin-sensitive afferent sensory nerves in guinea pigs (20) and reduces plasma extravasation induced by cigarette smoke in rats (21). These evidences and our results strongly suggest that the activation of afferent sensory nerves in airway tissue is modulated by N-type Ca$^{2+}$ channels and that the stimulation of irritant receptors evokes Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels and the release of tachykinins in airway afferent sensory nerves.

When excitatory capsaicin-sensitive afferent sensory nerves are stimulated, not only tachykinins but also other neuropeptides, e.g., calcitonin gene-related peptide, are released from their nerve endings and induce various respiratory reactions (22). The inflammatory effects of these peptides on airway tissues may be of pathological relevance in human bronchial hyperreactivity. We conclude that a N-type Ca$^{2+}$ channel antagonist, which inhibits the activation of capsaicin-sensitive afferent sensory nerves, will be a valuable tool for asthma research.

REFERENCES

1 Morimoto H, Fuji T and Senoh H: Plasma extravasation by chemical mediators in rat skin and trachea: A role of neurogenic agents on tracheal edema formation. Jpn J Pharmacol 49, 389–395 (1989)

2 Murai M, Morimoto H, Maeda Y and Fuji T: Effects of the tripeptide substance P antagonist, FR 113680, on airway constriction and airway edema induced by neurokinins in guinea pigs. Eur J Pharmacol 217, 23–27 (1992)

3 Murai M, Morimoto H, Maeda Y, Kiyotoh S, Nishikawa M and Fuji T: Effects of FK 224, a novel compound NK1 and NK2 receptor antagonist, on airway constriction and airway edema induced by neurokinins and sensory nerve stimulation in guinea pigs. J Pharmacol Exp Ther 262, 403–408 (1992)

4 Morimoto H, Yamashita M, Matsuda A, Miyake H and Fuji T: Effects of FR 113680 and FK 224, novel tachykinin antagonists, on cigarette smoke-induced rat tracheal plasma extravasation. Eur J Pharmacol 224, 1–5 (1992)

5 Mulkey RM and Zucker RS: Action potentials must admit calcium to evoke transmitter release. Nature 350, 153–155 (1991)

6 Franco-Cereceda A, Sarai A and Lundberg JM: Differential release of calcitonin-gene related peptide and neuropeptide Y from the isolated heart by capsaicin, ischemia, nicotine, bradykinin and ouabain. Acta Physiol Scand 135, 173–187 (1989)

7 Tsien RW, Lipscombe D, Madison DV, Bley KR and Fox AP: Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci 11, 431–438 (1988)