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Multiple calcium channels regulate neurotransmitter release from vagus nerve terminals in the cat bronchiole

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1 Twitch-like contractions and non-adrenergic non-cholinergic (NANC) relaxations evoked by electrical field stimulation (EFS) of the cat bronchiole were used to examine the voltage-activated calcium channels involved in excitatory and inhibitory neurotransmission in the cat bronchiole.

2 Nifedipine (50 μM), the L-type calcium channel antagonist, did not affect the twitch-like contraction and NANC relaxations. However, low concentrations of the N-type calcium channel blocker ω-conotoxin GVIA (ω-CgTX GVIA) (0.1 μM) irreversibly abolished twitch-like contractions evoked by trains of EFS $\leqslant$10 stimuli at 20 Hz.

3 After the prolonged treatment with 0.1 μM ω-CgTX GVIA, EFS evoked initial fast and later slow NANC relaxations in the presence of 5-HT (10 μM), atropine and guanethidine (1 μM each). However increased concentration of ω-CgTX GVIA (1 μM) completely suppressed the slow NANC relaxation without affecting the initial fast component.

4 ω-agatoxin IVA (100 nM), the P- and Q-type calcium channel inhibitor, and nimodipine (10 μM), the L- and T-type calcium channel blocker, did not affect the amplitude of the initial fast NANC relaxation in the absence or presence of ω-CgTX GVIA (1 μM).

5 The contraction or relaxation induced by exogenous acetylcholine (ACh) (0.5 μM) or the nitric oxide donor, s-nitroso-N-acetyl penicillamine (SNAP) (1 μM) were not affected by ω-CgTX GVIA (1 μM).

6 Taken together, these results suggest that generation of twitch-like contraction and later slow NANC relaxation are regulated by N-type calcium channels, whereas generation of the initial fast NANC relaxation possibly involves R-type calcium channel.

Keywords: Bronchiole; cholinergic excitatory neurotransmission; NANC inhibitory neurotransmissions; calcium channel antagonists; ω-CgTX GVIA; ω-agatoxin IVA; ω-CgTX MVIIC

Abbreviations: 5-HT, serotonin; ACh, acetylcholine; Cd^{2+}, cadminium; EFS, electrical field stimulation; EJPs, excitatory junction potentials; HVA, high voltage activated; IC_{50}, inhibitory concentration for 50%; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; SNAP, s-nitroso-N-acetyl pencillamine; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide; ω-CgTX GVIA, ω-conotoxin GVIA; ω-CgTX MVIIC, ω-conotoxin MVIIC

Introduction

The cranial parasympathetic nerves, which control airway smooth muscle, contain non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitters in addition to the excitatory neurotransmitter acetylcholine (ACh) (Coburn & Tomita, 1973; Ito & Takeda, 1982; Barnes, 1991; Belvisi *et al*., 1992). Although the neurotransmitters responsible for the NANC relaxation of the airway has not been conclusively identified, we have demonstrated that at least two substances, nitric oxide (NO) or NO-containing compounds for the fast relaxation and vasoactive intestinal polypeptide (VIP) or a VIP-like peptide for slow relaxation are involved (Matsuzaki *et al*., 1980; Palmer *et al*., 1986; Hakoda *et al*., 1991; Jing *et al*., 1995; Takahashi *et al*., 1995; Tanaka *et al*., 1996). Thus the excitation of parasympathetic nervous system releases at least three different neurotransmitters.

Voltage-gated Ca^{2+} channels mediate the increase in presynaptic Ca^{2+} concentration which plays a key role during the process of neurotransmitter release (Katz, 1969; Llinás *et al*., 1976). In recent years it has become clear that mammalian neurones express multiple types of pharmacologically and electrophysiologically distinct high-voltage-activated (HVA) Ca^{2+} channels (Randall & Tsien, 1995). The molecular characterization and functional expression of Ca^{2+} channel clones from a variety of species, have confirmed the existence of distinct L-, N-, P-, Q- and R-type calcium channels (Birnbaumer *et al*., 1994). Furthermore these calcium channels exhibit different sensitivity to specific neurotoxins (Wheeler *et al*., 1994; Dunlop *et al*., 1995). For example, ω-CgTX GVIA selectively inhibits N-type calcium channels and omega-conotoxin MVIIC (ω-CgTX MVIIC) inhibits N-, P-, Q-type channels.

In an attempt to study the subtypes of Ca^{2+} channels involved in the multiple neuro-transmitter release from the vagus nerve terminals, we observed the effects of various calcium channel blockers on the contraction and relaxation evoked by the activation of vagus nerve terminals in the cat bronchiole. In the cat airway smooth muscle, electrical field stimulation (EFS) evokes excitatory junction potentials (EJPs) followed by twitch-like contractions, but activation of inhibitory NANC nerve fibres does not cause a change in post-junctional membrane potential (Ito & Takeda, 1982; Takahashi *et al*., 1995).

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Methods

Tissue preparations

Adult mongrel cats of either sex (2–3 kg) were anaesthetized with sodium pentobarbitone (30–40 mg kg^{-1}, i.p.) and then bled. The trachea and whole pulmonary lobes were quickly resected from the main bronchus and placed in modified Krebs solution of the following composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was aerated with 97% O_2 and 3% CO_2 and the pH was 7.3–7.4. Small airways were carefully excised from the lung tissue and lung parenchyma, and pulmonary vessels running along the bronchiolar branch were carefully removed under microscopic observation. Since the branching pattern of the cat airways is not symmetrical and regular as in humans (Mortensen *et al*., 1983) and is similar to that of dogs (Amis & McKiernan, 1987), the diameter of the bronchi does not necessarily correlate with their order of branching, as described earlier (Takahashi *et al*., 1995). Segments of isolated bronchioles (0.5–2 mm o.d.) were cut into rings 2–3 mm wide. As much airway epithelium as possible was carefully removed by mechanical rubbing according to the method described earlier (Xie *et al*., 1992), since it is known that electric current (Xie *et al*., 1992) and superoxide anion radical (Matyas & Bauer, 1995) can release factor(s) from the airway epithelial cells which induce relaxation.

Mechanical recording

For measurement of mechanical responses, the ring preparations of bronchioles were hooked horizontally by a pair of right-angled fine needles in a 1 ml organ bath, through which the test solution flowed continuously at a rate of 2 ml min^{-1}. One needle was fixed to the wall of the chamber, the other was connected to a manipulator and to an isometric mechano-transducer (RCA-5734, Nihon Kohden) through a 1 mm-wide slit made in the other wall of the chamber (Takahashi *et al*., 1995). The strips were set up with an initial tension of 1–2 mN and mechanical activity was recorded with a pen recorder. Electrical field stimulation (EFS, 1, 3, 10, 20, 30 pulses of 0.5 ms duration at 20 Hz) was applied through a pair of Ag-AgCl plates fixed to both sides of the inner surface of the chamber, so that current pulse would pass transversely across the ring preparations of the bronchioles.

Chemicals and data analysis

The following drugs were used: N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Nakarai Chemicals), atropine sulphate, guanethidine monosulphate, 5-hydroxytryptamine creatinine sulphate, acetylcholine chloride, nifedipine and tetrodotoxin (Sigma), ω-CgTX GVIA, ω-CgTX MVIIC, ω-agatoxin IVA and nimodipine (Calbiochem), and s-nitroso-N-acetyl penicillamine (SNAP) (Dojin). Except nifedipine and nimodipine which were dissolved in DMSO, all drugs were dissolved in distilled water.

The mechanical recordings were carried out at 35–36°C. Results (amplitude of twitch-like contraction and NANC relaxation) are expressed as mean ± s.d. and were analysed for significance by Student's *t*-test.

Results

Effects of nifedipine and Cd^{2+} on twitch-like contraction and NANC relaxations of the cat bronchiole

The ring preparation of cat bronchioles was normally quiescent and lacked spontaneous mechanical activity. Single or repetitive electrical field stimulation (EFS) with short pulse duration (0.5 ms) at high frequency (20 Hz) evoked twitch like contraction, the amplitude of which was dependent on the number of stimuli at a constant stimulus frequency and intensity.

Firstly, we observed the effects of nifedipine, one of the L-type calcium channel blockers, on the twitch-like contraction and NANC relaxations, to examine the possible role of L-type calcium channels in excitatory and inhibitory neuro-effector transmission. As summarized in Figure 1a, nifedipine (1 μM) did not affect the twitch-like contraction, and at an increased

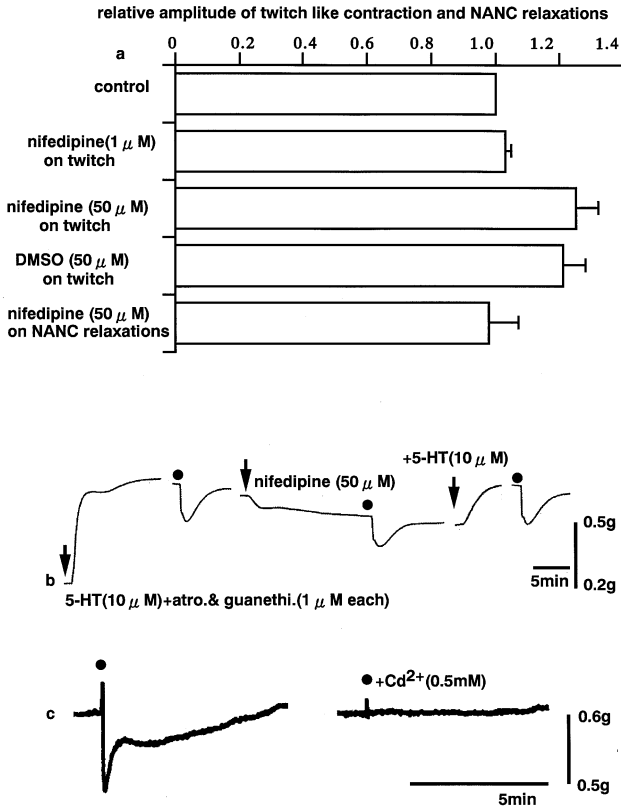

Figure 1 Effects of nifedipine and Cd^{2+} on twitch-like contractions and NANC relaxations of the cat bronchioles evoked by electrical field stimulation (EFS). (a) Effects of nifedipine (1 or 50 μM) and DMSO, the vehicle of nifedipine, on the contractions and NANC relaxation of the cat bronchioles. Twitch-like contractions were evoked by 30 stimuli at 20 Hz applied every 5 min. The amplitude before the application of chemicals was taken as 1.0. Each row represents the mean of 5–8 ring preparations of bronchioles, and horizontal bars s.d. (b) Actual traces of NANC relaxations evoked by EFS (30 stimuli at 20 Hz) in the presence of 5-HT (10 μM), atropine (atro.) and guanethidine (guanethi.) (1 μM each). Dots indicate the EFS. EFS evoked NANC relaxations with an initial fast and a following second slow component. Nifedipine (50 μM) reduced the muscle tone and the maximum amplitude of the NANC relaxation. However cumulative-application of 5-HT (10 μM) enhanced the muscle tone, and EFS evoked NANC relaxation, the amplitude of which was not significantly different from control. (c) Cd^{2+} (0.5 mM) completely abolished the biphasic NANC relaxations, evoked by EFS in the presence of 5-HT (10 μM), atropine and guanethidine (1 μM each).

concentration of 50 μM, it enhanced the amplitude of the contractions. DMSO (5 μM), the vehicle of nifedipine, also enhanced the amplitude of the twitch-like contraction to a similar extent as nifedipine (50 μM), thereby indicating that nifedipine has no effect on the excitatory neuro-effector transmission.

In the presence of atropine and guanethidine (1 μM each), 5-hydroxytryptamine (5-HT) (10 μM) increased the muscle tone, and EFS applied during the elevated muscle tone evoked biphasic NANC relaxations as reported previously (Takahashi *et al*., 1995; Tanaka *et al*., 1996) (Figure 1b). Application of nifedipine (10 μM) reduced the muscle tone induced by 5-HT indicating that L-type calcium channels are partly involved in the increase in the muscle tone of the cat bronchiole, and slightly reduced the maximum amplitude of the biphasic NANC relaxations. We previously reported that the amplitude of NANC relaxations depend on the level of the muscle tone of the cat bronchiole (Ito & Takeda, 1982). Cumulatively applied 5-HT (10 μM) increased the muscle tone (the maximum contraction was obtained by $5\times10^{-5}-10^{-4}$ M 5-HT), and EFS evoked NANC relaxations, the amplitude of which were not different from the control value (Figure 1a and b). These observations indicate that L-type calcium channels do not play any physiological role in the excitatory and inhibitory neuro-effector transmission in the cat bronchiole.

To confirm the involvement of Ca^{2+} channels in the parasympathetic neuro-effector transmission, we observed the effects of cadminium ions (Cd^{2+}) which block all known voltage-gated calcium channels involved in the twitch and NANC relaxations. Cd^{2+} (0.5 mM) completely abolished the twitch-like contraction (data not shown) and biphasic NANC relaxations evoked by trains of EFS (30 stimuli at 20 Hz) (Figure 1c), indicating that calcium influx into nerve terminals through voltage-gated calcium channels is necessary for the excitatory and inhibitory neuro-effector transmission in the cat bronchioles.

Effects of calcium channel blockers on twitch and NANC relaxations of the cat bronchiole

In an attempt to study the type of calcium channels involved in excitatory and inhibitory neuro-effector transmissions, we used various ω-conotoxins. Application of ω-CgTX GVIA (>0.5 nM) dose-dependently suppressed the amplitude of the twitch-like contractions evoked by 10 stimuli at 20 Hz. At a concentration of 0.1 μM this toxin gradually and progressively reduced the amplitude of the twitch-like contraction, and after some 60–90 min almost abolished it (Figure 2a and b). Additional application of tetrodotoxin (TTX, 0.1 μM) did not further reduce the amplitude of residual contractions evoked in the presence of ω-CgTX GVIA (0.1 μM), thereby indicating that ω-CgTX GVIA (0.1 μM) completely abolished the neurogenic contraction at $\leqslant10$ stimuli at 20 Hz in the cat bronchiole. However, when the number of stimuli was increased (30–50 stimuli at 20 Hz), trains of stimuli evoked small but distinct twitch-like contractions in the presence of 0.1 μM ω-CgTX GVIA (data not shown). The inhibitory effect of ω-CgTX GVIA was not reversed by prolonged washing (2 h). Figure 2b shows the dose-response curve of ω-CgTX GVIA on the twitch-like contractions evoked by 10 stimuli at 20 Hz. The half maximum inhibitory concentration (IC_{50}) and the Hill coefficient were 2.93 nM and 1.47 respectively.

We repeated similar experiments with ω-conotoxin MVIIC (ω-CgTX MVIIC), blocker of N-, P-, Q-type calcium channels, and found that this toxin also greatly suppressed the twitch-like contractions at a dose of 0.1 μM, but did not abolish it completely especially when more than 20 stimuli at 20 Hz were applied. Additional application of TTX (0.1 μM) further reduced the twitch amplitude, indicating that ω-CgTX MVIIC does not abolish the neurogenic response at a concentration of 0.1 μM. Figure 2c shows the effects of ω-CgTX GVIA and MVIIC (0.1 μM each) on the relationship between the number of stimuli at 20 Hz and relative amplitude of twitch-like contractions. After prolonged treatment of the tissue with ω-CgTX MVIIC (0.1 μM), trains of EFS ($\geqslant10$ stimuli) evoked small but distinct twitch-like contractions, which were sensitive to tetrodotoxin (TTX, 0.1 μM).

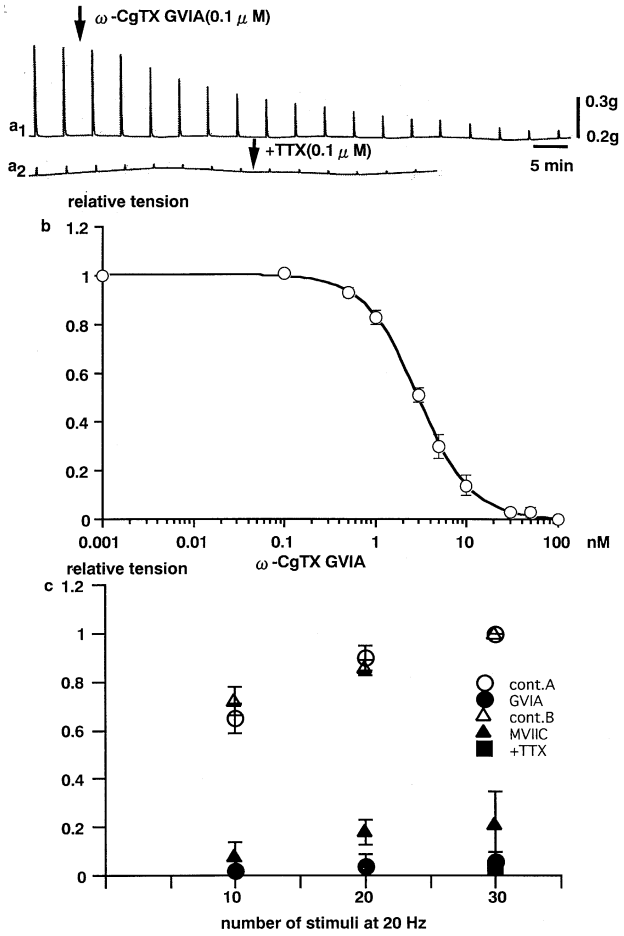

Figure 2 Effects of ω-CgTX GVIA and ω-CgTX MVIIC on twitch-like contractions of cat bronchiole. (a1 and a2) Actual trace of twitch like contractions evoked by 10 stimuli at 20 Hz applied every 5 min in the absence and presence of ω-CgTX GVIA (0.1 μM) with or without tetrodotoxin (0.1 μM). a1 and a2 are continuous recordings. (b) The relationship between the relative amplitude of twitch-like contractions and dose of ω-CgTX GVIA, after the prolonged (60–90 min) treatment of the tissue with the toxin. Each point represents the average of results from 5–12 ring preparations of bronchioles from eight cats. The data points in the concentration response curves were fitted to the logistic equation $A=1-(R_{max}XC^{nH})/(C^{nH}-IC_{50}{}^{nH})$ where A is the normalized amplitude of the twitch-like contractions, R_{max} is the maximum inhibition ratio, C is the concentration of ω-CgTX GVIA, IC_{50} is the concentration that produces 50% inhibition of the maximum response and nH is the Hill coefficient. (c) The relationship between the relative amplitude of twitch-like contractions of the cat bronchioles and number of stimuli (10–30) at 20 Hz in the absence and presence of ω-CgTX GVIA (0.1 μM), ω-CgTX MVIIC (0.1 μM) or TTX (0.1 μM), where the relative amplitude of twitch like contractions evoked by 30 stimuli at 20 Hz in the absence of chemicals was taken as 1.0. The amplitude of twitch-like contractions were measured after prolonged (60–90 m) treatment of the tissue with each toxins.

Effects of calcium channel blockers on NANC relaxations

During the course of experiments we found that biphasic NANC relaxations occur after prolonged (60–90 min) treatment with 0.1 μM ω-CgTX GVIA in response to EFS in the presence of 5-HT (10 μM), atropine and guanethidine (1 μM each) (Figure 3a). We also found that increased concentration of ω-CgTX GVIA (1 μM) selectively abolishes the second slow components of the NANC relaxation without affecting the amplitude of the initial fast NANC relaxation, which was sensitive to L-NAME (0.1 mM), one of nitric oxide synthase inhibitors (Figure 3a). Thus, to observe the effects of ω-CgTX GVIA on the second slow NANC relaxation, we repeated the experiments in the presence of L-NAME (0.1 mM) (Figure 3b). ω-CgTX GVIA (>10 nM) dose-dependently suppressed the amplitude of the second slow NANC relaxation and abolished it at a concentration of 1 μM (Figure 3d). Similarly, ω-CgTX MVIIC (1 μM) also completely suppressed the second slow component of the NANC relaxation evoked by EFS (Figure 3c). These observations indicate that the second slow but not the initial fast component of the NANC relaxation is sensitive to the higher concentration of ω-CgTX GVIA and ω-CgTX MVIIC (1 μM). Figure 3d shows the dose-response curve of ω-CgTX GVIA on the second slow NANC relaxation, indicating that half maximum inhibitory concentration (IC_{50}) and the Hill coefficient were 28.30 nM and 1.27 respectively.

Thus, it was of interest to observe the effects of ω-agatoxin IVA, which is known to inhibit P/Q type calcium channels specifically, on the initial fast component of the NANC relaxation. However, as shown in Figure 4a, ω-agatoxin IVA (100 nM) did not affect the amplitude of the initial fast component of the NANC relaxation in the presence of ω-CgTX GVIA (1 μM), although this component was sensitive to L-NAME (10^{-4} M) (Figure 4b), TTX (10^{-7} M) (Figure 4c) and Cd^{2+} (0.5 mM) (Figure 1c).

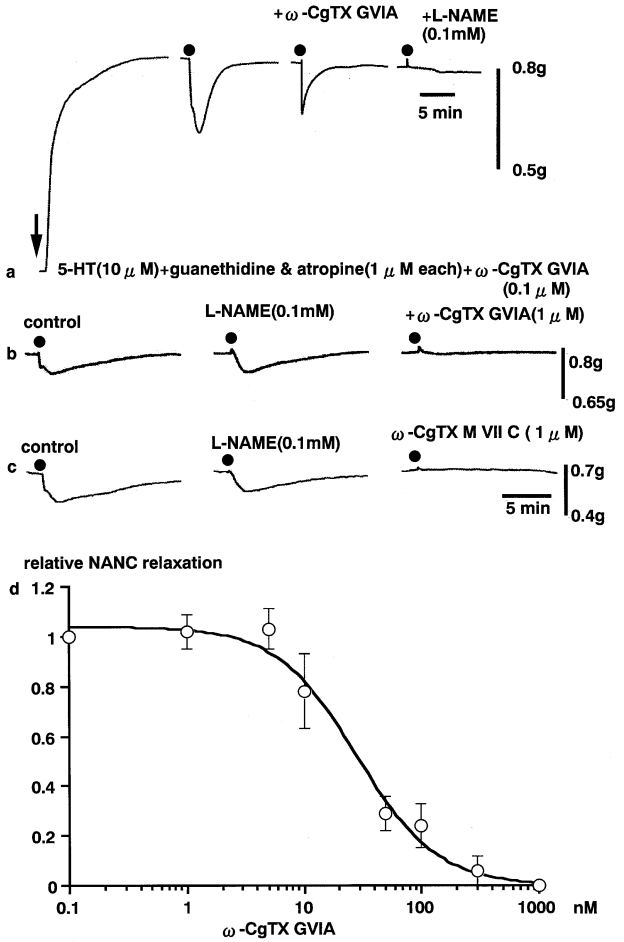

Figure 3 Effects of ω-CgTX GVIA and MVIIC on the NANC relaxations of the cat bronchiole. (a) Effects of ω-CgTX GVIA (0.1 and 1 μM) on NANC relaxations evoked by 30 stimuli at 20 Hz in the presence of 5-HT (10 μM), atropine and guanethidine (1 μM each). In the presence of 0.1 μM ω-CgTX GVIA, trains of EFS evoked NANC relaxations. Increased concentration of ω-CgTX GVIA (1 μM) suppressed the second slow component of the NANC relaxation, without affecting the initial fast component, which was abolished by L-NAME (0.1 mM). (b and c) The effects of prolonged (60–90 min) treatment of the tissue with ω-CgTX GVIA and ω-CgTX MVIIC on the second slow component of NANC relaxations. The drugs were applied after the initial relaxation was blocked by L-NAME (0.1 mM). Dots indicate the application of trains of EFS (30 stimuli at 20 Hz). (d) The relationship between the relative amplitude of second slow NANC relaxations and dose of ω-CgTX GVIA in the presence of L-NAME. Each point represents the average of results from 5–12 ring preparations of bronchioles from eight cats. The data points in the concentration response curves were fitted to the logistic equation $A = 1\text{-}(R_{max} x C^{nH})/(C^{nH} - IC_{50}{}^{nH})$ where A is the normalized amplitude of the second slow NANC relaxation, R_{max} is the maximum inhibition ratio, C is the concentration of ω-CgTX GVIA, IC_{50} is the concentration that produces 50% inhibition of the maximum response and nH is the Hill coefficient.

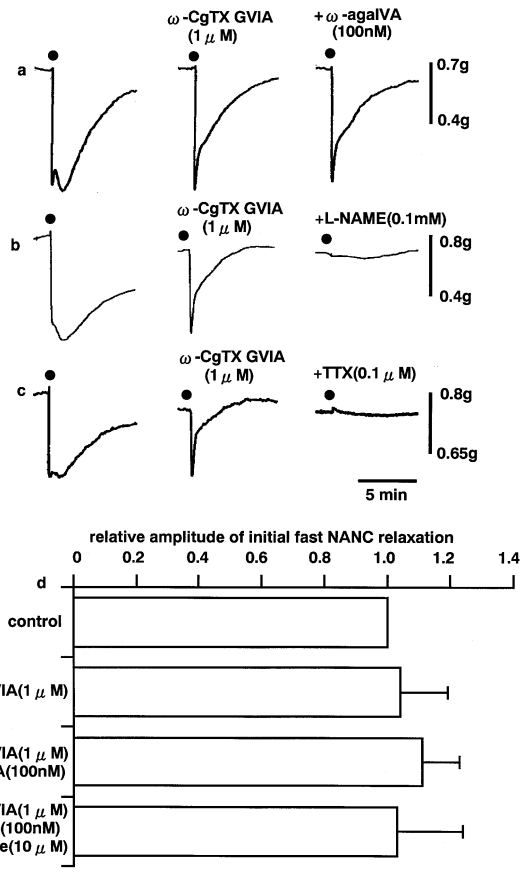

Figure 4 Effects of ω-agatoxin IVA (100 nM) on the initial fast NANC relaxation of the cat bronchiole. (a–c) Actual traces of NANC relaxations in the absence or presence of ω-CgTX GVIA (1 μM), ω-agatoxin IVA (100 nM), TTX (0.1 μM) and L-NAME (0.1 mM). (d) Relative amplitude of initial fast NANC relaxation in the presence of ω-CgTX GVIA with or without ω-agatoxin IVA (100 nM) and nimodipine (10 μM). Each row represents the mean value of 6–9 ring preparations, horizontal bars show s.d.

Further to characterize the calcium channels responsible for the generation of the initial fast NANC relaxation, we used nimodipine, a blocker of L- and T-type calcium channels (Randall & Tsien, 1997). Nimodipine (10 μM), however, did not affect the amplitude of the initial fast component of the NANC relaxations in the presence of ω-CgTX GVIA (1 μM) and ω-agatoxin IVA (100 nM) (Figure 4d). These results suggest that probably R-type Ca^{2+} channels are responsible for generation of the initial fast NANC relaxation in the cat bronchiole.

Lack of effects of ω-agatoxin IVA and nimodipine on the twitch-like contraction and the later slow NANC relaxation

Based on the inhibitory action of ω-CgTX GVIA and ω-CgTX MVIIC on twitch like contraction and later slow NANC relaxation (Figures 2 and 3), it seems that EFS evoked twitch-like contraction and later slow NANC relaxation are due to activation of presynaptic N-type calcium channels. To confirm this view we observed the effects of ω-agatoxin IVA (P-, Q-type calcium channel antagonist) and nimodipine (L-, T-type calcium channel antagonist) on the twitch like contraction and later slow NANC relaxation.

As summarized in Figure 5, nimodipine (10 μM) and ω-agatoxin IVA (10 μM) did not affect the amplitude of twitch-like contraction, at all. Increased concentration of ω-agatoxin IVA (100 nM), significantly suppressed the amplitude of twitch-like contraction, however the inhibitory effects was reversible (Figure 5b). It is known that the action of ω-agatoxin IVA on the calcium channels is irreversible, suggesting that the inhibitory action on the twitch like contraction is due to the non-specific action of the drug on pre- or post-junctional sites.

On the other hand, ω-agatoxin IVA (100 nM) showed no effect on the amplitude of later slow NANC relaxation. Application of nimodipine (10 μM) reduced the muscle tone induced by 5-HT as in the case of nifedipine (10 μM), and reduced the amplitude of later slow NANC relaxation, but did not abolish the generation (Figure 5). Cumulative application of 5-HT (10 μM) increased the muscle tone as shown in Figure 1b), and EFS evoked slow NANC relaxations, the amplitude of which were not different from the control value (Figure 5b). These results confirmed that ω-agatoxin IVA (P-, Q-type calcium channel antagonist) or nimodipine (L-, T-type calcium channel antagonist) – sensitive calcium channels are not involved in the generation of the twitch-like contraction and the later slow NANC relaxation.

Table 1 summarizes the effects of various calcium channel antagonists on twitch-like contraction and NANC relaxations.

Lack of effect of ω-CgTX GVIA on ACh-induced contraction and SNAP-induced relaxation

To examine whether the sensitivity of smooth muscle cells of the cat bronchiole to exogenous ACh or NO is affected by ω-CgTX GVIA, we observed the effects of ω-CgTX GVIA (1 μM) on contraction and relaxation evoked by exogenous ACh (0.5 μM) and SNAP (1 μM), an NO donor. The contraction or relaxation were not affected by ω-CgTX GVIA (1 μM) (0.96 ± 0.08 ($\pm$s.d., $n=5$, $P>0.05$) or 0.98 ± 0.06 ($\pm$s.d., $n=6$, $P>0.05$) of the control values respectively).

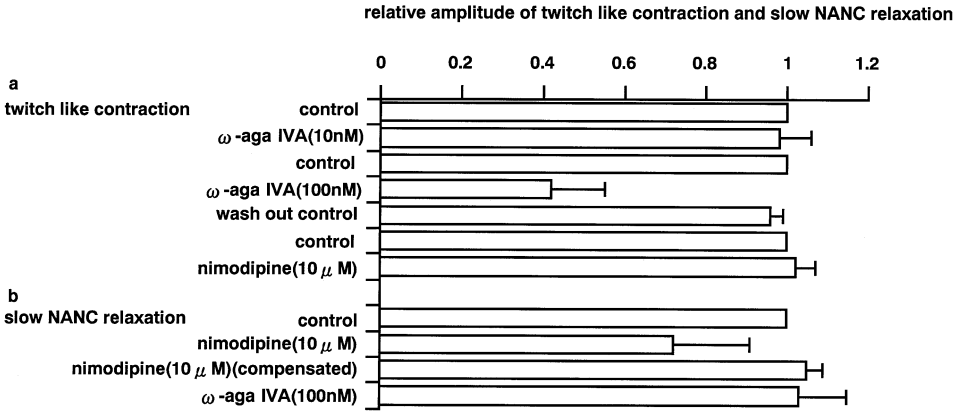

Figure 5 Lack of effects of ω-agatoxin IVA and nimodipine on the amplitude of twitch-like contraction and later slow NANC relaxation. (a and b) Effects of ω-agatoxin (10 and 100 nM) and nimodipine (10 μM) on twitch like contractions evoked by 10 stimuli at 20 Hz (a), and slow NANC relaxations evoked by 30 stimuli at 20 Hz in the presence of 5-HT (10 μM), atropine and guanethidine (1 μM each). Nimodipine (10 μM) reduced the muscle tone induced by 5-HT (10 μM) thereby indicating that L-type calcium channels are partly involved in the increase in the muscle tone. To compare the amplitude of slow NANC relaxation at a similar muscle tone, 5-HT (10 μM) were cumulatively applied, and then the amplitude of slow NANC relaxations were compared (indicated as 'compensated').

Table 1 Effects of various calcium channel antagonists on twitch-like contraction and NANC relaxations of the cat bronchiole

		Twitch like contraction	*Initial NANC relaxation*	*Later slow NANC relaxation*
Nifedipine (50 μM)	L-type calcium channel antagonist	–	–	–
Nimodipine (10 μM)	L-, T-type calcium channel antagonist	–	–	–
CD^{2+} (0.5 mM)	Nonspecific calcium channel antagonist	+	+	+
ω-CgTX GVIA (0.1 – 1 μM)	N-type calcium channel antagonist	+	–	+
ω-CgTX MVIIC (0.1 μM)	N-, P-, Q-type calcium channel antagonist	+	not tested	+
ω-agatoxin IVA (100 nM)	P-, Q-type calcium channel antagonist	–	–	–

+: effective, –: not effective.

Discussion

The main finding of this study was that the twitch-like contractions and second slow NANC relaxation of the cat bronchioles evoked by EFS were completely abolished by the selective, irreversible N-type calcium channel inhibitor, ω-CgTX GVIA, whereas the initial fast NANC relaxation was resistant to ω-CgTX GVIA, ω-CgTX MVIIC (1 μM each), ω-agatoxin IVA (100 nM) and nimodipine (10 μM). There is almost ten times difference in the sensitivity to ω-CgTX GVIA of the twitch-like contractions (0.1 μM) and second component of the NANC relaxation (1 μM) evoked by EFS (see Figures 2 and 3). The different kinetic and biophysical prolifes of ω-CgTX GVIA-sensitive calcium channels in neurones may suggest the existence of more than one type of N-type calcium channel. This idea may be supported by the existence of kinetically distinct currents encoded by α_{1B} splice variants (Lin *et al*., 1997). Diversity could also arise from different combinations of α_{1B} with ancillary channel subunits including $\alpha 2\delta$ and β-subunits, which differently modify both biophysical and pharmacological properties of the channels (see for example Nooney *et al*., 1997 for review).

In the present experiments 0.1 μM ω-CgTX GVIA required 60–90 min to abolish the twitch like contraction of cat bronchiole. Similar studies, on the other hand, have shown that 1 nM ω-CgTX GVIA blocks nerve-mediated twitch contractions of rat vas deferens in approximately the same time course (Maggi *et al*., 1988). Furthermore in the rat anococcygeus muscle, 50 nM ω-CgTX GVIA progressively reduced the amplitude of EJPs, and abolished the generation after some 20–30 min. Thus, it seems that the concentration of ω-CgTX GVIA required to abolish the twitch like contraction and later NANC relaxation of the cat bronchiole is relatively high and the time course of the drug action is rather slow, comparing to the other smooth muscle tissues examined so far. At present, the precise reason for this discrepancy is not known. It may be due to the tissue specificity which may interfere with the diffusion of the toxin to the nerve terminal, or relatively high concentration of ω-CgTX GVIA might exert non-specific action on the neurotransmissions. However, considering the IC_{50} on the twitch-like contraction and later slow NANC relaxation (2.93 nM and 28.30 nM), it seems that the effects of CgTX GVIA on twitch-like contraction and later slow NANC relaxation observed in the present experiments is due to the drug action on N-type calcium channels.

The twitch-like contractions and excitatory junction potentials (EJPs) of the cat bronchioles evoked by repetitive EFS at high stimulus frequency (20 Hz) are sensitive to atropine (1 μM) and TTX (0.1 μM), indicating that the EJP and twitch-like contraction is due to the action of acetylcholine (ACh) released from nerve terminals on smooth muscle cells (Ito & Tajima, 1981). We have also shown that at least NO or NO-containing compounds and VIP are responsible for initial fast and second slow NANC relaxations in the cat bronchiole, respectively (Jing *et al*., 1995; Takahashi *et al*., 1995; Tanaka *et al*., 1996). Therefore, the present results indicate that release of ACh and VIP are mediated solely by calcium influx through N-type high voltage activated (HVA) calcium channels distributed on the vagus nerve terminal, whereas the release of NO or NO-containing compounds probably involves mainly R-type calcium channels. It is known that 50 nM ω-agatoxin IVA or 5 μM nimodipine are the saturating concentration for inhibition of P/Q-type or T-type calcium channels in cerebellar synapses (Doroshenko *et al*., 1997) or in undifferentiated NG 108–15 cells respectively (Randall & Tsien, 1997).

In the central nervous system (CNS), it has been shown that multiple calcium channels play a role in neurotransmitter release in addition to the N-type calcium channel (see Dunlop *et al*., 1995; Miller, 1998 for reviews). In contrast to the CNS, it was generally thought that N-type calcium channels mainly control neurotransmitter release in peripheral sympathetic and cholinergic neurones (Lundy & Frew, 1994; Mudumbi & Leighton, 1994). However in recent years it has become clear that other calcium channels play a fundamental role in the peripheral nervous system. For example, in postganglionic sympathetic nerve terminals innervating the guinea-pig vas deferens, there is a component of the action potential-evoked neurotransmitter release, termed 'residual release', which is insensitive to the selective N-type calcium channel antagonist ω-CgTX GVIA (Smith & Cunnane, 1996). The 'residual release' (residual EJPs) were abolished by α,β-methylene ATP (1 μM), which desensitizes P_{2x} receptors, and therefore the residual EJPs resulted from the actions of neuronally released ATP. Similarly, in postganglionic sympathetic nerve terminals of the rat isolated anococcygeus muscle, the presence of 'residual release' was also revealed in the presence of 1 μM ω-CgTX GVIA. The 'residual release' can be inhibited by ω-Aga IVA and abolished by ω-CgTX MVIIC, suggesting the possible involvement of P/Q type in addition to N-type-calcium channels (Smith & Cunnane, 1997). In the present experiments, repetitive EFS (≥30 stimuli) evoked small but distinct twitch like contractions which is sensitive to TTX, after the prolonged treatment with ω-CgTX GVIA or ω-CgTX MVIIC (0.1 μM). This observation might suggest that there is 'residual release' in the cat bronchiole. In the submucosal plexus of the guinea-pig caecum, furthermore, release of noradrenaline from extrinsic nerve terminals (sympathetic origin) is regulatd by N-type calcium channels, whereas release of ACh from intrinsic nerve terminals (enteric origin) involves several types of calcium channels (Cunningham *et al*., 1998). Therefore, it seems that N-type calcium channels are the dominant channel controlling transmitter release in peripheral nervous system, but additional calcium channels are recruited especially when the stimulation frequency is increased.

The present study provides evidence that multiple calcium channels mediate excitatory and inhibitory parasympathetic neuro-effector transmission in the cat bronchiole. We have previously reported that there are at least four different types of calcium channels (L-, N-, P/Q- and R-type calcium channels) in the cell bodies of rat paratracheal ganglion cells according to the sensitivity to various calcium channel antagonists (Murai *et al*., 1998). ω-CgTX GVIA blocked the largest HVA calcium current (nearly 60%), and saturating doses of nifedipine and ω-CgTX MVIIC blocked about 15 and 6% of the whole-cell calcium current, respectively. These experimental facts indicate that neurones isolated from rat paratracheal ganglia have various populations of calcium channels corresponding to L-, N-, P-, Q- and R-type calcium channels (Murai *et al*., 1998). Therefore it seems reasonable to assume that multiple calcium channels present in the neuron of cat paratracheal ganglion, and that these play multiple roles in the excitatory and inhibitory neuro-effector transmission.

The vagus nerve terminals release at least three different neurotransmitters, namely ACh, NO or NO-containing compounds and VIP in response to the excitation of the parasympathetic nervous system. The present results may indicate that these neurotransmitters are released from three different nerve terminals, where three different subtypes of calcium channels are predominantly distributed. However, by use of immunofluorescence techniques, VIP immunoreactivity

has been localized to cholinergic nerves in cat exocrine glands (Lundberg, 1981). Furthermore immunohistochemical examination revealed that VIP and NO synthase (NOS) may co-exist in the same nerve terminals in the ferret (Dey *et al*., 1993). Thus, it appears that excitatory (ACh) and inhibitory (NO and VIP) neurotransmitters may co-exist together within some nerve terminals and may be co-released (Jing *et al*., 1995; Takahashi *et al*., 1995). In these nerve terminals, it is yet to be explained how the influxed calcium through different HVA calcium channels could regulate the release of different neurotransmitters.

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References

AMIS, T.C. & MCKIERNAN, B.C. (1987). Systemic identification of endobronchial anatomy during bronchoscopy in the dog. *Am. J. Vet. Res.,* **47,** 2649–2657.

BARNES, P.J. (1991). Neuropeptides and asthma. *Am. Rev. Respir. Dis.,* **143,** S28–S32.

BELVISI, M.G., STRETTON, C.D., MIURA, M., VERLEDEN, G.M., TADJKARIMI, S., YACOUB, M.A. & BARNES, P.J. (1992). Inhibitory NANC nerves in human tracheal smooth muscle: a quest for the neurotransmitter. *J. Appl. Physiol.,* **73,** 2505–2510.

BIRNBAUMER, K.P., CAMPBELL, K.P., CATTERALL, W.A., HARPOLD, M.M., HOFMANN, F., HORNE, W.A., MORI, Y., SCHWARTZ, A., SNUTCH, T.P., TANABE, T. & TSIEN, K.W. (1994). The naming of voltage-gated calcium channels. *Neuron,* **13,** 505–506.

COBURN, R.F. & TOMITA, T. (1973). Evidence for nonadrenergic inhibitory nerve in the guinea-pig trachealis muscle. *Am. J. Physiol.,* **224,** 1072–1080.

CUNNINGHAM, S.M.C., MIHARA, S. & HIGASHI, H. (1998). Presynaptic calcium channels mediating synaptic transmission in submucosal neurones of the guinea-pig caecum. *J. Physiol.,* **509,** 425–435.

DEY, R.D., MAYER, B. & SAID, S.I. (1993). Colocalization of vasoactive intestinal peptide and nitric oxide synthase in neurons of the ferret trachea. *Neurosci.,* **54,** 839–843.

DOROSHENKO, P.A., WOPPMANN, A., MILJANICH, G. & AUGUSTINE, G.J. (1997). Pharmacologically distinct presynaptic calcium channels in cerebellar excitatory and inhibitory synapses. *Neuropharmacology,* **36,** 865–872.

DUNLOP, K., LUEBKE, J.I. & TURNER, T.J. (1995). Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci.,* **18,** 89–98.

HAKODA, H., XIE, Z.Q., AIZAWA, H., INOUE, H., HIRATA, M. & ITO, Y. (1991). Effects of immunization against VIP on neurotransmission in cat trachea. *Am. J. Physiol.,* **261,** L341–L348.

ITO, Y. & TAJIMA, K. (1981). Actions of indomethacin and prostaglandins on neuro-effector transmission in the dog trachea. *J. Physiol.,* **319,** 379–392.

ITO, Y. & TAKEDA, K. (1982). Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. *J. Physiol.,* **330,** 497–511.

JING, L., INOUE, R., TASHIRO, K., TAKAHASHI, S. & ITO, Y. (1995). Role of nitric oxide in inhibitory and modulation of excitatory neuroeffector transmission in cat airway. *J. Physiol.,* **481,** 225–239.

KATZ, B. (1969). The release of neural transmitter substance. Sherrington Lectures, NO. 10, Liverpool, Liverpool University Press.

LIN, Z.X., HAUS, S., EDGERTON, J. & LIPSCOMBE, D. (1997). Identification of functionally distinct isoforms of the N-type Ca^{2+} channel in rat sympathetic ganglia and brain. *Neuron,* **18,** 153–166.

LLINÁS, R., STEINBERB, I.Z. & WALTON, K. (1976). Presynaptic calcium currents and their relation to synaptic transmission: Voltage clamp study in squid giant synapse and theoretical model for the calcium gate. *Proc. Natl. Acad. Sci. U.S.A.,* **73,** 2918–2922.

LUNDBERG, J.M. (1981). Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional studies. *Acta Physiol. Scand.,* **496** (Suppl), 1–57.

LUNDY, P.M. & FREW, R. (1994). Effects of ω-agatoxin IVA on autonomic neurotransmission. *Eur. J. Pharmacol.,* **261,** 79–84.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., LIPPE, I.T.H., GIULIANI, S., GEPPETTI, P., DELBIANCO, E., SELLERI, S. & MELI, A. (1988). The effect of omega conotoxin GVIA, a peptide modulator of the N-type voltage sensitive calcium channels, on motor responses produced by activation of efferent and sensory nerves in mammalian smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **338,** 107–113.

MATSUZAKI, Y., HAMASAKI, Y. & SAID, S.I. (1980). Vasoactive intestinal peptide; a possible transmitter of nonadrenergic relaxation of guinea-pig trachea. *Science,* **210,** 1252–1253.

MATYAS, S. & BAUER, V. (1995). Involvement of epithelium and arachidonic acid metabolites in the mechanisms of action of reactive oxygen species (ROS) on the guinea-pig trachea. *Pharmacol. Res.,* **31** (Suppl), 221.

MILLER, R.J. (1998). Presynaptic receptors. *Annu. Rev. Pharmacol. Toxicol.,* **38,** 201–229.

MORTENSEN, J.D., YOUNG, J.D., STROUT, L., STROUT, A., BAGLEY, B. & SCHAAP, R.N. (1983). A numerical identification system for airways in the lung. *Anat. Rec.,* **206,** 103–114.

MUDUMBI, R.V. & LEIGHTON, H.J. (1994). Effects of ω-conotoxin GVIA on electrical field stimulation and agonist-induced changes in cytosolic Ca^{2+} and tension in isolated rat anococcygeus muscle. *J. Auton. Pharmacol.,* **14,** 253–265.

MURAI, Y., ISHIBASHI, H., AKAIKE, N. & ITO, Y. (1998). Acetylcholine modulation of high-voltage-activated calcium channels in the neurones acutely dissociated from rat paratracheal ganglia. *Br. J. Pharmacol.,* **123,** 1441–1449.

NOONEY, J.M., LAMBERT, R.C. & FELTZ, A. (1997). Identifying neuronal non-L Ca^{2+} channels – more than stamps collecting? *Trends Pharmacol. Sci.,* **18,** 363–371.

PALMER, J.B., CUSS, F.M. & BARNES, P.J. (1986). VIP and PMI and their role in nonadrenergic inhibitory responses in isolated human airways. *J. Appl. Physiol.,* **61,** 1322–1328.

RANDALL, A. & TSIEN, R.W. (1995). Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebrallar granule neurones. *J. Neurosci.,* **15,** 2995–3012.

RANDALL, A. & TSIEN, R.W. (1997). Contrasting biophysical and pharmacological properties of T-type and R-type calcium channels. *Neuropharmacol.,* **36,** 879–893.

SMITH, A.B. & CUNNANE, T.C. (1996). ω-conotoxin GVIA-resistant neurotransmitter release in postganglionic sympathetic nerve terminals. *Neuroscience,* **70,** 817–824.

SMITH, A.B. & CUNNANE, T.C. (1997). Multiple calcium channels control neurotransmitter release from rat postganglionic sympathetic nerve terminals. *J. Physiol.,* **499,** 341–349.

TAKAHASHI, N., TANAKA, H., ABDULLAH, N., JING, L., INOUE, R. & ITO, Y. (1995). Regional difference in the distribution of L-NAME-sensitive and -insensitive NANC relaxations in cat airway. *J. Physiol.,* **488,** 709–720.

TANAKA, H., JING, L., TAKAHASHI, S. & ITO, Y. (1996). The possible role of nitric oxide in relaxations and excitatory neuroeffector transmission in the cat airway. *J. Physiol.,* **493,** 785–791.

WHEELER, D.B., RANDALL, A.D. & TSIEN, R.W. (1994). Role of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science,* **264,** 107–111.

XIE, Z.Q., HAKODA, H. & ITO, Y. (1992). Airway epithelial cells regulate membrane potential, neurotransmission and muscle tone of the dog airway smooth muscle. *J. Physiol.,* **449,** 619–639.

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