Influence of Ouabain on the Cholinergic Neurotransmission in the Canine Trachea

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Abstract—The effect of ouabain on the cholinergic neurotransmission of the trachea was investigated using isolated tracheal strips in dogs. Tracheal strips without epithelium were suspended in organ chambers filled with modified Krebs-Henseleit solution. Ouabain (3×10^{-7}–10^{-5} M) concentration-dependently caused a slow sustained tracheal contraction. The contractile response was significantly inhibited by 10^{-6} M atropine and was enhanced by 10^{-8} M physostigmine. The ouabain-induced tracheal contraction was unaffected by 10^{-7} M tetrodotoxin, but was significantly reduced by 10^{-3} M hemicholinium-3. In superfusion experiments, ouabain (10^{-5} M) produced an increase in the ACh release. Superfusion with Ca^{2+}-free solution almost eradicated the ACh release and abolished the tracheal contraction induced by ouabain. ω-Conotoxin (5×10^{-8} M), but not nicardipine (10^{-6} M), inhibited significantly the increase in ACh release induced by ouabain. These results suggest that the ouabain-induced tracheal contraction may be mainly due to acceleration of presynaptic ACh release by enhancing the influx of Ca^{2+}, and the Ca^{2+} entry may occur through the N-type Ca^{2+} channels in the canine airway presynaptic site.

Ouabain has been shown to inhibit Na^{+},K^{+}-ATPase activity and decrease the concentration gradient of Na^{+} and K^{+} across the smooth muscle cell membrane. Inhibition of the Na^{+} electrogenic pump has been shown to cause contraction of respiratory tract smooth muscle in dogs in vivo (1), guinea pigs in vitro (2) and humans in vitro (3). Although it is known that a Na^{+} electrogenic pump is present in the smooth muscle of guinea pig and bovine airways (4), whether the Na^{+} electrogenic pump is present in the canine trachea is not clear.

In the vascular smooth muscle of many species (5, 6) and rat kidney (7), ouabain causes an increase in the spontaneous release of norepinephrine. Ouabain stimulates the release of endogenous dopamine from rat hypothalamic tuberoinfundibular dopaminergic neurons (8). In contrast, removal of external K^{+} enhances both the resting and stimulated release of acetylcholine (ACh) from Auerbach's plexus of the guinea pig ileum (9). In the airway system, the influence of ouabain on the release of ACh is not clear. In a preliminary in vivo experiment using dogs, we confirmed that the tracheal contractile action of ouabain seems to be mediated by the prejunctional mechanism via ACh release, because the action is significantly inhibited by atropine. In the present study, we investigated the effect of ouabain on the cholinergic neurotransmission in the canine trachea.

Materials and Methods
Experimental procedures: Male mongrel dogs weighing between 10 and 18 kg were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and killed by exsanguination. The cervical trachea was rapidly removed and placed in oxygenated modified Krebs-Henseleit solution with the following composition: 117.6 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl_2, 0.57 mM MgSO_4, 1.03 mM
KH₂PO₄, 25.0 mM NaHCO₃ and 11.1 mM glucose. The solution always contained 20 μM choline chloride as a substrate for acetylcholine biosynthesis, 2 μM indomethacin as an inhibitor of cyclooxygenase for prostaglandin biosynthesis and 10 μM guanethidine as an adrenergic neuron blocker. It was then cleaned of extraneous tissue, cut longitudinally through the cartilage to form a flat sheet and transverse cuts made to form a strip of tissue.

Rectangular strips of the tracheal smooth muscle approximately 6-mm long and 3-mm wide were dissected from the trachea after removal of the epithelium. The tracheal strips were suspended in a 20-ml organ bath containing modified Krebs-Henseleit solution maintained at 37°C and gassed with 95% O₂-5% CO₂. The resting load was adjusted to the optimal level of 2 g. The tissues were equilibrated under the resting load for 90 min. Changes in developed tension were isometrically measured with a force-displacement transducer (Nihon Kohden, TB-611T) and recorded on a polygraph (Nihon Kohden, RM-6000) or a desk-top pen recorder (Yokogawa, 3021).

In some experiments, two platinum-ring electrodes were placed parallel to the tracheal strips for electrical field stimulation (ES). Electrical impulses (10 V, 0.5 msec, 10 Hz and 60 pulses) were delivered by a stimulator (Nihon Kohden, SEN-3301). Ca⁺⁺-free (containing 120.1 mM NaCl, 5.4 mM KCl, 0.57 mM MgSO₄, 1.03 mM NaH₂PO₄, 25.0 mM NaHCO₃ and 11.1 mM glucose) Krebs-Henseleit solution was substituted for the Krebs-Henseleit solution.

Procedures for examination of ACh release: To investigate the release of ACh by ouabain, the tracheal preparation was superfused at the rate of 0.4 ml/min with modified Krebs-Henseleit solution. All perfusates used in the superfusion study contained 10 mM methanesulfonyl fluoride, a cholinesterase inhibitor (10). After 60 min presuperfusion, fractions of perfusate were collected every 10 min (1.2 ml) before and after the start of the ouabain infusion and determined for ACh content by a radioimmunoassay (RIA). At the end of the experiment, all tissues used were weighted.

Procedures for RIA: RIA for ACh content in the superfusate was done using the procedure described by Kawashima et al. (11). A 400-μl portion of the collected superfusate was incubated overnight with 50 μl of the diluted antiserum in 0.15 M Tris-HCl buffer (pH 7.4) containing 0.3% bovine gamma-globulin, 0.05% isoflurophate and 50 μl of tritiated ACh at 4°C. The same volume of superfusion medium served as a blank. Antibody-bound tritiated ACh was separated from the free tritiated ACh by the ammonium sulfate method, and the radioactivity of the precipitates was quantitated in a liquid scintillation counter.

Drugs: The following drugs were used: ouabain octahydrate (Sigma), atropine sulfate (Sigma), physostigmine (eserine, Sigma), acetylcholine (Ovisot, Daiichi), tetrodotoxin (Sigma), hemicholinium-3 (Sigma), indomethacin (Sigma), choline chloride (Sigma), guanethidine sulfate (Tokyo Kasei), nicardipine (Taisho), α-conotoxin (Peptide Institute) and acetyl[methyl-³H]choline chloride (86 Ci/mmol, Amersham). Anti-ACh antiserum was kindly given to us by Prof. K. Kawashima. All drug concentrations were expressed as final bath concentrations.

Statistical analysis: The results shown in the figures are expressed as mean values±S.E. Multiple comparisons were performed using Scheffe’s test. The differences were considered significant at P<0.05.

Results

Typical recordings of the changes in tracheal tension by the treatment with ouabain are shown in Fig. 1. Ouabain had no effect on the resting tension of the tracheal smooth muscle at concentrations up to 10⁻⁷ M. At a concentration range from 3×10⁻⁷ to 10⁻⁵ M, ouabain induced, after a latency period, a slowly sustained contraction in a concentration-dependent manner (Fig. 1). Ouabain at the concentration of 10⁻⁵ M showed a trend of two phase contractions, but not in all preparations. The sustained contraction reached a peak within 60 min after the treatment of ouabain. Atropine (10⁻⁶ M) added after 60 min caused a rapid relaxation of the tracheal smooth muscle contracted by ouabain (Figs. 1 and 2).

Pretreatment of the tracheal muscle with
Fig. 1. Representative recordings of the response to varying concentrations of ouabain in the canine tracheal muscle. Treatment with ouabain induced, after a latency period, a slow, sustained contraction. Atropine was applied 60 min after ouabain.

Fig. 2. Effects of atropine on the tracheal contraction induced by ouabain. Atropine (10⁻⁶ M) was applied 60 min after ouabain. Each column shows the mean value with S.E. of results from five to six preparations. The changes are significant at *P<0.05 and **P<0.001 when compared to the value for the ouabain control (□□□□□).
10⁻⁶ M atropine inhibited significantly the tracheal contraction induced by 10⁻⁵ M ouabain. In contrast to atropine, 10⁻⁸ M physostigmine enhanced significantly the ouabain-induced tracheal contraction (Fig. 3). The biphasic responses observed by 10⁻⁵ M ouabain converted into monophasic responses after atropine and physostigmine, respectively.

Exogenously applied acetylcholine (10⁻⁶ M) induced a submaximal contraction of the trachea, which was equipotent in amplitude with the 10⁻⁵ M ouabain-induced contraction. Tetrodotoxin (TTX, 10⁻⁷ M) had no effect on the tracheal contractions induced by 10⁻⁵ M ouabain and exogenous 10⁻⁶ M acetylcholine, respectively. In contrast, the ES-induced tracheal contraction was completely blocked by the pretreatment with 10⁻⁷ M TTX (Fig. 4).

To deplete ACh in the cholinergic terminal, we treated the preparation with hemicholinium-3 (HC-3), an agent that inhibits the presynaptic neuronal uptake of choline (12–14). After incubation (1–1.5 hr) with HC-3, the tissues were repeatedly stimulated by prolonged ES with 10 Hz, which resulted in a progressive diminution of the contractile response to the ES as ACh stores became progressively depleted (data not shown). When no contractile response was further elicited with ES after HC-3, indicating marked depletion of intrinsic ACh stores, treatment with ouabain was initiated. At the concentration of 10⁻³ M, HC-3 completely abolished the ES-induced tracheal contraction, while the contraction to exogenous ACh was unaffected by the pretreatment with HC-3. The tracheal contraction induced by 10⁻⁵ M ouabain was significantly inhibited by the pretreatment with HC-3 (10⁻³ M), but was not completely abolished (Fig. 5). The two-phase responses observed by 10⁻⁸ M ouabain disappeared after HC-3.

Under the present experimental conditions, the amounts of spontaneous ACh release from the tracheal strips were approximately 0.9 pg/mg tissue/min (n=5) at the pre-control levels. At the concentration of 10⁻⁵ M, ouabain produced a tracheal contraction and an increase in the release of ACh from the tracheal strips in a similar time course (Fig. 6). Superfusion with Ca²⁺-free Krebs-Henseleit solution decreased spontaneous release of ACh, and it almost eradicated the ACh release induced by ouabain. Contractile re-

![Graph](image)

Fig. 3. Effects of atropine and physostigmine on the tracheal contraction induced by ouabain. Atropine and physostigmine were applied 20 min before treatment of ouabain. Each column shows the mean value with S.E. of results from six preparations. The changes are significant at **P<0.01 and ***P<0.001 when compared to the value for the ouabain control.
Fig. 4. Effects of tetrodotoxin on the tracheal contraction induced by acetylcholine (ACh), electrical field stimulation (E.S.) and ouabain. Tetrodotoxin ($10^{-7}$ M, $\square\square\square$) was applied 20 min before. The E.S.-induced tracheal contraction was abolished by tetrodotoxin. Each column shows the mean value with S.E. of the results from six preparations.

Fig. 5. Effects of hemicholinium-3 on the tracheal contraction induced by acetylcholine (ACh), electrical field stimulation (E.S.) and ouabain. Hemicholinium-3 ($10^{-3}$ M, $\square\square\square\square$) was pretreated during a period of 1–1.5 hr. The E.S.-induced tracheal contraction was abolished by hemicholinium-3. Each column shows the mean value with S.E. of results from five to six preparations. The changes are significant at ***$P<0.001$ when compared to the value for the ouabain control ($\square\square\square\square$).
sponse to ouabain disappeared upon superfusion with Ca++-free Krebs-Henseleit solution (Fig. 6).

**Fig. 6.** Effects of ouabain on the tracheal response (upper panel) and spontaneous acetylcholine (ACh) release (lower panel) from tracheal strips. Ouabain (---) was superfused at the concentration of 10^{-5} M. Superfusion with Ca++-free Krebs-Henseleit solution (----) decreased spontaneous release of ACh, and it almost eradicated the ACh release induced by ouabain. Contractile response to ouabain disappeared upon superfusion with Ca++-free Krebs-Henseleit solution (-----). Each point and column show the mean value with S.E. of results from five to six preparations. The changes are significant at *P<0.05, **P<0.01 and ***P<0.001 when compared to the value for the control (- - -).}

**Fig. 7.** Effects of nicardipine and \(\omega\)-conotoxin on the acetylcholine (ACh) release induced by ouabain. Nicardipine (10^{-6} M, [ ] ) and \(\omega\)-conotoxin (5 \times 10^{-8} M, [ ] ) were applied 30 min before infusion of ouabain. Each column shows the mean value with S.E. of results from five to six preparations. The changes are significant at *P<0.05 and **P<0.01 when compared to the value for the ouabain control ( [ ] ).
was reduced by nicardipine (10^{-6} M), and it was significantly inhibited by \(\omega\)-conotoxin (5\times10^{-8} M) (data not shown). \(\omega\)-Conotoxin (5\times10^{-8} M), but not nicardipine (10^{-6} M), significantly inhibited the release of ACh induced by ouabain (Fig. 7).

**Discussion**

The level of Na\(^{+}\),K\(^{+}\)-pump activity could play a role in determining the degree of bronchomotor tone (3). It has been demonstrated that digoxin and ouabain cause contractions of the canine bronchial (1) and tracheal (3) smooth muscle, respectively. The contractile responses have been explained by a direct effect of digitalis on the airway smooth muscle. In the present study, the tracheal contraction induced by ouabain was significantly inhibited by atropine, but not completely blocked. The remaining contraction after treatment of atropine may be due to a direct action on the tracheal smooth muscle. In contrast, the ouabain-induced tracheal contraction was enhanced by physostigmine. These findings suggest that the ouabain-induced tracheal contraction seems to be mediated by endogenous ACh.

It has been reported that ouabain causes an increase in the spontaneous release of norepinephrine in the vasculature and kidney (5-7), although this is not clear in the airway. In the present experiment, guanethidine was always added to Krebs-Henseleit solution as an adrenergic neuron blocker. The airway smooth muscle is thought to have mainly \(\beta\)-receptors, and the \(\alpha\)-receptors that subserve tracheal contraction can be revealed by a blockade of \(\beta\)-receptors by a \(\beta\)-blocker. Therefore, it appears that contraction mediated by \(\alpha\)-receptors is not involved in the present study.

TTX had no effect on the ouabain-induced tracheal contraction, indicating that the site of action of ouabain may be presynapses of the vagus nerves and, at least, a preganglionic site is not involved. To evaluate whether the contractile response to ouabain in the tracheal smooth muscle is related to presynaptic neuromodulation of ACh release, we investigated the effect of ouabain on the tracheal smooth muscle that was pretreated with HC-3. HC-3 inhibited significantly the ouabain-induced tracheal contraction, but did not completely block it. The remaining contraction may be due to a direct action on the tracheal smooth muscle. These findings suggest that the contractile response of the trachea to ouabain may be mainly associated with an enhanced release of ACh from presynapses of vagus nerves. In fact, this notion was substantiated by the finding that ouabain produced an increase in the ACh contents in the superfusate.

Inhibition of Na\(^{+}\),K\(^{+}\)-ATPase with agents such as cardiac glycosides promotes the release of catecholamines in a variety of tissues (15-19). However, the mechanism by which Na\(^{+}\),K\(^{+}\)-ATPase inhibition stimulates the release of norepinephrine is poorly understood. It has been reported that the increase in intracellular Na\(^{+}\) consequent to inhibition of Na\(^{+}\),K\(^{+}\)-ATPase brings about an increase in cytoplasmic free Ca\(^{++}\) as a result of either 1) depolarization due to loss of intracellular K\(^{+}\) leading to Ca\(^{++}\) influx (15), 2) Na\(^{+}\)-dependent Ca\(^{++}\) entry due to gain in intracellular Na\(^{+}\) (20), 3) Na\(^{+}\)-activated Ca\(^{++}\) release from intracellular stores (21) or 4) an interference with Ca\(^{++}\) efflux (22). However, there have been no investigations into the mechanism of ACh release by ouabain in the airway.

Sweadner (23) reported that the ouabain-induced norepinephrine release is independent of extracellular Ca\(^{++}\). In contrast, it has been suggested that ouabain in the presence of low K\(^{+}\) promotes the release of norepinephrine by enhancing the influx of Ca\(^{++}\) (7). In the present study, Ca\(^{++}\)-free solution almost eradicated the ouabain-induced ACh release, indicating that ACh release elicited by ouabain is dependent on extracellular Ca\(^{++}\).

Three types of voltage-dependent Ca\(^{++}\) channels, the L-, T- and N-types, characterized by differences in their conductance and drug sensitivity have been revealed in the neurons of the rat superior cervical ganglion (24). \(\omega\)-Conotoxin (25) blocked N-type Ca\(^{++}\) channels, whereas nicardipine blocked L-type Ca\(^{++}\) channels (24, 25). An important finding in the present study was that \(\omega\)-conotoxin, but not nicardipine, significantly inhibited the increase in ACh release induced by ouabain, suggesting that ouabain may...
enhance the influx of Ca\(^{++}\) through the N-type Ca\(^{++}\) channels.

We conclude that the tracheal contraction induced by ouabain may be mainly due to acceleration of presynaptic release of ACh by enhancing the influx of Ca\(^{++}\), and the Ca\(^{++}\) entry may occur through the N-type Ca\(^{++}\) channels in the canine airway presynaptic site.

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