Mechanism of Relaxant Effect of Clonidine in Isolated Bovine Tracheal Smooth Muscle

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ABSTRACT

The relaxant effect of clonidine and the possible involvement of imidazoline I₁ receptors in bovine tracheal smooth muscle (BTSM) were examined. Clonidine caused concentration-dependent significant relaxation in BTSM precontracted with 0.1 or 1 μM carbachol (CCh) but not in 72.7 mM KCl-induced contraction. The relaxation in CCh-contracted BTSM was inhibited by yohimbine (1 μM) and idazoxan (10 and 30 μM) but not by tetrodotoxin, indomethacin and other adrenoceptor antagonists. Oxymetazoline (0.1–100 μM) and phenotolamine (0.1–100 μM) caused concentration-dependent relaxation, which was attenuated by idazoxan (10 μM). Norepinephrine (0.1–100 μM) produced concentration-dependent relaxation, which was completely abolished by propranolol (10 μM) but not by yohimbine (1 μM). In fura-PE3/AM-loaded BTSM, CCh and 72.7 mM KCl increased intracellular calcium concentration ([Ca²⁺]i) followed by contraction. The high K⁺-induced increase in [Ca²⁺]i was not affected by clonidine. In CCh-stimulated BTSM, clonidine decreased [Ca²⁺]i and muscle force in parallel, whereas verapamil decreased [Ca²⁺]i more strongly than muscle force. Clonidine (100 μM) inhibited the transient increase in [Ca²⁺]i, induced by CCh but not by caffeine (20 mM) in Ca²⁺-free solution. Clonidine did not change the cAMP content in the presence of either 72.7 mM KCl or CCh. These results indicate that clonidine relaxes CCh-stimulated BTSM through the inhibition of CCh-induced increases in Ca²⁺-influx, Ca²⁺-release and intracellular signal transduction probably via imidazoline I₁ receptors.

Clonidine is a centrally acting A2R agonist that is used in clinical practice for its antihypertensive, sedative and analgesic effects. In the lower brain stem region, clonidine suppresses sympathetic outflow, resulting in a decrease in blood pressure when administered i.v. whereas clonidine administered i.v. causes acute hypertension because of systemic vascular constriction mediated by postsynaptic A2R activation (Kobinger, 1978).

In the airways, clonidine induces contraction in a canine in situ model (Leff and Munoz, 1981). Furthermore, clonidine enhances agonist-induced bronchoconstriction in conscious (Advenier et al., 1983) and anesthetized (Macquin-Mavier et al., 1988) guinea pigs as well as in conscious humans (Dinh Xuan et al., 1988). Furthermore, histamine-induced contraction was augmented by clonidine in isolated guinea pig trachea (Floch and Advenier, 1985). In contrast to these findings, clonidine has been demonstrated to attenuate the contraction evoked by electrical stimulation in isolated smooth muscles of guinea pig (Wikberg et al., 1982), canine (Tsuchiya et al., 1990) and equine (LeBlanc et al., 1993; Yu et al., 1993) airways. Moreover, in vivo studies have revealed antitussive and asthma prophylactic effects of clonidine in vagal-stimulated (Olsson and Ekdahl, 1985; Anderson et al., 1986) and citric acid-challenged guinea pigs (O’Connell et al., 1994). These complicated and, in part, contradictory results have caused difficulty in evaluating the therapeutic efficacy and safety of clonidine in asthmatic patients.

Although there have been a number of clinical trials investigating clonidine’s effects in patients with asthma (reviewed by Dinh Xuan and Lockhart, 1989), several factors, including dosing methods, made the results conflicting. In particular, it is noteworthy that, unlike oral or transdermal administration, only inhaled clonidine was reported to be beneficial to asthmatic patients (Lindgren et al., 1986). Therefore, clonidine is expected to have several direct peripheral antiasthmatic effects in the airways.

The present study was designed to examine the mechanisms of direct action of clonidine in bovine trachealis smooth muscle.
scope). The absolute Ca(2+) were measured using a fluorimeter (CAF-110, Japan Spectro-scoby 340-nm excitation (F340) and that induced by 380-nm excitation ratio for 40 to 60 min, high-K+(K+) obtained in vitro alternately (48 Hz) at excitation wavelengths (340-60 nm) and the amount of 500 ± 20-nm fluorescence induced by 340-nm excitation (F340) and that induced by 380-nm excitation (F380) were measured using a fluorimeter (CAP-110, Japan Spectroscopic). The absolute Ca(2+) concentration was not calculated in the present experiment because the dissociation constant (Kd) of the fluorescent indicator for Ca(2+) in cytosol may be different from that obtained in vitro (Karaki, 1989). After equilibration of the force and ratio for 40 to 60 min, high-K+(K+) solution (72.7 mM) was repeatedly applied until the response became stable. The ratios obtained in high-K+(K+) muscle strips were washed with PSS in a tissue bath at 37°C for more than 4 hr at room temperature. After the fura-PE3/AM-load-ing, muscle strips were washed with PSS maintained at 37°C.

Measurement of mechanical activity. Muscle contraction was recorded isometrically. One end of each muscle was attached by cotton thread to a force displacement transducer (Orientec, Tokyo, Japan), and the other end was tied to a glass holder situated parallel to the tissues with cotton thread under a resting force of 5 mN in a 20-ml tissue bath. After equilibration for 40 to 60 min until passive tension stabilized, high-K+(K+) solution (72.7 mM) was repeatedly applied.

Measurement of acetoxymethyl ester of fura-PE3 (furaPE3/AM) fluorescence. [Ca(2+)i] was measured simultaneously with muscle contraction as reported previously (Ozaki et al., 1987; Sato et al., 1988). Fura-PE3/AM was added to PSS to make a final concentration of 5 mM, together with noncytotoxic detergent, 0.02% cremophor EL. Muscle strips were loaded with fura-PE3/AM for concentration of 5 mM, together with noncytotoxic detergent, 0.02% cremophor EL. Muscle strips were loaded with fura-PE3/AM for more than 4 hr at room temperature. After the fura-PE3/AM-loading, muscle strips were washed with PSS in a tissue bath at 37°C for 30 to 40 min to remove the uncleaved fura-PE3/AM. One end of the muscle strip was connected to a force displacement transducer to monitor the mechanical activity. The muscle strips were illuminated alternately (48 Hz) at excitation wavelengths (340 ± 10 nm and 380 ± 10 nm), and the amount of 500 ± 20-nm fluorescence induced by 340-nm excitation (F340) and that induced by 380-nm excitation (F380) were measured using a fluorimeter (CAP-110, Japan Spectroscopic). The absolute Ca(2+) concentration was not calculated in the present experiment because the dissociation constant (Kd) of the fluorescent indicator for Ca(2+) in cytosol may be different from that obtained in vitro (Karaki, 1989). After equilibration of the force and ratio for 40 to 60 min, high-K+(K+) solution (72.7 mM) was repeatedly applied until the response became stable. The ratios obtained in resting state and in high-K+(K+) stimulation were taken as 0% and 100%, respectively. In these preparations, approximately 150 min was allowed for measurement because of the leakage of fura-PE3/AM. Therefore, application trials of the antagonists were limited to three times at most in order to obtain reliable data. For the same reason, the effects of drugs in Ca(2+)-free solution were observed immediately (approximately 1 min) after the ratio returned to base line by exchanging the high-K+(K+) solution for the Ca(2+)-free solution.

Measurement of adenosine 3’;5’-cyclic monophosphate (cAMP) content. After preincubation in PSS aerated with 95% O2-5% CO2 for 3 hr without resting tension, muscle strips were exposed to each drug for 5 min, frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation, trichloroacetic acid in the supernatant was removed by washing with water-saturated ether. cAMP was assayed by a competitive enzyme immunoassay with cAMP peroxidase conjugate.

Solutions and drugs. PSS contained (mM): NaCl 136.9, KCl 5.4, CaCl2 1.5, MgCl2 1.0, NaHCO3 23.8 and glucose 5.5. EDTA (0.01 mM) was added to chelate heavy metal ions contaminating the PSS. High-K+(K+) solution was made by substituting equimolar KCl for NaCl. Ca(2+)-free solution was made by removing CaCl2 from PSS and adding 0.5 mM EGTA. These solutions were aerated with 95% O2-5% CO2 mixture at 37°C and pH 7.4.

The following drugs, chemicals and apparatus were used: muscle (BTSM) contracted with muscarinic stimulation and to support its possible therapeutic use in patients with asthma.

Materials and Methods

Tissue preparation. Freshly excised bovine tracheae were obtained from a local abattoir. After cutting free of the cartilage rings, epithelium, fat and connective tissues were carefully removed from the smooth muscle tissues in PSS under a microscope. The smooth muscles were cut into small strips and suspended in PSS maintained at 37°C.

Fig. 1. Concentration-response curves of bovine tracheal strips precontracted with 0.1 μM CCh (○), 1 μM CCh (●) and 72.7 mM KCl (□) to clonidine. Each point represents the mean ± S.E.M. Asterisks indicate significant difference (* P < .05, ** P < .01) from control.

Fig. 2. Effects of 1 μM yohimbine (panel A, □), 10 μM idazoxan (B, ●) and 30 μM idazoxan (panel B, △) on the concentration-response curves of bovine tracheal strips precontracted with 0.1 μM CCh to clonidine compared with control data (● in both). Each point represents the mean ± S.E.M. IC50 values increase from 16.5 ± 2.1 μM to 42.8 ± 1.8 μM, 39.6 ± 1.3 μM and 52.2 ± 2.0 μM, respectively. Asterisks indicate significant difference from control curve († P < .05, †† P < .01) and between the two idazoxan groups († P < .05, †† P < .01).
Therefore, a combination of high K+ exposure for 15 min and interval for 15 min was repeated three times before the drug applications.

CCh at concentrations of 0.1 and 1 μM produced sustained contraction amounting to 111 ± 7.9% and 159 ± 11.4% of high K+ (72.7 mM)-induced contraction, respectively. Although no response was observed when it was administered alone, clonidine (0.1–100 μM) exhibited a concentration-dependent suppression of the force induced by 0.1 and 1 μM CCh, with IC50 values of 17.1 ± 2.0 μM and 100 ± 5.1 μM, respectively (n = 4 each). On the other hand, the relaxant effect of clonidine on high K+ (72.7 mM)-induced contraction was much weaker (fig. 1).

The A2R antagonists yohimbine (1 μM) and idazoxan (10 and 30 μM) shifted the concentration-response curve for clonidine to the right in parallel (fig. 2; n = 4 each). The dissociation constants (Kd) of these two antagonists were determined as 660 nM and 7,600–15,000 nM, respectively, from the relationship

\[
\log([D]/[D_o] - 1) = \log[I] - \log[K_d]
\]

where \([D]\) is the IC50 of clonidine in the presence of yohimbine or idazoxan, \([D_o]\) is the IC50 of clonidine alone and \([I]\) is the concentration of yohimbine or idazoxan. In contrast, clonidine-induced relaxation was not affected by an alpha-1a-adrenoceptor antagonist, WB 4101 (1 μM, n = 4), by an alpha-1 adrenoceptor antagonist, prazosin (0.01 μM, n = 5), by a nonselective beta-adrenoceptor antagonist, propranolol (20 μM, n = 5) or by a beta-2 adrenoceptor antagonist, butoxamine (1 μM, n = 4). A Na+ channel blocker, TTX (1 μM), and a cyclooxygenase inhibitor, indomethacin (10 μM), had no effect on clonidine-induced relaxation, either (n = 5 each).

The imidazoline agonists oxymetazoline (0.1–100 μM) and phentolamine (0.1–100 μM), also showed concentration-dependent relaxation in CCh (0.1 μM)-contracted BTSM, with IC50 values of 7.98 ± 2.1 μM and 37.8 ± 7.5 μM, respectively. Additionally, both of these relaxant effects were inhibited by idazoxan (10 μM), with IC50 values of 16.0 ± 3.4 μM and

Statistical analysis. The results of the experiments are expressed as mean ± S.E.M. Student’s t test or analysis of variance (ANOVA, when comparison involved more than two groups) was used for statistical analysis of the data. A P value less than .05 was considered significant.

**Results**

**Changes in muscle force.** High-K+ (72.7 mM) solution induced sustained contraction that reached plateau within 10 min in each exposure, and tension obtained in the third exposure trial that showed no significant difference from that in the fourth or later trials was adopted as the control value.
724.4 ± 80.9 μM, respectively, but not by yohimbine (1 μM, data not shown) (fig. 3, n = 4 – 5).

Norepinephrine (0.1–100 μM) exhibited a concentration-dependent relaxation in CCh (0.1 μM)-contracted BTSM, with IC50 of 6.6 ± 1.9 μM, which was completely attenuated by propranolol (10 μM) but not by yohimbine (1 μM) (fig. 4; n = 5 each).

Changes in [Ca2+]i and muscle force. In fura-PE3/AM-loaded trachea, high K+ (72.7 mM) induced sustained increases in [Ca2+]i and muscle force (n = 4 – 5). The control value of the ratio was determined approximately 10 min after high K+ application in the third trial in each strip, when the trace reached plateau. The center of the trace widths was adopted as the point of measurement. In KCl-stimulated tracheae, clonidine induced only slight relaxation without changing [Ca2+]i, (fig. 5A). CCh also induced sustained increases in [Ca2+]i and contraction. In CCh-stimulated tracheae, clonidine (10–100 μM) induced concentration-dependent decreases in [Ca2+]i and force (fig. 5B). Atropine (0.1–1.0 μM) also reduced both [Ca2+]i and force (fig. 5C). On the other hand, verapamil (0.01–0.1 μM) more strongly inhibited [Ca2+]i than force in the CCh-stimulated tracheae; although 1.0 μM verapamil almost completely inhibited CCh-stimulated [Ca2+]i, it inhibited CCh-induced contraction only by approximately 50% (fig. 5D).

Figure 6 summarizes the effects of clonidine, atropine and verapamil on the [Ca2+]i-force relationship in the presence of 0.1 μM CCh. CCh induced greater contraction than high K+ (72.7 mM) at a given [Ca2+]i. In the verapamil-induced relaxation, muscle force decreased less steeply than in the clonidine- or atropine-induced relaxation.

In Ca2+-free solution (with 0.5 mM EGTA), CCh (0.1 μM) induced a spike-like transient increase in [Ca2+]i (104.0 ± 11.2% of high K+-induced response, n = 5). Clonidine (100 μM) diminished the transient [Ca2+]i, rise elicited by CCh (0.1 μM) to 45.3 ± 17.5% (fig. 7, A and C). Caffeine (20 mM) also induced the transient increase in [Ca2+]i, in Ca2+-free solution (100.0 ± 9.0%, n = 4). Clonidine (100 μM) had no significant effect on the caffeine-induced Ca2+ transient (92 ± 18.5%, n = 4, fig. 7, B and C).

Discussion

In the bovine trachea, clonidine produced only slight suppression of high K+-induced increases in contraction and did not affect [Ca2+]i. In contrast, clonidine relaxed CCh-induced contraction in association with a decrease in [Ca2+]i. The effects of clonidine were not affected by α-1 or β-adrenoceptor antagonists, such as WB 4101, prazosin, propranolol and butoxamine, but were inhibited by yohimbine and idazoxan. TTX and indomethacin also had no effect on the clonidine-induced relaxation. These results suggest that clonidine directly inhibits smooth muscle contraction through A2Rs and/or I1Rs in bovine trachea without any influence of cyclooxygenase-related metabolites. In dogs (Barnes et al., 1983) and in guinea pigs (Floch and Advenier, 1985).
1985; Takayanagi et al., 1990), clonidine has been reported to contract isolated airway smooth muscle. By contrast, clonidine did not produce any direct contraction in BTSM, as shown in the present study. On the other hand, that the release of ACh from airways cholinergic nerves is reduced by A2R stimulation has been demonstrated in vitro in horses (Yu et al., 1993) and in guinea pigs (Baker et al., 1994). The variation in these results may be attributable to the difference in species. However, it has been reported that preapplied clonidine (10 and 100 μM) failed to reduce BTSM contraction induced by exogenous ACh (1 mM) (Manning and Brodstone, 1995). Their data seem to conflict with our results. The discrepancy may be caused by differences in the types and concentrations of muscarinic agonists. Potency order in these two agonists, ACh and CCh, varies with species and/or tissue type, which has been suggested to depend on the cholinesterase activity (Mitsui-Saito and Karaki, 1996). ACh and CCh have also been suggested to interact with different muscarinic receptors (Mitchelson and Ziegler, 1984). Furthermore, differences in tissue preparation may contribute to such a discrepancy. Epithelium and mucosa were left intact in their preparation but were removed in ours. Although the effects of clonidine on bovine airway epithelial function are unclear, several reports have indicated that agonist-stimulated epithelial cells modulate airway smooth muscle tone in various species (Farmer and Hay, 1991).

Clonidine stimulates not only A2Rs but also the I1Rs. The imidazoline receptors have been identified in several kinds of smooth muscle cells (Yablonsky and Dausse, 1991; Regunathan et al., 1995) and have been suggested to contribute to regulation of muscle tone. In the present study, the relaxant effects of clonidine were inhibited by either yohimbine (1 μM) or idazoxan (10 and 30 μM). However, the Ki for A2Rs of yohimbine and idazoxan have been reported to be 0.1–12 nM (Blaxall et al., 1994; Chruscinski et al., 1992) and 13–83 nM (Burke et al., 1995), respectively, both of which are approxi-
It has been reported that CCh augmented Ca\textsuperscript{++} sensitivity of the contractile element in the canine trachea (Ozaki et al., 1990). In the present study, we also revealed in the bovine trachea that CCh produced greater contraction than high K\textsuperscript{+} at a given [Ca\textsuperscript{++}], which suggests that CCh increased the Ca\textsuperscript{++} sensitivity of the contractile element. In Ca\textsuperscript{++}-free solution, 0.1 \textmu M CCh induced a transient increase in [Ca\textsuperscript{++}], that may be due to production of inositol-1,4,5-trisphosphate mediated by a muscarinic stimulation (Blüm et al., 1994). An activator of Ca\textsuperscript{++}-induced Ca\textsuperscript{++} release, 20 mM caffeine (Iino, 1990), also transiently increased [Ca\textsuperscript{++}], in Ca\textsuperscript{++}-free solution. Clonidine, at the concentration needed to inhibit the 0.1 \textmu M CCh-induced sustained increase in [Ca\textsuperscript{++}], and sustained contraction, inhibited significantly the CCh-induced transient increase in [Ca\textsuperscript{++}], in Ca\textsuperscript{++}-free solution, but not the effect of caffeine. These results suggest that clonidine inhibits the Ca\textsuperscript{++} release induced by inositol-1,4,5-trisphosphate but not the Ca\textsuperscript{++}-induced release of Ca\textsuperscript{++}. However, the effect of clonidine against the CCh stimulation was only partial (approximately 50%), which suggests that other mechanism(s) are involved. Because clonidine decreased force more steeply than verapamil in the [Ca\textsuperscript{++}]\textsubscript{o}-force relationship, it appears that clonidine inhibits not only Ca\textsuperscript{++} increase but also the other downstream mechanisms that enhance Ca\textsuperscript{++} sensitivity. Moreover, the relationship was not completely the same as that in the atropine-induced relaxation, which suggests that clonidine may inhibit not only the responses specific to muscarinic stimulation but also the other, nonspecific mechanisms. This hypothesis is supported by the results that clonidine slightly but significantly suppressed the force, but not [Ca\textsuperscript{++}], in high K\textsuperscript{+}-stimulated BTSM. Thus clonidine may exert its inhibitory effects by blocking the contractile mechanisms related to muscarinic stimulation and the other, nonspecific component(s) in CCh-contracted BTSM.

In summary, clonidine relaxes CCh-contracted BTSM by inhibiting the release of Ca\textsuperscript{++}, the increase in Ca\textsuperscript{++} influx and the increase in Ca\textsuperscript{++} sensitivity of contractile elements mainly via I\textsubscript{1}R.

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References


