

Mechanism of Relaxant Effect of Clonidine in Isolated Bovine Tracheal Smooth Muscle¹

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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 phentolamine (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 ([Ca⁺⁺]_i) followed by contraction. The high K⁺-induced increase in [Ca⁺⁺]_i was not affected by clonidine. In CCh-stimulated BTSM, clonidine decreased [Ca⁺⁺]_i and muscle force in parallel, whereas verapamil decreased [Ca⁺⁺]_i more strongly than muscle force. Clonidine (100 μM) inhibited the transient increase in [Ca⁺⁺]_i induced by CCh but not by caffeine (20 mM) in Ca⁺⁺-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 Ca⁺⁺-influx, Ca⁺⁺-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 p.o., 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 antibronchospastic 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 anti-spastic or spasmolytic effects in the airways.

The present study was designed to examine the mechanisms of direct action of clonidine in bovine trachealis smooth

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ABBREVIATIONS: A₂R, alpha-2 adrenoceptor; BTSM, bovine tracheal smooth muscle; PSS, physiological salt solution; fura-PE3/AM, acetoxymethyl ester of fura-PE3; [Ca⁺⁺]_i, intracellular calcium concentration; K_d or K_r, dissociation constant; EGTA, ethyleneglycol-bis-N,N,N',N'-tetraacetic acid; CCh, carbamylcholine chloride (carbachol); WB 4101, 2-[(2,6-dimethoxyphenoxy-ethyl)aminomethyl]-1,4-benzodioxane hydrochloride; TTX, tetrodotoxin; I₁R, imidazoline I₁ receptor.

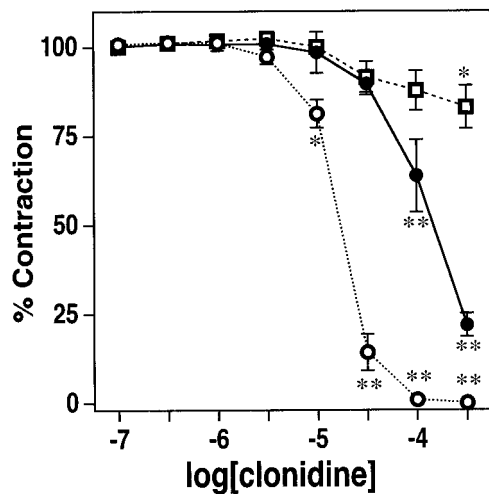


Fig. 1. Concentration-response curves of bovine tracheal strips precontracted with 0.1 μM CCh (\circ), 1 μM CCh (\bullet) and 72.7 mM KCl (\square) to clonidine. Each point represents the mean \pm S.E.M. Asterisks indicate significant difference (* $P < .05$, ** $P < .01$) from control.

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.

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^+ solution (72.7 mM) was repeatedly applied.

Measurement of acetoxymethyl ester of fura-PE3 (fura-PE3/AM) fluorescence. $[\text{Ca}^{++}]_i$ 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 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 (CAF-110, Japan Spectroscopic). The absolute Ca^{++} concentration was not calculated in the present experiment because the dissociation constant (K_d) of the fluorescent indicator for Ca^{++} 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^+ solution (72.7 mM) was repeatedly applied until the response became stable. The ratios obtained in resting state and in high- 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^{++} -free solution were observed immediately (approximately 1 min) after the ratio returned to base line by exchanging the high- K^+ solution for the Ca^{++} -free solution.

Measurement of adenosine 3':5'-cyclic monophosphate (cAMP) content. After preincubation in PSS aerated with 95% O_2 -5% CO_2 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, CaCl_2 1.5, MgCl_2 1.0, NaHCO_3 23.8 and glucose 5.5. EDTA (0.01 mM) was added to chelate heavy metal ions contaminating the PSS. High- K^+ solution was made by substituting equimolar KCl for NaCl. Ca^{++} -free solution was made by removing CaCl_2 from PSS and adding 0.5 mM EGTA. These solutions were aerated with 95% O_2 -5% CO_2 mixture at 37°C and pH 7.4.

The following drugs, chemicals and apparatus were used:

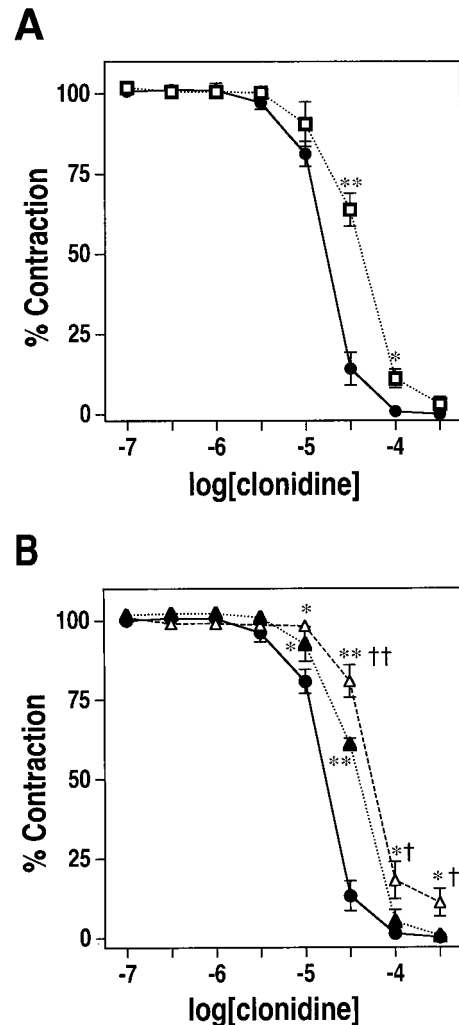


Fig. 2. Effects of 1 μM yohimbine (panel A, \square), 10 μM idazoxan (B, \blacktriangle) and 30 μM idazoxan (panel B, \triangle) on the concentration-response curves of bovine tracheal strips precontracted with 0.1 μM CCh to clonidine compared with control data (\bullet in both). Each point represents the mean \pm S.E.M. IC_{50} 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 ($\dagger P < .05$, $\dagger\dagger P < .01$).

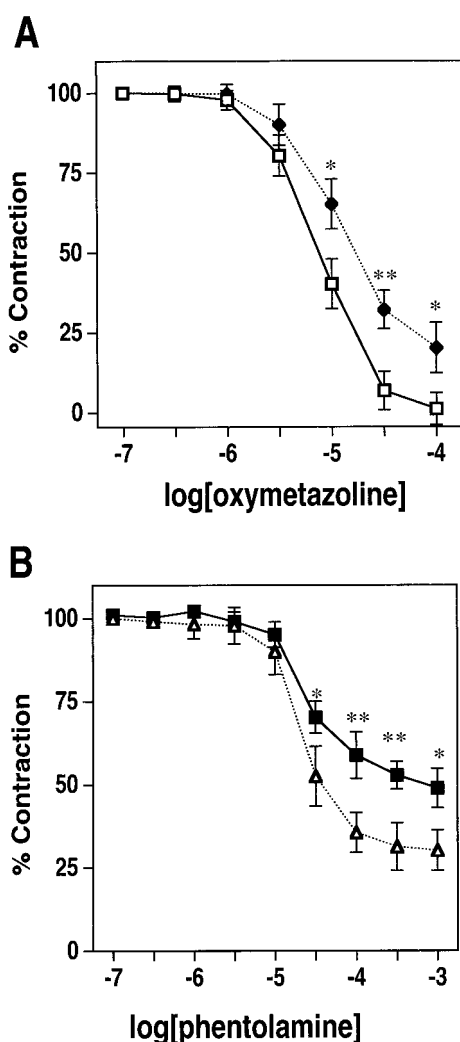


Fig. 3. Concentration-response curve of oxymetazoline (panel A) and phentolamine (panel B) in bovine tracheal strips precontracted with 0.1 μM CCh in the absence (\square and \triangle) and the presence (\blacklozenge and \blacksquare) of 10 μM idazoxan. Each point represents the mean \pm S.E.M. Asterisks indicate significant difference from control curve (* $P < .05$, ** $P < .01$).

clonidine hydrochloride, CCh, WB 4101, prazosin hydrochloride, yohimbine hydrochloride, idazoxan hydrochloride, phentolamine hydrochloride, indomethacin, verapamil hydrochloride, oxymetazoline hydrochloride, norepinephrine bitartrate, propranolol hydrochloride (Sigma Chemical, St. Louis, MO), anhydrous caffeine, atropine sulphate (Wako Junyaku, Japan), butoxamine hydrochloride (Junsei Chemical, Japan), forskolin (Calbiochem, Japan), fura-PE3/AM (Texas Fluorescence Laboratory, Austin, TX), cremophor EL (Nacalai Tesque, Kyoto, Japan), TTX (donated by Sankyo Co., Japan), and cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Statistical analysis. The results of the experiments are expressed as mean \pm 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.

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 $111 \pm 7.9\%$ and $159 \pm 11.4\%$ 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 IC_{50} values of $17.1 \pm 2.0 \mu\text{M}$ and $100 \pm 5.1 \mu\text{M}$, 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 (K_i) of these two antagonists were determined as 660 nM and 7,600–15,000 nM, respectively, from the relationship

$$\log([D]/[D_0] - 1) = \log[I] - \log[K_i]$$

where $[D]$ is the IC_{50} of clonidine in the presence of yohimbine or idazoxan, $[D_0]$ is the IC_{50} of clonidine alone and $[I]$ is the concentration of yohimbine or idazoxan. In contrast, clonidine-induced relaxation was not affected by an α_1 -adrenoceptor antagonist, WB 4101 (1 μM , $n = 4$), by an α_1 -adrenoceptor antagonist, prazosin (0.01 μM , $n = 5$), by a nonselective β -adrenoceptor antagonist, propranolol (20 μM , $n = 5$) or by a β_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 IC_{50} values of $7.98 \pm 2.1 \mu\text{M}$ and $37.8 \pm 7.5 \mu\text{M}$, respectively. Additionally, both of these relaxant effects were inhibited by idazoxan (10 μM), with IC_{50} values of $16.0 \pm 3.4 \mu\text{M}$ and

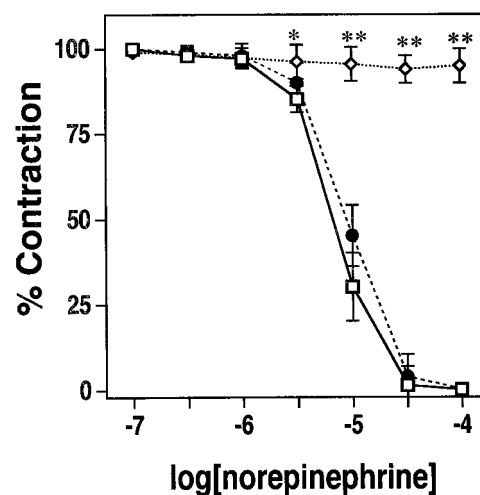


Fig. 4. Concentration-response curve of norepinephrine (\square) in bovine tracheal strips precontracted with 0.1 μM CCh and the effects of 10 μM propranolol (\diamond) and 1 μM yohimbine (\bullet). Each point represents the mean \pm S.E.M. Asterisks indicate significant difference from control curve (* $P < .05$, ** $P < .01$).

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 [Ca⁺⁺]_i and muscle force. In fura-PE3/AM-loaded trachea, high K⁺ (72.7 mM) induced sustained increases in [Ca⁺⁺]_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 [Ca⁺⁺]_i (fig. 5A). CCh also induced sustained increases in [Ca⁺⁺]_i and contraction. In CCh-stimulated tracheae, clonidine (10–100 μM) induced concentration-dependent decreases in [Ca⁺⁺]_i and force (fig. 5B). Atropine (0.1–1.0 μM) also reduced both [Ca⁺⁺]_i and force (fig. 5C). On the other hand, verapamil (0.01–0.1 μM) more strongly inhibited [Ca⁺⁺]_i than force in the CCh-stimulated tracheae; although 1.0 μM verapamil almost completely inhibited CCh-stimulated [Ca⁺⁺]_i, it inhibited CCh-induced contraction only by approximately 50% (fig. 5D).

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

In Ca⁺⁺-free solution (with 0.5 mM EGTA), CCh (0.1 μM) induced a spike-like transient increase in [Ca⁺⁺]_i (104.0 ± 11.2% of high K⁺-induced response, *n* = 5). Clonidine (100 μM) diminished the transient [Ca⁺⁺]_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 [Ca⁺⁺]_i in Ca⁺⁺-free solution (100.0 ± 9.0%, *n* = 4). Clonidine (100 μM) had no significant effect on the caffeine-induced Ca⁺⁺ transient (92 ± 18.5%, *n* = 4, fig. 7, B and C).

cAMP assay. Clonidine (100 μM) had no significant effect on cAMP concentration in the presence of CCh (0.1 μM) or KCl (72.7 mM). On the other hand, forskolin (10 μM) greatly increased cAMP concentration in this preparation (fig. 8, *n* = 4 each).

Discussion

In the bovine trachea, clonidine produced only slight suppression of high K⁺-induced increases in contraction and did not affect [Ca⁺⁺]_i. In contrast, clonidine relaxed CCh-induced contraction in association with a decrease in [Ca⁺⁺]_i. The effects of clonidine were not affected by *alpha*-1 or *beta* 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,

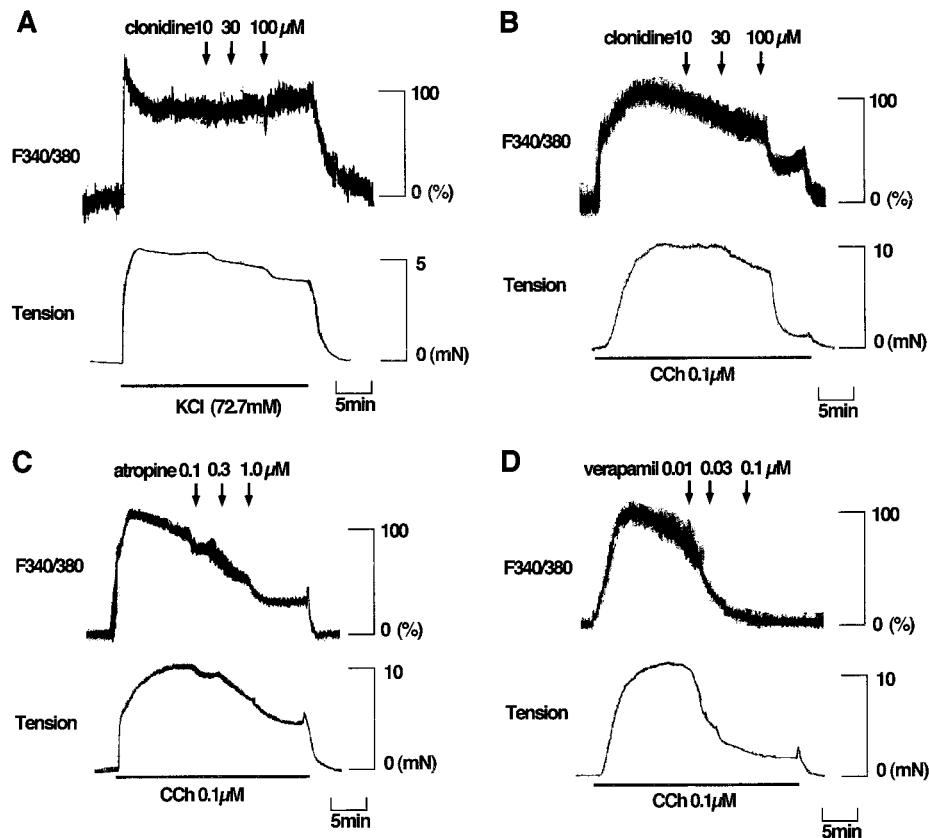


Fig. 5. Effect of cumulatively administrated clonidine on [Ca⁺⁺]_i (upper trace) and muscle force (lower trace) in fura-2-loaded bovine tracheal strips stimulated with high K (panel A), and effects of clonidine (panel B, 10–100 μM), atropine (panel C, 0.1–1.0 μM) and verapamil (panel D, 0.01–0.1 μM) on the strips precontracted with 0.1 μM CCh.

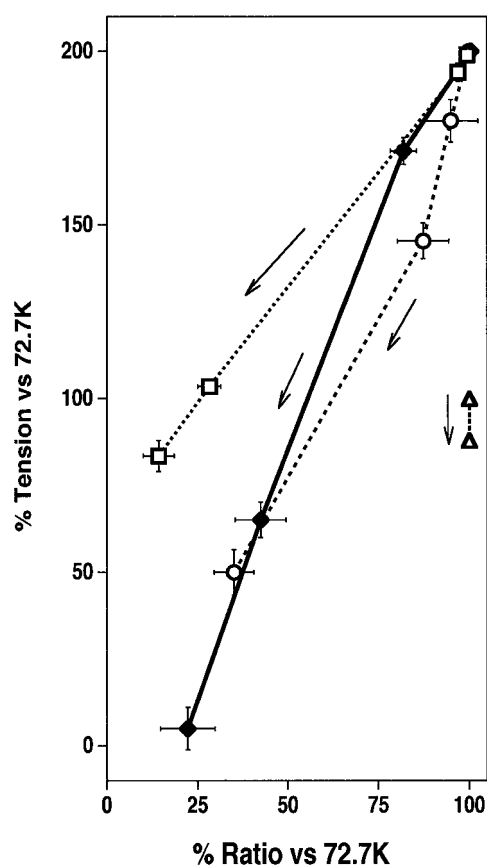


Fig. 6. Effects of cumulatively administered clonidine (10–100 μM , \blacklozenge), atropine (0.01–0.1 μM , \circ) and verapamil (0.1–1.0 μM , \square) on the $[\text{Ca}^{2+}]_i$ -force relationship on CCh-stimulated strips compared with the effect of clonidine on high K^+ -stimulated strips (\triangle). The relationship was obtained from the data in figure 5.

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).

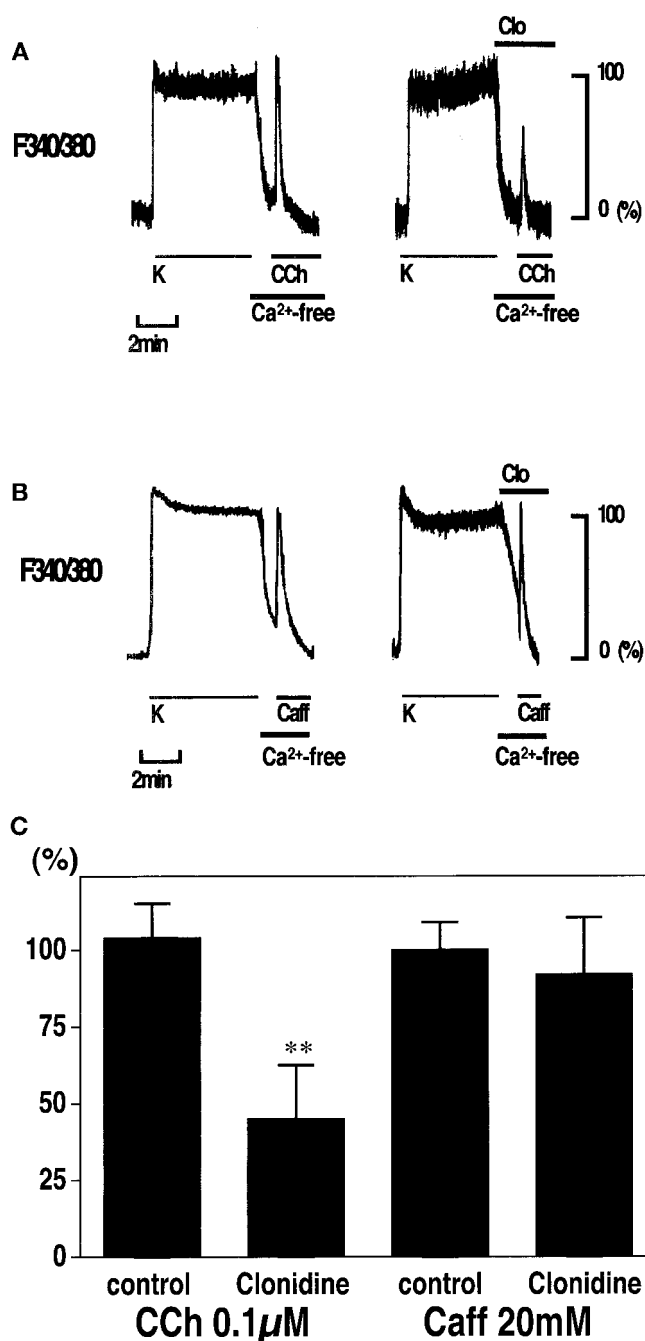


Fig. 7. Effects of clonidine on CCh (panel A)- and caffeine (panel B)-induced transient increase in $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution with 0.5 mM EGTA. 100% represents the 72.7 mM KCl-induced $[\text{Ca}^{2+}]_i$. CCh or caffeine was applied 1 min after removal of Ca^{2+} . The data are summarized in (panel C). Asterisk indicates significant difference (* $P < .01$) from control.

Clonidine stimulates not only A2Rs but also the I_1Rs . 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 K_i 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-

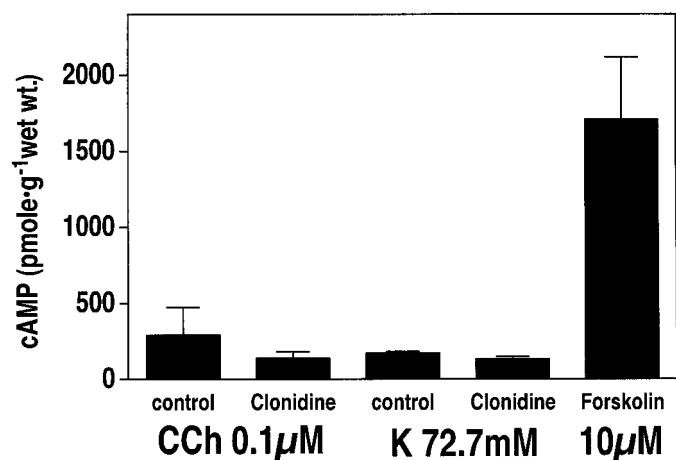


Fig. 8. Effects of clonidine on cAMP content in bovine tracheal strips pretreated with 0.1 μM CCh or 72.7 mM KCl compared with an effect of 10 μM forskolin.

mately 50 to 6600-fold lower than the value estimated in the present study. These findings make the involvement of A2Rs in the relaxation process questionable. Moreover, the I₁R agonists phentolamine (0.1–100 μM) and oxymetazoline (0.1–100 μM) also showed concentration-dependent relaxation in CCh-stimulated strips, and their effects were inhibited by idazoxan but not by yohimbine. Because the K_i values for I₁Rs of the two agonists, phentolamine and oxymetazoline, are similar to that of clonidine in platelet, *i.e.*, 11.4 nM, 6.2 nM and 55.0 nM, respectively (Piletz *et al.*, 1996), and because idazoxan has a much higher affinity for I₁Rs than does yohimbine (Szabo and Urban, 1995), these results strongly suggest involvement of I₁Rs in the relaxation in the CCh-contracted BTSM. Incidentally, the concentration-response curve for phentolamine was similar to that of partial agonists, indicating a difference between the selectivity of clonidine, and that of phentolamine, for I₁Rs. Additionally, the inhibitory effects of idazoxan on both phentolamine- and oxymetazoline-induced relaxation appeared to be noncompetitive, which suggests that the mechanisms of action of these two agonists do not involve exactly the same pathway as that of clonidine. The K_d of clonidine for A2Rs has been determined to be in the nanomolar range (7.3 nM) in vascular smooth muscle (Weiss *et al.*, 1983), whereas it is in the micromolar range for I₁R—for example, in liver (15,067 nM) (Zonnenchein *et al.*, 1990) and brain (2400 nM) (Bennai *et al.*, 1995). Therefore, the result that a relatively high concentration of clonidine was required to produce the maximal relaxation in the present study may support the hypothesis that the binding sites of clonidine are mainly I₁Rs in the BTSM. Additionally, norepinephrine-induced relaxation in CCh-stimulated strips was completely abolished by propranolol but not by yohimbine. Because the K_d for I₁Rs of norepinephrine was estimated to be 200 to 400-fold greater than that of clonidine (Bennai *et al.*, 1995), whereas the K_d for A2Rs is similar to that of clonidine (Hieble *et al.*, 1997), these results indicate that stimulation of the A2Rs is unlikely to induce relaxation in the BTSM. Moreover, increase in cAMP production *via* A2Rs has been reported in several types of cells (Åkerman *et al.*, 1997). In the present study, however, clonidine did not change the cAMP content in CCh-stimulated BTSM, which indicates that the A2Rs have little function in the preparation.

It has been reported that CCh augmented Ca⁺⁺ 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⁺ at a given [Ca⁺⁺]_i, which suggests that CCh increased the Ca⁺⁺ sensitivity of the contractile element. In Ca⁺⁺-free solution, 0.1 μM CCh induced a transient increase in [Ca⁺⁺]_i that may be due to production of inositol-1,4,5-trisphosphate mediated by a muscarinic stimulation (Blüml *et al.*, 1994). An activator of Ca⁺⁺-induced Ca⁺⁺ release, 20 mM caffeine (Iino, 1990), also transiently increased [Ca⁺⁺]_i in Ca⁺⁺-free solution. Clonidine, at the concentration needed to inhibit the 0.1 μM CCh-induced sustained increase in [Ca⁺⁺]_i and sustained contraction, inhibited significantly the CCh-induced transient increase in [Ca⁺⁺]_i in Ca⁺⁺-free solution, but not the effect of caffeine. These results suggest that clonidine inhibits the Ca⁺⁺ release induced by inositol-1,4,5-trisphosphate but not the Ca⁺⁺-induced release of Ca⁺⁺. 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⁺⁺]_i-force relationship, it appears that clonidine inhibits not only Ca⁺⁺ increase but also the other downstream mechanisms that enhance Ca⁺⁺ 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⁺⁺]_i, in high K⁺-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⁺⁺, the increase in Ca⁺⁺ influx and the increase in Ca⁺⁺ sensitivity of contractile elements mainly *via* I₁Rs.

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