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Effects of Clonidine on the Negative Chronotropic Response Induced by Vagal Stimulation in the Rat

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Abstract□The effects of clonidine on the negative chronotropic response induced by stimulation of vagus nerve were studied in the presence of propranolol in reserpinized and anesthetized rats. When the heart rate was decreased by stimulation of the vagus nerve, clonidine significantly inhibited vagally induced heart rate decrease (negative chronotropic response) in dose dependent manner. This inhibitory effect of clonidine was virtually abolished by phentolamine, α_1- and α_2-adrenoceptor antagonist, and partially antagonized by prazosin, α_1-adrenoceptor antagonist. On the other hand, when the heart rate was decreased by the infusion of bethanechol, a muscarinic parasympathetic stimulant, clonidine had no effect on the bethanechol-induced heart rate decrease. These results suggest that clonidine inhibits vagally induced negative chronotropic response by activation of presynaptic α-adrenoceptors located on the parasympathetic cholinergic nerve terminal in the heart and this effect of clonidine is more related to α_2-adrenoceptors than α_1-adrenoceptors.

Key words□Clonidine, Negative Chronotropic response, Presynaptic α-adrenoceptor, Parasympathetic cholinergic nerve terminal.

It is generally accepted that the systemic administration of clonidine causes bradycardic effect and a biphasic change in mean arterial blood pressure, an initial transient hypertension followed by a prolonged hypotension in experimental animals[1,2]. The initial hypertensive effect results from stimulation of postsynaptic vascular α-adrenoceptors[1-3] and the subsequent prolonged hypotension is due to the reduction in sympathetic discharge induced by stimulation of α-adrenoceptors at the level of brain stem[4-7]. The sustained bradycardia results from both the reduction in sympathetic discharge and an enhanced vagal outflow[2,8-12]. Peripherally, clonidine impairs adrenergic neurotransmission by activating inhibitory presynaptic α-adrenoceptors. This feedback mechanism has been extensively studied by measurement of the release of noradrenaline in numerous isolated organs[13,14] and intact animals[12,15,16].

While there are a considerable number of evidences for the existence of α-adrenoceptors on cholinergic nerve terminals in the myenteric plexus, the activation of which inhibits the release of acetylcholine[17-21], there are few and conflicting reports concerning presynaptic α-adrenoceptors on cholinergic nerve terminals in the heart. Starke found that the negative chronotropic response induced by stimulation of vagus nerve in the isolated rabbit heart was inhibited by oxymethazoline and naphazoline[22]. In contrast, noradrenaline and clonidine failed to inhibit bradycardia induced by field stimulation of isolated guinea-pig atria[23]. Similarly, clonidine and methoxamine did not significantly change the output of ^{3}H-acetylcholine in the isolated chicken heart in response to vagal stimulation[24]. Recently, Wetzel *et al.* reported that exogenous norepinephrine and epinephrine reduced ^{3}H-acetylcholine release induced by K^+ depolarization or by electrical field stimulation in isolated rat atria previously incubated with ^{3}H-choline and the inhibitory effect of norepinephrine was blocked much more potently by the α_1-antagonists than by the α_2-antagonists. With these results, they suggested that the adrenergic receptors modulating acetylcholine release in the rat heart are of α_1-subtype and that those receptors are located presynaptically on the vagal neuroeffector junctions[25-27]. Similar result was obtained by Benkirane *et al.*[28]. On the other hand, Loiacono *et al.* reported that the release of acetylcholine from the cholinergic nerve terminals in guinea-pig atria can be inhibited by a mechanism apparently involving prejunctional α_2-adrenoceptors; however, in their experimental condition, the negative chronotropic response induced by stimulation of vagus

nerve in isolated guinea-pig atria was not affected by the activation of the prejunctional α_2-adrenoceptors associated with the cholinergic nerve terminal[29].

The purpose of the present study was to investigate further whether α-adrenoceptors related to the inhibition of neurotransmitter release are on parasympathetic nerve innervating the heart. For this study, effect of clonidine, a selective α_2-adrenoceptor agonist, was examined on the negative chronotropic response induced by stimulation of vagus nerve or by infusion of bethanechol in reserpinized and vagotomized rats under urethane anesthesia.

EXPERIMENTAL METHODS

Male Spraque-Dawley rats weighing 250-300 g were maintained at a room temperature of 22-24 °C and given food (laboratory chow, Sam-Yang Co.) and tap water *ad libitum*. Reserpine (5.0 mg/kg i.p.) was administered 24 hr before the experiment for sympathetic denervation. Under urethane anesthesia (1.2 mg/kg i.p.), the left femoral vein and right femoral artery were cannulated for infusion of clonidine and measurement of blood pressure, respectively. Heart rate was recorded via needle electrodes connected to a Hi-Gain coupler (Narco 7180) and biotachometer on physiograph recorder (Narco MK-III-P). Blood pressure was simultaneously recorded from femoral artery by a pressure transducer (Narco RP-1500) connected to strain gauge coupler (Narco 7179) on physiograph recorder (Narco MK-III-P).

For vagal stimulation-induced negative chronotropic response, both vagi were carefully separated and cut at the cervical level. The distal end of either vagus nerve was placed on a pair of platinum electrode and stimulated with a train of square waves (3 Hz, 0.5 msec duration, 9 V) every 15 min for 30 sec. In order to produce bethanechol infusion-induced negative chronotropic response, bethanechol chloride (10 μg/kg/min i.v.) was infused through the right femoral vein for 120 min (infusion rate: 0.15 ml/min).

Clonidine (300 μg/kg) was infused through left femoral vein for 15 min at a rate of 0.07 ml/min using a syringe pump (Sage instruments). Propranolol (1.0 mg/kg i.p.) was administered 45 min before the infusion of clonidine to block β-adrenoceptors. Each antagonist, prazosin (1 mg/kg) or phentolamine (3.75 mg/kg) was intraperitoneally administered 30 min before clonidine infusion.

In these experiments, the inhibitory effect of clonidine on the negative chronotropic response induced by vagal stimulation or bethanechol infusion was expressed as percent increase in heart rate, which was calculated by dividing heart rate increase after clonidine infusion by the mean heart rate decrease induced by vagal stimulation or bethanechol infusion before clonidine infusion and multiplying 100. And blood pressure was expressed as mean femoral arterial pressure. All values were given as mean ± S.E.M. Student's *t*-test was used to test for statistical significance. Differences were taken to be significant for $P<0.05$.

The following drugs were used: clonidine (Boehringer-Ingelheim), reserpine (Sigma Co.), phentolamine hydrochloride (gifted by Ciba-Geigy), propranolol (Nakarai Chemicals), prazosin (Pfizer) and bethanechol chloride (Sigma Co.).

RESULTS

Effects of clonidine on negative chronotropic response induced by stimulation of the vagus nerve.

In reserpinized rats (reserpine 5 mg/kg i.p.), cervical vagal stimulation (0.5 msec duration, 9 V) caused frequency-dependent decrease of heart rate in the presence of propranolol (1 mg/kg i.p.) under urethane anesthesia (1.2 mg/kg i.p.). When the vagus nerve was stimulated for 30 sec at 3 Hz, heart rate was markedly decreased and reached a steady level within 15 sec. In this process, arterial blood pressure was also decreased simultaneously (Fig. 1). The decreased heart rate (negative chronotropic response) returned to the initial value within 15 min after cessation of stimulation. This negative chronotropic response was sustained for at least 120 min by the repeated stimulation for 30 sec at an interval of 15 min. In these experiments, heart rate and mean arterial pressure before vagal stimulation were 304.8 ± 7.7 beats/min and 81.5 ± 2.0 mmHg, respectively. During vagal stimulation, heart rate was decreased by 81.7 ± 4.4 beats/min and arterial blood pressure, by 19.4 ± 1.2 mmHg (n = 30).

When clonidine was infused every 30 min for 15 min in cumulative dose schedule, clonidine caused a dose-dependent inhibitory effect on the negative chronotropic response induced by vagal stimulation at 3 Hz: the inhibitions after the infusion of clonidine at an interval of 30 min were 12.7 ± 2.4% at 50 μg/kg, 19.5 ± 1.8% at 100 μg/kg, 31.5 ± 3.1% at 300 μg/kg and 37.5 ± 4.1% at 500 μg/kg (n = 6) (Fig. 2). In response to increasing frequency, the effect of clonidine (300 μg/kg i.v.) on the negative chronotropic response was attenuated in a frequency-dependent manner: the inhibitions were 42.9 ± 3.8% at 1 Hz, 34.0 ± 4.2% at 3 Hz and

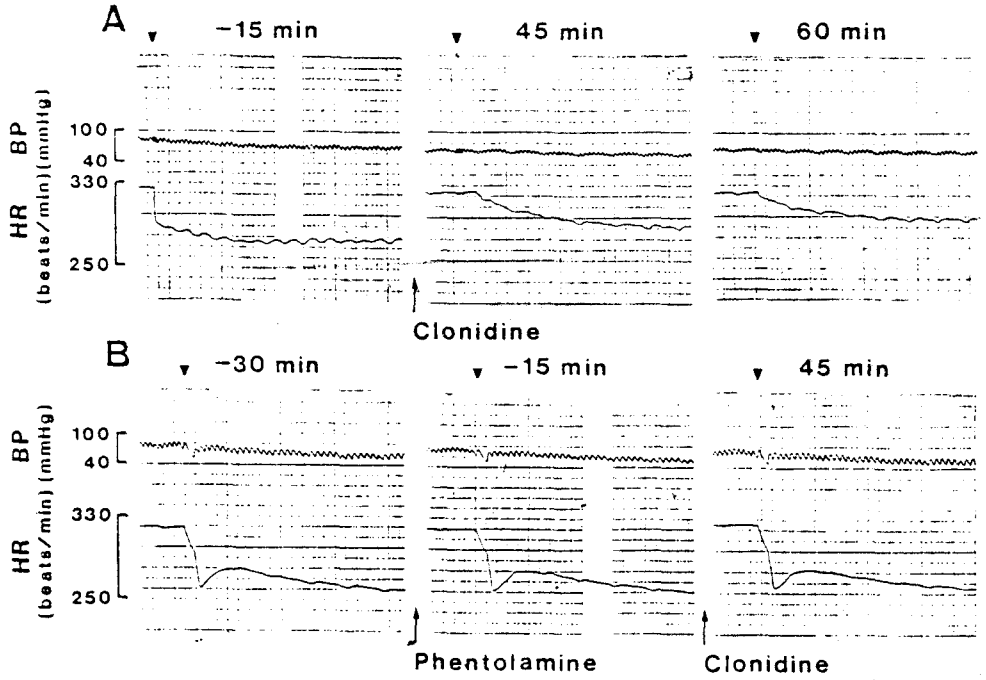

Fig. 1. A typical recording showing the effect of clonidine on the negative chronotropic response induced by vagal stimulation in the absence (A) and presence (B) of phentolamine.
Reserpine (5 mg/kg i.p.) was administered 24 hr before the experiment and propranolol (1 mg/kg i.p.) was administered 45 min before the infusion of clonidine. Phentolamine (3.75mg/kg i.p.) was administered 30 min before the infusion of clonidine (300 μg/kg/15min i.v.). Electrical vagal stimulation (▼) was applied at 3 Hz, 0.5msec duration and 9 V.

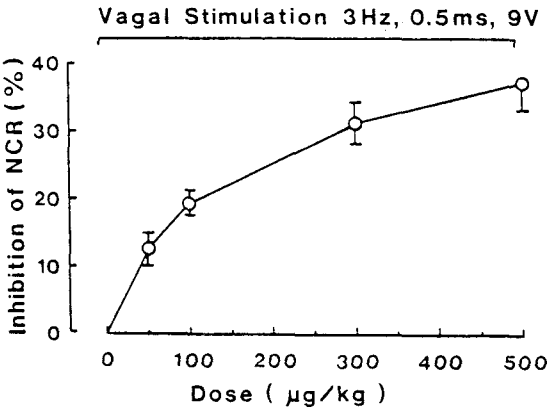

Fig. 2. Effect of doses of clonidine on the negative chronotropic response induced by vagal stimulation.
Clonidine was infused for 15 min cumulatively at an interval of 30 min. Other experimental conditions are the same as those discribed in Fig. 1. Ordinate: percent inhibition of negative chronotropic response (NCR: the decreased heart rate induced by vagal stimulation). Abscissa: dose of clonidine (μg/kg). Each point represents the mean ± S.E. of six experiments.

31.6 ± 4.7% at 5 Hz (n = 4) (Fig. 3). In further experiment, 300 μg/kg of clonidine was administered since the experimental animals were not maintained at regular physiological conditions at higher dose-level of clonidine (500 μg/kg or more) and the stimulation frequency of 3 Hz was employed since the decrease of heart rate at 1 Hz was not enough to examine the effect of clonidine which was expressed as percent inhibition of negative chronotropic response although the inhibitory effect of clonidine on the negative chronotropic response was more pronounced at 1 Hz than 3 Hz.

Clonidine (300 μg/kg i.v.) significantly inhibited the negative chronotropic response induced by vagal stimulation (Fig. 1,4A): the inhibition were 23.9 ± 6.9% at 15 min, 29.4 ± 5.0% at 30 min, 36.4 ± 4.4% at 45 min and 34.2 ± 8.4% at 60 min (Fig. 4A). Maximum inhibitory effect of clonidine was attained about 45 min after infusion of clonidine. Prior to the vagal stimulation, initial blood pressure was increased temporarily and reached maximum in 20 to 30 sec after clonidine infusion, and it was almost returned to the initial level

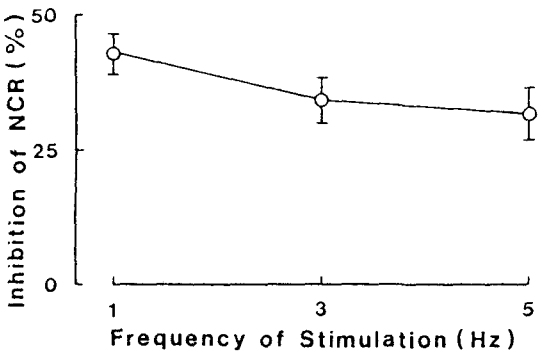

Fig. 3. Relationship of the stimulation frequency and clonidine effect on the negative chronotropic response induced by vagal stimulation.
Clonidine (300 μg/kg i.v.) was infused for 15 min. The stimulation conditions except frequency were fixed at 0.5 msec duration and 9 V. Other experimental conditions are the same as those discribed in Fig. 1. Ordinate: the same as Fig. 2. Abscissa: frequency of stimulation (Hz).

within 30 min. Clonidine did not affect the subsequent blood pressure (Table I, Fig. 4B).

Effects of phentolamine and prazosin on inhibition of the vagally stimulated negative chronotropic response by clonidine

In order to determine whether the inhibitory effect of clonidine on vagal stimulation-induced negative response involved α-adrenoceptor activation, the effect of clonidine was investigated in the presence of α-adrenoceptor antagonists. When phentolamine (3.7 mg/kg i.p.) or prazosin (1 mg/kg i.p.) was administered 30 min before the infusion of clonidine, the inhibitory effect of clonidine on the negative chronotropic response induced by the vagal stimulation was virtually abolished by phentolamine, α_1-and α_2-adrenoceptor antgonist, whereas it was partially antagonized by prazosin, α_1-adrenoceptor antagonist. The inhibition of negative chronotropic response induced by clonidine was reduced by 45.1% in the prazosin-treated rats (Table I, Fig. 4A). With respect to blood pressure, initial blood pressure was almost completely inhibited by phentolamine and partially by prazosin. The increase of initial blood pressure induced by clonidine was reduced by about 81.9% in the phentolamine-treated rats and by about 59.5% in the prazosin-treated rats (Table I).

Effects of clonidine and phentolamine on negative chronotropic response induced by infusion of bethanechol

To determine whether inhibitory effect of clonidine on the negative chronotropic response induced by vagal stimulation was caused by a blockade of the action of released acetylcholine on muscarinic receptors in the myocardial effector cells, the influence of clonidine on the negative chronotropic

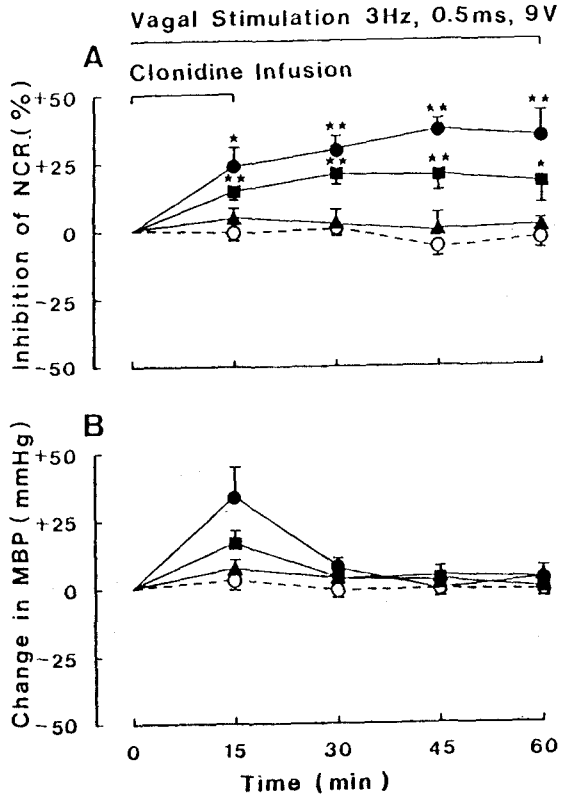

Fig. 4. Effect of clonidine on the negative chronotropic response induced by vagal stimulation (A) and the mean arterial blood pressure prior to vagal stimulation (B).
Clonidine (300 μg/kg i.v.) was infused for 15 min. Phentolamine (3.7 mg/kg i.p.) or prazosin (1 mg/kg i.p.) was administered 30 min before the infusion of clonidine. Other experimental conditions are the same as those discribed in Fig. 1. Ordinate A: the same as Fig. 2, B: change of mean blood pressure (mmHg). Each point represents the mean ± S.E. ○ - ○ : saline (n = 5), ● - ● : clonidine (n = 7), ■ - ■: clonidine after prazosin (n = 4), ▲ - ▲ : clonidine after phentolamine (n = 4), *P<0.05 and **P<0.01, compared with corresponding control rats (saline group).

Table I. The effect of -adrenoceptor antagonists on inhibition (%) of the vagally stimulated negative chronotropic response and increase of mean arterial pressure (mmHg) by clonidine (300 ug/kg i.v. infusion for 15 min).

	Saline (n = 5)	Clonidine 300 μg/kg (n = 7)[1]	Clonidine 300 μg/kg + Phentolamine[2] 3.75 mg/kg (n = 4)	Clonidine 300 μg/kg + Prazosin[2] 1 mg/kg (n = 4)
Inhibition (%) of negative chronotropic response[3]				
3 Hz	-5.8 ± 3.4[9]	36.4 ± 4.4[6]	0 ± 6.3[8]	20.0 ± 5.4[7]
Mean arterial pressure				
Initial increase[4]	8.8 ± 3.3	56.5 ± 6.6[6]	10.2 ± 3.7[8]	22.9 ± 3.0[8]
Subsequent decrease[5]	0.7 ± 2.4	0.6 ± 2.7	-3.4 ± 4.1[10]	-2.9 ± 0.5[10]

[1] n = number of rats.

[2] The antagonists were administered 30 min before the infusion of clonidine.

[3] Percentage change of inhibition of negative chronotropic response 45 min after infusion of clonidine.

[4,5] Changes in mean arterial pressure (mmHg) 20 sec and 45 min after infusion of clonidine, respectively.

[6] Statistical significance from control (Saline group): $^6P<0.01$

[7,8] Statistical significance from clonidine alone: $^7P<0.05$, $^8P<0.01$

[9] Increase in the negative chronotropic response.

[10] Subsequent increase in the arterial pressure though these values were not significant.

response induced by bethanechol infusion was examined. After the start of infusion of bethanechol (10 μg/kg/min), heart rate gradually decreased (negative chronotropic response), reached a steady level within 60 min and this level was maintained for at least 120 min. In this experiment, heart rate and mean arterial pressure before the infusion of bethanechol were 291.2 ± 5.5 beats/min and 81.1 ± 2.5 mmHg, respectively. 60 min after the infusion of bethanechol, heart rate was decreased by 86.4 ± 6.7 beats/min and arterial blood pressure, by 5.5 ± 1.9 mmHg 3 min after infusion of bethanechol (n = 15).

When clonidine (300 μg/kg i.v.) was infused for 15 min at 60 min after the infusion of bethanechol, clonidine did not provide any inhibition against bethanechol-induced negative chronotropic response. Furthermore, clonidine enhanced the negative chronotropic response though it is not significant (Fig. 5A, Table II). During the clonidine infusion, initial blood pressure also increased temporarilly and reached maximum in 20 to 30 sec after the infusion of clonidine and it was almost returned to initial level within 15 min. Clonidine slightly decreased subsequent blood pressure though it did not have statistical significance (Table II, Fig. 5B). When phentolamine (3.75mg/kg i.v.) was administered 30 min before the infusion of clonidine, initial blood pressure was virtually abolished while heart rate and subsequent blood pressure were not affected by treatment of phentolamine (Table II, Fig. 5A,B).

DISCUSSION

Parasympathetic transmission may be inhibited by a decrease of the release of acetylcholine from preganglionic or postganglionic nerve ending, or by a blockade of the action of released acetylcholine on ganglionic or myocardial receptors[22].

The negative chronotropic response induced by low frequency of vagal stimulation was significantly inhibited by clonidine in dose dependent manner (Fig. 2,3), whereas the negative chronotropic response induced by infusion of bethanechol, a muscarinic parasympathetic stimulant, was not decreased by clonidine (Fig. 5A, Table II). Therefore, clonidine seems to inhibit parasympathetic transmission in the rat heart not by a blockade of the action of liberated acetylcholine on muscarinic receptors of the myocardial effector cells, but by a decrease of the liberation of acetylcholine from pre- and/or postganglionic cholinergic nerve endings. No effort was made to distingusih between these possibilities. In this experiment, the inhibitory effect of clonidine on the negative chronotropic response induced by stimulation of vagus nerve was virtually abolished by phentolamine, α_1- and

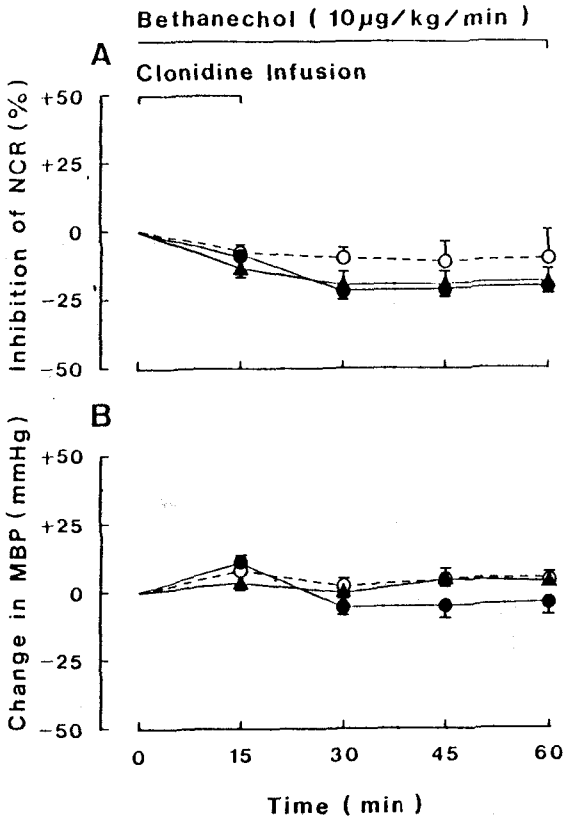

Fig. 5. Effect of clonidine on negative chronotropic response (A) and the mean blood pressure (B) induced by the infusion of bethanechol.

Sixty minutes after the start of infusion of bethanechol (10 μg/kg/min i.v.), clonidine (300 μg/kg i.v.) was infused for 15 min. Phentolamine (3.75 mg/kg i.p.) was administered 30 min before the infusion of clonidine. Other experimental conditions are the same as those discribed in Fig. 1. Each point represents the mean ± S.E. ○ – ○ : saline (n = 5), ● – ● : clonidine (n = 4), ▲ – ▲ : clonidine after phentolamine (n = 6). *P 0.05, compared with corresponding control rats (saline group).

α_2-adrenoceptor antagonist, whereas it was partially antagonized by prazosin, α_1-adrenoceptor antagonist (Fig. 4A, Table I). These results are considered to suggest that the effect of clonidine would be more related to α_2-adrenoceptors than α_1-adrenoceptors since clonidine has a higher affinity for α_2-adrenoceptors than α_1-adrenoceptors[14,19]. Influence of sympathetic nerve function does not appear to be involved in the inhibitory effect of clonidine since sympathetic nerve was denervated by reserpine and β-adrenoceptors were blocked by propranolol. Clonidine has little effect on ganglionic transmission[27,30], suggesting that clonidine produced inhibitory effect on the vagally induced negative chronotropic response by acting at α-adrenoceptors which are located on the postganglionic parasympathetic nerve terminal in the heart.

There are conflicting reports concerning presynaptic α-adrenoceptors on cholinergic nerve terminals in the heart. Starke reported that the negative chronotropic response induced by stimulation of vagus nerve in the isolated rabbit heart was inhibited by the α_2-adrenoceptor agonists oxymetazoline and naphazoline[22]. In contrast, in isolated guinea-pig atria, noradrenaline and clonidine failed to inhibit bradycardia induced by field timulation[23]. Recently, Wetzel et al. reported that exogenous norepinephrine and epinephrine reduced ^{3}H-acetylcholine release induced by K^+ depolarization or electrical field stimulation in isolated rat atria previously incubated with ^{3}H-choline and the inhibitory effect of norepinephrine was blocked much more potently by the α_1-antagonists than by the α_2-antagonists. With these results, they suggested that the adrenergic receptors modulating acetylcholine release in the rat heart are of α_1-subtype[25-27]. On the other hand, Loiacono et al. reported that the release of acetylcholine from the cholinergic nerve terminals in guinea-pig atria can be inhibited by a mechanism involving prejunctional α_2-adrenoceptors[29].

Clonidine has been shown to inhibit cholinergic transmission by activation of presynaptic α_2-adrenoceptors in the myenteric plexus in various preparation, with EC_{50} values in the range of 4 to 100 nmol/l[18,19,31], however, clonidine had no effect on the stimulation-induced efflux of the radioactivity from isolated atria[24,26,29]. Clonidine is generally considered to interact with both α_1-and α_2-adrenoceptors as a partial agonist though it has a higher affinity for α_2-adrenoceptors than α_1-adrenoceptors[14,18,19,32]. The intrinsic activity of clonidine in most studies is approximately 0.5 compared to that of norepinephrine (intrinsic activity = 1)[19,32]. Therefore, it is possible that the lack of effect of clonidine is due simply to its low intrinsic activity at the receptors modulating acetylcholine release[29]. In our experimental conditions, the negative chronotropic response to stimulation of vagus nerve was significantly inhibited by clonidine in the dose range of 100 to 500 μg/kg i.v. (Fig. 2). Thus, it is possible that cardiac preparations require higher doses of clonidine than external gland (from 10 to 60 μg/kg i.v.)[21,33] and myenteric plexus in order to activate presynaptic α-adrenoceptors.

Table II. The effect of phentolamine on inhibition (%) of the bethanechol-induced negative chronotropic response and increase of mean arterial pressure (mmHg) by clonidine (300 μg/kg i.v. infusion for 15 min).

	Saline (n = 5)	Clonidine 300 μg/kg (n = 4)[1]	Clonidine 300 μg/kg + Phentolamine[2] 3.75 mg/kg (n = 6)
Inhibition of negative chronotropic response[3] bethanechol (10 ug/kg/min infusion)	-11.0 ± 7.0[8]	-20.9 ± 2.4[8]	-19.3 ± 4.9[8]
Mean arterial pressure			
Initial increase[4]	8.2 ± 1.7	20.3 ± 2.0[6]	5.8 ± 1.9[7]
Subsequent decrease[5]	-4.8 ± 3.1[9]	4.5 ± 4.2	-4.7 ± 1.8[9]

[1-7] Same as Table 1.

[8] Increase in the negative chronotropic response though these values were not significant.

[9] Subsequent increase in the arterial pressure though these values were not significant.

With respect to blood pressure, prior to vagal stimulation initial blood pressure was increased temporarilly and reached maximum in 20 to 30 sec after clonidine infusion, and it was almost returned to initial level within 30 min. This initial blood pressure was almost completely inhibited by phentolamine and partially by prazosin (Table I, Fig. 4B). On the other hand, during bethanechol infusion initial blood pressure was also increased temporarilly and reached maximum in 20 to 30 sec after infusion of clonidine and it was almost returned to initial level within 15 min. This initial blood pressure was also virtually abolished by pretreatment of phentolamine (Table II, Fig. 5B). The hypertensive response to clonidine is due to a peripheral action on α-adrenoceptors as it is abolished by α-blocking agent and not by reserpine[1]. In the present experiment, initial hypertensive response to clonidine in reserpinized rat was abolished by α-adrenoceptor antagonist, indicating that it is due to a peripheral action on α-adrenoceptors. The hypertensive effect of clonidine was more prolonged in reserpinized rat. This prolonged response could have been due either to a development of denervation supersensitivity in peripheral α-adrenoceptors or it may represent an action of clonidine on the peripheral α-adrenoceptors unopposed by the hypotensive effect of the drug[2]. No effort was made to distinguish between these possibilities.

In summary, the negative chronotropic response induced by low frequency of vagal stimulation was significantly inhibited by clonidine in dose dependent manner, whereas the negative chronotropic response induced by infusion of bethanechol, a muscarinic parasympathetic stimulant, was not decreased by clonidine. These results suggest that clonidine inhibits vagally induced negative chronotropic response by activation of presynaptic α-adrenoceptors located on the parasympathetic cholinergic nerve terminal in the heart and this effect of clonidine is more related to α_2-adrenoceptors than α_1-adrenoceptors. The transient hypertensive effect during infusion of clonidine seems to be mediated by postsynaptic vascular α-adrenoceptors.

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