ELSEVIER

European Journal of Pharmacology 276 (1995) 291–296

ejp

Short communication

GABA-mediated inhibition of pacemaker neurons of rostral ventrolateral medulla by clonidine in vitro

Miao-Kun Sun *, Donald J. Reis

Division of Neurobiology, Department of Neurology and Neuroscience, Cornell University Medical College, 411 East 69th Street, New York, NY 10021, USA

Received 8 December 1994; revised 6 February 1995; accepted 10 February 1995

Abstract

In vitro, clonidine (1, 10, or 30 μM) dose-dependently and reversibly inhibited tonically active pacemaker neurons that correspond to the relatively fast-conducting reticulospinal vasomotor neurons of the rostral ventrolateral reticular nucleus of the medulla oblongata in rats. The clonidine-induced membrane-hyperpolarizing response of these neurons was abolished by either tetrodotoxin, bicuculline, a $GABA_A$ receptor antagonist, or 4,4′-diisothiocyano-1,2′-disulphonic stilbene acid, a Cl^- channel blocker. We conclude that the clonidine-induced inhibition of the pacemaker neurons of the rostral ventrolateral reticular nucleus is indirect, mediated by synaptic release of γ-aminobutyric acid (GABA) or GABA-like substances, which activate Cl^- channels of the pacemaker neurons of the rostral ventrolateral reticular nucleus.

Keywords: Clonidine; Rostral ventrolateral medulla; Vasomotor neuron; Sympathoexcitatory neuron; GABA (γ-aminobutyric acid)

1. Introduction

Clonidine, a centrally acting antihypertensive agent, lowers arterial pressure, at least partially, by inhibiting neurons in the rostral ventrolateral reticular nucleus of the medulla oblongata (Punnen et al., 1987; Ernsberger et al., 1990; Reis et al., 1992). At an intravenous hypotensive dose (reducing mean arterial pressure by about 20 mm Hg) in normotensive rats, it inhibits the spinal cord-projecting vasomotor neurons of the rostral ventrolateral reticular nucleus with slow-conducting axons (about 0.5 m/s) and a portion of the vasomotor neurons in the same area with fast-conducting axons (2–8 m/s) to the spinal cord (Sun and Guyenet, 1986; Haselton and Guyenet, 1989; Allen and Guyenet, 1993). These results, however, have often been interpreted as clonidine having no effect on the latter group of the vasomotor neurons, though it has never been directly examined whether these 'unresponsive' vasomotor neurons will respond to higher doses of clonidine, like those achieved in the rostral ventrolateral medulla after direct microinjections of the agent into the medulla (Bousquet and Schwartz, 1983).

We have therefore examined electrophysiologically in slices of rat medulla oblongata effects of clonidine on the pacemaker neurons of the rostral ventrolateral reticular nucleus, neurons that have been identified as reticulospinal (Sun et al., 1988b) and corresponding to spinal cord-projecting vasomotor neurons of the rostral ventrolateral reticular nucleus with fast-conducting axons to the spinal cord (Sun et al., 1988a,b; Sun and Reis, 1994). We demonstrate that high concentrations of clonidine reversibly inhibit these neurons indirectly, presumably by facilitating release of γ-aminobutyric acid (GABA).

2. Materials and methods

2.1. In vitro slice preparation

Male Sprague-Dawley rats (70–100 g) were deeply anaesthetized with halothane and decapitated. The

* Corresponding author. Division of Neurobiology, Cornell University Medical College, 411 East 69th Street (Room 410), New York, NY 10021, USA. Tel. (212) 570-2900, fax (212) 988-3672.

Elsevier Science B.V.
SSDI 0014-2999(95)00095-X

whole brain was quickly removed and placed in a cold, oxygenated solution (see below). The tissue was trimmed with a razor blade and fixed with cyanoacrylate glue to the chuck of a vibrating sectioning system (Vibratome). Coronal slices were taken at the posterior end of the facial motor nucleus. A 500 μm thick slice was transferred to a Haas-type interface chamber, where it rested on a nylon mesh. The slice was perfused at a rate of 2.0 ml/min, and the chamber maintained in an atmosphere of warmed 95% O_2-5% CO_2 saturated with water. The perfusion solution had the following compositions (in mM): NaCl, 124; KCl, 4.9; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 3.1; $NaHCO_3$, 25.6; glucose, 10; equilibrated with 95% O_2-5% CO_2 (pH 7.35). The slice was maintained at 31 ± 0.5° C and allowed to equilibrate for at least 1 h before commencement of recording (Sun et al., 1988a,b).

2.2. Recordings

Extracellular and intracellular recordings were obtained with glass microelectrodes filled with 2 M NaCl (4–8 MΩ) and 3 M potassium acetate or 2 M KCl (pH adjusted to 7.3; 65–120 MΩ), respectively. Electrodes were guided toward the rostral ventrolateral reticular nucleus under visual control with assistance of a dissecting microscope and advanced by using a WPI nanostepper microdrive. Signals were amplified with an Axoclamp-2A amplifier using standard techniques. Capacitance was optimally adjusted during discontinuous current-clamp mode before and after cell penetration to neutralize capacitance and reduce overshoot/undershoot errors as monitored on a second oscilloscope. For current- or voltage-clamping, discontinuous single-electrode current- or voltage-clamp mode was used, which employed a sampling rate of 3.0–5.0 kHz (30% duty cycle). Gain was usually set at 6–8 nA/mV, which was slightly below the maximum value without causing overshoot or instability in the step response to a repetitive 10 mV step command (Sun and Reis, 1994).

The pacemaker neurons in the rostral ventrolateral reticular nucleus were identified in slices during extracellular recording according to previously described criteria (Sun et al., 1988a,b), including their anatomical location, the duration and configuration of action potentials, and the presence of characteristic pacemaker firing pattern. In slices and in an in vitro vascularly perfused preparation, they exhibit the same type of regular rhythmic discharges as observed in vivo after intracisternal administration of kynurenate, an excitatory amino acid receptor antagonist (Sun et al., 1988a), which eliminates all detectable synaptic inputs. The pacemaker membrane potentials were then confirmed by observing intracellularly the presence of rhythmic pacemaker membrane potential cycles without the excitatory post-synaptic potentials and resetting the cycles with intracellular injections of currents (Sun et al., 1988b; Sun and Reis, 1994). The remarkable homogeneity of their responses to a variety of substances and hypoxia (e.g. Sun and Guyenet, 1986; Sun and Reis, 1994) further suggests that these tonically active neurons represent a very homogeneous cell group.

2.3. Drugs and chemicals

Agents were applied to the preparation by temporarily switching the perfusion solution from drug-free to drug-containing saline (compositions as described above). The volume of the chamber is about 0.5 ml and it is, thus, reasonable to assume that after a few minutes of equilibration, the concentration of these agents in the extracellular fluid surrounding the neurons should have closely approximated that present in the perfusion solution. The following drugs or chemicals were used: clonidine (Sigma), tetrodotoxin (Sigma), bicuculline methiodide (Sigma), 4,4′-diisothiocyano-1,2′-disulphonic stilbene acid (DDS, Sigma). The time delay between turning on the drug solution and its getting into the chamber was measured using fast green solution and subtracted in each experiment.

2.4. Data analysis

Whenever appropriate, statistical analysis was performed using the Student's t-test for paired data. All the values are expressed as means ± S.E.M. Differences were judged significant at $P < 0.05$.

3. Results

3.1. Effects of clonidine on the pacemaker neurons of the rostral ventrolateral reticular nucleus

Clonidine, added to the perfusate, reversibly reduced, at concentrations greater than 1 μM, the discharge of all the pacemaker neurons recorded extracellularly ($47.2 \pm 6.0\%$ inhibition to 10 μM clonidine, from their resting firing rate of 9.8 ± 2.3 spikes/s, $n = 6$, $P < 0.05$), while at 1 μM, no significant inhibition was observed ($4.6 \pm 3.7\%$, $n = 6$, $P > 0.05$). The neurons were either silenced or further inhibited by the application of 30 μM clonidine ($92.6 \pm 4.8\%$ inhibition on average, $n = 6$, $P < 0.05$).

Intracellular recordings demonstrated that spike configuration was not changed by clonidine (Fig. 1). The inhibition of neuronal firing of the pacemaker neurons by clonidine resulted from reduction in the rate of neuronal autodepolarization during the interspike interval, without significant alteration in their firing pattern and threshold for spike initiation.

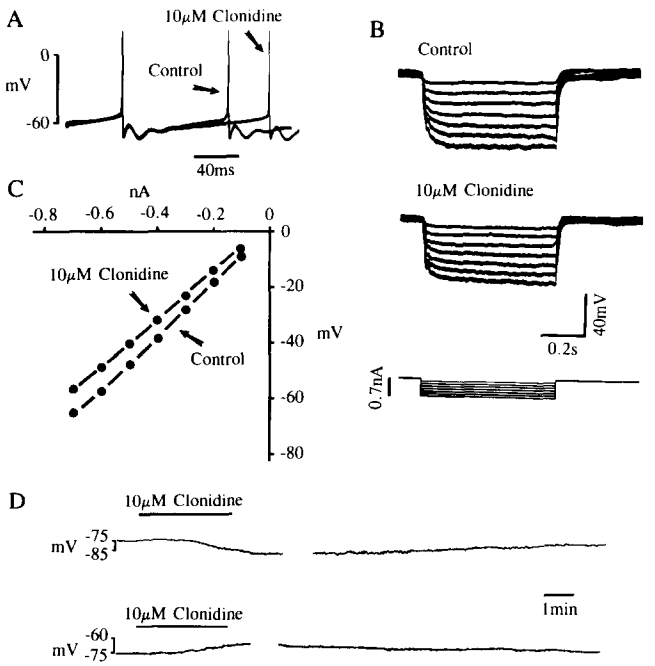

Fig. 1. Effects of clonidine on the pacemaker neurons of the rostral ventrolateral reticular nucleus. A: Single oscilloscope traces of membrane potential in the presence (10 μM clonidine) and absence (Control) of clonidine. B: Membrane responses to different intensities of current injection (bottom traces) in the absence (top traces) and presence (middle traces) of 10 μM clonidine. C: Current-voltage relationship constructed from the experiment illustrated in (B) of the same figure. Note the decrease in the input resistance during clonidine-evoked hyperpolarization (K acetate-filled electrodes). D: Clonidine (10 μM)-evoked membrane hyperpolarization (top trace) when recorded with K acetate-filled electrode and membrane depolarization (bottom trace) when recorded with KCl electrode. A 5 min segment of the recordings was omitted from each trace.

Resting membrane potentials of the pacemaker neurons could not be determined accurately because of continuous autodepolarization during the inter-spike interval. Thus, effects of clonidine on the membrane potentials were analyzed during discontinuous current-clamp recording with intracellular injection of hyperpolarizing currents to silence the cells (maintaining a membrane potential at −75 mV).

When recorded with electrodes filled with K acetate, the neuronal response to 10 μM clonidine was a maximum hyperpolarization of their membrane potentials of 9.6 mV (±1.0 mV, $n = 20$, $P < 0.05$; Fig. 1D), associated with a decrease in the membrane resistance (Fig. 1B and C). The responses were repeatable since a subsequent application of the same dose of clonidine evoked the same response 10 min after full recovery from a previous administration (not shown). However, when recorded with electrodes containing KCl, the polarity of the membrane potential response to 10 μM clonidine in 7 pacemaker neurons of the rostral ventrolateral reticular nucleus was reversed (Fig. 1D), suggesting that the response resulted from an activation of the Cl^- channels.

3.2. Effects of tetrodotoxin on clonidine-induced response

In 5 neurons, no changes in the membrane currents were evoked by 10 μM clonidine (not shown) when their membrane potentials were clamped at −70 mV in the presence of tetrodotoxin (10 μM), under conditions devoid of shifts or fluctuations in the membrane potentials. The results suggest that the clonidine-induced inhibition of the pacemaker neurons of the

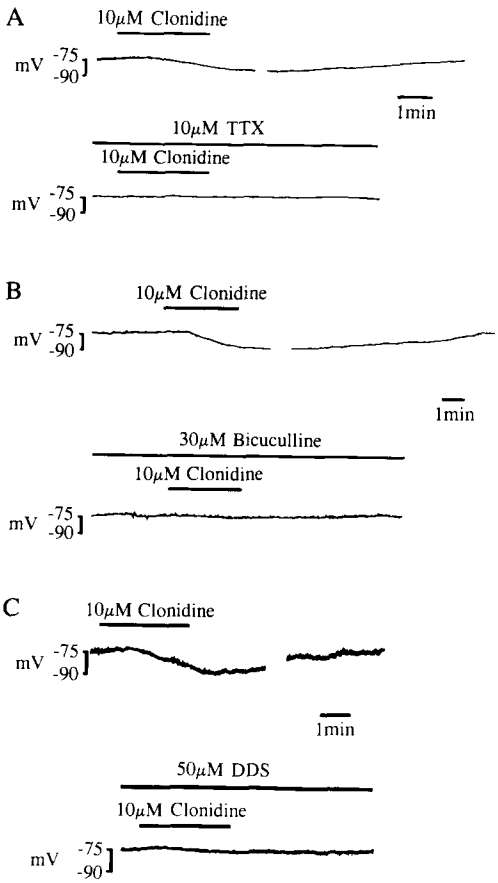

Fig. 2. Effects of tetrodotoxin, bicuculline, and DDS on membrane response of the pacemaker neurons of the rostral ventrolateral reticular nucleus to clonidine. A: Clonidine (10 μM)-evoked membrane hyperpolarization (top trace) was abolished by 10 μM tetrodotoxin (bottom trace). B: The membrane response to 10 μM clonidine (top trace) disappeared in the presence of 30 μM bicuculline (bottom trace). C: Application of 4,4′-diisothiocyano-1,2′-disulphonic stilbene acid (DDS; bottom trace) eliminated the clonidine (10 μM)-evoked hyperpolarization (top trace) of a pacemaker neuron of the rostral ventrolateral reticular nucleus. The membrane responses were observed under current clamp with intracellular injections of negative currents to silence the cell. A 5 min segment of the recordings was omitted from each trace.

rostral ventrolateral reticular nucleus is mediated indirectly via an activation of synaptic inputs.

We therefore examined the effect of tetrodotoxin on the neuronal response to clonidine in the same neurons under current-clamp (Fig. 2A). All the neurons were studied when their spontaneous discharges were eliminated by passing negative hyperpolarizing currents into the cells. Clonidine (10 μM) by itself hyperpolarized the neurons (8.6 ± 1.1 mV, $n = 6$, $P < 0.05$). Tetrodotoxin virtually blocked the responses ($98.6 \pm 1.3\%$, $n = 6$, $P < 0.05$). The results indicate that clonidine-induced inhibition of the pacemaker neurons of the rostral ventrolateral reticular nucleus is mediated by an activation of synaptic inputs onto these neurons.

3.3. Effects of bicuculline and DDS on the clonidine-induced inhibition

The fact that the neuronal membrane responses to clonidine were reversed by intracellular Cl^- and abolished by tetrodotoxin suggests that the responses might be mediated by release of GABA (or GABA-like substances). We, therefore, investigated effects of bicuculline, a $GABA_A$ receptor antagonist, and DDS, a selective Cl^- channel blocker (Mager et al., 1990) on the clonidine-induced inhibition of the pacemaker neurons of the rostral ventrolateral reticular nucleus. In 6 experiments, the membrane hyperpolarization of the pacemaker neurons elicited by 10 μM clonidine (9.2 ± 0.9 mV; $n = 6$, $P < 0.05$ from a membrane potential of -75 mV under current-clamp) was virtually eliminated in the presence of 30 μM bicuculline ($90.2 \pm 3.9\%$ blockade, $n = 6$, $P < 0.05$, Fig. 2B).

In 6 pacemaker neurons of the rostral ventrolateral reticular nucleus, clonidine (10 μM) produced an 8.8 mV (± 1.4 mV, $n = 7$, $P < 0.05$) hyperpolarization under current-clamp (from membrane potentials of -75 mV). The responses were eliminated by 50 μM DDS (100%, $n = 6$, $P < 0.05$; Fig. 2C).

4. Discussion

We have discovered that when applied in slices of rat medulla oblongata, clonidine dose-dependently and reversibly inhibits the pacemaker neurons. The inhibition results from a release of GABA or GABA-like substances, which then activate $GABA_A$ receptors and open Cl^- channels of the pacemaker neurons of the rostral ventrolateral reticular nucleus. First, the membrane response was abolished by tetrodotoxin, indicating that the response depends upon either synaptic inputs or an activation of tetrodotoxin-sensitive sodium channels of the neurons. Hyperpolarization associated with increases in membrane conductance, however, rules out the possibility of a direct involvement of sodium channels of the pacemaker neurons in the response. Second, neuronal inhibition by clonidine was blocked by bicuculline, a $GABA_A$ receptor antagonist. The involvement of GABA in the response is consistent with the observations that the neurons of the rostral ventrolateral reticular nucleus are directly innervated by GABAergic terminals and interneurons in the area and tonically and phasically inhibited by GABA at least in vivo (Willette et al., 1983; Sun and Guyenet, 1986) and that clonidine facilitates the release of GABA from synaptosomes of the rostral ventrolateral medulla (Tingley and Arnerić, 1990). Third, the clonidine-induced membrane response depends upon an activation of Cl^- channels since it was reversed in polarities when recorded with KCl-filled electrodes and blocked in the presence of DDS, an inhibitor of the Cl^- channels (Mager et al., 1990). It has been well established that an activation of Cl^- channel conductance is the ionic mechanism coupled to the $GABA_A$ receptors. It is important to emphasize that the evoked Cl^- current response was observed at the level of the pacemaker neurons of the rostral ventrolateral reticular nucleus, indicating that the affected GABAergic inputs synapse directly on the pacemaker neurons, while the general lack of spontaneous inhibitory post-synaptic potentials suggests that the acting sites might be distant to the recording tip and/or that many synapses were affected simultaneously. It remains to be determined whether the same GABA mechanism also underlies responses of the 'more sensitive' vasomotor neurons to clonidine. If so, sensitivity of the neuronal responses to clonidine will depend on the integrity and degrees of the inhibitory inputs that could be turned on at the moment and the densities of the inhibitory terminals in the affected field if clonidine is iontophoretically applied. The indirect mechanism may explain why not so many neurons were inhibited by iontophoresis of clonidine in vivo and why sensitivity of the pacemaker neurons to low doses of clonidine is not very impressive when examined in vitro.

The clonidine-induced inhibition of the pacemaker neurons in vitro was observed at micromolar concentrations, much higher than its effective concentrations (1–10 nM) in the blood. However, it is not defined whether clonidine accumulates in the rostral ventrolateral medulla after a systemic administration so that its concentrations in the extracellular environment of the rostral ventrolateral medulla are higher than those in the blood. In addition, due to factors such as short periods of application and diffusion restriction, it is not unusual that in vitro experiments often require the application of substances at higher concentrations. In the chromaffin system, clonidine was applied at 30 μM concentration to modulate catecholamine release or to increase cGMP production (cf. Reis et al., 1992). In

our slice preparation, the apparently low sensitivity of the pacemaker neurons to clonidine may also be partly due to the fact that an unknown portion of the GABAergic inputs into the vasomotor neurons (e.g., from the caudal ventrolateral medulla) had to be damaged so that higher concentrations of the agent appeared to be required to evoke a detectable membrane response. Thus, the observed inhibition of the pacemaker neurons at micromolar concentrations in vitro might well contribute to the antihypertensive action of clonidine and at least accounts for the hypotensive actions of clonidine when administrated at high doses in vivo (Bousquet and Schwartz, 1983; Punnen et al., 1987; Ernsberger et al., 1990), or a toxic effect.

When examined in vivo at an intravenous hypotensive dose (Sun and Guyenet, 1986), clonidine was found to inhibit all (5 out of 5) the vasomotor neurons of the rostral ventrolateral reticular nucleus with slow-conducting axons and a portion (1 out of 5; or 20%) of the vasomotor neurons in the same nucleus with relatively fast-conducting axons to the spinal cord. The results should be interpreted with caution. First, the original study (Sun and Guyenet, 1986) was intended for revealing responsive neurons of the rostral ventrolateral reticular nucleus at 'pharmacologically relevant' doses, at which clonidine produced a reduction in mean arterial pressure of some 20 mm Hg. The doses were not maximized and should not be taken as exclusive evidence. In addition, the possibility exists that the acute intravenous effective doses (ED_N) defined in normotensive rats based on the changes in mean arterial pressure may have been overestimated. An 'apparently maximal' response might be obtained at much lower doses due to the fact that gradually increased peripherally vasoconstricting effects at higher intravenous doses of clonidine offset the centrally induced hypotensive response. Second, the high sensitivity of the small portion of the spinal cord-projecting vasomotor neurons of the rostral ventrolateral reticular nucleus with relatively fast-conducting axons to low doses of clonidine may play a more important role in the hypotensive responses than the ones with slow-conducting axons, simply due to the number effect. The majority (more than 95%) of the vasomotor neurons recorded in the rostral ventrolateral reticular nucleus are the ones with relatively fast-conducting axons. The big difference in number of neurons of the two groups recorded more likely represents the actual abundance of the neurons and could not be explained by the sizes or location of cells or sampling bias. More vasomotor neurons (a small portion from a large pool) with fast-conducting axons than the ones with slow-conducting axons were inhibited even at the low doses of clonidine. Furthermore, the barosensitive neurons with slow-conducting axons are believed to project collaterally to other neural structures, such as the hypothalamus and the locus ceruleus (Haselton and Guyenet, 1989), probably representing part of ascending pathways relaying baroreceptor information. Direct relevance of this group of neurons in regulation of sympathetic nerve activity has, therefore, not been established.

Our study, however, does not define what receptor types mediate the clonidine-induced release of the inhibitory substance(s), i.e. whether the effects of clonidine could be attributed to an activation of α_2-adrenoceptors (Allen and Guyenet, 1993; Langer et al., 1980; Szabo et al., 1993), imidazoline receptors (Ernsberger et al., 1990; Punnen et al., 1987; Reis et al., 1992) or both. It also remains to be examined whether the receptors are actually located in the GABAergic interneurons that synapse on the spinal cord-projecting vasomotor neurons of the rostral ventrolateral reticular nucleus.

References

Allen, A.M. and P.G. Guyenet, 1993, α_2-Adrenoceptor-mediated inhibition of bulbospinal barosensitive cells of rat rostral medulla, Am. J. Physiol. 265, R1065.

Bousquet, P. and J. Schwartz, 1983, α-Adrenergic drugs: pharmacological tools for the central vasomotor control, Biochem. Pharmacol. 32, 1459.

Ernsberger, P., R. Giuliano, R.N. Willette and D.J. Reis, 1990, Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla, J. Pharmacol. Exp. Ther. 253, 408.

Haselton, J.R. and P.G. Guyenet, 1989, Electrophysiological characterization of putative C_1 adrenergic neurons in the rat, Neuroscience 30, 199.

Langer, S.A., I. Cavero and R. Massingham, 1980, Recent developments in noradrenergic transmission and its relevance to the mechanism of action of certain antihypertensive agents, Hypertension 2, 372.

Mager, R., S. Ferroni and P. Schubert, 1990, Adenosine modulates a voltage-dependent chloride conductance in cultured hippocampal neurons, Brain Res. 532, 58.

Punnen, S.R., R. Urbanski, A.J. Krieger and H.N. Sapru, 1987, Ventrolateral medullary pressor area: site of hypotensive action of clonidine, Brain Res. 422, 336.

Reis, D.J., S. Regunathan and M.P. Meeley, 1992, Imidazole receptor and clonidine-displacing substance in relationship to control of blood pressure, neuro-protection and adrenomedullary secretion, Am. J. Hypertens. 5, 51s.

Sun, M.-K. and P.G. Guyenet, 1986, Effect of clonidine and γ-aminobutyric acid on the discharges of medullo-spinal sympathoexcitatory neurons in the rat, Brain Res. 368, 1.

Sun, M.-K. and D.J. Reis, 1994, Hypoxia-activated Ca^{2+} currents in pacemaker neurones of rat rostral ventrolateral medulla in vitro, J. Physiol. (London) 476, 101.

Sun, M.-K., J.T. Hackett and P.G. Guyenet, 1988a, Reticulospinal pacemaker neurons of the rostral ventrolateral medulla exhibit pacemaker properties in the presence of a glutamate-receptor antagonist, Brain Res. 438, 23.

Sun, M.-K., B.S. Young, J.T. Hackett and P.G. Guyenet, 1988b, Reticulospinal pacemaker neurons of the rostral ventrolateral medulla with putative sympathoexcitatory function: an intracellular study in vitro, Brain Res. 442, 229.

Szabo, B., R. Urban and K. Starke, 1993, Sympathoinhibition by rilmenidine in conscious rabbits: involvement of α_2-adrenoceptors, Naunyn-Schmied. Arch. Pharmacol. 348, 593.

Tingley III, F.D. and S.P. Arnerić, 1990, Evidence for clonidine presynaptically modulating amino acid release in the rostral ventro medulla: role in the hypertension, Brain Res. 537, 175.

Willette, R.N., A.J. Krieger, P.P. Barcas and H.N. Sapru, 1983, Medullary gamma-aminobutyric acid (GABA) receptors and the regulation of blood pressure in the rat, J. Pharmacol. Exp. Ther. 226, 893.