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Mechanism of antinociceptive action of clonidine in nonmyelinated nerve fibres

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Abstract

Despite a large body of clinical evidence in favour of a local anesthetic effect of clonidine, the underlying mechanism has not yet been elucidated. In this study we have used the sucrose-gap method to measure the effects of clonidine on the electrophysiological properties of nonmyelinated nerve fibers in the rabbit vagus nerve. The results showed that clonidine enhanced the hyperpolarizing and reduced the depolarizing afterpotential that follow compound action potentials during electrical activity. We showed that summation of these afterpotentials shifts the membrane potential toward more negative values, thus creating a region of low safety conduction, where the local circuit currents might fail to depolarize the axonal membrane to the threshold value needed to open voltage-dependent Na^+ channels. Yohimbine did not reverse the inhibitory effects of clonidine on impulse propagation, indicating that the observed effects of clonidine relies on mechanisms not mediated by α_2-adrenoceptors.

Keywords: Clonidine; Antinociception; C-fibre; Sucrose-gap; Afterpotential

1. Introduction

Since the demonstration by Hille (1966) that lidocaine and procaine selectively depress the Na^+ current in axons, the local anesthetics have been considered as simple blockers of the voltage-dependent Na^+ channel. However, during the last decades, data accumulated showing that there is more than one way by which drugs may alter axonal excitability and thus interfere with impulse conduction. A typical example of such a substance is the α_2-adrenoceptor agonist clonidine which increases within a low dose range the duration and intensity of peripheral nerve blocks induced by local anesthetics (Singelyn et al., 1992, Eisenach et al., 1996).

The most likely hypothesis for explaining this effect seems to be that clonidine acts directly on the conduction of small-diameter unmyelinated C-fibres which are responsible for pain transmission. This idea is supported by several in vitro observations. Thus, clonidine was as effective as procaine in decreasing the amplitude of compound action potentials in the frog sciatic nerve (Starke et al., 1972). Moreover, in the desheathed rat sciatic nerve mounted in a sucrose-gap chamber, both clonidine and guanfacine, another α_2-adrenoceptor agonist, produced a reversible concentration-dependent tonic and phasic block of conduction (Butterworth and Strichartz, 1993). Finally, the effects of lidocaine on C-fibres compound action potentials in the rabbit vagus nerve were enhanced by low dose of clonidine (Gaumann et al., 1992). Despite these encouraging in vitro studies, the exact mechanism by which clonidine interferes with impulse traffic in peripheral nonmyelinated nerves fibers and finally interrupts signal transmission have not yet been identified. Therefore, the main objective of this work was to investigate the mechanism of action of clonidine on impulse propagation in nonmyelinated C-fibers. Previously, we have shown that clonidine increases the hyperpolarizing afterpotential which follows a single compound action potential (Gaumann et al., 1994). In the present study, we focused our attention on the effects of clonidine on the electrophysiological properties of C-fibers during repetitive stimulation. We observed that the summation of the hyperpolarizing after-

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potentials in the presence of clonidine shifts the membrane potential toward more negative values, thus creating a hyperpolarized region of low conduction safety, where the local circuit currents provided by the propagating impulse might fail to depolarize the axonal membrane to the threshold value needed to open voltage-dependent Na^+ channels.

2. Materials and methods

2.1. Nerve preparation

Rabbits (New Zealand White rabbits, 2 ± 0.5 kg; Wullschleger, Vordemwald, Germany) were housed and treated according to the guidelines of the Committee of the Swiss Agency for Animal Protection which also approved the study protocol. In order to avoid exposing the animals to neuroactive drugs and to keep the vagus nerve intact, the rabbits were sacrificed by a pistol shot into the neck. This approach resulted in the immediate death of the animals due to the destruction of the brainstem. Both cervical vagus nerves were immediately removed, placed in cold Locke's solution (4°C) in the refrigerator before being used for the experiments within 2 days. Before starting the experiments, nerves were desheathed using scissors under a microscope.

2.2. Stimulation and recording by the sucrose-gap apparatus

The mean diameter of nonmyelinated axons in the rabbit vagus nerve is in the order of 0.6 μm, too small to allow for a direct intracellular or patch clamp approach. Therefore, in this study we have used the method of the sucrose-gap, which allows measurements of monophasic compound action potentials as well as of changes in the resting membrane potential with extracellular electrodes. (Stämpfli, 1954; Jirounek and Straub, 1971; Jirounek et al., 1981). The experimental set-up has been described previously (Robert and Jirounek, 1994). Briefly, the nerve was placed across three compartments which were tightly sealed off by thin rubber membranes. In one of the lateral test compartments, the nerve was superfused by the drugs to be tested, in the other lateral compartment plain Locke's solution was used for perfusion. These two compartments were isolated from each other by a central compartment which was perfused with isotonic sucrose solution. The perfusion was by gravity at about 15 ml h^{-1}. With a width of 6 mm and a space constant of approximately 0.9 mm^{-1}, the central compartment rendered electronic interactions between the two lateral compartments negligible (Jirounek, 1978). The recording Ag–AgCl electrodes were placed at the outflow of the test and control compartments. The nerve was stimulated (Grass S48 stimulator) by short (0.4 ms) supramaximal depolarizing pulses delivered through two circular silver electrodes located in the test compartment. For repetitive stimulation, trains of pulses at 15 Hz during 1 s were used. The recording electrodes were connected to a differential electrometer. The measured signal was digitized by an A/D converter, displayed on the monitor of a PC and stored on its hard disk. All experiments were performed at room temperature (21°C ± 1°C).

The efficiency of our measurements was estimated by recording the modifications in membrane potential produced by changes in the concentration of K^+ in the range of 20 to 100 mM, where the membrane behave as a K^+ electrode and by comparing the experimentally obtained values with those expected from the Nernst equation. For this set-up, a value of 0.61 ± 0.05 (mean ± S.E.M.) was calculated ($n = 15$). However, the attenuation of the compound action potential was more important, because of the temporal dispersion occurring in a population of fibers with different conduction velocities (Ritchie and Straub, 1956).

2.3. Stimulation protocol

Before starting the experiment, the nerve was superfused with Locke's solution in order to achieve stable baseline and reproducible compound action potentials. Then, the preparation was exposed to the drug to be tested over a period of 30 min. In experiments examining the pharmacological interactions of clonidine and yohimbine, the initial equilibrium period was followed by a first test phase of 30 min during which nerves were exposed to 10 mM yohimbine in Locke's solution in order to attain a new steady state condition. During the next 30 min, different concentrations of clonidine were tested. The recovery was assessed during wash-out period of 60 min.

The changes in compound action potentials (amplitude, time-to-peak and the area under the curve) were recorded and measured. Tonic block was defined as the percent reduction in the compound action potential as compared with its base-line amplitude under steady state conditions during single stimulation (Butterworth and Strichartz, 1990). Phasic block was defined as the additional decline in compound action potential amplitude occurring during a train of 15 Hz over 1 s. Maximum effect on compound action potential amplitude was generally obtained within 20 min of drug exposure. For determination of tonic block induced by single nerve stimulation, only the 30-min values were included in the calculation. Data were expressed as mean ± S.E.M. The results were compared by multiple unpaired *t*-tests with Bonferoni's correction. The level of significance was $p < 0.05$.

2.4. Drugs and solutions

Clonidine hydrochloride was purchased from Fluka, Buchs, Switzerland and yohimbine hydrochloride was obtained from Sigma, Buchs, Switzerland. All chemicals

were of reagent grade or better. The drugs were dissolved in freshly prepared Locke's solution composed of 154 mM NaCl, 5.6 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot H_2O$ · 5 mM D(+)-glucose-monohydrate and 10 mM *N*-2-hydroxyethyl-piperazine-*N*,-2-ethanesulfonic acid (HEPES) buffer. The pH was adjusted to 7.40 ± 0.05 by addition of 1 N NaOH.

3. Results

The administration of clonidine at concentrations above 400 μM caused a consistent slight depolarization of the membrane potential ranging from 0.6 ± 0.1 mV at 400 μM (mean ± S.E.M., $n = 3$) to 1.47 mV ± 0.45 at 1600 μM ($n = 4$). Yohimbine, on the other hand, had no significant effects on the membrane potential in the concentration range tested in this study (5–320 μM).

3.1. Effects of clonidine on single compound action potential

Single isolated compound action potentials were elicited at intervals of 5 min. Under control conditions in plain Locke's solution, the amplitude of an isolated compound action potential decreased by 15% ± 3% ($n = 4$) over 60 min (Fig. 1, inset). This decline in compound action potential amplitude was accompanied by a similar decrease in the area under the compound action potential of 11% ± 4% ($n = 4$) over 60 min and a slight slowing in conduction velocity as evidenced by an increase in time to the peak of the compound action potential.

Addition of clonidine to the perfusion solution induced a further dose-dependent decrease in compound action potential amplitude in close association with a similar reduction of the area under the compound action potential, a dose-dependent slowing of impulse conduction and an increase of the time-to-peak (Fig. 1).

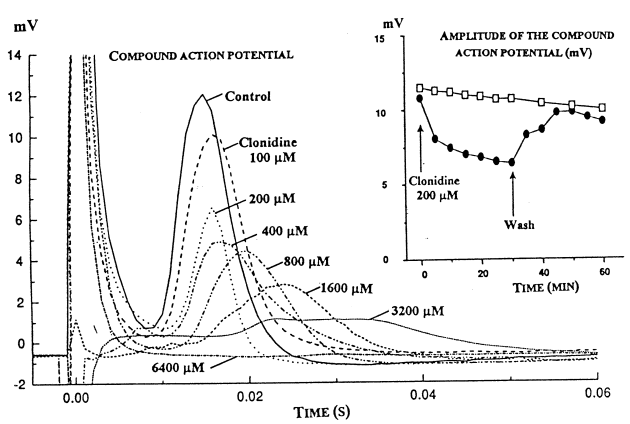

Fig. 1. Effects of clonidine on the compound action potential. The compound action potentials were elicited by short (0.4 ms) supramaximal depolarizing pulses delivered at intervals of 5 min. The inset shows the amplitude of the compound action potential in a control nerve (□) and in a nerve during and after treatment with 200 μM of clonidine (●).

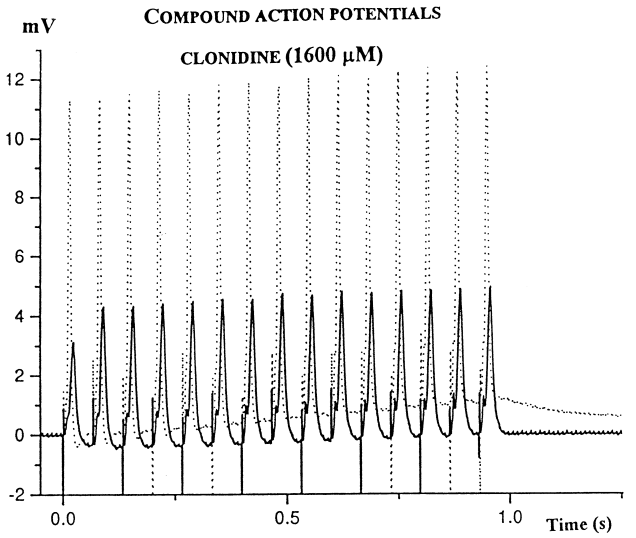

Fig. 2. Continuous recording of compound action potentials during repetitive stimulation at 15 Hz in a nerve under control conditions (dotted line) and in a nerve pretreated with 1600 μM clonidine.

3.2. Effect of clonidine during high-frequency stimulation

During 1 s repetitive stimulation at 15 Hz under control conditions in plain Locke's solution, a characteristic membrane depolarization of approximately 1 mV occurred without any perceptible change in the amplitude of the compound action potential (dotted line in Fig. 2).

In the lower dose range of clonidine (≤ 800 μM), a train at 15 Hz applied for 1 s did not further affect the compound action potential amplitudes observed during single stimulation. On the other hand, in nerves exposed to higher concentrations of clonidine, there was a step increase of compound action potential amplitudes between the 1st and 2nd volley (Fig. 2). This elevation in amplitude added up to 40% ± 12% (1600 μM; $n = 3$) and 52% (3200 μM; $n = 1$) to the compound action potential amplitude and was paralleled by an increase in the area under the curve of the compound action potential. Superposition of a pair of control and test recordings (1600 μM clonidine) is shown as an example (Fig. 2). The figure shows furthermore that clonidine abolished the activity-induced depolarization. As a consequence, the membrane potential following periods of activity was shifted to more negative values.

In Fig. 3 we show data calculated from a representative recording obtained during stimulation of the nerve at 15 Hz in plain Locke's solution and from recordings obtained 5, 15 and 30 min after adding low concentrations of clonidine (100 and 200 μM) to the perfusion solution. In all control experiments ($n = 9$), activity induced depolarization of the membrane developed almost immediately

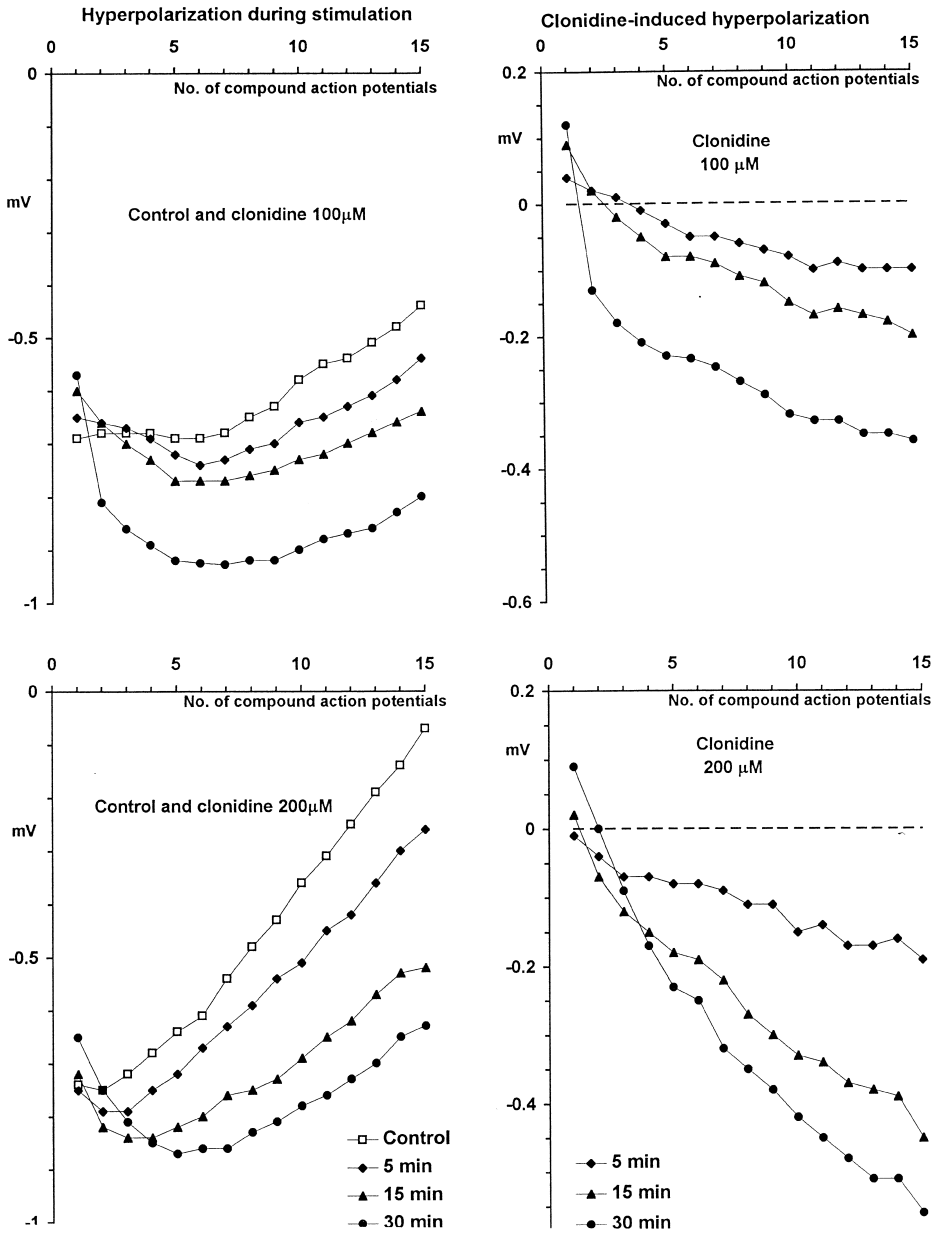

Fig. 3. Evolution of the membrane potential during repetitive stimulation in control conditions (□) and 5 (♦), 15 (▲) and 30 min (●) after application of clonidine 100 μM (at the top) and 200 μM (at the bottom). The points on the graphics at the left correspond to the difference between the resting membrane potential before the train and the membrane potential at the base of the compound action potentials during the train. The curves presented at the right show the clonidine-induced changes in membrane potential. They were obtained by subtracting the values of membrane potential observed during stimulation in the presence of clonidine from that observed in control experiments.

with the onset of stimulation. When clonidine was added to the perfusion solution, this membrane depolarization was preceded by a hyperpolarization which developed during the first three to 10 spikes of the train. Its duration depended on both the concentration of clonidine and the time of contact of the nerve with clonidine. The membrane potential during stimulation in presence of clonidine was thus invariably negative to that observed in controls. The curves presented on the right show this hyperpolarization in absolute values. They were obtained by subtracting the values of membrane potential observed during stimulation in presence of clonidine from that observed in control experiments.

3.3. Effect of clonidine during low-frequency stimulation

Nerve fibres during stimulation are known to undergo small changes in membrane potential that outlast the action potential and thus modulate excitability (Stys and Waxman, 1994). As described previously (Jirounek et al.,

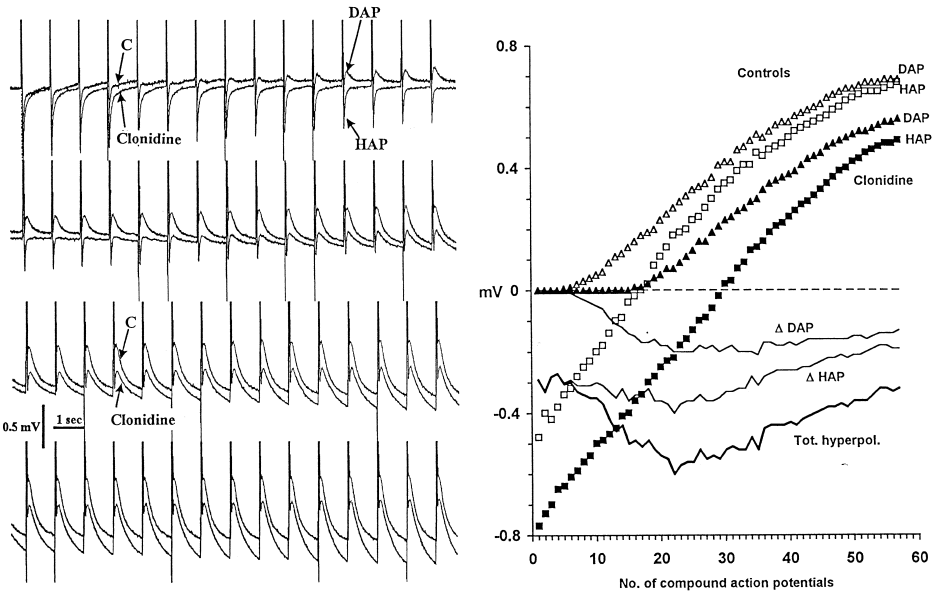

Fig. 4. At the left are superposed recordings of hyperpolarizing and depolarizing afterpotentials (DAP and HAP, respectively), observed during low-frequency stimulation from the same nerve before (C) and after addition of 200 μM clonidine. At the right are shown the amplitudes of the depolarizing afterpotentials (△) and hyperpolarizing afterpotentials (□) in controls and after addition of clonidine (▲) and (■), respectively. The clonidine-induced changes in membrane potential by the depolarizing and hyperpolarizing afterpotentials are represented by the solid line, as well as their addition, which represents the total clonidine-induced hyperpolarization.

1991), a single compound action potential in the rabbit vagus nerve is followed by a hyperpolarizing and a depolarizing afterpotential. The results presented above suggest that the clonidine-induced hyperpolarization might be due, at least in part, to a modulation of one or both these afterpotentials by clonidine.

On the left of Fig. 4 we present a superposition of afterpotentials that were continuously recorded at a rapid sampling rate during low-frequency (1 Hz) stimulation before and after application of 200 μM clonidine. The compound action potentials preceding each afterpotential were truncated because of the high amplification of the signal. These recordings show that during low-frequency stimulation in presence of clonidine, depolarizing afterpotentials appeared about 15 s later than in controls and that during the whole stimulation period hyperpolarizing afterpotentials were enhanced. The cumulative effect of both these actions resulted in a progressive hyperpolarization of the nerve during low-frequency stimulation. On the right of Fig. 4, the development of both afterpotentials is shown as well as the total change in membrane potential due to their conjoint effect.

3.4. Experiments with yohimbine

Clonidine is a well known α_2-adrenoceptor agonist. Therefore, in the next series of experiments, we tested whether the effects of clonidine could be antagonized by pretreatment of the nerve with the α_2-adrenoceptor antagonist yohimbine. We first assessed the effects of yohimbine alone on the compound action potentials evoked by a single isolated stimulus. The results showed that yohimbine is a potent blocker of impulse conduction per se, inducing a dose-dependent decrease in compound action potential amplitude, a decrease in the area under the curve of the compound action potential and an increase in the time-to-peak. In experiments with repetitive stimulation, yohimbine induced a dose-dependent progressive decrease in compound action potential amplitudes (Fig. 5), which was significant at doses above 10 μM. Using the 1-s

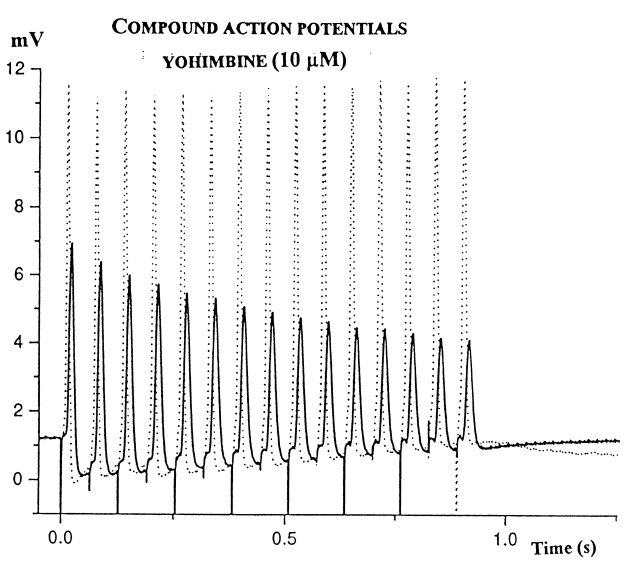

Fig. 5. Recordings of compound action potentials during a 1-s train at 15 Hz in a nerve before (dotted line) and after application of yohimbine (10 μM).

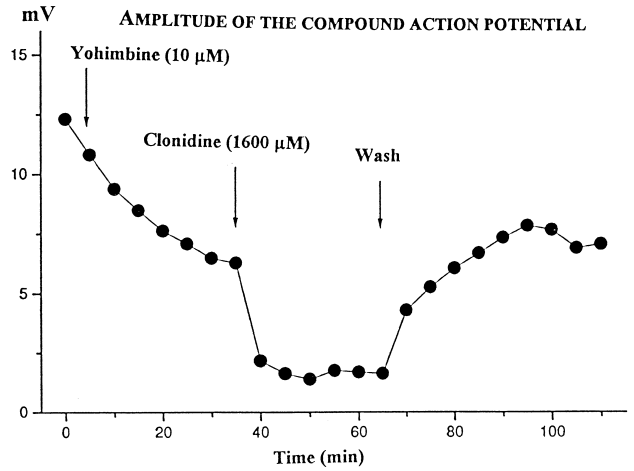

Fig. 6. Effect of yohimbine (10 μM) and yohimbine + clonidine on the amplitude of the compound action potential during single stimulation. The compound action potentials were elicited by short (0.4 ms) supramaximal depolarizing pulses delivered at intervals of 5 min. At the time labeled wash both yohimbine and clonidine were withdrawn from the superfusion solution.

15-Hz stimulation pattern, an average decline of compound action potential amplitude was observed extending from 2% ± 2% (5 μM; $n = 3$), 14% ± 6% (10 μM; $n = 3$), 60% ± 10% (20 μM; $n = 3$), 67% ± 6% (40 μM; $n = 3$), 77% ± 8% (80 μM; $n = 3$), 95% (160 μM; $n = 2$) to 98% (320 μM; $n = 2$). Of note, the ''frequency-dependent'' effects of yohimbine were not completely reversible even after a 60-min washing period.

In nerves pretreated during 30 min with a solution containing 10 μM yohimbine, experiments with single compound action potentials showed that addition of clonidine did not antagonize the inhibition of impulse conduction attributable to yohimbine, but even enhanced its inhibitory action (Fig. 6). Again, this effect on compound action potential was associated with a dose-dependent slowing of impulse conduction, as evidenced by an increase in time-to-peak.

During repetitive stimulation of nerves pretreated during 30 min with 10 μM yohimbine, supplemental clonidine exerted a dual action on trains of 15 compound action potentials which was characterized by a slight use-dependent inhibition in the lower concentration range (22% ± 9% and 16% ± 3% for 100 and 200 μM clonidine, respectively; $n = 3$), whereas a step increase of the compound action potential elevation was observed for higher drug concentrations (46% and 65% for 800 and 1600 μM clonidine, respectively; $n = 2$, data not shown).

4. Discussion

4.1. Nerve preparation and conditions of recording

In the rabbit vagus nerve, the group of slow conducting C-fibers comprises almost 90% of the total fiber population and thus represents the vast majority of its fibers (De Neef et al., 1982). The mammalian nociceptive system is based on afferent axons belonging to myelinated A_δ fibers and to some subpopulations of unmyelinated C-fibers. Therefore, pharmacological modulation of impulse traffic along the unmyelinated C-fibers may be, to some extent, equated with antinociceptive activity of the drugs that were tested (see Butterworth and Strichartz, 1990, for review).

Because of the small diameter of the nonmyelinated axons of vagus C-fibers, the use of patch clamp or intracellular electrodes was impossible. Therefore, for all experiments, a sucrose-gap apparatus was utilized, the design of which restricted optimum recording conditions to unmyelinated C-fibers. With a distance of 4 mm between the stimulation and recording electrodes, compound action potentials in slowly conducting C-fibers were clearly separated from faster conducting myelinated A- and B-fibers. In our set-up, an average C-fiber conduction velocity of 0.25 m/s was calculated under control conditions. This rather low value is almost certainly due to the specific experimental conditions that included room temperature as a part of the study protocol. The principal reason for conducting this study at room temperature was the increased stability of the preparation and the decrease in metabolic demands. We did not study whether low temperature per se had an influence on clonidine's action on impulse conduction. However, cooling has been shown to increase the potency of some local anesthetics (Bradley and Richards, 1984; Butterworth and Strichartz, 1990); therefore the possibility that room temperature may have enhanced the action of clonidine cannot be excluded.

4.2. Effects of clonidine on single spikes

Our data show that clonidine produced a tonic concentration-dependent conduction block in rabbit vagus C-fibers at a calculated ED_{50} of 647 μM. This result is in good agreement with our previous findings (Gaumann et al., 1992) which showed a 54% decrease in rabbit vagus C-fibers compound action potential at 500 μM clonidine. Furthermore, clonidine produced a concentration-dependent inhibition of compound action potentials in both myelinated and unmyelinated rat sciatic nerve fibers with an ED_{50} of 2.0 ± 0.8 mM for myelinated fibers and 0.45 ± 0.12 mM for nonmyelinated C-fibers, respectively (Butterworth and Strichartz, 1993).

The concentration-related inhibition of the amplitude of the compound action potential was mirrored by a parallel decrease in the area under the curve of the compound action potential, ranging from 11% ± 1% to 14% ± 2% at low concentrations (100 to 400 μM) to almost 100% at the highest concentration tested (6400 μM). The decrease in the area under the curve of the compound action potential indicate that the total number of conducting fibers was reduced – an observation suggestive of a true local anes-

thetic effect. Before almost complete extinction of electrical response to stimulation, there was also a progressive increase in latency, suggestive of both a slowing in conduction velocities and an increase in disparity among nerve fibers. The latency was increased by 14% ± 3% to 36% ± 5% within the lower concentration range (100 to 400 μM), whereas latencies of 47% ± 10% (800 μM) and 71% ± 11% (1600 μM) were observed in the higher concentration ranges. All these results show that clonidine possesses anesthetic properties, but only at much higher concentrations than those used clinically as admixture to lidocaine or other local anesthetics in order to prolong the peripheral nerve block.

4.3. Repetitive stimulation and potential mechanism of action

Gaumann et al. (1994) have shown that clonidine at subblocking concentrations (0.05–50 μM) increases the amplitude of the hyperpolarizing afterpotential which follows a single compound action potential in the rabbit vagus nerve (Jirounek et al., 1991). Here we extend these earlier observations by demonstrating that these clonidine-induced changes in afterpotentials are markedly enhanced during repetitive stimulation and that, in addition to changes in their magnitude, clonidine alters also their time-course. These observations are of importance, mainly in view of studies on partially blocked nerves, where failure of function occurs even when fibers are still capable of conducting impulses in response to electrical stimulation. For instance, sensation of skin cooling may be absent, even though A_δ fibers still respond to electrical stimulation (Mackenzie et al., 1975). It seems likely that such a failure of conducting fibres in transmitting information is due to alterations in membrane excitability that follows impulse conduction (Raymond, 1992). The different types of afterpotentials that follow action potentials in the vagus nerve (Jirounek et al., 1991) are responsible for oscillations of the membrane potential through a sequence of excitability changes, termed the recovery cycle (Stys and Waxman, 1994). This sequence far outlasts the duration of the action potential and modulates the excitability of an axon during several hundreds of milliseconds after the action potential has subsided (Raymond and Letvin, 1978). In an elegant study using sciatic frog nerves exposed to subblocking concentrations of local anesthetics, transient decreases in excitability and conduction velocity were observed in combination with a lower intrinsic propensity for oscillations of membrane excitability after impulse propagation (Raymond, 1992). Using computer simulation for mathematical modeling of impulse propagation, it has been shown that these effects were important enough to cause a distortion of the signal transmission without actually blocking impulse conduction whereby discharge patterns for peripheral fibers encoding sensory input information codes were degraded (Raymond, 1992). Here we showed that, in contrast to the effect of clonidine on single compound action potentials, the action of clonidine on afterpotentials during trains of impulses occurred at concentrations close to those used clinically. All these findings, together with the results presented in our study, strongly suggest that the ability of clonidine to potentiate the action of a local anesthetic is due to its action on afterpotentials thereby altering the axonal recovery cycle (Raymond and Letvin, 1978; Swadlow et al., 1980).

4.4. Are α_2-adrenoceptors involved?

With reference to studies which demonstrated that clonidine had an inhibitory action on peripheral nerve conduction, both interaction with Na^+ channels (Gaumann et al., 1992) or binding to α_2-adrenoceptors (Butterworth and Strichartz, 1993) were considered as possible mechanism. However, no data were available at that time indicating whether the effects of clonidine would be antagonized by α_2-adrenoceptor agonists.

Our results show that exposing the nerves to yohimbine (10 μM) did not reverse the inhibitory effects of clonidine on impulse propagation as assessed by the decrease in compound action potential amplitude, area under the curve of the compound action potential and time-to-peak. On the contrary, exposure to increasing concentrations of clonidine in combination with yohimbine resulted in additional inhibiting effects which were clearly concentration-dependent and exceeded those observed with clonidine alone. Failure to counteract the action of clonidine on impulse generation and propagation by yohimbine may be explained by the extremely low density of α_2-adrenoceptors on the rabbit vagus nerve as evidenced by lack of specific binding during autoradiographic examination (Erne-Brand et al., in preparation). This finding together with our results implies that the observed effects of clonidine probably relies on mechanisms not mediated by α_2-adrenoceptors. Consequently, it remains unclear at which target the two compounds act, or whether they act at the same site.

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