Br. J. Pharmac. (1985), **85**, 143–147

Clonidine and presynaptic adrenoceptor theory

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1 The effects of clonidine, a presumed selective presynaptic α_2-adrenoceptor agonist or partial agonist, were examined in guinea-pig atria.

2 Split left atrial preparations were stimulated transmurally at 2 Hz with 100 pulses of 0.5 ms duration and the efflux of ^{3}H-transmitter determined.

3 Clonidine inhibited efflux at 3×10^{-8}M to 3×10^{-7}M by about 30%. Yohimbine, at a concentration (10^{-6}M) which caused a 3 fold increase in the release of ^{3}H-transmitter during field stimulation, did not alter the ability of clonidine to inhibit transmitter efflux.

4 At 10^{-6}M clonidine alone had no significant effect on the stimulation-induced efflux of ^{3}H-transmitter, but in the presence of yohimbine (10^{-6}M), inhibited efflux by over 50%.

5 The inhibitory effect of noradrenaline (10^{-6}M) on ^{3}H-transmitter efflux was antagonized by clonidine at 10^{-6}M but not at 10^{-8}M, although neither concentration of clonidine alone inhibited transmitter efflux.

6 The present findings indicate that the effects of clonidine on the efflux of noradrenaline from sympathetic nerves cannot be accommodated within the currently held view that the compound is an agonist or partial agonist on presynaptic α_2-adrenoceptors. It appears that clonidine has multiple sites of action few of which are antagonized by a concentration of the prototypical presynaptic antagonist yohimbine, which enhances efflux 3 fold.

Introduction

The hypothesis that adrenergic neurosecretion is modulated by inhibitory presynaptic α_2-adrenoceptors subserving a feedback function relies, to a substantial extent, on the suitability of clonidine as a prototype of an α_2-adrenoceptor agonist. Previous work (Kalsner, 1982a) has shown that oxymetazoline, another imidazoline derivative presumed to have specific α_2-adrenoceptor properties, has actions not attributable to adrenergic mechanisms. The present study was done to evaluate clonidine as a presynaptic adrenoceptor agonist.

Methods

Left atria were removed from guinea-pigs, trimmed of adherent tissue and split lengthwise into two halves. They were incubated for 60 min with (−)-[7,8-^{3}H]-noradrenaline (10 μCi ml^{-1}, $7.6-10.0 \times 10^{-7}$M) in 4.0 ml of oxygenated (5% CO_2 in O_2) Krebs solution (composition in mM: NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1 and glucose 7.8) containing ethylenediamine tetraacetic acid (disodium salt; 0.03 mM) to retard heavy metal catalyzed oxidation of catecholamines (Kalsner, 1979). The tissues were then washed with fresh Krebs solution and mounted under 1 g tension between platinum wire electrodes. Tissues were superfused continuously with warmed (37°C) and oxygenated Krebs solution at a flow rate of 5 ml min^{-1}. Cocaine (8.8 or 29 μM, usually 8.8 μM) and normetanephrine (10 μM) were present routinely in the Krebs solution to block neuronal and extraneuronal uptake processes. Yohimbine and clonidine, when used, were made up in stock concentrations and added directly to the appropriate reservoirs of Krebs solution.

After a 90 min equilibration period, each tissue was stimulated transmurally with trains of 100 pulses of 0.5 ms duration and supramaximal voltage at the desired test frequencies. For example, to assess the effects of clonidine on the efflux of ^{3}H-transmitter, matching atrial halves were stimulated initially with 100 pulses at 2 Hz and 9 min later the stimulation was repeated so that the two efflux values could be averaged to obtain a single mean value for S_1. This procedure of duplicating each stimulation and averag-

ing the two values was utilized throughout the experiment. Sets of 2 stimulations each were repeated three more times in the course of the experiment (S_2, S_3, S_4), in the presence of progressively increasing concentrations of clonidine administered to one half of each atrium. The other half of each atrium served as a control for the treated half and was subjected to identical stimulations but it was not exposed to clonidine at any time during the course of the 4 stimulation cycles. Other stimulation protocols used in the present experiments are described at relevant points in the text. A maximum of 4 sets of stimulations at 30 min intervals (8 pulse trains of 100 pulses each) was received by any given atrial preparation.

The efflux of [^{3}H]-noradrenaline from the preparations was determined by counting 1.0 ml aliquots of the 15.0 ml superfusate collected in vials by a fraction collector which rotated every 3 min. The aliquots were transferred to vials containing 10 ml of Aqueous Counting Scintillant (Amersham) and counted in a Beckman LS-230 counter with automatic external standardization to determine efficiency. Basal efflux is expressed as d.p.m. and calculated as the total radioactivity detected in the 3 min sample collected immediately before stimulation. Transmural stimulation was always begun at the onset of a 3 min collection period. Stimulation-induced efflux was calculated as the difference between basal efflux and the total d.p.m. in the 3 min sample collected during and immediately after stimulation. As described above, each stimulation frequency was repeated twice and the efflux values averaged. Mean data are presented with their standard errors and Student's t test was used for all comparisons and a P value of less than 0.05 was considered significant.

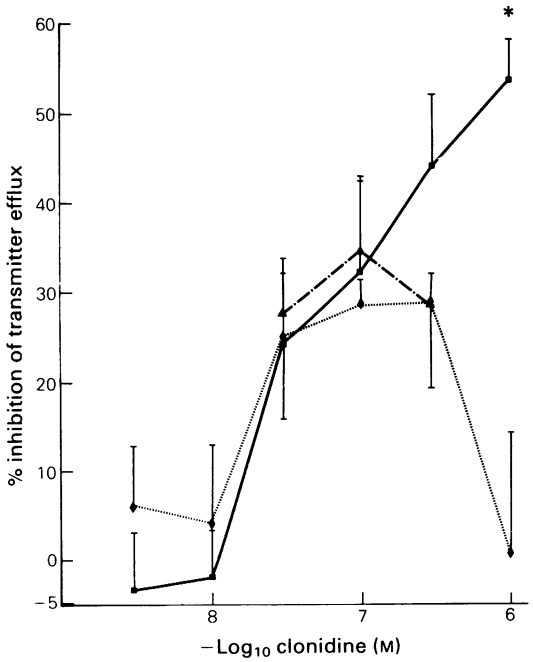

Figure 1 Effects of clonidine on the stimulation-induced efflux of [^{3}H]-noradrenaline from field stimulated guinea-pig atrial halves in the presence and absence of yohimbine (10^{-6}M) and cocaine (8.8×10^{-6}M). The percentage inhibition of ^{3}H-efflux induced by clonidine, at each test concentration, was determined as described in the text. Clonidine concentration-effect curve in the absence (◆.....◆) (5–7 atria at each point) and in the presence (■——■) (5–7 atria) of yohimbine and in the absence of yohimbine and cocaine (▲-.-.-▲) (6 atria).

Drugs

The drugs used and their sources were: clonidine hydrochloride (Boehringer Ingelheim), (−)-noradrenaline bitartrate hydrate (Calbiochem), yohimbine hydrochloride (Nutritional Biochemicals), (±)-normetanephrine hydrochloride (Sigma Chemical Co.) and cocaine hydrochloride (BDH Chemicals). The radioisotope, 1-[7,8-^{3}H]-noradrenaline hydrochloride (specific activity 10 to 13 Ci mmol^{-1}) was obtained from the Radiochemical Centre, Amersham. It was diluted to a stock concentration of 100 μCi ml^{-1} in (−)-ascorbic acid solution (50 μg ml^{-1}) and stored at 4°C in 5 ml aliquots under nitrogen gas. To obtain a final concentration of 10 μCi ml^{-1} (7.6 to 10.0×10^{-7}M) in the incubation medium, 0.4 ml of this stock solution was added to 3.6 ml of Krebs solution.

Results

Guinea-pig left atria which had been cut into 2 equivalent portions (Furchgott *et al.*, 1971) responded to field stimulation with 100 pulses at 2 Hz with effluxes of ^{3}H-transmitter well above background levels, as described previously (Kalsner *et al.*, 1980; Kalsner, 1983).

The efflux of tritium with 100 pulses averaged $30.3 \pm 2.7 \times 10^3$ d.p.m. above pre-stimulation basal values in 26 tests with 13 control atrial halves during the initial set of stimulations (S_1) and, in the absence of drug treatment ,declined to 24.1 ± 1.8, 21.3 ± 4.5 and $19.7 \pm 1.4 \times 10^3$ d.p.m. during subsequent stimulations (S_2, S_3 and S_4 respectively) yielding corresponding ratios of 0.81 ± 0.03, 0.73 ± 0.04 and 0.68 ± 0.04 for S_2/S_1, S_3/S_1 and S_4/S_1, respectively.

Clonidine

To rule out time dependent changes in efflux not related to the action of clonidine and to assess more

precisely assess the effects of clonidine, efflux values in the presence of clonidine during S_2, S_3 or S_4 were compared to initial efflux values (S_1) obtained in the same tissues in the absence of clonidine. The ratios obtained (S_n/S_1) were then compared with similar ratios for the matched atrial halves not treated with clonidine at any time during the experiment (see Methods) and expressed ultimately as a percentage inhibition of transmitter efflux as shown in Figure 1.

Clonidine at 3×10^{-11}, 1×10^{-10}, 3×10^{-10}, 3×10^{-9} and 1×10^{-8}M did not significantly inhibit efflux, but concentrations of 3×10^{-8}M, 1×10^{-7}M and 3×10^{-7}M clearly did. The magnitude of inhibition, however, was moderate, about 30%, and was similar in the presence or absence of cocaine (8.8×10^{-6}M) (Figure 1). Clonidine, at the higher concentration of 10^{-6}M did not significantly inhibit transmitter efflux (Figure 1), a surprising finding which was confirmed by experiments in an additional 4 sets of tissues.

Yohimbine and clonidine

Yohimbine increases stimulation-induced efflux of ^{3}H-transmitter and is widely assumed to do so by blockade of presynaptic α-receptors with a consequent interruption of on-going auto-inhibition. In the present experiments, the mean efflux value during S_1 with 100 pulses in the presence of yohimbine (10^{-6}M) was $84.8 \pm 4.1 \times 10^3$ d.p.m. in 48 tests on 24 tissues compared with $30.3 \pm 1.7 \times 10^3$ d.p.m. in 52 tests on 26 tissues without the antagonist present. This significant ($P < 0.001$) increase in efflux, to 2.8 times control values, confirmed the efficacy of the concentration of yohimbine used here, and is the maximal increment obtainable with the compound in guinea-pig atria under the present experimental conditions (Kalsner, 1983; Kalsner & Quillan, 1984).

To assess directly the relationship between yohimbine-induced enhancement of tritium efflux and clonidine-induced inhibition, the values for stimulation-induced efflux with 100 pulses at 2 Hz were first obtained (S_1) in matched atrial halves both pretreated for 30 min with yohimbine (10^{-6}M). Fifteen minutes later, without washout of yohimbine, one of each set of atria was exposed to progressively increasing concentrations of clonidine during S_2, S_3 and S_4. The stimulation sets (S_2, S_3 and S_4) were performed at 30 min intervals and the ratios (S_n/S_1) calculated as described above for experiments with no yohimbine present.

Clonidine clearly inhibited efflux in the presence of yohimbine. Comparisons of the percentage inhibitions of efflux by the agonist (3×10^{-8} to 3×10^{-7}M) in the presence of the antagonist with those in its absence revealed no significant differences (Figure 1). However, clonidine at 10^{-6}M, inhibited efflux substantially (54%) in the presence of the antagonist even though it did not do so in its absence; this inhibition was greater than that observed with any test concentration of the agonist, without yohimbine present.

Clonidine and noradrenaline

The effects of noradrenaline (1.8×10^{-6}M) and clonidine (10^{-8} or 10^{-6}M), alone or in combination, were determined by comparison of the efflux ratios (S_n/S_1) in the tissues exposed to one or both agonists during S_n to the similar ratios in the tissues not treated with either of the agonists at any point during the experiment, and the value in the treated tissue expressed as a percentage of the control. As shown in Figure 2, noradrenaline inhibited ^{3}H-efflux substantially, and

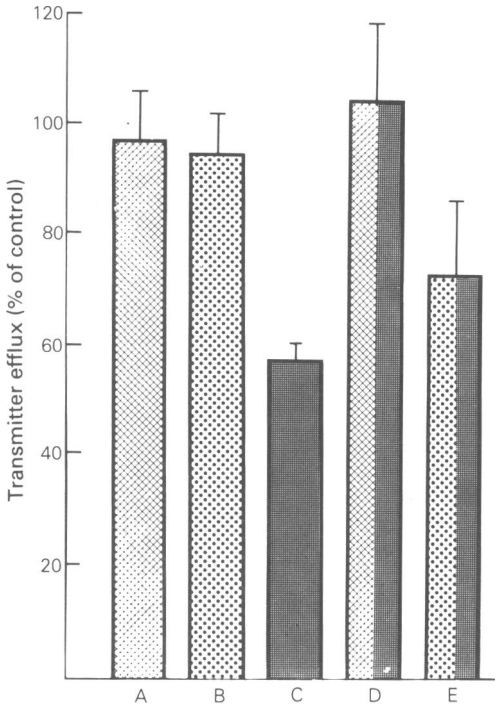

Figure 2 Noradrenaline and clonidine interactions on stimulation-induced efflux of ^{3}H-transmitter in guinea-pig atrial halves. Each column represents the mean transmitter efflux, with vertical lines showing s.e.means, during field stimulation with 100 pulses, as % of control values. Effect in the presence of noradrenaline (1.8×10^{-6}M) alone (10 atria) (column C); in the presence of clonidine 10^{-8}M (10 atria) (column B) or 10^{-6}M (10 atria) (column A); and in the simultaneous presence of noradrenaline (1.8×10^{-6}M) and clonidine 10^{-8}M (4 atria) (column E) or 10^{-6}M (5 atria) (Column D). Clonidine (10^{-6}M) + noradrenaline (1.8×10^{-6}M) vs noradrenaline (1.8×10^{-6}M), $P < 0.01$. Noradrenaline vs clonidine (10^{-8}M) or (10^{-6}M), $P < 0.01$.

this inhibition was not significantly diminished by the simultaneous presence of clonidine (10^{-8}M). The higher concentration of clonidine (10^{-6}M) antagonized significantly the inhibitory effect of noradrenaline, although it alone had no obvious effect on ^{3}H-efflux.

Discussion

Clonidine is considered by a number of investigators to be the prototypical presynaptic receptor agonist. However, the compound, originally developed as a topical vasoconstrictor, has a complex profile of action which makes such a status undeserved. The imidazoline derivative has been found to increase (e.g. Stjarne, 1975), decrease (e.g. Medgett *et al.*, 1978), or to be without significant effect on the stimulation-induced efflux of noradrenaline. By comparison, noradrenaline itself consistently decreases neurotransmitter secretion in an abundant variety of preparations (See review by Kalsner, 1982b).

Attempts have been made to reconcile the disparate observations about the effects of clonidine on transmitter efflux and retain the compound's actions within the framework of presynaptic theory by classifying it as a partial agonist (Medgett *et al.*, 1978; Sullivan & Drew, 1980). This would seem to allow for a variety of apparently contradictory effects on efflux contingent on the relative concentrations of agonist and antagonist. However, as pointed out by Ariëns *et al.* (1964), partial agonists exhibit predictable effects in the presence of full agonists and these expectations need to be applied to clonidine.

The present study examined the effects of clonidine on ^{3}H-efflux in guinea-pig atria and revealed a complex profile not easily explicable in terms of partial agonist action. Although clonidine only moderately inhibited stimulation-induced efflux, the effect did not increase over a 10 fold concentration range, and a higher concentration of the compound (10^{-6}M) had no inhibitory effect at all. This is not in keeping with the concentration-effect profile of a partial agonist with a lower intrinsic activity than noradrenaline, in the presence of a fixed amount of neurally liberated full agonist (i.e. noradrenaline). The inhibitory effect of a partial agonist on efflux should, with increasing concentration, progress towards and then sustain a plateau value equivalent to the maximal effect achievable with the compound's limited intrinsic activity (E_{B_m}). This maximal effect should have occurred when all the receptors were occupied by clonidine. Instead, a loss of efficacy with increased concentration was observed.

In the presence of the presumed selective presynaptic α-adrenoceptor competitive antagonist yohimbine, inhibition by a low concentration of clonidine should have been blocked and by higher concentrations substantially attenuated. Instead, yohimbine (10^{-6}M) failed to antagonize the inhibitory agonist action of clonidine at the lower concentrations (3×10^{-8} to 3×10^{-7}M) and paradoxically allowed the high concentration (10^{-6}M) to be more effective in inhibiting efflux than in the absence of yohimbine. According to receptor theory, and the dynamics of competition for a single occupation site, the agonist cannot produce a greater maximal response (E_{B_m}) in the presence of antagonist than in its absence. This points to a much more complex action than can be accommodated within presynaptic theory. This suggestion is made more likely by the additional finding that clonidine apparently competes for occupancy of the site through which noradrenaline inhibits transmitter efflux: although clonidine, at 10^{-6}M, did not itself inhibit or enhance transmitter efflux, except in the presence of yohimbine where it did the former, it prevented noradrenaline from inhibiting efflux. A likely explanation for the present results is the involvement of multiple loci in the actions of clonidine, few or none of which are antagonized by the presynaptic antagonist yohimbine.

It was found recently that yohimbine does not substantially reduce the inhibitory effects on ^{3}H-transmitter efflux of noradrenaline or the imidazoline derivative oxymetazoline in guinea-pig ureter (Kalsner, 1982a). Baker *et al.* (1984) noted the failure of yohimbine to antagonize substantially the inhibitory effects of clonidine in dog saphenous vein and also confirmed the relative ineffectiveness of yohimbine in blocking the efflux inhibitory effects of exogenous noradrenaline. Concern also has been expressed that we do not overlook other possible actions of clonidine unrelated to activation of presynaptic α_2-adrenoceptors (Stone & Taylor, 1978; Schmitt *et al.*, 1979; Karppanen, 1981; Kalsner, 1982b). These include histamine-like and opiate related actions. Although there is insufficient information in the present study to warrant a specific hypothesis on the locus or mechanism of clonidine action, it is clear that the currently held description of the compound as a partial agonist on neuronal α-adrenoceptors with dualistic properties of agonism and antagonism is inadequate to sustain it within the framework of presynaptic receptor theory.

This work was supported by grants from the Medical Research Council and the Ontario Heart Foundation. Mr James Carpenter provided excellent technical assistance.

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(Received September 18, 1984.
Revised December 4, 1984.
Accepted December 12, 1984.)