Br. J. Pharmacol. (1993), **108**, 336–341

Chloroethylclonidine: an irreversible agonist at prejunctional α_2-adrenoceptors in rat vas deferens

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1 The possibility that chloroethylclonidine (CEC) activates prejunctional α_2-adrenoceptors was studied in the isolated vas deferens of the rat. Tissues were stimulated electrically and both the stimulation-evoked overflow of tritium (after preincubation with [^{3}H]-noradrenaline) and the purinergic contraction component (isolated by prazosin 0.3 μM) were measured.

2 CEC (0.1–3 μM) concentration-dependently reduced the overflow of tritium evoked by trains of 6 pulses/100 Hz. The inhibition by CEC was not altered by prazosin (0.3 μM) but was prevented by pre-exposure to rauwolscine (0.3 μM). The inhibition, once established, did not fade upon washout of CEC, even when the washout fluid contained rauwolscine (0.3 μM).

3 CEC (0.1–3 μM) concentration-dependently reduced the purinergic component of contractions elicited by single pulses. The inhibition, again, was prevented by pre-exposure to rauwolscine (0.3 μM) and once established, did not fade upon washout of CEC, even when the washout fluid contained rauwolscine (0.3 μM).

4 CEC (3 μM) reduced the overflow of tritium evoked by 20 pulses/10 Hz, did not alter the overflow evoked by 100 pulses/10 Hz and increased the overflow evoked by 500 pulses/10 Hz.

5 CEC (3 μM) reduced the early peak, but increased the late plateau phase, of purinergic contractions elicited by 100 pulses/10 Hz.

6 It is concluded that CEC reduces the release of noradrenaline and a purinergic co-transmitter by irreversible activation of prejunctional α_2-adrenoceptors. CEC seems to be a partial α_2-agonist with an efficacy lower than that of noradrenaline. The prejunctional inhibitory effect limits the suitability of CEC for the characterization of postjunctional α_1-adrenoceptors mediating responses to sympathetic nerve stimulation.

Keywords: Chloroethylclonidine; rauwolscine; prejunctional α_2-adrenoceptors; α_1-adrenoceptor subtypes; co-transmission; purinergic transmission; irreversible agonism; rat vas deferens

Introduction

The clonidine derivative *N*-β-chloroethyl-*N*-methylaminomethylclonidine (chloroethylclonidine; CEC) was first described by Leclerc *et al.* (1980). The authors showed that CEC (i) inhibited brain synaptosomal binding of [^{3}H]-clonidine, (ii) caused a phentolamine-sensitive contraction of rat aorta that persisted during washout, (iii) caused a long-lasting blood pressure increase in pithed rats and (iv) lowered the blood pressure of anaesthetized rats upon intracerebroventricular injection. They concluded that CEC alkylates and thereby persistently activates α-adrenoceptors, 'the first example of an α-agonist with an irreversible effect'. Retrospectively, a long-lasting activation of α_1-adrenoceptors seems a likely interpretation, since only α_1-adrenoceptors mediate contraction in rat aorta (see Flavahan & Vanhoutte, 1986; Docherty, 1989). Activation of α_2-adrenoceptors also seems possible since this subtype mediates (much of) the central hypotensive effect of clonidine-like drugs (see Kobinger, 1986).

CEC was not studied further until several years later, when it was introduced as an irreversible α_1-adrenoceptor antagonist, and one that blocked the α_{1B} subtype selectively (Johnson & Minneman, 1987; Minneman *et al.*, 1988). Since then it has become a key compound to distinguish α_1 subtypes, including subtypes of postjunctional receptors mediating adrenergic responses to sympathetic nerve stimulation (Muramatsu, 1991; Sulpizio & Hieble, 1991; Mallard *et al.*, 1992). The possibility that the blockade by CEC of responses to nerve stimulation might in part be due to activation of prejunctional α_2-adrenoceptors has not so far been taken into account.

We investigated the effect of CEC on prejunctional α_2-adrenoceptors in the rat vas deferens. Both effects on tritium overflow from tissues prelabelled with [^{3}H]-noradrenaline and effects on purinergic neurogenic contractions were examined.

Methods

Male Wistar rats (240–340 g) were decapitated. The vasa deferentia were removed and cleaned of adherent tissue. Unless stated otherwise, the medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Tritium overflow

Vasa deferentia were split open longitudinally. Four pieces of about 10 mg each were cut from the prostatic portions. The eight pieces were incubated for 30 min in medium with reduced Ca^{2+} ($CaCl_2$ 0.2 mM; see Limberger *et al.*, 1992) containing 0.2 μM (−)-[^{3}H]-noradrenaline, specific activity 56.9 Ci mmol^{-1}. They were then rinsed, and one piece was transferred to each of six 0.16 ml superfusion chambers. The tissue was held by a polypropylene mesh between platinum wire electrodes 6 mm apart and was superfused with [^{3}H]-noradrenaline-free medium at 2 ml min^{-1} ($CaCl_2$ 2.5 mM). A Stimulator I (Hugo Sachs Elektronik, Hugstetten, Germany) operating in the constant voltage mode was used for electrical stimulation (1 ms pulse width, 36 V cm^{-1} voltage drop between the electrodes of each chamber, yielding a current of 60 mA). An initial stimulation period (180 pulses, 1 Hz) was applied after 30 min of superfusion; it was not used for

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determination of tritium overflow. The superfusate was collected in 2 or 10 min periods from 116 min of superfusion onwards. Two different stimulation protocols were then used. In the first protocol, tissues were stimulated five times (S_1 to S_5), after 120, 146, 172, 198 and 224 min of superfusion; each of the five stimulation periods consisted of a train of 6 pulses/100 Hz. In the second protocol, tissues were stimulated twice (S_1 and S_2), after 120 and 150 min of superfusion; each of the two stimulation periods consisted of a train of either 20, 100 or 500 pulses at a frequency of 10 Hz; S_1 and S_2 had the same pulse number in a single piece of tissue. At the end of an experiment, tissues were solubilized in 1 ml Soluene-350 (Packard Instrument, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized tissues by liquid scintillation spectrometry.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of outflow, min^{-1}). The stimulation-evoked overflow was calculated as the difference between the total outflow of tritium in the 4 min (first protocol) or 6 min (second protocol) following the start of a stimulation period, and the estimated basal outflow during this time; the basal outflow was assumed to decline linearly from the 2 min interval before, to the interval 4–6 min (first protocol) or 6–8 min (second protocol) after, the start of stimulation; the difference (nCi) was expressed as a percentage of the amount of tritium in the tissue (nCi) at the start of stimulation. For further evaluation of basal efflux, ratios were calculated of the fractional rate of outflow in the 2 min before each stimulation period S_n (b_n) over that in the 2 min before S_1 (b_1). Ratios S_n/S_1 were calculated for further evaluation of the stimulation-evoked overflow.

Contraction

The vasa deferentia were suspended vertically in an organ bath with 5.7 ml medium. The medium contained prazosin (0.3 μM) in order to isolate the purinergic contraction component. The bath fluid was exchanged every 13 to 26 min. The lower end of the vas deferens was fixed and the upper end attached to an isometric force transducer (K30; Hugo Sachs Elektronik) under an initial tension of 9.8 mN. The tissues were allowed to relax to a resting tension of approximately 3 mN during a 60 min equilibration period. This final resting tension remained constant during the course of the experiment. The tension was recorded on a Graphtec thermal pen recorder (Ettlingen, Germany). A Stimulator II (Hugo Sachs Elektronik) operating in the constant current mode was used for electrical stimulation (0.3 ms pulse width, current strength 100 mA). The platinum electrodes were located at the top and the bottom of the organ bath. Two protocols were used. In the first protocol, tissues were stimulated five times (S_1 to S_5), 60, 86, 112, 138 and 164 min after the preparation had been set up; each of the five stimulations consisted of a single pulse. In the second protocol, tissues were stimulated three times (S_1 to S_3), 60, 90 and 120 min after the preparation had been set up; each of the three stimulation periods consisted of a train of 100 pulses/10 Hz. In the case of biphasic contractions (100 pulses/10 Hz), amplitudes of both the initial and the secondary phase were measured. Amplitude ratios S_n/S_1 were calculated for further evaluation.

Some additional experimental details are described in the Results section.

Materials

The following drugs were used: chloroethylclonidine dihydrochloride (purity > 99.9%; Biotrend, Köln, Germany); clonidine hydrochloride (Boehringer, Ingelheim, Germany); (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 56.9 Ci $mmol^{-1}$ (Du Pont, Dreieich, Germany); prazosin hydrochloride (Pfizer, Karlsruhe, Germany); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); suramin hexasodium salt (Bayer, Wuppertal, Germany); tetrodotoxin (Sigma, Deisenhofen, Germany). Tetrodotoxin was dissolved in 0.1 M sodium acetate buffer pH 4.85. Other drugs were dissolved in distilled water. In tritium overflow experiments, drug solutions were added to the superfusion medium reservoir; in contraction experiments, they were added to the organ bath in volumes not exceeding 30 μl.

Statistics

The effect of a drug, added after S_1, on an S_n/S_1 ratio is expressed as a percentage of the average S_n/S_1 value obtained in a control group without addition of this drug but otherwise treated identically. For example, the effect of CEC (1 μM) added before S_4 in the continuous presence of rauwolscine (0.3 μM), on S_4/S_1 is expressed as a percentage of the average S_4/S_1 value obtained in experiments with rauwolscine alone.

Arithmetic means ± s.e.mean are given throughout, except for IC_{50} values which are geometric means with 95% confidence intervals (c.i.). IC_{50} values of CEC were interpolated as the concentrations producing 50% inhibition. Differences between means were tested by the Mann-Whitney test. They were taken to be statistically significant when the error probability was $P < 0.05$. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Results

Tritium overflow

In the first series of experiments, vasa deferentia prelabelled with [^{3}H]-noradrenaline were stimulated electrically five times, each stimulation period (S_1 to S_5) consisting of a train of 6 pulses/100 Hz. In the absence of drugs, the fractional rate of tritium outflow immediately before S_1 (b_1) was $0.00133 \pm 0.00002\ min^{-1}$, and the overflow evoked by S_1 amonted to $0.043 \pm 0.002\%$ of the tritium content of the tissue ($n = 50$). The evoked overflow remained approximately constant from S_1 to S_5 in control experiments without any drug (for example, ratio $S_5/S_1 = 1.10 \pm 0.13$; $n = 7$). Tetrodotoxin (0.3 μM), when added 20 min before S_5, almost abolished the evoked overflow ($S_5/S_1 = 0.10 \pm 0.02$; $n = 6$).

Cumulative addition of increasing concentrations of CEC (0.1–3 μM) after S_1 produced increasing inhibition (Figure 1a), with an IC_{50} of 0.4 (95% c.i. 0.3–0.6) μM. The basal efflux of tritium was not changed (based on b_n/b_1 ratios). The same concentrations of CEC were used in the same cumulative protocol in order to examine its interaction with prazosin or rauwolscine. Prazosin (0.3 μM), when added 60 min before S_1 and kept for the remainder of the experiment, increased the basal efflux of tritium ($b_1 = 0.00221 \pm 0.00004\ min^{-1}$; $P < 0.01$) but did not by itself change the evoked overflow ($S_1 = 0.042 \pm 0.003\%$; $n = 14$). As in the absence of any drug, the evoked overflow remained approximately constant when prazosin alone was present (for example, $S_5/S_1 = 0.91 \pm 0.11$; $n = 7$). Prazosin (0.3 μM) did not affect the concentration-dependent inhibition produced by CEC (for example, $S_5/S_1 = 0.10 \pm 0.03$ when CEC, 3 μM, was administered at S_5 in the presence of prazosin as the highest concentration of the concentration-response curve; $n = 7$). Rauwolscine (0.3 μM), when added 60 min before S_1, did not change basal tritium efflux (b_1) but slightly increased the evoked overflow ($S_1 = 0.050 \pm 0.002\%$ of tissue tritium; $n = 16$; $P < 0.01$). In the presence of rauwolscine (0.3 μM) alone, the evoked overflow again remained stable (for example, $S_5/S_1 = 0.94 \pm 0.10$; $n = 8$). Rauwolscine completely prevented the inhibitory effect of CEC (Figure 1a).

The reversibility of the effect of CEC was tested by addi-

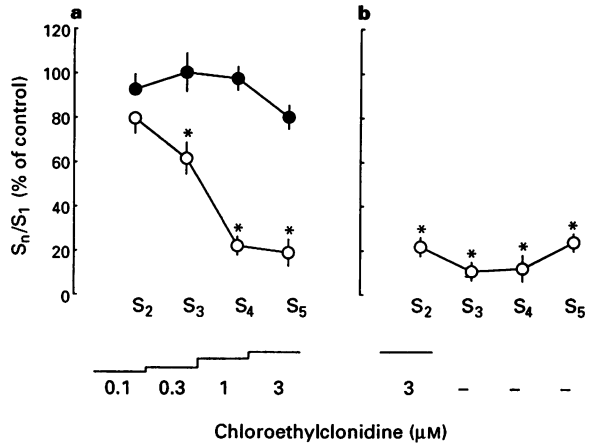

Figure 1 Effect of chloroethylclonidine (CEC) on tritium overflow evoked by brief pulse trains, and interaction with rauwolscine. Tissues were stimulated five times (S_1 to S_5). Each stimulation period consisted of 6 pulses/100 Hz. In (a), CEC was administered at increasing concentrations (0.1–3 μM), in a cumulative manner, 20 min before S_2 to S_5, either in the absence (○) or in the presence of rauwolscine 0.3 μM (●); rauwolscine was added 60 min before S_1 and was present for the remainder of the experiment. In (b), CEC (3 μM) was added from 20 min before until 6 min after S_2 and then washed out. Ordinate scale shows S_n/S_1 ratios obtained in the presence of CEC, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls. Means and s.e.mean (vertical lines) from 7–9 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

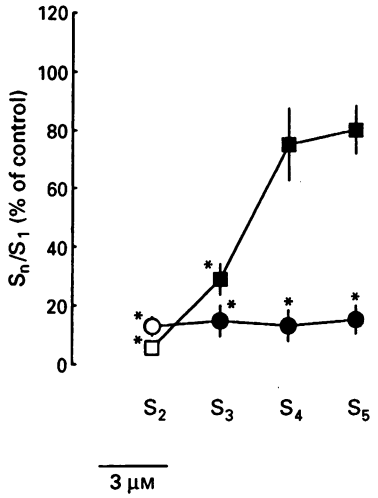

Figure 2 Reversibility of the effects of chloroethylclonidine (CEC) and clonidine on evoked tritium overflow. Tissues were stimulated five times (S_1 to S_5). Each stimulation period consisted of 6 pulses/100 Hz. CEC (circles) or clonidine (squares) was added at a single concentration (3 μM) from 20 min before until 6 min after S_2 and then washed out. Throughout the washout phase (i.e., from 6 min after S_2 to the end of the experiment), rauwolscine (0.3 μM) was added to the superfusion medium (indicated by solid symbols). Ordinate scale shows S_n/S_1 ratios obtained in experiments with CEC and clonidine, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls (rauwolscine only from S_3 to S_5). Means and s.e.mean (vertical lines) from 6–8 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

tion of CEC (3 μM) at S_2 only. The ensuing reduction of evoked tritium overflow was similar to that obtained in the cumulative concentration-response curve (Figure 1b). The inhibition persisted, despite superfusion with CEC-free medium, throughout the stimulation periods S_3 to S_5, i.e., even after 72 min of washout (S_5; Figure 1b). To examine further the reversibility, rauwolscine, at the concentration (0.3 μM) that had prevented the effect of CEC when given before the latter (Figure 1a), was added during the phase after S_2 when CEC was washed out. The inhibition by CEC persisted throughout S_3 to S_5 even during exposure to the antagonist (Figure 2). Rauwolscine (0.3 μM) alone, added after S_2, did not change the evoked overflow of tritium (for example, $S_5/S_1 = 1.09 \pm 0.14$; $n = 7$).

The reversibility of the effect of an equimolar concentration of clonidine (3 μM) was tested for comparison. When added 20 min before S_2, clonidine (3 μM) virtually abolished the evoked overflow of tritium (Figure 2). However, this inhibition disappeared upon superfusion with clonidine-free medium containing rauwolscine (0.3 μM; Figure 2).

In the second set of experiments, two trains of 20, 100 or 500 pulses and a frequency of 10 Hz were applied to each vas deferens (S_1, S_2). The overflow of tritium evoked by S_1 was 0.070 ± 0.005% (20 pulses; $n = 13$), 0.366 ± 0.022% (100 pulses; $n = 14$) and 1.126 ± 0.064% (500 pulses; $n = 13$) of tissue tritium, respectively. In the absence of drugs, the overflow evoked by S_2 was similar to S_1 (Figure 3). Tetrodotoxin (0.3 μM), when added 20 min before S_2, abolished the overflow of tritium evoked by 500 pulses/10 Hz ($S_2/S_1 = 0.00$ and 0.01 in 2 experiments). CEC (3 μM), also added 20 min before S_2, reduced by about 46% the overflow evoked by 20 pulses/10 Hz (train length 2 s), did not change the overflow evoked by 100 pulses/10 Hz (train length 10 s), and increased by about 25% the overflow evoked by 500 pulses/10 Hz (train length 50 s; Figure 3).

Contractions

These experiments were devised to mirror the tritium overflow experiments (pulse pattern, drug administration, reversibility). The purinergic component of neurogenic contractions was isolated by prazosin (0.3 μM; Bourreau *et al.*, 1991; Mallard *et al.*, 1992).

In the first series of experiments, each of five stimulation periods (S_1 to S_5) consisted of a single pulse. The pulses elicited rapid monophasic contractions in the presence of prazosin, the average force developed at S_1 amounting to 17.4 ± 0.7 mN ($n = 31$). The twitches remained constant in control experiments without other drugs (for example, $S_5/S_1 = 1.01 \pm 0.05$; $n = 6$). Tetrodotoxin (0.3 μM; $n = 2$) or suramin (300 μM; Dunn & Blakeley, 1988; $n = 3$), when added after S_5 of control experiments in which further

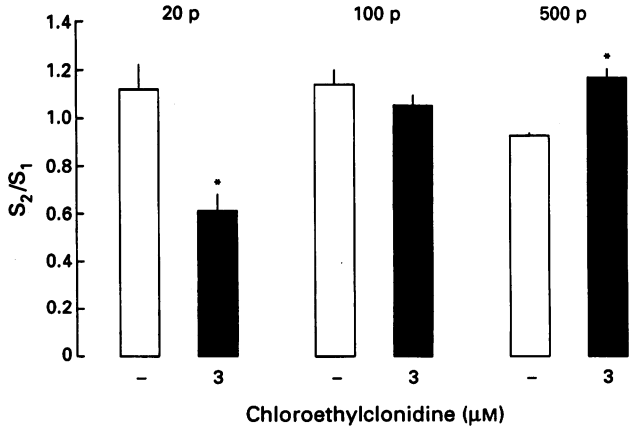

Figure 3 Effect of chloroethylclonidine (CEC) on tritium overflow evoked by long pulse trains. Tissues were stimulated twice (S_1 and S_2). Each stimulation period consisted of either 20, 100 or 500 pulses (p) as indicated and a frequency of 10 Hz. CEC (3 μM) was added 20 min before S_2 and was present for the remainder of the experiment. Ordinate scale shows S_2/S_1 ratios obtained in control experiments (open columns) and in experiments with CEC (solid columns). Means and s.e.mean (vertical lines) from 5–8 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

stimulation periods were applied, abolished the twitches, thus confirming their neurogenic and purinergic character.

Cumulative addition of increasing concentrations of CEC (0.1–3 μM) after S_1 did not change the basal tension of the tissue but produced increasing inhibition of electrically evoked twitches (Figure 4a), with an IC_{50} value of 1.6 (0.7–3.5) μM. Rauwolscine (0.3 μM), when present in the medium in addition to prazosin, did not by itself alter the twitches (S_1 = 18.5 ± 1.1 mN; n = 8), and as in the absence of rauwolscine, twitches remained constant from S_1 to S_5 (for example, S_5/S_1 = 1.00 ± 0.05; n = 4). Rauwolscine prevented the inhibition by CEC (Figure 4a).

As in the tritium overflow experiments, the reversibility of the effect of CEC was tested by exposure to CEC (3 μM) at S_2 only. The ensuing inhibition was similar to that obtained in the cumulative concentration-response curve (Figure 4b). The inhibition persisted, despite repeated washing with CEC-free medium, throughout the stimulation periods S_3 to S_5, i.e., even after 72 min of washout (S_5; Figure 4b). The inhibition by CEC persisted even when rauwolscine (0.3 μM) was added to the CEC-free medium during washout (Figure 5). Rauwolscine alone, added after S_2, did not change the twitch contraction (for example, S_5/S_1 = 0.99 ± 0.04; n = 5).

The reversibility of the effect of an equimolar concentration of clonidine (3 μM) was again tested for comparison. When added 20 min before S_2, clonidine (3 μM) abolished the twitch responses (Figure 5). This inhibition was markedly attenuated upon washout with clonidine-free medium containing rauwolscine (0.3 μM; Figure 5).

The three trains of 100 pulses/10 Hz applied in the second series of experiments elicited biphasic tetanic contractions: an initial peak within the first 2 s was followed by a secondary plateau with a maximum beyond 4 s of stimulation (Figure 6). The responses remained approximately constant from S_1 to S_3 in control experiments with no drug added except prazosin (for example, S_2/S_1 for initial peak = 1.05 ± 0.01; for plateau = 1.12 ± 0.02; Figure 6a). Tetrodotoxin (0.3 μM; n = 2) and suramin (300 μM; n = 3), when added after S_3 of control experiments in which further stimulation periods were applied, abolished or almost abolished the contractions. CEC (3 μM), when added before and during S_2 and then washed out, reduced the initial peak (S_2/S_1 = 0.80 ± 0.04; $P<0.01$) but enhanced the plateau (S_2/S_1 = 1.32 ± 0.04; $P<0.01$; n = 5; Figure 6b). These changes were not attenuated after 30 min of washout (Figure 6b).

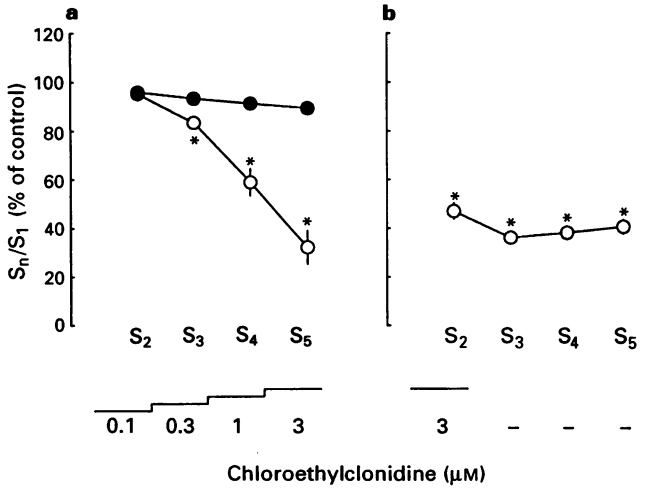

Figure 4 Effect of chloroethylclonidine (CEC) on purinergic contractions evoked by single pulses, and interaction with rauwolscine. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated five times (S_1 to S_5). Each stimulation consisted of a single pulse. In (a), CEC was administered at increasing concentrations (0.1–3 μM), in a cumulative manner, 20 min before S_2 to S_5, either in the absence (○) or in the presence of rauwolscine 0.3 μM (●); rauwolscine was present in the medium from the beginning, i.e., 60 min before S_1. In (b), CEC (3 μM) was added from 20 min before until 6 min after S_2 and then washed out by replacement of the bath fluid every 13 min. Ordinate scale shows S_n/S_1 ratios obtained in the presence of CEC, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls. Means and s.e.mean (vertical lines) from 4 or 5 experiments. *denotes a significant difference from corresponding control ($P<0.01$).

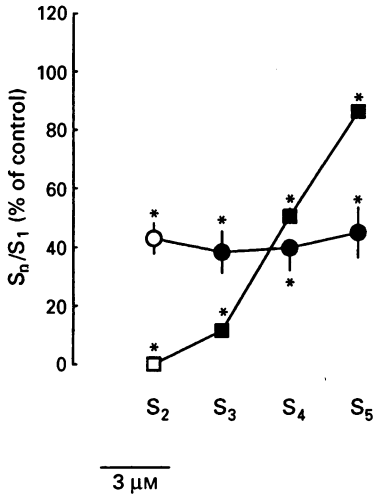

Figure 5 Reversibility of the effects of chloroethylclonidine (CEC) and clonidine on purinergic contractions. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated five times (S_1 to S_5). Each stimulation consisted of a single pulse. CEC (circles) or clonidine (squares) was added at a single concentration (3 μM) from 20 min before until 6 min after S_2 and then washed out by replacement of the bath fluid every 13 min. Throughout the washout phase (i.e., from 6 min after S_2 to the end of the experiment), rauwolscine (0.3 μM) was added to the medium (indicated by solid symbols). Ordinate scale shows S_n/S_1 ratios obtained in experiments with CEC and clonidine, expressed as a percentage of the average S_n/S_1 ratio obtained in corresponding controls (rauwolscine only from S_3 to S_5). Means and s.e.mean (vertical lines) from 5 or 6 experiments. *denotes a significant difference from corresponding controls ($P<0.01$).

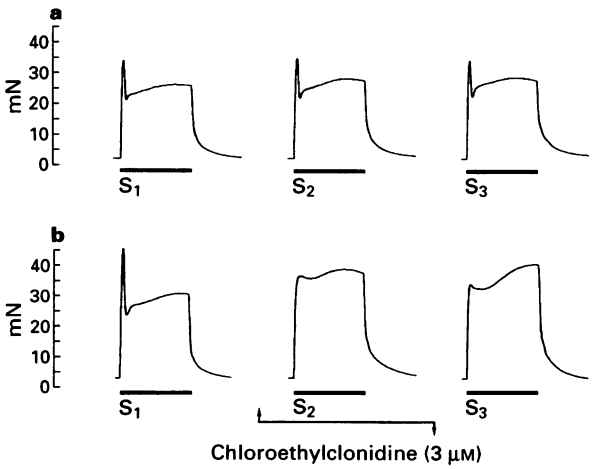

Figure 6 Effect of chloroethylclonidine (CEC) on purinergic contractions evoked by long pulse trains. Prazosin (0.3 μM) was present in the medium from the beginning. Tissues were stimulated three times (S_1 to S_3). Each stimulation period consisted of 100 pulses/10 Hz (10 s; horizontal bars). (a) Shows a control experiment without CEC; (b) shows an experiment in which CEC (3 μM) was added 20 min before S_2 and was washed out immediately afterwards. Representative tracings from 5 experiments each.

Discussion

CEC concentration-dependently reduced the overflow of tritium (and presumably the release of [^{3}H]-noradrenaline; Starke, 1977) elicited by brief (50 ms) trains of high frequency. Release of transmitter evoked by such brief trains resembles release by a single pulse and is free, or almost free, from prejunctional autoinhibition (Singer, 1988; Limberger *et al.*, 1992), as confirmed here by the failure of rauwolscine to cause any major increase. The IC_{50} of CEC (0.4 μM) was close to its K_i value for inhibition of the binding of [^{3}H]-clonidine to brain synaptosomes (1.7 μM; Leclerc *et al.*, 1980). Moreover, its effect was not changed by prazosin (0.3 μM) but was prevented by pre-exposure to rauwolscine (0.3 μM). The effect, hence, was mediated by α_2-adrenoceptors, specifically, the α_2-autoreceptor recently characterized as α_{2D} in rat vas deferens (Limberger *et al.*, 1992; Smith & Docherty, 1992). CEC, hence, is an agonist at α_2-adrenoceptors.

Noradrenaline and adenosine 5'-triphosphate (ATP) (or a related nucleotide) are postganglionic sympathetic co-transmitters in rat vas deferens (French & Scott, 1983; Bourreau *et al.*, 1991; Mallard *et al.*, 1992; see Burnstock, 1990; von Kügelgen & Starke, 1991). If CEC is an α_2-agonist, it should also inhibit the release of ATP. In order to examine this possibility, we isolated the rapid purinergic component of single pulse-evoked contractions by prazosin. In fact, CEC concentration-dependently decreased the purinergic twitches, and again the effect was prevented by pre-exposure to rauwolscine. CEC was less potent at inhibiting the purinergic twitches (IC_{50} 1.6 μM) than the release of noradrenaline (0.4 μM). More or less marked differences in the sensitivity of purinergic postjunctional effects (or ATP overflow) on the one hand, and adrenergic postjunctional effects (or noradrenaline overflow) on the other hand, to α_2-adrenoceptor agonists have been noticed previously (Brown *et al.*, 1983; Hammond *et al.*, 1988; Bültmann *et al.*, 1991; von Kügelgen & Starke, 1991; but see Msghina *et al.*, 1992); the reason is not known.

The effects of CEC on tritium overflow and single pulse-evoked purinergic twitches persisted, not only during washout with normal buffer but even upon washout with buffer containing rauwolscine at a concentration that, when given before CEC, had completely prevented the effect of the latter. In this respect, CEC contrasted sharply with clonidine, despite the fact that, in contrast to CEC, clonidine was given at a greatly supramaximal concentration (as little clonidine as 10 nM inhibited the evoked overflow of tritium to the same extent as did CEC, 3 μM, under the conditions of Figure 1; R. Bültmann, unpublished observation). An irreversible activation of α_1-adrenoceptors by CEC has been observed repeatedly (Leclerc *et al.*, 1980, in rat aorta as explained in the Introduction; Muramatsu *et al.*, 1991, in rat aorta; Schwietert *et al.*, 1991, in rat portal vein). Our results demonstrate that CEC is an irreversible α_2-adrenoceptor agonist as well, presumably because it alkylates the α_2-adrenoceptor (Leclerc *et al.*, 1980). CEC may irreversibly activate both α_1- and α_2-adrenoceptors in dog saphenous vein (Nunes & Guimarães, 1992). The mechanism of irreversible activation by covalent binding is not known. It has been suggested that the phenomenon argues against the rate theory of receptor activation (Lohse *et al.*, 1986). In the case of CEC, however, the alkylating β-chloroethylamino group may be sufficiently remote from the region of the molecule responsible for agonism (see Tian *et al.*, 1990) to permit successive attachment and detachment of that region to and from the receptor, as the rate theory postulates.

Clonidine acts as a partial α_2-agonist on many cells including noradrenergic neurones (Medgett *et al.*, 1978; Cichini *et al.*, 1986; see Starke, 1987). The same seems to be true for CEC in rat vas deferens. It reduced the release of noradrenaline elicited by 6 pulses/100 Hz and also 20 pulses/10 Hz, caused no change for 100 pulses/10 Hz, and increased the release elicited by 500 pulses/10 Hz. This is a pattern predicted for a prejunctional α_2-agonist with lower efficacy than noradrenaline (Starke *et al.*, 1974): the agonist effect prevailed early in trains of pulses, up to 2 s in our experiments; later in the 10 Hz trains, sufficient noradrenaline presumably accumulated in the autoreceptor biophase to reveal antagonism against noradrenaline; early inhibition and later facilitation of release cancelled each other in the 10 s trains, but facilitation prevailed in the 50 s train. Results obtained on purinergic contractions, in this case those elicited by 100 pulses/10 Hz, again confirm the observations on tritium overflow, and in fact show the change from agonism to antagonism directly. Just as CEC diminished the release of noradrenaline elicited by 2 s stimulation periods (Figure 3), it markedly reduced the initial purinergic contraction peak (Figure 6); just as CEC increased the release of noradrenaline later in the trains (Figure 3), it increased the secondary plateau with its maximum beyond 4 s.

The fact that CEC is an irreversible agonist at α_2-adrenoceptors, specifically at α_2-autoreceptors, limits its suitability for the distinction of postjunctional α_1-adrenoceptor subtypes mediating responses to nerve stimulation (see Introduction). Muramatsu (1991) administered CEC in the presence of an α_2-antagonist and, hence, CEC possibly did not act at α_2-adrenoceptors in his study. However, CEC may have activated prejunctional α_2-autoreceptors in other investigations (Sulpizio & Hieble, 1990; Mallard *et al.*, 1992).

Mallard *et al.* (1992) showed, in the rat vas deferens, that CEC (0.1 to 3 μM) selectively blocked the slow, adrenergic but not the rapid, purinergic component of single pulse-evoked twitches; they also reported that CEC (3 μM) blocked the early peak (<2 s) but not the late plateau (>4 s) of the adrenergic component of the tetanus elicited by 100 pulses/10 Hz; they concluded that the adrenergic component of the single pulse twitches and of the early tetanic peak is mediated by α_{1B}-adrenoceptors whereas the adrenergic component of the secondary tetanic plateau is mediated by α_{1A}-adrenoceptors. The presynaptic α_2-agonist effect of CEC does not disprove, but questions, this conclusion. In conditions when CEC depresses adrenergic contractions (Mallard *et al.*, 1992), it also reduces the release of noradrenaline (Figures 1 and 3). Late in a 10 Hz train, when CEC fails to attenuate the adrenergic contraction plateau (Mallard *et al.*, 1992), it also fails to reduce the release of noradrenaline (Figure 3). Strikingly, just as CEC (3 μM) abolishes the early peak of the adrenergic response to 100 pulses/10 Hz (Mallard *et al.*, 1992), it also abolishes the early peak of the purinergic response to 100 pulses/10 Hz (Figure 6), a clear indication of prejunctional inhibition. In unpublished experiments (R. Bültmann), direct evidence has been obtained for at least a contribution of prejunctional α_2-adrenergic inhibition to the blockade of postjunctional adrenergic responses by CEC: CEC (1 and 3 μM) abolished the adrenergic component of single pulse twitches, isolated by nifedipine (10 μM) in the absence of rauwolscine, but reduced them only by 70% when prejunctional α_2-adrenoceptors were blocked by rauwolscine (0.3 μM). One finding might argue against prejunctional inhibition in the study of Mallard *et al.* (1992): CEC did not reduce the purinergic component of single pulse-evoked twitches in their experiments, in contrast to the present work. Mallard *et al.* (1992) did not pharmacologically isolate the purinergic component, and the ensuing purinergic-adrenergic overlap may have obscured the inhibitory effect of CEC. Moreover, we also find that the purinergic twitch is slightly less sensitive to CEC than the release of noradrenaline. However when an antagonist is used to identify postjunctional adrenoceptor types mediating neurogenic responses, it is effects on the release of noradrenaline that impede interpretation.

This study was supported by the Jung Foundation (Hamburg) and the Deutsche Forschungsgemeinschaft (SFB 325). We thank Bayer, Boehringer and Pfizer for gifts of drugs.

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(Received August 27, 1992
Accepted September 23, 1992)