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AN ENDOGENOUS BRAIN SUBSTANCE, CDS (CLONIDINE-DISPLACING-SUBSTANCE), INHIBITS THE TWITCH RESPONSE OF RAT VAS DEFERENS

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The effect of CDS, an endogenous brain substance that specifically displaces bound [^{3}H]clonidine and [^{3}H]rauwolscine in rat brain membranes and human platelets, has been tested in isolated, field-stimulated rat vas deferens. CDS, obtained after an extensive purification procedure as a single peak from an HPLC sizing column, inhibited the electrically stimulated rat vas deferens similarly to the inhibitory action of clonidine, an α_2-agonist. The effective dose of CDS as an inhibitor of the vas deferens is equivalent to its effective dose in displacing specifically bound [^{3}H]-clonidine in rat brain membranes. Furthermore, the CDS inhibition of the twitch response is reversed by two α_2-adrenergic antagonists, yohimbine and phentolamine. From these results, it is suggested that CDS extracted from brain, with affinity for clonidine sites, may be involved in the non-adrenergic fast response of the sympathetic transmission of the vas deferens.

Norepinephrine (NE) is the motor transmitter of the post-ganglionic sympathetic neurons of the rat vas deferens (1-4). The contractile response elicited in the rat vas deferens is composed of two distinct phases: a slow adrenergic phase (5-7) and a fast "twitch" response which is triggered by an unknown neurotransmitter (5-10).

The pharmacological profile of the prejunctional regulation of motor transmission in the rat vas deferens is α_2-adrenergic. However, the different effects of imidazoline-type agonists (*i.e.* clonidine) and phenylethylamine-type agonists (*i.e.* α-methylnoradrenaline) on the twitch response, suggest that there might be more than one population of α_2-adrenoceptors (clonidine sites *vs.* norepinephrine sites) (11-13).

Recently, an endogenous clonidine-displacing-substance (CDS) has been isolated from calf brain (14,15) and purified to an apparent homogeneity in a series of HPLC columns; the determination of its structure is under

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study. CDS binds selectively to sites labeled with clonidine and yohimbine (both are α_2-adrenergic ligands), and upon intracerebral injection produces an increase in the mean arterial pressure (16).

In view of the α_2-characteristics of this new endogenous substance, we decided to study its effect on the twitch response of field-stimulated rat vas deferens. In the present work, we (a) tested the effect of CDS on the field-stimulated twitch response; (b) compared the potency of CDS on the twitch response to its potency in displacing of specifically-bound [^{3}H]clonidine to rat brain membranes; and (c) tested the specificity of the CDS effect by the ability of α_2-antagonists to reverse the CDS effect on the twitch.

MATERIALS AND METHODS

[^{3}H]Clonidine (66 Ci/mmole) was purchased from New England Nuclear (USA); prazosin was a gift from Pfizer (USA); phentolamine was a gift from Ciba (USA); guanabenz was synthesized as previously described (17); (–)norepinephrine (NE) and (–)propranolol were obtained from Sigma (USA). All chemicals were of reagent grade; CDS was isolated from calf brain (14,15).

Rat brain membranes

Rat brain membranes (for binding assays) were prepared according to a procedure described previously (15).

Displacement of [^{3}H]clonidine by CDS

CDS, in concentrations indicated (0.1-2.0 units), was added to 250 µg of brain membrane protein in 50 mM Tris-HCl buffer, pH 7.6, with 2 mM $MgCl_2$ (0.25 ml final volume). The incubation was initiated by adding 1-2 nM of [^{3}H]clonidine. Non-specific binding was determined in the presence of 10 µM NE. After 40 minutes of incubation at 25°C, the samples were rapidly filtered through GF/C filters. The filters were washed with 3x4 ml of 10 mM Tris-HCl buffer, pH 7.5, at 4°C, and counted with a scintillation liquid with a 50% efficiency. Non-specific binding was defined as the amount of [^{3}H]-clonidine bound in the presence of 10 µM (–)NE.

Electrically stimulated twitch response

Male Albino rats (∿ 250 g) were slightly anaesthesized and killed by decapitation. The vas deferens was removed and the prostatic ends (2-3 cm long) were mounted between two ring electrodes (platinum) in organ bath solution. The composition of the organ bath solution, a Mg-free Krebs-bicarbonate medium, was as follows (mM): NaCl, 112; KCl, 4.7; $CaCl_2$, 2.6; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0; glucose, 11.1; ascorbic acid, 0.11. The solution (2.5 ml) was kept at 37°C with bubbling of 95% oxygen and 5% CO_2 throughout the experiment. The initial tension on the preparation was 0.5 g and the preparation was left to equilibrate for 30 minutes before starting the stimulation. Stimulation was obtained by a square wave stim-

ulator (Grass, model S4) with constant current of 2 mS duration, at a maximum voltage of 40 V at a frequency of 0.1 Hz. The isometric tension changes were recorded as twitches of the vas deferens on a two-pen recorder (Rika Denki). (−)Propranolol (1 μM) and prazosin (0.3 μM) were present in the medium throughout the assay.

Cumulative concentration response curves were obtained for CDS with a contact time of 20 minutes at each concentration. The results were expressed as percentage of the inhibition of the twitch relative to the response before the addition of CDS. In competition studies, (a) yohimbine and phentolamine were added to the CDS-inhibited twitch and the recovery of the twitch was recorded; (b) preincubation of the vas deferens in the presence of α_2-antagonists was followed by the addition of CDS.

RESULTS

Displacement of [^{3}H]clonidine by CDS

One unit of activity of CDS is defined as the amount needed to displace 50% of specifically bound [^{3}H]clonidine in a 250 μl assay containing 250 μg membrane protein and 1-2 nM [^{3}H]clonidine (14).

As shown in Figure 1, 1 μl of a pure fraction eluted from an HPLC sizing column, which is pure CDS , as judged by a single peak from three different HPLC columns with different mobile phases (16), represents one unit of CDS activity. We have used the same CDS solution in the vas deferens assay in order to compare the potency of CDS in the two assays. Specific binding under the assay conditions used was 7.1 fmol/mg protein, and non-specific binding (with 10 μM (−)norepinephrine) was 20% of the total [^{3}H]-clonidine bound.

Dose-response curve of CDS on vas deferens

The low frequency (0.1 Hz) used to stimulate the vas deferens, produced a reproducible twitch which was reduced as a function of increasing concentrations of CDS (1-25 units/2.5 ml) (Fig. 2). An IC_{50} = 7 units (in 2.5 ml organ bath) was obtained, which is comparable to 0.7 μl of the same fraction used in the binding assay; see above). Thus, the effective dose of CDS for the inhibition of the electrically stimulated twitch response and for the displacement of specifically bound [^{3}H]clonidine is of the same order of magnitude.

Unlike the rapid response of the vas deferens to clonidine, where 4 minutes gave a maximal effect of inhibition (with an IC_{50}=1.6±0.2 nM, data

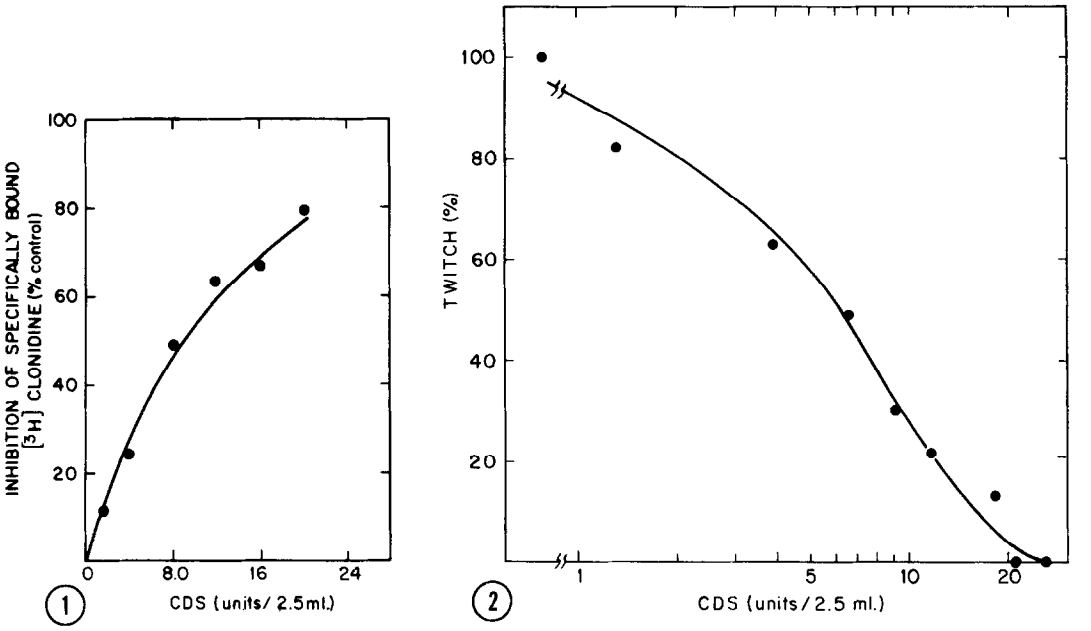

Fig. 1. Displacement of [^{3}H]clonidine by CDS purified on an HPLC sizing column in rat brain membranes. Rat brain membranes (250 µg protein) were incubated with increasing concentrations of CDS eluted from an HPLC sizing column (after lyophilization). The binding assay was initiated by adding 1.2 nM of [^{3}H]clonidine in a final volume of 0.25 ml. Total binding capacity of the membranes under the assay conditions of 40 minutes at 25°C was 11.5 fmol/assay. Non-specific binding was determined in the presence of 10 µM of NE (4.2 fmol/assay). Specific binding at the indicated [^{3}H]clonidine concentration was 7.1 fmol/assay. One unit of CDS activity is defined as the concentration needed to displace 50% of the specifically bound [^{3}H]clonidine under the assay conditions described above. The IC_{50} observed for CDS was 1.0 µl/250 µl assay, and the same solution was used for the cumulative dose response in the rat vas deferens.

Fig. 2 A cumulative dose response curve of CDS measured on the electrically-stimulated rat vas deferens. Rat vas deferens was stimulated at 0.1 Hz with a starting tension of 0.5 g in a 2.5 ml organ bath. CDS was added to the bath and allowed to equilibrate for 20 minutes. The results, which are the mean ± S.M.E. of 3 preparations, are expressed as the percentage of the original twitch response. The IC_{50} (the amount of CDS needed to cause 50% inhibition of the twitch) was 7 µl of the same solution used in the binding assay (where 1.0 µl in 250 µl assay was the IC_{50}).

not shown), the maximal response to CDS was obtained after about 20 minutes. A prolonged inhibitory effect of the twitch response was also observed for guanabenz, a potent α_2-agonist (18-20) (data not shown). All three ligands fully inhibited the twitch response of the vas deferens.

Reversal of the inhibitory effect of CDS

Antagonism of the CDS effect was tested in two ways: (a) addition of an α_2-antagonist to the CDS-inhibited vas deferens, and (b) preincubation

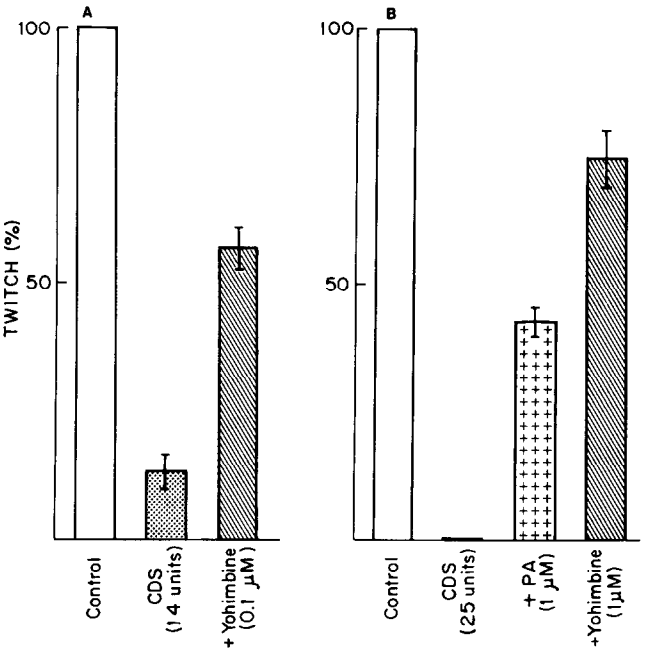

Fig. 3 Partial competition between CDS and α_2-adrenergic antagonists on twitch response. A. CDS (14 units in 2.5 ml bath) inhibited the twitch response by 86%. Addition of 0.1 μM yohimbine partially antagonized the inhibition of the twitch by CDS to 42% of the original twitch. B. CDS, 25 units/2.5 ml, fully inhibited the twitch response. Partial recovery of the twitch was obtained by the addition of 1.0 μM phentolamine (50% of the original twitch) or 1.0 μM yohimbine (75% of the original twitch). The results are the mean ± S.M.E. of 3 separate experiments.

of the vas deferens with an α_2-antagonist for 20 minutes, followed by the addition of CDS.

(a) Inhibition of 84% of the field-stimulated rat vas deferens by 14 units/2.5 ml of CDS was partially reversed by 0.1 μM yohimbine, to 42% of the original twitch (Fig. 3A). Yohimbine, at higher concentrations (1.0 μM), restored 75% of the completely inhibited twitch observed with 25 CDS units/2.5 ml (Fig. 3B). Phentolamine, even though slightly less effective than yohimbine, reversed the complete inhibition of the twitch (in the presence of 25 units CDS/2.5 ml) to 50% of the original twitch (Fig. 3B).

(b) Pre-exposure of the vas deferens to yohimbine (1.0 μM), followed by 30 units of CDS, decreased the inhibition of the twitch to 10% as compared with the full inhibition in the absence of yohimbine (data not shown).

DISCUSSION

Our results show that an endogenous, non-catecholamine substance (CDS), purified from bovine brain, inhibits the twitch response of the rat vas deferens to an electrical stimulation at a low frequency.

CDS competes with yohimbine and clonidine in binding assays (14,15) and, as shown in the present study, inhibits the fast phase of the twitch response. The effective dose of CDS in displacing specifically bound [^{3}H]clonidine to rat brain membranes is comparable to its effective dose for the inhibition of the low frequency-stimulated rat vas deferens. Similarly, the clonidine concentration (1.6±0.2 nM) which causes 50% inhibition of the field-stimulated rat vas deferens, is comparable to its dissociation constant calculated from direct binding to rat brain membranes (1.4 nM) using radiolabeled clonidine (20,21).

Both α_2-antagonists, *i.e.* yohimbine and phentolamine, partially reversed the inhibitory effect of CDS on the twitch response. Preincubation of the vas deferens in the presence of 0.1 μM yohimbine or 0.1 μM phentolamine prevented almost 90% of the inhibitory effect of CDS. Reversal of the inhibitory action of CDS on the stimulated vas deferens by α_2-adrenergic blockers,corroborates the binding data for overlap of specificity of CDS with α_2-adrenergic receptors.

In conclusion, our data demonstrate that CDS, purified from bovine brain, is an inhibitor of the electrically-stimulated rat vas deferens, with a potency equivalent to binding studies in rat brain membranes, and its inhibitory action is reversed by α_2-adrenergic antagonists. In view of these results and of binding studies using [^{3}H]clonidine and [^{3}H]rauwolscine, we suggest that CDS represents a transmitter or a neuroeffector for the control of the fast twitch of the rat vas deferens with specificity for clonidine sites. The overlap of affinity of CDS for α_2-adrenergic receptors adds credence to the proposal (22) that α_2-adrenergic receptors are further subdivided into two distinct sites exemplified by imidazolines and phenylethylamines.

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