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Effects of α_2-adrenoceptor agonists on locus coeruleus firing rate and brain noradrenaline turnover in *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-treated rats

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Summary. Previous studies have shown that a low dose of the alkylating compound *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) reduces the density of α_2-adrenoceptors in rat cerebral cortex and antagonizes the effects of an α_2-adrenoceptor agonist on noradrenaline release in rat cortical slices. In the present study, a corresponding dose of EEDQ (1 mg/kg, s.c., 24 h) was shown to reduce the effect of the α_2-adrenoceptor agonists clonidine and guanfacine on noradrenaline turnover in rat brain while not affecting the inhibitory effect of clonidine on locus coeruleus (LC) cell firing. When considerably higher doses of EEDQ were administered (10 and 20 mg/kg, s.c., 24 h) not only the biochemical but also the electrophysiological effects of clonidine were markedly reduced (or even reversed). The data support the notion that EEDQ decreases the responsiveness of brain α_2-adrenergic receptors; moreover, they indicate that α_2-adrenoceptors regulating LC activity are characterized by a larger receptor reserve or are less sensitive to the influence of alkylation than are the population of α_2-adrenoceptors regulating noradrenaline utilization.

Key words: Locus coeruleus – *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EEDQ – Clonidine – Guanfacine – α_2-Adrenoceptors

Introduction

Brain α_2-adrenoceptors may be classified on the basis of their localization and function. Thus, while the majority of α_2-adrenoceptors are probably postsynaptically located at non-noradrenergic neurons (U'Prichard et al. 1979), α_2-adrenergic receptors also serve as autoreceptors situated either presynaptically at noradrenergic nerve terminals (Farnebo and Hamberger 1971; Langer 1977; Starke 1977) or somatodendritically in the region of noradrenergic cell bodies (Svensson et al. 1975). The latter α_2-adrenergic autoreceptors strongly influence the firing rate of the major noradrenergic nucleus, the locus coeruleus (LC), via a collateral inhibitory system (Cedarbaum and Aghajanian 1976; Ennis and Aston-Jones 1986). Thus, the α_2-receptor agonist clonidine, systemically or microiontophoretically administered, inhibits LC activity (Svensson et al. 1975; Engberg et al. 1982), whereas α_2-receptor antagonists, like yohimbine or idazoxan, induce excitation of the LC neurons (Svensson 1978; Marwaha and Aghajanian 1982; Freedman and Aghajanian 1984).

Systemic administration of clonidine to rat leads not only to a decrease in the LC firing rate (Svensson et al. 1975) but also to a marked reduction of brain noradrenaline turnover (Andén et al. 1976). Partly, the latter effect may be secondary to the decrease in firing rate obtained by activation of somatodendritic autoreceptors (cf. Salzman and Roth, 1979). However, the effects of α_2-adrenoceptor agonists on transmitter turnover may also be due to activation of the presynaptic α_2-adrenergic autoreceptors situated in the terminal region (see Curet et al. 1987). Thus, the relative importance of somatodendritic and presynaptic α_2-adrenoceptors for the decrease in noradrenaline utilization induced by administration of α_2-adrenergic agonists is a matter of controversy.

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) is an alkylating compound that inactivates different populations of neurotransmitter receptors, including α_2-adrenoceptors in the CNS (see Crocker and Cameron 1989). Recently, Adler et al. (1987) showed that pretreatment with a comparatively low dose of EEDQ antagonizes the decrease in noradrenaline release obtained by the full α_2-adrenoceptor agonist UK-14304 in rat cortical slices in vitro (see also Nasseri and Minnemann 1987). In the present study the effect of various doses of EEDQ on α_2-adrenoceptor agonist-induced decrease in rat LC firing rate and brain noradrenaline utilization was examined in vivo.

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Methods

Animals. In all experiments male Sprague-Dawley rats weighing 200–250 g were used.

Single unit recording. The electrophysiological experiments were performed essentially as has been previously described (Engberg et al. 1982). Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic apparatus. After exposing the dorsal surface of the skull, a 3 mm burr hole was drilled with its center located approximately 1.1 mm posterior to lambda and 1.1 mm lateral to the midline. The dura was carefully removed and a micropipette, broken back under microscopic control to obtain a tip diameter of approximately 1–2 µm and subsequently filled with 2 *M* NaCl saturated with fast green, was lowered by means of a hydraulic microdrive into the region of the LC, according to stereotaxic coordinates as interpolated from the atlas of König and Klippel (1970). The in vitro impedances of the electrodes were 3–6 MΩ, measured in saline at 135 Hz. Single unit potentials were passed through a high input impedance amplifier and filters. The impulses were discriminated from background noise by a window discriminator and fed into a digital counter, which was reset every 10 s, and finally displayed on a storage oscilloscope, an audiomonitor and a strip chart recorder. The neurophysiological characteristics of the cells were identical to those previously described for noradrenaline neurons of the rat LC. The body temperature of the animals was maintained at 37°C by means of a thermostatically controlled heating pad. During registration of the cell firing rate additional chloral hydrate and cumulative doses of drugs were administered i.v. via a lateral tail vein. The position of the electrode was marked at the end of each experiment by iontophoretic ejection of fast green. The rats were then perfused with 10% buffered formaldehyde solution and the brains were subjected to conventional histological procedures. Only cells within the LC were included in this study.

Biochemical experiments. For estimation of the effect of clonidine and guanfacine on brain noradrenaline utilization the disappearance of noradrenaline after administration of the catecholamine synthesis inhibitor α-methyl-paratyrosine (α-MT; 250 mg/kg) in combination with various simultaneously administered doses of either of the two α_2-adrenoceptor agonists was investigated (cf. Andén et al. 1976). The rats were decapitated 50 min after drug administration and the brains were quickly removed and placed on an ice-cold glass plate. Biochemical analyses of whole-brain noradrenaline were performed by means of standard high-performance reversed-phase ion-pair liquid chromatography with electrochemical detection (LCEC), as previously described (Felice et al. 1978; Nissbrandt et al. 1988). Briefly, the brains were homogenized with an Ultra-Turrax homogenizer in 0.1 *M* $HClO_4$ containing 4.5 m*M* Na_2EDTA and 1.6 m*M* reduced glutathione. After centrifugation (8000–9000 *g*, 0°C for 10 min), 0.4 ml of the supernatant was taken for analysis of noradrenaline and 20 mg of acid washed Al_2O_3 was added. Under vigorous stirring, 0.5 ml 3.0 *M* Tris buffer (pH 8.6) was added. After mixing for 10 min the samples were washed twice with distilled water and finally eluted with 200 µl of a solution containing boric acid (0.25 *M*) and citric acid(0.125 *M*). Injection volume was 75 µl. The detections were carried out electrochemically by means of a thin layer cell (TL-3, Bioanalytical Systems, West Lafayette, Ind, USA) with a single glassy carbon working electrode (TL-5A, Bioanalytical Systems), an Ag/AgCl reference electrode and an amperometric detector. The detector was operated at +0.75 V. The current produced was monitored using an integrator (Spectra-Physics model SP 4270, San José, Calif., USA). The mobile phase for the LCEC system consisted of 0.015 *M* K_2HPO_4, 0.035 *M* citric acid, 0.054 m*M* Na_2-EDTA, 0.43–0.52 m*M* Na-octyl-sulfate and 9–11% methanol (pH 2.75–2.85). The flow rate was 1.5–2 ml/min.

Drugs. Chloral hydrate (Merck, Darmstadt, FRG), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; Sigma, St. Louis, Mo., USA), clonidine (Sigma), guanfacine (Sandoz, Basel, Switzerland), idazoxan (Reckitt and Colman, Hull, UK) and α-methyl-para-tyrosine (α-MT; Sigma) were used.

Statistics. Differences between groups in the biochemical experiments were statistically evaluated by means of analysis of variance followed by Fisher's protective least significant difference test using Macintosh Statview II software. All values are given as means ± SEM.

Results

Pretreatment with a relatively low dose of EEDQ (1 mg/kg, s.c., 24 h) did not affect clonidine-induced inhibition of LC activity (Fig. 1). However, pretreatment with EEDQ in a higher dose (10 mg/kg, s.c., 24 h) markedly reduced the ability of clonidine to decrease the firing rate of LC neurons (Fig. 1). The non-competitive nature of the EEDQ-induced inhibition of clonidine-induced decrease in LC firing is evident from the significant change in the slope of the dose-response curve of clonidine in rats given EEDQ 10 mg/kg; in contrast pretreatment with competitive α_2-adrenoceptor antagonists, such as idazoxan (Fig. 2), induces a parallel rightward shift in the dose-response curve for clonidine.

In rats pretreated with 20 mg/kg of EEDQ relatively high doses of clonidine were required to produce inhibition of LC firing rate compared with the doses causing LC inhibition in control rats (Figs. 1, 3A, B). However,

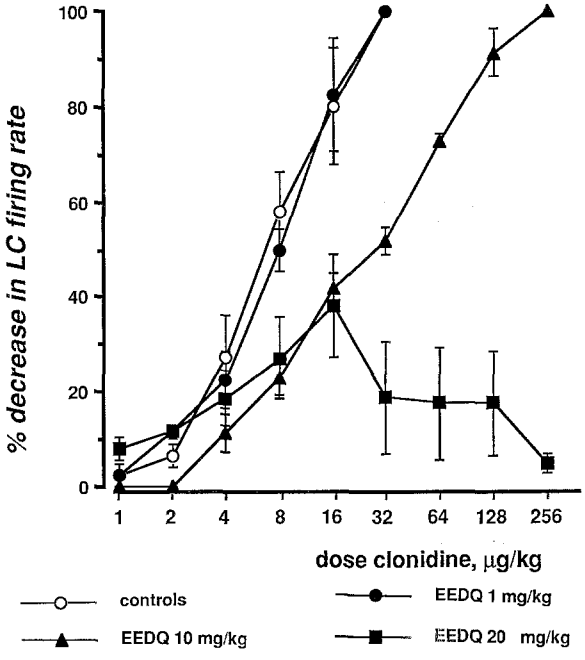


Fig. 1. Logarithmic dose-response curve for the inhibitory effect of clonidine on noradrenergic LC neurons in control rats and rats pretreated with EEDQ (1, 10 or 20 mg/kg, s.c., 24 h). Each value represents mean ± SEM from 4 cells in 4 rats. The slopes of the 4 individual control curves and the 4 individual curves obtained using 10 mg/kg of EEDQ differ significantly; for each curve the regression coefficient was calculated using probit values. The mean of the regression coefficients for the EEDQ (10 mg/kg) curves differed significantly from that of the control curves (Student's *t*-test, $P < 0.025$)

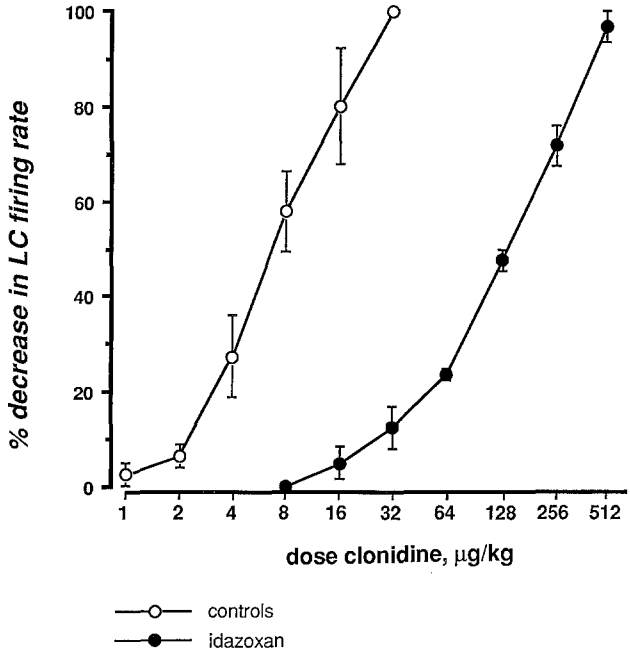


Fig. 2. Logarithmic dose-response curve for the inhibitory effect of clonidine on noradrenergic LC neurons in control rats and rats pretreated with idazoxan (1 mg/kg, i.p., 20 min). Each value represents mean ± SEM from 4 cells in 4 rats. Curves are near parallel; regression coefficients, calculated from 4 individual curves, are 1.46 ± 0.16 and 1.71 ± 0.17, respectively

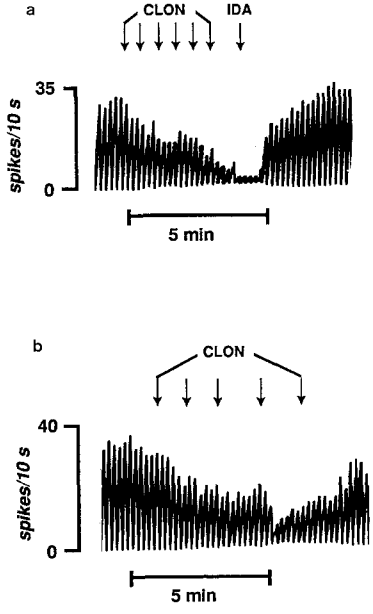


Fig. 3. Representative examples of the action of EEDQ on the firing rate of single noradrenergic LC neurons. a) Low doses of clonidine (CLON, 1 + 1 + 2 + 4 + 8 + 16 µg/kg, i.v., at *arrows*) totally inhibits the firing rate of an C neuron in an untreated rat. This action is rapidly reversed by administration of idazoxan (IDA, 2 mg/kg, i.v., at *arrow*). b) Following EEDQ pretreatment (20 mg/kg, s.c., 24 h) relatively high doses of clonidine (*CLON*, 5 + 5 + 10 + 20 + 40 µg/kg, i.v., at *arrows*) is required to decrease the neuronal activity. Note the increase in firing rate following administration of higher doses of clonidine

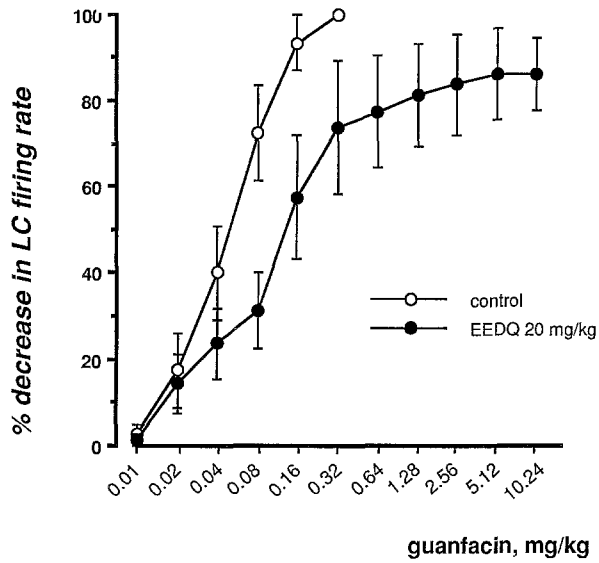


Fig. 4. Logarithmic dose-response curve for the inhibitory action of guanfacin on the firing rate of noradrenergic neurons in the LC in control rats and in rats treated with EEDQ (20 mg/kg, s.c., 24 h). Each value represents mean ± SEM from 4 cells in 4 rats

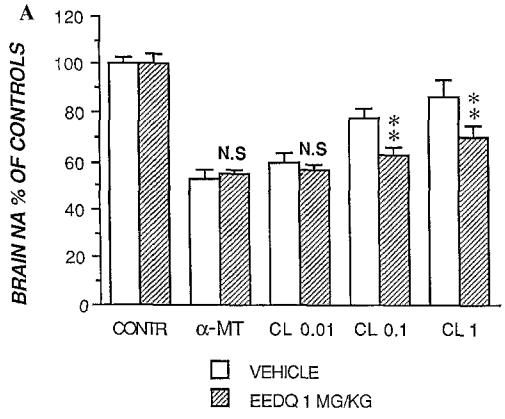
doses higher than 80 µg/kg were frequently associated with an increase in LC activity, i.e. a return to baseline level (Figs. 1, 3B). In rats pretreated with a high dose of EEDQ (20 mg/kg, s.c., 24 h) the ability of guanfacin to inhibit LC firing rate was also attenuated (Fig. 4); however, no paradoxical increase in LC firing rate after high doses of guanfacine was observed.

EEDQ pretreatment did not significantly affect the basal firing rate of the LC neurons; following EEDQ pretreatment with 0, 1, 10 or 20 mg/kg s.c. the average basal firing rate, expressed as spikes/10 s, was 22.7 ± 2.6 ($n = 7$), 23.0 ± 4 ($n = 7$), 27.4 ± 4 ($n = 5$) and 25.3 ± 3.1 ($n = 11$), respectively. Also, acute administration of EEDQ (0.25–4 mg/kg, i.v., $n = 3$) did not significantly alter the firing rate of LC noradrenergic neurons (data not shown).

Both clonidine and guanfacin antagonized the disappearance rate of brain noradrenaline in α-MT treated rats. These effects were attenuated by pretreatment with EEDQ, (1, 10 or 20 mg/kg) as shown in Figs. 5–7.

Discussion

In a previous study, pretreatment with EEDQ (0.8–1.6 mg/kg) was reported to antagonize the effect of a full α_2-adrenoceptor agonist at terminal, presynaptic α_2-adrenoceptors exerting an inhibitory influence on the release of noradrenaline from rat cortical slices in vitro (Adler et al. 1987). The present finding that a similar dose of EEDQ (1 mg/kg) decreases the inhibitory influence of the α_2-adrenoceptor agonists clonidine and guanfacine on whole brain noradrenaline utilization demonstrates that EEDQ also interacts with brain α_2-adrenoceptor mechanisms in vivo. However, the same dose of EEDQ failed to antagonize clonidine-induced inhibition of LC



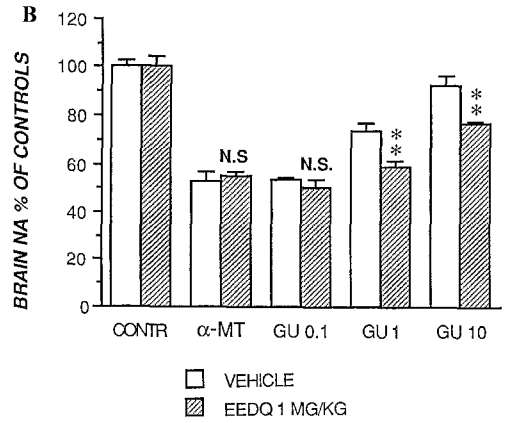
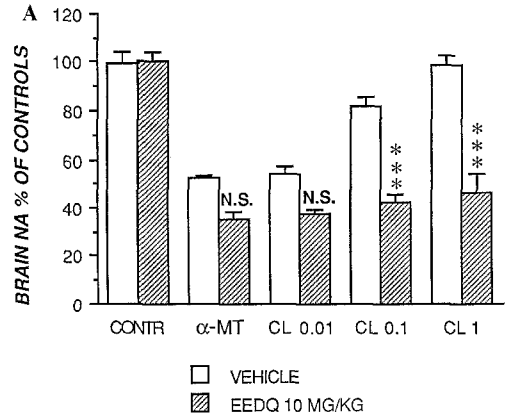


Fig. 5 A, B. Effects of EEDQ (1 mg/kg, 24 h) **(A)** on clonidine and **(B)** guanfacine-induced inhibition of the disappearance of noradrenaline in the whole brain of rats given the noradrenaline synthesis inhibitor α-MT. Brain noradrenaline levels in controls not given α-MT were 425 ng/mg (SEM 19) (EEDQ) and 534 ng/mg (SEM 15) (vehicle), respectively. All values are presented as % of these control values. In vehicle-treated animals the group receiving α-MT differed significantly from controls (*CONTR*) ($P < 0.001$) and from rats given α-MT + clonidine 0.1 mg/kg (*CL 0.1*) ($P < 0.001$), α-MT + clonidine 1 mg/kg (CL 1) ($P < 0.001$), α-MT + guanfacine 1 mg/kg (*GU 1*) ($P < 0.001$) or α-MT + guanfacine 10 mg/kg (GU 10) ($P < 0.001$). In EEDQ-treated animals the group receiving α-MT differed significantly from controls ($P < 0.001$) and from rats given α-MT + clonidine 1 mg/kg ($P < 0.01$) or α-MT + guanfacine 10 mg/kg ($P < 0.001$). The levels of significance for differences between rats treated with vehicle and EEDQ, respectively, are indicated in the figure. NS, not significant; ** = $P < 0.01$



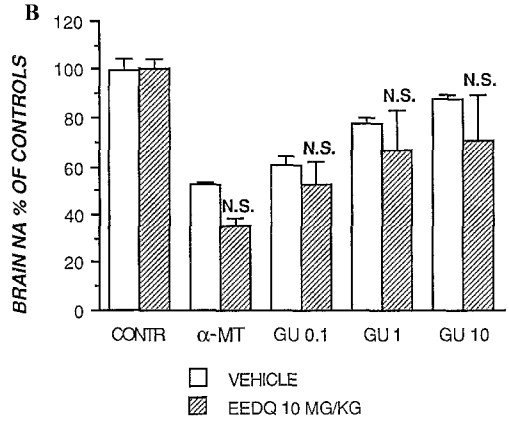


Fig. 6. Effects of EEDQ (10 mg/kg, 24 h) on clonidine (A) and guanfacine (B) induced inhibition of the disappearance of noradrenaline in the whole brain of rats given the noradrenaline synthesis inhibitor α-MT. Brain noradrenaline levels in controls not given α-MT were 375 ng/mg (SEM 17) (EEDQ) and 537 ng/mg (SEM 23) (vehicle), respectively. All values are presented as % of these control values. In vehicle treated animals the group receiving α-MT differed significantly from controls (*CONTR*) ($P < 0.001$) and from rats given α-MT + clonidine 0.1 mg/kg (*CL 0.1*) ($P < 0.05$), α-MT + clonidine 1 mg/kg (*CL 1*) ($P < 0.001$), α-MT + guanfacine 1 mg/kg (*GU 1*) ($P < 0.05$) or α-MT + guanfacine 10 mg/kg (*GU 10*) ($P < 0.01$). In EEDQ-treated animals the group receiving α-MT differed significantly from controls ($P < 0.001$) and from α-MT + guanfacine 1 mg/kg) ($P < 0.01$) or α-MT + guanfacine 10 mg/kg ($P < 0.01$). The level of significance for differences between rats treated with vehicle and EEDQ, respectively, are indicated in the figure. NS, not significant; *** = $P < 0.001$

cell firing; hence it may be concluded that the effect of α_2-adrenoceptor antagonists on noradrenaline turnover is not primarily related to a decrease in cell firing but rather mediated by release-regulating presynaptic receptors in the terminal region (cf. Curet et al. 1987).

Previous radioligand-binding studies have shown that EEDQ reduces the density of α_2-receptor binding sites in the central nervous system; a maximal reduction of 85% of cortical α_2-receptor binding sites was observed after administration of 1.6 mg/kg of the drug (Adler et al. 1985). In previous studies using EEDQ as an inactivator of brain catecholamine receptors, the decreased receptor responsiveness observed after administration of EEDQ has generally been attributed to an EEDQ-induced decrease in receptor density leading to a reduction of the amount of spare receptors (Meller et al. 1986; Adler et al. 1987; Nasseri and Minnemann 1987). If a similar mechanism is also responsible for the decreased responses to clonidine and guanfacine observed in the present study, the finding that higher doses of EEDQ were required in order to inactive α_2-adrenoceptors regulating LC firing than those exerting an inhibitory influence on transmitter utilization would indicate that somatodendritic autoreceptors are characterized by a considerably larger receptor reserve than are the turnover-regulating receptors (tentatively situated in the terminal region) (cf. Meller et al. 1986; Adler et al. 1987; Nasseri and Minnemann 1987). However, the possibility that the relatively low sensitivity of somatodendritic compared with terminal α_2-adrenoceptors, as observed following EEDQ pretreat-

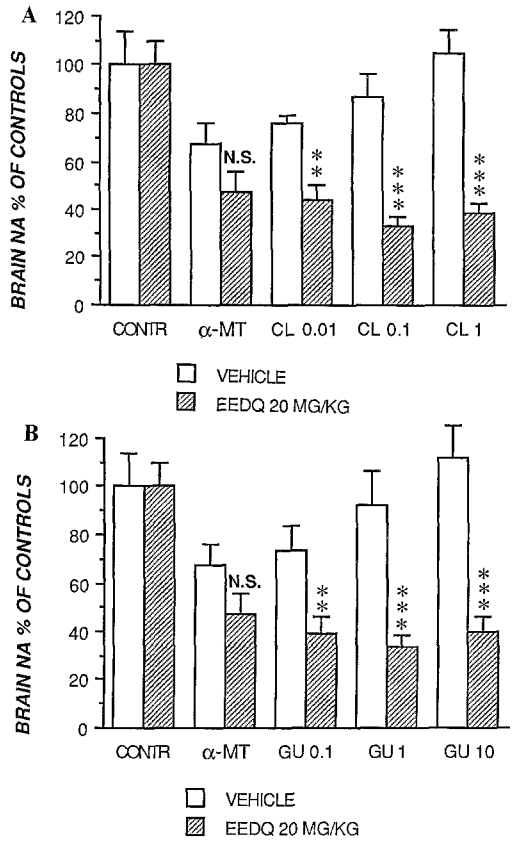


Fig. 7A, B. Effects of EEDQ (20 mg/kg, 24 h) on **(A)** clonidine and **(B)** guanfacine induced inhibition of the disappearance of noradrenaline in the whole brain of rats given the noradrenaline synthesis inhibitor α-MT. Brain noradrenaline levels in controls not given α-MT were 396 ng/mg (SEM 36) (EEDQ) and 432 ng/mg (SEM 60) (vehicle), respectively. All values are presented as % of these control values. In vehicle-treated animals the group receiving α-MT differed significantly from controls (*CONTR*) ($P < 0.05$) and from rats given α-MT + clonidine 1 mg/kg (*CL 1*) ($P < 0.01$), α-MT + guanfacine 1 mg/kg (*GU 1*) ($P < 0.05$) or α-MT + guanfacine 10 mg/kg (*GU 10*) ($P < 0.01$). In EEDQ-treated animals the group receiving α-MT differed significantly from controls ($P < 0.001$). The level of significance for differences between rats treated with vehicle and EEDQ, respectively, are indicated in the figure. NS, not significant; ** = $P < 0.01$; *** = $P < 0.001$

ment, is due to a difference in the structure of the respective receptor complexes rather than to differences in receptor number cannot be excluded. Indeed, recent pharmacological experiments as well as receptor cloning studies support the notion of different subpopulations of α_2-adrenoceptors in brain (Raiteri et al. 1983; Bylund 1988).

At doses considerably higher than those previously reported as maximal with respect to reducing α_2-adrenoceptor density in the cerebral cortex (Adler et al. 1985; Pilc et al. 1989), EEDQ also antagonized clonidine-induced inhibition of LC firing rate (as illustrated by an unparalleled rightward shift of the dose-response curve). While the effect of EEDQ on terminal α_2-adrenoceptors regulating turnover may well be related to a decrease in receptor density, to what extent the influence of high doses of EEDQ on antagonist-induced suppression of LC activity is due to a decrease in receptor number may thus be questioned. Alternatively, high doses of EEDQ may decrease the responsiveness of somatodendritic α_2-adrenoceptors by changing the structure of the receptor molecule or other subunits of the receptor complex (cf. Ekman and Eriksson 1991).

Like clonidine, guanfacine induced a total suppression of firing rate of LC neurons in control animals. In this regard, guanfacine was ten times less potent than clonidine; a similar difference in potency between the two agonists has previously been reported with respect to other α_2-adrenoceptor-mediated effects in the peripheral nervous system as well as in brain (Takeushi et al. 1987; Scholtysik 1980). In rats given a high dose of EEDQ the maximal response to guanfacine was only moderately decreased, while the efficacy of clonidine was clearly reduced, or even reversed. This difference is in line with the concept that clonidine is a partial agonist with lower intrinsic efficacy than guanfacine (Takeushi et al. 1987).

In conclusion, the results of the present study show that high doses of EEDQ reduce the responsiveness of α_2-adrenergic autoreceptors regulating the firing rate of brain noradrenergic neurons as well as the utilization of noradrenaline. The finding that a low dose of EEDQ antagonizes the effect of clonidine on noradrenaline disappearance, but not the decrease in LC firing rate induced by the drug, suggests that the effect of clonidine on noradrenaline disappearance is not due to the decrease in LC firing induced by the drug but probably exerted by α_2-adrenoceptors exerting a more direct influence on noradrenaline utilization. To what extent the effect of high doses of EEDQ on the influence of agonist-induced inhibition of LC firing is related to a decrease in receptor density or to other mechanisms is not clear.

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