Receptor Reserve and Turnover of Alpha-2 Adrenoceptors that Mediate the Clonidine-Induced Inhibition of Rat Locus Coeruleus Neurons In Vivo

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ABSTRACT
The population of reserve alpha-2 adrenoceptors that mediate the inhibitory effect of clonidine on the activity of locus coeruleus neurons has been studied using extracellular recordings in anesthetized rats. Animals were pretreated with the irreversible receptor antagonist N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). In rats pretreated with EEDQ (1, 2 and 6 mg/kg, i.p., 6 hr before experiment), there was an increase in firing rate, a reduction in firing regularity (i.e., increased variation coefficient) and an increase in burst firing of locus coeruleus neurons. Partial receptor inactivation with EEDQ (1 and 2 mg/kg, i.p.) caused a dose-dependent shift to the right of dose-effect curves for i.v. administered clonidine together with a reduction in its maximal effect. Higher doses of EEDQ (6 mg/kg, i.p.) completely abolished the effect induced by clonidine. This blockade was associated with a progressive decrease in the number of remaining receptors (noninactivated receptors). The pseudo-constant of dissociation for the drug-receptor complex was calculated to be approximately 70 μg/kg. The receptor occupancy-effect relationship was hyperbolic giving a value of only ~4% occupancy at 50% maximal effect. Estimates of noninactivated receptors and percentage of receptor occupancy at 50% of maximal effect were comparable when locally administered clonidine was used. After complete receptor inactivation with EEDQ (6 mg/kg), dose-effect curves for clonidine recovered gradually. The inhibitory effect of clonidine returned faster (half-life = 14 hr) than the receptor pool (half-life = 37 hr). These results indicate that locus coeruleus neurons have a large reserve of α2 adrenoceptors that in addition, are rapidly turned over.

The “receptor reserve” theory postulates that the relationship between drug effect and receptor occupancy is a nonlinear function (Furchgott, 1966; Stephenson, 1956). According to this hypothesis, systems with a large pool of reserve receptors require only a small fraction of receptors to be occupied to elicit a near maximal response. Classically, quantification of receptor reserve has been performed by comparing the effect of an agonist before and after a fraction of receptors is inactivated (Furchgott, 1966). In particular, reserve of monoamine receptors can be analyzed using Furchgott’s methodology applied to the irreversible receptor blocker EEDQ (Belleau et al., 1968), an alkylating agent that binds to adrenoceptors, dopamine receptors and 5-hydroxytryptamine receptors (Meller et al., 1985; 1988). In the CNS, a large pool of reserve monoamine receptors has been reported including alpha and beta adrenoceptors (Adler et al., 1987; Atkinson and Minneman, 1992), dopamine receptors (Cox and Waszak, 1989; Meller et al., 1987) and 5-hydroxytryptamine receptors (Cox et al., 1993; Meller et al., 1990).

It is now widely accepted that alpha-2 adrenoceptors that are located on presynaptic noradrenergic nerves, mediate negative feedback inhibition of norepinephrine release (Starke, 1987). There is a large reserve population of alpha-2 adrenoceptors mediating this inhibitory effect in the rat cerebral cortex (Adler et al., 1987; Agneter et al., 1993). In addition, receptor reserve for other regulatory effects mediated by brain alpha-2 adrenoceptors has been reported, including mydriasis (Menargues et al., 1991), hypotension (Hamilton and Reid, 1985), sedation and hypothermia (Durcan et al., 1994). The LC nucleus, which represents the major noradrenergic cluster in the brain, contains somatodendritic alpha-2 adrenoceptors (Cedarbaum and Aghajanian, 1976; 1977; Williams et al., 1985). In in vivo studies, systemic administration of the alpha-2 adrenoceptor agonist clonidine inhibits the firing rate of LC neurons by a mechanism that can be mimicked by local administration of this agonist (Svensson et al., 1975). Engberg and Eriksson (1991) have reported that alpha-2 adrenoceptors regulating LC cell
activity are characterized by a large receptor reserve. This suggestion is based on the observation that a proportion of alpha-2 adrenoceptors can be blocked by EEDQ without affecting the maximal effect of clonidine on LC neurons.

The purpose of this study was to quantify in vivo the reserve pool of alpha-2 adrenoceptors that regulate the activity of LC neurons. Dose-effect curves for the inhibitory effect of clonidine on the firing rate of LC neurons were compared in control rats and in rats subjected to blockade of alpha-2 adrenoceptors with EEDQ. In addition, the turnover of alpha-2 adrenoceptors was studied by evaluating the recovery of the clonidine effect after complete inactivation of alpha-2 adrenoceptors with EEDQ.

Materials and Methods

Materials. Clonidine hydrochloride and EEDQ were purchased from Sigma Chemical Co. (St. Louis, MO). EEDQ was dissolved in ethanol and then diluted sequentially in propylene glycol and water (0.25:0.25:0.50, v/v/v). Clonidine was dissolved in 0.9% NaCl to be administered i.v.

Animals and drug treatments. Adult male Sprague-Dawley rats (220-330 g) were treated with EEDQ (1, 2 and 6 mg/kg, i.p., at a volume of 1 ml/kg, 6 hr before the experiments) to produce a partial or complete block of alpha-2 adrenoceptors. To study the recovery of alpha-2 adrenoceptors, animals were treated with EEDQ (6 mg/kg, i.p., 12, 24, 48 and 96 hr before the experiments). In control experiments, an equivalent volume (1 ml/kg) of vehicle was administered. Extracellular single-unit recordings of LC neurons were performed as described previously (Pineda et al., 1993). The animals were anesthetized initially with chloral hydrate (400 mg/kg, i.p.) and additional doses of the anesthetic were administered through a catheter via the jugular vein as needed. Rats were placed in a stereotaxic frame with the head oriented 15° below the horizontal plane, and a 3-mm burr hole was drilled 3.7 mm posterior to lambda and 1.1 mm lateral to the midline (Paxinos and Watson, 1986). Rat body temperature was maintained at 36 to 37°C by means of a heating pad. Omegadot glass micropipettes that had been filled with 2% Pontamine sky blue in 0.5% sodium acetate (in vitro impedance 2-6 MΩ) were lowered to 5.6 mm below the cortical surface. The extracellular signal was amplified and then monitored on an oscilloscope and an audiometer. Firing rates, which were obtained by an electronic rate meter triggered by individual neuronal spikes, were displayed on a pen chart recorder as consecutive 10-sec histograms. The mean and variation coefficient of firing rate and the percentage of burst firing were calculated with a PC-based computer that created interspike-time-interval histograms. The variation coefficient (SD/mean interspike interval) is a measure of the regularity of firing. Burst onset was indicated by an interval < 80 msec and burst termination was signaled by an interval > 160 msec. LC neurons were identified by standard criteria which included: 1) a regular firing rate at 0.5 to 5 Hz; 2) a characteristic spike with positive-negative waveform and 3) a biphasic excitation-inhibition response to pressure applied on the contralateral hind paw ("paw pinch") (Cedarbaum and Aghajanian, 1976). Additional clues to locate the LC were a zone of relative electrical silence just dorsal to the LC (corresponding to the IVth ventricle) and the presence just lateral of the LC of the mesencephalic nucleus of the Vth nerve, whose cells were activated by displacement of the mandible. The recording sites were marked at the end of each experiment, by passing a 5 μA cathodic current through the recording electrode for 10 min, thereby depositing a blue spot at the location of the electrode tip. The rats were perfused transcardially with 10% formaldehyde and serial 50 μm frozen sections of the brain were cut and stained with neutral red to be examined microscopically. Brains with labeled cells located outside the LC were discarded from this study.

Intracereovertical pressure microinjections. A thick-wall pipette with a calibrated narrow inner diameter was broken back (tip diameter ~ 40 μm) and filled with a solution of clonidine (1 or 10 μM) in Dulbecco’s buffered saline solution (NaCl 136.9 mM, KCl 2.7 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM, MgCl₂ 5 mM and CaCl₂ 9 mM, pH ~ 7.4). Pipettes made in this manner were glued adjacent to a recording microtipette using the procedure described by Akaoka et al. (1992). Drug ejection was performed by applying one or more pressure pulses (50-150 msec) by a solenoid-controlled pneumatic pressure device (Picospritzer, General Valve Corp., Fairfield, NJ) driven by synthetic air. The volume of each ejection pulse, measured by monitoring the meniscus movement in the calibrated pipette, was ~3.2 nl. Doses of clonidine were calculated as the volume of solution locally administered (i.e., number of ejection pulses multiplied by 3.2 nl/pulse) multiplied by the concentration of clonidine in the pipette solution.

Analysis of dose-effect curves. The alpha-2 adrenoceptor agonist clonidine was administered i.v. at cumulative doses of 0.6 to 640 μg/kg (2x) (at 1-min intervals for each successive dose) until a maximal response was reached. For local applications in the LC, clonidine was applied via the microinjection pipette at cumulative doses of 3.3 to 13.2 fmol (2x) or 31.4 to 2009 fmol (2x), every 30 sec, until the maximal effect was achieved. The inhibition of LC cells induced by clonidine was quantified as the percentage reduction from the basal firing rate. Baselines were considered as the mean firing rate recorded for 3 to 8 min before the experiment. Experimental data from each group were pooled and analyzed using the computer program GraFit (v. 2.08, Erithacus Software Ltd., Staines, Middlesex, UK) (Leatherbarrow, 1990) for the best simple nonlinear fit to the three-parameter logistic equation $E = E_{max}/(1 + (ED_{50}/A)^n)$, where $E$ is the effect induced by a certain dose of clonidine (A), $E_{max}$ is the maximal effect, $ED_{50}$ is the dose of clonidine needed to elicit a 50% of $E_{max}$ and $n$ is the slope factor of the dose-effect curve (Parker and Waud, 1971). $ED_{50}$, $E_{max}$ and n were estimated by this analysis.

Analysis of receptor affinity and receptor reserve. Receptor affinity, which in vivo is referred to as the pseudo-dissociation constant ($K_D$) (Meller et al., 1990), was calculated using the method of Furchgott (1966), modified for nonlinear regressions by James et al. (1989). Using this method, the native receptors remaining after partial receptor inactivation were studied. Dose-effect curves for clonidine-induced inhibition of the LC after vehicle pretreatments were compared to dose-effect curves after EEDQ pretreatments as follows: data from the vehicle group were fitted to $E = E_{max}/(1 + (ED_{50}/A)^n)$ and simultaneously, data from the EEDQ group were fitted to $E = K_A/([A] + K_A A)/(1 + [A] K_A A)$, where $E$ is the effect induced by a certain dose of clonidine (A') after EEDQ pretreatment, $q$ is the fraction of receptors not inactivated by EEDQ, $K_A$ is the pseudo-dissociation constant of the receptor, and $E_{max}$, $ED_{50}$ and $n$ are as described above. $ED_{50}$, $E_{max}$, $q$, and $K_A$ were estimated by this analysis. When receptor inactivation by EEDQ was not sufficient to reduce the maximal effect, Furchgott’s analysis could not be applied, and the $q$ value was estimated as the ratio between $ED_{50}$ of dose-effect curves after vehicle or EEDQ pretreatments (i.e., $ED_{50}$ after vehicle/$ED_{50}$ after EEDQ) (Minneman and Abel, 1984). The $K_A$ values were used to calculate the percentage receptor occupancy at a particular dose of clonidine (A) from the law of mass action as follows: occupancy = $100 A/(A + K_A)$. Receptor occupancy was plotted against effect for each dose and analyzed for the best fit to $E = E_{max}/(1 + (K_A Oc)^n)$, where Oc is the receptor occupancy at a certain dose of clonidine, $K_A$ is the percentage of receptors needed be occupied to elicit a 50% of the maximal effect, and $E_{max}$ and n are as described above (Black and Leff, 1983).

Analysis of receptor turnover. Receptor turnover was evaluated using the method described by Mauger et al. (1982), which analyzes the recovery of newly synthesized receptors after inactivation of the total receptor pool. After total blockade of alpha-2 adrenoceptors with EEDQ (6 mg/kg), recovery of dose-effect curves for clonidine-inhibited induction of LC neurons was followed at various...
cells with a potency (ED$_{50}$ = 2.7 μg/kg) which is consistent with previously reported values (Lacroix et al., 1991; Marwaha and Aghajanian, 1982; Svensson et al., 1975) (fig. 1A; table 2). Pretreatments with EEDQ (1 and 2 mg/kg) induced a progressive shift to the right of dose-effect curves for clonidine, resulting in ED$_{50}$ values that were increased by 14 fold (P < .05) and 27-fold (P < .001), respectively (figs. 1B and C and 2; table 2). In addition, EEDQ administered at 2 mg/kg caused a reduction in the maximal effect induced by clonidine (E$_{max}$ = 55%, P < .001), but no significant change in the maximal effect was found after EEDQ administration at 1 mg/kg (figs. 1B, 1C and 2; table 2). Pretreatments with a higher dose of EEDQ (6 mg/kg) completely blocked the effect induced by clonidine (figs. 1D and 2; table 2).

Analysis of receptor reserve revealed that pretreatments with EEDQ (1 and 2 mg/kg) produced a progressive reduction in the number of alpha-2 adrenoceptors (q = 0.10 and 0.04, respectively) (table 2). The pseudo-dissociation constant (K$_A$) for the alpha-2 adrenoceptor, as estimated with EEDQ (2 mg/kg), was 73 μg/kg. This value was not significantly different from the K$_A$ calculated with a lower dose of EEDQ (1 mg/kg) (K$_A$ = 71 μg/kg) (table 2), consistent with the idea that estimates of K$_A$ are independent of the fraction of receptors that are inactivated by EEDQ. Receptor occupancy was derived from the K$_A$ value of 73 μg/kg and plotted against the effect, resulting in a hyperbolic occupancy-effect relationship (fig. 3). In this occupancy-effect curve, 50 and 95% of the maximal response occurred at only 3.8% (K$_E$) and 17% receptor occupancy, respectively.

To rule out a possible cross-talk between the actions of EEDQ and clonidine outside the LC, dose-effect curves for local applications of clonidine were studied in control rats and rats pretreated with EEDQ. In control rats, locally applied clonidine completely inhibited the activity of LC neurons (fig. 4A; table 3). Pretreatment with EEDQ (2 mg/kg, i.p.) produced a 57-fold increase in the ED$_{50}$ for clonidine (P < .001), with a reduction in the maximal effect of this agonist (E$_{max}$ = 38%) (figs. 4A and B; table 3). The fraction of alpha-2 adrenoceptors not inactivated by EEDQ (2 mg/kg) (q = 0.047) was equivalent to previous estimates using systemic application of clonidine (see above; tables 2 and 3). The K$_A$ was calculated to be 72 fmol (table 3). The receptor occupancy-effect relationship was hyperbolic with a K$_E$ of 5.6%.

**Alpha-2 adrenoceptor turnover in LC neurons.** After complete inactivation of alpha-2 adrenoceptors with EEDQ (6 mg/kg), which occurs 6 hr after drug administration (see above), recovery of dose-effect curves for clonidine occurred gradually within a period of 12 to 96 hr (figs. 5 and 6). The recovery of the maximal effect of clonidine was more rapid (24 hr after EEDQ) than the recovery of ED$_{50}$ values (96 hr after EEDQ) (table 4; fig. 6).

Analysis of dose-effect curves after partial receptor reappearance (Furchgott, 1966) indicated that the newly synthesized receptors (q) recovered to near 100% of control levels within 96 hr (table 4). The K$_A$ of newly synthesized receptors (52 μg/kg) was similar to the K$_A$ of native receptors (tables 2 and 4). The time course of receptor recovery was fitted to an exponential equation (Mahan et al., 1987) to calculate the rate constants of receptor reappearance (r = 2.27%R$_{inf}$/hr) and receptor degradation (k = 0.02/hr) (fig. 7). The relative proportion of newly synthesized receptors at steady state (R$_m$ = 114% of control) was not significantly different from

### Results

All EEDQ doses were administered 6 hours before each experiment, except where stated otherwise.

**Effect of EEDQ on the activity of LC neurons.** The mean firing rate, the variation coefficient of firing and the percentage of burst firing of LC neurons were evaluated in rats pretreated with vehicle (control) or EEDQ (1, 2 and 6 mg/kg) (receptor inactivated). In rats pretreated with vehicle, LC neurons had an average firing rate of ~ 2 Hz, a regular firing pattern (variation coefficient 43.5%) and a small proportion of spikes in bursts (1.46%) (table 1). In rats pretreated with EEDQ at 6 mg/kg, the following modifications with respect to controls were observed: 1) the firing rate of LC neurons increased by 76% (P < .05); 2) there was an increase in the variation coefficient of cell firing (P < .01) indicating a reduced regularity of discharge and 3) the amount of spikes in bursts also increased (P < .05) (table 1).

**Alpha-2 adrenoceptor reserve in LC neurons.** The presence of alpha-2 adrenoceptor reserve was examined by establishing dose-effect curves for the alpha-2 adrenoceptor agonist clonidine in rats pretreated with vehicle or EEDQ (1, 2 and 6 mg/kg). In rats pretreated with vehicle, i.e. application of clonidine completely inhibited the firing rate of LC neurons with a potency (ED$_{50}$ = 2.7 μg/kg) which is consistent with previously reported values (Lacroix et al., 1991; Marwaha and Aghajanian, 1982; Svensson et al., 1975) (fig. 1A; table 2). Pretreatments with EEDQ (1 and 2 mg/kg) induced a progressive shift to the right of dose-effect curves for clonidine, resulting in ED$_{50}$ values that were increased by 14 fold (P < .05) and 27-fold (P < .001), respectively (figs. 1B and C and 2; table 2). In addition, EEDQ administered at 2 mg/kg caused a reduction in the maximal effect induced by clonidine (E$_{max}$ = 55%, P < .001), but no significant change in the maximal effect was found after EEDQ administration at 1 mg/kg (figs. 1B, 1C and 2; table 2). Pretreatments with a higher dose of EEDQ (6 mg/kg) completely blocked the effect induced by clonidine (figs. 1D and 2; table 2).

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### Tables

**Table 1**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Firing Rate (Hz)</th>
<th>Variation Coefficient (%)</th>
<th>Burst Firing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.83 ± 0.34</td>
<td>43.5 ± 4.1</td>
<td>1.46 ± 0.67</td>
</tr>
<tr>
<td>EEDQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>2.78 ± 0.53</td>
<td>48.1 ± 3.0</td>
<td>4.31 ± 1.77</td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>3.04 ± 0.35</td>
<td>47.6 ± 2.1</td>
<td>2.93 ± 1.34</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>3.22 ± 0.18b</td>
<td>67.9 ± 6.2b</td>
<td>13.78 ± 3.69b</td>
</tr>
</tbody>
</table>

*a* Vehicle (control) and EEDQ were administered i.p. 6 hr before experiments; values are mean ± S.E.M. of n cells (see "Materials and Methods" for calculations).

$^b$ P < .05, $^c$ P < .01 compared to control by one-way analysis of variance followed by Scheffe’s test.

$^d$ P < .05 compared to control by Kruskal Wallis’s test followed by Mann Whitney’s test.
the native situation (i.e., 100%). The \( \alpha_2 \) adrenoceptor half-life for receptor recovery (\( t_{1/2} = 37 \) h) was slower than that for functional recovery (\( t'_{1/2} = 14 \) h).

**Discussion**

Our study demonstrates that there is a large reserve of \( \alpha_2 \) adrenoceptors that mediate the inhibition of the activity of LC neurons. This may explain why the inhibitory responses to clonidine and guanfacine in LC cells have been proposed to be relatively insensitive to EEDQ inactivation (Engberg and Eriksson, 1991).

To investigate the affinity of \( \alpha_2 \) adrenoceptors, we used the method described by Furchgott (1966) and modified by James et al. (1989) for nonlinear regressions. Furchgott’s analysis has the advantage in *vivo* over other approaches (Furchgott and Bursztyn, 1967; Waud, 1969) in that it minimizes the existence of pseudo-equilibrium reactions of reversible binding. Nonlinear regressions provide more precise and direct estimates of affinity than linear transformations (James et al., 1989). This methodology requires the availability of an irreversible antagonist and an agonist for the receptor under investigation. Clonidine was chosen as the agonist because it produces a potent and complete inhibition of firing of LC neurons through somatodendritic \( \alpha_2 \) adrenoceptors (Cedarbaum and Aghajanian, 1977; Svensson et al., 1975; Williams et al., 1985). Intravenous administration of clonidine has been shown to be a reliable way to construct reproducible dose-effect curves (Lacroix et al., 1991; Marwaha and Aghajanian, 1982). EEDQ is an irreversible antagonist of both \( \alpha_2A \) and \( \alpha_2B \) adrenoceptors (Bar- turten and García-Sevilla, 1992; Pile et al., 1989; 1992) that has been used extensively to calculate \( \alpha_2 \) adrenoceptor reserve (Adler et al., 1987; Agneter et al., 1993).

High doses of EEDQ also block other neurotransmitter receptors (Meller et al., 1985; 1988). The order of sensitivity of several rat brain receptors to EEDQ has been evaluated in radioligand binding studies by obtaining the maximal degree of inactivation after the administration of this agent (EEDQ 6 mg/kg, s.c., 24 hr): \( \alpha_2 \)-adrenoceptors (95%) > \( \alpha_1 \)-adrenoceptors (80%) > D2/D1 receptors (70%) > 5-HT2/5-HT1 receptors (60%) > \( \beta_1 \) (25%) > muscarinic (10%) (Meller et al., 1985). Thus, the highest dose of EEDQ used in our study (6 mg/kg) would be expected to inactive \( \alpha_2 \) adrenoceptors and, to a lesser extent, \( \alpha_1 \) adrenoceptors and other receptors. Moreover, binding experiments have demonstrated that \(^{3}H\)clonidine is a highly selective ligand for \( \alpha_2 \) adrenoceptors, with a moderate affinity for \( \alpha_1 \) adrenoceptors (Timmermans et al., 1984). The major \(^{3}H\)clonidine binding component that is inactivated by

**TABLE 2**

Parameters of clonidine dose-effect curves and of \( \alpha_2 \) adrenoceptor reserve after EEDQ pretreatments. (Intravenous applications of clonidine)*

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose-Effect Curves</th>
<th>Noninactivated Receptors</th>
<th>Receptor Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E_{\text{max}} ) (%)</td>
<td>( ED_{50} ) (µg/kg)</td>
<td>( q_{100} )</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>2.7 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>EEDQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>92 ± 5</td>
<td>39 ± 5(^{c})</td>
<td>9.1 ± 1.3</td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>55 ± 8(^{d})</td>
<td>73 ± 21(^{c})</td>
<td>4.1 ± 1.7(^{d})</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Vehicle (control) and EEDQ were administered i.p. 6 hr before experiments; values are the best fit ± S.E. obtained by nonlinear regressions of \( n \) cells (see “Materials and Methods” for calculations).

\(^{c}\) Indicates that no value could be measured.

\(^{d}\) Indicate that no value could be measured.

\(^{a}\) Indicates that no value could be measured.

\(^{b}\) Indicates that no value could be measured.
EEDQ has the pharmacology of an alpha-2 adrenoceptor in rat brain (Barturen and García-Sevilla, 1992). However, alpha-1 adrenoceptors do not seem to play an important role in the LC (Marwaha and Aghajanian, 1982; Nicholas et al., 1996). Therefore, the possibility that altered cross-talk between receptors could influence the quantification of alpha-2 adrenoceptor reserve is unlikely, although cannot yet be ruled out. Finally, the selectivity of clonidine for alpha-2 adrenoceptors in the LC of EEDQ-pretreated animals was confirmed in one experiment in which the selective alpha-2 adrenoceptor antagonist RX821002 (400 µg/kg, i.v.; Uhlen and Wikberg, 1991) was able to completely reverse the inhibitory effect induced by clonidine (data not shown).

In agreement with binding and functional studies (see above), EEDQ had the typical profile of an irreversible antagonist of alpha-2 adrenoceptors in the LC; EEDQ dose-dependently blocked the inhibitory effect induced by...
clonidine, and the clonidine effect was abolished after the highest dose of this blocker (6 mg/kg). According to Furchgott's analysis, the fraction of receptors not inactivated by EEDQ \( q \) decreased with higher doses of the antagonist. The value of \( q \) after EEDQ administration (2 mg/kg) as assessed by i.v. clonidine was similar to the value of \( q \) that was calculated using locally applied clonidine. This suggests that the major component of the inhibition induced by systemic administration of clonidine is mediated by \( \alpha-2 \) adrenoceptors located at the somatodendritic level, ruling out an altered interaction at a different level. Furthermore, the basal firing rate of LC neurons was increased by EEDQ administration, resembling the effect of other \( \alpha-2 \) adrenoceptor antagonists in the LC (Cedarbaum and Aghajanian, 1976; 1977). This is consistent with the idea that LC neurons are inhibited tonically by norepinephrine acting on \( \alpha_2 \) adrenoceptors. In addition, the increased spontaneous impulse activity of LC neurons found in EEDQ-pretreated rats could be due in part to indirect effects of this compound outside of the LC (e.g., changes in blood pressure). EEDQ also deregularized the firing pattern of LC neurons (i.e., increased variation coefficient), which suggests that \( \alpha-2 \) adrenoceptors might tonically maintain a certain degree of regularization of firing in LC neurons. Indeed, a role for \( \alpha-2 \) adrenoceptors in regularizing cell firing patterns has been proposed for neurons in the LC (Murase et al., 1992) and in the substantia nigra (Grenhoff and Svensson, 1988).

In vivo analysis of receptor affinity for clonidine in the LC yielded a pseudo-constant of dissociation \( K_A \) equal to 73 \( \mu g/kg \). This value is comparable to receptor affinity for other in vivo functions mediated by central \( \alpha-2 \) adrenoceptors such as clonidine-induced mydriasis \( K_A = 76 \mu g/kg \); Menargues et al., 1991). Certain departures from the traditional assumptions of Furchgott's analysis should be considered in the interpretation of \( K_A \) data. First, responses to clonidine after either vehicle or EEDQ were measured in separate groups of animals. Hence, calculations of receptor reserve may be less accurate than in experiments in which the effect of agonist is evaluated on the same tissue both before and after the blocker. This problem might have been minimized by simultaneously analyzing the dose-effect curves in the presence or absence of the blocker with the method described by James et al. (1989). Second, the in vivo systemic administration of both EEDQ and clonidine does not provide for equilibrium conditions and leads to unknown concentrations of these drugs at the receptor sites. However, in our study, estimates of \( K_A \) were independent of the fraction of receptors not inactivated by the blocker (i.e., EEDQ at 1 mg/kg, \( q = 0.09, K_A = 71 \mu g/kg \); EEDQ at 2 mg/kg, \( q = 0.04, K_A = 73 \mu g/kg \)), thus supporting the idea that under our conditions a pseudo-equilibrium state may have been achieved. In similar in vivo studies of the dopamine system, \( K_{A50} \) obtained with Furchgott's method have been used as valid parameters in calculations of occupancy-response relationships (Cox and Waszczak, 1989; Meller et al., 1987). Third, Furchgott's method is considered applicable only in the theoretical situation where a drug acts on a single population of receptors to produce a single response. Although both clonidine and EEDQ have been reported to bind to different subtypes of \( \alpha-2 \) adrenoceptors (Barturen and García-Sevilla, 1992; Gleason and Hieble, 1991), LC neurons contain mostly the \( \alpha-2A \) subtype (Ruiz-Ortega and Ugedo, 1993; Scheinin et al., 1994). Finally, values of receptor affinity obtained by functional methodologies could be overestimated systematically (Kenakin, 1990; Leff et al., 1990). However, this systematic error may not be important if partial agonists such as clonidine are used or if there is a large pool of reserve receptors as is the case in the LC (Leff et al., 1990; Mackay, 1988a, b). The fact that changing the proportion of functional receptors by two different doses of EEDQ resulted in equivalent receptor affinities (see above) argues against an overestimation of \( K_A \) (Leff et al., 1990).

The reduction in the maximal effect of clonidine induced by EEDQ was less sensitive than the decrease in the pool of \( \alpha-2 \) adrenoceptors \( q \) caused by the blocker. This discrepancy suggests that a fraction of \( \alpha-2 \) adrenoceptors

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**Fig. 5.** Firing-rate recordings of LC neurons showing the recovery of the inhibitory effect of clonidine after complete receptor inactivation with EEDQ (6 mg/kg, i.p.). Experiments were performed at the following time points after EEDQ administration: 12 hr (A), 24 hr (B), 48 hr (C) and 96 hr (D). Clonidine was administered i.v. (arrows) at increasing (2x) cumulative doses (as indicated) until the maximal effect was reached. Vertical lines represent extracellularly recorded firing rates that were displayed on a chart recorder as integrated time histograms. The time scale is identical for all traces.
can be inactivated without affecting the intrinsic activity of the system. In addition, the dose of clonidine that elicited 50% of the maximal effect (ED50 = 2.7 μg/kg, for intravenous clonidine; ED50 = 4.3 fmol, for local clonidine) was smaller than the dose needed to occupy 50% of total receptors (Ka = 73 μg/kg, for i.v. clonidine; Ka = 72 fmol, for local clonidine), which indicates that less than 50% of receptor occupancy is able to produce 50% of the maximal effect. Finally, quantification of receptor reserve revealed that the occupancy-effect relation for the inhibitory effect of clonidine in the LC is hyperbolic. The hyperbolic nature of this relationship is typical of systems with large fractions of reserve receptors (Black and Leff, 1983). Only 3.6% of total receptors were needed to be occupied by clonidine (systemically administered) to elicit 50% of the maximal effect (Ka). This value was similar when the fraction of receptor reserve was calculated with local administration of clonidine (Ka = 5.6%), suggesting that LC neurons contain a large population of reserve alpha-2 adrenoceptors. Reserve receptors constituted 83% of total receptors at submaximal responses (95%) to clonidine. A smaller receptor brain alpha-2 adrenoceptor reserve has been reported for the inhibitory effect of clonidine on norepinephrine release (40%) in the cortex (Agneter et al., 1993) and for the mydriatic effect of clonidine (22%) (Menargues et al., 1991). This smaller reserve of receptors might be due to differences in receptor number or to variations in the amplification capabilities of these receptors in different tissues (Kenakin, 1993).

Turnover of alpha-2 adrenoceptors was studied by analyzing the recovery of the response to clonidine after irreversible receptor inactivation by EEDQ (6 mg/kg). This method is less toxic than other approaches which evaluate receptor disappearance (Mahan et al., 1987). After complete blockade of alpha-2 adrenoceptors (EEDQ, 6 mg/kg), dose-effect curves for clonidine progressively recovered within 2 to 3 days. Comparisons between control and EEDQ-pretreated groups by Furchgott’s methodology revealed that the affinity of newly synthesized alpha-2 adrenoceptors (Ka = 52 μg/kg) was equivalent to the affinity of native receptors (see above). In the CNS, similar affinities of native and newly synthesized alpha-2 adrenoceptors have also been found after total receptor inactivation by EEDQ (Adler et al., 1987; Barturen and García-Sevilla, 1992). Parameters of receptor recovery were determined by the method described by Mauger et al. (1982) and modified for nonlinear regressions (exponential) (Mahan et al., 1987). This analysis assumes that receptor production is constant during the entire period of reappearance of receptors (r = 2.27%RA/hr) and that degradation of these receptors is, at any time, proportional to the density of receptors in the cell (k = 0.02/hr). The half-life of alpha-2 adrenoceptor recovery was calculated to be 37 hr. This value is similar to
that obtained in the brainstem (as estimated from functional studies) (Hamilton and Reid, 1985; Menargues et al., 1991), but lower than that found in the anterior brain (as estimated from functional and binding studies) (Adler et al., 1985; Agneter et al., 1993; Barturen and García-Sevilla, 1992). These discrepancies suggest that receptor recovery may be delayed in brain projecting areas, presumably because alpha-2 adrenoceptors are synthesized in the cellular soma and then transported to distal areas (Levin, 1984). In addition, the half-life of alpha-2 adrenoceptor recovery was slower than the half-life of functional recovery (t½0.5 = 14 hr), which may reflect again the presence of a large receptor reserve for these adrenoceptors. An interesting aspect of the recovery of LC alpha-2 adrenoceptors after EEDQ inactivation is that the limit for this process (n/k parameter = 114%) was equivalent to the steady-state density before blockade (100%). This observation together with the similarity of K values (see above) indicate that alpha-2 adrenoceptors which are newly synthesized after EEDQ blockade are similar to native receptors.

In conclusion, the present study indicates that alpha-2 adrenoceptors that regulate the firing rate of cells in the LC are characterized by a high proportion of reserve receptors. The existence of an abundant receptor reserve might be of interest in relation to certain physiological conditions and pathological states (Kenakin, 1993). In addition, our data support the notion that alpha-2 adrenoceptors regulating LC activity are turned over in a relatively rapid manner.

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