[\textsuperscript{3}H]CLONIDINE BINDS AT MULTIPLE HIGH AFFINITY STATES IN HUMAN PREFRONTAL CORTEX

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[\textsuperscript{3}H]Clonidine binds at particulate membrane fractions of human prefrontal cortex in a process that demonstrates high affinity, saturability, reversibility, \(\alpha_2\)-adrenergic selectivity and the existence of multiple affinity states. At 37°C maximal specific [\textsuperscript{3}H]clonidine binding was briefly attained at 10 and lasted only until 15 min, while at 21°C maximal binding was maintained from 20 to 90 min. At 21°C, rate dissociation studies and saturation analyses were at least biphasic, and adrenergic competitors decreased [\textsuperscript{3}H]clonidine binding with Hill coefficients < 0.70. Analysis of these data showed at least two affinity states with apparent \(K_D\)s of 0.34 and 6.0 nM, and the order in which ligands decreased [\textsuperscript{3}H]clonidine binding was clonidine > (−)-epinephrine > (−)-norepinephrine > yohimbine > (+)-norepinephrine > (±)-isoproterenol > prazosin > serotonin.

\(\alpha_2\)-Adrenoceptors  Human brain  [\textsuperscript{3}H]Clonidine

1. Introduction

The binding of [\textsuperscript{3}H]clonidine to a variety of tissues, including both the central and peripheral nervous systems, platelets and internal organs has been well established in animals such as the rat, cow, chicken and man (U’Prichard et al., 1979; Braunwald et al., 1981; Shattil et al., 1981; Steer and Atlas, 1982; Randall et al., 1983). In literature describing these findings, [\textsuperscript{3}H]clonidine has been shown to bind at the \(\alpha_2\)-adrenoceptor (U’Prichard and Snyder, 1979). The receptor site has been postulated to occur pre- and post-synthetically in different tissues (Unnerstall et al., 1984). The existence of high and low affinity states of the \(\alpha_2\)-adrenoceptor with \(\alpha_2\)-adrenoceptor agonists preferentially binding at the former and antagonists at the latter, has also been suggested (U’Prichard et al., 1979). The two affinity states have been proposed to be non-interconverting independent sites (Greenberg et al., 1978). [\textsuperscript{3}H]Clonidine binding is down-regulated in the presence of GTP and its analogues and also monovalent cations (Greenberg et al., 1978; Glossman and Presek, 1979). The binding of this ligand is up-regulated in the presence of divalent cations (Glossman and Presek, 1979; Rout et al., 1980) and further up-regulated in the presence of a precise combination of GTP analogues and divalent cations (Rout et al., 1980).

[\textsuperscript{3}H]Clonidine binding in the human has been studied primarily in platelets (Garcia-Sevilla et al., 1981; Shattil et al., 1981; Steer and Atlas, 1982; Cameron et al., 1984) although there have been two reports using the brain (Weinreich et al., 1980; Cash et al., 1985). One of the goals of the platelet studies has been to compare the \(\alpha_2\)-adrenoceptor binding in normal and depressed individuals in order to quantitate changes in binding parameters with depression. Another method of studying the

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relationship between the $\alpha_2$-adrenoceptor and depressive disorders is to study the brain, the presumed site of pathology in depressive illness. With that in mind, the goal of this study was to establish the binding parameters of $[^3]$H]clonidine in normal human brain.

2. Materials and methods

Postmortem human brain was obtained at the time of autopsy from individuals who died sudden accidental or cardiovascular death with no history of psychiatric or neurologic illness, with no unrelated pathological findings, and with negative toxicological screens. A total of 14 specimens were used for this study, with an average age of 47 $\pm$ 8 years (range 30-59 years) and an average postmortem interval of 12.6 $\pm$ 5.6 h (range 2-26 h). Prefrontal cortex (PFC) was isolated, minced in a buffer consisting of 20 mM Na-Hepes (pH 7.4 at 4°C) plus 2 mM MgSO$_4$, quick-frozen in plastic vials in a dry-ice acetone bath and stored at $-70^\circ$C. Minces were quickly thawed at 37°C, diluted to 10 volumes (v/v) in ice-cold buffer, and homogenized with 10-15 strokes in a glass-teflon homogenizer. The homogenate was diluted to 50 volumes (v/v) of the original tissue and centrifuged at 39,000 $\times$ g at 4°C for 20 min. This washing procedure was repeated three more times in fresh buffer. The final pellets were resuspended to 1.5 volumes of the original tissue (v/v), quick frozen and stored at $-70^\circ$C. Protein concentration was determined by the method of Lowry et al. (1951) with BSA as the standard in the Hepes-MgSO$_4$ buffer.

$[^3]$H]Clonidine (52.3 Ci/mmol) was obtained from New England Nuclear, assayed by thin layer chromatography (3:1 acetone:methanol, Gelman Silica Acid glass fiber sheets) to be $>97\%$ pure, and stored in 7:3 ethanol:water at $-20^\circ$C. The following drugs were the generous gifts of the indicated companies: clonidine (Boehringer, Ingelheim), spiroperidol and ketanserin (Janssen Pharmaceuticals), metiamide (Smith, Kline and French), (+)-epinephrine and (+)-norepinephrine (Sterling-Winthrop) and prazosin (Pfizer). All other drugs and chemicals were purchased commercially (Sigma). Drug solutions were made fresh daily.

Binding of $[^3]$H]clonidine was assayed in duplicate in borosilicate glass test tubes. Aliquots of $[^3]$H]clonidine in ethanol/water were dried under $N_2$, resuspended in 200 mM Na-Hepes or Tris HCl (pH 7.4 at temperature of assay) and 1 mM Na-ascorbate, and equilibrated for 20 min at 4°C. A typical assay tube contained 100 $\mu$l of the $[^3]$H-ligand resuspension, 100 $\mu$l of competing drug or water and 700 $\mu$l of water. The binding reaction was initiated by the addition of 100 $\mu$l of particulate membrane suspension (quickly thawed PFC particulate fractions diluted 1:10 in 20 mM Na-Hepes or Tris HCl pH 7.4 at temperature of assay). Final concentrations of reagents were 0.1-10.0 nM $[^3]$H]clonidine, 2 mM Na-Hepes or Tris HCl, 0.1mM Na-ascorbate, and 0.3-0.8 mg protein/ml assay. Following incubation for the times stated, the binding reaction was terminated by rapid filtration on Schleicher and Schuell No. 30 glass fiber filters under reduced pressure. The tubes and filters were washed with three 5 ml aliquots of ice-cold 5 mM Na-Hepes (pH 7.4 at 4°C) plus 2 mM MgSO$_4$ (time for filtering and washing < 6 s). Filters were placed in plastic vials with 4.0 ml of Beckman EP, and radioactivity was determined by liquid scintillation spectrophotometry at an efficiency of 30%. When the $[^3]$H]clonidine concentration was below 0.2 nM, the assay volumes were tripled (to 3 ml), and when the $[^3]$H]clonidine concentration was $>3.0$ nM the assay volumes were halved. Sufficient counts were obtained to effect a counting error of $<5\%$. All experiments were repeated at least three times on different postmortem specimens. There was $<7\%$ coefficient of variation between duplicate samples within an individual experiment. Data is reported, unless otherwise stated, as the mean $\pm$ the standard deviation. Saturation analyses were analyzed by LIGAND (Munson and Rodbard, 1980).

3. Results

Specific $[^3]$H]clonidine binding to PFC particulate fractions at 21°C and 2.0 nM $[^3]$H]clonidine represented 75$\pm$5% of total binding when specific binding was determined as the difference between binding in the absence and presence of 1
μM clonidine. However, in preliminary studies it was noted that at a concentration of $[^3H]$clonidine $>1.5$ nM, sufficient competed cpm were accumulated to the filter alone to influence the presentation of the data. These studies showed that a linear relationship resulted between the fmol of $[^3H]$-ligand filtered (x) and fmol specifically bound to the filter (y) in the absence of PFC ($y = 2.1E - 3(x) - 2.280$). The data presented below, with the exception of the dose-response data, has been corrected for the effect of this filter binding at all concentrations of $[^3H]$clonidine $>1.5$ nM. Specific $[^3H]$clonidine binding was linear with protein concentration in the range 0.2-1.0 mg/ml assay (not shown). There were no differences in data obtained in Tris-HCl or Na-Hepes (see below).

Rate association of $[^3H]$clonidine to PFC particulate fractions is shown in fig. 1A. At 37°C, binding quickly reached a maximum at 10 min, but specific binding decreased 50% by 60 min. At 21°C a steady-state was attained after 20 min and was maintained for at least 70 min. Addition of 1 μM clonidine to the 21°C assay at 60 min incubation resulted in a $>99\%$ loss of specific binding after approximately 30 min. A plot of log total bound (%) versus time (fig. 1B) was biphasic and plateaued at the level of non-specific binding in each experiment. At 2.0 nM $[^3H]$clonidine, the rapidly dissociating component had a $t_{1/2}$ of $1.6 \pm 0.7$ min and the slowly dissociating component had a $t_{1/2}$ of $9.3 \pm 1.1$ min (N = 3).
Rodbard, 1980) showed that a two-site fit was superior to either a one- or three-site fit. For the high affinity component the apparent $K_A$ was $3.0 \times 10^9 \text{M}^{-1}$ ± a S.E.M. of $7.4 \times 10^8 \text{M}^{-1}$ ($K_D = 0.34 \text{nM}$), and the $B_{\text{max}}$ was $49.6 \pm 12.1 \text{fmol/mg}$ protein. The apparent $K_A$ and $B_{\text{max}}$ for the low affinity component were $1.7 \pm 2.5 \times 10^8 \text{M}^{-1}$ ($K_D = 6.0 \text{nM}$) and $48.8 \pm 20.7 \text{fmol/mg}$ protein ($N = 4$).

The binding of $[^3\text{H}]$clonidine to PFC was effectively decreased by $\alpha_2$-adrenoceptor agonists and antagonists, as shown in fig. 3 and table 1. The IC$_{50}$s of a variety of competitors were calculated by least squares linear regression from the Hill plots of the dose-response data, and are given in table 1 along with the Hill coefficients. Acetylcholine and $\gamma$-aminobutyric acid, at 1 mM were unable to decrease $[^3\text{H}]$clonidine binding (not shown). The IC$_{50}$s of histamine and metiamide which were approximately 1 mM ($N = 3$). As shown in fig. 3 and table 1, the rank order of ligands in decreasing $[^3\text{H}]$clonidine binding was clonidine > ($-$)-epinephrine) > ($-$)-norepinephrine > yohimbine > ($+$)-norepinephrine > ($+$)-epinephrine > ($\pm$)-isoproterenol > prazosin. Almost all Hill coefficients (table 1) were < 0.80, and those of the $\alpha_2$-adrenergic agents were < 0.70.

4. Discussion

Specific $[^3\text{H}]$clonidine binding sites with characteristics of $\alpha_2$-adrenoceptors, are present in human PFC and exist in more than one affinity state. $[^3\text{H}]$Clonidine binding in human PFC occurs rapidly (fig. 1A), is reversible (fig. 1B), is of high affinity, occurs at sites of limited density (fig. 2) and is $\alpha_2$-adrenergic selective with stereoselectivity preserved (fig. 3 and table 1). The evidence for multiple affinity states is the biphasicity of rate dissociation and saturation data and the relatively low Hill coefficients of the dose-response data.

The data of this study are in reasonable agreement with two published studies of $[^3\text{H}]$clonidine binding in human cortex. Weinreich et al. (1980) reported two affinity states with $K_D$s of 0.98 and 8.4 nM, and $B_{\text{max}}$s of 32 and 118 fmol/mg protein, respectively. Cash et al. (1985) reported one $K_D$ of

**TABLE 1**

IC$_{50}$ values and Hill coefficients for inhibitors of $[^3\text{H}]$clonidine binding in human prefrontal cortex. At least six concentrations of various competitors were used in dose-response assays consisting of 2.0 nM $[^3\text{H}]$clonidine and other assay ingredients. All experiments were performed at least three times on different postmortem specimens. The IC$_{50}$s were determined from least squares linear regression of Hill plots of the dose-response data. Correlation coefficients for the regression analyses were > 0.97.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ (nM)</th>
<th>Hill coefficient</th>
</tr>
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<tbody>
<tr>
<td>Clonidine</td>
<td>5.87 ± 1.34</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td>($-$)-Epinephrine</td>
<td>8.70 ± 2.10</td>
<td>0.63 ± 0.22</td>
</tr>
<tr>
<td>($-$)-Norepinephrine</td>
<td>30.70 ± 4.50</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>53.30 ± 9.20</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>Dopamine</td>
<td>251.00 ± 80.00</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>($+$)-Epinephrine</td>
<td>536.00 ± 112.00</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>($+$)-Norepinephrine</td>
<td>613.00 ± 140.00</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>2800.00 ± 1000.00</td>
<td>0.72 ± 0.18</td>
</tr>
<tr>
<td>Spiroperidol</td>
<td>3540.00 ± 1390.00</td>
<td>0.71 ± 0.17</td>
</tr>
<tr>
<td>Prazosin</td>
<td>5040.00 ± 1360.00</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td>($\pm$)-Propranolol</td>
<td>16600.00 ± 4200.00</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>Serotonin</td>
<td>23500.00 ± 9600.00</td>
<td>1.13 ± 0.09</td>
</tr>
</tbody>
</table>
2.2 nM and a $B_{max}$ of 48.2 fmol/mg protein. However, since the rate dissociation data in this report were biphasic, and the majority of the Hill coefficients of dose-response data were all $< 0.6$, the authors concluded that multiple affinity states were present. Three factors may have prohibited observation of the high affinity component in the saturation studies reported by these authors. First, the average age of the postmortem specimens used was 80 years as compared to our average age of 47 years. Since decreases in density have been reported for many receptor systems with increasing age, perhaps not enough of the high affinity sites was present for its observation (Maggi et al., 1979; Severson et al., 1982; Marcusson et al., 1984). Secondly, the tissue was less extensively washed as compared to our studies, which may influence the observation of high affinity states (Andorn and Maguire, 1980). Lastly, the lowest $^3$H-ligand concentration employed by these authors was 0.2 nM which may not have yielded enough points sufficiently below the $K_D$ of the highest affinity state. In contrast, we routinely employed four points between 0.1-0.3 nM (fig. 2). Additionally, multiple affinity states for $^3$Hclonidine binding sites have been routinely observed in lower mammalian brain (U’Prichard et al., 1979; Salama et al., 1982).

Multiple affinity states have been observed in some of the studies examining $\alpha_2$-adrenoceptor agonist binding in human platelets (Mooney et al., 1982; Steer and Atlas, 1982). In both of these studies, the highest affinity state had a $K_D < 1.0$ nM. Other authors have reported only one affinity state as determined by equilibrium saturation analysis with $K_D$ ranging from 5 to 24 nM (Garcia-Sevilla et al., 1981; Shattil et al., 1981; Smith et al., 1983; Cameron et al., 1984). The higher affinity state may not have been observed in these studies for some of the methodological reasons given above. For example, $^3$Hclonidine concentrations below 1.0 nM were not reportedly used in these studies (Garcia-Sevilla et al., 1981; Shattil et al., 1981; Smith et al., 1983; Cameron et al., 1984), whereas concentrations as low as 0.3 nM were used by the investigators who described the higher affinity state (Mooney et al., 1982; Steer and Atlas, 1982). Nevertheless, the differences in the number of affinity states observed does not negate the finding of increased total platelet $^3$Hclonidine binding site density in depressed patients (Garcia-Sevilla et al., 1981; Smith et al., 1983; Cameron et al., 1984). We have shown here data for normal postmortem human cortical $^3$Hclonidine binding on which to predicate further studies in brains from depressed individuals.

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