Clonidine-Specific Antisera Recognize an Endogenous Clonidine-Displacing Substance in Brain

Mary P. Meeley, Andrew C. Towle, Paul Ernsberger, Lynette K. Char, Phillip M. McCauley, and Donald J. Reis

An endogenous substance in brain, clonidine-displacing substance, binds to the same receptor populations as clonidine and is biologically active. Since receptor binding sites can be modeled by using specific antiligand antibodies, we tested the hypothesis that polyclonal antibodies raised in rat and rabbit against the clonidine analog p-aminoclonidine coupled to hemocyanin would recognize compounds structurally related to clonidine, including clonidine-displacing substance. Binding to anti-p-aminoclonidine antibodies was examined by using a competitive radioimmunoassay with tritiated p-aminoclonidine as the radioligand. Central vasodepressor agents that, like clonidine, are known to bind with high affinity to both imidazole sites and α2-adrenergic receptors in brain inhibited radioligand binding to anti-p-aminoclonidine antibodies. All of these agents contain imidazol(in)e and phenyl ring moieties as part of their chemical structures (e.g., oxymetazoline); a number of other compounds without one or both of these rings failed to cross-react with the antisera. Clonidine-displacing substance, partially purified from bovine brain, also inhibited specific radioligand binding to anti-p-aminoclonidine antibodies. The inhibition was dose dependent and high affinity (IC50, 4 Units). The endogenous substance had no effect on the apparent affinity of the antibodies for the radioligand, but blocked a specific number of binding sites. Immunoprecipitation experiments showed that authentic clonidine-displacing substance, that which displaces tritiated p-aminoclonidine binding to membrane receptors, is recognized by anti-p-aminoclonidine antibodies. We conclude that a unique subset of structural determinants required for ligand interaction with both imidazole and α2-adrenergic receptors is critical for binding to anti-p-aminoclonidine antibodies, and that since clonidine-displacing substance is recognized by highly clonidine-specific antisera, it may also contain these determinants within its structure, namely the imidazol(in)e and phenyl ring systems. (Hypertension 1989;13:341-351)

Clonidine, a synthetic phenyl imidazolidine, is a highly potent antihypertensive agent that lowers arterial pressure largely by its action within the C1 area of the rostral ventrolateral medulla oblongata (VLM).1-4 It has been widely assumed that the vasodepressor and other central actions of clonidine, such as sedation5,6 and antiwithdrawal effects,7,8 occur as a result of its role as an α2-adrenergic receptor partial agonist. However, we have shown recently that clonidine binds with high affinity, not only to α2-adrenergic receptors, but to a newly described population of sites in brain that are distinct from both adrenergic and histaminergic receptors.9 These imidazole binding sites are present in the VLM in rats9,10 and in humans11 and, in fact, may be functional receptors that mediate the hypotensive actions of clonidine and other imidazole agents in this region.3,12

The natural ligand for imidazole receptors is unknown. One possibility is an endogenous clonidine-displacing substance (CDS) isolated from bovine brain.13,14 CDS appears to be a relatively hydrophobic, positively charged, low molecular weight substance that displaces the binding of tritiated clonidine13 or the high-affinity clonidine analogue tritiated p-aminoclonidine ([3H]PAC)14-16 to membranes from bovine and rat brains. Like clonidine, CDS binds to both α2-adrenergic and imidazole receptors in VLM, with a 30-fold relative selectivity for the latter.15,16

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This clonidine-like substance is biologically active. Microinjection of CDS into the rostral VLM of the rat14 and the cat17 results in dramatic changes in arterial pressure. In addition, CDS inhibits the twitch response in the vas deferens of the rat,18 facilitates aggregation of human platelets,19 and causes a dose-related contraction of rat fundic smooth muscle.20 Recently, the endogenous substance itself has been measured in some peripheral tissues, such as the adrenal gland, gastric fundus, and serum of the rat21 and in sera from both normotensive patients and patients with pregnancy-induced hypertension.22 The chemical structure of this clonidine-like substance is, as yet, undetermined.

Recently, it has been shown that antibodies raised against selective drugs can be used as models for specific recognition sites of receptor proteins.23-26 We reasoned, therefore, that antibodies to a conjugate of \( \beta \)-aminoclonidine (PAC) may serve as probes for the recognition sites of \( \alpha_\text{2} \)-adrenergic or imidazole receptors, or both. Since CDS binds to these same receptor populations, the endogenous substance may also be recognized by anti-PAC antibodies. If so, then the results of studying the ligand specificity of the antibodies may yield clues as to the chemical structure of CDS. Thus, we sought to produce polyclonal anti-PAC antisera in rats and in rabbits, examine the ligand-binding specificity of the antisera relative to receptors labeled by \( \text{[^3H]} \)PAC in the VLM, and to determine whether anti-PACs bind CDS from brain.

A preliminary report of related findings with a different antiserum has appeared.27

**Materials and Methods**

**Production of Polyclonal Antisera**

Immunogen was prepared as described previously.27 The clonidine analogue PAC was coupled to hemocyanin (from *Limulus polyphemus*) with glutaraldehyde as the cross-linking agent. Sampling of O\( \text{2} \) blown over the nose) and bled via cardiac puncture (rat) or femoral artery catheterization (rabbit). Blood samples were allowed to clot overnight at 4° C; the antisera were centrifuged at 300g and stored as 1 ml aliquots at -20° C.

**Preparation of Immunoglobulin Fractions From Antisera**

For most experiments, a total protein fraction was precipitated from rat (anti-PAC\(_1\)) and rabbit (anti-PAC\(_2\)) antisera with 50% (wt/vol) ammonium sulfate.27 Precipitates were reconstituted in a minimum amount of 0.9% saline, dialyzed, adjusted to serum-equivalent volumes, and then stored frozen at -20° C until radioimmunoassay.

Alternatively, anti-PAC\(_2\) immunoglobulin Gs were affinity purified from serum by Protein A Sepharose chromatography. Anti-PAC\(_2\) serum (12 ml) was loaded onto a Protein A Sepharose (Pharmacia Biotechnology Prods., Piscataway, New Jersey) column (10x0.9 cm, i.d.) that had been equilibrated at 4° C with phosphate-buffered saline (pH 7.0). The column was washed with phosphate-buffered saline at a flow rate of approximately 20 ml/hr until the optical density at 280 nm (OD\(_{280}\)) of the effluent was less than 0.1. Immunoglobulin Gs were eluted in a 0.1 M glycine buffer, pH 3.0, and fractions of 1 ml were collected and immediately neutralized with 0.1 M Tris base. Elution fractions containing greater than 0.1 OD\(_{280}\) were pooled and the immunoglobulin Gs concentrated by precipitating with 50% ammonium sulfate. Saline-dialyzed aliquots of the anti-PAC\(_2\) immunoglobulin Gs were stored as 3x concentrates (relative to original serum immunoglobulin G content) at -20° C.

**Tritiated \( \beta \)-Aminoclonidine Radioimmunoassay**

To examine the binding properties of anti-PAC polyclonal antibodies, a radioimmunoassay with unconjugated \( \text{[^3H]} \)PAC as radioligand was adapted as described27 from the method of Sangameswaran and deBlas.28 Briefly, an aliquot of ammonium sulfate-precipitated anti-PAC immunoglobulins was thawed, then diluted to a 10x stock solution with bovine serum albumin (1 mg/ml). The anti-PAC stock was added to 50 mM Tris-HCl (pH 7.4) containing the compound to be tested or CDS (see below) to give the final dilution indicated and was preincubated at 25° C for 15 minutes. To define nonspecific binding, unlabeled PAC (20 \( \mu \text{M} \)) was included in parallel preincubations. \( \text{[^3H]} \)PAC (1 nM, or as specified) was added to all samples (incubation volume, 0.5 ml) that were then vortexed and incubated for 15 minutes at 25° C, followed by 1 hour on ice. The reaction was terminated by precipitating with carrier bovine gamma globulin (0.125% wt/vol) and ice-cold polyethylene glycol 8000 (7.5% wt/vol, final volume, 1.0 ml). Samples were vacuum-filtered over Whatman GF/B filters using a modified cell harvester (Brandel, Gaithersburg, Maryland). The filters were washed with 6-8 ml of 8% polyethylene glycol in Tris-HCl, pH 7.4, then transferred to scintillation vials, covered with 5 ml of scintillation cocktail, and counted at 45-50% efficiency.
Protein was measured by the Coomassie Blue dye binding method of Bradford, as modified by Read and Northcote, with bovine gamma globulin as standard.

Isolation of Clonidine-Displacing Substance

Extracts of calf brain containing CDS were prepared according to methods described elsewhere, with slight modification. Fresh brains, obtained from a local slaughterhouse (maximum 2 hours postmortem), were cleaned of major blood vessels and membranes, and the cerebellum was removed. The tissue was chopped into small pieces, then homogenized in three volumes of boiling distilled water using a Polytron (Brinkman Instruments, Westbury, New York). The homogenate was centrifuged at 100,000g for 30 minutes at 4°C. The supernatant then was denatured in a microwave oven by heating just to boiling and recentrifuged for 15 minutes; the resulting supernatant was freeze-dried overnight. The solids were reconstituted in distilled water (50–100 ml/2 brain equivalents) and were transferred to a dialysis bag made of H45 membrane tubing (Thomas Scientific, Philadelphia, Pennsylvania) with a cutoff of 3,500 MW. Dialysis was carried out overnight at 4°C against 3x20 volumes (vol/vol) of distilled water. The dialysis media (diffusates) were combined and freeze-dried. The low molecular weight solids were extracted with 20 volumes (vol/wt) of high-purity methanol by bath sonicating for 3 minutes at room temperature. The extract was decanted and the residue re-extracted with 5–10 volumes of fresh methanol. The extracts were pooled, filtered over Whatman (Clifton, New Jersey) No. 1 paper, and then aliquoted and dried in a Speed-Vac (Savant). The dialysates were combined and freeze-dried. The amount of CDS in these preparations was asayed by competition in radioreceptor binding experiments. Inhibition of the binding of [3H]PAC to membrane receptors by CDS was measured by using washed P2 membranes prepared from bovine frontal cortex as previously described. A Unit of CDS activity has been defined as the amount of extract required to inhibit [3H]PAC binding by 50%.

Data Analysis

Competition curves from studies of radioligand binding to antibody recognition sites were analyzed by linear regression of Hill plots. More complex data and data from multiple experiments were analyzed with programs originally designed for quantification of membrane receptor–ligand interactions: the Equilibrium Binding Data Analysis (EBDA) program of McPherson, which provides initial parameter estimates, followed by subsequent input into the LIGAND program for the final linear and nonlinear multivariate least-squares analysis.

Materials

[3H]PAC (New England Nuclear; 43 Ci/mmol) was stored in ethanol at -20°C; subsequently, it was diluted with distilled water and stored at 4°C for up to 2 weeks before use. Detomidine, medetomidine, MPV 295, and MPV 830 were the generous gift of J.-M. Savola, Department of Pharmacology, University of Oulu, Finland. Chloroethylnicotinidine was supplied by Research Biochemicals Inc. (South Natick, Massachusetts), idazoxan by Reckitt & Coleman (Kingston-upon-Hull, United Kingdom), and phen-tolamine by Ciba-Geigy (Summit, New Jersey). Benzimidazole and 1-benzylimidazole were from Chemical Dynamics Corporation (South Plainfield, New Jersey); all other compounds were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Results

Binding of Tritiated p-Aminoclonidine to Anti-p-Aminoclonidine Immunoglobulins

To determine whether antibodies to PAC were produced in rat or rabbit after immunization with the synthetic PAC-glutaraldehyde-hemocyanin conjugate, the specific binding of unconjugated [3H]PAC to anti-PACs was examined. Saturation binding experiments were carried out by using the rapid-filtration radiolmmunoassay technique detailed in Materials and Methods. Saturation data for binding to rat anti-PACi (1:500 final dilution) at [3H]PAC concentrations ranging over nearly three orders of magnitude are shown in Figure 1A. The total amount of [3H]PAC bound increased with radioligand concentration. Nonspecific binding, measured in the presence of 20 μM unlabeled PAC, accounted for 29±2% of the total binding at 0.6 nM [3H]PAC (n=3). Specific [3H]PAC binding to anti-PACi was of high affinity and saturable; as the radioligand concentration was more than tripled from 17 to 56 nM, specific binding increased by only 20% (Figure 1A; note discontinuous abscissa for complete representation). A Scatchard plot of this saturation data (Figure 1B) was distinctly curvilinear. Nonlinear computerized curve-fitting analysis (LIGAND) for anti-PACi and for anti-PAC2 is summarized in Table 1.

For the range of radioligand concentrations examined, rat anti-PACi appeared to contain at least two classes of antibody binding sites for [3H]PAC: one with an apparent equilibrium binding constant (Kd) of 0.56 nM and one with somewhat lower affinity, having a Kd of 11 nM. Thus, immunoglobulins with different affinities for free PAC were present in the ammonium sulfate–precipitated fraction of the polyclonal antiserum. Anti-PAC2 from rabbit was assayed using the same radioligand concentrations as with anti-PACi, but at a 1:10,000 final antiserum dilution to give a similar level of total specific [3H]PAC binding at 1 nM. Anti-PAC2 exhibited a single class of antibody binding sites, with an affinity nearly identical (Kd, 0.49 nM) to the high-affinity
The titer (calculated as the total number of antibody binding sites) was significantly greater for rabbit anti-PAC compared with the rat antiserum. The rabbit antiserum, however, showed a considerably higher titer of anti-PAC than the antiserum raised in rat and appeared to contain a single class of specific antibodies.

**Specificity of Binding to Anti-p-Aminoclonidine Antiserum**

Anti-PAC antiserum were examined for specificity of binding by measuring the cross-reactivity of PAC, clonidine, and a series of other compounds that are either chemically or functionally related to clonidine or are known to interact with imidazole sites or a2-adrenergic receptors, or both. \(^{1,9,12,16}\) Cross-reactivity was measured as inhibition in a competitive radioimmunoassay with \(^{3}H\)PAC.

Increasing concentrations of clonidine potently and completely inhibited the total specific binding of \(^{3}H\)PAC to anti-PAC, in a manner indistinguishable from that of PAC itself (Figure 2). Chlороethyl- clonidine (2-[2,6-dichloro(N-β-chloroethyl-N-methyl)-4-methylamino]phenylmimino-2-imidazoline) also completely inhibited \(^{3}H\)PAC binding, though at somewhat higher concentrations. In addition, several other imidazolines cross-reacted with anti-PAC antiserum. Naphazoline (2-[1-naphthylmethyl]imidazoline; see Figure 6 for structure), oxymetazoline (2-[4-tert-butyl-2,6-dimethyl-3-hydroxybenzyl]-2-imidazoline), and tolazoline (2-benzyl-2-imidazoline) all inhibited binding to anti-PAC. The displacement curves for these agents were shifted still further to the right relative to PAC and clonidine, indicating that much higher concentrations were required for inhibition.

The binding affinities (IC50) of these and several other clonidine-related imidazole compounds at anti-PAC, and at anti-PAC recognition sites are given in Table 2. PAC and clonidine were nearly equipotent (2–5 nM) in cross-reacting with both antiserum; chlороethylclonidine was approximately 10-fold less potent. Up to 10% \(\times \) the IC50 for clonidine was required to give 50% inhibition at anti-PAC or anti-PAC sites with the cross-reactants oxymetazoline, naphazoline, and tolazoline. The aryalkyl imidazoles detomidine (4(5)-[2,3-dimethylbenzyl]-imidazole), medetomidine (4(5)-[α,2,3-trimethylbenzyl]imidazole), and MPV 830 (4(5)-[α-hydroxy-2,6-dimethylbenzyl]imidazole) were weakly cross-reactive with anti-PAC, and noncross-reactive with the anti-PAC antiserum. In general, the order of potency of binding of these compounds was similar for the rat and rabbit antiserum. However, anti-PAC appeared more specific for clonidine.

**Pseudo-Hill coefficients** for all of the compounds, except naphazoline, were near unity. This suggests that, at the high affinity recognition sites labeled by 1 nM \(^{3}H\)PAC, these agents exhibit an apparent single-site interaction. (A higher radioligand concentration would presumably have revealed multisite interactions that included low affinity binding, which were not observed in the present experiments.)

### Table 1. Saturation Binding Parameters for the Interaction of Tritiated p-Aminoclonidine with Anti-p-Aminoclonidine Immunoglobulins

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>(K_d) (nM)</th>
<th>(B_{max}) (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-PAC1</td>
<td>(a) 0.36±0.39</td>
<td>0.95±0.55</td>
</tr>
<tr>
<td></td>
<td>(b) 1±9</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>anti-PAC2</td>
<td>0.49±0.12</td>
<td>31±3</td>
</tr>
</tbody>
</table>

*Values (mean±SEM) are the result of combined analysis of data from four rat anti-p-aminoclonidine (anti-PAC1) and three rabbit (anti-PAC2) experiments using the nonlinear curve-fitting program (LIGAND) of Munson and Rodbard.\(^{32}\) Six concentrations of tritiated PAC, ranging from 0.2 to 56 nM were incubated in triplicate with the ammonium sulfate-precipitated immunoglobulin fraction of each antiserum, at a 1:500 dilution of anti-PAC (16 μg total protein) and a 1:10,000 dilution of anti-PAC (1 μg total), as outlined in Materials and Methods. Nonspecific binding was determined using 20 μM unlabeled PAC.

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**FIGURE 1.** Saturation binding of tritiated p-aminoclonidine (\(^{3}H\)PAC) to rat (anti-PAC1) immunoglobulins. The saturation isotherm (note discontinuous abscissa) (panel A) and corresponding Scatchard plot (panel B) were obtained by incubating the ammonium sulfate-precipitated immunoglobulin fraction of anti-PAC1 antiserum at a 1:500 final dilution with increasing concentrations of radioligand (0.2–56 nM) in 50 mM Tris-HCl, pH 7.4, for 15 minutes at 25°C, then 1 hour on ice. Nonspecific binding was defined by parallel preincubation of the immunoglobulins with 20 μM unlabeled PAC at 25°C for 15 minutes. Data are mean values of triplicate samples from four experiments. Nonlinear multivariate least-squares analysis of the data using LIGAND\(^{32}\) resulted in a two-component best fit as illustrated by the dashed lines on the Scatchard plot B. Actual values for binding constants are given in Table 1.
Antibodies to p-Aminoclonidine Recognize CDS

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Ethylimidazole) significantly inhibited binding to either anti-PAC₁ or anti-PAC₂, or both, at $10^{-4}$ to $10^{-3}$ M concentration. However, none of these agents inhibited binding by 50% or more. Finally, although polyclonal antisera may be expected to contain antibodies produced to the protein portion of hapten-protein conjugates, hemocyanin (1 mg/ml, final concentration), the carrier protein used in synthesizing the PAC immunogen, did not interfere with [³H]PAC binding to the antibodies (data not shown), that is, it had no effect in this assay system.

Thus, binding to polyclonal anti-PAC antibodies raised in two different species was highly specific. Only those agents that, like clonidine, contain phenyl and imidazole(ino) ring moieties in their structures competed for binding to antibody recognition sites.

Recognition of Clonidine-Displacing Substance by Anti-p-Aminoclonidine Antibodies

Given the strict structural requirements for cross-reactivity, we sought to determine whether CDS was truly clonidine-like in that it also was recognized by anti-PAC antibodies. As shown in Figure 3, increasing amounts of bovine brain extract containing CDS inhibited [³H]PAC binding to anti-PAC₁ in a concentration-dependent manner. The IC₅₀ value for the interaction of CDS with the antiserum was 4.2±0.2 Units. As with PAC itself, this interaction appeared to occur at a single class of [³H]PAC recognition sites since the pseudo-Hill coefficient was 0.88±0.32 ($r>0.9$; $n=3$). Experiments carried out with the rabbit antiserum gave similar results (data not shown).

To further characterize the apparent cross-reactivity of CDS with anti-PAC antibodies, anti-PAC₂ immunoglobulin G that had been affinity purified on Protein A Sepharose (see Materials and Methods) was used. The immunoglobulin G was preincubated either in the presence or absence of a fixed amount of CDS (approximating its IC₅₀ at anti-PAC₁ sites; average, 3.2 Units), and then saturation binding of [³H]PAC was examined. Scatchard analysis of the results (Figure 4) showed that CDS had no effect on the radioligand binding affinity of anti-PAC₂: the slopes for data obtained in either condition were parallel, giving nearly identical $Kₐ$ values. However, a significant number of [³H]PAC binding sites were blocked by CDS as indicated by the shift in Scatchard x intercept (Bₘₐₓ) in the presence of CDS. The results are typical of a noncompetitive interaction and are expected for antibody binding, which is an essentially irreversible process, that is, primarily association and not dissociation dependent. Nonspecific binding was unaffected by coincubation with CDS.

Since the experiments described here involved the use of partially purified extracts of brain containing CDS and since CDS is defined in all of our studies¹⁴-¹⁶,²⁷ as a substance that displaces the binding of [³H]PAC to bovine frontal cortex membrane receptors, it was critical to determine whether...
the displacing substance found in brain extract was in fact responsible for the inhibition of binding to anti-PAC antibodies. To do this, aliquots of a reconstituted CDS-containing extract were pretreated with logarithmically increasing amounts of affinity-purified anti-PAC\textsubscript{2} immunoglobulin G, then total immunoglobulin G was immunoprecipitated using \textit{Staphylococcus aureus} cells (Pansorbin; Calbiochem, San Diego, California). The supernatants were assayed for remaining CDS by displacement of \textit{[3H]}PAC in the standard radioreceptor assay with bovine frontal cortex membranes (Figure 5). Incubation with increasing amounts of immunoglobulin G resulted in a decrease in the amount of CDS measured in the supernatant. At a 1:100 dilution of anti-PAC\textsubscript{2}, 83\% of the displacing activity present (1.3±0.2 Units; \(n=7\)) was removed from the medium. In contrast, incubation of CDS with equivalent dilutions (determined spectrophotometrically by OD\textsubscript{280}) of nonspecific affinity-purified total rabbit immunoglobulin G (Sigma Chemical Co.) had no effect.

In a separate series of experiments, anti-PAC\textsubscript{2} immunoglobulin G was used to detect purified CDS (bovine brain extract) from different preparations. Extracts of whole bovine brain, bovine VLM, and whole rat brain were serially diluted, blotted (3.0–0.006 Units) onto filter paper, and processed.\textsuperscript{34} The immunoblots clearly indicated positive staining with anti-PAC\textsubscript{2}, which was detectable to approximately 0.06 Units (data not shown). Non-specific staining in the absence of primary immunoglobulin G was near background. The presence of CDS in rat brain as well as bovine brain was thus confirmed.\textsuperscript{13} This is critical since a number of the biological actions described for partially purified CDS have been in rat.\textsuperscript{14,18,20} In addition, bovine VLM, presumably containing the rostral clonidine-sensitive zone, showed strong, concentration-dependent staining with anti-PAC\textsubscript{2} immunoglobulin G.

### Discussion

Polyclonal antisera were produced against a PAC-glutaraldehyde-hemocyanin conjugate in rat and rabbit. The antisera exhibit different anti-PAC titers, yet have comparable high affinities for free \textit{[3H]}PAC as measured by radioimmunoassay. These affinities are nearly identical to that of an antiserum raised against a derivative of 4-hydroxyclonidine for tritiated clonidine (0.58 nM).\textsuperscript{35} Binding to anti-PAC antibody recognition sites is highly specific. In addition to PAC itself, anti-PAC binds clonidine and chloroethylclonidine with high affinity. The phenyl-imidazolines naphazoline, oxymetazoline, and tolazoline, and the imidazoles detomidine, medetomidine, and MPV 830 bind with intermediate and low affinities, respectively. Several other imidazole-containing compounds also weakly interact with the antisera. In general, of the synthetic and naturally occurring compounds tested, only those with a substituted phenyl group linked via a single-atom spacer to an imidazol(in)e ring cross-react with anti-PAC.

In the present study, we have demonstrated that endogenous CDS\textsuperscript{13} in brain is recognized by anti-PAC antisera. As with the other cross-reactants, CDS (bovine brain extract) inhibits \textit{[3H]}PAC binding to anti-PAC in a competitive radioimmunoassay. This inhibition is dose dependent and of high affinity. CDS isolated from rat brain and bovine ventrolateral medulla are also recognized by anti-PAC. It is authentic CDS, and not some other component of the brain extract, that cross-reacts with the antisera since the substance that displaces the binding of the clonidine analogue \textit{[3H]}PAC from membrane receptors is fully immunoprecipitated with anti-PAC immunoglobulin G.

#### Recognition by Anti-p-Aminoclonidine Antibodies: Structure–Activity Relations

The results presented here indicate that, as with other polyclonal and monoclonal antibodies, certain structural requirements must be fulfilled for an agent to exhibit cross-reactivity with anti-PAC antibodies. The necessary structural elements are, not

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (nM)*</th>
<th>(n_H\textsuperscript{t})</th>
<th>IC\textsubscript{50} (nM)*</th>
<th>(n_H\textsuperscript{t})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p)-Aminoclonidine</td>
<td>3.7±0.5</td>
<td>0.92±0.06</td>
<td>1.8±0.2</td>
<td>0.95±0.05</td>
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<tr>
<td>Clonidine</td>
<td>4.8±1.0</td>
<td>0.89±0.14</td>
<td>3.6±0.4</td>
<td>0.87±0.04</td>
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<tr>
<td>Chloroethylclonidine</td>
<td>85±6</td>
<td>1.1±0.1</td>
<td>13±2</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Detomidine</td>
<td>420,000±31,000</td>
<td>1.2±0.1</td>
<td>&gt;1,000,000</td>
<td>...</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>270,000±23,000</td>
<td>0.97±0.06</td>
<td>&gt;1,000,000</td>
<td>...</td>
</tr>
<tr>
<td>MPV 830</td>
<td>251,000±32,000</td>
<td>0.80±0.07</td>
<td>&gt;1,000,000</td>
<td>...</td>
</tr>
<tr>
<td>Naphazoline</td>
<td>28,000±14,000</td>
<td>0.68±0.17</td>
<td>33,000±9,000</td>
<td>0.72±0.10</td>
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<tr>
<td>Oxymetazoline</td>
<td>3,400±1,200</td>
<td>0.75±0.23</td>
<td>140,000±20,000</td>
<td>0.88±0.04</td>
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<tr>
<td>Tolazoline</td>
<td>130,000±30,000</td>
<td>0.85±0.12</td>
<td>530,000±50,000</td>
<td>1.3±0.2</td>
</tr>
</tbody>
</table>

An aliquot of the ammonium sulfate–precipitated immunoglobulin fraction from anti-\(p\)-aminoclonidine (anti-PAC\textsubscript{2}) (1:500) or anti-PAC\textsubscript{2} (1:10,000) was incubated with 1 nM anti-\(p\)-aminoclonidine (\textit{[3H]}PAC) and six to nine concentrations of the cross-reactive drug as described in the legend to Figure 2. The concentration of drug giving 50% inhibition of radioligand binding (IC\textsubscript{50})\textsuperscript{*} and pseudo-Hill coefficients\textsuperscript{t} were obtained from linear regression analysis of Hill plot transformations of the inhibition curves; mean \(r>0.9\) in all cases. Values represent mean±SEM of three to six experiments, except for PAC (\(n=24–26\)).
The p-amino group of PAC that, presumably, center of the aromatic ring has been reported as and nonperpendicular. The distance between a result, in solution the two ring systems are aplanar (see Figure 6). The drug is protonated (85%) clonic (imidazolidine) tautomer as the predominant atom to a substituted phenyl ring, with the exocy-
dazoline) ring covalently bound through a nitrogen ure 6) contains a partially saturated imidazole (imi-
cally, the clonidine portion of the immunogen. (control); p<0.05, unpaired / test.

**TABLE 3. Compounds That Did Not Effectively Cross-react (IC₅₀ > 10⁻³ M) With Anti-p-Aminonidolidine Antibodies**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Anti-PAC₁ %</th>
<th>Anti-PAC₂ %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylethylamines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>24±3 (7)</td>
<td>-4.3±0.2 (7)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>7.9±0.4 (4)</td>
<td>10±2 (5)</td>
</tr>
<tr>
<td>Tyramine</td>
<td>2.1±0.1 (4)</td>
<td>-3.7±0.1 (11)</td>
</tr>
<tr>
<td>Imidazoles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclo (histidyl-prolyl)</td>
<td>16±4 (4)</td>
<td>7.5±0.7 (3)</td>
</tr>
<tr>
<td>Histamine</td>
<td>14±1 (3)</td>
<td>4.5±0.1 (3)</td>
</tr>
<tr>
<td>Histidine</td>
<td>35±4 (5)</td>
<td>14±2 (3)</td>
</tr>
<tr>
<td>Histidyl-tyrosine</td>
<td>15±1 (4)</td>
<td>30±6 (9)</td>
</tr>
<tr>
<td>Imidazole-4-acetic acid</td>
<td>7.1±0.2(4)</td>
<td>17±4 (9)</td>
</tr>
<tr>
<td>Thyrotropin releasing hormone</td>
<td>9.6±0.9 (14)</td>
<td>32±6 (11)</td>
</tr>
<tr>
<td><strong>Other Compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>-1.6±0.1 (6)</td>
<td>3.6±0.2 (6)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.6±0.1 (3)</td>
<td>-9.2±0.2 (4)</td>
</tr>
<tr>
<td>GABA</td>
<td>2.1±0.3 (3)</td>
<td>0.4±0.01 (3)</td>
</tr>
<tr>
<td>Guanidine (HCl)</td>
<td>-5.3±0.4 (3)</td>
<td>6.6±0.8 (4)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.0±0.1 (2)</td>
<td>n.t.</td>
</tr>
<tr>
<td>Serotonin (10⁻⁴ M)</td>
<td>3.6±0.2 (3)</td>
<td>-1.4±0.1 (3)</td>
</tr>
<tr>
<td><strong>Synthetic Imidazol(in)es</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzimidazole</td>
<td>n.t.</td>
<td>0.4±0.01 (6)</td>
</tr>
<tr>
<td>1-Benzylimidazole</td>
<td>4.4±0.4 (4)</td>
<td>3.0±0.1 (9)</td>
</tr>
<tr>
<td>Cimctidine</td>
<td>41±4 (16)</td>
<td>21±2 (9)</td>
</tr>
<tr>
<td>Idazoxan (10⁻⁴ M)</td>
<td>35±2 (6)</td>
<td>6.4±0.2 (8)</td>
</tr>
<tr>
<td>MPV 295 (10⁻⁴ M)</td>
<td>-19±0.5 (3)</td>
<td>13±1 (5)</td>
</tr>
<tr>
<td>Phentolamine (10⁻⁴ M)</td>
<td>19±3 (3)</td>
<td>13±1 (4)</td>
</tr>
<tr>
<td><strong>Other Compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yohimbine (10⁻⁴ M)</td>
<td>-0.03±0.00 (3)</td>
<td>0.8±0.03 (3)</td>
</tr>
<tr>
<td>Guanabenz</td>
<td>71±5 (3)</td>
<td>55±9 (3)</td>
</tr>
</tbody>
</table>

*Anti-p-aminonidolidine (anti-PACO and anti-PAC₂ were incubated in triplicate with 1 nM [³H]PAC and a single concentration (10⁻³ M, or as indicated) of the competing compound under standard conditions. Data (mean±SEM) are reported as percent inhibition of total specific [³H]PAC binding, number of experiments (n) in parentheses, n.t., not tested.

†Significantly different from total specific [³H]PAC binding (control); p<0.05, unpaired / test.

unexpectedly, derived from PAC or, more specifically, the clonidine portion of the immunogen. Clonidine (2-[2,6-dichloroanilino]-2-imidazoline; Figure 6) contains a partially saturated imidazole (imidazoline) ring covalently bound through a nitrogen atom to a substituted phenyl ring, with the exocyclic (imidazolindoline) tautomer as the predominant form (see Figure 6). The drug is protonated (85%) at neutral pH due to its net positive charge and, as a result, in solution the two ring systems are aplanar and nonperpendicular. The distance between the one nitrogen of the imidazoline ring and the center of the aromatic ring has been reported as 5 Å. The p-amino group of PAC that, presumably, serves as the point of attachment of the hapten to the carrier appears to play little or no role in ligand recognition. In fact, chloroethylclonidine contains a bulky (Af-/3-chloroethyl-N-methyl)-methylamino substituent at the para position, yet still binds with high affinity to anti-PAC antibodies.

Based on our ligand specificity studies, the following structural elements of clonidine appear to be imprinted within the antibody recognition site. (1) Both ring structures are necessary. Compounds possessing only an imidazole ring (e.g., histamine, cimctidine) or phenyl ring (epinephrine, norepinephrine) do not cross-react with anti-PAC. (2) Replacing the ring-linking nitrogen of clonidine with a carbon atom and changing or removing the phenyl ring substituents (see Figure 6, naphazoline; also oxymetazoline, tolazoline) causes a decrease in affinity, but does not abolish binding. These tetrahedral benzyl-imidazolines are moderately cross-reactive. (3) Connecting a substituted phenyl ring via a carbon spacer to an unsaturated imidazole ring at the carbon four (five) position (detomidine, see Figure 6; medetomidine and MPV 830) allows only low affinity binding. (4) Attachment of the imidazole ring at the position one nitrogen, as in 1-benzylimidazole, results in a complete loss of binding to anti-PAC. (5) The ring-to-ring distance or a tolerance in the flexibility of conformation of the ring systems, appears critical for interaction at anti-PAC recognition sites. Benzimidazole (collapsed ring distance: no spacer), idazoxan (dioxan structure, see Figure 6), MPV 295 and phentol-
amino (extended ring distances: two-atom spacers), and histidyl-tyrosine (seven-atom spacers) fail to cross-react effectively and exhibit only a weak interaction with anti-PAC antibodies. Thus, in general, the spatial orientation of the phenyl and imidazol(in)e rings, and perhaps their degree of substitution, constitute the critical chemical determinants of the clonidine epitope. Since they are not readily available for study, it is unclear whether unsaturated (imidazole) analogues of the partially saturated imidazolines, linked at ring position two (i.e., substituted 2-benzylimidazoles), would be as effective as clonidine in binding to anti-PAC antibodies.

Recognition by Anti-p-Aminoclonidine Antibodies: Antihypertensive Agents and Their Receptors

Two types of receptors are localized in the VLM that are labeled by the clonidine derivative [3H]PAC. It is an α2-adrenergic receptors in the C1 area of this region that clonidine has traditionally been thought to act as a partial agonist to lower arterial pressure.1,39 However, clonidine binds with even higher affinity to a newly discovered class of putative imidazole receptors in VLM, which are distinct from adrenergic and histaminergic receptors.9 Recent evidence from our laboratory suggests that the vasodepressor potency of clonidine and related compounds correlates best with binding affinity at imidazole receptor sites, not α2-adrenergic receptors.12 Alternatively, vasodilator potency may result from concerted effects via both receptor types. This dual action would be structurally represented by the phenyl moiety of phenylethylamine adrenergic agents along with the imidazol(in)e moiety of histaminergic agents, both of which are present in clonidine-like agents. These possibilities raise the question as to whether the recognition sites of clonidine-specific antibodies resemble classic α2-adrenergic sites, imidazole sites, or a hybrid of both receptors.

Specific anti-PAC antibodies cross-react with clonidine and only a few other agents, all of which bind...
with high affinity (nanomolar range) to both imidazole and α2-adrenergic receptor populations (see Reference 9; detomidine, medetomidine, MPV 830, and tolazoline, unpublished observations). Thus, anti-PAC sites resemble a hybrid of imidazole and α2-adrenergic receptor sites. Significantly, of the agents tested thus far in vivo, all of those that cross-react with anti-PAC antibodies elicit hypotension when administered directly into the central nervous system. Specifically, clonidine and PAC both bind with high affinity to anti-PAC recognition sites, and both are exquisitely potent central vasodepressor agents.1,3-12,40 Naphazoline and oxymetazoline also lower arterial pressure on central administration,41 but only at doses that exceed those of clonidine by two orders of magnitude. These two compounds also exhibit a substantially lower affinity for anti-PAC sites compared with clonidine. In addition, detomidine, medetomidine, and MPV 830 are potent sedatives that act as vasodepressor agents via central mechanisms.42-43 These sedative agents are α2-selective (P. Ernsberger, unpublished observation) and only weakly cross-reactive with anti-PAC antibodies. Thus, binding to anti-PAC antibodies defines a unique subset of agents with affinities for anti-PAC sites that appear to be qualitatively related to their effectiveness in lowering arterial pressure within the central nervous system. Although several noncross-reactive compounds show moderate central hypotensive actions, such as guanabenz (α2-adrenergic), and idazoxan and cimetidine (imidazole),12 binding to anti-PAC antibodies may be useful in providing information for the design of novel centrally acting antihypertensive agents.

**Recognition by Anti-p-Aminoclonidine Antibodies: Clonidine-Displacing Substance**

The recognition of CDS by antibodies raised against the clonidine analog PAC is added evidence that this endogenous substance from brain is truly clonidine-like. The fact that anti-PAC antibodies exhibit such strict structural requirements for binding, and yet CDS is also recognized by these antibodies, strongly suggests that the natural substance shares some of the same chemical elements as clonidine and the other cross-reactants, namely, the phenyl and imidazol(in)e ring moieties. The reported finding that CDS is relatively hydrophobic and positively charged at neutral pH13 is consistent with the presence of these structural elements. This observation may provide useful clues for the structural analysis and identification of purified CDS.

Binding to clonidine-specific antibodies also supports our proposal that CDS is a unique substance in brain.16 CDS appears to be distinct from a number of endogenous compounds, including the catecholamines and other neurotransmitters such as acetylcholine, GABA, histamine, and serotonin, since these do not bind effectively to anti-PAC. Furthermore, CDS is one of a small and unique set of agents that bind to the imidazole and α2-adrenergic receptor populations in VLM15,16 as well as to anti-PAC antibodies. This finding may account for the clonidine-related central cardiovascular actions of CDS.14-17 We cannot, of course, rule out the possibility that more than one substance in our partially purified CDS preparation combines in the antibody recognition site to result in a unique profile of inhibition of [3H]PAC binding. However, the dose-response relation for clonidine and for CDS appear to be similar, based on pseudo-Hill coefficients. In addition, receptor binding experiments using the same CDS preparation described here indicate that these extracts contain a single active species.16 Furthermore, our data show that more than 85% of this inhibitory species can be immuno-precipitated with anti-PAC antibodies.
Recognition of endogenous CDS by anti-PAC antibodies is probably the result of a relatively weak interaction. That is, CDS binds with lower affinity to antibody recognition sites (IC_{50} 4 Units) than to α2-adrenergic (1 Unit) or imidazole (0.03 Units) receptor sites labeled by [3H]PAC. This pattern is similar to that of the low affinity cross-reactants naphazoline and oxymetazoline, but contrasts with that for the high affinity clonidine epitope. Clonidine binds with sixfold higher affinity to anti-PAC antibodies than to receptors in VLM. Once identified, the chemical structure of CDS as it compares with clonidine will help to explain these observed differences in binding affinities. For example, CDS may contain an imidazole ring and not the partially saturated imidazoline ring that is present in synthetic compounds such as clonidine and naphazoline. The degree of saturation of this ring may be important for high affinity binding to a clonidine-specific antibody and less critical for interaction with receptors, which have a broader specificity.

**Recognition by Anti-α-Aminoclonidine Antibodies: Quantifying Clonidine-Displacing Substance Using Radioimmunoassay**

The production of polyclonal antisera against PAC has provided us with a tool for further evaluating the ligand binding specificities of imidazole and α2-adrenergic receptors and with indirect information on the chemical structure of endogenous CDS, since it also binds to anti-PAC. In addition, we now have a highly specific means of measuring relative amounts of CDS in brain extracts or other biological samples. Despite being somewhat less sensitive than the [3H]PAC radioreceptor assay, the [3H]PAC radioimmunoassay has the advantage of being a more accurate method for determining levels of CDS. Various salts or other factors that are known to interfere with or displace [3H]PAC binding to receptors, which may be present in partially purified preparations of CDS, do not affect binding to anti-PAC antibodies. Thus, it is likely that radioimmunoassay values represent a truer measure of CDS activity in crude preparations than the radioreceptor assay estimates of CDS Units used heretofore. Furthermore, used in tandem with radioreceptor or bioassays for CDS, the radioimmunoassay ensures positive identification and accurate tracking of the substance during purification.

Furthermore, in addition to determining relative levels of CDS activity based on extent of inhibition, absolute amounts of CDS can be estimated using saturation binding data from the radioimmunoassay. For example, the results of the saturation binding of [3H]PAC to anti-PAC immunoglobulin G carried out in the presence of CDS (refer to Figure 4) can be used to estimate the molar quantities of CDS in bovine brain. As its IC_{50} concentration, only approximately 2% of the [3H]PAC that was added to the assay tubes bound to the antibodies. CDS, at a concentration near its IC_{50}, blocked 29 pmol of [3H]PAC binding sites per milligram immunoglobulin G. Assuming that, as with the radioligand, about 2% of the CDS added was bound to these sites and that the interaction of CDS at anti-PAC sites is at least a thousandfold weaker than that of PAC itself, there, to a first approximation, no more than 0.002% of the known amount of CDS added was bound to recognition sites. If the assumptions are valid, then 1 Unit of CDS is equivalent to 110 pmol of CDS. On this basis, given a molecular mass of 590 Daltons, a yield of 130 ng of CDS per gram of bovine brain tissue is obtained routinely in our preparations. This estimated level is on the order of that of several transmitter substances known to be localized in discrete regions of the brain, such as norepinephrine, epinephrine histamine, and a number of neuropeptides (for reviews, see Reference 49).

We conclude that highly specific antisera produced against the clonidine analogue PAC bind an endogenous CDS from brain and may serve as useful immunological tools for localization, purification, and structural identification of this substance. In addition, anti-PAC, along with other anti-drug antibodies, may be useful as highly specific models for the receptor subpopulations responsible for mediating the antihypertensive, sedative, and anti-withdrawal effects of clonidine.

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