Aminotriarylmethane Dyes Are High-Affinity Noncompetitive Antagonists of the Nicotinic Acetylcholine Receptor

MONICA M. LURTZ and STEEN E. PEDERSEN

Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas

Received July 6, 1998; accepted September 22, 1998

ABSTRACT

A series of aminotriarylmethane dyes were examined for binding to the nicotinic acetylcholine receptor (AChR) from Torpedo californica. Several compounds were found to bind to the non-competitive antagonist site of the AChR as demonstrated by inhibition of [3H]acetylcholine binding; apparent KD values ranged from 50 nM to >100 μM. One dye with high affinity, crystal violet, revealed a greater than 200-fold fluorescence enhancement upon binding the AChR. Using fluorescence to measure binding, we determined that one crystal violet bound per receptor with a dissociation constant of 100 nM; in the presence of the agonist carbamylcholine this value decreased to 10 nM. The KD for [3H]acetylcholine binding likewise was decreased in the presence of crystal violet. These results are consistent with preferential binding of crystal violet to the desensitized conformation of the AChR. Crystal violet binding blocked agonist-induced 22Na ion efflux from AChR-rich vesicles. It is concluded that crystal violet and other dyes of similar structure bind to the high-affinity noncompetitive antagonist site of the AChR associated with the channel lumen. Because of their optical properties, crystal violet and several of the other homologous dyes are likely to be useful ligands for further characterization of the AChR channel. Structure-activity comparison of the various dyes suggests the importance of non-quaternary nitrogens in binding the pore. Additional steric bulk on amines or at meta positions increase or have neutral effect on affinity, suggesting that steric considerations alone do not limit high affinity for the binding site.

The nicotinic acetylcholine receptor (AChR) from Torpedo californica electric organ is a pentameric, ligand-gated, non-specific cation channel composed of four distinct, homologous subunits with a stoichiometry of α2βγδ (Raftery et al., 1980; Noda et al., 1983; Unwin, 1993). Each subunit possesses four putative transmembrane segments termed M1, M2, M3, and M4. Studies of chimeric AChRs (Imoto et al., 1986, 1988) have produced substantial evidence that the M2 domain lines the pore of the channel. Affinity-labeling experiments further identified this sequence as the site of high-affinity noncompetitive antagonist binding. Meproadifen mustard labeling was localized to αGlu-262 in M2 (Pedersen et al., 1992); chlorpromazine and triphenylmethylphosphonium specifically label homologous residues within the M2 sequences of each subunit (Giraudat et al., 1986, 1987, 1989; Hucho et al., 1986). Labeling studies with TID, an uncharged noncompetitive antagonist, identified reactive sites within M2 for AChR...
ifen, chlorpromazine, triphenylmethylphosphonium, and quinacrine, preferentially bind the desensitized configuration of AChR at equilibrium (Krodel et al., 1979; Heidmann et al., 1983; White et al., 1991; Wu et al., 1994; Lurtz et al., 1997).

Fluorescent NCAs such as ethidium and quinacrine have been used to examine the structure and environment of the AChR. These compounds increase their quantum yield upon binding the NCA site. Fluorescence measurements have shown that ethidium becomes immobilized and shielded from solvent upon binding the AChR (Herz et al., 1987; Herz and Atherton, 1992). Quenching and fluorescence energy transfer measurements are potentially useful tools for further characterization of the properties of the NCA site. The utility of these two fluorophores is somewhat limited by their affinity, the sensitivity of their affinities to ionic strength changes (Lurtz et al., 1997), and nonspecific interaction with the lipid bilayer. We desired to carry out diffusion-enhanced energy transfer experiments (Stryer et al., 1982) using NCA site ligands as the energy transfer acceptors. The sensitivity of such experiments is strongly dependent on the extinction coefficient of the ligand. We therefore investigated the ability of strongly absorbing dyes to act as NCAs of the AChR.

We present data that the aminothiatriamethane dyes, compounds with structures similar to the NCA, triphenylmethylphosphonium, are a novel family of NCAs. Several members of this family bind with $K_B$ values in the low nanomolar range, particularly crystal violet (CrV). Further analysis of CrV demonstrated that crystal violet binds the noncompetitive antagonist site in the pore of the AChR with high affinity and a significant, concomitant fluorescence enhancement. Its high affinity and fluorescence properties further make CrV, in particular, a good probe for future fluorescence quenching and fluorescence energy transfer experiments. The high affinity of this compound and the existence of many structurally related basic dyes will provide an approach to a more detailed structure-activity relationship of the NCA site. Here we present an analysis of the binding relationships of these ligands.

**Experimental Procedures**

**Materials.** Crystal violet hydrochloride, methyl violet 2B, brilliant green, malachite green carbinol hydrochloride, ethyl violet hydrochloride, methyl green zinc chloride, pararosaniline chloride, new fuchsin, patent blue VF, Victoria pure blue BO, leuco rhodamine, methyl violet 2B, bril-...
ensure that binding was at equilibrium. To minimize dilution effects, volume changes were kept at less than 2% of the total by adding concentrated CrV solutions. The CrV binding-site concentration was determined from the intercepts of the limiting slopes of the initial data points and of the final data points. The initial points in the titration reflect binding of nearly all the added CrV to the AChR. Therefore, the fluorescence intensity $F_i = Q_b \cdot L$, where $L$ is the concentration of CrV added and $Q_b$ is the quantum yield of CrV when bound to the AChR. At the final data points in the titration, the binding site is saturated and any further increase in fluorescence reflects increases in nonspecific binding. The equation for the fluorescence intensity is as follows: $F_i = Q_b \cdot R_0 + Q_b (L - R_0)$, where $R_0$ is the total concentration of CrV-binding sites and $Q_b$ is the quantum yield of the fluorescence due to nonspecific interactions. At the intercept of these lines $(F_i - F_0) / R_0 = L$, and the CrV binding-site concentration can be read directly from the CrV concentration axis.

**22Na Efflux Assay.** $^{22}$Na efflux assays were conducted using the procedure of Neubig and Cohen (1980) essentially as described by White et al. (1991); all manipulations were carried out at 4°C. Briefly, a concentrated suspension of AChR-rich vesicles was incubated with $^{22}$Na overnight in HTPS. The excess $^{22}$Na was removed by passing the vesicles over a 3-ml Dowex 50W-X8 column, and the vesicles were diluted immediately with HTPS. After a 20-min incubation to permit passive release of $^{22}$Na to reach a slow, steady-state level, the assays were initiated by addition of 0.3 mM phenyltrimethyleneammonium and the suspension was filtered through Whatman GF/F filters 20 s later. The filters had been pretreated with 1% polyethyleneimine and washed with 10 ml HTPS before filtration. To measure block of efflux by NCAs, the vesicles were preincubated for 10 min with the NCA before initiating release. Maximal $^{22}$Na efflux from *Torpedo californica* vesicles was determined by 10-min incubation with gramicidin D. Data were normalized to the release observed in the presence of gramicidin.

**Determination of Partition Coefficients.** AChR-rich membranes were incubated for 1 h at ambient temperature in the presence of 500 nM CrV and 0.1 mM carbamylcholine in the presence or absence of 1 mM PCP. Membranes were sedimanted in a Hitachi System Gold, with a model 166 variable-wavelength detector (254 nm used routinely), and data collection was carried out with System Gold software. The separation was obtained in pure form, it was selected for further characterization. Because CrV possessed high binding affinity and interesting optical properties, and could be more readily obtained in pure form, it was selected for further characterization. The stoichiometry experiments described below establish that the properties of crystal violet were not due to minor contamination by methyl violet.

**Fluorescence and Absorbance Spectra of Crystal Violet.** CrV is essentially nonfluorescent in aqueous solution (Fig. 3A) but had been reported to acquire fluorescence upon binding protein (Anderson et al., 1996; Baptista and Indig, 1998). Therefore, we examined the fluorescence of three higher-affinity dyes in the presence of the AChR: CrV, ethyl violet, and new fuchsia. Preliminary experiments indicated that CrV had substantial specific fluorescence enhancement upon binding the AChR. The other two compounds also exhibited fluorescence when added to AChR-rich membranes, but their fluorescence was less susceptible to inhibition by PCP. This suggested less specific fluores-
cence upon binding the NCA site or substantial fluorescence due to nonspecific interactions.

Excitation and emission spectra collected for 10 μM CrV in the absence of AChR confirmed the weak intrinsic fluorescence (Fig. 3A, spectra 3 and 6). In contrast, a 200-fold-lower concentration of 50 nM CrV bound to an excess of AChR (traces 1 and 4) shows 2- to 3-fold-higher fluorescence intensity. Blocking the NCA site with PCP (spectra 2 and 5) can substantially decrease this fluorescence. The residual fluorescence observed in the presence of PCP likely represents fluorescence enhancement of CrV due to nonspecific interaction with the lipid bilayer or with other proteins; the contribution from CrV free in solution was negligible at these concentrations, as indicated by spectra 3 and 6 at a 200-fold higher concentration. Upon binding the AChR, CrV fluorescence increases more than 200-fold with accompanying shifts in the peak excitation and emission wavelengths. The excitation maximum for free CrV shifts from 592 to 599 nm when bound; the emission maximum shifts from 636 nm to 626 nm. Addition of PCP displaces about 75% of the fluorescence signal, indicating that most of the fluorescence enhancement is due to CrV binding to the NCA site of the AChR.

The absorbance spectra of free and AChR-bound CrV (Fig. 3B) show a maximum absorbance at 600 nm for bound CrV (spectrum 2), whereas free CrV (spectrum 1) peaks at 591 nm. The red-shift of 9 nm is consistent with that seen in the corresponding fluorescence excitation spectra (Fig. 3A). Displacement of the bound crystal violet by PCP yields an absorbance maximum similar to AChR-bound CrV (data not shown). At the high membrane concentrations needed to record the absorbance spectrum of bound CrV, a significant fraction of the unbound CrV will interact nonspecifically with the membrane. It is likely that these interactions produce a shift in the absorbance spectrum similar to the shift that occurs upon binding the AChR. The extinction coefficient of CrV does not change substantially upon binding the AChR nor upon interaction with membranes. Therefore, the change in fluorescence intensity represents a change in the quantum yield of the CrV rather than a change in its absorbance properties.

**Interaction of Crystal Violet with Membranes.** Although the majority of the fluorescence enhancement observed upon CrV binding the AChR was inhibitable by PCP, there was substantial residual fluorescence likely due to nonspecific interactions with the lipid bilayer or with other proteins. Therefore, we used HPLC to quantitate nonspecific interaction with AChR-rich vesicles; this was carried out in

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pararosaniline</td>
<td>NH₂</td>
<td>NH₂</td>
<td>NH₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Rosaniline</td>
<td>NH₂</td>
<td>NH₂</td>
<td>NH₂</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>New fuchsine</td>
<td>NH₂</td>
<td>NH₂</td>
<td>NH₂</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
</tr>
<tr>
<td>Malachite green</td>
<td>H</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>H</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Methyl violet</td>
<td>NHCH₃</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Ethyl violet</td>
<td>N(CH₃CH₂)₂</td>
<td>N(CH₃CH₂)₂</td>
<td>N(CH₃CH₂)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Methyl green</td>
<td>N'(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Victoria pure blue BO</td>
<td>N(CH₃CH₂)₂</td>
<td>N(CH₃)₂</td>
<td>N(CH₃CH₂)₂</td>
<td>(CH₃)₂-R6</td>
<td>H</td>
<td>(CH₃)₂-R4</td>
</tr>
<tr>
<td>Crystal violet lactone</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>COO⁻</td>
</tr>
<tr>
<td>Leuco crystal violet</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>N(CH₃)₂</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Dpmsm</td>
<td>N(CH₃)₂</td>
<td>OCH₃</td>
<td>N(CH₃)₂</td>
<td>SO₄⁻</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

**Fig. 1.** Structures of aminotriarylmethane dyes. The figure shows the structure of crystal violet and other dyes examined in this study.
high concentrations of blocking agents to prevent specific interaction with the high-affinity NCA site on the AChR (see Experimental Procedures). We measured the partition coefficient of CrV in both physiological buffer (HTPS, high ionic strength) and at low ionic strength (20 mM HEPES). CrV has a high partition coefficient of $2.62 \pm 0.26 \text{ (mg/ml)}^{-1}$ ($n = 4$) in physiological buffer. Compare these values with the much lower values determined for ethidium, PCP, and quinacrine (Lurtz et al., 1997) that range from 0.05 to 0.17 (mg/ml)$^{-1}$. In low-ionic-strength buffer, the partition coefficient is $7.48 \pm 0.86 \text{ (mg/ml)}^{-1}$ ($n = 4$), a value similar to quinacrine, but somewhat higher than ethidium [2 (mg/ml)$^{-1}$] or PCP [0.11 (mg/ml)$^{-1}$. Thus, under typical conditions used for measuring binding and fluorescence to the AChR (~0.1 mg/ml AChR-rich vesicles), we expect a significant amount (~20%) of nonspecific interaction with the vesicles.

**Crystal Violet Binding Measured by Fluorescence Enhancement.** The relatively high concentration of AChR (40 nM) limited the ability to measure true $K_D$ values less than this value by inhibition of $[^3H]$PCP binding. This concentration was near the $K_{app}$ values observed for CrV, methyl violet, and perhaps ethyl violet. In these cases, the $K_{app}$ value may reflect titration of the binding sites rather than the true $K_D$. Therefore, we took advantage of the fluorescence of CrV to measure its $K_D$ for interaction with the AChR by fluorescence enhancement in varying concentrations of CrV (Fig. 4).

Because of its high partition coefficient and its propensity to interact with the surfaces of glass- and plasticware, the free concentration of CrV could not simply be assumed based on the amount of CrV added to the solution. We developed a method to determine directly the solution concentration of CrV using the detergent CHAPS, which also enhances the fluorescence of CrV. After removal of CrV bound to AChR-rich vesicles by centrifugation (see Experimental Procedures), the supernatant CrV concentration was readily measured by adding CHAPS to a final concentration of 1% and comparing the fluorescent intensity with a standard curve. This provided a simple means of monitoring free CrV concentrations.

In the presence of excess agonist (Fig. 4A), CrV has a $K_D$ for the AChR of $10 \pm 3 \text{ nM}$; in the absence of agonist the affinity is near $100 \pm 50 \text{ nM}$ (Fig. 4B; summarized in Table 2). In each case, the data were fit to the binding isotherm (eq. 2). The data saturate and are consistent with the presence of a single binding site. The determination of the $K_D$ in the absence of agonist was substantially more variable. This was due to the higher nonspecific fluorescence encountered at the higher CrV concentrations needed to approach saturable binding. Similar experiments conducted for CrV in low-ionic-strength buffer indicate $K_D$ values of less than 1 nM (data not shown); however, accurate dissociation constants were not obtained because the CHAPS method for determining free CrV concentrations could not accurately measure CrV below 1 nM. Nonetheless, the ionic strength effects on the CrV $K_D$ are consistent with a substantial charge dependence of binding, as was observed for ethidium, PCP, quinacrine, and

![Image](image_url)
meprofadin (Lurtz et al., 1997), and with competition for channel-permeant ions.

**Crystal Violet Interaction with the Agonist-Binding Sites.** The approximately 10-fold decrease in the $K_D$ value for CrV in the presence of agonist suggested that a reciprocal effect of CrV on agonist binding should exist. We examined the affinity of $[3^H]$ACh in varying concentrations of CrV (Fig. 5). Using low concentrations of AChR and low concentrations of high specific radioactivity $[3^H]$ACh, we measured the ratio of bound to free $[3^H]$ACh at increasing concentrations of CrV or proadifen (Fig. 5). At conditions in which only a small percentage of the sites are bound with ligand, the bound-to-free ratio of $[3^H]$ACh is proportional to the affinity of $[3^H]$ACh (i.e., inversely proportional to the $K_D$). The bound-to-free ratio increases nearly 10-fold in the presence of either added ligand, consistent with an increase in $[3^H]$ACh affinity to the AChR. The CrV curve is to the left of the proadifen curve, indicating that CrV was more potent, consistent with its higher affinity. The decrease in the bound-to-free ratio of $[3^H]$ACh observed at the higher concentrations of NCA likely reflects direct competition for binding at the agonist sites. CrV appears to interact with the ACh sites with an $IC_{50}$ of 30 $\mu$M and with proadifen with an $IC_{50}$ of $\sim$300 $\mu$M.

**Crystal Violet Binds Competitively with the PCP.** Fluorescence binding data (Fig. 4) and $[3^H]$PCP inhibition data (Fig. 2) were consistent with CrV binding to the high-affinity noncompetitive antagonist site of the AChR. On this basis alone, however, an allosteric mechanism of inhibition could not be ruled out altogether. Other NCAs have multiple sites of binding associated with the AChR (Heidmann et al., 1983) and may be able to influence the conformation of the AChR through nonspecific interactions (Boyd and Cohen, 1984). We therefore examined the PCP inhibition of CrV binding at several CrV concentrations, using CrV fluorescence as an indicator of binding. For each curve, the CrV concentration was held constant and the fluorescence was measured in the presence of increasing PCP concentrations (Fig. 6). Each set of data was fit to the model for single site inhibition, and the $K_{app}$ values for PCP were derived. The $K_{app}$ values for PCP were replotted as a function of CrV concentration (Fig. 6, inset). For a competitive mechanism of binding the replot is expected to be linear where $K_{app} = K_{app}^{PCP} + [CrV](K_{app}^{PCP}/K_{app}^{CrV})$. The corresponding plot for an allosteric mechanism of inhibition has a hyperbolic form that will saturate at high CrV concentrations (Wang et al., 1996). For the competitive mechanism the slope of the line is the ratio of the $K_D$ values for PCP and for CrV; the measured linear slope from the replot in Fig. 6 is 15, a value that corresponds well to the value of 30 obtained from the ratio of $K_D$ values of PCP ($\sim$300 nM; Lurtz et al., 1997) and CrV (10 nM, Table 2). The linear replot of the $K_{app}$ values is consistent with inhibition by PCP by binding at the same locus. Although the data cannot absolutely exclude an allosteric mechanism, the high concentrations of CrV used (2- to 40-fold higher than its $K_D$)

### Table 2

Comparison of $K_{app}$ and $IC_{50}$ values for crystal violet interaction with the AChR in the presence and absence of agonist.

<table>
<thead>
<tr>
<th>Method</th>
<th>$K_{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carb</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$100 \pm 18$ (5)</td>
</tr>
<tr>
<td>$[3^H]$Phencyclidine</td>
<td>$1200 \pm 140$ (2)</td>
</tr>
<tr>
<td>$[3^H]$Acetylcholine</td>
<td>$860 \pm 120$ (3)</td>
</tr>
<tr>
<td>$^{22}$Na flux</td>
<td>$1700 \pm 840$ (4)</td>
</tr>
</tbody>
</table>

Fig. 4. Equilibrium-binding crystal violet as measured by fluorescence intensity. AChR-rich membranes (20 nM AChR, 1.5 ml) were incubated in HTPS at ambient temperature with varying concentrations of crystal violet in the absence (○) or presence (□) of 0.1 mM PCP. The experiments were either in the presence (A) or in the absence (B) of 100 $\mu$M carbamylcholine. The fluorescence intensity was measured for each sample as described in Experimental Procedures. After removal of bound crystal violet by centrifugation, free crystal violet was quantified by fluorescence violet in the absence (○) or presence (□) of 0.1 mM PCP. The experiments were either in the presence (A) or in the absence (B) of 100 $\mu$M carbamylcholine. The fluorescence intensity was measured for each sample as described in Experimental Procedures. After removal of bound crystal violet by centrifugation, free crystal violet was quantified by fluorescence.
would require extremely strong interactions between two independent sites such that binding to one causes greater than 40-fold changes in the affinity of the second ligand. This result, therefore, strongly suggests that CrV fluorescence enhancement occurs by binding the same site as PCP to the AChR.

**Stoichiometry of Crystal Violet Binding to the AChR.** Binding data from both radioligand inhibition assays and direct binding experiments are consistent with CrV binding to a single class of sites on the AChR. To quantitate the number of CrV-binding sites on the AChR associated with the fluorescence enhancement, CrV was titrated into a concentrated suspension of AChR-rich vesicles and the fluorescence was measured (Fig. 7). At low concentrations, nearly all of the CrV added bound to the AChR and the fluorescence increased linearly; the slope of this line reflects the fluorescence yield (per nM CrV) due to binding the AChR. At higher concentrations of CrV, the binding sites become saturated, and the fluorescence signal increased linearly with a lower slope. A similar slope was observed at the higher CrV concentrations titrated into AChR-rich vesicles in the presence of PCP to block binding to the NCA site. The limiting slope at the higher CrV concentrations, therefore, reflects the fluorescence increase due to nonspecific interactions. At the intersection of these lines, the added CrV concentration equals the total binding-site concentration (see Experimental Procedures). This value can be compared with the AChR concentration added based on [3H]ACh binding, which routinely is used to quantitate various AChR-rich vesicle preparations. The ratio is 1.06 ± 0.17 CrV binding sites per AChR for four independent determinations carried out as shown in Fig. 7. This value suggests that CrV binds a single high-affinity site on the AChR, which is consistent with binding to the central ion pore.

**Crystal Violet Blocks 22Na Efflux from AChR-Rich Vesicles.** To determine the effect of CrV on AChR function, we examined the ability of CrV to block 22Na efflux from AChR-rich vesicles. Efflux was elicited by the addition of the partial agonist phenyltrimethylammonium (PTMA). Preliminary experiments established that 0.3 mM PTMA yielded maximum agonist-stimulated efflux, a level of efflux that was usually 60 to 70% of that released by the ionophore gramicidin. Preincubation of AChR-rich vesicles with a CrV concentration of 1 to 10 μM blocked agonist-stimulated efflux with an average IC50 of 1.7 ± 0.8 μM (Fig. 8; Table 2). This value can be compared to the average IC50 of 19 ± 6 μM for PCP. This IC50 was consistent with that observed previously by others (White et al., 1991).

At higher concentrations, there is apparent loss of the block of efflux by CrV. Control experiments established that the 22Na efflux observed at high levels was independent of the addition of agonist and could not be blocked by the antagonist d-tubocurarine. At these concentrations, CrV appears to cause 22Na efflux from the vesicles independent of its interaction with the AChR. This observation is consistent with the strong ability of CrV to interact with the membrane as measured by its partition coefficient.

**Discussion**

We have shown that various basic aminotriarylmethane dyes bind to the NCA site of the AChR. By characterizing one of these dyes, CrV, in detail we have shown that it possesses the characteristics of many high-affinity noncompetitive antagonists that bind the M2 region of the AChR and block activity by steric hindrance of ion passage through the pore of the AChR. These characteristics include high-affinity binding (~10 nM) for a single site on the AChR, higher affinity in the presence of agonist than for the resting conformation, competitive binding with PCP, the ability to allosterically interact with the agonist-binding sites, and the ability to block 22Na efflux from AChR-rich vesicles. The Kapp values for these properties are summarized in Table 2. There is good agreement between the potencies of CrV to inhibit 22Na efflux, enhance [3H]ACh affinity, and block [3H]PCP binding in the absence of agonist (data not shown), all at concentrations near 1 μM. The primary difference is between those values and the directly measured KD in the absence of agonist (100 nM, Table 2). The reason for this difference is not entirely clear, but may reflect difficulty in obtaining precise data for the KD by fluorescence enhancement because of the

---

**Fig. 6.** Inhibition of crystal violet fluorescence by phencyclidine. AChR-rich membranes (20 nM) were incubated in HTPS in the presence of 0.1 mM carbachol, with 25 nM (●), 50 nM (○), 100 nM (■), 200 nM (□), or 400 nM (▲) crystal violet and increasing concentrations of PCP. The fluorescence intensity at each PCP concentration for each crystal violet concentration is plotted. Symbols represent the average of duplicate determinations. Each curve was fit to a binding isotherm for a single site inhibition. Kapp values for PCP were replotted as a function of CrV concentration (inset). The slope of the replot was determined by linear regression.

**Fig. 7.** Stoichiometry of crystal violet binding to the AChR. AChR-rich membranes (375 nM AChR) were incubated in the presence of 0.1 mM carbachol in HTPS (●). CrV was titrated into the membranes, and the fluorescence intensity was monitored. The titration was repeated in the presence of 1 mM PCP (○) to define nonspecific fluorescence. Linear regressions were applied to initial and ending linear components of the titration in the absence of PCP (0–200 nM CrV and 650–1000 nM CrV, respectively). The intercept from these two sets of points is 376 nM and defines the concentration of CrV-binding sites (see Experimental Procedures).
higher nonspecific binding at the CrV concentrations required to measure binding in the absence of agonist. The discrepancy between the $K_{app}$ measured by fluorescence enhancement and by [3H]PCP binding in the presence of agonist (Table 2) simply reflects the lower limit of the [3H]PCP-binding assay; the value determined by fluorescence likely reflects the true $K_p$. Comparison of the $K_{app}$ values in the absence of agonist versus those in the presence of agonist suggests that CrV binding strongly favors the desensitized conformation of the AChR because this is the conformation stabilized by agonists at equilibrium. Nonetheless, CrV potentially could be acting as an open channel blocker or trapping the AChR in a distinct conformation with high affinity for agonist. These possibilities may be resolved by examination of the kinetics of binding using either fluorescence methods or by single channel measurements of blockade. The preferential binding in the presence of agonist is consistent with the observation that larger NCAs preferentially bind the desensitized state of the AChR, whereas ligands that prefer binding to the resting conformation, such as tetracaine or TID, tend to be smaller (Cohen et al., 1986; White and Cohen, 1992). This trend also favors the model of Furois-Corbin and Pullman and of White and Cohen that suggests that the pore of the AChR expands at the synaptic end upon desensitization by tilting of the M2 a-helices away from the central axis of the AChR (Furois-Corbin and Pullman, 1989; White and Cohen, 1992).

Several of the other dyes bind with affinities comparable to CrV, and many of the dyes display similar properties, including higher affinity for the desensitized state as measured by inhibition of [3H]PCP binding (brilliant green, malachite green, rosaniline, ethyl violet; data not shown). Therefore, the affinities of the various dyes can be compared in terms of binding a single site on the AChR. The detailed structure-activity relationship of these dyes will be particularly interesting as they represent a large class of compounds with well developed chemistry. This will permit a closer examination of the structure of the pore of the AChR than has been possible with the previously known heterogeneous collection of ligands.

**Structure Activity Relationship of the Basic Dyes.**

The dyes examined have several discrete, small changes in structure that cause significant changes in affinity (see Fig. 1 for reference). The six methyls on the CrV-amines contribute substantially to affinity. Pararosaniline has none of these methyls and binds with ~200-fold lower affinity (compare $K_{app} = 2200$ nM for pararosaniline, Table 1, with $K_p = 10$ nM for CrV, Table 2). Removal of one complete dimethylamino group also substantially lowers affinity (compare malachite green, $K_{app} = 3.9$μM, with CrV, $K_p = 10$ nM). Increasing the size of the amine substituents from methyl to ethyl groups improves affinity: compare the $K_{app}$ values of malachite green and brilliant green. Likewise, ethyl violet has a $K_{app}$ similar to CrV, but because the $K_{app}$ estimate was limited by the AChR concentration, a precise comparison will require further experiments.

Substitutions at the positions ortho and meta to the central carbon also increase affinity. Comparison of pararosaniline, rosaniline, and new fuchsin shows enhanced affinity with methylation at the positions meta to the central carbon. As a caveat, however, it should be noted that rosaniline contained at least 14 separable components when examined by HPLC (data not shown). The compound Victoria blue BO includes an additional fused aromatic ring. The added bulk appears to inhibit binding only moderately as it binds with a $K_{app}$ slightly higher than its congeners, ethyl violet and CrV. The increased bulk and hydrophobicity in this region does not, therefore, substantially interfere with binding and, in the case of the pararosaniline series, enhances binding. CrV bears a single, delocalized positive charge. The zwitterionic analog, Dpmsm, binds with very low affinity ($K_{app} > 300$ μM). This compound has one of the dimethylamino groups replaced by a methoxy group and a sulfate moiety in a position meta to the central carbon (Fig. 1). Although the methoxy may contribute to some of the affinity loss, a significant amount likely is due to the presence of the negative charge added. In contrast, methyl green is dicationic with an additional N-ethyl group and binds 700-fold more weakly ($K_{app} ~ 7000$ nM, Table 1) than CrV. It appears that addition of either the ethyl group or the second charge alters the interaction substantially.

However, as argued above, increased steric bulk on the amines does not dramatically decrease affinity. Therefore, it seems unlikely that the steric bulk of the added ethyl group on methyl green, as compared with CrV, would substantially affect binding. A similar observation was made for analogs of $d$-tubocurarine: addition of a single methyl group at the 2'-nitrogen of tubocurarine (converting it to $d$-tubocurarine) decreased the affinity for the NCA site ~100-fold (Pedersen and Papineni, 1995). This addition, however, did not alter the net charge of +2 on the curare compound. The distances between the two charged nitrogens on $d$-tubocurarine (10.5 Å) is similar to the distance between two dimethylamines on CrV (9.8 Å) and the distance between the two arylamines of ethidium (9.8 Å). These observations suggest the importance of nonquaternary amines in a particular arrangement, perhaps to act as hydrogen bond acceptors or participate in other specific interactions with the pore of the channel.
Fluorescence of Crystal Violet. The fluorescence enhancement that accompanies binding of CrV to the AChR is greater than 200-fold and is due to a change in quantum yield, as shown by the small changes in the absorbance spectrum (Fig. 3). This large enhancement is possible because CrV is only weakly fluorescent in aqueous solution. The weak fluorescence is attributed to rapid de-excitation of the excited state by collision with water molecules or by internal conversion.


Pedersen SE, Dreyer EB and Cohen JB (1986) Location of ligand-binding sites on the M2 sequence of the AChR that comprise the high-affinity NCA-binding site. In addition to structure-activity analysis, the dyes we have examined are likely to have substantial utility in future experiments to characterize the AChR pore. The high-affinity dyes (new fuchsin, brilliant green, crystal violet, and ethyl violet) have large molar extinction coefficients across a broad portion of the visible spectrum. They can serve as excellent fluorescence energy transfer acceptors from a variety of donors. The properties of CrV make it suitable for fluorescence quenching studies to examine the environment of the pore of the AChR, whereas other fluorescent ligands such as ethidium and quinacrine have been limited by their moderate affinity. Another intriguing possibility is to use this ligand as a source of heat or free radicals for site-directed protein modification. Malachite green has been shown to cause local heating that results in protein damage (Indig et al., 1992). Preliminary experiments show that CrV indeed sensitizes the AChR to free radical damage in the presence of light (W. H. Vila-Carriles and S.E.P, unpublished observations).

One drawback to using CrV is its substantial nonspecific interactions with the membrane, with plastic surfaces, and with glass. This creates difficulties in quantitation of CrV itself. However, most of these problems can be overcome by explicit measurements of CrV concentration by the CHAPS assay or by HPLC. Treatment of glass with polyethyleneimine or cationic silanes also prevents CrV from sticking to assay or by HPLC. Treatment of glass with polyethyleneimine or cationic silanes also prevents CrV from sticking to

Acknowledgments

We thank Arlene Samano for excellent technical assistance in carrying out many ligand-binding assays.

References


Huc O, Oberthur W and Lootspeich F (1986) The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M1 of the receptor subunits. FEBS Lett 202:137–141.


Send reprint requests to: Dr. Steen E. Pedersen, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: pedersen@bcm.tmc.edu