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INTERACTION OF *p*-AMINOPHENYLDICHLOROARSINE, AN ARSENICAL WITH SPECIFICITY FOR VICINAL CYSTEINES, WITH [^{3}H]CYTISINE BINDING SITES IN RAT BRAIN MEMBRANES

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Abstract—The arsenical compound *p*-aminophenyldichloroarsine (APA) is selective for spatially close thiols with which it forms a stable complex. The α subunits of nicotinic acetylcholine receptors are defined by the presence of a pair of adjacent cysteines close to the agonist binding site. Here the interaction of APA with [^{3}H]cytisine binding sites, which correspond to the major subtype of nicotinic receptors in rat brain has been examined. Incubation of brain membranes with 10 μM APA abolished [^{3}H]cytisine binding. The action of APA was dependent on prior reduction of sulphydryls with dithiothreitol. APA effects could not be reversed by oxidizing agents but could be reversed by the anti-arsenical reagent 2,3-dimercapto-1-propane sulphonic acid. Under the conditions used, the concentration of APA producing a half-maximal decrease in binding was 130 nM. The loss of [^{3}H]cytisine binding was due to a decrease in the number of binding sites (B_{max}) with no effect on affinity for the radioligand (K_d). Nicotinic ligands failed to protect against the reduction and arsenylation of neuronal receptor sites. These observations are consistent with the potent interaction of APA with this neuronal nicotinic receptor.

Key words: neuronal nicotinic receptors; sulphydryl reduction; dithiothreitol

nAChR‡ belong to the superfamily of ligand gated ion channels. The subunits of these pentameric proteins share a common topology, each having four hydrophobic putative membrane spanning segments and a large extracellular N-terminus. The α subunits of *Torpedo* and muscle nAChR possess a pair of adjacent cysteine residues in this N-terminal domain, at positions 192 and 193, which are unusual in forming a disulphide bond [1]. Reduction of this disulphide bond and affinity alkylation with reagents such as bromacetylcholine and MBTA indicated that Cys 192 and Cys 193 were very close to the agonist binding site. Numerous nAChR subunits have subsequently been identified in nervous tissues and the presence of an analogous pair of adjacent cysteines is definitive of their designation as α subunits; neuronal subunits lacking the adjacent cysteines are termed "β" [2]. By analogy with *Torpedo* and muscle nAChR, the neuronal α subunits are considered to be largely responsible for agonist binding.

Two major classes of nAChR in rat brain can be distinguished on the basis of radioligand binding. Tritiated agonists, such as nicotine and cytisine, bind with nanomolar affinity to a nAChR, probably comprising α4 and β2 subunits [3, 4], while [^{125}I]-αbungarotoxin labels a discrete population of binding sites that correlate with the α7 subunit. The binding of both types of radioligand is decreased by disulphide reduction and affinity alkylation [5–9], and MBTA has been shown to label specifically the α4 subunit (formerly called "β" because of its large mass on polyacrylamide gels) in an immunoisolated α4 β2-nAChR from chicken brain [10].

Trivalent arsenical reagents interact with thiol groups. Alkyldihaloarsines and alkylarsenoxides are selective for spatially close thiols and form a stable cyclic dithioarsinite [11]. This reaction can, however, be reversed by reactive dithiol reagents like British Anti Lewisite or DMPS. Arsenicals have been used to explore the role of vicinal cysteines in enzymes, such as dihydrolipoamide dehydrogenase [11–13]. Arsenical reagents are attractive because bifunctional reagents can be generated to study residues in the vicinity of the vicinal cysteines. As a prerequisite to adopting such an approach to studying the agonist binding site of nAChR, it is necessary to establish that the monofunctional arsenical interacts with the receptor. Pike and Loring [9] and Rossant *et al.* [14] have shown that this is the case for immunoisolated nAChR from chicken brain labelled with tritiated agonists. Here we report that [^{3}H]cytisine binding to rat brain membranes is prevented by reduction and reaction with APA (for structure see insert, Fig. 2) in a manner consistent with a specific modification of the pair of vicinal cysteines in the α subunit.

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‡ Abbreviations: APA, *p*-aminophenyldichloroarsine; DTT, dithiothreitol; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); DMPS, 2,3-dimercapto-1-propane sulphonic acid; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium; DHβE, dihydroβerythroidine; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor.

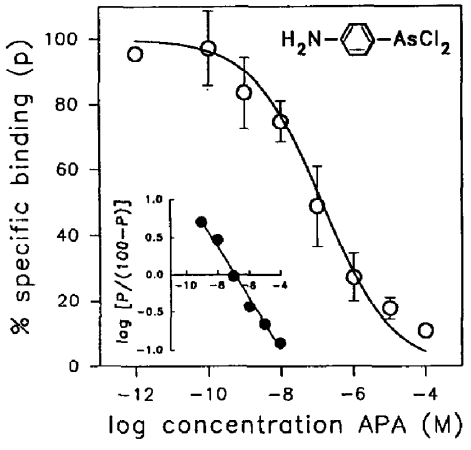

Fig. 2. Concentration dependence of APA effects on [^{3}H]-cytisine binding to rat brain membranes. P2 membranes were incubated with DTT (2 mM, 30 min, 4°) followed by incubation with APA at the concentrations indicated. [^{3}H]Cytisine specific binding is expressed as a % of control where APA was absent from the second incubation. Values are the mean ± SEM of three or more determinations. Linear transformation of the data points (insert, bottom) gives a value of 130 nM APA for the half-maximal effect under these assay conditions (the irreversible nature of APA binding invalidates the application of conventional analyses for competitive interactions). Insert, top: chemical structure of APA.

[^{3}H]cytisine binding to rat brain P2 membranes are summarized in Fig. 1. Pre-incubation at alkaline pH in the absence of added reagents had no effect on the equilibrium binding of [^{3}H]cytisine, whereas the addition of 2 mM DTT significantly decreased binding. The effect of DTT was rather variable between assays, and the incomplete inhibition probably reflected the auto-oxidation of the receptors during subsequent incubations, as suggested by Pike and Loring [9]. Lukas and Bennett [5] reported that DTT had no permanent effect on [^{3}H]αbungarotoxin binding to brain membranes, although pre-treatment with DTT was essential for successful alkylation of these sites with MBTA or bromacetylcholine. In the present study the arsenical reagent APA, tested at 10 μM, decreased [^{3}H]cytisine binding by more than 95% (Fig. 1). This result was dependent on prior treatment of the tissue with DTT, consistent with a complete reduction of sulphydryl groups by DTT, followed by covalent modification with APA.

Next the effects of the oxidizing agent DTNB were examined. DTNB alone had no effect on [^{3}H]cytisine binding. However, it reversed the decrease in binding produced by DTT, as expected [6]. This was also evident from its dramatic effects on the action of APA: incubation of DTT-treated membranes with DTNB prior to exposure to APA protected against the APA-dependent decrease in [^{3}H]cytisine binding. Protection was not afforded if exposure to APA preceded DTNB treatment. In contrast, the anti-arsenical oxidizing agent DMPS was able to reverse the effects of both DTT and APA, as previously reported for *Torpedo* and chick brain nAChR [9, 19].

The concentration-dependence of the effect of APA on [^{3}H]cytisine binding was examined, after first reducing receptors with DTT (Fig. 2). Increasing concentrations of APA from 10^{-12} to 10^{-4} M progressively decreased the subsequent specific binding of [^{3}H]cytisine. Under the assay conditions used, the half-maximal effect was observed at 130 nM APA.

Saturation binding experiments for [^{3}H]cytisine binding to rat brain membranes pre-treated with DTT and various concentrations of APA (0.1–100 μM) demonstrated that the effect of APA was entirely attributable to an apparent decrease in the number of sites (B_{max}) with no effect on binding affinity (K_d; Table 1).

Incubations with DTT were carried out in the presence of nicotinic ligands to see if occupation of the agonist binding site would protect against reduction and subsequent covalent attachment of APA. Neither the agonists (−)nicotine and cytisine, competitive antagonists DHβE and MLA nor the non-competitive antagonists mecamylamine and MK801 had any effect on the loss of [^{3}H]cytisine binding produced by 10 μM APA (Table 2). All ligands were examined at 10 μM, a concentration at which each of them would be expected to interact with the neuronal receptor. However, (−)nicotine was also tested over a wide concentration range (10 nM to 1 mM) but was without effect even at the highest concentrations (data not shown).

DISCUSSION

The arsenical APA reacted with reduced nAChR to form a stable product that could no longer bind [^{3}H]cytisine. This was supported by the insensitivity of APA inhibition to the oxidizing agent DTNB but reversal by the anti-arsenical DMPS. APA is a very potent sulphydryl reagent, half-maximal effects on [^{3}H]cytisine binding occurring at submicromolar concentrations of APA, confirming observations on chicken nAChR [9, 14]. This contrasted with the IC_{50} for DTT inhibition of [^{3}H]nicotine binding measured under similar conditions, which was >100 μM [6, 9].

Table 1. Effect of APA on K_d and B_{max} for [^{3}H]cytisine binding to brain membranes

APA concentration (μM)	0	0.1	1.0	100
B_{max} (fmol/mg protein)	45.2 ± 12.2 (N = 3)	29.1	9.8	3.5
K_d (nM)	3.1 ± 0.5 (N = 3)	2.6	4.3	2.3

Table 2. Effect of nicotinic ligands on DTT reduction and APA interaction with [^{3}H]cytisine binding sites

	Drug (10 μM)	[^{3}H]cytisine binding (% control)*†
	(None)	16.2 ± 3.2
Agonists:	(−)Nicotine	18.2 ± 5.5
	Cytisine	21.3 ± 4.3
Competitive antagonists:	DHβE	17.8 ± 5.1
	MLA	14.1 ± 4.4
Channel blockers:	Mecamylamine	15.8 ± 2.2
	MK801	12.2 ± 3.6

* [^{3}H]Cytisine binding to controls (no drug or APP) = 51.4 ± 4.7 fmol/mg protein.
† Mean ± SEM (N = 3).

The most plausible interpretation of these data is that APA formed a stable dithiol arsenite bond with the reduced vicinal cysteines equivalent to positions 192, 193 in the *Torpedo* α sequence, the unequivocal target of affinity alkylating agents [1]. Pike and Loring [9] and Rossant *et al.* [14] demonstrated that APA could protect [^{3}H]agonist sites against irreversible alkylation by bromacetylcholine, indicative of a common site of action. Although all nAChR subunits possess a second disulphide bond, creating a loop between Cys 128 and Cys 142 (*Torpedo* numbering), these residues remained cross-linked following mild reduction and were not labelled by MBTA [1].

Pre-incubation with nicotinic ligands before and during treatment with DTT failed to protect against reduction of the nAChR. Similarly Schwartz and Kellar [6] found that 100 μM nicotine or acetylcholine were unable to prevent the DTT-induced loss of [^{3}H]acetylcholine binding to brain membranes. In a study of [^{125}I]αbungarotoxin binding to PC12 cells, unlabelled toxin was unable to protect against DTT reduction and MBTA alkylation [7]. However, high concentrations (greater than millimolar) of nicotinic ligands are reported to retard the rate of alkylation by MBTA and BAC of brain αbungarotoxin binding sites [5], whereas agonists but not antagonists are claimed to protect against the reduction of the critical disulphide in *Torpedo* nAChR [20]. The lack of protection observed in the present experiments may reflect subtle differences between the agonist binding sites of neuronal and peripheral nAChR.

Current models of the nicotinic agonist binding site, based on affinity labelling experiments with *Torpedo* nAChR and mutagenesis experiments, propose the involvement of aromatic residues belonging to three different regions of the N-terminal extracellular domain, leading to the proposition of a "3 loop" model [21]. These residues are conserved in all neuronal α subunits (with the exception of α5). The vicinal cysteines may not contribute directly to agonist binding, but the disulphide bond between them may impose a critical constraint on the conformation of the binding site. Potent arsenical reagents based on APA could prove useful in further mapping the binding pocket: bifunctional arsenicals could be tethered to the vicinal cysteines allowing subsequent alkylation of neighbouring residues by other functional groups on the arsenical. This approach has been exploited in the study of lecithin–cholesterol acyltransferase, for example [22]. The stable high affinity binding of APA to vicinal cysteines in the nAChR, and the ability of DMPS to reverse this interaction with recovery of agonist binding, could also be exploited for affinity chromatography; this strategy was used successfully in the isolation of lipoic acid [23]. Thus, arsenical reagents promise to be valuable additions to the arsenal of chemical probes for studying nAChR.

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