Ganglionic nAChRs and high-affinity nicotinic binding sites are not equivalent

George Kemp and Barbara J. Morley*

Neurosciences Program, University of Alabama School of Medicine, Birmingham, AL 35294 and **Research Division, Boys Town National Institute, 555 North 30th Street, Omaha, NE 68131, USA*

Received 21 July 1986

High-affinity ($K_d \simeq 10$ nM) binding sites for nicotine and acetylcholine (ACh) have recently been identified in vertebrate brain. It has been suggested that these sites are desensitized ganglionic (C6) nicotinic acetylcholine receptors (nAChRs). We have tested the pheochromocytoma cell line PC12, which is known to contain well-expressed C6 nAChRs, to determine if these nAChRs are associated with high-affinity [^{3}H]ACh-binding sites. We found that the high-affinity nicotinic [^{3}H]ACh-binding site is absent in PC12 cells. We also found that the concentration of nicotine or ACh necessary to desensitize carbamylcholine-stimulated Na^+ flux was at least two orders of magnitude greater than the concentrations used in binding experiments. We conclude that high-affinity nicotinic binding sites are not equivalent to C6 ganglionic receptors.

Acetylcholine receptor *Nicotinic receptor* *Nicotine* *Acetylcholine* *Na^+ flux*

1. INTRODUCTION

A high-affinity nicotinic binding site in mammalian brain has been identified in several laboratories using radiolabeled acetylcholine [1] and nicotine [2] that has a high affinity for cholinergic agonists but not antagonists and is unrelated to the brain α-bungarotoxin (Butx) binding component [3,4]. It has been suggested that the high affinity of this site is associated with a desensitized receptor state that results from the use of agonists as ligands [5]. Time-dependent increases in agonist affinities are known to occur with *Torpedo* [6] and muscle [7] nAChR, and this phenomenon reconciles the apparent discrepancy between agonist binding constants measured by equilibrium methods and the much higher concentrations known to be necessary for channel activation.

Two kinds of mammalian nAChRs have been characterized by electrophysiological techniques. Those found at the neuromuscular synapse are blocked by Butx. Nicotinic receptors insensitive to Butx and several other neurotoxins are found at the spinal Renshaw synapse and at several ganglionic synapses (review [8]). These nAChRs are termed ganglionic or C6 receptors. Since the binding of nicotine and ACh to high-affinity sites is not displaced by Butx, it is possible that these receptor sites are C6 nAChRs. Although electrophysiological studies are not in complete agreement, ganglionic blockers such as mecamylamine typically antagonize central nicotinic cholinergic activity [9], suggesting that at least some brain nAChRs have characteristics of C6 receptors.

To test the hypothesis that C6 receptors and high-affinity nicotinic [^{3}H]ACh-binding sites are equivalent, we measured high-affinity [^{3}H]ACh

Abbreviations: ACh, acetylcholine; DFP, diisopropyl fluorophosphate; nAChR, nicotinic acetylcholine receptor

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

binding to the clonal sympathomimetic PC12 cell line, which is known to express nAChRs that have been extensively characterized in Na^+ flux experiments as C6 receptors [10–12]. We also determined the concentrations of ACh and nicotine necessary to activate and desensitize PC12 cells in order to assess whether the concentration of ACh or nicotine used in binding studies would be sufficient to produce desensitization.

2. MATERIALS AND METHODS

2.1. *[^{3}H]ACh-binding assay*

The concentration of [^{3}H]ACh-binding sites was determined by a modification of the method of Schwartz et al. [1] as described by Larsson et al. [13]. Rats were decapitated and the brains rapidly removed. Individual brain areas were rapidly dissected, frozen in liquid nitrogen, and stored at −70°C. Brain membranes were prepared by repeated (3×) homogenization and centrifugation (39000×g, 10 min) in ice-cold buffer containing 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5 μM atropine. PC12 cells were prepared in the same manner except that some were homogenized between centrifugations and some were sonicated between centrifugations. All samples were incubated in 100 μM DFP for 20 min prior to assay.

Tissue samples (500–700 μg protein in a volume of 200 μl) were incubated with 10 nM [^{3}H]ACh, synthesized from choline (New England Nuclear, spec. act. 80 Ci/mmol) as described by Schwartz et al. [1], for 45 min at 2°C in a total of 500 μl buffer. Non-specific binding was determined by adding carbamylcholine to control tubes.

2.2. *Flux experiments*

Ion flux experiments were carried out in buffer containing 0.8 μCi/ml $^{22}Na^+$ (Amersham, spec. act. 13500 Ci/mol), 2 mM carbamylcholine, and 5 mM ouabain. Cells were incubated for 1 min at 25°C, then rapidly rinsed in 3 ice-cold 1-l beakers of buffer. The control flux obtained in the absence of desensitization treatment ranged from 180 to 260 nmol/mg per min, similar to that reported in [10]. A background flux of approx. 25 nmol/mg per min, obtained in the absence of carbamylcholine, was subtracted from all fluxes.

$^{22}Na^+$ influx experiments were done essentially as described by Stallcup [10]. Cells were desensitized in buffer containing ACh or nicotine for 40 min at 25°C. In experiments where ACh was used to desensitize the $^{22}Na^+$ flux, cells were pretreated for 20 min with 10^{-4} M DFP, and 10^{-4} M DFP and 10^{-4} M neostigmine were added to the incubation buffer.

3. RESULTS AND DISCUSSION

The PC12 cell line, first developed by Greene and Tischler [14] from a rat pheochromocytoma, has become an established model for sympathetic neurons in culture. PC12 cells express C6 nAChRs which are characterized by their insensitivity to Butx [11] and their blockade by the ganglionic antagonist, mecamylamine [12].

Table 1 shows that PC12 cells do not contain high-affinity [^{3}H]ACh-binding sites. To confirm the validity of our assay, the amount of high-affinity [^{3}H]ACh binding to a number of rat brain areas was simultaneously determined. Both the absolute and relative amounts of high-affinity [^{3}H]ACh binding are in essential agreement with those reported by Schwartz et al. [1]. It is not likely that our results reflect a site density too low to measure in the [^{3}H]ACh-binding assay but still sufficient to give rise to the observed Na^+ flux. The concentration of C6 nAChRs in PC12 cells can be estimated by the comparison of flux in PC12 cells with that of a muscle cell line where the concentration of nAChRs can be assayed by [^{125}I]Butx binding. A flux of 380 nmol Na^+/mg protein per min and a concentration of 750 fmol nAChRs/ mg protein have been measured in BC3H-1 cells [7]. If the conductance properties of PC12 nAChRs are reasonably similar to muscle nAChRs, then PC12 cells would be expected to contain approximately 400 fmol nAChRs/mg protein. This concentration of nAChRs is considerably greater than the amount of the [^{3}H]ACh binding in a rat brain homogenate and far greater than the limit of sensitivity of the [^{3}H]ACh assay, which is about 2 fmol/mg.

Early studies that characterized purified [15] and membrane-associated [16] electric fish and electric eel nAChR reported very high (K_d = 2–60 nM) binding affinities for ACh. These were several orders of magnitude greater than the $K_{0.5}$ (concentration of agonist required to produce a 50% of

Table 1

The concentration of [^{3}H]ACh nicotinic binding sites in several regions of rat brain and PC12 cells

Tissue	N	Specific binding (fmol/mg protein, $\bar{X}$ ± SE)	Specific binding (pmol/g original wet wt, $\bar{X}$ ± SE)	Data taken from Schwartz et al. [6] (pmol/g original wet wt)
Thalamus	2	31.4 ± 0.08	2.70 ± 0.10	2.66
Cortex	3	30.7 ± 0.85	2.70 ± 0.43	1.61
Hippocampus	6	18.0 ± 2.10	1.40 ± 0.17	0.44
Hypothalamus	2	13.0 ± 0.25	0.90 ± 0.02	1.05
Cerebellum	2	11.0 ± 0.01	0.72 ± 0.09	1.03
Inferior colliculus	3	9.3 ± 0.32	0.68 ± 0.04	0.51
PC12 cells (intact)	2	0	0	0
PC12 cells (sonicated)	2	0	0	0
PC12 cells (homogenized)	2	0	0	0
Liver		0	0	0
Heat-denatured cortex	2	0	0	0

N, number of different samples. Each sample was assayed in triplicate as described in section 2

maximum response) values observed in ion flux experiments [6]. It was ultimately shown that exposure to agonists resulted in a time-dependent increase in the affinity of the receptor for the agonist [17].

When prolonged exposure to agonists causes receptors to become desensitized, these receptors cannot participate in the mediation of ion flux. The concentration dependence of the consequent reduction in conductance should then reflect the affinity of the desensitized receptor (K_{des}) for agonists. This expectation has been rigorously confirmed by Sine and Taylor [7] in the muscle cell line BC3H-1, where K_{des} values were found to agree closely with binding constants obtained from [^{125}I]Butx competition experiments where the cells were pretreated with agonists. We therefore applied this strategy to ACh and nicotine-mediated ion flux in PC12 cells. Determination of the affinity of PC12 cell nAChRs for nicotine and ACh by this method consequently provided an independent means for establishing whether PC12 nAChRs have K_{des} values in the 10–100 nM range that would correspond to high-affinity ACh and nicotine sites.

The concentration of agonist necessary to achieve half-maximal activation (K_{act}) of PC12 nAChRs is 7.7×10^{-5} M for nicotine and 8.2×10^{-5} M for ACh. These values are similar to those reported for the muscle cell line, BC3H-1, where the K_{act} for nicotine is 2.32×10^{-4} M and the K_{act} for carbamylcholine is 9.49×10^{-5} M [7].

Fig.1 shows dose-response curves obtained for desensitization of carbamylcholine-stimulated PC12 Na^+ flux by nicotine and acetylcholine. K_{des} values obtained in these experiments were 3.3×10^{-5} M for nicotine and 6.4×10^{-5} M for ACh. Since these values reflect the affinity of desensitized nAChRs for these ligands, we conclude that desensitized PC12 nAChRs do not bind ACh or nicotine with affinities consistent with the

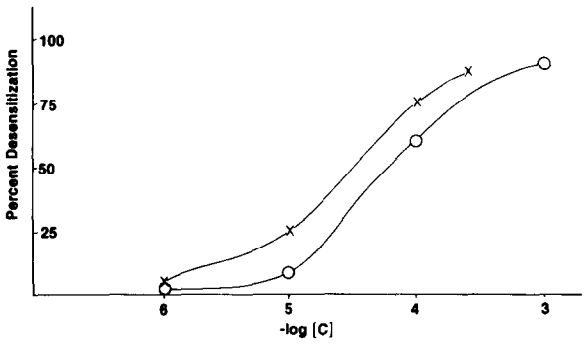

Fig.1. Desensitization of $^{22}Na^+$ flux in PC12 cells by ACh (○—○), and nicotine (×—×). Sodium flux experiments were carried out as described in section 2.

high-affinity binding sites measured for these ligands in brain.

We conclude that the C6 nAChRs present in PC12 cells do not possess high-affinity binding sites for ACh or nicotine and suggest that high-affinity nicotinic ACh and nicotine binding sites and C6 nAChRs are not equivalent. The function of high-affinity [^{3}H]ACh- and [^{3}H]nicotinic binding sites is not known but they are apparently pre-synaptic in several areas of mammalian brain [3,18]. Clearly, the high-affinity nicotinic receptors are distinct from the central Butx binding component which is a presumed post-synaptic nicotinic receptor with binding sites for ACh and peripheral antagonists [8,19]. Ganglionic antagonists do not displace the binding of [^{3}H]ACh, [^{3}H]nicotine nor [^{125}I]Butx to neural tissue, suggesting the possibility of an additional nicotinic receptor site. The diversity of receptor subtypes in other systems is consistent with the concept that multiple forms of nicotinic receptors may exist.

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