ELSEVIER

Biochemical Pharmacology 61 (2001) 733–740

Biochemical Pharmacology

Investigation of nicotine binding to THP-1 cells: evidence for a non-cholinergic binding site

Deri Morgan, Mike E. Parsons, Cliff J. Whelan*

Department of Biosciences, CP Snow Building, University of Hertfordshire, College Lane, Hatfield, Hertfordshire, AL10 9AB, UK

Received 6 March 2000; accepted 9 August 2000

Abstract

Nicotine is known to modulate immune function, but reports have produced conflicting evidence as to whether nicotinic acetylcholine (nACh) receptors are responsible for these effects. This study was designed to examine the identity of nicotine-binding sites on immune cells using a human leukaemic monocytic cell line, THP-1, that is known to have functions that are modulated by nicotine. Binding studies were performed on THP-1 whole cells using [^{3}H]nicotine as a probe to analyse any possible nicotine-binding sites on these cells. Saturation analysis of THP-1 cells revealed the presence of 2 distinct binding sites; one with a K_{d1} of 3.5 ± 2.1 × 10^{-9} M and a B_{max1} of 4100 ± 560 sites/cell (designated the high-affinity site) and the other with a K_{d2} of 27 ± 9.2 × 10^{-9} M and a B_{max2} of 11,600 ± 630 sites/cell (low-affinity site). Competition analysis revealed that one site had an affinity to a range of cholinergic ligands including epibatidine and cytisine. When saturation analysis of [^{3}H](−)-nicotine to THP-1 cells was performed in the presence of 1 × 10^{-6} M epibatidine, only one binding site was detected. Comparisons of K_d and B_{max} values showed that the high-affinity site was not occluded by epibatidine. No drugs tested displayed any affinity for the high-affinity site except the two enantiomers of nicotine. The high-affinity site was shown to be stereoselective for the (+)-enantiomer of nicotine as shown by K_i values produced by competition analysis in the presence of 1 × 10^{-6} M epibatidine. These values were 5.7 ± 0.32 × 10^{-11} M and 1.9 ± 4.9 × 10^{-9} M for (+)-nicotine and (−)-nicotine, respectively. This study presents evidence for a possible non-cholinergic binding site that may play a role in the mechanism of immunomodulation by nicotine.

Keywords: Nicotine; Binding studies; Monocytes; Cholinergic; Non-cholinergic; Receptors

1. Introduction

Nicotine is one of the most pharmacologically active components of cigarette smoke, and its action on the brain and neuromuscular junction nicotinic acetylcholine receptors has been well described [1–3]. These effects of nicotine are mediated through its actions on nACh receptors, a class of multisubunit ligand-gated ion channels belonging to a supergene family of receptors that include glutamate, glycine, and serotonin receptors [4]. To date, the diversity of vertebrate nACh receptors numbers 16 subunits: 9 α-subunits, 4 β-subunits, and γ, δ-, and ϵ-subunits [5,6]. Structurally, these receptors are believed to form a pentameric conformation with a central pore that allows the passage of cations upon opening [3,7,8]. nACh receptors were until recently classed as muscular and neuronal nACh receptors with the muscular receptor comprised of $\alpha1\beta1\epsilon\delta$ in adult vertebrate ($\alpha1\beta1\gamma\delta$ in the embryo) and the other subunits being found in the brain. Recently, however, nACh receptors have also been shown to exist in non-neuronal tissues including keratinocytes, chromaffin cells, and immune cells, and receptors are thus now named by subunit identity [9–11]. Agonist-binding sites are generally associated with α-subunits, although β-subunits are known to alter ligand affinities and efficacy [12,13]. Channels composed of subunits $\alpha2$–6 form heteromeric receptors with $\beta2$–4 subunits, whereas $\alpha7$–9 can form homomeric receptors [6,14,15]. All subunits are known to exist in human tissues with the exception of $\alpha8$ and $\alpha9$, which have only been discovered in chick retina and rat cochlear hair cells [6,16].

There is now evidence that nicotine may have actions at sites other than the brain and nervous system and in particular on the immune system. *In vitro* studies of nicotine on the immune system have demonstrated that nicotine has

* Corresponding author. Tel.: +44-1707-285.139; fax: +44-1707-285.258.

E-mail address: c.j.whelan@herts.ac.uk (C.J. Whelan).

Abbreviations: nACh, Nicotinic acetylcholine; DMPP, 1,1-dimethyl piperazine; and TNFα, tumor necrosis factor alpha.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00587-6

effects on immune cells such as inhibition of apoptosis [17], the potentiation of superoxide production [18], and an increase in chemotaxis in human neutrophils [19]. Nicotine causes a modulation of immunoglobulin synthesis in murine splenic lymphocytes [20], an increase in intracellular calcium mobilisation [21,22], and an inhibition of the production of interleukin-2 and TNFα from human lymphocytes [23]. The production of TNFα from human leukaemic monocytes in response to lipopolysaccharide is also inhibited by nicotine [24].

However, the mechanism by which nicotine produces these effects is not known. It has been demonstrated that nicotinic nACh receptors exist in human blood leukocytes, peripheral lymphocytes, and a number of lymphocyte cell lines using reverse transcriptase-polymerase chain reaction and radioligand binding techniques [10,25]. Evidence that these nACh receptors are functional comes from studies showing that they may play a role in proliferation. It has been demonstrated that concentrations of acetylcholine (1–10 nM) inhibited lymphocyte proliferation. This effect was blocked by the nicotinic antagonists α-bungarotoxin (1 μM) and d-tubocurarine (1 μM), indicating that the receptors responsible were nACh [26,27], but it is not known whether these receptors possess the capabilities to modulate the inflammatory response. A previous study found that conventional nicotinic antagonists did not alter the inhibitory effect of nicotine on the release of cytokines from peripheral blood mononuclear cell fractions [28], suggesting that "atypical" or non-cholinergic nicotine receptors may be involved.

Binding studies on polymorphonuclear leukocyte membranes and whole cells have produced conflicting data as to the identity of [^{3}H]nicotine-binding sites. Two studies demonstrated the presence of a nicotine-binding site on the surface of human polymorphonuclear whole cells that was not competed for by conventional nicotinic ligands [29,30]. In contrast, Lebargy *et al*. showed the presence of a nicotine-binding site on human polymorphonuclear leukocyte membranes that have a structure–affinity profile similar to that found in nicotine-binding sites in the brain [31]. However, both studies only demonstrated a single binding site on leukocytes from normal volunteers, and the identity and possible function of this binding site remain obscure.

In an attempt to address the question of the identity of nicotine-binding sites on immune cells, we chose to examine THP-1 cells, a monocyte cell line in which cytokine release is inhibited by nicotine [24]. The experiments described below use radiolabelled nicotine to examine the possibility of a nicotinic binding site that is not typically cholinergic. We also looked at this cell line because monocytes, unlike lymphocytes and neutrophils, have not yet been analysed for the presence of nicotine-binding sites. The discovery of a non-cholinergic nicotinic receptor on immune cells that has an immunomodulatory effect would be a potentially valuable target for therapeutic drugs.

2. Materials and methods

2.1. Materials

(−)-*N*-Methyl-[^{3}H]nicotine (85 Ci/mmol) was obtained from New England Life Sciences. Nicotine bitartrate, (+)-nicotine, cytisine, α-bungarotoxin, carbachol, hexamethonium chloride, DMPP, dihydro-β-erythroidine, atropine, and (1)-polylysine were obtained from Sigma. Epibatidine was purchased from Tocris. Whatman GF/B filters were obtained from Merck. Skatron 12-well filter tube strips were obtained from Camo Ltd. THP-1 cells were obtained from the European Collection of Cell Cultures. RPMI-1640 medium, penicillin, streptomycin, and foetal bovine serum were purchased from GIBCO. Scintillation vials and Liquiscint were obtained from National Diagnostics.

2.2. THP-1 maintenance and preparation

Cells were grown as a suspension culture and maintained in RPMI-1640 media with 10% foetal bovine serum and 200 μg/mL of penicillin and streptomycin at a cell density of 2–9 $\times$ 10^5 cells/mL at 37°, 5% CO_2. The cells were split and the media were changed three times a week. Before each experiment, cell viability was checked using trypan blue exclusion.

Cell suspensions were harvested by centrifugation for 5 min at 4°, 300 *g*. Cells were then washed once with assay buffer (Hanks' balanced salt solution: 5 mM KCl, 0.5 mM KH_2PO_4, 0.13 M NaCl, 0.3 mM Na_2HPO_4, and 5 mM D-glucose without Ca^{2+} or Mg^{2+} plus 20 mM HEPES and 1 mM EDTA, pH 7.4) and resuspended in assay buffer at a concentration of 2 $\times$ 10^7 cells/mL before being assayed.

2.3. Binding assays

Binding assays were carried out on cell suspensions using a filtration assay with the aid of a Skatron cell harvester. [^{3}H](−)-Nicotine was used as the tritiated ligand and was stored at −20° in aliquots of ethanol. In each assay, 175 μL of cell suspension was added to the wells of 12-well microtubes containing 25 μL of a predetermined concentration of [^{3}H](−)-nicotine, 25 μL of assay buffer with or without 1 $\times$ 10^{-5} M unlabelled (−)-nicotine, and 25 μL of a given concentration of the competing ligand. Cell suspensions were incubated at 4° for 20 min (except when a time-course was being studied) before cells were collected by rapid filtration onto Whatman GF/C filters. Filters were presoaked with 0.1% (w/v) poly-L-lysine to reduce nicotine binding to the filter [32]. Filters were washed with 5 mL of ice-cold assay buffer and placed into scintillation with 4 mL of Liquiscint. Samples were then allowed to solubilise overnight before counting in a Packard 2500 liquid scintillation counter.

For occlusion of nicotine with epibatidine or α-bungarotoxin, the drugs were added to the stock solution of

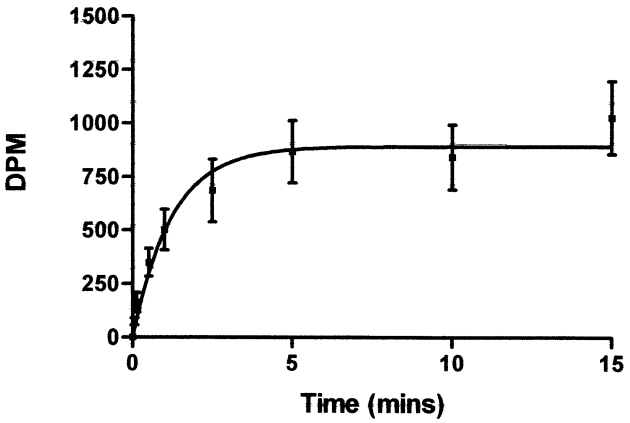

Fig. 1. A time-course for [^{3}H](−)-nicotine binding to THP-1 whole cells. The experiment was conducted at 4° and [^{3}H](−)-nicotine concentration was 20 nM. Experimental details are as stated in Methods and $T_{1/2}$ was found to be 0.52 ± 0.08. Each point represents the mean ± SEM of 4 triplicate determinations.

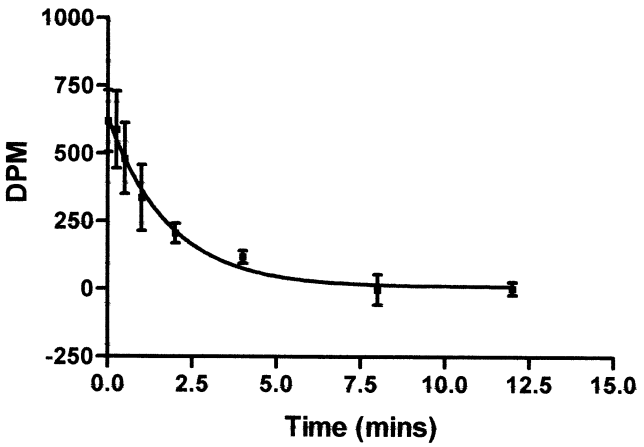

Fig. 2. A dissociation time-course for binding of [^{3}H](−)-nicotine (20 nM) bound to THP-1 cells. Dissociation was determined by the reduction of nicotine bound at different time points after addition of an excess of unlabelled nicotine (1×10^{-5} M). Data are the means ± SEM of 3 triplicate determinations. $T_{1/2}$ was calculated to be 1.14 ± 0.56 min.

unlabelled nicotine so that 25 μL of the solution would produce a final concentration of 1×10^{-6} M epibatidine or 1×10^{-5} M α-bungarotoxin and 1×10^{-5} M nicotine. Non-specific binding was determined in the presence of 1×10^{-5} M nicotine. Specific binding was the difference between total and non-specific counts.

2.4. Experimental analyses

The GraphPad Prism® program was used for data manipulations, graphical representations, and statistical analysis. All values including B_{max}, K_d, half-life, and K_i values were determined by this program. Unpaired Student's *t*-test was used to determine significance, and values are represented by means ± SEM of the number of experiments stated.

3. Results

3.1. Characterisation of nicotine binding to THP-1 cells

All the cell suspensions used in these experiments were < 95% viable as determined by trypan blue exclusion. [^{3}H](−)-Nicotine (20 nM) binding was performed at 4°, as preliminary binding studies on lymphocyte membranes showed that the ratio of total: non-specific binding was lowest at this temperature (ratio at 4° = 1.6:1, ratio at 37° = 1.2:1). Binding of nicotine at 4° displayed a half-time of association of 0.52 ± 0.08 min and reached maximum levels after 5 min (Fig. 1). This level of binding persisted up to 60 min, after which the specific binding declined rapidly (data not shown).

When an excess of unlabelled nicotine was added to cell suspensions that had been preincubated with 20 nM [^{3}H]nicotine long enough for equilibrium to be reached (20 min), specific binding of the radioligand decreased in a time-dependent fashion. The half-life of dissociation from the binding to THP-1 cells was 1.14 ± 0.56 min (Fig. 2). Both association and dissociation curves could only be fitted with a monoexponential curve.

3.2. Saturation analysis of [^{3}H](−)-nicotine

Saturation studies on whole THP-1 cells using concentrations of nicotine from 1×10^{-10} M to 4×10^{-8} M revealed the presence of two saturable binding sites (Fig. 3a). Non-linear regression analysis of nicotine binding produced a K_{d1} value of 3.5 ± 2.1 × 10^{-9} M and a B_{max1} of 4100 ± 560 sites/cell for the high-affinity site, while the lower-affinity binding site exhibited a K_{d2} of 27 ± 9.2 × 10^{-9} M and a B_{max2} of 11,600 ± 6,300 sites/cell. Scatchard analysis of the binding data also suggested two binding sites, as described by the curved appearance of the points on the graph (Fig. 3b).

3.3. Competition studies

A number of compounds were used in an attempt to investigate the nature of the binding sites for nicotine on THP-1 cells. The data presented in Table 1 summarise the K_i values from studies with (−)-nicotine, cytisine, epibatidine, hexamethonium, atropine, and carbachol. (−)-Nicotine displayed a biphasic competition curve showing two separate K_i values: 6.6 ± 4.3 × 10^{-10} M and 1.6 ± 1.5 × 10^{-7} M. However, all other drugs showed monophasic competition curves, and there was always some residual specific binding comprising approximately 20% of the total specific binding. Hill coefficients supported the hypothesis that all drugs except nicotine competed at one site (Table 1). Epibatidine displayed the highest affinity of the competing

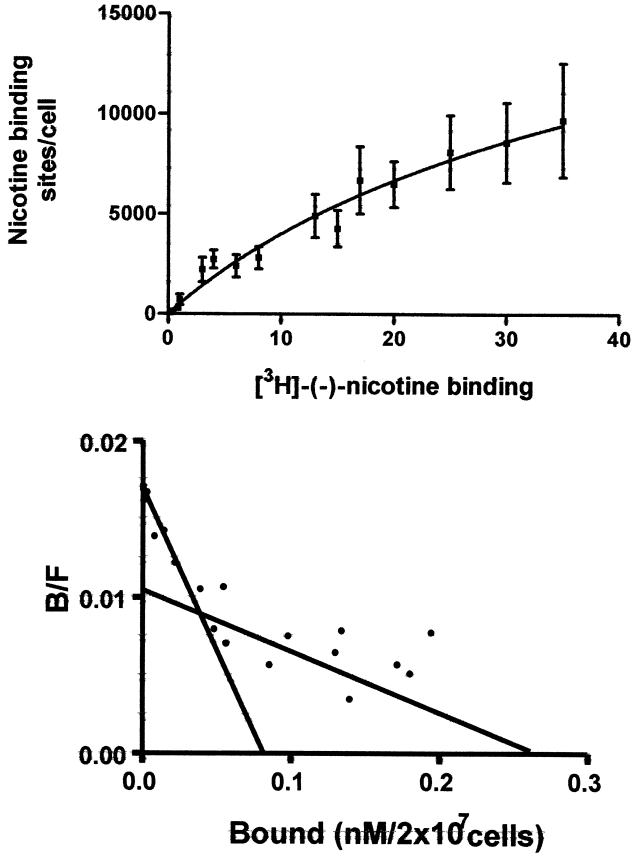

Fig. 3. (a) Saturation binding of [^{3}H](−)-nicotine to THP-1 whole cells. The graph displays the specific binding of nicotine to THP-1 cells as determined in the presence and absence of unlabelled nicotine. The results were obtained from a filtration assay conducted at 4°. Data represent the means ± SEM of 6 triplicate determinations. The B_{max} of the higher-affinity site was 4100 ± 560 sites/cell with a K_d of 3.5 ± 2.1 × 10^{-9} M. The low-affinity site displayed a B_{max} of 11,600 ± 630 sites/cell and a K_d of 27 ± 9.2 × 10^{-9} M. (b) Scatchard plot of [^{3}H](−)-nicotine binding to THP-1 whole cells. The graph depicts the presence of 2 binding sites as displayed by the 2 lines. Data display the means of 6 experiments.

ligands (K_i values shown in Table 1), and relative potencies for the competing ligands were epibatidine > cytisine > atropine > hexamethonium > carbachol (Fig. 4). Drugs that

Table 1
K_i values and Hill slopes for the different ligands used in the competition assay

Competing drug	K_i (M)	Hill slopes
Nicotine high affinity	6.4 ± 4.8 × 10^{-10}	−0.43 ± 0.15[a]
Nicotine low affinity	1.6 ± 1.5 × 10^{-7}	
Epibatidine	4.3 ± 2.5 × 10^{-9}	−1.03 ± 0.14
Cytisine	4.4 ± 1.8 × 10^{-8}	−1.07 ± 0.09
Atropine	5.6 ± 0.9 × 10^{-7}	−0.82 ± 0.27
Hexamethonium	3.3 ± 3.7 × 10^{-5}	−0.87 ± 0.19
Carbachol	1.9 ± 6.5 × 10^{-4}	−1.22 ± 0.36

All values were as calculated from computer analysis of the data (Prism) and are the means ± SEM of 3 triplicate determinations.

[a] Hill slope calculated for nicotine reflects binding at two sites.

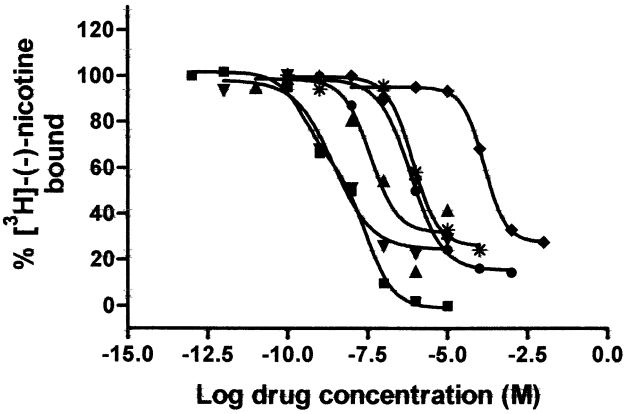

Fig. 4. Competition binding of [^{3}H](−)-nicotine to THP-1 cells by cholinergic drugs. Cells were incubated with 15 nM [^{3}H](−)-nicotine in the presence of a serial dilution of each drug for 20 min. Points are the means of at least 3 triplicate determinations. Error bars have been removed for clarity. ◆ carbachol, ▲ cytisine, ▼ epibatidine, ∗ hexamethonium, ● atropine, and ■ (−)-nicotine.

showed no competition for either site were α-bungarotoxin, dihydro-β-erythroidine, and DMPP (maximum concentration tested = 1 × 10^{-3} M).

3.4. Occlusion studies

To ascertain which site was being competed for in the competition studies, saturation experiments were performed in the presence of the drugs epibatidine and α-bungarotoxin. α-Bungarotoxin showed no affinity for either site (data not shown), whereas epibatidine (1 × 10^{-6} M) succeeded in blocking the lower-affinity site only. In the presence of epibatidine, the K_d for nicotine was 5.71 ± 3.3 × 10^{-9} M and the B_{max} was 4030 ± 800 sites/cell (Fig. 5). Neither of

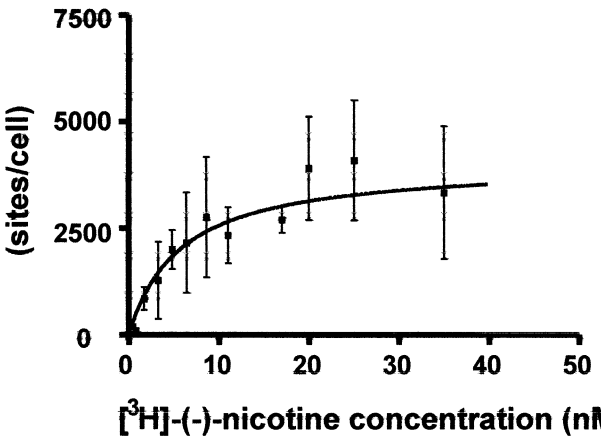

Fig. 5. Saturation binding of [^{3}H]nicotine in the presence of epibatidine as a displacing agent. The assay was conducted as previously with the addition of 1 × 10^{-6} M epibatidine in the reaction buffer. Experiments were conducted at 4° and incubated for 20 min. B_{max} was calculated as 4.03 ± 0.8 sites/cell and K_d was calculated as 5.71 ± 3.3 nM. Data are expressed as the means ± SEM of 4 triplicate determinations.

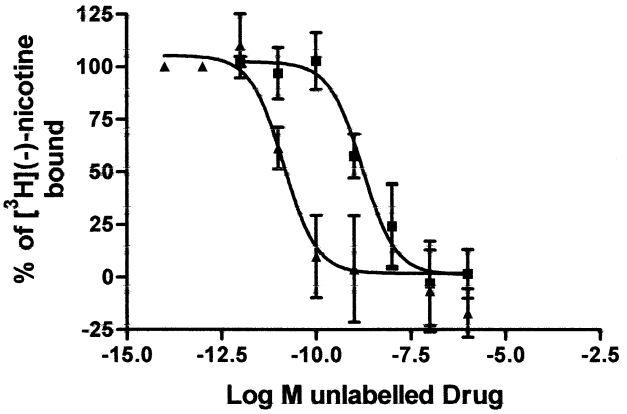

Fig. 6. Competition binding of [^{3}H](−)-nicotine for the enantiomers of nicotine in the presence of 1×10^{-6} M epibatidine. Cells were incubated with 6 nM labelled nicotine for 20 min, 4°. K_i values were $5.7 \pm 0.32 \times 10^{-11}$ M for (+)-nicotine and $1.9 \pm 4.9 \times 10^{-9}$ M for (−)-nicotine. Data are the means ± SEM of 3 triplicate determinations. ▲ (+)-nicotine, ■ (−)-nicotine.

these values was significantly different from those obtained in the initial studies on the high-affinity site in the absence of epibatidine ($P > 0.05$).

3.5. Competition studies in the presence of epibatidine

Competition analysis was performed in the presence of 1×10^{-6} M epibatidine to identify the ligands that would compete for the high-affinity site. The 3 compounds used were (+)-nicotine, (−)-nicotine, and the peptide Pro-Lys-Arg-NH_2. The high-affinity site displayed a higher affinity for the (+)-enantiomer than the (−)-nicotine enatiomer, with K_i values of $5.7 \pm 4.8 \times 10^{-11}$ M and $1.9 \pm 4.9 \times 10^{-9}$ M, respectively (Fig. 6). The peptide showed no affinity for the site up to a concentration of 1×10^{-3} M, the highest concentration tested.

4. Discussion

In this study, we have shown the presence of specific nicotine-binding sites on THP-1 whole cells. Association experiments showed a half-life of 0.52 ± 0.08 min and an equilibration time for binding of 5 min; this is faster than values previously published on granulocyte membranes, which reported equilibration times of an hour [31], but similar to those seen on granulocyte whole cell preparations that achieved maximal binding in 8 min [29]. The specific binding for the site remained at peak levels for only 60 min, after which the specific binding fell to approximately one-third of the maximum level. Conformational changes in nACh receptors are known to alter the affinities of ligands to binding sites, and this may be the reason for the reduction in specific binding over time [33]. Nicotine binding to THP-1 cells was found to be reversible, as specific binding was depleted fully over time when an excess of unlabelled nicotine was added (half-life of dissociation = 1.14 ± 0.56 min). The dissociation kinetics of binding is faster than those from nicotine-binding studies performed on human polymorphonuclear cells, human brain, and rat brain, which quoted half-life of dissociation of 5, 17, and 20 min, respectively [29,34,35].

The [^{3}H]nicotine-binding data presented show the presence of two binding sites with different binding characteristics. The saturation curve was analysed for a non-linear regression two-site model and resulted in the calculation of two separate K_d and B_{max} values. The curved appearance of the Scatchard plot supports this interpretation, since the data points obtained can be represented by two separate lines. K_d values for (−)-nicotine binding to the high- and low-affinity sites were 3.5 ± 2.1 nM and 27 ± 9.2 nM, respectively. These values are similar to those for nicotine-binding sites found in the brain and in the periphery, which have quoted K_d values for nicotine between 2 and 43 nM [29,31,36].

Competition binding experiments for [^{3}H](−)-nicotine resulted in a biphasic competition consisting of two K_i values, $6.6 \pm 4.3 \times 10^{-10}$ M and $1.6 \pm 1.5 \times 10^{-7}$ M. These two separate values are consistent with the data obtained from saturation experiments, which also described two independent binding sites specific for nicotine. Data obtained from competition studies with other ligands suggest that at least one of the sites may be cholinergic in nature. nACh receptor subunit mRNA have recently been found in human lymphocytes [10,25], but their presence has not previously been described in monocytes. Cytisine, epibatidine, hexamethonium, and carbachol all show affinities for nicotinic receptors [33,37], and their competition at at least one of these binding sites indicates a cholinergic nature. Cytisine and hexamethonium displayed K_i values similar to those previously reported in other nicotine-binding experiments [33]. Epibatidine is known to have greater affinities than nicotine for cholinergic sites [37,38], and in this study epibatidine had a 200-fold greater affinity than nicotine for the low-affinity site. This 200-fold greater affinity is comparable to the affinity epibatidine shows for binding sites in the brain, which have been recorded as being 100- to 1000-fold the value for nicotine affinity. In the present study, carbachol appeared to have a low affinity for the binding to THP-1 cells, but similar observations have also been seen previously in other agonist-binding studies [38]. The lack of affinity for DMPP and dihydro-β-erythroidine is unusual, as they have a high affinity for neuronal nicotine receptors [33]. A number of nicotinic cholinergic subunit configurations are known to exist [39], and the possibility of other more elaborate configurations and novel subunits cannot be excluded. As different configurations of nACh subunits are known to influence the pharmacological characteristics of nACh receptors and significantly alter affinities that ligands have for these receptors [5,13], it may be that these cells express subunit configurations different from those in the nervous system. Furthermore, cholinergic

receptors of unusual properties have been discovered in animal cells [6,40], and the possibility exists that receptors of this nature may be present in human tissues. The α9-subunit is known to display mixed muscarinic and nicotinic properties as defined by functional studies performed in rat cochlear hair cells, and a similar "mixed" binding site was found in guinea pig cochlear outer hair cells. Furthermore, Atweh *et al*. [41] reported the binding of [^{3}H]quinuclidinyl benzilate, a muscarinic antagonist, to murine lymphocytes that was blocked by the nicotinic antagonist curare. The unusual binding characteristics may be explained by the presence of a receptor containing or being similar in properties to the α9-subunit, as atropine displayed high affinity for one of the binding sites in this report ($K_i = 5.6 \pm 0.6 \times 10^{-7}$) compared to its affinity for other nicotinic binding sites, which have previously reported values in excess of 1×10^{-5} M [31,34].

In the present study, the identity of the binding site at which the drugs used in this study competed was demonstrated by performing a saturation curve in the presence of 1×10^{-6} M epibatidine. In this experiment, the presence of epibatidine blocked the lower-affinity site but did not significantly effect the higher-affinity site. Comparisons of the K_d and B_{max} values from saturation experiments in the absence or presence of epibatidine showed no significant difference, indicating that the higher-affinity site was indeed left unoccupied. The same experiment was performed in the presence of α-bungarotoxin, which failed to compete for either site so that the specific binding was the same as seen in the absence of the drug. Nicotinic cholinergic receptors that have high-affinity sites for α-bungarotoxin are known to contain only α1- and α7-receptors [42,43], and these subunits are thus not likely to make up the binding site demonstrated in this study. It can also be deduced that the low-affinity site resides on the cell surface, as hexamethonium competed for binding. Hexamethonium is a highly charged quaternary amine that does not readily pass through the membrane and therefore would have to compete at a site on the cell surface.

In contrast to the lower-affinity site, the higher-affinity site displayed a binding nature that was different from known cholinergic receptor patterns. The fact that none of the drugs studied, except nicotine, completely displaced the radiolabelled nicotine suggests that the nature of this site is unusual. Binding data displaying atypical characteristics have been published previously [29,30], but the site found on THP-1 cells appears to be different to the binding site found previously. In particular, the peptide Pro-Lys-Arg-NH_2 was shown to displace nicotine from a binding site on human granulocytes but did not compete for nicotine binding at the site on THP-1 cells, suggesting that sites on THP-1 cells have different characteristics to those on granulocytes. However, in agreement with the data published by Davies *et al*., the binding site was stereoselective for the (+)-nicotine enantiomer. This is further evidence that this site may be non-cholinergic in nature, since cholinergic sites are selective for the natural (−)-isomer [37]. The apparent 150-fold greater affinity of the (+)-enantiomer for the binding site is larger than the differences in affinity of the two isomers seen in other binding studies, which have quoted values from 30- to 60-fold greater affinity for the (+)-enantiomer. Although the difference obtained may be due to the characteristics of a novel binding site, part of this large difference may also be due to errors in the values as a result of high non-specific binding. An example of the errors encountered are the two different K_i values obtained for the high-affinity binding of (−)-nicotine in the absence and presence of epibatidine. The two values, while not significantly different from each other ($p > 0.05$), were numerically different, being $6.6 \pm 4.3 \times 10^{-10}$ M and $1.9 \pm 4.9 \times 10^{-9}$ M, respectively. In order to clarify this difference, the use of [^{3}H](+)-nicotine as a radioligand would be beneficial as it is more potent than the (−)-enantiomer, but it is unavailable. Furthermore, altering the isomer would not prevent the large degree of non-specific binding seen with nicotine as the radioligand.

The use of membrane preparations of cells is a common method for reducing non-specific binding in radioligand-binding studies in the CNS, and this method was attempted by our laboratory. However, preliminary experiments with membrane preparations showed no improvement in the level of non-specific binding of nicotine when performed on lymphocytes (data not shown), following a previously cited method [31]. The most obvious method for reducing non-specific binding is to use a different ligand, but the lack of competing ligands does not allow this. No functional studies on either of these binding sites were performed, making the function of these binding sites unknown; the possibility of the site being an enzyme or a different conformation/allosteric site of the same receptor exists. Nicotine interacts directly with thromboxane synthase, and it is possible that the binding site may be an enzyme [44]. Preliminary studies conducted in our laboratory have indicated that nicotine inhibits TNFα release from THP-1 cells at low doses. At 1×10^{-11} M, nicotine caused a 50% reduction in the release of TNFα, and as yet no antagonist for this effect has been found. These data could indicate a possible function for the higher-affinity binding site in THP-1 cells.

In summary, we have identified two nicotine-binding sites in THP-1 whole cells. One site appears to be cholinergic, whereas the second higher-affinity site displays characteristics distinct from typical cholinergic receptors. To unequivocally determine the presence of nACh receptors in THP-1 cells, more work is needed using molecular techniques such as reverse transcription–polymerase chain reaction or immunostaining. The identity of the higher-affinity site remains unknown and requires the discovery of other competing ligands to reveal more evidence of its nature. The presence of nicotine-binding sites on THP-1 cells suggests a possible mechanism for the immunomodulatory effect of nicotine.

References

[1] Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, Wells G. Structure and function of neuronal nicotinic acetylcholine receptors. Prog Brain Res 1996;109:125–37.

[2] Gotti C, Fornsari D, Clementi F. Human neuronal nicotinic receptors. Prog Neurobiol 1997;53:199–237.

[3] Arias HR. Topology of ligand binding sites on nicotinic acetylcholine receptors. Brain Res Brain Res Rev **25:** 133–191, 1997.

[4] Karlin A, Akabas MH. Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. Neuron 1995;15: 1231–44.

[5] Sargent PB. The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 1993;16:403–443.

[6] Elgoyhen A, Johnson D, Boulter J, Vetter D, Heinemann S. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 1994;79:705–715.

[7] Anand R, Conroy WC, Schoepfer R, Whiting P, Lindstrom J. Neuronal nicotinic receptors expressed in *Xenopus oocytes* have a pentameric quaternary structure. J Biol Chem 1991;266:11192–98.

[8] Cooper E, Courtier S, Ballivet M. Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. Nature 1991;350:235–8.

[9] Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 1998;77:59–79.

[10] Toyabe S, Iiai T, Fukuda M, Kawamura T, Suzuki S, Uchiyama M, Abo T. Identification of nicotinic acetylcholine receptors on lymphocytes in the periphery as well as thymus in mice. Immunology 1997;92:201–205.

[11] Grando S, Horton R, Mauro T, Kist D, Lee T, Dahl M. Activation of keratinocyte nicotinic cholinergic receptors stimulates calcium influx and enhances cell differentiation. J Invest Dermatol 1996;107:412–418.

[12] Chavez-Noriega LE, Crona J, Washburn M, Urrutia A, Elliott K, Johnson E. Pharmacological characterisation of recombinant human neuronal nicotinic acetylcholine receptors hα2β2, hα2β4, hα3β4, hα4β4 and α7 expressed in Xenopus oocytes. J Pharmacol Exp Ther 1997;280:346–356.

[13] Luetje CW, Patrick J. Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. J Neurosci 1991;11:837–845.

[14] Peng X, Katz M, Gerzanich V, Anand R, Lindstrom J. Human acetylcholine receptor: cloning of the α7 subunit from the SH-SY5Y cell line and determination of pharmacological properties native receptors and functional alpha 7 homomers expressed in *Xenopus oocytes.* Mol Pharmacol 1994;45:546–554.

[15] Gerzanich V, Anand R, Lindstrom J. Homomers of alpha 8 and alpha 7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties Mol Pharmacol 1994;45:212–220.

[16] Keyser K, Britto L, Schoepfer R, Whiting P, Cooper J, Conroy W, Brozozowska-Prechtl A, Karten A, Lindstrom J. Three types of alpha bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. J Neurosci 1993;13:442–454.

[17] Aoshiba K, Nagai A, Yasui S, Konno K. Nicotine prolongs neutrophil survival by suppressing apoptosis. J Lab Clin Med 1996;127: 186–194.

[18] Jay M, Kojima JM, Gillespie MN. Nicotine potentiates superoxide generation by human neutrophils. Toxicol Appl Pharmacol 1996;86: 484–487.

[19] Totti N, McCusker KT, Campell EJ, Griffin GL, Senior RM. Nicotine is chemotactic for neutrophils and enhances neutrophil responsiveness to chemotactic peptides. Science 1984;223:69–171.

[20] Fischer A, Konig W. Modulation of *in-vitro* immunoglobulin synthesis of human peripheral blood mononuclear cells by nicotine and cotinine. Clin Invest 1994;72:225–32.

[21] Sopori ML, Kozak W, Savage SM, Geng Y, Soszynski D, Kluger MJ, Perryman EK, Snow GE. Effect of nicotine on the immune system: possible regulation of immune responses by central and peripheral mechanisms. Psychoneuroendocrinology 1998;23:189–204.

[22] Sopori ML, Kozak W, Savage SM, Geng Y, Kluger M. Nicotine-induced modulation of T-cell function. Adv Exp Med Biol 1998;437: 279–289.

[23] Madretsma S, Wolters LM, van Dijk JP, Tak CJ, Feyerabend C, Wilson JH, Zijlstra FJ. *In-vivo* effect of nicotine on cytokine production by human non-adherent human mononuclear cells. Eur J Gastroenterol Hepatol 1996;8:1017–1020.

[24] Sykes AP, Brampton C, Klee S, Chander CL, Whelan C, Parsons ME. An investigation into the effects and mechanisms of action of nicotine in inflammatory bowel disease. Inflamm Res 2000;49:311–319.

[25] Sato K, Fujii T, Watanabe Y, Yamada S, Ando T, Kazuko F, Kawashima K. Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukaemic cells. Neurosci Lett 1999;266:17–20.

[26] Richman DP, Arnason BG. Nicotinic acetylcholine receptor: evidence for a functionally distinct receptor on human lymphocytes. Proc Natl Acad Sci USA 1979;76:4632–5.

[27] Richman DP, Antel JP, Burns JB, Arnason BG. Nicotinic acetylcholine receptor on human lymphocytes. Ann N Y Acad Sci 1981;377: 427–435.

[28] Zijlstra FJ. Smoking and nicotine in inflammatory bowel disease: good or bad for cytokines. Mediators Inflamm 1998;7:153–155.

[29] Davies BD, Hoss W, Lin J-P, Lionetti F. Evidence for a noncholinergic nicotine receptor on human phagocytic leukocytes. Mol Cell Biochem 1982;44:23–31.

[30] Hoss W, Lin JP, Matchett S, Davies BD. Characterisation of noncholinergic nicotine receptors on human granulocytes. Biochem Pharmacol 1986;35:2367–72.

[31] Lebargy F, Benhammou K, Morin D, Zini R, Urien S, Bree F, Bignon J, Branellec A, Largue G. Tobacco smoking induces expression of very-high-affinity nicotine binding sites on blood polymorphonuclear cells. Am J Resp Crit Care Med 1996;153:1056–63.

[32] Romano C, Goldstein A. Stereospecific nicotine receptors in rat brain membranes. Science 1980;210:647–50.

[33] Lena C, Changeux JP. Allosteric modulations of the nicotinic acetylcholine receptor. Trends Neurosci 1993;16:181–186.

[34] Shimohama S, Taniguchi T, Fujiwara M, Kameyama M. Biochemical characterisation of the nicotinic cholinergic receptors in human brain: binding of (−)-[^{3}H]-nicotine. J Neurochem 1985;47:604–610.

[35] Lippiello PM, Fernandes, KG. The binding of L-[^{3}H]nicotine to a single class of high affinity sites in rat brain membranes. Mol Pharmacol 1986;29:448–454.

[36] Wonnacott S. Brain nicotine binding sites. Hum Toxicol 1987;6:343–353.

[37] Gerzanich V, Peng X, Wang F, Wells G, Anand R, Fletcher S, Lindstrom J. Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. Mol Pharmacol 1995;48:774–782.

[38] Houghtling RA, Davila-Garcia MI, Kellar KJ. Characterization of (±)-[^{3}H]epibatidine binding to nicotinic cholinergic receptors in rat and human brain. Mol Pharmacol 1995;48:280–287.

[39] Lukas RJ, Changuex JP, Novere NL, Alberquerque EX, Balfour DJ, Berg DK, Bertrand D, Chiappinelli VA, Clarke PB, Collins AC, Dani JA, Grady SR, Kellar KJ, Lindstrom JM, Marks MJ, Quik M, Taylor PW, Wonnacott S. International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol Rev 1999;51:397–401.

[40] Lawoko G, Jarlebark L, Heilbronn E. Ligand-binding properties of an unusual nicotinic acetylcholine receptor subtype found on isolated outer hair cells from guinea pig cochlea. Neurosci Lett 1995;195: 64–68.

[41] Atweh SF, Grayhack JJ, Richman DP. A cholinergic receptor site on murine lymphocytes with novel binding characteristics. Life Sci 1984;35:2459–2469.

[42] Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 1990;5:847–856.

[43] Schopfer R, Conroy WG, Whiting P, Gore M, Lindstrom J. Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. Neuron 1990;5:35–48.

[44] Goerig M, Ullrich V, Schettler G, Foltis C, Habenicht A. A new role for nicotine: selective inhibition of thromboxane formation by direct interaction with thromboxane synthase in human promyelocytic leukaemia cells differentiating into macrophages. Clin Investig 1992;70:239–43.