Characterisation of nicotine receptors on human peripheral blood mononuclear cells (PBMC)

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Running title nAChR on human PBMC

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Abstract

Aim and objective

The aim of the work was to characterise the nAChRs on human PBMC.

Method

PBMC were isolated from human blood buffy coats provided by the blood transfusion service and were used for radioligand binding studies with [^{3}H]-nicotine. RT-PCR experiments were used to determine nAChR subunit expression while immunoblotting experiments were used to confirm that nAChR subunits identified by RT-PCR were translated into protein.

Results

Binding studies suggested the presence of one binding site for (-)- nicotine on human peripheral blood lymphocytes. Competition studies showed that only (-)-nicotine, epibatidine and α-bungarotoxin, displaced radiolabelled nicotine from cells. RT-PCR studies demonstrated mRNA for α4, α5, α7, β1and β2 nAChRs subunits in PBMC. Expression of α5 mRNA subunit of nAChR was observed in all lymphocyte samples tested. In contrast, the expression pattern of mRNAs for α4, α7, β1, and β2 mRNAs subunits of nAChRs, varied between samples. Western blot analysis showed that protein for α4, α5, and α7 and β2 nAChR subunits was expressed in most, but not all of the PBMC samples tested but some of the bands obtained were faint.

Conclusion

The results obtained suggest that human PBMC contain nAChRs containing α4β2, α4β2α5, and/or α7 subunits.

Introduction

We, and others, have previously shown that nicotine inhibits TNBS-induced colitis in rats [1, 2]. We have also shown that nicotine, at concentrations likely to be found in the blood of tobacco users [3] suppresses TNF release from a monocytic cell line (THP-1 cells) [2]. In addition, nicotine has a small inhibitory effect on TNF-induced IL-8 release from an epithelial cell line (HT-29) [4]. Furthermore, the inhibitory effect of nicotine on IL-8 release appeared to be mediated by α7 homomeric nicotinic acetylcholine receptors (nAChR), in that the inhibitory effect of nicotine on HT-29 cells was blocked by an antagonist of the α7 nAChR, α-bungarotoxin. In addition, RT-PCR and western blotting experiments demonstrated the presence of mRNA and protein for this receptor in HT-29 cells [4].

A criticism of the above work is that many of the *in vitro* studies were conducted using artificial cell lines and the data may not be relevant to man. Lymphocytes are key cells in inflammatory responses and modulation of their function by nAChR agonists, including nicotine derived from the use of tobacco, represents a potential target for therapeutic intervention as well as an explanation for tobacco-induced disorders. Published work has shown that nicotine and nAChR ligands suppress lymphocyte proliferation [5], modulate cytokine release [6, 7] or increase T cell-mediated cytotoxicity [8]. However, the identity of the receptor(s) mediating these effects is not clear with some studies claiming they are mediated by non-cholinergic nicotine receptors [9] while others provide evidence for a nAChR mediated effect [10]. Furthermore, there is no consensus as to the composition of the nAChR on human lymphocytes with some studies demonstrating expression of nAChR subunits that were not identified by others [11, 12,13]. Collectively, this work suggests the presence of functional receptors for nicotine on PBMC and these observations have led to the proposal that the α7 nAChR is a potential therapeutic target [14,15]. However, the therapeutic potential of ligands for the α7 nAChR depends on the contribution made by other nAChR receptor subtypes to the effects of nicotine.

One explanation for these findings may be that a genetic variation leads to variability in nAChR expression on human PBMC. Alternatively, different findings may result from the different endpoints measured in different studies. In view of the significance of these data to the

therapeutic potential of nAChR, we decided to characterise nAChR on human PBMC using a combination of radioligand binding, RT-PCR and western blotting in an attempt to resolve these discrepancies.

Method

Materials

Buffy coat cells were obtained from the National Blood Transfusion Service (NBTS; Colindale, UK). These cells were a by-product of blood processed for clinical use and no details as to tobacco use or ethnicity were provided. [^{3}H]-nicotine was purchased from NEN Life Sciences, (Zaventum, Belgium). RNA STAT-60^{TM} kit for RNA extraction was purchased from Biogenesis, (Poole, UK), reagents for RT-PCR were purchased as a kit (Access RT-PCR) from Promega (Southampton, UK). Primers for RT-PCR were obtained from Invitrogen Life Technologies (Paisley, UK) and brain RNA from Origene Technologies Inc., (Cambridge, UK). Antibodies for the α4, α5 and β2 nAChR subunits were purchased from Covance Research Products (Berkley, CA.,USA) and the antibody for the α7 nAChR subunit from Research Diagnostics Inc. (Flanders, NJ., USA). Histopaque 1077, Aprotinin, Leupeptin and PBS-tween 20 were purchased from Sigma-Aldrich (Poole, UK).

Isolation of PBMC

On receipt, buffy coats were diluted with phosphate buffered saline (PBS) to a total leukocyte count of 1 x 10^7 cells/ml. Aliquots (25ml) of this suspension were layered over an equal volume of histopaque 1077. Gradients were subjected to centrifugation (1000 x g for 25 min at room temperature) and PBMC were recovered from the PBS/histopaque interface. Finally, PBMC were washed with PBS and resuspended in Hanks balanced salt solution (5mM KCl; 0.5mM KH_2PO_4; 0.13 mM NaCl; 0.3 mM Na_2PO_4; 5mM glucose; 20mM HEPES and 1mM EDTA) at a density of 1 x 10^7 cells/ml.

Radioligand binding studies

Radioligand binding studies were performed on intact PBMC as described previously for THP-1 cells [16]. Briefly, for saturation studies PBMC were incubated with [^{3}H]-nicotine (0.9 x 10^{-9} M to 4 x 10^{-8} M) at 4°C for 50 minutes. For competition experiments, a single concentration of [^{3}H]-nicotine (1.5 x 10^{-8} M) was used. In experiments where association and dissociation times were determined, a concentration of 2.0 x 10^{-8} M [^{3}H]-nicotine was used and the incubation time varied between 1 and 120 minutes. Non-specific binding was determined by the addition of an excess of unlabelled nicotine (1 x 10^{-5} M). Incubations were terminated by filtration of the cells

in a cell harvester (Skatron) followed by washing with 5ml ice cold HBSS. The [^{3}H]-nicotine content of each filter was determined by liquid scintillation counting.

Isolation of total RNA and RT-PCR

Total RNA was extracted from PBMC using a single-step protocol (RNA STAT-60^{TM}). RNA was extracted from PBMC homogenates by the addition of 20% v/v chloroform followed by centrifugation at 12000 x g for 15 min at 4°C. The colourless upper aqueous phase was harvested and mixed with 0.5 volume of isopropanol and the RNA precipitate collected by centrifugation (12000 x g, 1 hr, 4°C). After washing twice with cold ethanol, by centrifugation at 7500 x g for 45 mins at, 4°C, the RNA was subjected to RT-PCR. RNA concentration and quality were assessed based on 260 nm/280 nm absorbance ratios.

RT-PCR experiments were conducted using a commercially available kit. Human PBMC RNA was used as an experimental template and human brain RNA was used as a positive control. Primer pairs for nAChR subunits (α1-α7, α10, β1 and β4) were designed as described previously [4] and were obtained commercially.

Each primer (final concentration 1µM) was mixed with total RNA (final concentration 1µg) and subjected to RT-PCR. A protocol where, for the first strand cDNA synthesis, 1 cycle at 48ºC for 45 minutes (reverse transcription), and 1 cycle at 94ºC for 2 minutes (AMV RT-inactivation, RNA/cDNA/primer denaturation) was used. For the second strand synthesis and PCR amplification, a further 35 cycles at 94ºC for 30 seconds (denaturation), 55º C for 1 minute (annealing), and an extension step at 68º C for 2 minutes. The final extension was for 1 cycle at 68º C for 7 minutes, and the last 1 cycle at 4º C (soak).

Following RT-PCR, aliquots of the reaction mixture were subjected to electrophoresis on agarose (1.5%) at a constant voltage (50V) for 3h in a TAE buffer (Tris base 0.04M; 0.0.001M EDTA; pH 8.0 with glacial acetic acid). The size of PCR products was estimated by comparison of bands with DNA molecular weight markers (New England, BioLabs Inc., Hitchin, UK) used as a reference in each gel.

Immunoblotting

Human PBMC (1 x 10^7 cells) were suspended in 0.5 ml of cell lysis buffer (125 mM Tris HCl, pH 6.8; 2% w/v sodium dodecyl sulphate; 10% v/v glycerol) containing aprotinin (60μg/ml; Sigma) and leupeptin (10 μg/ml; Sigma) as described by Laemmli [17]. Cells were incubated on ice for 5 mins followed by incubation at 95°C for 5-10 mins to reduce the viscosity of the lysate. Following centrifugation at 14000 x g, for 10 min at room temperature, the lysate (supernatant) was recovered and the protein concentration of determined.

Rat brain protein was used as a positive control and was prepared according to a protocol provided by Pharmingen Ltd., (B.D.Biosciences UK, 2003). Briefly, brains were homogenised in 50mM Tris/HCl buffer (pH 7.4) containing 10% w/v sucrose, 5mM EDTA, leupeptin (10 μg/ml) and aprotinin (60 μg/ml). Following centrifugation (800 x g, 4°C, 20 min), pellets were resuspended in sucrose (320 mM) and rehomogenised. The resulting homogenate was centrifuged (16000 x g, 4°C, 30 min). This process was repeated, the pellet suspended in Tris/ acetate buffer (pH 7.4) and the protein concentration determined.

Protein samples were subjected to SDS-PAGE electrophoresis in a discontinuous Tris/glycine buffer as described by Laemmli [17]. Stacking and resolving gels contained 5 and 10% SDS-PAGE respectively. Protein samples (20μg/well) were added and gels subjected to electrophoresis at 200V for 30-40 min at room temperature. Following electrophoresis, gels were either stained with Coomassie brilliant blue to identify protein bands or transferred to a blotting buffer sandwich for transfer to nitrocellulose membranes and immunoblotting. Electrophoretic transfer of protein from acrylamide gels to nitrocellulose membranes was performed as described by Tobin *et al*., [18].

Following transfer of proteins to nitrocellulose membranes, membranes were blocked by immersion for 2h at room temperature in 5% w/v semi-skimmed milk (Marvell, Cadbury, UK) in PBS containing 0.05% Tween 20 (Sigma UK). Membranes were then incubated with monoclonal antibodies for nAChR subunits overnight at 4°C. The final dilution of monoclonal antibodies used was 1:1000 for anti-α4 nAChR antibody and 1:500 for anti-α5, anti-α7 and anti-

β2 nAChR antibodies. Following washing with PBS-tween 20 (4 x 5 min), membranes were incubated with goat anti-rat IgG horseradish peroxidase (dilution 1:5,000) (for experiments with α4, α5 and β2 nAChR subunits) or goat anti-mouse IgG horseradish peroxidase (for experiments with α7 nAChR subunits) (dilution 1:5,000) for 2h. Immunoreactivity was detected using the enhanced chemiluminescence (ELC) system (Amersham Life Sciences, UK).

Statistical analysis

Data obtained from radioligand binding studies were analysed using commercially available software (Prism, Graphpad, San Diego, USA). Data were analysed using a non-linear curve fitting procedure and all values were derived from this analysis. Values quoted represent the mean ± s.e.mean of the number of observations stated. K*d* and IC_{50} values were determined from each individual experiment and the values stated represent the mean and standard error of the number of replicate experiments conducted.

Results

Isolation of PBMC

The number of lymphocytes recovered from a sample of blood differed from donor to donor and was dependent, to an extent, on the initial volume of the buffy coat provided. However, usually 30 x 10^7 cells were recovered from a single buffy coat giving 30 ml of cell suspension when suspended at a concentration of 1 x 10^7 cells/ml. All lymphocyte cell suspensions used in these experiments had viability >95 % as determined by trypan blue exclusion tests. The lymphocyte purity was >99% as determined from staining cytospin preparations followed by a differential count under oil immersion.

Radioligand binding

Preliminary experiments showed that [^{3}H]-(-)-nicotine bound to PBMC at 4˚C. The half time of association was 12.02 ± 5.15 minutes and binding reached equilibrium after 25 minutes. However, considerable intersubject variation was experienced and specific binding of [^{3}H]-(-)-nicotine was usually low and was <30% of total binding in most experiments.

[^{3}H]-(-)-nicotine (0.9 x 10^{-9}M - 4.0 x 10^{-8}M) bound to PBMC in a concentration-related manner and was saturable (Figure 1). From these data the presence of a single binding site for nicotine was identified on human lymphocytes with a K*d* of 15.00 ± 5.76 nM (1.5 ± 0.58 x 10^{-8}M) and a B*max* of 2253 ± 409.00 sites/cell.

In competition binding experiments using a single concentration of [^{3}H]-(-)-nicotine (1.5 x 10^{-8}M), a number of compounds such as (-) - nicotine, α-bungarotoxin, cytisine, d-tubocurarine, epibatidine, hexamethonium, atropine, and carbachol were used to investigate the nature of nicotine binding sites on human lymphocytes. Of these, only nicotine, epibatidine (IC_{50} = 10.48 ± 2.14 nM, n=5) and α-bungarotoxin (IC_{50} = 13.30 ± 2.83 nM, n=5) competed with [^{3}H]-(-)-nicotine for the binding site on PBMC (Figure 2) while cytosine, d-tubocurarine, hexamethonium, atropine and carbachol did not consistently displace [^{3}H]-(-)-nicotine in a concentration-related manner (data not shown).

RT-PCR

Following electrophoresis and ethidium bromide staining, in each experiment a PCR product was observed with a molecular size that corresponded to the predicted size of the PCR product if nAChR subunit mRNA was present in the human PBMC. The predicted size of these products was 190 bp for α1, 349 bp for α2, 474 bp for α3, 387 bp for α4, 556 bp for α5, 356 bp for α6, 451 bp for α7, 589 bp for β1, 309 bp for β2, 418 for β3, and 220 bp for β4. All human PBMC tested expressed mRNA for α4, α5, and β1, nAChR. No mRNA for α1, α2, α3, α6, α10, β3 or β4 nAChR subunits was detected in any of the samples studied (Table 2). The expression of mRNA for α7 (present in 8 out of 10), and β2 (5 out of 10), nAChR subunits was not seen in all samples (Table 1). The presence or absence of mRNA for these subunits did not appear to correlate with blood group (Table 1).

Immunoblotting

When human PBMC protein was subjected to SDS-PAGE electrophoresis and stained with Coomassie Brilliant Blue, a range of protein bands were detected with molecular sizes from 10 to 250 kD. Rat brain protein (the positive control) also showed the same range of protein bands as human lymphocytes.

However, not all PBMC samples tested appeared to express the same nAChR subunits. Human PBMC expressed proteins with immunoreactivity suggesting the presence of α4 (70 kD), α5 (53 kD), α7 (56 kD), and β2 (57 kD) nAChR subunit protein (Table 2). Protein bands for α7 nAChR protein subunits were detected with a molecular size of 56 kD in all samples tested. However, the bands varied in intensity from subject to subject (Figure 3) although, in many of these experiments, the band for the positive control (rat brain protein) was also less distinct than that obtained for other nAChR subunits.

Expression of β2 nAChR protein subunits was detected in 6 out of the 12 samples tested but the observed bands were faint. (Table 2).

Discussion

The aim of the present study was to extend previous findings from our laboratory, and those published by others [10, 11, 12, 13], by characterising the nAChR on human PBMC. In attempting to do so we found that there were relatively few nAChR on these cells, as suggested by low specific binding of radiolabelled nicotine, and that the nature of this binding was subject to intersubject variation, a finding that was not explicitly suggested by some previously published work[12, 19]. Since further purification of cells into lymphocyte subsets would have reduced the number of cells obtained to a level where radioligand binding studies would have been impractical. Thus, these studies were performed on cells that were a mixture of lymphocyte subsets (PBMC). In order to be consistent, RT-PCR and immunoblotting experiments were also performed on cells purified to the same extent.

Initial studies utilised radioligand binding in order to identify the number of binding sites for nicotine on PBMC. Preliminary experiments revealed that non-specific binding of [^{3}H]-nicotine comprised a high percentage of the total binding and that this was reduced if whole cells were used rather than using cell membrane preparations. This high degree of non-specific binding has been reported by others [16, 20, 21] and may reflect the lipophilic nature of nicotine. In spite of the high degree of non-specific binding the data obtained from kinetic experiments indicate that nicotine interacts with a single binding site. Furthermore, binding of nicotine to PBMC occurs at concentrations of nicotine likely to be encountered in the blood of tobacco users [3] suggesting that this binding may be clinically relevant.

Competition studies showed that binding of [^{3}H]-nicotine was substantially reversed by epibatidine and α-bungarotoxin suggesting that the binding site for [^{3}H]-nicotine was a nAChR, eliminating the possibility that PBMC used in the samples studied contained a non-cholinergic nicotine receptor as reported by others [9]. α-bungarotoxin is a ligand which binds preferentially to the α7-homomeric nAChR [22, 23]. Epibatidine also has an affinity for nAChR containing α4, and α7 subunits [24, 25]. Thus, it is possible that human PBMC contain a mixture of these two nAChR subtypes although the low specific binding and intersubject variabilty did not enable a definitive identification to be made. Others have also studied the nicotine receptors on human

PBMC [20, 21, 26, 27] although no consensus is apparent. One group found a number of nicotine binding sites in PBMC but the [^{3}H] nicotine-binding site was not saturable [20]. Many groups have reported data suggesting the presence of the α7 homomeric nAChR [28, 29]. It has also been reported that d-tubocurarine (a nAChR antagonist) inhibited radiolabeled ACh binding by lymphocytes suggesting that the receptor present on PBMC contained α7 nAChR subunits [30]. These findings conflict with those obtained in the present study although the relatively poor affinity of d-tubocurarine for the α7nAChR [31], coupled with the high degree of non-specific binding experienced in the present study, may have rendered competition difficult to detect. Furthermore, others, [12, 32] identified α4 nAChR subunits in human circulating lymphocytes from healthy volunteers, a finding confirmed by *in situ* hybridisation in one study [32]. However in a study of lymphocytes from 7 subjects, Sato *et al* [11] did not find expression of this nAChR subunit. Many of these earlier studies did not report the degree of intersubject variability seen in the present study with regard to expression of α nAChR subunits but one group [11, 13] did find intersubject variation in β2 nAChR subunit expression.

The source of this intersubject variability is of importance in order to understand the therapeutic potential of selective nAChR agonists. Such variability may result from induction of nAChR subunit expression in smokers compared to non-smokers as has been noted in leukocytes [18, 33]. Alternatively, the variability may be genetic in origin and linked to some other factor. Clearly it would be preferable to conduct experiments on PBMC taken from subjects whose background was known, particularly with regard to tobacco exposure but the source of PBMC used, and the volume of blood required for radioligand binding studies, precluded this in the present study. However, the blood cells used in the present study were provided by NBTS whose primary purpose is to provide blood products for clinical use and the smoking status of donors is not gathered at the time of donation.

In an attempt to further characterise the nAChR present on PBMC, and to understand the intersubject variability observed, we used molecular biology to determine nAChR subunit expression at the gene and protein level. Thus, RNA from PBMC was analysed to see if a wide range of nAChR subunits were expressed and membrane protein examined by immunoblotting to see which of the nAChR subunits, identified by ligand binding and RT-PCR were present in the

cell membrane. In the present study, of the 10 subjects, all expressed mRNA for the β1 nAChR and half the subjects also expressed mRNA for β2 nAChR. However, expression of the β2 nAChR subunit appeared to be low since only faint bands were seen in some, but not all, samples examined by Western blotting, a finding described previously by others [12]. These findings differ from those published by some groups in which lymphocytes also expressed mRNA for the α2 nAChR subunit but did not express the β1 subunit [11]. In contrast, work published by others failed to find mRNA for the α2, β1 or the β2 nAChR subunit [34, 35] although Benhammou *et al.* reported expression of mRNA and protein for α4 and β2 nAChR in human blood leukocytes[12].

There are many possible explanations for these observations and further work may be necessary to determine the reason for these differences. Because the presence of nAChRs containing β1 subunits was not suggested by the radioligand binding data, and no mRNA for the α1 nAChR was detected, we elected not to immunoblot gels for this subunit since this subunit was unlikely to form a functional receptor. The majority of subjects expressed mRNA and protein for α7 nAChR subunits suggesting the presence of the α7 homomeric nAChR on human PBMC, a finding that is supported by the radioligand binding data and confirms previously published work [28]. In addition, mRNA for α4 and α5 nAChR was detected by RT-PCR in most of the subjects studied and also appears to result in expression of subunit protein. Thus, the most likely explanation for our findings is that PBMC from most subjects express α7 nAChR but that many subjects also express other receptors such as the α4β2 and α4α5β2 nAChR. Although this work was based on a limited number of blood samples the variation in nAChR seen may correlate with blood group, suggesting a genetic basis for the intersubject variation seen. However, further studies with blood samples taken from a greater number of donors, with a known history is required to confirm this hypothesis.

The findings of the present study are consistent with those of others who have used molecular biology to study nAChR on PBMC [11, 12, 28, 29, 32] although few reports have mentioned the intersubject variability observed in the present study [11, 28], possibly because cells were harvested from a relatively small number of subjects. In studies where samples from a limited

number of subjects was used the investigators may have inadvertently selected samples that were similar in composition and responded similarly to nicotine [19]. In contrast, in other studies [11, 28,] a more diverse group of subjects may have been selected and the intersubject variation was apparent.

It has been proposed that acetylcholine receptors on leukocytes have a physiological role [13]. Lymphocytes, and other cells, are able to synthesise acetylcholine [13] which is released and can modify T and B cell activity [36]. While it is possible that such processes may modulate lymphocyte activity in situations where cells are in close proximity to each other, it is also possible that nicotine and nAChR ligands may modulate lymphocyte activity. Thus, nAChR ligands may have both therapeutic potential as well as the potential to promote pathologies that result from immune cell malfunction.

In conclusion, the experiments described above show that human PBMC, taken from a wide range of subjects, possess nicotine receptors that bind nicotine in a manner consistent with the presence of a nAChR. Furthermore, this receptor also binds epibatidine and α-bungarotoxin suggesting the presence of $\alpha 7$ homomeric nAChR or/and $\alpha 4\beta 2$ nAChR. However, RT-PCR and immunoblotting experiments using PBMC from a number of subjects revealed a degree of intersubject variation that may impact on the therapeutic utility of nAChR ligands.

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Figure 1. Saturation binding of [^{3}H]-(-)-nicotine to human lymphocytes. The graph shows specific binding of [^{3}H]-(-)-nicotine to human lymphocytes. The results were obtained from a filtration assay conducted at 4 ºC for 50 minutes. Data represented are the mean ± SEM of 5 experiments and each experiment was performed in triplicate. The B*max* of binding site was 2253 ± 409 sites/cells and K*d* was 15.00 ± 5.76 nM (1.5 ± 0.58 x 10^{-8}M).

Figure 2. Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by A unlabeled-(-)-Nicotine, B epibatidine and C α-bungarotoxin. Cells were incubated with 15 nM (1.50 x 10^{-8}M) [^{3}H]-(-)-nicotine in the presence of a serial dilution of (-)-nicotine for 35 minutes, at 4ºC. Each chart is a single experiment in graphical form. Each point is the mean of a single concentration of drug performed in triplicate.

Figure 3. Western blot analysis of 12 samples of human lymphocyte protein was screened for the presence of nAChR α7 subunit protein using a monoclonal antibody against the α7 nAChR subunit. Lanes 1-12 were loaded with 20 μg of human PBMC protein. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. M = Perfect Protein HRP Western Makers (Novagen, Nottingham, UK), R = rat brain protein membrane, Lanes 1-12 = human lymphocytes (← = predicted molecular weight).

Table 1. The expression of mRNAs encoding nicotinic acetylcholine receptor subunits in human peripheral blood lymphocytes. The predicted products were observed by 1.5 % agarose gel electrophoresis. (+) = mRNA for subunit present in human lymphocytes, (-) = mRNA for subunit was not detected in human lymphocytes, (+/-) = mRNA for subunit was detected, but the product bands were faint.

Table 2. Individual nAChR subunit profiles determined by Western blotting of 12 human PBMC protein samples. Each lane was loaded with 20 μg of protein. Samples were tested for subunits where mRNA expression was detected by RT-PCR experiments. (+) = Protein detected; (-) = no protein detected; (+/-) = faint expression detected.