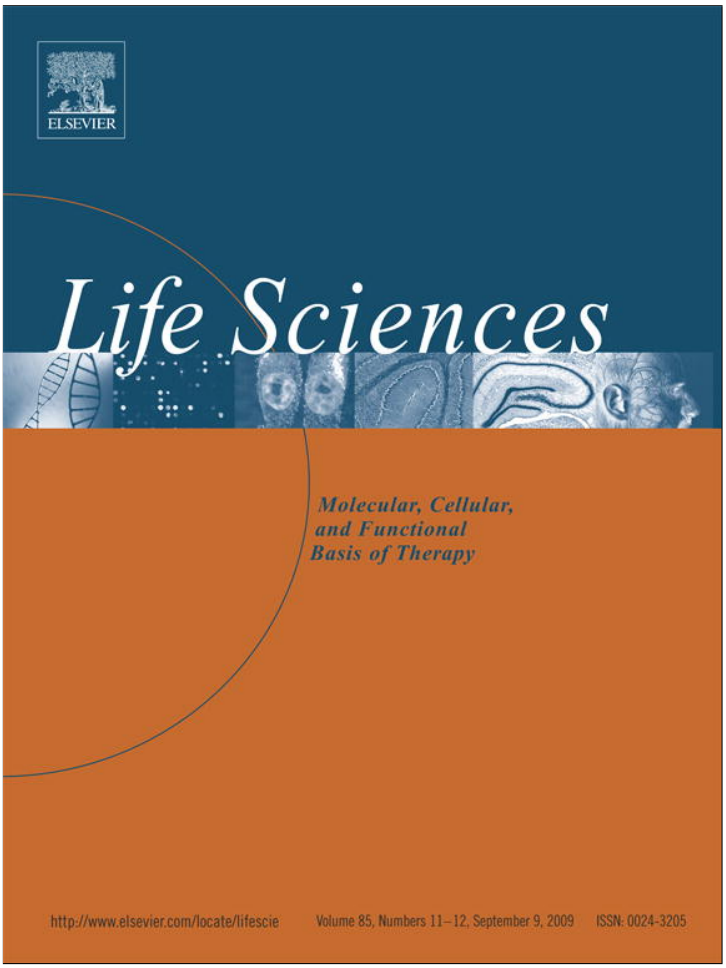

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Alpha 7 nicotinic acetylcholine receptor modulates lymphocyte activation

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ABSTRACT

Aims: Even though the presence of α7 nicotinic receptor (nAChR) in lymphocytes has been demonstrated, its functional role still remains elusive. The aim of our study was to characterize α7 nAChRs in human lymphocytes upon phytohemagglutinin (PHA) stimulation.
Main methods: Lymphocytes were activated with the mitogen PHA. α7 nAChRs were studied by reverse transcription-polymerase chain reaction (RT-PCR), real time PCR, flow cytometry and confocal laser scanning microscopy. The effects of nicotinic drugs on PHA-induced proliferation was evaluated by the [^{3}H]-thymidine incorporation assay.
Key findings: We show that the expression of functional α7 receptors increases after PHA stimulation. The activation of peripheral lymphocytes by PHA increases 2.2-fold the α7 subunit mRNA expression and 4-fold the binding of the antagonist α-bungarotoxin (α-BTX) with respect to non activated lymphocytes. By measuring the increase of intracellular calcium in response to nicotine we determine that α7 receptors in lymphocytes are functional. Nicotinic drugs differentially modulate T cell activation. While nicotine tends to inhibit proliferative responses, specific α7 antagonists, such as α-BTX and methyllycaconitine, enhance cell division.
Significance: This study reveals that the α7 receptor modulates lymphocyte activation and contributes to clarifying the role of the non neuronal cholinergic system in the immune response.

Introduction

Nicotinic receptors (nAChR) are ligand-gated ion channels, members of the Cys-loop receptor superfamily. They are composed of five identical (homopentamers) or different (heteropentamers) subunits. In muscle, nAChR plays a major role in neuromuscular transmission, initiating the action potential that ends in muscle contraction. In neuronal tissues, nAChRs contribute to a wide range of brain activities, influence a number of physiological functions, and act as modulators of synaptic and cellular signaling (Gotti and Clementi 2004; Gotti et al. 2006; Sharma and Vijayaraghavan 2002).

nAChRs are also present in non neuronal cells, such as keratinocytes (Zia et al. 2000), blood cells (Sato et al. 1999; Skok et al. 2003), vascular endothelial cells (Wang et al. 2001), and epithelial cells (Wessler et al. 1998). Experimental evidence suggests that nAChRs on non excitable cells might play roles in several processes such as proliferation, differentiation, migration and cell–cell contact (Wessler and Kirkpatrick 2008).

Of special interest is the presence of nAChRs in lymphocytes because these cells produce acetylcholine (ACh) which may exert its effects through autocrine or paracrine transmission (Rinner et al. 1998). First evidence for the expression of the α7 nAChR in human lymphocytes has been reported by Sato et al. (1999). We have demonstrated that this nicotinic receptor in lymphocytes is involved in the modulation of the cortisol-induced apoptosis (De Rosa et al. 2005). Several studies have focused on the role of nAChR on the immune response. Nicotine is known to inhibit the production of pro-inflammatory cytokines from macrophages through the stimulation of the α7 nAChRs (Wang et al. 2003). It has been shown that nicotine, probably by acting through the α7 receptors, inhibits the expression of adhesion molecules, cytokine production and T cell proliferation during human mixed lymphocyte reaction (Takahashi et al. 2007). Fujii et al. (2007a) described enhanced serum antigen-specific IgG1 and pro-inflammatory cytokines in the α7 nAChRs gene knock-out mice.

Given the experimental evidence suggesting that immune cell function might be regulated by its own cholinergic system, we here characterize the role of the α7 nAChR in T cell activation and the modulation of this process by nicotinic drugs.

Materials and methods

Reagents

Nicotine ((-)-nicotine hydrogen tartrate salt), α-bungarotoxin (α-BTX), methyllycaconitine (MLA) and phytohemagglutinin (PHA) were purchased from Sigma (USA).

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Isolation and culture of human peripheral lymphocytes

The experiments on human subjects were conducted in accordance with the Declaration of Helsinski. All procedures were carried out with the adequate understanding and written consent of the subjects. Lymphocytes were obtained from healthy non-smokers volunteers (22–40 years old) as described before (De Rosa et al. 2005). Blood (20 ml) was withdrawn from the antecubital vein using EDTA as anticoagulant. The obtained blood was loaded on 3 ml Ficoll separating solution (Amersham Biosciences, AB, Sweden) and centrifuged for 20 min at 2000 rpm. Cells were washed with PBS and then resuspended in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal calf serum (FCS). Macrophages were discarded by the plastic adherence method. Lymphocytes were cultured in RPMI-1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere at 5% CO_2.

RT-PCR analysis

Total RNA was isolated by the acid guanidium–phenol–chloroform method. For samples to be amplified with real time PCR, total RNA was extracted using SV Total RNA isolation System (Promega, USA). RNA was converted into cDNA using the Molony murine leukaemia virus reverse transcriptase (MLV-RT; Promega, USA) and random primers (Promega, USA).

End point PCR was run for 40 cycles in a Mini Cycler^{tm} (MJ Research, USA). The choline acetyl transferase (ChAT) primer sequences used were: sense 5′CCACCAACCGGGACTCGC3′; and antisense 5′GCATCAGGGCTGCATTTCTGC3′. The α7, α1 and α9 primer sequences were described somewhere else (De Rosa et al. 2005).

Quantitative real time PCR was performed using a Rotor-Gene 6000 (Corbett Research, Australia). Amplification included 2 min at 94 °C followed by 40 cycles of a three-step loop: 20 s at 94 °C, 40 s at 56 °C and 40 s at 72 °C. The results are expressed as the fold increase of the gene expression in samples from PHA-treated cells above those from control non treated cells. The results for the gene expression were normalized for the *18S rRNA* gene, the expression of which was not changed under stimulation conditions. The 18S rRNA primer sequences used were: sense 5′TCGAGGCCCTGTAATTGGAA3′; and antisense 5′CCCTCCAATGGATCCTCGTT3′.

α-BTX staining and confocal microscopy

Lymphocytes were incubated with PHA (10 μg/ml) for 48 h. After fixation in paraformaldehyde, cells were incubated in the absence (total binding) or in the presence (non specific binding) of 1 μM α-BTX (Sigma, USA) for 30 min at room temperature (RT). Then cells were incubated with Alexa 594-labeled α-BTX (1 μg/ml) (Molecular Probes, USA) in PBS at RT for 1 h. Cultures were then analyzed by phase and fluorescence microscopy, using a Nikon Eclipse E600 microscope, and by laser scanning confocal microscopy (LSCM; Leica DMIRE2) with a 63 water objective. *X–y* (top to bottom) and *x–z* sections were collected and processed with LCS software (Leica) and PHOTOSHOP 8.0 (Adobe Systems, San Jose, CA).

α-BTX staining and flow cytometry

Lymphocytes were cultured in the presence or absence of PHA (10 μg/ml). After 48 h, cells were harvested and stained with 100 nM Alexa 488-conjugated α-BTX for 1 h. Leukocytes surface markers were also analyzed using the following antibodies: anti-CD8-PE (Leu-2a) and anti-CD3-PerCP (SK7) (Becton Dickinson, USA). Cells were processed by flow cytometry (FACSCalibur, Becton-Dickinson, USA). The data were analyzed using the program Paint a Gate (Becton Dickinson, USA).

Measurement of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in lymphocyte cultures

Lymphocytes were incubated for 48 h in the presence of PHA (10 μg/ml) and transferred to cover-glass bottomed culture dishes coated with poly-L-ornithine for 4 h. $[Ca^{2+}]_i$ changes were measured using the Ca^{2+}-sensitive fluorescent dye fura-2/pentaacetoxymethyl ester (fura-2/AM) (Sigma Chemical Co, USA) as previously described by Katz et al. (2006). Excitation of fura-2/AM was performed at 340 and 380 nm employing a dual excitation monochromator from an SLM-Aminco 8100 spectrofluorimeter connected to the epifluorescence port of the microscope through an optic fiber. Emitted cellular fluorescence was collected at 510 nm and ratios from 340/380 nm signals were obtained.

$[Ca^{2+}]_i$ was imaged in human lymphocytes using a confocal laser scanning microscope and the Ca^{2+}-sensitive fluorescent indicator fluo-3/AM (Molecular Probes, USA). After poly-L-ornithine treatment, cells were incubated with 3 μM fluo-3/AM. Fluorescent images of living cells in buffer were acquired using a Nikon Eclipse E600 microscope and by LSCM (Leica DMIRE2). Fluo-3/AM loaded cells were excited at 488 nm, and the fluorescence emission above 530 nm was imaged using a 63 water objective. Images of confocal fluorescence were obtained at a rate of one frame every 1.4 s. Nicotine (500 μM) was added directly to the samples. Basal fluorescence (F0) was calculated prior to the addition of nicotine. Data were normalized to F0 (F/F0 ratio) in order to control for variations in basal fluorescence.

[^{3}H]-thymidine incorporation assay

2×10^5 cells were seeded in 200 μl RPMI medium per well in 96-well plates. After 48 h, cells were exposed for 16 h to [^{3}H]-thymidine. Radioactivity was quantified in a liquid scintillation counter. The percentage of cell proliferation for each condition was related to that achieved in the presence of PHA concentration, which was considered as 100% of proliferation.

Statistical analysis

Experimental data are shown as mean ± S.D. Statistical comparisons were made using the Student's *t*-test. A level of $p<0.05$ was considered significant.

Results

Expression of α7 nAChR in resting and activated human lymphocytes

We have previously shown the expression of neuronal α7 nAChR subunit in freshly isolated lymphocytes (De Rosa et al. 2005). In the present study, we investigated the presence of α7 mRNA in activated lymphocytes. RT-end point PCR was carried out 48 h after the induction of the T cell activation by the mitogen PHA (10 μg/ml). α7 mRNA was detected in activated cells as well as in non activated ones (Fig. 1A). Relative quantitative RT-real time PCR revealed that α7 nAChR expression increases 2.2-fold (±0.1, $n=3$) upon PHA activation compared to the non activated cells (Fig. 1B).

To determine the surface expression of α7 nAChRs, isolated human lymphocytes were first stained with Alexa 594-labeled α-BTX. Binding of the antagonist was clearly detected on the surface of PHA-stimulated lymphocytes under confocal microscopy, while it was faintly detected in non stimulated ones (data not shown). Given that α-BTX also binds to nAChR subtypes containing α1 and α9 subunits, we performed RT-end point PCR using mRNA of stimulated lymphocytes to detect the presence of these subunits. No mRNA corresponding to the α1 and α9 subunits was detected. Thus, Alexa-α-BTX label corresponds to the α7 nAChRs. We discard that label

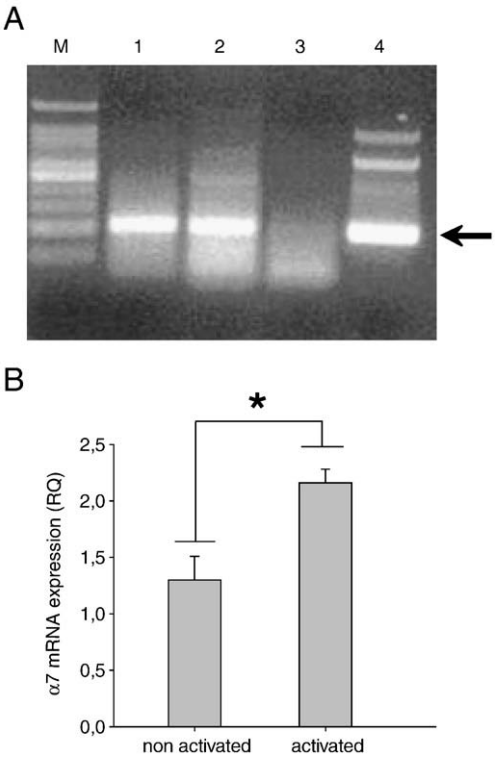

Fig. 1. α7 subunit mRNA expression in activated and non activated lymphocytes. Lymphocytes were incubated with and without PHA (10 μg/ml) for 48 h. (A). Agarose gel electrophoresis of the RT-end point PCR reaction products. Transcripts of α7 nAChR were detected as a band of 190 bp (arrow). Sample 1: 48 h incubation without PHA; sample 2: 48 h incubation with PHA (10 μg/ml); sample 3: PCR negative control; sample 4: PCR positive control (human α7 cDNA PCR product). M: DNA marker (100 bp ladder). (B) Relative quantification (RQ) of α7 mRNA expression. Real time PCR was carried out according to Materials and methods. The results are the means ± S.D. of three independent experiments. *$p<0.05$.

corresponds to the so called hybrid-α7 described in lymphocytes because the latter subunit lacks exons 1 to 4 that encode part of the α-BTX binding site (Villiger et al. 2002).

To further characterize the surface expression of α7 nAChRs, resting and activated lymphocytes were stained with Alexa 488-conjugated α-BTX. Cells were also analyzed for the binding of antibodies to the leukocytes surface markers CD8 and CD3. Fig. 2 shows that a significant fraction of the activated lymphocytes bound α-BTX. Binding was also detected on the non activated cells. The fluorescence intensity was approximately 4–5 times higher in the activated lymphocytes compared to the resting cells. No difference was observed between the subsets of CD3+ CD8− and CD3+ CD8+ lymphocytes.

Functional α7 nAChR in human lymphocytes

To ascertain whether the α7 receptor is functional in lymphocytes, we evaluated Ca^{2+} oscillations in activated lymphocytes in response to nicotinic drugs. Acute stimulation of loaded-fura-2/AM cells with the agonist nicotine (500 μM) elicits an increase in the fluorescence ratio (340/380 nm). The response is blocked by preincubation with 1 μM α-BTX, demonstrating that α7 nAChR is responsible for the nicotine-induced Ca^{2+}-signaling in activated lymphocytes (Fig. 3A). In addition, we also determined individual cell responses to nicotine using a confocal laser scanning microscope with the Ca^{2+}-sensitive fluorescent indicator fluo-3/AM. The increment in $[Ca^{2+}]_i$ due to the exposure to the agonist was detected as a 1.5–2 fold increase in fluorescence intensity (Fig. 3B). We also observed spontaneous $[Ca^{2+}]_i$ increments in the activated lymphocytes, which were detected as changes in the fluo-3/AM fluorescence in the absence of nicotine. These spontaneous signals were of variable intensity and duration and were not detected in the resting lymphocytes.

Expression of ChAT mRNA in resting and activated human lymphocytes

We further investigated the induction of mRNA for ChAT, the enzyme that catalizes ACh synthesis, upon PHA-stimulation. ChAT mRNA was detected by RT-end point PCR in lymphocytes that were incubated 48 h with PHA (10 μg/ml). In contrast, ChAT mRNA was not detected in resting lymphocytes ($n=3$) (Fig. 4). We conclude that stimulation of mononuclear leukocytes (MNL) by PHA enhances the expression of ChAT mRNA, which, in turn, may increase ACh synthesis.

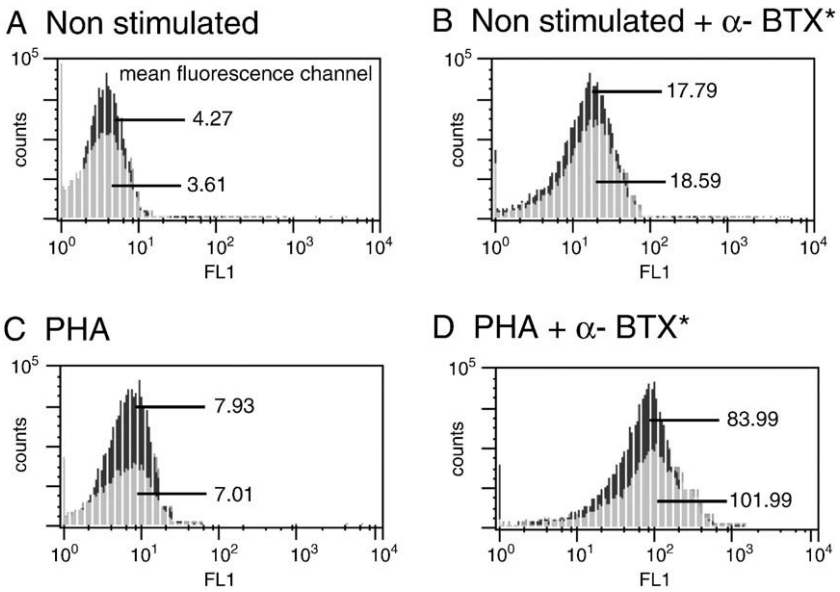

Fig. 2. Binding of α-BTX to nAChR on lymphocytes. Lymphocytes were incubated with and without PHA (10 μg/ml) for 48 h and stained with fluorescence Alexa 488-conjugated α-BTX and antibodies against CD3 and CD8 surface markers. Then cells were analyzed by flow cytometry. A) untreated non stimulated lymphocytes, B) non stimulated lymphocytes stained with Alexa 488-conjugated α-BTX (α-BTX*), C) untreated PHA-stimulated lymphocytes, D) PHA-stimulated lymphocytes stained with Alexa 488-conjugated α-BTX. Grey histogram: CD3+ CD8+, Black histogram: CD3+ CD8−. A typical result is represented.

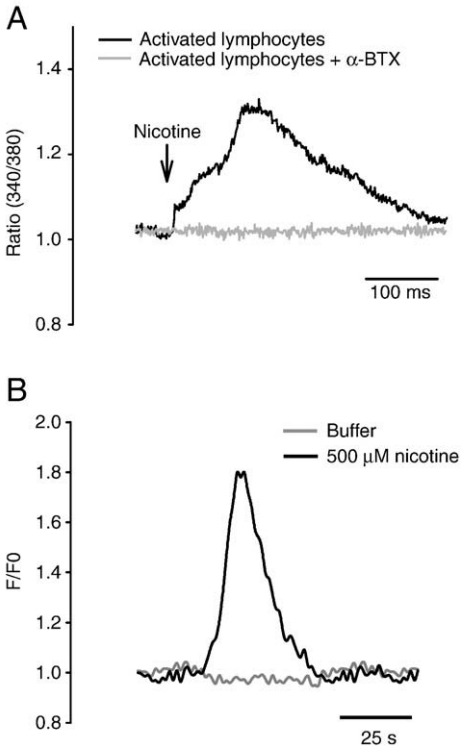

Fig. 3. Nicotine-evoked changes in $[Ca2+]_i$ in human lymphocytes. A- Activated lymphocytes were loaded with fura-2/AM and the fluorescence ratio at 340/380 nm was measured. Nicotine (500 μM) application is indicated by the arrow. Nicotine stimulation was also carried out in activated cells preincubated 10 min with α-BTX (1 μM). B- PHA-activated cells were loaded with fluo-3/AM. Time course of the $[Ca^{2+}]_i$ represents the changes in the fluo-3/AM fluorescence intensity when a representative activated lymphocyte was exposed to buffer alone (——) or containing 500 μM nicotine (——). Data points were obtained every 1.4 s. Figure traces are representative of 3–5 determinations at each condition.

Modulation of lymphocyte proliferation by agonists and antagonists of α7 nAChR

We studied the effects of nicotinic drugs on T cell activation. Cells were incubated with PHA in the presence of nicotine. The agonist was tested under two different mitogen concentrations: an optimal concentration (10 μg/ml) and a suboptimal one (5 μg/ml). The presence of nicotine during optimal PHA stimulation seemed to inhibit proliferation (Fig. 5). However, the changes were not significant even

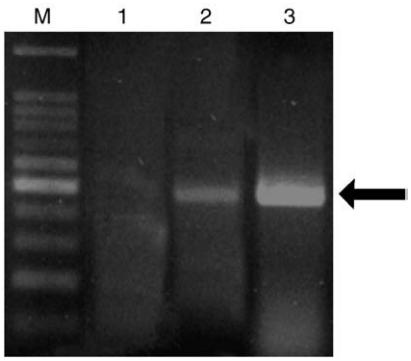

Fig. 4. ChAT mRNA expression in activated and non activated lymphocytes. Lymphocytes were incubated with and without PHA (10 μg/ml) for 48 h. Agarose gel electrophoresis of the RT-end point PCR reaction products. Transcripts of human ChAT were detected as a band of 484 bp (arrow). Sample 1: 48 h incubation without PHA; sample 2: 48 h incubation with PHA; sample 3: positive control (Jurkat cells). M: DNA marker (100 bp ladder). The results are representative of at least 3 different experiments.

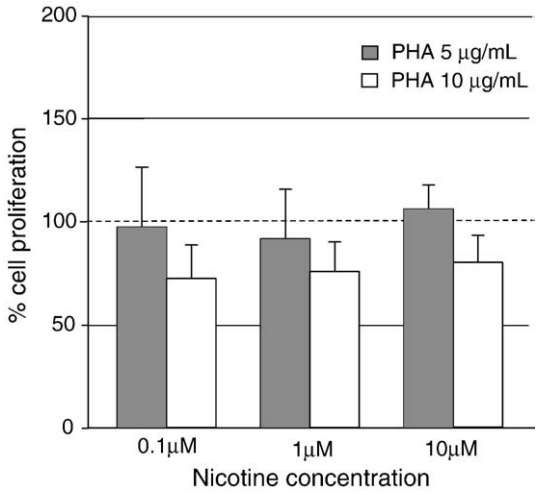

Fig. 5. Effect of nicotine on lymphocyte proliferation. Human MNL were activated with optimal PHA and suboptimal PHA concentrations (10 and 5 μg/ml respectively). After 72 h, cell proliferation was evaluated by $[^3H]$-thymidine incorporation. The degree of proliferation for each condition was related to that achieved in the presence of PHA alone, which was considered as 100% of proliferation (discontinued line). Results are expressed as mean ± S.D. of at least 4 experiments. No statistically significant effect ($p>0.05$) was found between treated samples and PHA- stimulated cells (control).

for a 100-fold concentration range (0.1–10 μM). Nicotine also failed to affect cell proliferation when the suboptimal PHA concentration was used to activate lymphocytes (Fig. 5). When lymphocytes were cultured in the presence of the antagonist α-BTX (0.5 and 1 μM) at optimal PHA concentrations, no changes were observed (data not shown). By contrast, the presence of α-BTX and PHA at suboptimal concentration increased in about 50% the number of lymphocytes. Similarly, MLA, another antagonist, enhanced suboptimal PHA induced-cell proliferation (Fig. 6). Cells incubated with antagonists alone did not proliferate, indicating that these drugs do not have mitogenic properties by themselves.

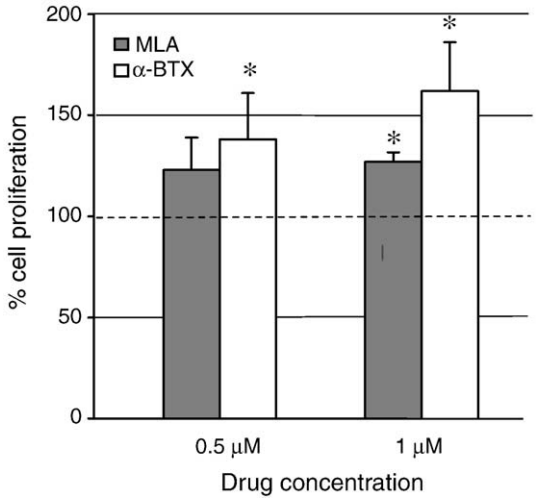

Fig. 6. Effect of nicotinic antagonists on lymphocyte proliferation. Human MNL were activated with PHA (5 μg/ml) in the presence of the nicotinic antagonists α-BTX and MLA. After 72 h, cell proliferation was evaluated by $[^3H]$-thymidine incorporation. The degree of proliferation for each condition was related to that achieved in the presence of PHA alone, which was considered as 100% of proliferation (discontinued line). Results are expressed as mean ± S.D. of at least 4 experiments. *$p<0.05$ compared to PHA stimulated cells (control).

Discussion

In the present study, we show that the expression level of mRNA corresponding to α7 is higher in activated than in resting lymphocytes. The increased α7 mRNA is accompanied by an increased expression of surface receptors, as evidenced by α-BTX binding. These observations are in agreement with previous reports, showing that the expression of α7 receptors in non activated lymphocytes is low, and that the binding of radiolabeled α-BTX can be detected only after the α7 receptor up-regulation induced by nicotine (De Rosa et al. 2005).

Razani-Boroujerdi et al. (2007) have demonstrated that the activation of α7 nAChR raises $[Ca^{2+}]_i$ in the Jurkat cell line. We present evidence that surface α7 nAChRs are functional in human activated lymphocytes, as shown by the increase in $[Ca^{2+}]_i$ in response to nicotine. As expected, cation movements are also elicited by merely incubation with the mitogen, thus indicating that other receptors or calcium channels are also active during T cell proliferation. Ca^{2+} is a highly versatile signaling agent that regulates T cell activation. In fact, a critical role in the Ca^{2+} signaling has been assigned to the muscarinic receptor (mAChR). This receptor mobilizes intracellular calcium and its activation enhances T cell responses (Fujino et al. 1997; Razani-Boroujerdi et al. 2008).

We also demonstrated that PHA stimulation induces expression of ChAT mRNA, and consequently increases ACh. Although we did not measure directly the ACh release, other investigators (Rinner et al. 1998) have reported in rat lymphocytes that stimulation with PHA increases its synthesis as well as its release.

Several lines of experimental evidence support the anti-inflammatory activity mediated by the cholinergic system, specifically by ACh and α7 nAChRs (Shytle et al. 2004; Wang et al. 2003). Nizri et al. (2006, 2008) reported the suppression of lymphocyte proliferation and the reduction in pro-inflammatory cytokines production upon treatment with inhibitors of the acetylcholinesterase (AChEI). The authors attributed this effect to nAChRs since the inhibition of the T cell proliferation was observed after treatment with the agonist nicotine. We show that nicotine slightly inhibits proliferation only when lymphocytes are activated at an optimal concentration of PHA. Under suboptimal conditions this effect was not observed, probably because the sensitivity of the assay does not allow the detection of a slight degree of inhibition.

This study shows that antagonists of the α7 nAChR, such as α-BTX and MLA, enhance T cell proliferation. The modulation by antagonists is neither detected at saturation of proliferation achieved by optimal PHA concentrations nor in the resting lymphocytes. The fact that this effect is only observed when T cells are activated by suboptimal PHA concentrations indicates that the blockade of α7 modulates positively the lymphocyte proliferation triggered by PHA. In line with this observation, Freedman et al. (1993) reported that α-BTX binding to receptors on the hippocampal interneurons increased the expression of growth factors like nerve growth factor and brain-derived neurotrophic factor. Further studies are needed to determine if similar mechanisms are elicited in the activated lymphocytes. It might be possible that the gene expression of cytokines that control growth and differentiation of lymphocytes differs upon exposure to the α7 antagonists.

Cholinergic up-regulation is not always associated with anti-inflammatory signaling. As mentioned above, mAChRs are involved in the proliferation and activation of lymphocytes (Nizri et al. 2008). It has been reported that the synthesis of the antigen-specific IgG1 and interleukin-6 (IL-6) is diminished in mAChR knockout mice, while enhanced production was described in the α7 AChR subunit knockout mice (Fujii et al. 2007a,b). It seems that the cholinergic system in the lymphocytes might have different outcomes depending on which cholinergic receptor type, i.e. muscarinic or nicotinic, is activated. The α7 nAChR might function as an autoreceptor that modulates the synthesis and release of the ACh. The activation of the autoreceptors is known to be important in the modulation of the presynaptic transmitter secretion in the peripheral and central nervous systems. In fact, nicotinic autoreceptors have been proposed to regulate the ACh release at motor nerve terminals (Bowman et al. 1988), in cortical and hippocampal slices (Araujo et al. 1988) and in brain cholinergic neurons and interneurons (Azam et al. 2003).

We propose that the α7 receptors act as modulators affecting the amount of the ACh released available to interact with other cholinergic receptors, such as the mAChRs. It might be possible that depending on the levels of ACh, the actions are selectively mediated by the α7 nAChRs or the mAChRs. A similar explanation has been suggested before by Nizri et al. (2006). The authors reported that the inhibition of the lymphocyte proliferation observed at low ACh concentrations is abolished after increasing its concentration by the inhibitors of AChEI.

Without disregarding the complexity of the immune response, here we propose a model that points out the importance of ACh as an immunomodulator, which in turn binds to two receptors with antagonistic roles. According to the levels of ACh, these receptors contribute to restrict or promote T cell proliferation. It is probable that mAChRs act as effector receptors since their activation promotes directly IL-2 production and T cell proliferation (Fujino et al. 1997). On the other hand, signaling through nicotinic receptors is likely to exert a modulating, tuning effect on the immune processes. Their actions might be indirect by affecting the amount of the ACh released available to interact with other cholinergic receptors, such as the mAChRs.

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