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Relationship between α7 nAChR and apoptosis in human lymphocytes

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Abstract

The presence of nicotinic receptors (nAChRs) in blood cells has been demonstrated. However, little is known about their functional roles. We have detected mRNA of α7 nAChR in peripheral human lymphocytes and determined that its expression is highly variable among individuals and within the same individual at different times. Upregulation of α7 is systematically observed after incubation of lymphocytes with nicotine or α-bungarotoxin. In addition, the incubation with these drugs decreases the percentage of apoptotic cells induced by the exposure to cortisol. Our results suggest that α7 nAChRs are involved in the modulation of cortisol-induced apoptosis.

Keywords: Lymphocyte; α7 nicotinic receptor; Apoptosis; Nicotine

1. Introduction

Acetylcholine (ACh) plays an important role as a neurotransmitter of the central and peripheral nervous systems. There is increasing experimental evidence that ACh is also widely expressed in non-neuronal cells. The presence of both muscarinic (mAChRs) and nicotinic receptors (nAChRs) in various blood cells, including lymphocytes, has been demonstrated (Kao and Drachman, 1977; Toyabe et al., 1997; Sato et al., 1999; Skok et al., 2003). The expression of these neurotransmitter receptors on the surface of immunocompetent cells is indicative of the existence of a link between the nervous and the immune systems. However, little is known about the functional role of this extraneuronal cholinergic system. Recent investigations have provided evidence that lymphocytes have most of the essential components needed to constitute an independent, non-neuronal cholinergic system, and that ACh synthesized and released from lymphocytes acts as an immunomodulator via mAChR and nAChR (Khan et al., 2001; Kawashima and Fujii, 2003). Activated immune cells may use the released ACh in self-modulating autocrine and paracrine loops. ACh detected in lymphocytes appears to be involved in the regulation of the activation and clonal expansion of these cells (Wessler et al., 1999).

mRNAs encoding several mAChRs subtypes have been detected in peripheral lymphocytes as well as in several leukemic cell lines. Stimulation of T lymphocytes by muscarinic agonists has been shown to cause an increase in GMPc, gene expression, and protein synthesis and to alter immune functions (Wessler et al., 1999).

Different nAChR subunits have been detected in blood cells but their patterns of expression as well as their functional roles remain unknown. Studies of the expression of the neuronal α7 nAChR, which can form functional homopentamers (Karlin, 2002), as well as that of different heteropentameric nAChRs (neuronal and muscle) have lead to controversial conclusions.

Nicotine, probably acting through α7 nAChRs, has been shown to elicit transient increases in Ca^{++} in MNLs (mononuclear leukocytes) and human leukemic cell lines, but the role of nAChRs in the regulation of lymphocyte function is still unknown (Kawashima and Fujii, 2000).

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In neuronal cells, long-term exposure to nicotine has been shown to induce upregulation of α7 nAChRs (Schwartz and Kellar, 1985; Wonnacott, 1990; Pauly et al., 1991; Marks et al., 1992; Jonnala and Buccafusco, 2001). In addition, this upregulation has been implicated in neuroprotective functions (Dani and Heinemann, 1996; Jonnala and Buccafusco, 2001).

The aim of the present study was to further characterize nAChRs in lymphocytes and to investigate the effect of nicotine on these cells. We show that nicotine upregulates α7 nAChRs in peripheral lymphocytes and acts as an immunomodulator due to its capacity to decrease the cortisol-induced apoptosis.

2. Materials and methods

2.1. Isolation and culture of human peripheral lymphocytes

Lymphocytes were obtained from healthy volunteers (aged 22–40 years old) as described by Hirano et al. (1997). To avoid potential effects produced by chronic exposure to nicotine, all volunteers were non-smokers. 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 (Iiai et al., 1992). Lymphocytes were cultured in RPMI-1640 medium supplemented with 10% FCS at 37 °C in a humidified atmosphere at 5% CO_2.

The percentage of contaminating monocytes and platelets in the cultures was determined by May Grumwald–Giemsa staining. Monocytes were less than 1% of mononuclear leukocytes before the adherence depletion and only one monocyte was observed in the complete slide after this procedure. The relationship lymphocyte/platelet in the culture was 1:5 before the adherence depletion, 1:3 after the adherence depletion, and 1:1 after 48 h incubation. The presence of nAChRs on platelets has not been demonstrated to date. In addition, it is unlikely that the contaminating platelets affect our results given that the decrease in this cell population during incubation does not follow the observed increase in α7 (see Results).

2.2. Isolation of rat splenic and peripheral lymphocytes

Wistar rats aged 3–7 months were used. Blood samples were obtained by intracardial puncture of rats under Equitesin anesthesia (2.5 ml/kg ip) using EDTA as anticoagulant. Lymphocyte cells either from spleen or blood samples were obtained by Ficoll density gradient centrifugation and cultured as described above. Animals were treated in accordance with the National Guidelines for the treatment of laboratory animals, which are similar to those established by the United States National Institute of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. RT-PCR analysis

Total RNA was isolated from 1×10^6 cells by the acid guanidium–phenol–chloroform method. RNA was converted into cDNA using the Molony murine leukaemia virus reverse transcriptase (MLV-RT; Promega, USA) and random primers (Promega, USA).

Polymerase chain reaction (PCR) was run for 35 cycles in a Mini Cycler™ (MJ Research, USA). RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The primers of the different nAChRs subunits were designed to prime in different exons to differentiate by length cDNA amplification from genomic DNA amplification (Table 1). Semiquantitative RT-PCR analysis was carried out by using β-actin mRNA amplification as an internal standard.

Table 1
List of primers used for PCR amplification of the indicated genes

Gene	Sense primer (5′→3′) (exon number)	Anti-sense primer (5′→3′) (exon number)	Predicted RT-PCR product (bp)
Human α1	GCCAGGGCCTCCACCCATGG (exon 8)	GATAAGTGCGAGTGGAGC (exon 9)	350
Human β1	CTGCCCCTGATCTGCGGCG (exon 10)	GGGGTCTGGAGGGGGCAAG (exon 11)	260
Human ε	CACCGGCAGGGGACCTGG (exon 10)	CACGGCGCGTAGGGGAGATC (exon 12)	271
Human δ	GGGCTGGCCAGGCGCCTC (exon 10)	GGCTGGGGTGGTGGCTGG (exon 11)	277
Human γ	GGGCTGAGCCAGTTCTGTG (exon 11)	GCAGGTAGGGGCGTGGATC (exon 12)	273
Human α7	CCGACGGGGGCAAGATGCC (exon 8)	GGCCGCGGAAGCCGATGTAC (exon 9)	190
Human α9	GCCGGCCTCAGAAAATGTGCC (exon 4)	GCCCAGTGTGGCACCGGCCGG (exon 5)	130
Rat α1	CCGGGGCCTCCACCTATGG (exon 8)	GGTAAGCGCGAAAGGATTC (exon 9)	334
Rat β1	GGGCCATGGCCTTAGGGGC (exon 1)	GGTCTAAGTACACCTTTGTGC (exon 3)	260
Rat ε	GGCTCTGCTTGGGACCC (exon 1)	CCAATCCAGACACTGGTGG (exon 3)	271
Rat δ	GGGGCTGCTGGCTGCCC (exon 1)	GGTGGTGAGGGTCTCCTCC (exon 3)	277
Rat α7	CTGATGGTGGCAAAATGCC (exon 8)	GGCCTCGGAAGCCAATGTAG (exon 9)	190

2.4. DNA ladder visualization

Apoptotic DNA was isolated from 1×10^6 cells according to Herrmann et al. (1994). DNA bands were visualized with ethidium bromide after electroforesis in a 1.5% agarose gel.

2.5. Cytochemical analysis

For the cytochemical analysis of the nuclei, lymphocytes were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. After fixation, cultures were permeated with 0.1% triton X-100 for 15 min, and stained with 10 μg/ml of the DNA-specific fluorochrome 4′, 6-diamino-2-phenylindole (DAPI). Finally, cells were observed with a NIKON fluorescence microscope. Apoptotic cells were identified by their condensed or fragmented nucleus. The mean percentage of apoptotic cells was obtained after counting two times >500 cells in random microscopic fields.

2.6. MTT reduction assay

For MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] analysis, 2×10^5 cells were plated in 200 μl medium per well in 96-well plates. After 48 h, 20 μl of 5 mg/ml MTT stock was added to each well and then incubated for another 4 h at 37 °C and 5% CO_2. Then 100 μl of a solution containing 0.04 N isopropanol–HCl was added. Absorption values were determined at 570 nm (Guan et al., 2001).

2.7. [^{125}I]α-bungarotoxin binding essays

Cells were incubated with 20 nM [^{125}I]α-bungarotoxin either in the absence (total binding) or presence (nonspecific binding) of 1 μM α-bungarotoxin for 1.5 h. The cells were then washed with cold K^+ Ringer by centrifugation.

2.8. 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. Dose–response curves were fitted by non-linear regression analysis using the SigmaPlot 2001 software.

3. Results

3.1. α7 in human lymphocytes is upregulated by nicotine

We have studied the expression of the neuronal α7 nAChR in human lymphocytes from healthy donors. The results shown in Table 2 provide evidence that the α7 gene expresses in 70% of the samples corresponding to freshly isolated lymphocytes.

To test if nicotine influences the expression of α7 mRNA, lymphocytes were incubated 48 h in the absence and presence of 10 and 100 μM nicotine (Table 2). The results show that nicotine induces the transcription of α7 mRNA in all tested samples, suggesting that nicotine upregulates the expression of the α7 subunit in human lymphocytes. In some cases, the incubation in the absence of nicotine also altered the expression pattern with respect to that of freshly isolated lymphocytes. However, the changes were variable as either the appearance or the disappearance of α7 mRNA could be observed (Table 2).

To analyze the level of α7 expression, a semiquantitative RT-PCR was carried out. The results reveal an increase of α7 mRNA in lymphocytes incubated with nicotine with respect to freshly isolated lymphocytes or lymphocytes incubated in the absence of the agonist (Fig. 1). These results give full support to the increase of the expression of the α7 subunit in lymphocytes after incubation with nicotine.

Surface expression of α7 was confirmed by [^{125}I]α-bungarotoxin binding. Specific binding could be detected only in lymphocytes previously incubated with nicotine. The number of toxin sites was 166±20 fmol/1.500.000 cells (n=3). Given such a low expression, it is probable that the method is not sensitive enough to allow detection in freshly isolated lymphocytes.

3.2. Functional role of α7 nAChRs in human lymphocytes

Given that α7 has been shown to have a protective effect on neuronal tissues (Li et al., 1999; Guan et al., 2001; Jonnala and Buccafusco, 2001), we tested its role in the immune cells. To this end, we investigated whether nicotine has the capacity to attenuate cortisol-induced apoptosis in lymphocytes. Lymphocytes were first incubated with nicotine for 24 h and then cortisol was added for an additional period of 24 h. The degree of apoptosis was first determined by DNA fragmentation observation. As shown in Fig. 2A, the level of apoptosis, revealed by the presence of DNA fragments, was more significant in the

Table 2
Expression of α7 subunit in human lymphocytes

N	Volunteer	FI	WN	N
10 μM	m, 24 years	nd	–	+
	m, 27 years	nd	–	+
	w, 40 years	+	–	+
	m, 27 years	+	+	+
100 μM	m, 28 years	+	+	+
	m, 26 years	–	–	+
	m, 24 years	+	+	+
	m, 27 years	+	+	+
	w, 37 years	–	+	+

mRNA corresponding to α7 was detected by RT-PCR. The predicted product was observed by agarose gel electrophoresis.
N: nicotine; FI: freshly isolated; WN: without nicotine; w: woman; m: man; nd: not determined.

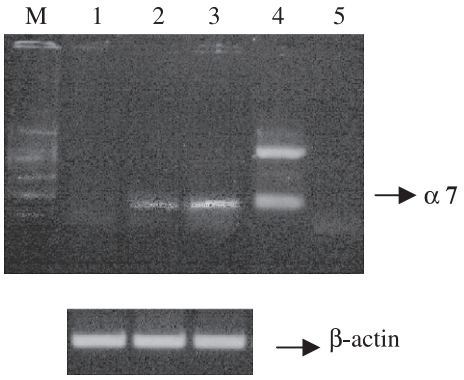

Fig. 1. Agarose gel electrophoresis of the semiquantitative RT-PCR reaction products from total RNA isolated from human lymphocytes. Total RNA was isolated from freshly human lymphocytes and from lymphocytes incubated in the presence and absence of nicotine (100 μM). Transcripts of the α7 nAChR (190 bp) were detected using RT-PCR. Samples were compared using β-actin mRNA expression as an internal standard. Sample 1: freshly isolated lymphocytes; sample 2: 48 h incubation without nicotine; sample 3: 48 h incubation with 100 μM nicotine; sample 4: PCR positive control (human α7 cDNA PCR product); sample 5: PCR negative control; M: DNA marker (100 bp ladder). The results are representative of 3 different experiments.

cortisol-treated samples than in those preincubated with nicotine before the addition of cortisol. This result suggests that nicotine protects lymphocytes from cortisol-induced apoptosis.

The ability of nicotine to reverse the apoptotic conditions produced by cortisol was also tested. To this end, lymphocytes were exposed 24 h to cortisol and 100 μM nicotine was then added for an additional period of 24 h. As shown in Fig. 2B, no significant inhibition of apoptosis was observed with respect to the cortisol-treated samples.

In order to quantify the level of apoptotic cells, we determined the percentage of cells that showed nuclear condensation or fragmentation by DAPI staining (Fig. 2C). Non-treated samples as well as those incubated with nicotine alone showed less than 8% of apoptotic cells. This percentage increased to about 25–30% when cells were treated with cortisol. However, if samples were preincubated with 100 μM nicotine before the addition of cortisol, the percentage of apoptotic cells decreased to about 13–18%. This decrease corresponds to an inhibition of apoptosis with respect to cortisol-treated samples of about 55% (Fig. 3). Fig. 3 shows that the inhibition of apoptosis is dependent on the concentration of nicotine. The EC_{50} calculated from the data is 90 μM. Thus, the results reveal a correlation between the exposure to nicotine and the decrease in the percentage of apoptotic cells. The reversion of cortisol-induced apoptosis by nicotine was also quantified. Cells were first treated with cortisol and 24 h later nicotine was added. The inhibition of apoptosis was 25±5%. Thus, the maximal effect of nicotine is achieved when it is present before the incubation with cortisol. It might be that either the stimuli of cortisol triggers an irreversible cell-death pathway or that the incubation for a period of 24 h is too long to be reversed by nicotine.

In order to determine if nicotine has a mitogenic effect on lymphocytes, a MTT assay was performed. No significant changes in the number of cells were observed in lymphocytes exposed to nicotine under the present conditions compared to control wells without treatment. The absorb-

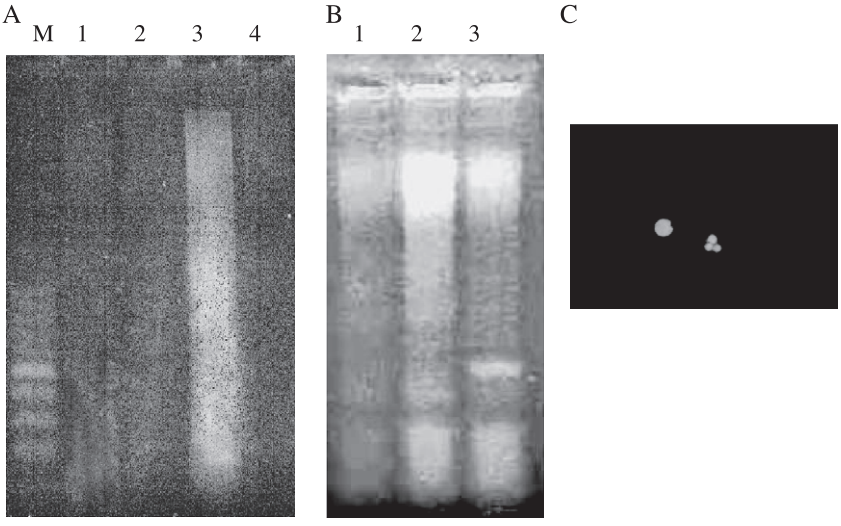

Fig. 2. Apoptosis evaluation by DNA fragmentation. Apoptotic DNA was extracted according to Methods. (A) Lymphocytes were incubated in the absence and presence of nicotine (100 μM) for 24 h and then exposed to cortisol (10 μM) for another 24 h. Sample 1: 48 h incubation without drug addition; sample 2: 48 h incubation with only 100 μM nicotine; sample 3: 24 h incubation without nicotine followed by 24 h incubation with cortisol; sample 4: 24 h incubation with 100 μM nicotine followed by 24 h incubation with cortisol and nicotine. (B) Lymphocytes were incubated in the presence of 10 μM cortisol for 24 h and 100 μM nicotine was then added for another 24 h. Sample 1: 48 h incubation without drug addition; sample 2: 48 h incubation with cortisol; sample 3: 24 h incubation with 10 μM cortisol followed by 24 h incubation with 100 μM nicotine. (C) Comparison of nuclei from non-apoptotic (left) and apoptotic (right) lymphocytes labeled with DAPI. The results are representative of 5 different experiments.

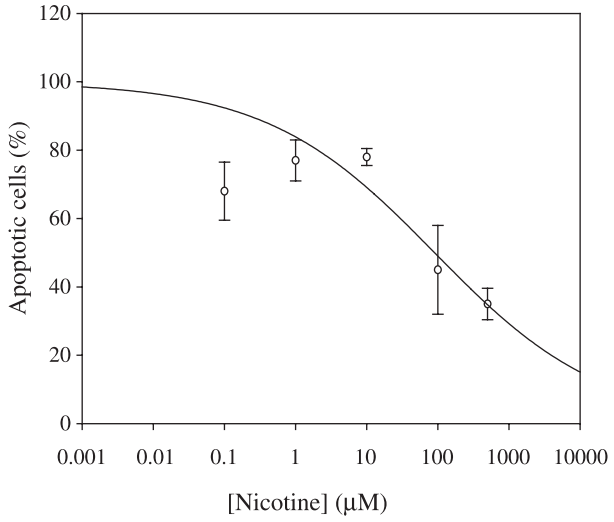

Fig. 3. Dose-dependent inhibition of cortisol-induced apoptosis by nicotine. Lymphocytes were incubated 24 h in the absence or presence of different nicotine concentrations (0.1, 1, 10, 100, and 500 μM) and then exposed to 10 μM cortisol. Twenty-four hours later, cells were stained with DAPI. The degree of apoptosis for each condition was related to that achieved in the presence of cortisol alone, which was considered 100% apoptosis for the entire experiment. Results are expressed as mean±S.D. of 4 independent experiments.

ance values (O.D.) measured after incubation with 10 and 100 μM nicotine were 0.362±0.05 and 0.359±0.04, respectively, while the O.D. in untreated cells was 0.351±0.04 (n=4).

The apoptosis protection was also detected when lymphocytes were incubated with α-bungarotoxin (α-BTX), which is an antagonist for α7 nAChRs. As described above for nicotine, after 24 h incubation of human lymphocytes with α-BTX (0.5 μM) an increase in α7 expression (Fig. 4A) as well as protection of apoptosis were observed (Fig. 4B). When lymphocytes were incubated with both nicotine (100 μM) and α-BTX (0.5 μM) before the addition of cortisol, the level of protection did not increase with respect to that observed with the individual drugs (Fig. 4B).

3.3. Study of α7 nAChR in rat lymphocytes

To study α7 in immune cells of different tissues, we determined its expression pattern in rat splenic and peripheral lymphocytes. In contrast to human cells, no α7 mRNA was detected in these cells. Moreover, α7 was not detected after incubation of the cells with nicotine. Interestingly, DAPI staining and DNA fragmentation analysis of these lymphocytes that do not express α7 nAChR revealed that cortisol-induced apoptosis was not attenuated by the presence of nicotine (10 and 100 μM). For example, in rat spleen cells, the percentage of apoptotic cells related to that obtained in the presence of cortisol alone (100%) was 93.6±3.7% and 108±6.0% (n=3), when 10 and 100 μM nicotine, respectively, were added before cortisol. Similar results were observed in peripheral lymphocytes. These data provide new evidence that the presence of α7 receptor may be in some way involved in the protection of lymphocytes against cortisol-induced apoptosis.

3.4. Detection of mRNA encoding other nAChR subunits

We have also studied the expression of muscle (α1, β, δ, ε, and γ) and neuronal α9 nAChR subunits in human lymphocytes from healthy donors. Lymphocytes from all tested samples contained at least one muscle nAChR subunit. The mRNAs patterns varied among individuals as well as among samples from the same individual obtained at different times. Surprisingly, mRNAs encoding for the different muscle subunits were not all present in the same individual at a given time (Table 3). No γ and α9 subunit mRNAs were detected in freshly isolated lymphocytes from adult donors.

Similar to what was observed in human lymphocytes, the expression pattern of the muscle subunits varied in rat

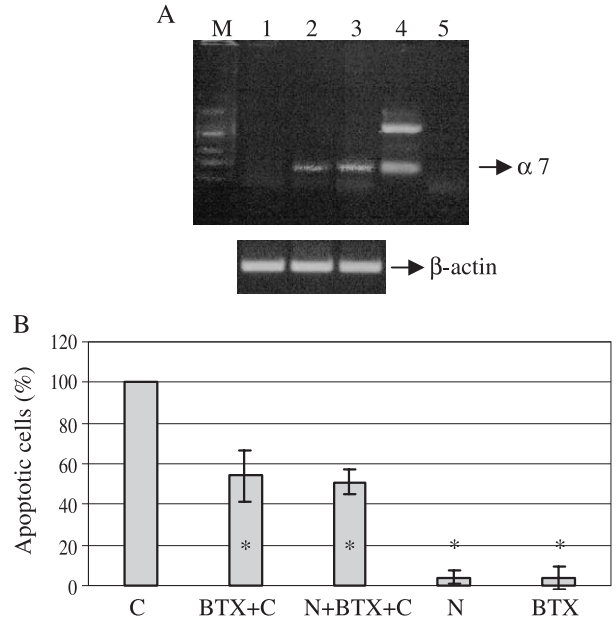

Fig. 4. Effect of α-BTX on lymphocyte apoptosis. (A) Agarose gel of the RT-PCR products from RNA isolated from freshly human lymphocytes and from lymphocytes incubated in the presence and absence of α-BTX (0.5 μM). Transcripts of the α7 nAChR were detected as a band of 190 bp. For semiquantification, β-actin mRNA amplification was used as an internal standard. Sample 1: freshly isolated lymphocytes; sample 2: 48 h incubation in the absence of drugs; sample 3: 48 h incubation with α-BTX (0.5 μM); sample 4: PCR positive control (human α7 cDNA PCR product); sample 5: PCR negative control; M: DNA marker (100 bp ladder). (B) Lymphocytes were first incubated with 0.5 μM α-BTX, and 0.5 μM α-BTX plus 100 μM nicotine (N) and 24 h later were exposed to 10 μM cortisol (C). As a control, cells were also incubated with 100 μM nicotine and 0.5 μM α-BTX but not exposed to cortisol. The degree of apoptosis for each condition was related to that achieved in the presence of cortisol, which was considered 100%. Results are expressed as mean±S.D. of 5 independent experiments. *p<0.05 compared to cortisol-treated cells (C). Apoptosis was evaluated by DAPI staining.

Table 3
Expression of muscle nAChR subunits in human and rat lymphocytes

Sample	α1			δ			ε			β		
	FI	WN	N	FI	WN	N	FI	WN	N	FI	WN	N
Human, w, 40 years, peripheral lymphocytes*	–	–	–	+	+	+	+	+	+	nd		
Human, w, 40 years, peripheral lymphocytes*	–	–	–	–	–	+	nd			+	+	+
Human, w, 27 years, peripheral lymphocytes	–	–	–	+	+	+	–	–	–	nd		
Rat, f, 4 months, spleen	–	+	+	+	+	+	+	+	+	+	+	+
Rat, f, 5 months, spleen	+	+	+	+	+	+	+	+	+	–	–	–
Rat, m, 7 months, peripheral lymphocytes	–	–	–	+	+	+	+	+	+	–	–	–

FI: freshly isolated; WN: without nicotine; N: 100 μM nicotine; w: woman; f: female; m: male; nd: not determined.
*Samples corresponding to the same individual taken at different times.
mRNA corresponding to each subunit was detected by RT-PCR. The predicted product was observed by agarose gel electrophoresis.

lymphocytes (Table 3). Again, not all muscle subunits were present in the same sample of freshly isolated lymphocytes.

In contrast to the results described for human α7, in most of the samples (90%) the expression of muscle subunits (α1, ε, β, and δ) was not influenced by the presence of nicotine (Table 3). The former observation is valid for human and rat lymphocytes. Therefore, upregulation by nicotine seems to be specific for the α7 nAChR.

4. Discussion

In the present study we have detected mRNAs encoding different subunits of the nAChRs in human peripheral lymphocytes and we have observed a high variability in the expression pattern among individuals and within the same individual at different times. Such variability suggests that the level of expression may not only be genetically determined but also regulated by environmental factors, such as infection and physiological stress. In addition, we show that the expression pattern of nAChRs varies among species since α7 nAChR is present in human lymphocytes but not in the corresponding rat cells. Controversial results about the type of nAChR subunits expressed in blood cells can be found throughout the literature (Hiemke et al., 1996; Navaneetham et al., 1997; Sato et al., 1999; Kuo et al., 2002). Due to this high variability, the presence of nAChRs in lymphocytes has remained controversial and the identification of the role of this extraneuronal cholinergic system turns out to be quite difficult.

We have also observed the expression of muscle nAChR subunits in freshly isolated lymphocytes. Controversial results either showing (Toyabe et al., 1997) or discarding (Sato et al., 1999) the presence of muscle nAChR subunits in lymphocytes have been reported before. Although we detected muscle subunits in lymphocytes, the reason why some but not all subunits are transcribed in a given sample remains intriguing given that the four different subunits are necessary for surface expression of the muscle nAChR. This observation is similar to that showing the presence of isolated α6 subunit in the absence of the complementary subunits needed to form functional receptors (Sato et al., 1999). Moreover, muscle nAChR subunits also appear to be expressed in incomplete sets in the thymus (Navaneetham et al., 2001). The expression of γ and α9 subunits was not detected in freshly isolated human lymphocytes. In muscle, the γ subunit is expressed only in embryonic and denervated muscle. After maturation of the end plate, the γ subunit is replaced by the ε subunit. It seems likely that the lack of this subunit in lymphocytes is due to the fact that our study was restricted to adult blood cells. However, a direct correlation between muscle and blood cells cannot be established, since nerve-dependent trophic factors have been described as regulators of the expression of γ and ε subunits in the muscle junction (Witzemann et al., 1996). An alternative explanation can be related to the high variability observed in the nAChRs expression, as it occurs for other subunits. In contrast to our findings, the presence of α9 has been reported previously in peripheral blood lymphocytes (Lustig et al., 2001). Taken together, these results are indicative of a considerable diversity and variability in the expression of nAChR subunits among individual subjects and species. It would be interesting to determine in future work if the nAChR expression is restricted to a specific subset of lymphocytes. In this regard, Richman and Arnason (1979) suggested expression of nAChR in "suppressor" cells. They proposed that the activation of this receptor might result in proliferation or activation of these cells and the subsequent suppression of proliferation in the overall lymphocyte population.

The α7 subunit was detected in most of the tested samples. This observation is in agreement with previous reports (Kawashima and Fujii, 2000). The more reproducible expression of this type of nAChR allowed us to study its role in these immune cells.

On the one hand, we observed that incubation of lymphocytes with nicotine increases α7 expression. Upregulation of neuronal nAChRs due to long-term exposure to nicotine has been described in detail in many neuronal cells (Wonnacott, 1990; Jonnala and Buccafusco, 2001). To our knowledge, the present study is the first report showing that extraneuronal α7 nAChRs are also upregulated by nicotine. On the other hand, we observed that incubation with nicotine protects lymphocytes from cortisol-induced apoptosis, and that this protection is dependent on the concentration (Fig. 3). The observation of a nicotine-induced

protection against apoptosis is in accordance with the neuroprotective effect associated with the presence of the α7 nAChR in primary hippocampal cultures (Li et al., 1999; Dajas-Bailador et al., 2000; Guan et al., 2001; Jonnala and Buccafusco, 2001). According to our results, it is not possible to determine if both phenomena, nicotine-induced α7 upregulation and apoptosis protection, occur in parallel or if the latter is the consequence of the increase in α7 expression.

Although the induction of apoptosis by glucocorticoids in thymocytes is one of the earliest recognized forms of apoptosis, the total genes that mediate cell death have not been identified to date (Distelhorst, 2002). The precise mechanism by which nicotine inhibited the apoptotic induction by cortisol has not been addressed in this study although various hypotheses might be suggested by extrapolating data obtained from other cells. Recent findings have supported the role for the multicatalytic proteasome in corticosteroid-induced apoptosis. Proteasome activation in dexamethasone-treated thymocytes is inhibited by the overexpression of the antiapoptotic protein Bcl-2. In human lung carcinoma cells, nicotine has been shown to stimulate cell survival by regulating Bcl-2 (Mai et al., 2003). The mechanism for this regulation is not completely clear, but a novel nicotine-stimulated survival signal pathway has been suggested (Mai et al., 2003). A relationship between Bcl-2 and nicotine has been also reported by Kihara et al. (2001). This study shows that nicotine, acting through α7 nAChRs, protects neurons from glutamate cytotoxicity. An increase in the levels of Bcl-2 through activation of the phosphatidylinositol 3-kinase cascade is involved in this effect. It would be interesting to determine in future works if Bcl-2 in human lymphocytes is involved in the protection from the cortisol-induced apoptosis mediated by nicotine. In PC12 cells, α7 nAChRs have been shown to mediate antiapoptotic effects through the activation of calcium-dependent protein kinase C (Li et al., 1999). Thus, α7 nAChRs may act through different intracellular transduction processes to modulate cell viability.

Interestingly, we have also observed α7 upregulation as well as apoptosis protection after incubation with α-BTX, a specific antagonist of α7 and muscle nAChRs. This finding led us to suggest that α7, and not other neuronal nAChRs that respond to nicotine but not to α-BTX, is the nAChR subtype involved in the observed effects. However, our findings do not agree with those reports on neurons showing upregulation of α7 but not protection from trophic factor withdrawal toxicity upon exposure to α-BTX (Jonnala and Buccafusco, 2001; Hejmadi et al., 2003). The explanations for this controversy can relay on the capacity of lymphocytes to synthesize ACh (Kawashima et al., 1998). One possibility is that the new nAChRs induced after α-BTX incubation can be activated by the endogenous ACh before being blocked by α-BTX; this activation could be the starting point for the apoptosis inhibition. Under this hypothesis, the activation of α7 would be related to its protective effect. However, newly expressed nAChRs may be instantly blocked by α-BTX. Therefore, a more plausible explanation can be that the long-term exposure to nicotine induces desensitization of the nAChR, thus resulting in the same final effect as the incubation with an antagonist. Under this hypothesis, the blockade of α7 would be involved in the apoptosis protection. α7 nAChRs might act as modulators of the secretion of ACh, which in turn, may have an effect on apoptosis by acting through mAChRs or other types of nAChRs. Regarding this, there is evidence that the stimulation of mAChRs induces Ca^{2+} oscillations and upregulates c-*fos* gene expression in both T- and B-lymphocytes. (Fujii and Kawashima, 2000). Given that c-*fos* is degraded by the proteasome in the early steps of cortisol-induced apoptosis (Distelhorst, 2002), its upregulation could be involved in the apoptosis protection.

The results obtained from our study of rat lymphocytes emphasize the role of α7 in the apoptosis modulation. We did not detect inhibition of apoptosis after incubation with nicotine in rat splenocytes and peripheral lymphocytes that do not express the α7 mRNA. This is an indirect evidence of the action of this neuronal receptor. Apoptosis is a process of paramount importance in maintaining homeostasis, especially in systems where cells are continually being generated and destroyed, such as the immune system. This study suggests that α7 nAChRs in lymphocytes are involved in this process.

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