

## Lymphocytes prime activation is required for nicotine-induced calcium waves

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## 1. ABSTRACT

Lymphocytes are reported to express nicotinic acetylcholine receptors (nAChR). However, no data are available on the expression of these nAChR on activated lymphocyte relatively to resting lymphocytes. In this study, we examined nAChR subunits expression in PHA-stimulated versus un-stimulated lymphocytes, and four leukemic cell lines. Cell stimulation with nicotine triggered calcium responses only in some experiments conducted with PHA-stimulated lymphocytes. Likewise, only the Jurkat and HL-60 cell lines displayed calcium waves upon nicotine stimulation, whereas the Raji and CCRF-CEM did not. All responding cells displayed an active form of the nicotinic  $\alpha 7$  nAChR. Indeed, use of 2 different sets of primers for the corresponding mRNA showed that expression of the full-length  $\alpha 7$  subunit mRNA was only present in PHA-stimulated lymphocytes for which calcium waves had been evidenced. Microscopy analysis of lymphocytes structure showed a direct relationship between their size, their  $\alpha 7$  nAChR expression, and calcium release upon nicotine stimulation. Then, this relationship suggested that lymphocytes need a prime activation to express the  $\alpha 7$  nAChR, and therefore to release calcium in response to nicotine.

## 2. INTRODUCTION

Nicotinic acetylcholine receptors (nAChR) are members of the family of ligand-gated ion channels. Eleven genes encoding neuronal nAChR different subunits have been identified in the human genome including  $\alpha 2$ - $\alpha 7$ ,  $\alpha 9$ ,  $\alpha 10$  and  $\beta 2$ - $\beta 4$  (1). These subunits associate into functional homopentamers composed exclusively of  $\alpha$  subunits, or heteropentamers, which result from the combinatorial association of three  $\alpha$  and two  $\beta$  subunits (2-5).

These members of the superfamily of ligand cation channels form the predominant excitatory neurotransmitter receptors on muscles and nerves. For many years, it has been showed that  $\text{Ca}^{2+}$  influx was observed after activation of nAChR in post-junctional membrane. All nAChR form ion channels but the ionic permeability is dependent upon the subunit composition of the receptor. Homomeric nAChR are believed to have the largest calcium permeability among the family of nAChR, although this function is also displayed by heteromeric nAChR. The type of nAChR  $\alpha$  subunits seems to play an important role in calcium flux. For instances, calcium permeability of heteromeric receptors is enhanced by presence of regulator subunit such as  $\alpha 5$  (6), whereas the most completely characterized homopentamer constituted

## $\alpha$ -7 nAChR expression in activated lymphocyte

of  $\alpha$ 7 subunit display a high permeability for calcium (7;8). This discrimination of effects bound to the  $\alpha$ 7 subunit among others was achieved through specific inhibition using low dose of  $\alpha$ -bungarotoxin (1).

The physiological effects of nicotine have been genuinely and mostly described in the human brain with particular focus on their role in tobacco addiction and mental illness (9). However recent advances on their physiological functions have provided a better insight into possible functional roles for nAChR in non-excitabile cells (10-13). For instances, neuronal nicotinic receptors in non-neuronal tissues have been proposed to modulate cell proliferation, differentiation or apoptosis (14-18). In addition to the central nervous system, studies of the last decade also convincingly demonstrated the presence of these receptors in various tissues such as the respiratory tract, skin vascular and immune tissues (19-23) suggesting that nAChR display other function than those genuinely described.

Nicotine inhaled in cigarette smoke quickly diffuse in blood circulation, and interacts with myeloid or lymphoid derived cells. Presence of nAChR on various blood cells, including lymphocytes, has been suggested by the binding of cholinergic radioligands and analysis of different subunit mRNA expression. Lymphocytes stimulation with nicotine modulates apoptosis and cell cycle entry (18;24). We recently demonstrated that stimulation of nAChR on HL60 leukemia cell line by nicotine led to an alteration of the chromatin structure and was associated with an increase of the early gene expression *c-fos* (25). Other studies showed that nicotine prevents activation in T and B cells (26; 27) and promotes proliferation of normal mouse lymphocytes (28). Hence, discrepancies between these reports could be linked to changes in nAChR expression.

Despite the determination of nAChR subunits mRNA expression on peripheral blood lymphocytes and leukemic cell lines, no data are available on the expression of these nAChR on activated lymphocyte relatively to resting lymphocytes. Therefore, we performed experiments to determine whether nAChR expression is modulated following T-cell activation following PHA stimulation. To improve the knowledge about the role of these nAChR in lymphocytes functions, we also investigated in this study the effect of nicotine on calcium release, a relevant property regarding leukocyte activation. Calcium variations following nicotine treatment of stimulated PBL, but not quiescent PBL, were related to the expression of  $\alpha$ 7-nAChR subunits at both mRNA and protein levels. This relationship was further confirmed using 4 leukemic cell lines.

## 3 MATERIALS AND METHODS

### 3.1. Reagents

Nicotine tartrate and alpha-bungarotoxin were purchased from Sigma Chemical Co. (St. Louis, MO,

USA). Phytohemagglutinin (PHA) is directly present in culture medium PB-Karyotyping (GIBCO-BRL, Grand Island, NY).

### 3.2. Cell culture

After giving informed consent, healthy no smokers blood donors underwent mononuclear cell leukapheresis using a continuous-flow cell separator (Spectra, COBE laboratory, Lakewood, CO, USA) and ACD-A anticoagulant (Maco Pharma, Tourcoing, France). Human peripheral blood lymphocytes were obtained from leukapheresis sediments using countercurrent elutriation (29).  $5 \times 10^6$  PBL (106 cells/mL) were cultivated in RPMI 1640 medium containing 1% glutamax (GIBCO-BRL, Grand Island, NY) supplemented with 10 % fetal calf serum (FCS) in a well of a 6-well plate during 7 days (PB-Max medium diluted to 1/5) at 37°C in a humidified 95% air, 5% CO<sub>2</sub> atmosphere in the presence or not of PHA during 7 days.

The human T-cell lymphoma-derived cell lines Jurkat and CCRF-CEM, the Raji (Burkitt's-derived) B-cell line and the human promyelocytic leukemia cell line HL-60 were obtained from ATCC, Bethesda, Md. (ATCC TIB-152, ATCC CCL-119, ATCC CCL-86 and ATCC CCL 240). The Cell lines were grown in RPMI 1640 medium containing 1% glutamax supplemented with 10 % (CCRF-CEM and Jurkat cells), 15 % (Raji cells) and 20 % (HL-60 cells) of FCS, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin and 0.025  $\mu$ g/mL amphotericin B (GIBCO-BRL) at 37°C in 5% CO<sub>2</sub> atmosphere.

In each experiment and at each step of the procedure, viable cells were evaluated by trypan blue exclusion test (200 cells were numbered).

### 3.3. Calcium influx assay

The concentration of  $(Ca^{2+})_i$  in different blood cells and four leukemic cell lines was measured following nicotine stimulation ( $10^{-2}$ M) with the calcium-sensitive Fura-2 acetoxymethyl ester by a modified fluorescence ratioimetric method (30). At the end of culture, cells were loaded for 50 min with 3  $\mu$ mol/L Fura-2 acetoxymethyl ester (Sigma Aldrich) in culture medium containing 10 mmol/L HEPES, washed in the same medium, and allowed to recover in a 5% CO<sub>2</sub> incubator at 37°C for 60 min. The chamber slide was placed on the preheated stage (37°C) of an inverted Nikon TE300 microscope equipped with an incubator chamber. The Fura-2 fluorochrome was excited every 4 s during 120 seconds and sequentially at wavelengths of 340 and 380 nm generated by a Polychrome II monochromator (TILL Photonics, Planegg, Germany); the emission at 510 nm was detected with a x40 Plan Fluor objective (Nikon) and a cooled charge-coupled device camera (Micromax; Roper Scientific, Evry, France). In all experiments, increments of  $(Ca^{2+})_i$  evoke a positive signal at 340 nm and a negative signal at 380 nm. The wavelengths of excitation and emission, the time course of image acquisition, and the image treatment were controlled by the computer software Metafluor (Universal Imaging, West Chester, PA). The fluorescence was quantified by averaging pixel intensities in the cell area of interest to calculate the ratio  $R = F_{340}/F_{380}$ .

**Table 1.** nAChR subunits and GAPDH RT-PCR primers and conditions

nAChR subunits	Optimal temperature	Annealing	Product size (bp)	Forward primers 5'-3'	Reverse primer 5'-3'
alpha 2	55°C		466	CCGGTGGCTTCTGATGA	CAGATCATTCCAGCTAGG
alpha 3	55°C		409	GCTGGTGAAGGTGGATGAAGTAAA	CTCGCCGCTCTCCCAATAGTCC
alpha 4	55°C		399	TTCTTCGGTTACAACAAGTGGTC	CTGCTGGTCGAAGGGGAAGA
alpha 5	55°C		265	TCATGTAGACAGGTACTTC	ATTGCCCATTATAAATAA
alpha 6	57°C		413	GGCCTCTGGACAAGACAA	AAGATTTTCCTGTGTTCCTCC
5'-end alpha 7	55°C		399	GACCAGCCTCCGTAAGACCAG	GGCCAATGACTCGCAACCACCT
3'-end alpha 7	60°C		490	GGCAGATATCAGTGGCTATATATC	CTTCATTGGCAGGAACC
Beta 2	55°C		347	CAGCTCATCAGTGTGCA	GTGCGGTCTAGGTCCA
Beta 4	52.8°C		310	CTGAAACAGGAATGGACT	CCATGTCTATCTCCGTGT
GAPDH	60°C		110	TGGGGAAGGTGAAGGTCGGA	GAAGGGGTCATTGATGGCAA

### 3.4. RNA isolation, Reverse Transcriptase RT-PCR

Total RNA was isolated from  $5.10^6$  cells by guanidine thiocyanate-phenol chloroform method (Tri Reagent, Sigma Chemical Co.). Samples (1  $\mu$ g) were reverse-transcribed using reverse transcriptase and oligo (dT)<sub>12-18</sub> primers (GIBCO-BRL). Polymerase chain reactions (PCR) were performed in a Perkin-Elmer 9600 thermal cycler using the Fast Start Taq DNA polymerase (Roche Diagnostics). Reactions were started with 95°C for 5 min, followed by 40 cycles (nAChR subunits) or 30 cycles (*GAPDH*) of 95°C for 30 s, annealing temperature for 30 s, 72°C for 90 s, and 72°C for 7 min, and finally kept at 4°C. The gene specific oligonucleotides primers used to determine mRNA expression of different nAChR subunits and the corresponding annealing temperature were described in table 1. The expression of the housekeeping *GAPDH* gene was used as control to ensure the similarity in quality and quantity of RNA samples amplified by RT-PCR. PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide. A 100 base pair DNA Ladder (MBI Fermentas) was used as molecular weight standard. Non-reverse-transcribed RNAs were used as a template to control some eventual contaminations at every step of the procedure. Bands were visualized by UV transillumination using ethidium bromide (Sigma Chemical Co) staining prior to photography and analysed by photoimaging (Appligen, Oncor).

### 3.5. Immunostaining

For detection of different nAChR subunits, cells were spun and air-dried. Then cells were fixed, first in methanol (5 min at -20°C) then in acetone (3 min at -20°C). The quenching endogenous peroxidase activity was performed with a solution of 3 % hydrogen peroxyde in PBS supplemented with 2 % goat IgG-Fc (Jackson ImmunoResearch Lab., West Grove, PA, USA) for 20 min. Non-specific sites were blocked for 5 min with 4 % goat IgG-Fc in PBS. Then the antibody against  $\alpha$ 7 nAChR subunit (R & D Ab, Benicia, CA, USA) (1:100 in PBS + 2 % goat IgG-Fc) was applied (2h). The immunoperoxidase staining was revealed (Vectastain Elite ABC kit) with NovaRED<sup>TM</sup> substrate (Vector Laboratories).

## 4. RESULTS

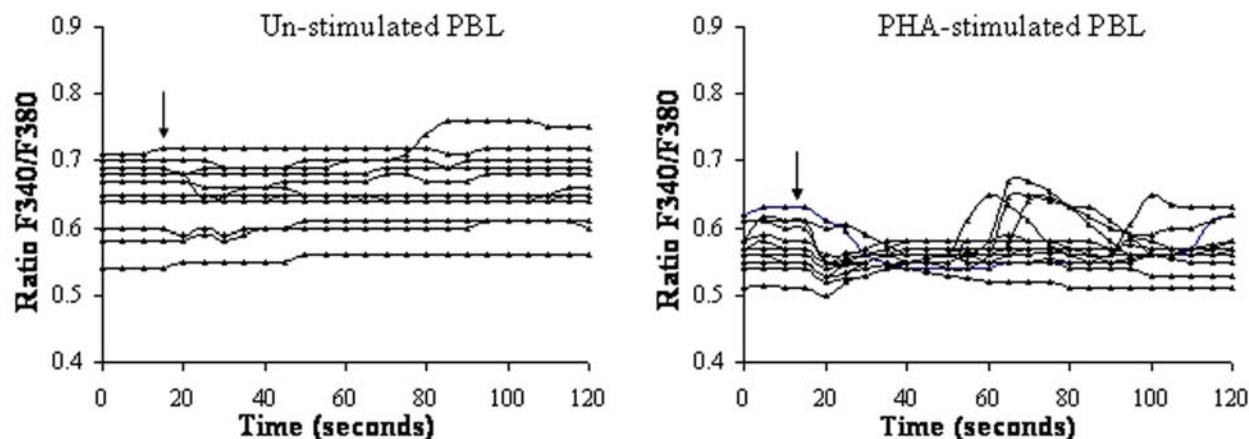
### 4.1. Nicotine induces calcium influx in PHA-stimulated lymphocyte

Analysis of intracellular calcium content with the calcium-sensitive Fura-2 probe showed that calcium basal level slightly varied from cell to cell, but not significantly

between un-stimulated cells and PHA-stimulated cells ( $0.65 \pm 0.06$  and  $0.57 \pm 0.03$  respectively, Figure 1). However, only some of the PHA-stimulated PBL displayed a calcium wave in the presence of nicotine, whereas no variation of the basal calcium signal was detected in un-stimulated PBL (Figure 1). This effect observed in PHA-stimulated PBL could not be reproduced with PBL of all donors (data not shown). Considering the key role that calcium play in leukocyte activation, it was therefore of importance to deeper investigating the link between calcium increase in PHA-stimulated PBL upon nicotine treatment and expression of the different nAChR. Then, we next aimed at determining nAChR expression by lymphocyte stimulated or not.

### 4.2. Stimulation of PBL by PHA modulates nicotinic receptor expression

Distribution of nAChR was determined at the mRNA level by RT-PCR, using the *GAPDH* gene as an internal control (Figure 2). Analysis performed on 5 donors demonstrated that nAChR subunits expression varied following PBL stimulation with PHA compared to un-stimulated PBL (Figure 2 lanes 1-5 compared to lanes 6-10). In non-stimulated PBL, PCR products corresponding to the  $\alpha$ 2,  $\alpha$ 3 and  $\alpha$ 7 (3'-end) -subunit mRNAs were observed, whereas the  $\alpha$ 6 and  $\beta$ 2 subunits were weakly expressed, and the  $\alpha$ 4,  $\alpha$ 5 and  $\beta$ 4 subunit mRNAs were not (Figure 2). In all experiments, all PCR products were of the expected sizes. A positive control from human cultured motoneurons (provided by D. Petite, INSERM 583-“Instituts des Neurosciences de Montpellier”, France) was used for the  $\alpha$ 4 subunit to verify that the lack of expression of this nAChR subunit in human PBL was not linked to troubleshooting in the experiments. Compared to un-stimulated PBL, the main difference in nAChR expression observed in PHA-stimulated PBL consisted in an important increase of the  $\alpha$ 6 subunit mRNA and, to a lesser extent, of the  $\alpha$ 5 and  $\beta$ 4 subunit mRNAs. The  $\alpha$ 7 (3'-end) -subunit mRNAs were also observed in all PHA-stimulated lymphocyte. The presence of an inactive  $\alpha$ 7-nAChR form has been previously described by Gault and coll. (31). According to these authors, the 2 forms of the  $\alpha$ 7-nAChR can be discriminated at the mRNA level by RT-PCR using a set of primers located in the N-terminal segment of the corresponding protein. In this setting, an inconsistent expression of  $\alpha$ 7 subunit mRNA was observed in stimulated PBL when defined with a set of primers localised in the 5' end of the  $\alpha$ 7 mRNA (one case out of 5 in PHA-stimulated PBL, Fig2, lane 6), whereas no expression was observed un-stimulated lymphocyte (Figure 2).



**Figure 1.** Effect of nicotine on intracellular calcium wave generation in human peripheral blood lymphocytes. PBL were stimulated or not with PHA. Nicotine ( $10^{-2}$ M) (arrow) was added 15 sec after the beginning of calcium measures. The level of intracellular calcium was estimated by the *ratio* of fluorescence emission signals after Fura2-acetoxymethyl ester excitation at 340 and 380 nM. Each curve represents variations of intracellular calcium level in one cell. The data presented are representative of 3 individual experiments.

#### 4.3. Calcium influx and expression of nicotinic receptors by various leukemic cell lines

The relationship between calcium waves and nicotine receptors expression was further investigated through analysis of the Jurkat, CCRF-CEM, HL-60 and Raji cell lines. A rapid and transient variation in calcium influx upon nicotine treatment was observed in the T-lymphocyte Jurkat cell line (Figure 3 A). In our experimental conditions, this effect was not cell lineage-dependent since another T-cell line (CCRF-CEM) did not display calcium variation in response to nicotine stimulation (Figure 3 B). In this setting, calcium waves were also observed in the promyelocyte HL-60 cell line (Fig3 C), although the profile of the calcium curves was different than those observed in Jurkat cells (Figure 3 C). Stimulation of HL-60 by nicotine resulted in a series of repetitive oscillations, whereas Jurkat cells displayed a single wave (Fig 3 A and C). Finally, no variation in calcium content of the B-lymphocyte Raji cell line was observed upon nicotine treatment (Figure 3 D).

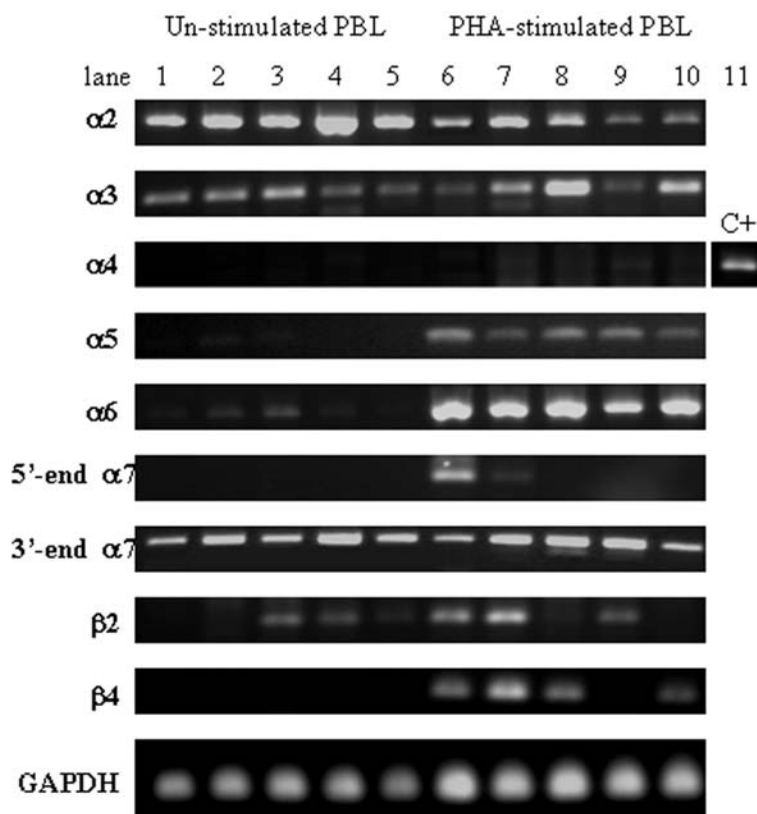
Analysis of nAChR subunit expression in these 4 cell lines revealed different nicotinic subunits pattern (Figure 4). The expression pattern of nAChR subunits mRNA showed that the  $\alpha$ 2,  $\alpha$ 5 and  $\alpha$ 6 subunits were expressed in all cell lines, whereas the  $\alpha$ 3,  $\beta$ 2 and  $\beta$ 4 were hardly expressed in HL-60 cells, eliminating those subunits as mediators of calcium response (Fig4A). We also performed RT-PCR experiments with the two set of primers related to the  $\alpha$ 7 subunit protein. Both set revealed that  $\alpha$ 7 mRNA subunit expression was mainly (3'-end) or exclusively (5'-end) in Jurkat and HL-60 cells, the two cell lines that displayed a nicotine-mediated calcium effect. Pre-treatment of Jurkat cells with  $\alpha$ -bungarotoxin, an inhibitor of  $\alpha$ 7 nicotinic subunit abolished nicotine-induced calcium influx in those cells (Figure 4B).

#### 4.4. Expression of the $\alpha$ 7 nicotinic receptor protein by leukemic cell lines and human PBL

We then examined the  $\alpha$ 7-nAChR subunit protein expression by immunohistochemistry on the 4 cell types (Figure 5A) and in PBL (Figure 5B). The Jurkat and HL-60 cell lines largely expressed the  $\alpha$ 7 protein, whereas both Raji and CCRF-CEM cells labelling was rather weak and diffuse (Figure 5A). However, using human PBL, the  $\alpha$ 7 nAChR subunit protein was observed in PHA-stimulated as well as in resting lymphocytes (Figure 5B). These  $\alpha$ 7-nAChR subunit protein expressions were in keeping with the 3'-end  $\alpha$ 7 nAChR subunit mRNA expression observed in these cells (Figure 2 and 4).

#### 4.5. Nicotine stimulates calcium influx through activation of the $\alpha$ 7 subunit in stimulated PBL

To further investigate the link between the  $\alpha$ 7 nAChR subunit expression and calcium response to nicotine stimulation, we then examined the expression of the 5'-end  $\alpha$ 7-nAChR mRNA in PHA-activated PBL from 3 donors selected according to their capacity to generate calcium waves upon nicotine stimulation. Figure 6A shows that following PHA-activation, PBL from donor 1 displayed a larger PCR band than PBL from donor 2, whereas no PCR product could be observed in PBL from donor 3. Calcium response following nicotine stimulation from PHA-activated PBL also varied accordingly to donors, with a higher effect in PBL from donor 1 compared to donors 2 and 3 (Figure 6B). Concomitantly, we observed that only a fraction of PHA-activated PBL responded to nicotine by generating a calcium wave. Microscopy analysis of PBL activation by PHA revealed heterogeneous structural changes in these cell populations. To get a deeper insight into this structural/activity relationship, we combined data about microscopy structure analysis of the different PHA-stimulated PBL subpopulation and data about calcium release. In PBL from donor 1, which



**Figure 2.** Expression of nAChR subunits mRNA in human peripheral blood lymphocytes. PBL were cultured as described in figure 1. Lanes 1 to 5, and 6 to 10 correspond to un-stimulated-PBL and PHA-stimulated PBL respectively from 5 different donors. Expression of nAChR mRNA subunits was performed by RT-PCR using specific primers (see table 1 for the expected size of amplified fragments). cDNA of human motoneurons was used as a positive control (C+) for  $\alpha$ 4 subunit expression of nAChR (line 11). GAPDH mRNA was used as control.

strongly expressed the 5'-end  $\alpha$ 7 mRNA, we found a positive correlation between PBL surface area and PBL intracellular calcium release (Figure 6C). The regression curve revealed a linear correlation coefficient of  $r = 0.85$ . However, no such correlation could be drawn from donors 2 and 3 (Figure 6C).

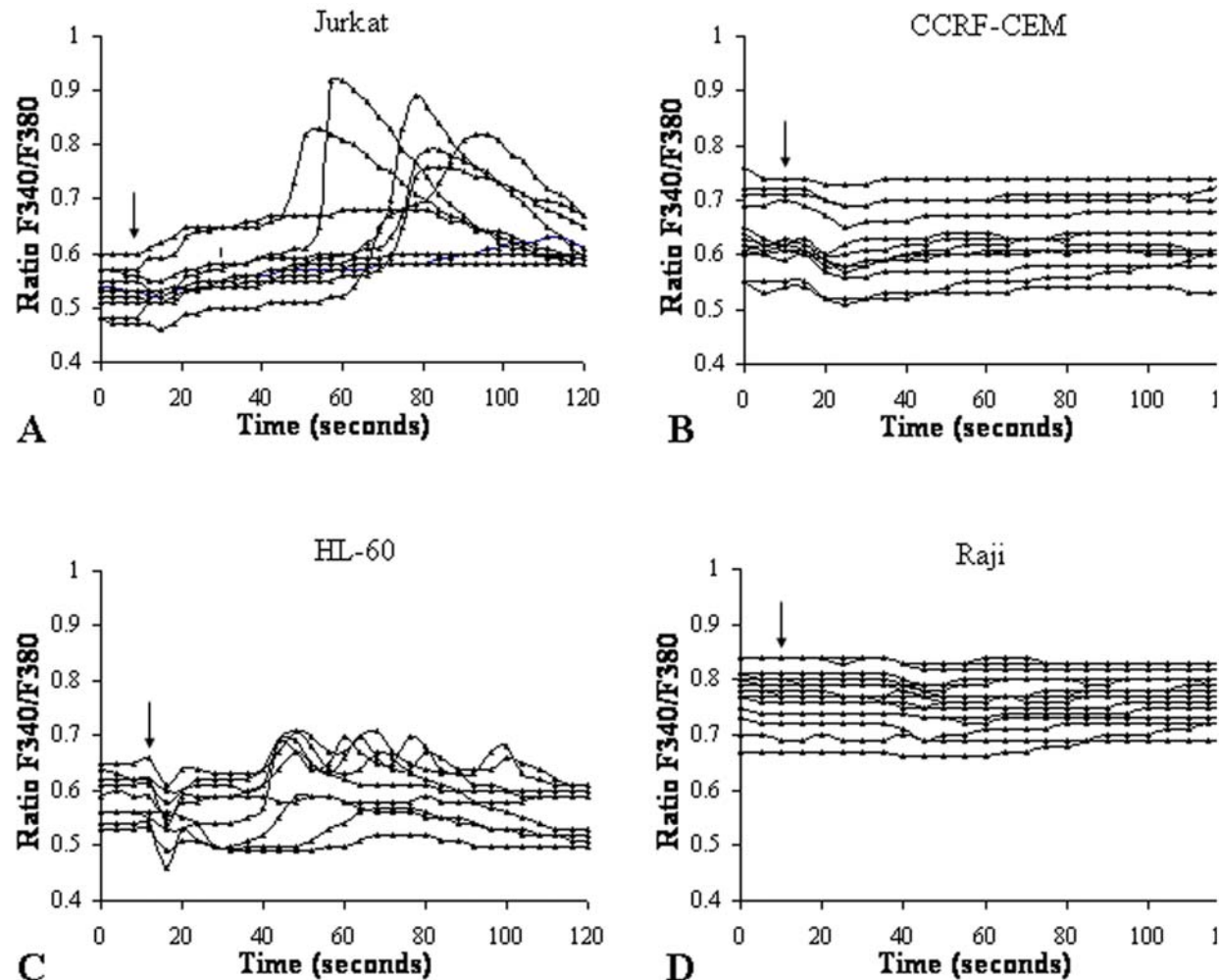
## 5. DISCUSSION

In this study, we have examined the effects of nicotine on calcium flux in PHA-activated lymphocytes compared to un-stimulated lymphocytes. With this regard, we delineated a close relationship between the  $\alpha$ 7 subunit expression, lymphocyte activation, and the ability of stimulated lymphocytes to generate a calcium signal.

PHA stimulation of leukocytes resulted in a decrease of the  $\alpha$ 2 and an increase of the  $\alpha$ 5,  $\alpha$ 6 and  $\beta$ 4 subunits compared to resting lymphocytes. Although nAChR subunits had been previously described on human mononuclear (23;32), this is to our knowledge, the first report demonstrating that expression of these receptors differs according to cell stimulation with PHA. A similar effect was observed when PBL were stimulated with IL-2, although the magnitude in the effects was not as important

as those observed in PHA-stimulated PBL (data not shown). This is in setting with an increased IL-2 expression following Lymphocyte activation by PHA (33). Thus, differential nAChR expression according to cell activation could explain the different nAChR patterns described in the literature. For instances, expression of the  $\alpha$ 2 but not the  $\alpha$ 6 subunits reported in the study from Sato and Colleagues is consistent with the fact that nicotinic receptors were defined in non-stimulated cells. However, they also reported the presence of the  $\alpha$ 5 subunit in PBL from each donor, whereas expression of this subunit was restricted to stimulated PBL in our study, hence suggesting that other parameters might influence NACHR expression. Several possibilities might account for differences of expression such as genetic origin (Asian versus European) and influence of environmental factors (smokers versus non-smokers). Although further experiments will be required to unravel the consequence of variation in nAChR expression, the difference of expression observed is of medical importance since recent reports demonstrated that nicotine receptors are associated to the development of genetically transmitted diseases (34-39)

Calcium waves occurred only occasionally in PHA and IL-2 stimulated PBL, discarding a direct link with

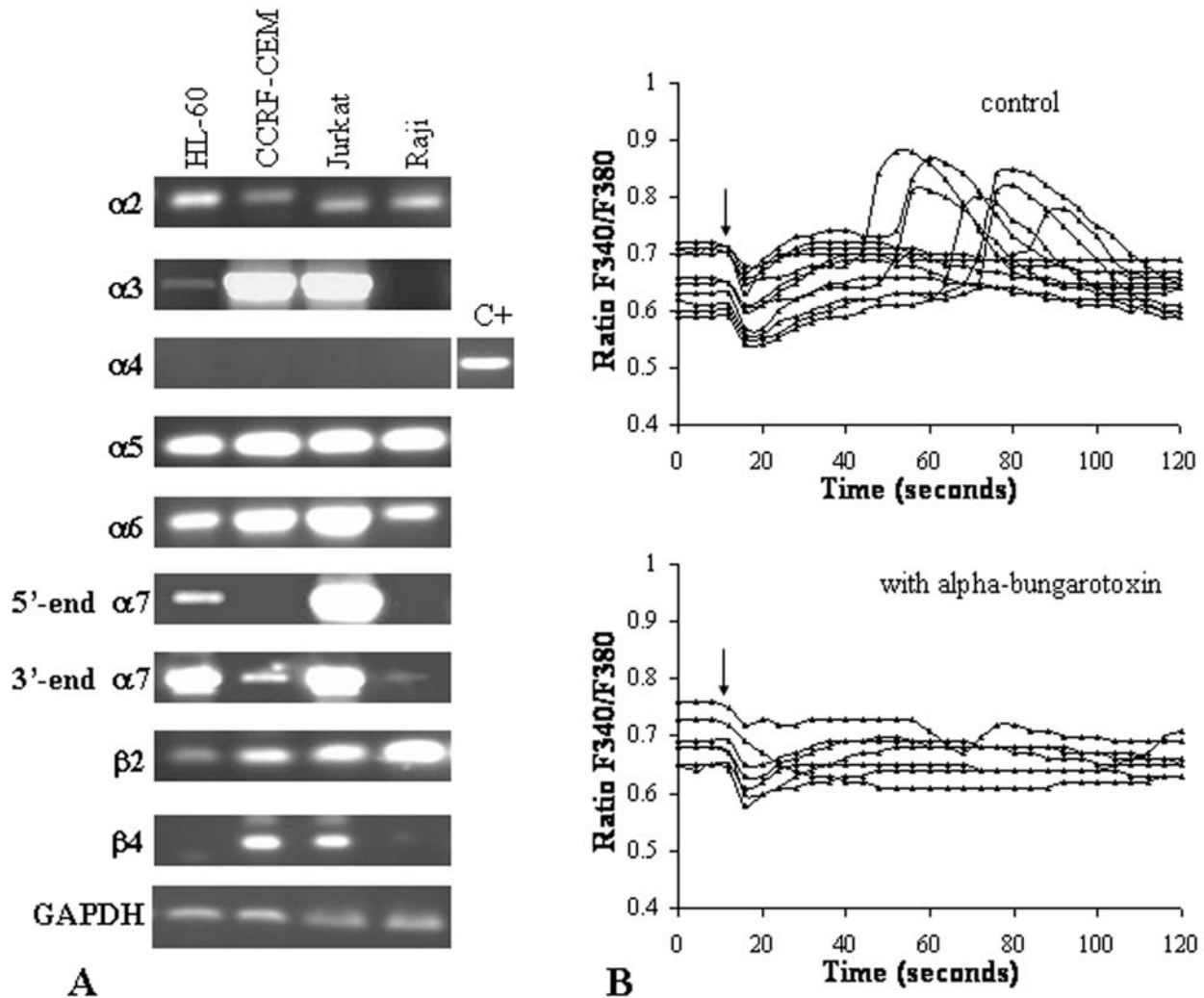


**Figure 3.** Effect of nicotine on intracellular calcium wave generation in human leukemic cell lines. The experiments have been conducted like in figure 1 on four different cell lines derived from T lymphocytes (Jurkat (A) and CCRF-CEM (B)), promyelocytes (HL-60 (C)) and B lymphocytes (Raji (D)). Each curve represents variations of intracellular calcium level in one cell. The data presented are representative of 3 individual experiments.

the  $\alpha$ 2,  $\alpha$ 5,  $\alpha$ 6, and  $\beta$ 4 subunits expression whose expressions were constant inside each group. This is in agreement with previous data showing that heteromeric receptors containing the  $\alpha$ 2 and the  $\alpha$ 6 subunits are less permeable to calcium than homopentameric receptors (40). The main prototype of homomeric pentamer receptor related to calcium signal is constituted of the  $\alpha$ 7 subunit (8). In our study, the  $\alpha$ 7 subunit mRNA displayed a specific pattern of expression, when determined with a set of primers designed against the related 5'-end of mRNA (N-ter of the protein). Like for calcium results, these PCR products were only present in some donors and only after lymphocyte stimulation. Conversely, immunohistochemistry of the  $\alpha$ 7 nAChR subunit revealed the presence of the  $\alpha$ 7 protein in stimulated as well as in non-stimulated lymphocytes. Then, this protein expression, obtained with an  $\alpha$ 7 antibody raised against the C-ter extremity of the protein fit with the results observed in PCR experiments obtained with the 3'-end set of primers. In the

literature, detection of the  $\alpha$ 7-nAChR subunit mRNA was also controversial until a partial duplication of the gene was described leading to an inactive form of  $\alpha$ 7-nAChR (31). The corresponding dupalpha7 protein was previously shown to be expressed in leukocytes but its physiological role could not be identified (41). In our model, the differential expression of the  $\alpha$ 7-nAChR subunit mRNA in lymphocytes observed with the different sets of primers is in accordance with the expression of a constitutive inactive and of an induced active forms of the  $\alpha$ 7-nAChR.

In order to delineate a relationship between the  $\alpha$ 7 subunit expression and calcium flux, we alternatively used leukemic cell line models. Among the 4 cell lines studied, the Jurkat cells showed intense and similar quantities of PCR product with both set of primers, suggesting that mainly the genuine  $\alpha$ 7-nAChR subunit mRNA was expressed in this cell type. Conversely, HL-60 cells expressed predominantly the dupalpha7 form as



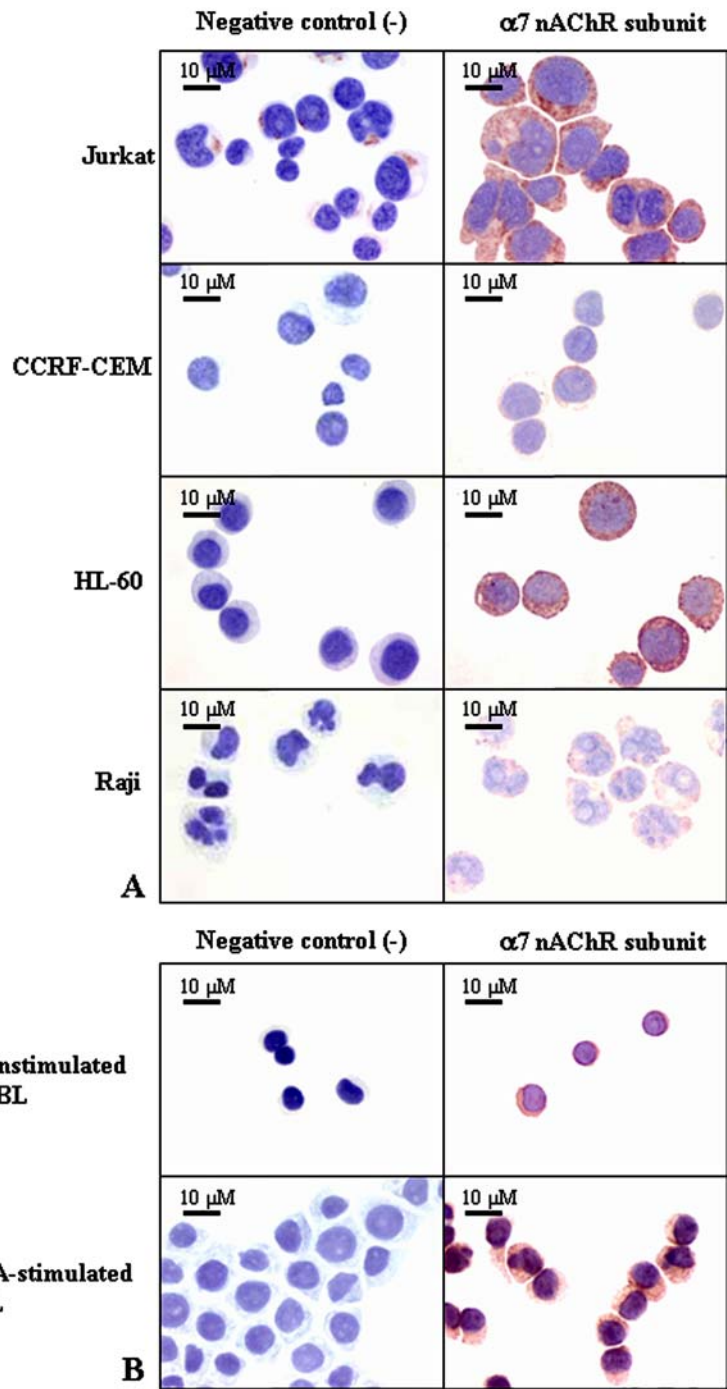
**Figure 4.** Relationship between nAChR expression and calcium release in human leukemic cell lines. A. Expression of nAChR mRNA subunits in leukemic cell lines. Expression of nAChR mRNA subunits was performed by RT-PCR using the same RT-PCR conditions as in figure 2. Data are representative of 2 independent experiments. B. Effect of alpha-bungarotoxin on intracellular calcium release in Jurkat cells following nicotine stimulation. Calcium experiments were conducted as described in figures 1 and 2 apart from the fact that cells were pre-incubated with alpha-bungarotoxin ( $10^{-5}$ M) during 30 minutes before nicotine addition. Each curve represents variations of intracellular calcium level in one cell. The data presented are representative of 3 individual experiments.

demonstrated by a strong PCR band with the 3'-end set of primers, whereas the 5'-end set of primers gave only a weak band. Beside, Jurkat cells also were associated with calcium waves following nicotine stimulation. Abrogation of nicotine effects on calcium variation by  $\alpha$ -bungarotoxin in Jurkat cells confirmed that the  $\alpha$ 7 subunit was expressed under an active form. Indeed, the  $\alpha$ 7 nicotinic receptor antagonist  $\alpha$ -bungarotoxin binding domain is located in the extracellular N-Terminal domain of the protein, which is missing in the duplicated inactive form (41). Accordingly, expression of the genuine  $\alpha$ 7 nAChR subunit was only present in PBL that displayed a calcium wave upon nicotine stimulation, whereas the dupalpha7 protein was present in all lymphocytes. Then, our results on leukemic cell lines support the fact that the dupalpha7 protein subunit

is constitutively expressed in PBL but under an inactive form with regard to calcium flux. This also underscores the Jurkat cells as a model cell line to investigate the functions associated to the active  $\alpha$ 7 nicotinic receptor. Besides, HL-60 which expresses both  $\alpha$ 7 nicotinic receptor forms, also responded to nicotine by generating a series of repetitive oscillations. However, this specific pattern might not be linked to interferences between both forms of the  $\alpha$ 7 receptor since this co-expression, also observed in human lymphocytes, did not lead to multiple calcium oscillations upon nicotine stimulation.

In this study, we demonstrated that nicotine initiates a rise in intracellular calcium concentration only in cells that have been pre-activated by PHA, suggesting that



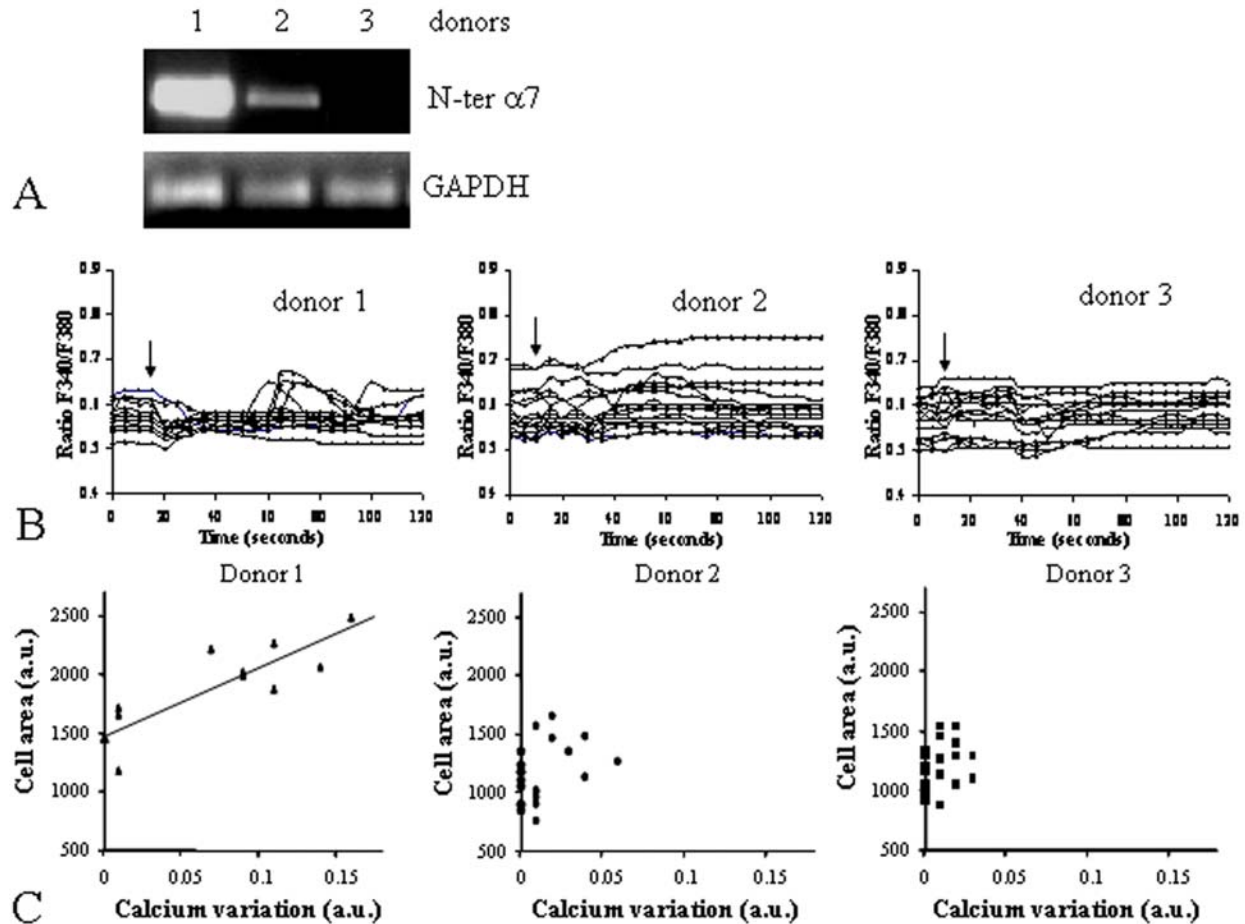


**Figure 5.** Expression of  $\alpha$ 7 nAChR protein subunit in human PBL and leukemic cell lines in various cell lines (A) and in human peripheral blood lymphocytes (B). The  $\alpha$ 7 nAChR protein subunit expression was visualized in the four leukemic cell lines (A) and in PHA-stimulated or not PBL (B) by immunocytochemistry. Cells were stained with  $\alpha$ 7 nAChR antibody (brown reaction product), and counterstained with hematoxylin. Panels displayed on the left column show negative control (-) realized without primary antibody.

T-cells need a primo-activation to express the functional  $\alpha$ 7 nicotinic receptors. PBL stimulated with PHA displayed reduced intracellular basal calcium level compared to unstimulated cells. Similarly, basal calcium level was lower in

Jurkat cells than in HL-60 and the two others cells lines that did not respond to nicotine by displaying a calcium wave (average ratio F340/F380: 0.53; 0.59; 0.65; 0.76 for the Jurkat, HL-60, CCRF-CEM and Raji cells respectively).





**Figure 6.** Correlation between PBL surfaces, calcium waves and 5'-end  $\alpha$ 7 mRNA expression in PHA-stimulated PBL. A. Expression of 5'-end  $\alpha$ 7 mRNA in PHA-stimulated PBL. B. Intracellular calcium release upon nicotine stimulation in human PHA-stimulated PBL. Levels of intracellular calcium of PHA-stimulated PBL were estimated as described in figure 1. C. Dispersion diagram between PHA-stimulated PBL areas and intracellular calcium variation induced by nicotine addition. Each point represents intracellular calcium variation induced by nicotine addition in one cell.

A low intracellular calcium level could represent a required step for PBL to respond to nicotine. However this low calcium level was not the only prerequisite step, as all PHA-stimulated PBL did not display a calcium wave following nicotine stimulation. *In vitro*, PHA mimics lymphocyte B and T proliferation and activation by antigen, and trigger these cells into mitotic division. This activation, followed by cell proliferation, is characterized by an increase of nuclear and cellular morphology, which is concomitant with an increase in gene expression and protein synthesis (42;43). We also found from microscopy experiments that expression of the  $\alpha$ 7 subunit was associated with an increase in PBL size. Furthermore, compared to non-responding cell, only cells responding positively to nicotine (as measured by calcium input) showed an increase in cell surface from  $1506 \pm 195$  a.u. to  $2111 \pm 208$  a.u. ( $p < 0.05$ ). This permits us to delineate a relationship between the PBL size, the  $\alpha$ 7 subunit receptor expression and the transient calcium increase following nicotine stimulation. Calcium is generally assumed to be an

essential messenger for T-cell activation (44). Physiologically, one of the events that follow calcium signal is the production of the autocrine growth factor IL-2, which converts T-cells from a stimulus dependent to an autonomous replicative cell (45). Then, although nicotine effects on calcium release are transient, they could potentiate IL-2 effects and generate a commitment period required to initiate a proliferation process (46). Accordingly, we demonstrated in a previous study that a short-term treatment of HL-60 with nicotine at concentration observed in peripheral blood of smokers induced chromatin structural changes, an epigenetic modification associated with the expression of the transcription factor AP-1 (25).

Even so the molecular mechanisms responsible for the expression of the active form of the  $\alpha$ 7 subunit still need to be unravelled (genetic, epigenetic, environmental...), our results demonstrate that lymphocytes need a prime activation process to express the active  $\alpha$ -7 nicotinic receptor

## 6. ACKNOWLEDGMENTS

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**Abbreviations:** bp : base pair , C-ter : C-terminal region, DNA : deoxyribonucleic acid , ER: Endoplasmic reticulum, f : forward, FCS : foetal calf serum, IgG-Fc : Fc region of immunoglobulin G, IL-2 : interleukin 2,  $\mu$ g : microgram , mRNA : messenger ribonucleic acid, nAChR : nicotinic acetylcholine receptors , N-ter : N-terminal region, PBL : peripheral blood lymphocytes, PHA : phytohemagglutinin, r : reverse, RNA : ribonucleic acid, RT-PCR : reverse-transcriptase polymerase chain reaction , UV: ultraviolet

## **$\alpha$ -7 nAChR expression in activated lymphocyte**

**Key Words:** Lymphocytes, Nicotine, Nicotinic Receptor, Calcium

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