Biochimie 95 (2013) 2376–2384

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

$GABA_A$ receptor plasticity in Jurkat T cells

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ARTICLE INFO

Article history:
Received 29 May 2013
Accepted 25 August 2013
Available online 4 September 2013

Keywords:
$GABA_A$ receptors
Lymphocytes
Plasticity
GABA

ABSTRACT

$GABA_A$ receptors ($GABA_AR$) mediate inhibitory neurotransmission in the human brain. Neurons modify subunit expression, cellular distribution and function of $GABA_AR$ in response to different stimuli, a process named plasticity. Human lymphocytes have a functional neuronal-like GABAergic system with $GABA_AR$ acting as inhibitors of proliferation. We here explore if receptor plasticity occurs in lymphocytes. To this end, we analyzed human T lymphocyte Jurkat cells exposed to different physiological stimuli shown to mediate plasticity in neurons: GABA, progesterone and insulin. The exposure to 100 μM GABA differently affected the expression of $GABA_AR$ subunits measured at both the mRNA and protein level, showing an increase of α1, β3, and γ2 subunits but no changes in δ subunit. Exposure of Jurkat cells to different stimuli produced different changes in subunit expression: 0.1 μM progesterone decreased δ and 0.5 μM insulin increased β3 subunits. To identify the mechanisms underlying plasticity, we evaluated the Akt pathway, which is involved in the phosphorylation of β subunits and receptor translocation to the membrane. A significant increase of phosphorylated Akt and on the expression of β3 subunit in membrane occurred in cells exposed 15 h to GABA. To determine if plastic changes are translated into functional changes, we performed whole cell recordings. After 15 h GABA-exposure, a significantly higher percentage of cells responded to GABA application when compared to 0 and 40 h exposure, thus indicating that the detected plastic changes may have a role in GABA-modulated lymphocyte function.

Our results reveal that lymphocyte $GABA_AR$ are modified by different stimuli similarly and by similar mechanisms to those in neurons. This property is of significance for the development of future therapies involving pharmacological modulation of the immune response.

1. Introduction

Inhibitory neurotransmission is vital to maintaining the balance between excitatory and inhibitory inputs in the brain. GABA is the principal inhibitory neurotransmitter in the human brain and exerts its effects through ionotropic $GABA_AR$ and metabotropic $GABA_B$ receptors. $GABA_AR$ are neurotransmitter-gated ion channels that belong to the Cys-loop receptor family, which in vertebrates include cationic channels, nicotinic acetylcholine and $5\text{-}HT_3$ receptors, and anionic channels, glycine and $GABA_A$ receptors. They are pentameric proteins containing a large extracellular domain, that carries the neurotransmitter-binding sites; a transmembrane domain, that forms the pore and the channel gate; and a large cytoplasmic domain containing phosphorylation sites for Protein kinase B (Akt), cAMP-dependent protein kinase (PKA), Protein kinase C (PKC), Ca^{2+}/calmodulin dependent kinase (CaMKII) and tyrosine kinases of the Src family [1–3]. $GABA_AR$ can form receptors composed of five identical (homopentamers) or different (heteropentamers) subunits arranged around an axis perpendicular to the membrane.

$GABA_AR$ subunits are encoded by 19 different genes grouped into eight subclasses based on sequence homology (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) [4]. $GABA_AR$ with different subunit compositions exhibit distinct pharmacology and channel gating properties, they are differentially expressed and are subject to differential regulation. Thus, dynamic changes in the expression and function of $GABA_AR$ are implicated in the regulation of virtually all aspects of brain function. Neurons modify subunit expression, cellular distribution and function of $GABA_AR$ in response to different stimuli, or environmental signals, a process named plasticity. For example, the exposure to the neurotransmitter GABA reduces the steady-state levels of mRNAs encoding α1, β2 and γ1 subunits in neurons [5]. Similarly, neuroactive steroids mediate changes in the expression of specific $GABA_AR$ subunits [6,7]. Other plasticity mechanisms involve the translocation of receptors to/from the cell surface by post-translational modifications like phosphorylation, ubiquitination and palmitoylation. Phosphorylation and Ca^{2+}-dependent signaling cascades affect the activity of kinases and phosphatases

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0300-9084/$ – see front matter
http://dx.doi.org/10.1016/j.biochi.2013.08.023

Table 1
Primers used for RT-qPCR analysis.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product (bp)
α1	GGATTGGGAGAGCGTGTAACC	TGAAACGGGTCCGAAACTG	66
β3	ACCGTTCAAAGAGCGAAAGC	TTCCAGCGATGTCAACAGAATATT	65
δ	GAGGCCAACATGGAGTACAC	TTCACGATGAAGGTGTCGG	145
γ2	GCACAACTTTCCAATGGATGAA	CGTGGATAGCCATAACTGGAGAA	63
ρ2	ATGCTGTCCTGGGTGTCCTT	CCGTGATGATGGTGGTCATG	100
β-actin	CGGAACCGCTCATTGCC	ACCCACACTGTGCCCATCTA	289

that alters the phosphorylation state of $GABA_AR$ β and γ subunits [2]. For example, insulin stimulates $GABA_AR$ insertion into the cell surface membrane via Akt-mediated phosphorylation of the $GABA_AR$ β-subunit [8]. All these examples represent adaptive modifications to confront environmental changes to ensure an appropriate neuronal function.

We have previously reported that human lymphocytes contain components to constitute a neuronal-like GABAergic system [9]. We have determined that lymphocytes are capable of synthesizing, uptaking and catabolizing the neurotransmitter GABA. Moreover, these immune cells express functional $GABA_AR$ whose activation inhibits lymphocyte proliferation. We have observed variability in some $GABA_AR$ subunit expression in isolated lymphocytes among individuals and even within the same individual. We here explored if $GABA_AR$ subunit expression in lymphocytes changes by different stimuli, a phenomenon that has been described for neuronal cells and associated to neuronal plasticity. To this end, we determined the expression, in Jurkat T-cell line, of mRNA and protein $GABA_AR$ subunit levels in response to different stimuli: GABA, progesterone and insulin, and the probable underlying mechanism for the expression changes.

2. Materials and methods

2.1. Cell culture

Jurkat cells were kindly provided by Dr. M. Giordano (Instituto de Medicina Experimental, Academia Nacional de Medicina-CONICET, Argentina). Cells were grown at an optimal concentration of 0.5×10^6 cells/mL in RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere at 5% CO_2.

2.2. Reverse transcription and end point PCR (RT-PCR) and real time PCR (RT-qPCR) analysis

Total RNA was isolated from 3×10^6 cells by the acid guanidium-phenol-chloroform method. mRNA was converted into cDNA using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT; Promega, USA) and random primers (Promega, USA).

RT-PCR was run in a Mini Cycler™ (MJ Research, USA). The Superscript human brain cDNA library (female, 36 years) (Invitrogen) was used as positive control. Primer sequences were described before [9]. Semiquantitative RT-PCR analysis was carried out by using β-actin mRNA amplification as an internal standard. RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Relative quantification of mRNA by qPCR was performed using a Rotor-Gene 6000 (Corbett Research, Australia). The gene-specific primer sequences for RT-qPCR are listed in Table 1. The comparative CT method was used to determine the relative gene expression [10,11]. The expression of the gene of interest is presented relative to an internal control gene. The advantage of the comparative CT method is the ability to present the data as "fold change" in expression. The CT values of target mRNA were normalized with the CT value of the housekeeping gene β-actin using the formula $\Delta CT = CT\,(target) - CT\,(\beta\text{-actin})$ and the data is presented as $2^{-\Delta CT}$. The method was further used to determine the relative expression of the same gene from two samples: $\Delta\Delta CT = \Delta CT\,(sample\text{-}A) - \Delta CT\,(sample\text{-}B)$. The data is presented as $2^{-\Delta\Delta CT}$ [11].

2.3. Western blot analysis

4×10^6 cells were washed with PBS buffer and lysed in RIPA buffer (10 mM Tris HCl, 150 mM NaCl, 1% TX100, 5 mM NaF, 1 mM Na_3VO_4, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). After centrifugation, the supernatant was collected and proteins were quantified by the Bradford method. Lysate proteins were separated on SDS–polyacrylamide gels and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween 20), the membranes were first incubated with the primary antibody in TBST plus 5% BSA and then with horseradish peroxidase-conjugated secondary antibody in TBST plus 5% BSA. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

The following antibodies were used in the Western Blot experiments: Goat anti-α1 (1/500, overnight at 4 °C), rabbit anti-β3 (1/500, overnight at 4 °C), rabbit anti-Akt (1/1000, 2 h at room temperature), rabbit anti-GAPDH (1/5000, 2 h at room temperature), polyclonal horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1/2000, 1 h at room temperature) and polyclonal horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1/2000, 1 h at room temperature) all of them provided by Santa Cruz Biotechnology, Inc. Rabbit anti-p-Akt (Ser 473) (1/1000, 2 h at room temperature) was provided by Cell Signaling.

2.4. Immunocytochemistry and wide field fluorescence microscopy

Jurkat cells were seeded into 35 mm glass discs coated with poly-L-ornithine (Sigma) and fixed with 2% paraformaldehyde in PBS. After fixation, cultures were incubated with a rabbit anti-β3 antibody (1/100) during 1 h at 4 °C and then with the secondary antibody FITC $F(ab')_2$ goat anti-rabbit IgG (1/50) (Jackson ImmunoResearch, West Groove, PA, USA) for 45 min. Cells were observed by phase and fluorescence microscopy using a Nikon Eclipse E-600 microscope. Imaging was done with an SBIG Astronomical Instruments (Santa Barbara, CA, USA) model ST-7 digital charge-coupled device camera (765 × 510 pixels, 9.0 × 9.0-μm pixel size). The ST-7 CCD camera was driven by the CCDOPS software package (version 5.02, SBIG Astronomical Instruments). For all experiments, ×100 (1.4 numerical aperture) oil immersion objectives were used. 16-bit TIFF images were exported and further analyzed by *FIJI* program.

2.5. Macroscopic current recordings

Cells were seeded into 35 mm plastic dishes coated with poly-L-ornithine. Currents were recorded in the whole-cell patch clamp

configuration at −70 mV. Pipette solution contained 140 mM KCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 8 mM EGTA, and 10 mM HEPES, pH 7.3. Extracellular solution (ECS) contained 120 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM HEPES and 10 mM Glucose pH 7.3. A series of pulses (0.5 s) of ECS containing GABA was applied to the cells. Given that the chloride concentration was similar for the intracellular and extracellular solutions, negative pipette voltages (−70 mV) are expected to induce outward chloride movements. Currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Inc., CA) and filtered at 5 kHz. Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, Oregon). Currents were aligned with each other at the point where the current reached 50% of its maximum. Peak currents correspond to the value obtained by extrapolation of the decay current to this point. Current decays were fitted by the exponential function:

$$I_{(t)} = I_0(\exp(-t/\tau_d)) + I_\infty \quad (1)$$

where I_0 and I_∞ are peak and the steady state current values, respectively, and τ_d is the decay time constant [12].

2.6. Statistical analysis

All of the data and results were confirmed through at least three independent experiments done in triplicate. The values shown represent the means ± SEM. The significance of the differences was identified using one way ANOVA, post hoc Tukey Multiple Comparison test. For cell surface β3-staining in Figs. 4b and 5, Student's *t*-test was used. All of the statistical analysis were performed using the GraphPad Prism 5.01 software (GraphPad software, San Diego, USA).

3. Results

3.1. Jurkat cells express $GABA_AR$ subunits

The variability in the expression pattern of $GABA_AR$ subunits in human lymphocytes among individuals [9] turns rather difficult to evaluate changes in subunit composition in these cells in response to a specific stimulus. Therefore, Jurkat cells, which correspond to a human leukemic T-cell line, were chosen as a model in which plasticity could be explored. The first step was to determine if these cells express $GABA_AR$ subunits and if the pattern of subunit expression is constant in different cultures in the absence of exogenous stimulus. By using RT-PCR, we determined that these cells express mRNA subunits detected previously in human lymphocytes: α1, β3, δ, γ2, and ρ2 [9], thus showing a similar subunit expression pattern with slight variations in some subunits with respect to isolated human lymphocytes (Table 2). Unlike human lymphocytes, we did not detect α3 and α6 mRNAs in Jurkat cells. The comparison of the subunit mRNAs detected in 3 different cultures of Jurkat cells from different passage numbers showed that the expression pattern was invariable. Thus, the fact that the expression pattern of $GABA_AR$ subunit genes is stable in Jurkat cells compared to the expression in human peripheral lymphocytes makes this cell line suitable to explore $GABA_AR$ plasticity in T cells.

3.2. GABA exposure modifies mRNA levels of $GABA_AR$ subunits in Jurkat cells

It was reported that neurons exposed to the neurotransmitter GABA change their $GABA_AR$ subunit mRNA expression [5]. Moreover, cell surface expression of $GABA_AR$ can be promoted by GABA exposure [13]. Therefore, we evaluated whether changes in $GABA_AR$ subunits occur in Jurkat cells upon exposure to 100 μM GABA. RT-PCR for α1, β3, δ, γ2 and ρ subunits was done on total RNA isolated from Jurkat cells to determine subunit expression in a wide range of incubation time: 0, 3, 15, 25 and 40 h. Semiquantitative analysis was carried out using β-actin mRNA amplification as an internal standard. In this first screening, we detected changes in the expression of α1, β3 and γ2 subunits during GABA incubation. The α6 subunit was initially absent (0 h), but was detected after 3 h of GABA incubation. In contrast, δ and ρ2 subunit levels did not change along drug incubation (Table 3, Fig. S1). These results show that GABA exposure produces changes in mRNA $GABA_AR$ subunit expression.

To relatively quantify the subunit expression changes previously observed, RT-qPCR for α1, β3, δ, γ2 and ρ subunits were done after 0, 15 and 40 h of GABA incubation. We selected 15 h- and 40 h-GABA exposure because at these time points there is a clear increase and decrease, respectively, of mRNA subunits evaluated by RT-PCR (Table 3). Applying the ΔCT method (see Methods), we determined the change in the level of expression of each subunit over time. Each sample was related to the internal control gene β-actin and the sample at time 0 h was defined as the calibrator. β-actin mRNA levels resulted unalterable during drug exposure to Jurkat cells, so this gene was suitable to use as a valid housekeeping gene. The samples at 15 and 40 h were compared to the calibrator to

Table 2
Expression of $GABA_AR$ subunits in human lymphocytes and Jurkat cells.

Subunits	Human lymphocytes[a]		Jurkat cells
	R[b]	A[c]	
α1	+[d]	+	+
α 2	−[e]	−	−
α3	+/−[f]	+/−	−
α4	−	−	−
α5	−	−	−
α6	+/−	+/−	−
β3	−	+	+
δ	+	+	+
γ2	+/−	+/−	+
ρ1	−	−	−
ρ2	+	+	+
ρ3	−	−	−

[a] Data extracted from Dionisio et al., 2011.
[b] R: resting cells.
[c] A: 10 μg/ml PHA-activated cells.
[d] '+' indicates presence of PCR bands.
[e] '−' indicates absence of PCR bands.
[f] '+/−' indicates variability of expression between donors ($n = 5$).

Table 3
Expression of $GABA_A$ receptor subunits after incubation with 100 μM GABA at different time-points in Jurkat cells.

Subunit	0 h	3 h	15 h	25 h	40 h
α1	+[a]	+	++[b]	++	(+)[c]
α2	−[d]	−	−	−	−
α3	−	−	−	−	−
α4	−	−	−	−	−
α5	−	−	−	−	−
α6	−	+	+	+	+
β3	+	+	++	++	++
δ	+	+	+	+	+
γ2	+	++	++	++	(+)
ρ1	−	−	−	−	−
ρ2	+	+	+	+	+
ρ3	−	−	−	−	−

[a] '+' indicates presence of PCR bands.
[b] '++' indicates presence of the most intensely stained PCR bands.
[c] '(+)' indicates presence of faintly stained PCR bands.
[d] '−' indicates absence of PCR bands.

calculate the subunit expression fold change [11] (see Methods). We observed more than a 30-fold increase for α1 and γ2 subunit mRNA expression after 15 h of GABA incubation compared to the calibrator (0 h). The expression level of β3 at 15 h was about 8-fold higher than at 0 h. No differences in the expression of δ and ρ2 subunits were detected after 15 h of GABA incubation. At 40 h, the only subunit that increased its expression is the ρ2 subunit (about 6-fold change), the rest of them remained unalterable or decreased at this time-point (Fig. 1). These results confirm that the levels of mRNA of $GABA_AR$ subunits change differentially upon neurotransmitter incubation, thus suggesting a selective modulation of the $GABA_AR$ subunit expression by GABA in Jurkat cells.

3.3. Progesterone and insulin also induce mRNA $GABA_AR$ subunit expression changes in Jurkat cells

We evaluated if other stimuli reported to cause plastic changes in $GABA_AR$ in neurons are also active at immune cells. To test if progesterone also triggers changes in $GABA_AR$ expression, Jurkat cells were exposed to 0.1 μM progesterone during 0, 15 and 40 h and then RT-qPCR was performed to relatively quantify the δ subunit mRNA expression level. Although δ mRNA level did not change significantly after GABA exposure (Section 3.2), we chose this subunit because δ-containing $GABA_ARs$ are more sensitive to neurosteroids than those that lack this subunit [14]. Furthermore, δ subunit mRNA expression decreases in neurons upon the increase of progesterone in pregnant mice [15]. Interestingly, we found a correlation with these findings since δ subunit mRNA expression decreased after 15 h progesterone incubation and restored to control levels at 40 h of drug exposure (Fig. 2a). We also checked by RT-PCR if α4 and α6 subunits, which are common partners of δ subunit and endow $GABA_AR$ with high sensitivity to neurosteroids [16], change their expression during progesterone exposure. These subunits were not initially detected in Jurkat cells (Table 3) or after 15 h and 40 h progesterone incubation ($n = 3$), thus indicating that this stimulus is selective for certain subunits.

We also explored the effect of insulin, which has been shown to induce $GABA_AR$ plasticity in neurons. By RT-qPCR we detected an increase in β3 mRNA after 15 and 40 h of insulin incubation compared to control levels, thus demonstrating that insulin also induces changes in $GABA_AR$ subunit expression (Fig. 2b).

3.4. Protein expression of α1 and β3 subunits changes upon GABA exposure

To determine if the differential expression of mRNA detected in Jurkat cells during GABA exposure is also detected at the protein level, we performed western blot analysis for α1 and β3 subunits. We studied these subunits because they are required to form functional $GABA_AR$. We performed western blot analysis of Jurkat cells cultured with 100 μM GABA for 0, 15 and 40 h. As shown in Fig. 3, higher expression of α1 and β3 proteins was observed at 15 h of GABA incubation compared to the initial levels and to the levels at 40 h of incubation. The increase in α1 subunit was higher than the one obtained for β3 subunit, in agreement with the levels of mRNA expression described above (Section 3.2). These results suggest a possible modulation in the number and/or composition of $GABA_AR$ in Jurkat cells by external stimuli.

3.5. Mechanisms underlying $GABA_AR$ plasticity in Jurkat cells

We further explored potential mechanisms involved in plasticity. Activation of Ca^{2+}-PI3K/Akt pathway has been shown to modulate the membrane surface level of $GABA_AR$. In particular, it has been postulated that GABA activates this pathway in pancreatic beta cells [17] and that phosphorylation of $GABA_AR$ subunits mediated by Akt increases the number of receptors on the membrane surface in neurons [18]. Therefore, we tested if similar processes occur in Jurkat cells during GABA incubation. First, we evaluated by western blot the presence of activated (phosphorylated) Akt (p-Akt) in Jurkat cells incubated with 100 μM GABA at the same time-points in which we observed changes in $GABA_AR$ subunits: 0, 15 and 40 h. The results show that exposure of Jurkat cells to GABA stimulates Akt phosphorylation (Fig. 4a). Higher p-Akt/Akt ratios were observed in cells at 15 h of GABA incubation compared to non incubated cells (0 h).

To test if GABA leads to an increase in $GABA_AR$ expression on plasma membrane through the Akt pathway, we performed immunofluorescence staining of Jurkat cells membrane surface with an antibody against the β3 subunit. We detected stronger surface staining in cells incubated 15 h with GABA compared to non incubated cells (Fig. 4b, top). Effectively, fluorescence quantification confirmed a significant increase in $GABA_AR$ surface expression at 15 h of GABA exposure (Fig. 4b, bottom), suggesting that activated Akt is responsible for phosphorylation-dependent translocation of $GABA_AR$ to cell membrane (Fig. 4b). Furthermore, we exposed cells to 10 μM LY294002, a PI3K inhibitor, and evaluated membrane expression of β3 subunit by immunocytochemistry. As shown in Fig. 4c, after 15 h incubation with GABA and LY294002, there is a decrease in β3-staining, confirming that the PI3K/Akt pathway is involved in GABA stimulated-membrane receptor increase.

These results show that GABA activates PI3K/Akt pathway and probably leads to $GABA_AR$ phosphorylation and translocation to plasma membrane in Jurkat cells.

Another plasticity mechanism described in neurons is the rapid recruitment of $GABA_AR$ to the cell membrane mediated by insulin-

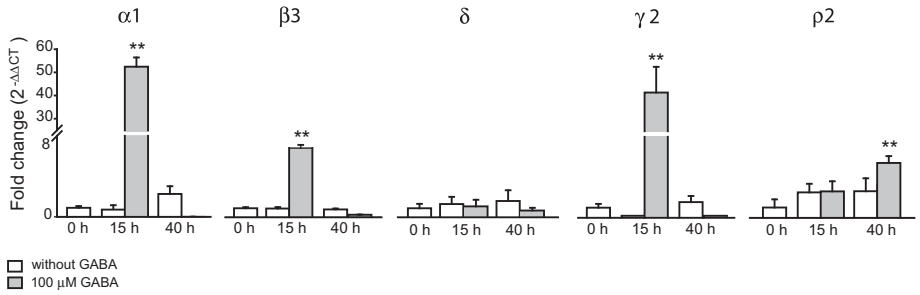

Fig. 1. Relative expression of $GABA_AR$ subunits mRNA at different time points of GABA exposure. Jurkat cells were incubated with 100 μM GABA and after 0, 15 and 40 h of drug incubation, total RNA was extracted and RT-qPCR was performed. The fold change of each subunit was calculated using $2^{-\Delta\Delta CT}$. Subunit mRNA expression was referred to β-actin gene and compared to the calibrator (0 h) (See Methods). Data are represented as means ± SEM from three independent experiments done in triplicate ($n = 3$). **, $p < 0.01$ compared with the result at 0 h, one-way ANOVA and post hoc Tukey test.

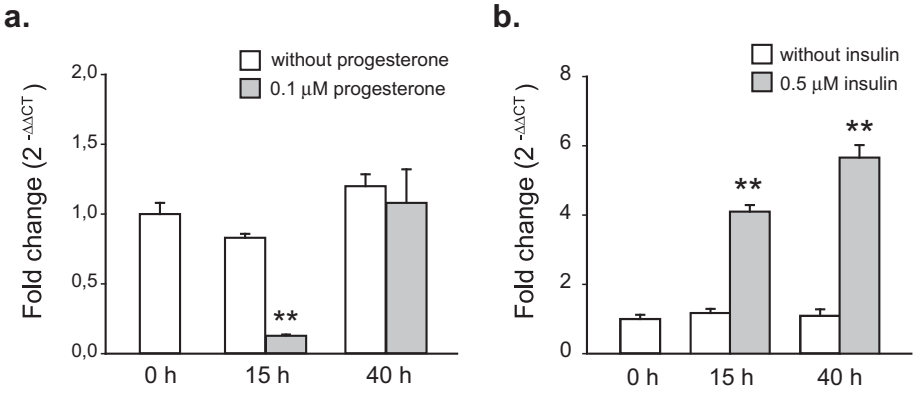

Fig. 2. Others stimuli induce mRNA changes of $GABA_AR$ in Jurkat cells. After 0, 15 and 40 h of 100 μM GABA incubation, total RNA was extracted from Jurkat cells and RT-qPCR was performed. Relative expression of (a) δ subunit mRNA after different time points of 0.1 μM progesterone exposure and of (b) β3 subunit mRNA after different time points of 0.5 μM insulin exposure in Jurkat cells. The fold change was calculated using $2^{-\Delta\Delta CT}$. Subunit mRNA expression was referred to β-actin gene and compared to the calibrator (0 h) (See Methods). Data are represented as means ± SEM from three independent experiments done in triplicate ($n = 3$). **, $p < 0.01$ compared with the result at time 0 h, one-way ANOVA and post hoc Tukey test.

activated Akt pathway [19]. This process is dependent on Akt phosphorylation of β subunits and is observed rapidly after insulin incubation [8,18–20]. We therefore investigated whether insulin recruits $GABA_AR$ to the surface membrane of immune cells. To this end, we exposed Jurkat cells to 0.5 μM insulin for 10 min and then performed immunofluorescence staining of cell surface $GABA_AR$ using an antibody against the β3 subunit. We observed an increment of $GABA_AR$ β3 subunit staining in the cell membrane after insulin incubation compared to control levels, which is confirmed by quantification of fluorescence intensity (Fig. 5). This result suggests that insulin induces a rapid $GABA_AR$ membrane translocation in Jurkat cells.

3.6. GABA exposure leads to an increase in the number of detectable GABA-responding cells

We next explored whether the described changes in subunit levels during incubation with the neurotransmitter are reflected in functional changes. Therefore, using the patch-clamp technique, we measured whole-cell currents elicited by GABA in Jurkat cells incubated with 100 μM GABA, which was shown to mediate differential subunit expression. 500 μM GABA-elicited currents were detected in 5% of the tested cells from cultures not exposed to GABA (0 h, control, $n = 50$). The percentage of active cells showed a significant increase after 15 h-GABA incubation (21%, $n = 50$ cells), and decreased to levels similar to the control at 40 h-GABA exposure (8%, $n = 50$) (Fig. 6a). These results demonstrate that the increment of expression of specific $GABA_AR$ subunits detected at 15 h of exposure to GABA (Figs. 1, 3 and 4b) is accompanied by an increment in the percentage of cells that respond to GABA. As described for peripheral lymphocytes [9], at least two types of GABA-elicited currents can be clearly distinguished on the basis of their decay rates. One type corresponds to fast desensitizing currents and the other type, to slow desensitization ones (Fig. 6b). Although GABA incubation increases the number of cells responding to GABA, both types of currents were recorded after 0, 15 and 40 h GABA-incubation. For example from 9 detected currents at 15 h, 4 were fast and 5 were slow, whereas at 40 h, 2 were fast and 1 was slow.

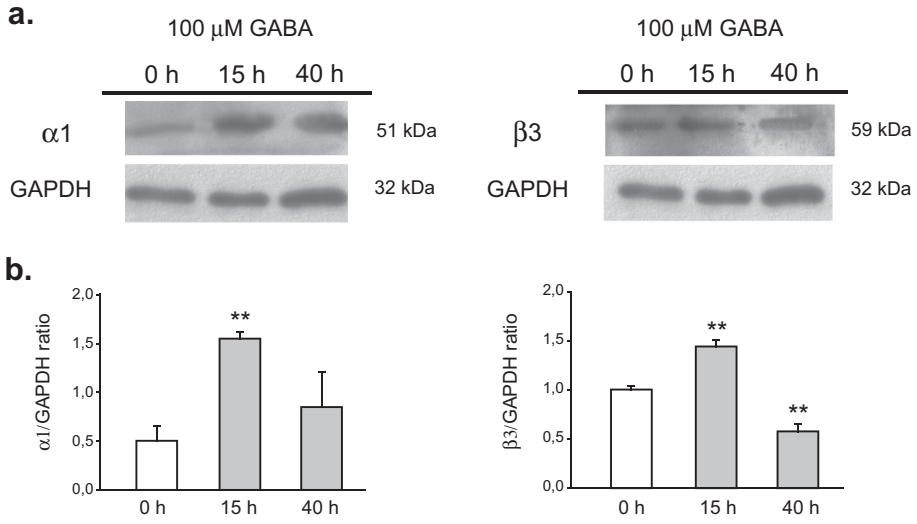

Fig. 3. Expression of α1 and β3 subunit proteins in Jurkat cells upon GABA exposure. Jurkat cells were incubated with 100 μM GABA for 0, 15 and 40 h. Cell lysates were prepared for western blot as described in Methods. (a) Western blots of cell lysates using the indicated antibodies on the left. GAPDH was used as internal standard. The western blot in each case is representative of three different experiments. (b) Quantification of bands from the corresponding western blots was determined by scanning densitometry. Data are represented as means ± SEM from three independent experiments done in triplicate ($n = 3$). **, $p < 0.01$ compared with the result at time 0 h, one-way ANOVA and post hoc Tukey test.

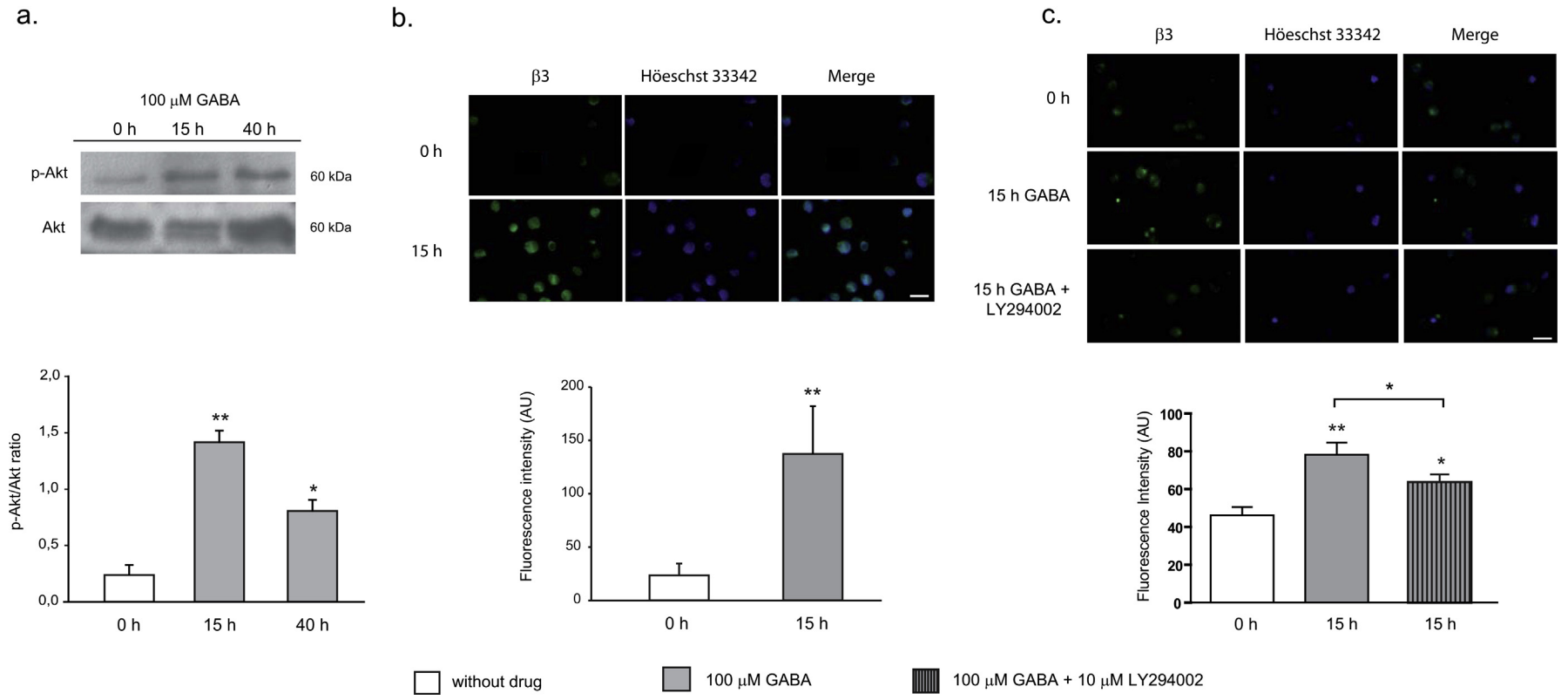

Fig. 4. Mechanisms underlying $GABA_AR$ plasticity in Jurkat cells. (a) Jurkat cells were incubated with 100 μM GABA for 0, 15 and 40 h. Cell lysates were prepared for western blot as described in Methods. *Top*, western blot of cell lysates using the antibodies indicated on the left. The western blot is representative of three different experiments. *Bottom*, Quantification of bands from the corresponding western blots was determined by scanning densitometry. Data are represented as means ± SEM from three independent experiments done in triplicate ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$ compared with the result at time 0 h, one-way ANOVA and post hoc Tukey test. (b) Expression of cell surface of β3 subunit upon GABA exposure. Jurkat cells were incubated with 100 μM GABA for 0, 15 h and 40 h and processed for immunocytochemistry using an antibody against β3 subunit. Höeschst 33342 was used as nuclear marker. *Top*, Representative pictures showing immunocytochemistry of surface β3 subunit and nuclear staining from three different experiments are shown. Bar, 10 μm. *Bottom*, fluorescence quantification of immunocytochemistry from β3-positive cells was determined using *FIJI* program. Data are represented as means ± SD ($n = 29$ for 0 h, $n = 20$ for 15 h). **, $p < 0.01$ compared with the result at time 0 h, Student's *t*-test. (c) Effect of LY294002, a PI3K inhibitor, on β3 membrane expression. Jurkat cells were incubated with 10 μM LY294002 for 30 min. Then 100 μM GABA was added to the cultures and cells were incubated for 15 h. Finally, cells were processed for immunocytochemistry using an antibody against β3 subunit. Höeschst 33342 was used as nuclear marker. *Top*, Representative pictures showing immunocytochemistry of surface β3 subunit and nuclear staining from three different experiments are shown. Bar, 10 μm. *Bottom*, fluorescence quantification of immunocytochemistry from β3-positive cells was determined using *FIJI* program. Data are represented as means ± SEM ($n = 20$ for 0 h, $n = 19$ for 15 h GABA and $n = 21$ for 15 h GABA + LY294002). *, $p < 0.05$ and **, $p < 0.01$ compared with the result at time 0 h, one-way ANOVA and post hoc Tukey test.

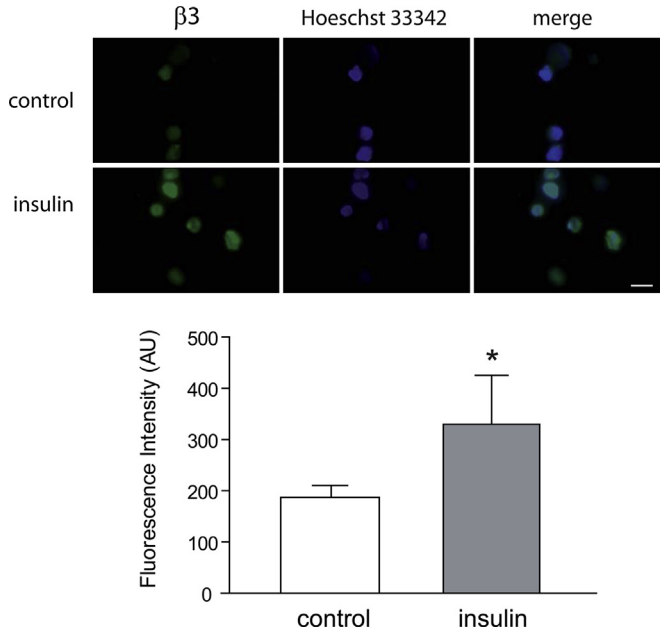

Fig. 5. Insulin-mediated recruitment of $GABA_AR$ to the cell membrane in Jurkat cells. Jurkat cells were incubated with 0.5 μM insulin for 10 min and processed for immunocytochemistry using an antibody against β3 subunit. Höeschst 33342 was used as nuclear marker. *Top*, Representative pictures showing immunocytochemistry of surface β3 subunit and nuclear staining from three different experiments are shown. Bar, 10 μm. *Bottom*, fluorescence quantification of immunocytochemistry from β3-positive cells was determined using *FIJI* program. Data are represented as means ± SEM ($n = 16$ for control, $n = 13$ for insulin exposure). *, $p < 0.05$ compared with the result at time 0 h, Student's *t*-test.

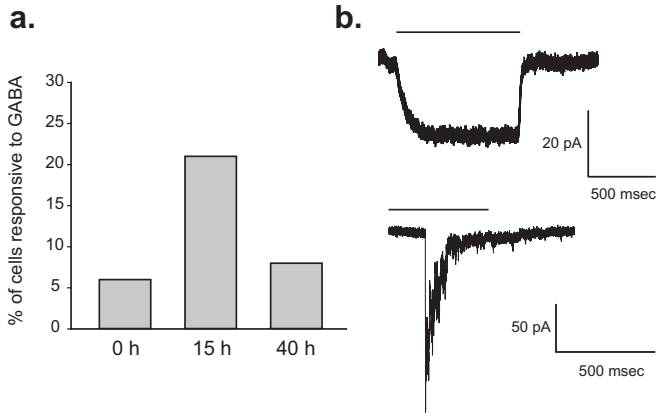

Fig. 6. Macroscopic currents evoked from Jurkat cells. Jurkat cells were incubated with 100 μM GABA for 0, 15 and 40 h. Then, cells were seeded into 35 mm plastic dishes coated with poly-L-ornithine during the last 2 h of incubation time and whole-cell patch clamp configuration was applied to obtain macroscopic currents. (a) Percentage of currents elicited per number of obtained patches en each condition ($n = 50$) in Jurkat cells after 0, 15 and 40 h of incubation with 100 μM GABA. (b) Representative whole-cell currents elicited by 500 μM GABA are shown. The solid line above each current trace indicates the duration of drug application. The pipette potential is −70 mV.

Unfortunately, the relatively low number of detected currents in addition to the high variation in the proportion of the two types of currents does not allow statistical comparison for both types of currents.

In conclusion, the detected mRNA and protein changes are translated into functional changes, which in turn, may have a role in lymphocyte activity.

4. Discussion

We have previously reported that lymphocytes express an intrinsic GABAergic system that includes functional $GABA_AR$ [9]. There is high variability in the $GABA_AR$ subunit expression profile in lymphocytes among individuals and even within the same individual at different times, indicating that several not yet identified factors influence receptor expression, such as age, sex, hormone milieu, drug exposure, stress, etc [9]. Such variation led us to infer that lymphocyte $GABA_AR$ may exhibit plasticity properties as described for neuronal $GABA_AR$. Because subunit expression is too variable in lymphocytes we here explored plasticity in response to different stimuli in Jurkat cells, a human leukemic T cell line. We showed that these cells express a subunit profile comparable to that of primary cultured lymphocytes and that the subunit expression pattern does not change during culture incubation, a crucial issue for our experimental design.

Our results reveal that $GABA_AR$ in immune cells, as in neurons, exhibit plasticity, which is evidenced by the change in $GABA_AR$ subunit expression when exposed to a given stimulus. Since under certain conditions, such as antigen/mitogen-activation, lymphocytes are capable of synthesizing, and, probably, releasing the neurotransmitter [9], the GABA levels achieved at the proximity of lymphocyte $GABA_AR$ are probably higher than those detected in blood. Therefore, the GABA concentration chosen to study this phenomenon was higher than the blood concentration in healthy individuals (0.1 μM, [21]) but was similar or lower to that used by others to explore plasticity [5,13]. The changes detected at mRNA level after GABA incubation revealed a differential modulation of subunit expression. The α1, β3 and γ2 subunits showed a peak in their mRNA levels at 15 h GABA exposure while the δ subunit remained unalterable over time. The magnitude of the fold change of mRNA β3 subunit was lower than the α1 mRNA subunit (Fig. 1), although the relative expression levels were similar for both subunits at 15 h GABA exposure (Fig. S2). At this time point, α1 and β3 subunits achieved similar and the highest expression levels while the other subunits levels remained low (Fig. S2). This result correlates with the increase at 15 h of GABA exposure of protein expression of α1 and β3 subunit and with the higher percentage of cells responding to the agonist in whole-cell recordings. In agreement with our results, it was reported that GABA acts promoting cell surface expression of $GABA_AR$ in transiently transfected cells [13]. In Jurkat cells, we detected macroscopic currents elicited by GABA, revealing that ionotropic GABA receptors are functional. Two types of detected currents can be clearly distinguished by the rate at which they decay in the presence of the agonist, which, in turn, is probably related to the rate of desensitization. This suggests that at least more than one subtype of $GABA_AR$ is present on Jurkat cells membrane, in agreement with the detection of different types of subunits. A similar pattern of GABA-elicited currents has been described previously for human lymphocytes [9]. Unfortunately, it is not possible to correlate the increase in the number of cells responding to GABA with the prevalence of a type of current since statistic analysis cannot be done due to the great variability even at control conditions. In addition, the relatively low number of active cells and the profound run-down effect impair detailed pharmacological studies.

Altogether our observations indicate that exposure to GABA modulates gene and protein expression. This regulation could be mediated by signal transduction pathways associated to $GABA_AR$ activation. In the adult brain, GABA induces fast inhibition in neurons as a consequence of Cl^- influx that results in membrane hyperpolarization. In the developing brain, however, activation of $GABA_AR$ induces membrane depolarization, causing Ca^{2+} influx which regulates neuronal cell proliferation and maturation [22].

Intracellular concentration of Cl^- ion in immune cells has been shown to be elevated [23], and therefore opening of $GABA_AR$ probably leads to an output of Cl^- causing membrane depolarization. Indeed, it has been postulated that the depolarization induced by GABA in lymphocytes may lead to opening of calcium channels and, in turn, to the inhibition of the proliferative response [9,23–25]. Thus, it is possible that in Jurkat cells, which correspond to T cells, $GABA_AR$ activation also causes depolarization of the membrane, thus resembling the excitatory effect of GABA described for immature neurons. This effect has been also described in pancreatic beta cells in which GABA, through activation of $GABA_AR$ produces membrane depolarization and Ca^{2+} influx, leading to activation of Akt-mediated cell growth and survival signaling pathways [17]. Recently, it has been shown that Akt up-regulates gene expression during T cell activation [26].

Here we present evidence that $GABA_AR$ activation by GABA in Jurkat cells changes the expression of receptor subunit genes and leads to Akt activation. Therefore, it could be possible that in Jurkat cells, activation of $GABA_AR$ induces membrane depolarization that activates Ca^{2+} channels, thus increasing intracellular calcium and subsequently promotes activation of different signaling pathways including the Ca^{2+}/PI3K/Akt signaling pathway. Although we cannot discard that metabotropic $GABA_B$ receptor may be involved in transduction pathways, several studies have shown that GABA, in lymphocytes, acts mainly through $GABA_AR$ [23,24].

An important feature of neuronal $GABA_AR$ is their plasticity in response to neuroactive steroids and steroid hormones. It is now well recognized that progesterone and its metabolites are potent allosteric modulators of $GABA_AR$ function through a direct, non-genomic interaction with specific receptor subtypes, being the δ-containing receptor especially sensitive to them [27]. However, fluctuations in the concentration of neuroactive steroids affect not only $GABA_AR$ function but also the expression of receptor subunits [6]. It has been shown a decrease in the expression of δ-containing $GABA_AR$ during late pregnancy which may constitute a homeostatic mechanism to maintain a steady level of inhibition throughout pregnancy [6]. In Jurkat cells we have observed that δ subunit expression changes upon exposure to 0.1 μM progesterone, a concentration similar to that reached in blood during pregnancy. As plasticity represents an adaptive mechanism in response to a certain stimulus, it is not expected to observe changes at physiological blood concentrations. This is the first evidence that plastic changes of $GABA_AR$ in response to steroids also occurs in immune cells. Modulation of the immune system by progesterone in women has been suggested [28], but the mechanism of such action is unknown. This study provides a novel potential mechanism for progesterone action as a regulator of the immune response.

Finally, we have shown that insulin causes an increase in β3 subunit expression in Jurkat cells by two different time-scale mechanisms. The fast one involves a rapid recruitment of $GABA_AR$ to the plasma membrane surface after insulin exposure. In line with this observation, it has been reported that insulin stimulates postsynaptic $GABA_AR$ insertion into the cell surface membrane via Akt-mediated phosphorylation of the β subunit in neurons [18–20]. The slow mechanism, involves a transcriptional mechanism increasing β3 mRNA expression levels upon insulin incubation after long periods of time. This transcriptional change might also be triggered by Akt activation, similar to the mechanism proposed above for GABA-induced plasticity.

In conclusion, we demonstrate that several stimuli induce $GABA_AR$ changes in lymphocytes, thus revealing a property that the lymphocytic GABAergic system shares with the neuronal system: receptor plasticity. Since we have previously described that activation of $GABA_AR$ inhibits lymphocyte proliferation, it would be interesting to further study the role of the described plastic changes in T-cell response regulation. Probably, receptor plasticity arises as an adaptive mechanism to contribute to the balance of immune homeostasis in response to hormone and drug fluctuations.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

Acknowledgments

We thank Dr. Gravielle (Instituto de Investigaciones Farmacológicas, UBA-CONICET, Argentine) for kindly providing primary antibodies against $GABA_AR$ subunits α1 and β3. Special thanks to Dr. Melina Mateos and Dr. Virginia Borroni for helpful advice. This work was supported by grants from Universidad Nacional del Sur (MCE, CB), Agencia Nacional de Promoción Científica y Tecnológica (CB) and CONICET (CB).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2013.08.023.

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