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An intrinsic GABAergic system in human lymphocytes

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ABSTRACT

γ-Amino butyric acid (GABA) is an ubiquitous neurotransmitter in the central nervous system and it is also present in non-neuronal cells. In this study we investigated the presence of neuronal components of the GABAergic system in lymphocytes and its functional significance.

By using RT-PCR we detected mRNA expression of different components of the GABAergic system in resting and mitogen-activated lymphocytes: i) GAD67, an isoform of the enzyme that synthetizes GABA; ii) VIAAT, the vesicular protein involved in GABA storage; iii) GABA transporters (GAT-1 and GAT-2); iv) GABA-T, the enzyme that catabolizes GABA; and v) subunits that conform ionotropic GABA receptors. The presence of VIAAT protein in resting and activated cells was confirmed by immunocytochemistry. The functionality of GABA transporters was evaluated by measuring the uptake of radioactive GABA. The results show that [^{3}H]GABA uptake is 5-fold higher in activated than in resting lymphocytes. To determine if GABA subunits assemble into functional channels, we performed whole-cell recordings in activated lymphocytes. GABA and muscimol, a specific agonist of ionotropic GABA receptors, elicit macroscopic currents in about 10–15% of the cells. Finally, by using [^{3}H]thymidine incorporation assays, we determined that the presence of agonists of GABA receptor during activation inhibits lymphocyte proliferation.

Our results reveal that lymphocytes have a functional GABAergic system, similar to the neuronal one, which may operate as a modulator of T-cell activation. Pharmacological modulation of this system may provide new approaches for regulation of T-cell response.

1. Introduction

Several reports concerning the presence of typical neurotransmitter systems in non-neuronal cells have displaced the concept that these molecules are exclusively expressed in nervous system. For example, it has been described that lymphocytes possess a complete cholinergic system as well as receptors for serotonin, histamine and dopamine (Akdis and Simons, 2006; De Rosa et al., 2005; Kawashima and Fujii, 2003; Kirillova et al., 2008; Yin et al., 2006). However, the physiological relevance of these extra-neuronal systems is not completely understood. It has been proposed that they control cell proliferation, differentiation and cell–cell contact (De Rosa et al., 2009; Gladkevich et al., 2006; Wessler and Kirkpatrick, 2008). GABA, the principal inhibitory neurotransmitter in central nervous system, has been also detected in immune cells like monocytes and macrophages (Stuckey et al., 2005). It has been also postulated that GABA is involved in the progression of autoimmune diseases, such as Type-1 diabetes and multiple sclerosis (Bhat et al., 2010; Bjurstom et al., 2008; Tian et al., 2004).

The concentration of GABA in the brain is controlled by three main events: the synthesis by the enzyme glutamate decarboxilase (GAD), the catabolism by the enzyme GABA transaminase (GABA-T) and the uptake of released GABA by GABA transporters (GATs). At least two isoforms of GAD, GAD65 and GAD67, exist in mammals (Metzeler et al., 2004). Once synthesized, the vesicular inhibitory amino acid transporter (VIAAT) mediates the uptake of GABA into secretory vesicles. After being released, the synaptic action of GABA ends by the recapture of the neurotransmitter by specific high-affinity transporters (GATs). Four different GAT subtypes (GAT-1–3 and BGT-1) have been identified in humans. GAT-1 and GAT-3 are abundantly expressed in CNS and absent from the periphery, whereas BGT-1 and GAT-2 are expressed in human kidney, brain, lung and testis (Christiansen et al., 2007).

Once released, GABA exerts its effects through $GABA_A$, $GABA_B$ and $GABA_C$ receptors. Whereas $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels permeable to chloride ions, $GABA_B$ receptors are associated to G-proteins. We here focused on ionotropic GABA receptors. Sixteen subunits (α1-6, β1-3, γ1-3, δ, ε, π

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and θ) can differently combine to form heteropentameric $GABA_A$ receptors. Most of the $GABA_A$ receptors contain at least α and β subunits. The rho subunits (ρ1–3) combine as homo or heteropentamers to conform $GABA_C$ receptors (Birnir and Korpi, 2007; Ong and Kerr, 2000).

Our study shows that lymphocytes contain the necessary components to constitute an independent GABAergic system. Furthermore, we describe the effects of GABA and muscimol on proliferation of mitogen-stimulated lymphocytes.

2. Materials and methods

2.1. Isolation and culture of human peripheral lymphocytes

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

The percentages of contaminating monocytes and platelets in the cultures were determined by May Grumwald–Giemsa staining. Monocytes were less than 1% of mononuclear leukocytes after the adherence depletion procedure. Furthermore, the relationship lymphocyte:platelets in the culture was 1:5 before adherence depletion, 1:3 after adherence depletion and 1:1 after 48 h incubation (De Rosa et al., 2005).

Since the relation between T and B lymphocytes in peripheral blood is about 3 to 1 and the isolated PBMC are stimulated with a specific T-cell-mitogen, phytohemagglutinin (PHA), it is expected that the studied cell population is mainly conformed by T cells.

2.2. RT-PCR analysis

Total RNA was isolated from 3×10^6 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).

End-point PCR was run in a Mini Cycler™ (MJ Research, USA). The gene-specific primer sequences are described in Table 1. For VIAAT, the product of the first PCR was used as template for a second PCR with nested primers. The Superscript human brain cDNA library (female, 36 years) (Invitrogen) was used as positive control.

Relative quantification of GABA-T mRNA by real time PCR was performed using a Rotor-Gene 6000 (Corbett Research, Australia). The results are expressed as the fold increase of gene expression in samples from PHA-treated cells above those from control non-treated cells and normalized for the 18S ribosomal RNA gene. Primer sequences are described in Table 1.

2.3. [^{3}H]GABA uptake assay

Isolated lymphocytes (3×10^6) were incubated with [^{3}H]GABA (12 nM) at 37 °C for 20 min. Cells were washed with a buffer containing: 119 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11 mM Glucose, 25 mM Tris–HCl and 1.7 mM KCl. Radioactivity was quantified in a liquid scintillation counter.

Previous to [^{3}H]GABA addition, protein content was determined in an aliquot (0.3 ml) using the Lowry assay.

2.4. [^{3}H]thymidine incorporation assay

Isolated lymphocytes (2×10^5/well) were seeded in 96-well plates. After 48 h, cells were exposed to [^{3}H]thymidine for 16 h. Cells were then harvested on Whatman paper discs, washed with Ringer buffer plus BSA (30 mg/l) and dried. Radioactivity was quantified using a liquid scintillation counter.

2.5. Macroscopic current recordings

Currents were recorded in the whole-cell patch-clamp configuration at a pipette potential of −50 or −70 mV. Pipette solution contained 140 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, and 10 mM HEPES, pH 7.3. Extracellular solution (ECS) contained 150 mM NaCl, 5.6 mM KCl, 0.2 mM $CaCl_2$, and 10 mM HEPES pH 7.3. Different extracellular solutions containing different Ca^{2+} and Mg^{2+} concentrations were tested (0.1 mM, 0.2 mM, 1.8 mM Ca^{2+}, with or without 10 mM Mg^{2+}). A series of pulses (0.5 s) of ECS containing GABA or muscimol was applied to the cells as described before (Bouzat et al., 2008). Briefly, the perfusion system consisted of solution reservoirs suspended on a pole for gravity-driven flow, switching valves, a solenoid-driven pinch valve, and two tubes oriented into the culture dish. One tube contained ECS without agonist and the other contained ECS with agonist. When the pinch valve was switched, solution flowed from only one side at a time, and switched from one side to the other. This perfusion system allowed for a rapid exchange of the solution bathing the patch. The solution exchange time was estimated by stepping the patch held at 0 mV in bath solution and then applying a 500-ms pulse of bath solution diluted 1:2 with water. Typical exchange times ranged from 0.1 to 1 ms (Corradi et al., 2009). Usually, the delay time for the response after opening the valve calculated by the open pipette method is of about 20–50 ms. We determined the delay time for GABA and muscimol responses by measuring the time at which the current starts after opening the valve and substracting the delay time obtained with the open pipette for each cell.

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. Currents were fitted by the exponential function:

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

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

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product (bp)	Ref
α1	GGTTATGCATGGGATGGC	CGGTCAATTTTGCTGACAC	240	(Alam et al., 2006)
α2	AGGATGGACTTGGGATGG	TTCTTTGCTTCAGCTGGC	207	(Alam et al., 2006)
α3	AGTTGGGCTTGGGAAGGC	AGGTCTTGGTCTCAGTCG	247	(Alam et al., 2006)
α4	GAAAGACATCAAAGCCCC	TGTGCCAGATCCAGAAGG	487	(Alam et al., 2006)
α5	CTTTACCAAGAGAGGCTG	GCTTTCAGAAGTCTTCTC	216	(Alam et al., 2006)
α6	TCTTCAGACACAGAAGGC	GGTCTATTTTACTGGTGC	269	(Alam et al., 2006)
β3	CAAGGCAAAGAATGACCG	CACATCGGTTAGATCAGG	295	(Alam et al., 2006)
δ	GAGGCCAACATGGAGTACAC	TTCACGATGAAGGTGTCGG	145	–
γ2	CTCCAGTTATGGCTATCCACG	GATCCAGAAAGACACCCAGG	265	–
ρ1	CATCACGGGCGTGAATGCC	TCCAGCATTGCAGTGCG	215	(Enz and Cutting, 1999)
ρ2	CATCACGGGCGTGAATGCC	GTACCCAGCCAGGCTGTT	257	(Enz and Cutting, 1999)
ρ3	CCCTTTCTCAGTTCTTCATTG	GGAAACTCTTGCAGGAACAG	214	–
GAD67	TGGAAGTGGTGGACATACTCC	AAGTACTTGTAGCGAGCAGCC	531	(Yoshioka et al., 1997)
GAD65	GGACTACCGGGTTTGAAGC	CGAGAGGCGACTCATTCTC	156	–
GAT-1	CCGTGGAGTGACGCTGCCCG	GCTCTCTGCGGTTGCGGAGG	497	–
GAT-2	GGTGACGTTGCCTGGGGCAGC	CCAAGGATGAGGACTTCCCTCCG	500	–
VIAAT	CCGTGTCCAACAAGTCCCAG	TCGCCGTCTTCATTCTCCTC	490	(Metzeler et al., 2004)
VIAAT(nested)	GCAGATGGACATCCTGAAAG	CGGCGAAGATGATGAGAAAC	293	(Metzeler et al., 2004)
GABA-T	GACGGAAGTCCCAGGGCC	CGAGAGCAAGGACTGCCG	307	–
18S	TCGAGGCCCTGTAATTGGAA	CCCTCCAATGGATCCTCGTT	50	(Nizri et al., 2006)

Table 2
mRNA expression of GAD67, VIAAT and GABA-T in human lymphocytes.

	GAD67		VIAAT		GABA-T	
	Resting	Activated	Resting	Activated	Resting	Activated
M, 30 years	−	+	+	+	nd	nd
W, 24 years	+	+	−	+	nd	nd
M, 31 years	+	+	nd	nd	+	+
W, 23 years	+	+	+	+	+	+
W, 28 years	−	+	+	+	+	+
M, 26 years	+	+	+	+	+	+

M: man, W: woman, (+) indicates the presence of PCR bands and (−) indicates absence of bands. nd: not determined.

where I_0 and $I\infty$ are peak and the steady state current values, respectively, and τ_d is the decay time constant (Corradi et al., 2009).

2.6. Immunocytochemistry of VIAAT

Anti-VIAAT staining was performed as previously described by Dumoulin et al. (1999). Lymphocytes were fixed with 2% paraformaldehyde in PBS for 30 min and washed with PBS at room temperature. After fixation, cultures were permeated with 0.1% Triton X-100 for 15 min, washed with PBS and then incubated with anti-VIAAT (1:200, gift from Dr B. Gasnier, Institut de Biologie Physico-Chimique, Paris, France) for 1 h. After washed, cells were incubated with the secondary antibody Cy-3 goat anti-rabbit immunoglobulin G (1:500; Jackson Immunoresearch) for 45 min, washed and mounted. Finally, cells were observed by phase and fluorescence microscopy, using a Nikon Eclipse E600 microscope, and by LSCM (Leica DMIRE2).

2.7. Statistical analysis

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

3. Results

3.1. Expression of GAD, VIAAT and GABA-T in human lymphocytes

Using RT-PCR we first explored if GAD isoforms (GAD67 and GAD65), VIAAT and GABA-T are expressed in resting and mitogen-activated lymphocytes. Human peripheral lymphocytes were incubated for 72 h in the absence and presence of PHA (10 μg/ml). No GAD65 mRNA expression was detected in samples of either resting or activated lymphocytes ($n = 3$). By contrast, mRNAs corresponding to GAD67 and VIAAT were detected in 70–80% of samples corresponding to resting cells and 100% of those of activated lymphocytes (Table 2, Fig. 1A). The presence of the VIAAT protein was confirmed by immunocytochemistry in resting and activated lymphocytes. VIAAT immunoreactivity shows bright spots in either resting or activated cells that might be coincident with vesicular compartments (Fig. 1B and C). The intensity of fluorescence was higher in activated than in resting cells (Fig. 1D).

GABA-T mRNA is detected in all samples, corresponding to resting and activated cells (Table 2, Fig. 2A). To determine if GABA-T gene is differentially expressed between resting and activated cells, we carried out a relative quantification by real time PCR. The results reveal that GABA-T mRNA levels increase about 3-fold in activated cells compared to resting cells (Fig. 2B). Thus, positive regulation of GABA-T expression occurs upon immunological activation, suggesting an increment of GABA catabolism.

These results reveal that lymphocytes contain proteins involved in the synthesis, vesicle storage and catabolism of GABA, similar to the nervous system. They also demonstrate that VIAAT and GABA-T increase upon mitogen stimulation.

3.2. Lymphocytes express functional GABA transporters

Regarding GABA transporters, we studied mRNA expression of GAT-1, which is mainly present in the CNS, and GAT-2, which is present in peripheral tissues.

GAT-1 mRNA was detected in 50% of samples from resting lymphocytes whereas GAT-2 mRNA was not detected in these samples. In contrast, 100% of samples from activated cells express at least one of the two types of transporters (Table 3).

To determine if GABA transporters are functional, we performed [^{3}H]GABA uptake assays. After 72 h incubation in the presence and absence of PHA (10 μg/ml), the radioactive neurotransmitter was added in the presence of Na^+. We detected a 5-fold increment in

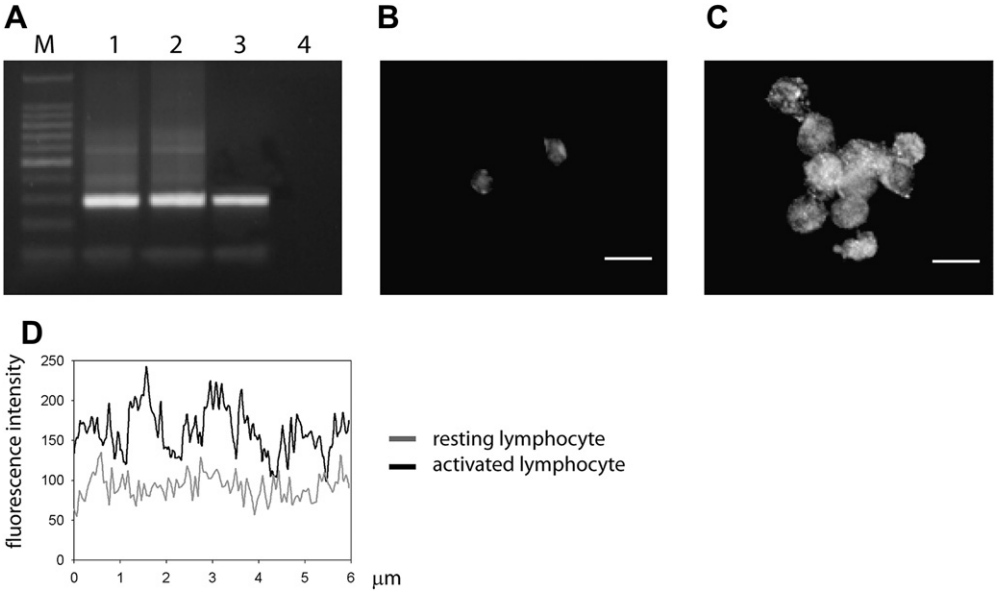

Fig. 1. Expression of VIAAT in resting and activated lymphocytes. (A) Agarose gel electrophoresis of the RT-nested PCR reaction products. Transcripts of VIAAT were detected as a band of 293 bp. Sample 1: 72 h incubation without PHA; sample 2: 72 h incubation with PHA (10 μg/ml); sample 3: PCR positive control (human brain); sample 4: negative control. M: DNA marker (100 bp ladder). (B and C) Expression of VIAAT analyzed by indirect immunofluorescence using confocal microscopy. B – corresponds to resting lymphocytes and C – corresponds to activated lymphocytes. Bars, 12 μm. (D) Example of fluorescence intensity of resting (grey) and activated lymphocytes (black). An arbitrary 6 μm line was traced inside the cells and a curve of fluorescence intensity was constructed for each point of the line.

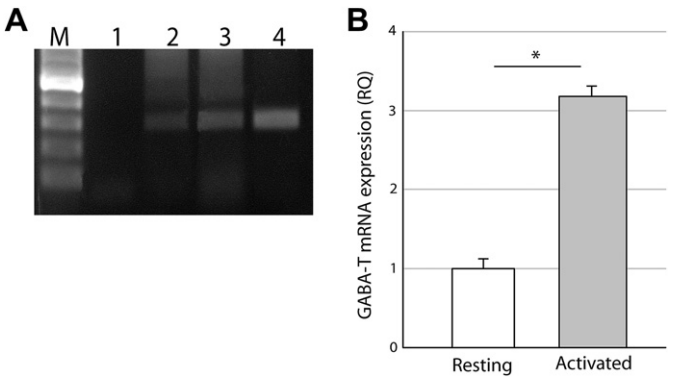

Fig. 2. Upregulation of GABA-T mRNA expression upon lymphocyte activation. (A). Agarose gel electrophoresis of the RT-end-point PCR reaction products. Transcripts of GABA-T were detected as a band of 307 bp. Sample 1: negative control; sample 2: 72 h incubation without PHA; sample 3: 72 h incubation with PHA (10 μg/ml); sample 4: PCR positive control (human brain). M: DNA marker (100 bp ladder). (B) Relative quantification (RQ) of GABA-T mRNA expression. The results are the means ± SD of three independent experiments (*$p < 0.05$).

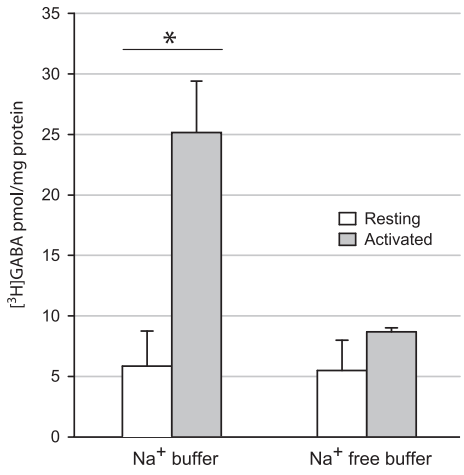

Fig. 3. [^{3}H]GABA uptake in human lymphocytes. [^{3}H]GABA uptake assay was performed as described in M&M. The same experiment was performed in Na^+ depletion to test Na^+ dependency of GABA transporters. Data are given as [^{3}H]GABA pmol/mg protein and are means ± S.D. of 3 independent experiments (*$p < 0.05$).

GABA uptake in activated lymphocytes compared to resting ones (Fig. 3). No changes in GABA uptake in activated cells were detected when the assay was performed at 0 °C, temperature at which the transporter is inactive (data not shown). We also investigated the Na^+-dependence of the transport by measuring GABA uptake in a Na^+-free buffer. Removal of Na^+ reduced the uptake significantly (Fig. 3), indicating that GABA transport is dependent on Na^+, as expected for this type of transporter.

3.3. Ionotropic GABA receptor subunits are expressed in human lymphocytes

We studied mRNA expression of $GABA_A$ receptor subunit subtypes (α1–α6, β3, γ2 and δ) and $GABA_C$ subunit subtypes (ρ1–ρ3) by RT-PCR. RNA was isolated from lymphocytes after 72 h incubation in the presence and absence of PHA (10 μg/ml). Resting and activated lymphocytes expressed at least one GABA subunit subtype (Table 4). The patterns of expression varied among individuals as well as among different samples from the same individual (data not shown). Furthermore, subunit expression changed whether cells were activated or not. For example, β3 subunit was detected only in activated cells (Table 4). α1, δ and ρ2 subunits seem to show the most constant pattern of expression between resting and activated cells. Regarding $GABA_C$ receptor subunits, only ρ2 subunit was detected either in resting or activated lymphocytes.

3.4. Ionotropic GABA receptors on lymphocytes are functional

To determine if GABA subunits assemble into functional channels, we performed whole-cell recordings in activated lymphocytes. When applied to whole cells, clamped at a pipette potential of −50 or −70 mV under almost symmetrical chloride solutions, GABA (0.5–10 mM) elicits macroscopic currents in about 10–15% of the cells. Two types of currents were observed on the basis of their decay time constants (Fig. 4). One type corresponds to currents that activate rapidly (rise time briefer than 1 ms) and decay more rapidly (Fig. 4A). For most currents, the decays could be fitted by a single exponential, with a mean time constant of ~75 ms. The second type includes currents that decay to less than 50% within the 0.5-s GABA pulse and show a mean decay time constant of ~2.5 s (Fig. 4B).

We measured the delay times of the agonist-elicited responses respect to the delay of the system determined by the open pipette method (see Materials and Methods, Section 2.5). We observed that fast desensitizing currents show delays of 250 ± 87 ms and slow-desensitization currents, delays of 7 ± 5 ms. (Fig. 4).

To determine if these currents correspond to ionotropic GABA receptors, we performed similar experiments using muscimol. This drug behaves as a full agonist of $GABA_A$ receptors and partial agonist of $GABA_C$ receptors. As shown in Fig. 4C, 50–100 μM muscimol is also capable of activating macroscopic currents, which decay with rates of ~70 ms ($n = 5$). Typically, the currents show delays of about 250–300 ms, similar to that of fast currents elicited by GABA.

Unfortunately, important rundown of the currents is observed, and currents disappear completely usually after 5 pulses of agonist. This fact restricts the performance of complete pharmacological studies.

This set of experiments shows that lymphocytes express functional ionotropic GABA-activated channels, and suggest that at least two different types of GABA receptors are present.

Table 3
mRNA expression of GAT-1 and GAT-2 in human lymphocytes.

	GAT-1		GAT-2	
	Resting	Activated	Resting	Activated
M, 30 years	−	−	−	+
W, 24 years	−	+	−	+
M, 31 years	+	+	−	−
W, 28 years	+	+	−	+

M: man; W: woman, (+) indicates the presence of the PCR bands and (−) indicates absence of bands.

3.5. GABA inhibits lymphocyte proliferation

To determine the role of the GABAergic system in lymphocyte proliferation, we incubated cells in the presence of PHA (10 μg/ml) and GABA (0–1000 μM) for 72 h. The PHA-induced T-cell proliferation response considerably decreased in the presence of GABA. The inhibition reached values of about 20–30% for GABA concentrations ≥1 μM. At 0.1 μM no significant effect was observed (Fig. 5). No changes were observed in the presence of GABA in resting cells, indicating that GABA neither stimulates cell proliferation nor has toxic effects.

Table 4
mRNA expression of ionotropic GABA receptor subunits.

A. Expression of $GABA_A$ receptor subunits.

	α1		α2		α3		α4		α5		α6		β3		γ2		δ	
	R	A	R	A	R	A	R	A	R	A	R	A	R	A	R	A	R	A
M, 30 years	+	+	−	−	−	+	−	−	−	−	+/−	+/−	−	+	−	+	+	+
W, 23 years	+	+	−	−	+/−	+/−	−	−	−	−	+	+	−	+	+	+	+	+
M, 28 years	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+
W, 24 years	+	+	−	−	−	−	−	−	−	−	−	+/−	nd	nd	nd	nd	nd	nd

B. Expression of $GABA_C$ receptor subunits.

	ρ1		ρ2		ρ3	
	R	A	R	A	R	A
M, 30 years	−	−	+	+	−	−
W, 23 years	−	−	+	+	−	−
M, 28 years	−	−	+	+	−	−

R: resting; A: activated; M: man; W: woman, nd: not determined '+' indicates the presence of the most intensely stained PCR bands, '+/−' indicates the presence of faint bands and '−' indicates absence of bands.

3.6. The immuno-suppressive effect of GABA is principally mediated by ionotropic GABA receptors

To ascertain if the effect of GABA on proliferation is mediated by ionotropic GABA receptors, we performed proliferation assays in the presence of muscimol, an agonist for these GABA receptors. Muscimol, similarly to GABA, inhibits lymphocyte proliferation at concentrations ≥1 μM. The inhibition level was about 20% for all tested concentrations. No effect was observed at concentrations lower than 1 μM (Fig. 5) or in resting cells (data not shown).

Thus, these data suggest that ionotropic GABA receptors are the main modulators of the immune-suppressive effects of GABA.

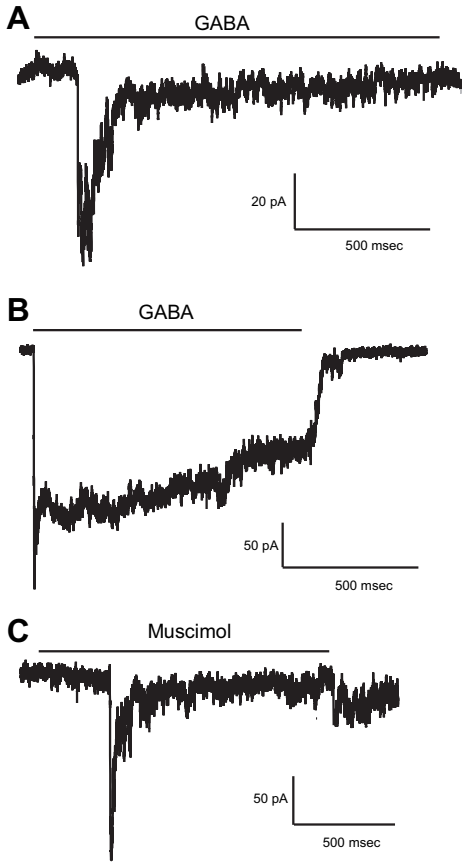

Fig. 4. Macroscopic currents evoked from lymphocytes. Representative whole-cell currents elicited by 10 mM GABA (A, B) or 50 μM muscimol (C) from activated lymphocytes are shown. The solid line above each current trace indicates the duration of drug application. The pipette potential is −50 mV (A, B) and −70 mV (C).

4. Discussion

Our results reveal the presence of a GABAergic system in human peripheral lymphocytes. We determine GAD and VIAAT mRNA expression, which are indicators of GABA-producing cells. Blood GABA concentration in healthy individuals is around 0.1 μM, however the precise origin of this molecule is not known (Bjork et al., 2001). Our results suggest that peripheral lymphocytes are a source of plasmatic GABA. RT-PCR experiments show that mRNA corresponding to GAD67 is present in lymphocytes, whereas GAD65 mRNA could not be detected. These two GAD isoforms are expressed in neurons. While GAD65 is associated to nerve

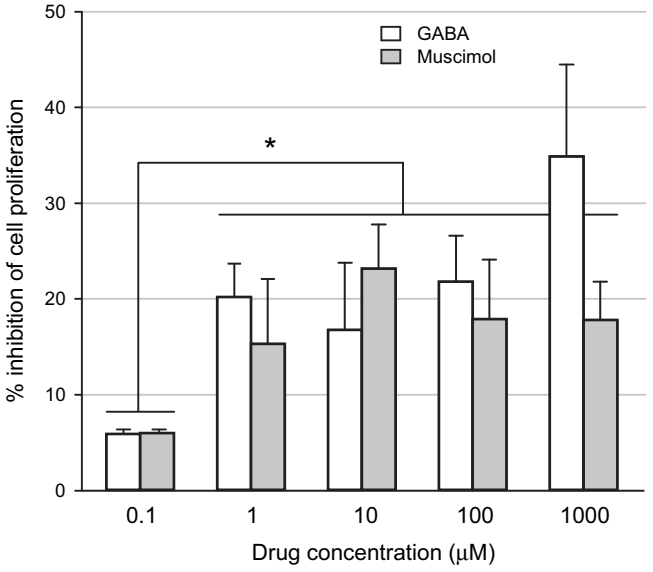

Fig. 5. Inhibition of lymphocyte proliferation by GABA and muscimol. Lymphocyte proliferation was evaluated by [^{3}H]thymidine incorporation assay as described in M&M. Percentage of inhibition was calculated as the difference between percentage of maximal proliferation (PHA) and percentage of PHA-induced-proliferation in the presence of different drug concentrations. Results are expressed as mean ± S.D. of 6 independent experiments (*$p < 0.05$).

terminals and it produces GABA for neurotransmission, GAD67 is spread evenly throughout the neuronal cytoplasm and the generated GABA is utilized for purposes other than neurotransmission, such as serving as a signaling molecule during development, source of energy and regulator of redox potential during oxidative stress (Buddhala et al., 2009). However in GAD65 knockout mice, GAD67 can play a similar role as GAD65, providing GABA for neurotransmission (Wu et al., 2007). Parallel expression of GAD67 and VIAAT has also been characterized in other non-neuronal tissues, like pancreatic islet (Chessler et al., 2002). Since this GAD isoform is non-membrane associated, some authors proposed that this association is necessary to allow an adequate GABA entry into synaptic-like microvesicles. Our results are in agreement with this statement since expression of VIAAT was detected in lymphocytes in parallel with expression of GAD67.

The existence of vesicles for neurotransmitters in lymphocytes is controversial. Some authors revealed immunoreactivity for specific neurotransmitter vesicle transporters like VAChT for acetylcholine and VMAT for monoamines, but others were not able to detect mRNA of these proteins (Amenta et al., 2001; Fujii et al., 1998; Tayebati et al., 2002). Here we demonstrate the presence of VIAAT mRNA in resting and activated lymphocytes. Besides, VIAAT staining reveals punctual fluorescence distribution suggesting the presence of vesicles. However, it may be possible that these are not typical neurotransmitter vesicles as the ones described in neurons. In immune cells, secretory lysosomes are dual-function organelles that harbour degrading and secretory properties at the same time (Lettau et al., 2007). Regarding this, there is evidence that dendritic cells store the neurotransmitter serotonin in lysosomal vesicles (O'Connell et al., 2006). Further investigation is needed to confirm the nature of these GABA vesicles in lymphocytes.

We also demonstrate the functionality of GATs and their dependence on Na^+, in agreement with previous reports (Christiansen et al., 2007; Loo et al., 2000). The increment in GAT activity observed in activated lymphocytes is in line with previous studies that demonstrated that after antigen triggered T-cell activation, GAT-1 expression is upregulated (Wang et al., 2008). Recently, it has been proposed that GAT-1 negatively regulates T-cell activation and survival by modulating protein kinase C-mediated signaling pathways by a still unknown mechanism (Wang et al., 2009).

According to other studies that described an increment in GABA-T activity in thymus of patients under immune stimulation (Cavallotti et al., 2000), we observed an increment in GABA-T mRNA levels 72 h after lymphocyte activation. This could be explained by a necessity of catabolizing GABA to restore its physiological levels. Although measurements of neurotransmitter levels during activation are required to explain the final effect on GABA content during activation, the increases in VIAAT fluorescence intensity, GABA-T expression and GAT activity show that this process is accompanied by changes in intracellular GABA content. Besides, others have demonstrated a functional coupling between GABA synthesis and vesicular GABA transport (Jin et al., 2003). Another explanation to the increased in GABA-T mRNA levels may be that during activation GABA is not only being used as a classical neurotransmitter but also as a source of energy. GABA can be transaminated with pyruvate to yield alanine or with 2-oxoglutarate to yield glutamate, generating succinic semialdehyde, which can then be metabolized to succinate. The conversion of glutamate to succinate is known as the "GABA shunt" affording an alternative pathway for glutamate entry into the Krebs cycle. Therefore, through the action of GABA-T, GABA provides ATP required in many processes during activation, for example phosphorylation of T-cell receptor signaling proteins.

The presence of GABA-T and functional GATs in lymphocytes is relevant not only because of the participation of GABA during immune response but also because these proteins are drug targets for treatment of CNS disorders like epilepsy (Schachter, 1999). This might explain why treatments with antiepileptic drugs have been associated with certain immune aberrations (Basta-Kaim et al., 2008).

The lymphocytic GABAergic system might be involved in the aetiology of autoimmune diseases. For example, antibodies to GAD65 are found in Type-1 Diabetes Mellitus (Falorni and Brozzetti, 2005). It could be that if GABA release has a local immunosuppressive effect, particularly on T-cell function, the defective GABA release could be expected to impact on disease progression in this T-cell driven autoimmune disease.

Macroscopic current recordings reveal that ionotropic GABA receptors in lymphocytes are functional. Two main types of GABA-elicited currents could be distinguished on the basis of their decay rates and extent of desensitization. This suggests that at least more than one subtype is present on membrane lymphocytes, in agreement with the fact that different types of subunits were detected in most of the tested samples. Moreover, it has been determined that currents from receptors with different subunit composition show different activation and desensitization rates (Feng et al., 2004). Further experiments are required to characterize the functional subtypes of ionotropic GABA receptors on lymphocytes and to determine the existence of expression specificity among different lymphocyte subtypes. However, the profound rundown of the currents makes the pharmacological characterization very difficult.

We have observed variability in the expression pattern of receptor subunits among individuals (Table 4). It could be possible that after immune stimulation, the receptors change their composition and acquire different pharmacological and kinetic characteristics. The plasticity of GABA receptors has been extensively studied in the nervous system. GABA chronic exposure in cortical neurons changes subunit mRNA levels, indicating that changes in GABA receptor number and composition are initiated by GABA binding and/or subsequent receptor activation (Lyons et al., 2000).

Further pharmacological studies are needed to clarify the nature of the GABA receptors in lymphocytes. We can speculate that they assemble into similar receptors to the ones described in CNS. However, even in the brain the molecular composition of GABA receptors is still a matter of debate. Early studies supported the view that $GABA_C$ receptors were composed exclusively of ρ subunits. More recent studies suggest that hybrid $GABA_A$ subunit and $GABA_C$ subunit receptors are also able to assemble (Harvey et al., 2006; Milligan et al., 2004).

We demonstrate a negative modulation of human peripheral lymphocyte proliferation by GABA. Similar effects were reported in mouse encephalitogenic T-cell lines, lymph node and splenic mouse cells (Bjurstom et al., 2008; Tian et al., 1999). The maximal inhibition of proliferation is about 20% and there is not a gradual dose response by increasing the doses of GABA. It may be possible that only a subset of lymphocytes is sensitive to GABA. Although inhibition is achieved at higher GABA concentrations than the plasmatic ones, it could be possible that these high concentrations are reached at the receptors neighbourhood as it occurs in neuronal synapses (Bjork et al., 2001). The results from the proliferation assays with muscimol suggest that the inhibitory effect is principally mediated by ionotropic GABA receptors. These data is in accordance with Tian et al. (1999) who demonstrated that muscimol and not baclofen, the metabotropic $GABA_B$ receptor agonist, inhibits mouse antigen primed T-cell proliferation. It also agrees with the detection of muscimol-activated currents. It has been proposed that activation of depolarizing GABA (Cl^-) channels would decrease the entry of calcium into the cell. This would reduce all Ca^{2+} dependent processes involved in lymphocyte activation, like cytokine production and expression of mitogen genes

like c-fos and c-jun, and consequently cell proliferation (Bjurstom et al., 2008; Tian et al., 2004).

In conclusion, our study reveals the presence of an intrinsic GABAergic system in human lymphocytes. This system seems to be similar to that expressed in neurons and functionally operates as a modulator of cell activation. Our findings suggest that pharmacological modulation of the GABAergic system may provide new approaches for regulation of T-cell response.

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