International Journal of Pharmacology

ISSN 1811-7775

science alert

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.205.211

Research Article
Expression and Function of Non-neuronal GABA Transporters in Human Lymphocytes

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Abstract

Background: Neurotransmitters not only play a key role in neuronal communication but also coordinate cell functions in non-neuronal cells, such as immune cells. Previous research reported a complete GABAergic system in human lymphocytes. In this study, focus is put on GABA transporters (GATs) and their physiological role during lymphocyte activation. **Materials and Methods:** Using RT-PCR and [^{3}H]-GABA uptake assays, GAT expression and activity was evaluated under basal conditions and in cells exposed to phytohemagglutinin (PHA) or GABA. To study GAT role, cell proliferation was evaluated by [^{3}H]-thymidine incorporation in the presence of Nipecotic Acid (NA), a GAT inhibitor. Finally, using HPLC GABA levels were analyzed in culture supernatants. **Results:** In lymphocytes under any condition, at least one GAT subtype was detected. No GAT-3 was found. The PHA and GABA-treatment increased both expression and activity. In addition, GAT blockade inhibited PHA-induced lymphocyte proliferation. The GABA was detected only in PHA-treated cell supernatants, thus indicating GABA secretion at least during proliferation. **Conclusion:** These findings demonstrate that extraneuronal GATs affect lymphocyte functionality. Characterization of GAT roles in immune response and as a link between nervous system and immune system will provide new therapeutic targets and could help reduce side effects of current therapies.

Key words: GABA transporters, GABA, T-cell proliferation, pharmacological modulation, lymphocytes, neuroimmune interactions, GAT expression, non-neuronal tissues

Received: August 04, 2016 Accepted: November 06, 2016 Published: January 15, 2017

Citation: L. Dionisio, H. Caldironi and M.J. De Rosa, 2017. Expression and function of non-neuronal GABA transporters in human lymphocytes. Int. J. Pharmacol., 13: 205-211.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the Nervous System (NS). Once released, as GABA is not inactivated by any enzyme GABAergic signal termination depends on clearance by GABA transporters (GATs) which quickly remove the neurotransmitter from the synaptic cleft. This modulation of GABA levels, highlight the importance of GATs as emerging targets to regulate many physiological processes in which GABAergic transmission is involved. GABAergic drugs are widely used in medicine for several NS pathologies. They are applied as anticonvulsants, anxiolytics and anesthetic drugs. At present, GATs blockers, such as tiagabina are used in epilepsy treatment given its anticonvulsant properties[1-3]. The GABA is nonetheless far from being restricted to NS as it is also produced in several non-neuronal cells, such as pancreas and immune cells[4,5]. In these extraneuronal tissues, GABA physiology is less understood.

The GATs belong to the SLC6 family which includes Na^+/Cl^- dependent transporters, such as dopamine, serotonin glycine and norepinephrine transporters[6]. Transporters can work in any direction depending on electrochemistry gradient and cell membrane potential. In spite of their typical role of removing GABA from the synaptic cleft, several reports described that transporter reversion can also occur under physiological conditions, being responsible for GABA leakage in NS[7,8]. In humans, there are 4 different GAT subtypes (GAT 1-3 and BGT-1), each with typical tissue distribution and pharmacological properties[6,9]. Although, GAT-1 and GAT-3 have been considered to be exclusively expressed in NS, previous research confirmed the presence of GAT-1 and GAT-2 in human lymphocytes[10]. On the other hand, GAT-2 and BGT-1 subtypes were also described in non-neuronal cells such as kidney, liver, lungs and testicles[9,11].

Neurotransmitters not only play a key role in neuronal communication but also coordinate cell functions in non-neuronal cells, such as immune cells[12]. Several studies on the brain have shown the involvement of neurotransmitter systems in the development of neuroinflammation in autoimmune diseases, such as multiple sclerosis and neurodegeneration processes observed in Alzheimer and Parkinson disease[13-15]. In this respect, Wang *et al.*[16,17] reported that GAT-1 deficiency exacerbates experimental autoimmune encephalomyelitis progression. In addition, an increase in GAT-2 expression was observed in multiple sclerosis patients' brains[18]. All these findings underscore the role of GATs in several diseases with an immune component, probably through the regulation of GABA levels.

Previously research from our lab characterized the presence of a complete functional GABAergic system in human lymphocytes. This endogenous system is functional since exposure to GABA negatively regulates PHA-induced proliferation[10]. In the present study, the focus is on the study of GATs expression in human lymphocyte and their physiological role during lymphocyte activation. These studies demonstrated that GATs could represent a new pharmacological target for several diseases in which the immune response and/or GABA are involved.

MATERIALS AND METHODS

Isolation and culture of human peripheral lymphocytes: Samples used in this study were obtained after informed consent following the declaration of Helsinki and after the ethical approval of Hospital Municipal de Agudos, Bahía Blanca, Argentina. All procedures were followed with the adequate understanding and written consent of the subjects involved in the present study. Lymphocytes were obtained from healthy volunteers (22-40 years old) essentially as described before[19]. Blood (20 mL) was withdrawn from the antecubital vein using EDTA as anticoagulant and loaded on 3 mL ficoll (Amersham Biosciences, AB, Sweden) separating solution to isolate mononuclear cells. Macrophages were discarded by the plastic adherence method. Lymphocytes were cultured in RPMI-1640 (Hyclone, USA) medium supplemented with 10% Fetal Calf Serum (FCS) at 37°C in a humidified atmosphere at 5% CO_2. Lymphocytes were incubated in either the absence (resting) or presence of mitogen phytohemagglutinin (PHA) or γ-aminobutyric acid (GABA) during 72 h.

RT-PCR analysis: Total RNA was isolated from 3×10^6 cells by using trizol (1 mL). The RNA was converted into cDNA using the molony murine leukaemia virus reverse transcriptase (MLV-RT, Promega, USA) and random primers (Promega, USA). End-point Polymerase Chain Reaction (PCR) was run in a Mini Cycler™ (MJ Research, USA) for 35 cycles. Specific primers of each GAT subtypes were used (Table 1). Primers were designed in two different exons to distinguish from genomic amplification. Superscript human brain cDNA library (female, 36 years) (Invitrogen) was used as positive control. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

[^{3}H]-GABA Uptake assay: The [^{3}H]-GABA (12 nM) was added to the culture dishes and cells were incubated at 37°C for 20 min. Cells were subsequently washed with an ionic buffer

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

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
GAT-1	CCGTGGAGTGACGCTGCCCG	GCTCTCTGCGGTTGCGGAGG	497
GAT-2	GGTGACGTTGCCTGGGGCAGC	CCAAGGATGAGGACTTCCCTCCG	500
GAT-3	CATCATCATCCTGGCATGG	GCTGTAGTTGCTCACATTCA	136
BGT-1	CTACTACTTGAAGCCAGAT	GTTGTTGTGATACTTGTTGT	139

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 determined in a scintillation counter. A Lowry assay was performed to measure protein content and to normalize radioactivity.

[^{3}H]-thymidine incorporation assay: About 2×10^5 cells were seeded in 200 µL medium per well in 96-well plates. After 48 h cells were exposed to [^{3}H]-thymidine for 16 h. Cells were harvested on Whatman paper discs, washed with Ringer buffer plus BSA (30 mg L^{-1}) and subsequently dried at room temperature. Radioactivity was quantified in a liquid scintillation counter.

High Performance Liquid Chromatography (HPLC): The GABA present either in lymphocyte supernatant or in the standard curve was determined using HPLC and fluorometric detection (Varian Instr., Palo Alto, CA, USA) under the described condition. Briefly, samples were derivatized with phenyl isothiocyanate (PICT), dried under N_2 at room temperature. Residues were dissolved in ethanol:water:triethylamine (TEA) (2:2:1) and dried under N_2. Ethanol:water:TEA:PICT (7:1:1:1) was added to the residues and allowed to react for 20 min at room temperature. An 80% solution A (sodium acetate 100 mM, 0.5 mL TEA and 0.7 mL acetic acid, pH 5.5) and 20% acetonitrile was used as mobile phase. Separations were performed on a RP-18 column (250×4 mm, particle size 5 µm). The mobile phase was pumped at 0.7 mL min^{-1} at 30°C and detection was done at 244 nm. The GABA (SIGMA) standard curve was prepared by dissolving solid drug in ultrapure water. The stock solution was subsequently diluted to reach different concentrations (2, 5 and 25 µM).

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.

RESULTS

GAT expression in lymphocytes can be modulated pharmacologically: The GABA transporter subtype (GAT-1-3 and BGT-1) expression was studied in human lymphocytes. Lymphocytes from at least three healthy volunteers were incubated for 72 h and samples were further analyzed by RT-PCR. The expression of GAT-1, GAT-2 and/or BGT-1 was detected but GAT-3 was not detected in resting cells. Percentages of positive samples were relatively low for GAT-1 and GAT-2 (being approximately 20 and 12.5%, respectively). The BGT-1 expression was higher than the other subtypes detected in approximately 66% of untreated samples (Table 2).

Table 2: mRNA expression of GAT subtypes in human lymphocytes

GABA transporter subtype	Resting	PHA	GABA
GAT-1	2/9	4/9	0/5
GAT-2	1/8	7/8	3/4
GAT-3	0/3	0/3	0/3
BGT-1	2/3	3/3	3/3

GAT subtypes were studied in lymphocytes incubated in the absence (resting) or presence of each drug during 72 h, mRNA corresponding to each subtype was detected by RT-PCR and the predicted product was observed by agarose gel electrophoresis. Ratio represents positive samples/total samples analyzed

The regulation of GAT expression by drugs was analyzed. The effect of the presence of the mitogen PHA (10 µg mL^{-1}), which induces lymphocyte activation and the presence of GABA (100 µM), the endogenous GAT substrate was studied. Expression of the three subtypes detected increased after PHA treatment. Positive samples were 45% for GAT-1, 88% for GAT-2 and 100% for BGT-1. In addition, the expression of GAT-2 and BGT-1 subtypes also increased in 75 and 100% of the samples analyzed, respectively by GABA treatment. Once again, no mRNA GAT-3 was detected under any condition. Table 2 summarizes the results obtained.

Activity regulation of endogenous GATs in human lymphocytes: To study if the mRNA modulation observed represents an increase in GAT activity, [^{3}H]-GABA uptake assays was performed in human lymphocytes incubated with PHA (10 µg mL^{-1}) and GABA (100 µM) during 72 h. The results showed that incubation with both drugs increased GABA uptake. According to previous results, an increment of approximately 5-fold in GABA uptake in PHA-treated lymphocytes (5.3±1.2, n = 3) was detected, whereas, GABA incubation was observed to induce a 2-fold increment (1.8±0.5, n = 3), (Fig. 1). As previously reported, no changes in GABA uptake were detected either when the assay was performed at 0°C, a temperature at which this type of transporters are inactive, or was significantly reduced by using Na^+-free buffer[10].

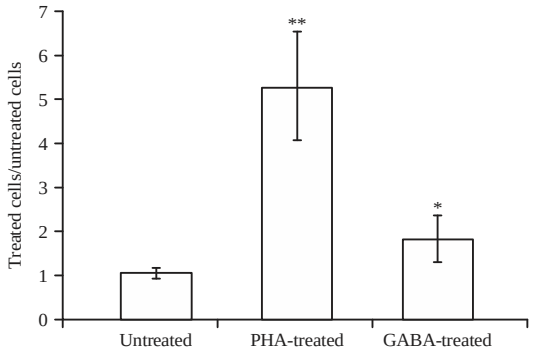

Fig. 1: GAT activity is increased in PHA or GABA-treated lymphocytes. The [^{3}H]-GABA uptake assay was performed as described in Methods section. Data are given as the ratio between PHA or GABA treated vs untreated samples (comparing [^{3}H]-GABA pmol mg^{-1} protein). For each condition the Mean±SD of 3 independent experiments is shown, **p<0.005, *p<0.05

Taken together, these results indicate that incubation with mitogen PHA and the endogenous GAT substrate can increase GABA uptake in lymphocytes.

GATs blockade modulates lymphocyte proliferation: To study a functional role of GATs expressed in human lymphocytes, the focus was on investigate their participation in cell proliferation induced by mitogen PHA. To this end, cells were exposed to both, PHA (5 μg mL^{-1}) and a characterized GAT blocker, Nipecotic Acid ((NA) from 1-500 μM for 72 h. Cell proliferation was subsequently evaluated by [^{3}H]-thymidine incorporation. Figure 2 shows that, all NA concentrations evoked an inhibition of PHA-induced lymphocyte proliferation (Fig. 2a). The effect reached approximately 30-40% of inhibition and exhibited no concentration dependence. In addition, no significant changes in the number of cells were observed in the presence of NA alone, thus indicating no toxic effect even at 1000 μM (Fig. 2b).

Lymphocytes are able to secrete GABA: Previous study demonstrated that lymphocytes have all the necessary components for GABA synthesis[10]. However, whether or not these cells have the ability to release the neurotransmitter and the mechanism involved in this secretion is still unclear. Thus, GABA detection in lymphocyte supernatant cultures was analyzed.

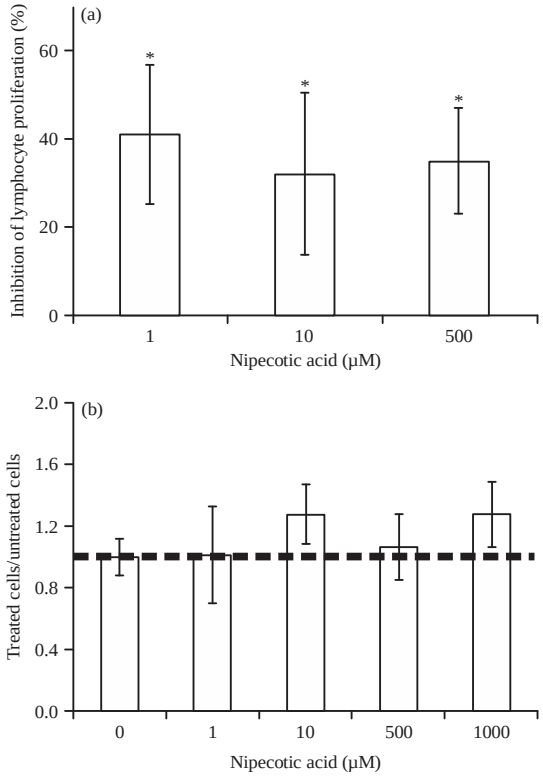

Fig. 2(a-b): GAT blockade inhibits PHA-induced lymphocyte proliferation. Lymphocyte proliferation was analyzed by [^{3}H]-thymidine incorporation assay as described in Methods section, (a) Nipecotic Acid (NA) inhibites PHA-induced cell proliferation. Percentage of inhibition was calculated as the difference between percentage of maximal proliferation (PHA-treated samples) and percentage of PHA induced-proliferation in the presence of NA at different concentrations and (b) NA has no toxic effect in lymphocyte cultures. Cell number is expressed as the ratio between NA-treated versus untreated cells. Disrupted line represents basal lymphocyte proliferation (untreated samples). Results are expressed as Mean±SD of 3 independent experiments (*p<0.05)

Lymphocytes were incubated in the presence or absence of mitogen PHA (10 μg mL^{-1}) for 72 h and the supernatants were subsequently collected and analyzed by HPLC. The PHA-treated samples showed a peak with the same retention time as GABA (Fig. 3). The characteristic peak was only detected in activated lymphocytes. The GABA concentration was 129±43 nmol mL^{-1} (n = 4). These results indicate that lymphocytes have the ability to synthesize and release the neurotransmitter at least during activation.

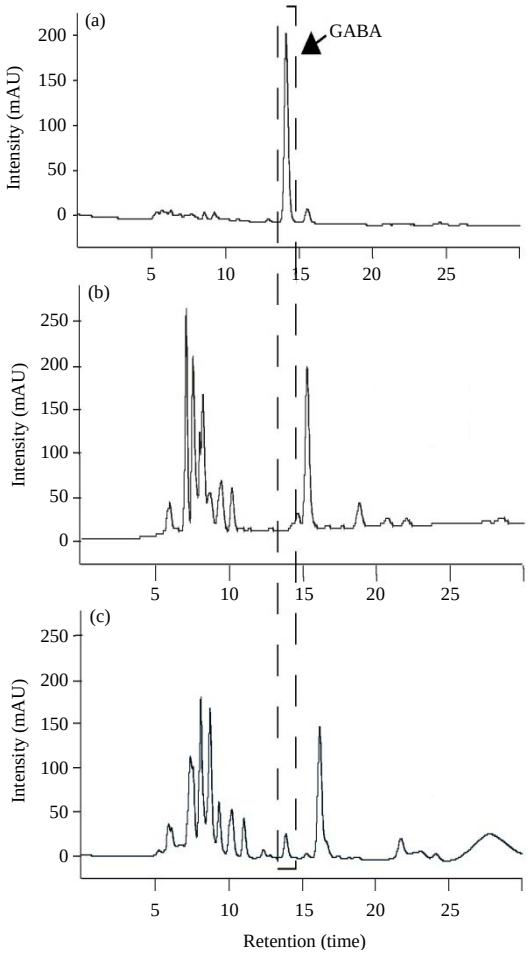

Fig. 3(a-c): PHA induces GABA release in lymphocytes. HPLC traces of supernatants of human lymphocyte cultures incubated for 72 h either in the absence or presence of PHA, (a) GABA (2 μM) was used as standard, (b) Untreated samples and (c) PHA-treated samples

DISCUSSION

This study shows that GAT expression can be regulated *in vitro* either by drugs, such as mitogens, that affect the immune system, or by molecules with a main function in the NS like the neurotransmitter GABA. This effect was also reported in GATs expressed in the brain, as their expression can be modulated by immune molecules, such as cytokines[20]. Therefore, the GABA transporter system of both neuronal and immune cells is regulated by chemical mediators that act under physiological or pathological conditions. It would thus be promising to study the functional consequences of the expression regulation in each tissue.

Previous research has demonstrated that the pattern of expression of GABA receptor subunits and other components of the GABAergic system varies among individuals[10]. A similar variability was observed in this analysis of GAT expression levels. As any of each subset cells was not dissected, such as B-cells, $CD4^+$, $CD8^+$ or regulatory T-cells, it could be possible that each lymphocyte subset expresses different GAT subtypes and that the particular immunological state of each volunteer represents an increment of this lymphocyte subset. In agreement with other researchers who demonstrated that GAT-3 subtype is exclusively expressed in the brain, the presence of GAT-3 in human lymphocyte samples was not detected[6].

An increment in GAT mRNA expression in neurons does not necessarily represent an increment in GAT activity[21]. To clarify the effect of induction of GAT expression on human lymphocyte cultures, GAT activity by [^{3}H]-GABA uptake assays was studied. According to our results PHA and GABA increase both, expression and activity. Further experiments may help identify GAT subtype contribution to this uptake increment as well as determine if there is/are any specific transporter/s involved that could be particularly modulated in lymphocytes.

As GABA is synthesized from glutamate by only one enzyme, the Glutamic Acid Decarboxylase (GAD), the presence of this enzyme suggests that lymphocytes can synthetize GABA[10]. In this study, GABA detection in supernatant cultures, at least after mitogen stimulus is the first line of evidence that human lymphocytes synthetize and release this neurotransmitter. Neurotransmitter release mechanism in these cells is unknown on account of the fact that classical neurotransmitter vesicles structures have not been described to date. Some alternative GABA release mechanisms were reported in glia cells. The latter share with lymphocytes two characteristics points: They have no neurotransmitter vesicles and they are non-excitable cells. A GABA release mechanism in glia cells seems to involve proteins called bestrophines (Best 1)[22]. These proteins are specialized Ca^{2+}-activated anion-channels which permeate GABA and also contribute to tonic GABAergic currents[23]. However, this hypothesis was discarded because Best 1 mRNA in resting or activated lymphocytes was not detected (data not shown). Another non-vesicle related mechanism of GABA release reported in glia cells was observed to involve GATs[7]. It could thus be possible that under particular conditions the intracellular versus the extracellular balance of GABA concentrations or even ionic variations allows the release of GABA through GATs[7,8,24]. The same could also be possible in human lymphocytes, for example during activation, when GABA synthesis is increased.

These findings demonstrate that GAT activity affects lymphocyte functionality. They also show that the presence of a GAT blocker inhibits PHA-induced proliferation, independently of its concentration. Taking into account that exogenous GABA in lymphocyte cultures decreases PHA-induced proliferation, GABA transporter blockade could lead to a build-up of endogenous GABA in culture supernatants, thus producing a similar inhibitory effect[10].

This does not discard the hypothesis that even if GATs were involved in GABA release, GABA accumulation could be possible on account of the fact that whereas, NA has no effects on the intracellular side of transporters[25], a possible mechanism involved in neurotransmitter release, it does have effects on the extracellular side of transporters, a mechanism involved in GABA uptake[25].

The fact that GAT blockade affects lymphocyte physiology is important as GAT blockade is used in clinic for the treatment of epilepsy, thus being a molecular basis of some immunological problems reported for GABAergic therapies[26-28].

Lymphocyte-secreted GABA could have an autocrine and paracrine effect on immune cells. Although in plasma, GABA reaches a low concentration (submicromolar)[29], it could be possible that during the immune response, a tight cellular contact interface-known as immunological synapse-allows cells to be exposed to higher GABA levels, activating the GABA receptors observed in lymphocytes, macrophages and dendritic cells, thus affecting cell physiology, mainly by inhibition[10,30-32]. All this merits further investigation, because GAT modulation may affect GABA levels during the immune response, thus acting as an immunoregulatory molecule.

CONCLUSION

In this study the presence and functionality of GABA transporters were characterized in human lymphocytes. GATs can affect lymphocyte physiology. The PHA-induced cell proliferation was negatively modulated by GAT blockade. These results also show that lymphocytes can secrete GABA at least during proliferation.

The lymphocyte endogenous GABAergic system may act either independently or as a link between NS and IS to receive and transmit signals that neuronal as well as immune cells can decipher. These results lead us to conclude that the modulation of extraneuronal GAT expression and functionality could not only become a new therapeutic strategy for the treatment of diseases that affect systems other than the NS, but also contribute to preventing and reducing the side effects of current therapies.

ACKNOWLEDGMENTS

We thank Dr. D. Rayes and Dr. C. Bouzat (both from the Instituto de Investigaciones Bioquímicas de Bahía Blanca, Argentina) for their discussion, help and support in the present study. We also thank V. Soler for her technical assistance in controlling the use of English in this ms.

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