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Stimulation of group I metabotropic glutamate receptors evokes calcium signals and *c-jun* and *c-fos* gene expression in human T cells

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Abstract

To study if the activation of group I mGlu receptors in human T cells modifies intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell function, we measured $[Ca^{2+}]_i$ on cell suspensions (spectrofluorimetric method) or single cell (digital Ca^{2+} imaging system) using fura-2 as indicator. Early-inducible gene (*c-jun* and *c-fos*) expression was studied by reverse transcriptase-polymerase chain reaction assay as representative of Ca^{2+}-sensitive gene expression. (1*S*,3*R*)-ACPD (100 μM), the selective mGlu receptor agonist, evoked a significant increase (34.1 ± 4.9%) of $[Ca^{2+}]_i$, pharmacologically characterized as mediated by group I mGlu receptors, since both (*S*)-3,5-DHPG (100 μM), a selective group I mGlu receptor agonist and CHPG (1 mM), the specific $mGlu_5$ receptor agonist, reproduced the effects, that were abolished by AIDA (1 mM), a selective group I mGlu receptor antagonist. (*S*)-3,5-DHPG-induced a rapid $[Ca^{2+}]_i$ rise (initial phase) followed by a slow decrease (second phase) to the baseline. Both extracellular Ca^{2+} and Ca^{2+} released from intracellular stores contribute to the $[Ca^{2+}]_i$ increase which depend on PLC activation. In a Ca^{2+}-free buffer, the second phase rapidly return to the baseline; $LaCl_3$ (1–10 μM), an inhibitor of extracellular Ca^{2+} influx, significantly reduced the second phase only; thapsigargin (1 μM), by discharging intracellular Ca^{2+} stores, U 73122 (10 μM) and D609 (300 μM), by inhibiting PLC activity, prevented both phases. In our system, PTX pre-treatment increased (*S*)-3,5-DHPG effects, demonstrating that PXT-sensitive $G_{i/o}$ proteins are involved. Finally, specific stimulation of these receptors in Jurkat cells upregulates *c-jun* and *c-fos* gene expression, thus activating multiple downstream signalling regulating important T cell functions.

1. Introduction

L-Glutamate is both a ubiquitous amino acid and a chemical transmitter not only in the mammalian CNS, but also in the peripheral tissues. In the CNS, L-glutamate is the principal excitatory neurotransmitter, which binds to and activates a variety of receptors. These receptors have been fully characterized and classified as ionotropic (iGlu) and metabotropic (mGlu) receptors [1]. iGlu receptors are oligomeric, ligand-gated ion channels that mediate fast

Abbreviations: iGlu, ionotropic glutamate; mGlu, metabotropic glutamate; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PLC, phospholipase C; AC, adenylate cyclase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; mAb, monoclonal antibodies; PHA, phytohemagglutinin; ERK, extracellular signal-regulated kinase; PBMC, peripheral blood mononuclear cells; fura-2/AM, fura-2 acetoxymethyl ester; PTX, pertussis toxin; TG, thapsigargin; (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; AIDA, (*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid; CHPG, (RS)-2-chloro-5-hydroxyphenylglycine; (*S*)-3,5-DHPG, (*S*)-3,5-dihydroxyphenylglycine; LY 367385, (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; U 73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U 73343, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-pyrrolidine-2,5-dione; D609, tricyclodecan-9-yl xanthogenate; RT-PCR, reverse transcriptase-polymerase chain reaction; *GAPDH*, glyceraldehydes-3-phosphate dehydrogenase; VGCC, voltage-gated Ca^{2+} channels; TRPC, canonical transient receptor potential; HBSS, Hank's balanced salts solution; EAAT, excitatory amino acid transporters; APC, antigen-presenting cells

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synaptic responses [2]. They are pharmacologically defined as *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. mGlu receptors are members of the family 3 (or C) of G protein-coupled receptors, including Ca^{2+} and Mg^{2+} receptors, $GABA_B$ receptors, a number of receptors found in the vomeronasal organ and receptors for sweet molecules [3]. They mediate a wide variety of functions, such as learning and memory processes, motor coordination and pain. Eight mammalian genes coding for mGlu receptors has been identified so far. These receptors are divided into three groups according to their primary sequence identity, namely group I: $mGlu_1$ and $mGlu_5$ receptors, group II: $mGlu_2$ and $mGlu_3$ receptors, and group III: $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors. Group I mGlu receptors are preferentially coupled to the $G_{q/11}$/phospholipase C (PLC) transduction pathway, leading to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase, whereas the others are coupled to the $G_{i/o}$/adenylate cyclase (AC) pathway, leading to changes in intracellular cAMP accumulation [4].

In non-neuronal peripheral tissues, the existence of a glutamate-mediated transmission has been demonstrated in several systems [5,6], including immune system. Here specific high affinity L-glutamate binding sites are found on the surface of human T cells [7], while the expression of both iGlu and mGlu receptors is demonstrated in cells of the T lineage (thymocytes and lymphocytes) [8–12]. Through these receptors L-glutamate modulates lymphocyte functions: (i) it potentiates T cell responses ($[Ca^{2+}]_i$ rise) to specific stimuli [anti-CD3 monoclonal antibodies (mAb) and phytohemagglutinin (PHA)] by acting on NMDA and non-NMDA iGlu receptors [13]; (ii) it increases lymphocyte adhesion to extracellular matrix proteins and cell motility by acting on AMPA iGlu receptors [10]; (iii) it induces reactive oxygen species formation by acting on NMDA iGlu receptors [12,14].

Concerning the transduction pathways linked to the activation of mGlu receptors in lymphocytes no conclusive data have been published yet. In fact, Storto et al. [8] reported that mGlu receptor stimulation significantly increases phosphoinositide hydrolysis, enhances Ins(1,4,5)P_3 formation, and reduces forskolin-induced cAMP formation in mouse thymocytes. Pacheco et al. [11] have since shown that group I mGlu receptor stimulation does not increase $[Ca^{2+}]_i$ but significantly raised cAMP intracellular levels, and activates extracellular signal-regulated kinases (ERK)1/2 in human lymphocytes and Jurkat leukemic T cells. Therefore, further studies are needed to fully characterize these signals.

The aim of this study was to further examine the transduction pathways activated by mGlu receptor stimulation in human T cells, specifically regarding Ca^{2+} signalling.

Our results clearly show that group I mGlu receptor stimulation evokes calcium signals and immediate early gene (*c-jun* and *c-fos*) expression in human peripheral blood mononuclear cells (PBMC) and Jurkat cells.

2. Materials and methods

2.1. Materials and chemicals

Ficoll-Paque PLUS was obtained from Amersham Bioscience (Uppsala, Sweden). BSA, EDTA, EGTA, fura-2 acetoxymethyl ester (fura-2/AM), HEPES, penicillin, pertussis toxin (PTX), poly-L-lysine, probenecid, streptomycin and thapsigargin (TG), were obtained from Sigma–Aldrich (Milan, Italy). (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD], (*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG], (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), 2-methyl-6-(phenylethynyl)-pyridine (MPEP), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U 73122), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-pyrrolidine-2,5-dione (U 73343), tricyclodecan-9-yl xanthogenate (D609), verapamil, diltiazem and nifedipine were from Tocris Cookson (Bristol, UK). RPMI 1640, and foetal bovine serum were purchased from Gibco (Milan, Italy). All other reagents were analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Cell culture

Heparinized peripheral blood (15–20 ml) was collected from healthy donors after informed consent. PBMC were separated by centrifugation at 450 × *g* for 30 min at room temperature over a Ficoll-Paque PLUS gradient as described by Boyum [15]. After washing, the cells were resuspended in RPMI 1640 supplemented with heat inactivated foetal bovine serum (10%, v/v), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and incubated on dishes for 1 h in a 37 °C, humidified 5% CO_2–95% air incubator. Non-adherent cells were collected and maintained in supplemented RPMI 1640 medium in a 37 °C, humidified 5% CO_2–95% air incubator. Cell viability, evaluated at the end of the cell isolation by Trypan blue dye exclusion test, was always >98%.

Jurkat cells (clone E6-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in supplemented RPMI 1640 medium in a 37 °C, humidified 5% CO_2–95% air incubator.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from Jurkat cells with the GeneElute Mammalian Total RNA Kit (Sigma–Aldrich) and treated (1 h, 25 °C) with 5 U DNase I, RNase-free (Roche Diagnostics, Penzberg, Germany). DNase was then inactivated by heating for 5 min at 95 °C. Resulting RNA was reverse-transcribed with oligo(dT) primers

Table 1
Oligonucleotides used

PCR primers		Amplicon size (bp)
c-fos		
Forward	5′-GGAATAAGATGGCTGCAGCCAAATGCC-3′	388
Reverse	5′-GGGAACAGGAAGTCATCAAAGGGCTC-3′	
c-jun		
Forward	5′-CTGCAAAGATGGAAACGACCT-3′	240
Reverse	5′-GGATTATCAGGCGCTCCAG-3′	
GAPDH		
Forward	5′-GGTCGGAGTCAACGGATTTGG-3′	1000
Reverse	5′-ACCACCCTGTTGCTGTAGCCA-3′	

using 10 μg of total RNA and the ThermoScrip RT-PCR System (Invitrogen, Milan, Italy). PCR was performed in a 25 μl reaction mixture containing 20 μg of cDNA, 2.5 μl of ×10 buffer, 1.5 μl of 50 mM $MgCl_2$, 0.5 μl of a 10 mM dNTPs mix (Invitrogen), 2.5 U of *Taq* DNA polymerase (Invitrogen) and 2.5 μl of each primer (Table 1). The amplification conditions were at 95 °C for 30 s, 70 °C for 90 s and 72 °C for 60 s for 35 cycles (*c-fos*); at 95 °C for 30 s, 61 °C for 90 s and 72 °C for 60 s for 35 cycles (*c-jun*); and at 96 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s for 25 cycles (glyceraldehydes-3-phosphate dehydrogenase; *GAPDH*). RT-PCR amplicons were resolved in a 2% agarose gel by electrophoresis, and signals were quantified with densitometric analysis software (NIH Image 1.32; National Institutes of Health, Bethesda, MD, USA). Data are expressed as the ratio of the signal obtained for each gene in one sample divided by that obtained for the reference gene (*GAPDH*) in the same sample.

2.4. Intracellular Ca^{2+} measurements

These were performed on cell suspensions (spectrofluorimetric method) or single cells (digital Ca^{2+} imaging system).

For the spectrofluorimetric measurements, cells (1×10^6 cells/ml) were loaded with fura-2/AM (1 μM) for 1 h at 37 °C, washed with Hank's balanced salts solution (HBSS) (in mM: NaCl, 137; KCl, 5.8; $MgCl_2$, 1.0; $CaCl_2$, 2.5; D-glucose, 5.0; and HEPES, 10 pH 7.4), supplemented with probenecid (2.5 mM final concentration) to avoid fura-2 leakage. For Ca^{2+} free experiments, HBSS buffer (Ca^{2+}-free buffer) was prepared by omitting $CaCl_2$ and by adding 1 mM EGTA, an extracellular calcium chelator. After centrifugation (200 × *g*, for 5 min), to remove unincorporated dye, cells were resuspended in HBSS. Fluorescence was monitored throughout each experiment at 37 °C with a spectrofluorimeter (FP-777; Jasco, Tokyo, Japan) at an emission wavelength of 505 nm and an excitation wavelength of 340 nm. Ca^{2+} signals were digitally converted using the software developed by Dr. R. Gindro (DISCAFF, Novara, Italy) and expressed as the pseudoratio calculated as $\Delta F/F = (F - F_{base})/F_{base}$ where F and F_{base} are the measured fluorescence intensity of the Ca^{2+} indicator after and before stimulation, respectively [16].

For the single-cell measurements, fura-2-loaded cells (1×10^6 cells/ml) were firmly attached (15 min at 37 °C) to poly-L-lysine (0.1 mg/ml)-coated thin (0.2 mm) round glass coverslips (4 cm), washed, transferred to a perfusion chamber (Bioptechs, Butler, PA, USA) and mounted on an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan). Experiments were performed at a chamber temperature of 37 °C. The cells were continuously superfused by means of a gravity microperfusion system combined with electrovalves to allow switching between different solutions. All measurements were taken at 40-fold magnification. The light source was a 150 W xenon-arc lamp. Excitation wavelengths were alternately selected at 340 and 380 nm by a monochromator system (Polychrome IV; TILL Photonics, Gräfelfing, Germany). Fluorescence, filtered at 505 nm, was taken with a grey-scale CCD camera (SensiCam; PCO, Kelheim, Germany). Hardware was controlled and images were acquired and analyzed with Axon Imaging Workbench 4.0 software (Axon Instruments, Union City, CA). Data were expressed as the ratio of fluorescence emitted by excitation at 340 and 380 nm.

2.5. Data analysis

Results are expressed as means ± S.E.M. of n experiments. Significance was assessed with Student's t-test for paired varieties with $P \leq 0.05$ as the cut-off. Data were fitted as sigmoidal concentration-response curves and analyzed with a four-parameter logistic equation. The molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist (EC_{50}) and the molar concentration of an antagonist that reduces the response to an agonist by 50% (IC_{50}) values were determined with a nonlinear regression model using the software Origin version 6.0 (Microcal Software, Northampton, MA, USA).

3. Results

3.1. Stimulation of mGlu receptors evokes a $[Ca^{2+}]_i$ increase in human T cells

To determine whether the specific stimulation of mGlu receptors evokes a $[Ca^{2+}]_i$ increase in human T cells, we spectrofluorimetrically measured the fluorescence emitted by fura-2-loaded cells. Exposure of human PBMC ($n = 8$) (Fig. 1A) or Jurkat cell ($n = 10$) (Fig. 1B) suspensions to (1*S*,3*R*)-ACPD (100 μM), the prototype selective mGlu receptor agonist, resulted in a 34.1 ± 4.9% increase of fura-2 fluorescence within few seconds and faded during the drug application. At the single-cell level, fluorescence

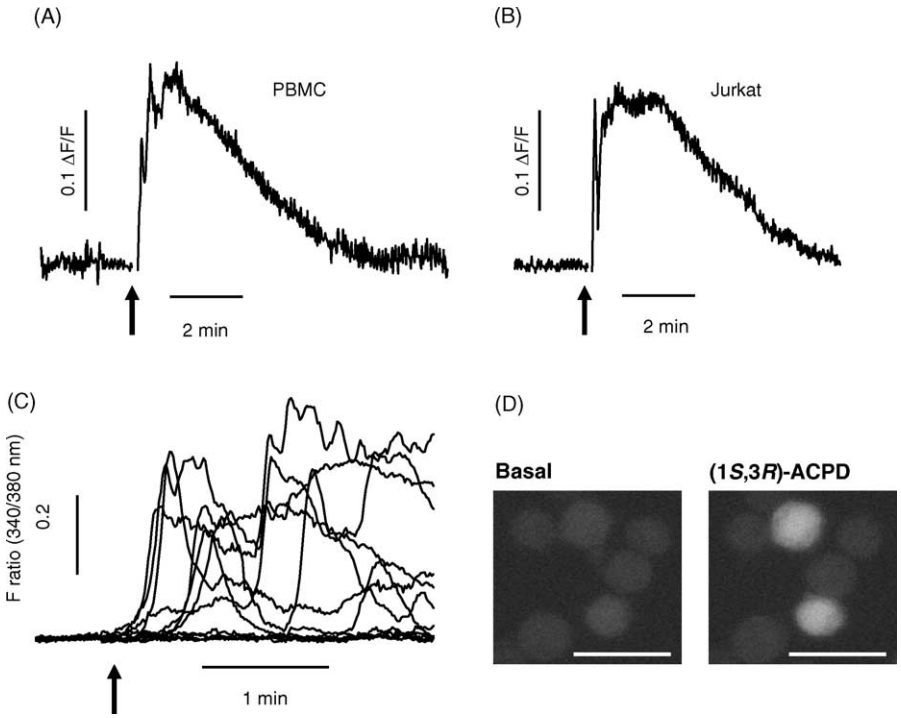

Fig. 1. Ca^{2+} responses evoked by mGlu receptor stimulation in human PBMC and Jurkat cells. Fura-2/AM loaded cells were placed in the measuring chambers at 37 °C. (1*S*,3*R*)-ACPD (100 μM) was added at the time indicated by the black vertical arrow, and maintained throughout the experiment. The emitted fluorescence was monitored either from cell suspensions (A and B) or single cells (C and D) (see Section 2). Upper panels show the representative Ca^{2+}-responses of PBMC (A) or Jurkat (B) cell suspensions. The data are representative of at least eight recordings. Panel C shows the representative Ca^{2+}-responses of single Jurkat cells (number of cells displayed, $n = 15$). Panel D shows the images of single Jurkat cells before and after (30 s) (1*S*,3*R*)-ACPD exposure (40-fold magnification, scale bar, 15 μm).

monitored in Jurkat cells with a digital imaging system revealed a 49.8 ± 10.8% increase in 28.1% of the cells (41 of 146 cells monitored) exposed to (1*S*,3*R*)-ACPD (100 μM) (Fig. 1C and D). Analysis of the image data showed that the kinetic effects of (1*S*,3*R*)-ACPD responses varied among different cells. In fact, the latency of cell response occurred within few seconds to 1 min. Moreover, the $[Ca^{2+}]_i$ increased in an oscillatory manner: either two to several irregular Ca^{2+} spikes or a combination of spikes and sustained elevated Ca^{2+} levels were, indeed, observed. Exposure to (*S*)-3,5-DHPG (100 μM), a selective group I mGlu receptor agonist, led to an increase (95.8 ± 13.2%) of fluorescence in 23.0% of the cells (31 of 135 cells monitored) (data not shown). These results demonstrate

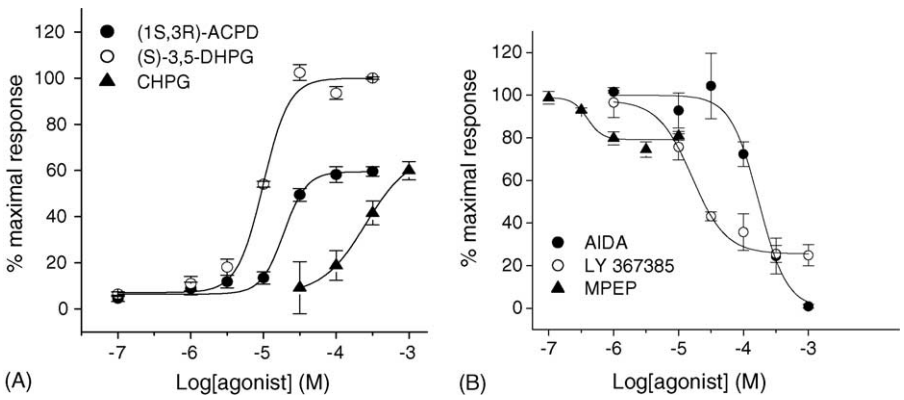

Fig. 2. Concentration-response curves of the effects of mGlu receptor agonists or antagonists on Ca^{2+} responses in Jurkat cells. Increasing concentrations of (1*S*,3*R*)-ACPD (0.1–300 μM), (*S*)-3,5-DHPG (0.1–300 μM) or CHPG (30–1000 μM) were added to the measuring chamber at the beginning of the experiments and maintained thereafter. For experiments with mGlu receptor antagonists, cells were pre-treated with increasing concentrations of AIDA (1–1000 μM), LY 367385 (1–1000 μM) or MPEP (0.1–10 μM) 5 min before (*S*)-3,5-DHPG (30 μM). The fura-2 emitted fluorescence was spectrofluorimetrically monitored (see Section 2). The effects were expressed as amplitude of the Ca^{2+} responses calculated as difference between the maximal value of the emitted fluorescence after drug addition and the baseline. Data are the percentage over (*S*)-3,5-DHPG (300 μM)-induced responses, and are the mean ± S.E.M. of at least five experiments.

that stimulation of mGlu receptors evokes $[Ca^{2+}]_i$ increase in human T cells, and suggest that this is mediated by group I mGlu receptors.

3.2. Pharmacological characterization of the mGlu receptor-mediated $[Ca^{2+}]_i$ increase in human T cells

The maximum fluorescence increase over the baseline of fura-2-loaded Jurkat cell suspension exposed to increasing concentrations of (1*S*,3*R*)-ACPD (0.1–300 μM), (*S*)-3,5-DHPG (0.1–300 μM) or the specific $mGlu_5$ receptor agonist CHPG (30–1000 μM) ($n = 8$) (Fig. 2A) was considered to determine which group I mGlu receptor subtypes are involved in these effects. (*S*)-3,5-DHPG (300 μM) was the most effective mGlu receptor agonist in increasing $[Ca^{2+}]_i$, and it was thus used as the reference drug in our experiments. Its EC_{50} was 9.84 μM. The maximum effects were: 59.6 ± 1.8% and 59.9 ± 4.1% of (*S*)-3,5-DHPG for (1*S*,3*R*)-ACPD (300 μM) and CHPG (1 mM), respectively. The EC_{50} were 19.12 μM for (1*S*,3*R*)-ACPD and 257.21 μM for CHPG. The overall results suggest that both $mGlu_1$ and $mGlu_5$ receptor subtypes mediate the Ca^{2+} responses observed in Jurkat cells.

The soundness of this conclusion was assessed in cells pre-treated with either AIDA (1–1000 μM), a selective group I mGlu receptor antagonist, LY 367385 (1–1000 μM), a specific $mGlu_1$ receptor antagonist or MPEP (0.1–10 μM), a specific $mGlu_5$ receptor antagonist 5 min

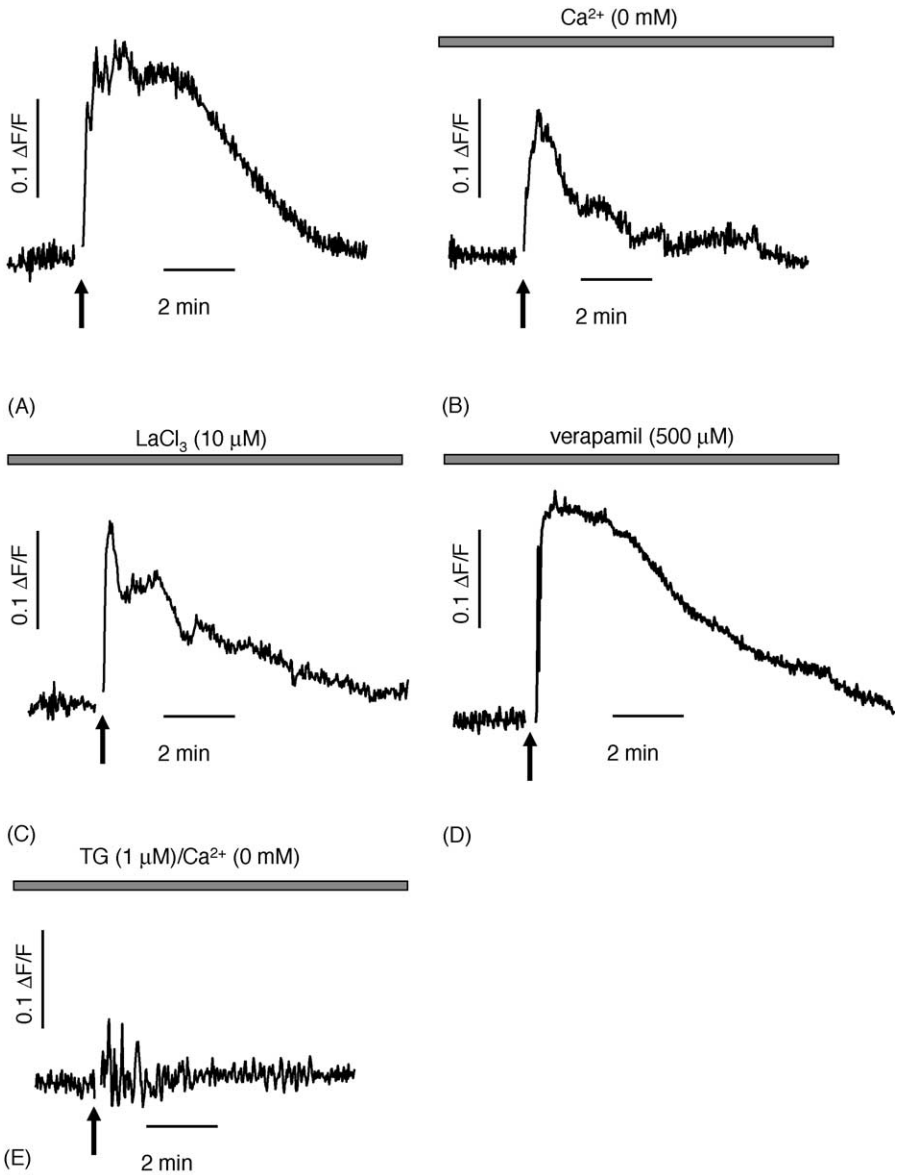

Fig. 3. Analysis of Ca^{2+} signals in Jurkat cells. Fura-2/AM loaded cells were placed in the measuring chambers. (*S*)-3,5-DHPG (100 μM) was added at the time indicated by the black vertical arrow, and maintained thereafter. The emitted fluorescence was spectrofluorimetrically monitored (see Section 2). Each panel shows the representative Ca^{2+}-response of Jurkat cells suspended in: Ca^{2+}-containig HBSS (A); Ca^{2+}-free HBSS (B); HBSS supplemented with $LaCl_3$ (10 μM) (C); HBSS supplemented with verapamil (500 μM). Panel (D) shows the representative of Ca^{2+}-response of cells pre-treated (15 min) with TG (1 μM) in Ca^{2+}-free buffer. The data are representative of at least five recordings.

before exposure to (*S*)-3,5-DHPG (30 μM). All three compounds antagonized the agonist-induced effects in a concentration-dependent manner. The IC_{50} were 171.82 μM and 43.51 μM for AIDA and LY 367385, respectively ($n = 5$) (Fig. 2B). These results confirm the involvement of both group I mGlu receptor subtypes in the Ca^{2+} responses.

AIDA (1 mM) abolished the effect of (*S*)-3,5-DHPG, LY 367385 (1 mM) and MPEP (10 μM), however, only partially antagonized its effects. Comparison of the maximum inhibitions they evoked showed that LY 367385 is more effective ($74.5 \pm 3.2\%$ inhibition) than MPEP ($20.7 \pm 1.9\%$), suggesting a predominant contribution for $mGlu_1$ relative to $mGlu_5$ receptors in Jurkat cells.

3.3. Analysis of Ca^{2+}-signals

Both extracellular Ca^{2+} and Ca^{2+} released from intracellular stores contribute to the $[Ca^{2+}]_i$ increase evoked by specific stimulation of mGlu receptors [4,17]. In Jurkat cell suspension, (*S*)-3,5-DHPG (100 μM) evoked a rapid $[Ca^{2+}]_i$ rise (initial phase) within 30 s, followed by a prolonged shoulder (second phase) returning to the baseline within 10 min (Fig. 3A). To determine the contribution of extracellular Ca^{2+} influx, we repeated these experiments either in a virtually Ca^{2+}-free buffer (see Section 2) or in the presence of extracellular Ca^{2+} influx inhibitors ($LaCl_3$ or $CdCl_2$). As shown in Fig. 3B, the initial phase evoked by (*S*)-3,5-DHPG (100 μM) was significantly lower ($36.3 \pm 2.7\%$) in Ca^{2+}-free buffer than in Ca^{2+}-containing buffer and followed by a rapid return to the baseline ($n = 5$). $LaCl_3$ (1–10 μM) added to HBSS buffer markedly reduced the second phase of the Ca^{2+} response, while it had no effect on the initial phase ($n = 5$) (Fig. 3C). Similar results were obtained with $CdCl_2$ (100 μM) (data not shown). Ca^{2+} released from intracellular stores may thus be supposed to determine the initial $[Ca^{2+}]_i$ rise, whereas the extracellular Ca^{2+} influx sustains the $[Ca^{2+}]_i$ increase for 4–6 min after application of the agonist. Since the extracellular Ca^{2+} influx is mostly due to activation of different voltage-gated Ca^{2+} channels (VGCC) and T cells express different subunits of these channels [18–20], we studied the effects of verapamil, diltiazem and nifedipine, well-known L-type VGCC blockers, on (*S*)-3,5-DHPG-induced responses. As shown in Fig. 3D verapamil (500 μM;

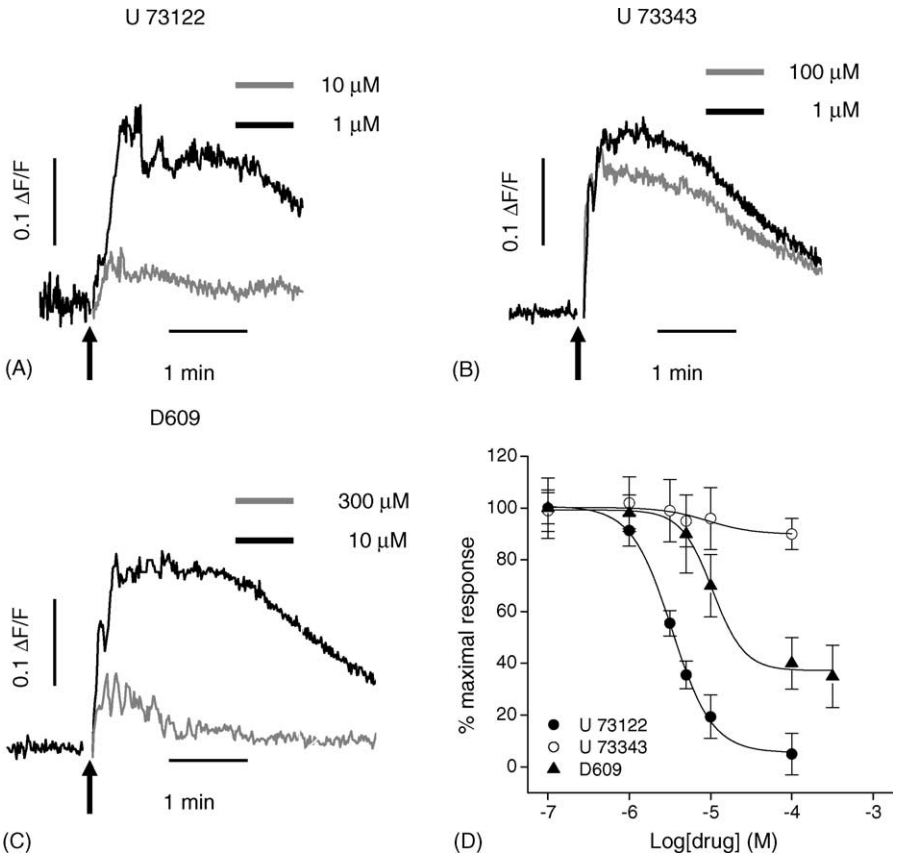

Fig. 4. Effects of the PLC inhibitors on Ca^{2+}-responses in Jurkat cells. Fura-2/AM loaded cells were exposed to increasing concentrations of U 73122 (0.1–100 μM; 10 min), U 73343 (0.1–100 μM; 10 min) or D609 (0.1–300 μM; 30 min) before (*S*)-3,5-DHPG (100 μM). The emitted fluorescence was spectrofluorimetrically monitored (see Section 2). Each panel shows the representative Ca^{2+}-response (the traces are superimposed for clarity) of Jurkat cells exposed to U 73122 (A); U 73343 (B) or D609 (C). Panel (D) shows the concentration-response curves of U 73122, U 73343 and D609 on Ca^{2+}-responses. The effects were expressed as amplitude of the Ca^{2+} responses calculated as the difference between the maximal value of the emitted fluorescence after drug addition and the baseline value. Data are the percentage over (*S*)-3,5-DHPG (300 μM)-induced responses in drug-untreated cells, and are the mean ± S.E.M. of at least four experiments.

5 min before (*S*)-3,5-DHPG) did not modify cell responses, and similar results were obtained with diltiazem or nifedipine (data not shown).

To evaluate the contribution of Ca^{2+} released from intracellular stores, we thus measured the (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ increase in cells pre-treated with TG, an irreversible inhibitor of Ca^{2+}-ATPase of the sarco- and endoplasmic reticula (SERCA), to deplete intracellular Ca^{2+} stores. In the absence of extracellular Ca^{2+}, TG causes a depletion of internal stores resulting from the unopposed leakage of Ca^{2+} followed by Ca^{2+} extrusion across the plasma membrane. TG (1 μM) was added to Ca^{2+}-free buffer 15 min before (*S*)-3,5-DHPG exposure [21]. TG abolished the (*S*)-3,5-DHPG-induced $[Ca^{2+}]_i$ increase ($n = 5$) (Fig. 3D), confirming the contribution of Ca^{2+}-released from TG-sensitive intracellular stores on the initial phase of cell response.

3.4. Analysis of mGlu receptor transduction pathways

It is generally assumed that the group I mGlu receptor-induced $[Ca^{2+}]_i$ increase requires PLC activation. To determine the contribution of PLC in our system, we pre-treated the cell suspensions with increasing concentrations of U 73122 (0.1–100 μM; 10 min), an inhibitor of PLC activity [22], U 73343 (0.1–100 μM; 10 min), an inactive structural analogue of U 73122 or D609 (0.1–300 μM; 30 min) [23], a selective phosphatidylcholine-specific PLC inhibitor, before (*S*)-3,5-DHPG (100 μM). U 73122 and D609, but not U 73343, decreased in a concentration-dependent manner (the IC_{50}s calculated were 3.48 and 11.5 μM, respectively) both the initial and the second phases of the cellular responses. U 73122 at 100 μM almost abolished the Ca^{2+} responses ($n = 5$), while the maximal inhibition of D609 was 65.2 ± 12% (Fig. 4 A–D). These results demonstrate the involvement of PLC isoenzimes on group I mGlu receptor-mediated Ca^{2+} responses in Jurkat T cells.

Group I mGlu receptor-induced PLC activation preferentially couples to PTX-insensitive $G_{q/11}$ family proteins [17]. However, in different cellular models, $mGlu_{1\alpha}$ receptors couple to PTX-sensitive $G_{i/o}$ proteins [24–26]. To assess if $G_{i/o}$ proteins play a functional role in our system, we exposed Jurkat cells to PTX (100 ng/ml, 24 h) prior to the measurement of the Ca^{2+} responses evoked by increasing concentrations (0.001–300 μM) of (*S*)-3,5-DHPG. PTX pre-treatment resulted in a significant increase (~82-fold) in the potency of (*S*)-3,5-DHPG (EC_{50} were 11.51 and 0.14 μM in the absence or presence of PTX, respectively). A 19.3 ± 4.9%, increase in the agonist efficacy was also observed ($n = 5$) (Fig. 5 A and B).

These results are consistent with those reported by Carruthers et al. [25] and Hermans et al. [26] in baby hamster kidney (BHK) cells expressing $mGlu_{1\alpha}$ receptors and demonstrate that group I mGlu receptors functionally couple also to $G_{i/o}$ proteins in Jurkat cells.

3.5. Studies on c-fos and c-jun gene expression

Ca^{2+}, as intracellular second messenger, is able to activate several enzymes (i.e., protein kinase C, protein kinase A, Ca^{2+}/calmodulin-dependent protein kinase), acting on down- or upstream targets and modulating different cell functions, including gene expression [27]. We, therefore, studied whether group I mGlu receptor stimulation modifies early-inducible gene expression. *c-fos* and *c-jun* gene transcriptions were determined by using a semiquantitative RT-PCR assay. Jurkat cell suspensions were exposed to (*S*)-3,5-DHPG (100 μM) for 15–180 min or PHA (10 μg/ml) plus PMA (50 ng/ml) for 60 min, two molecules commonly used to induce both $[Ca^{2+}]_i$ increase and protein kinase C activation. Low and constitutive levels of gene expression were detected in the control cells. Both PHA/PMA and (*S*)-3,5-DHPG induced a significant ($P < 0.05$; $n = 4$) upregulation of the *c-fos* (339.1 ± 65.2% and 155.0 ± 55.5% for

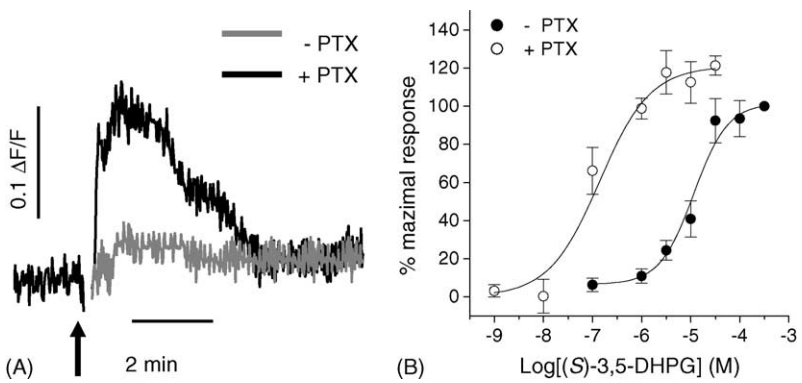

Fig. 5. Effect of PTX pre-treatment on the Ca^{2+} signals evoked by group I mGlu receptor stimulation in Jurkat cells. Jurkat cells were pre-treated (24 h) with PTX (100 ng/ml). The emitted fluorescence was spectrofluorimetrically monitored (see Section 2). Panel (A) shows the representative Ca^{2+}-response of Jurkat cells treated or untreated with PTX and exposed to (*S*)-3,5-DHPG (10 μM) (the traces are superimposed for clarity). Panel (B) shows the effect of PTX on the concentration-response curves of the effects of (*S*)-3,5-DHPG on Ca^{2+}-responses. The effects were expressed as amplitude of the Ca^{2+} responses calculated as the difference between the maximal value of the emitted fluorescence after drug addition and the baseline. Data are the percentage over (*S*)-3,5-DHPG (300 μM)-induced responses in PTX-untreated cells, and are the mean ± S.E.M. of at least five experiments.

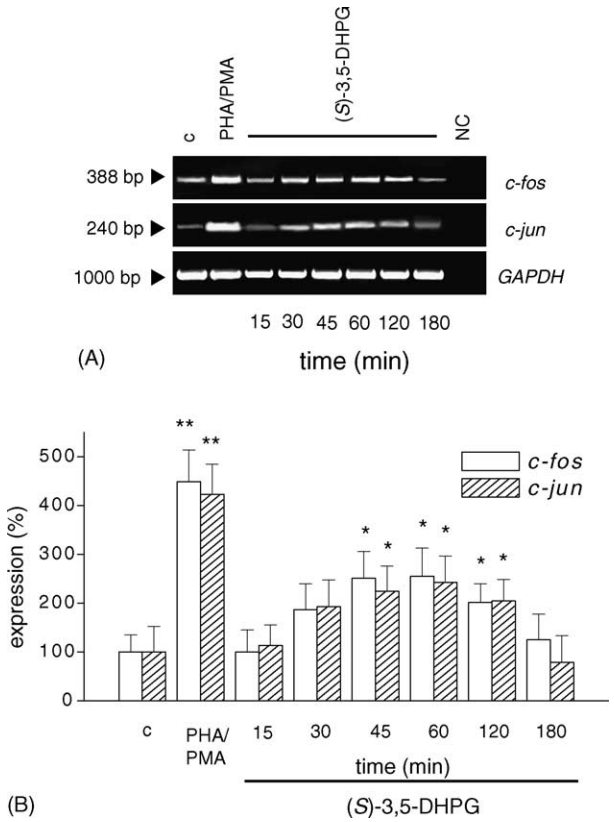

Fig. 6. Effect of group I mGlu receptor stimulation on early gene expression in Jurkat cells. Jurkat cells were exposed either to PHA (10 μg/ml) plus PMA (50 ng/ml) for 1 h, to (*S*)-3,5-DHPG (100 μM) for 15–180 min or vehicle alone (c) and *c-jun* and *c-fos* mRNA transcriptions were analysed by semiquantitative RT-PCR (see Section 2) (A). The signals were densitometrically analysed and data, calculated as mean ± S.E.M. of at least four determinations, are expressed as the ratio of the signal obtained for each sample divided by that obtained for *GAPDH* in the same sample to permit between-sample comparison of RNA species (B). $^{*}P < 0.05$; $^{**}P < 0.01$; significantly different from Jurkat exposed to vehicle alone, paired Student *t*-test.

PHA/PMA and (*S*)-3,5-DHPG, respectively) and *c-jun* (323.1 ± 62.2% and 142.6 ± 56.2% for PHA/PMA and (*S*)-3,5-DHPG, respectively) gene transcription at 60 min of exposure. The effect of (*S*)-3,5-DHPG was time-dependent, with a significant increase after 45 min, following by a return to the baseline after 180 min (Fig. 6).

These results demonstrate that the agonist-induced group I mGlu receptor stimulation modulates *c-fos* and *c-jun* gene expression.

4. Discussion

Our findings show that specific stimulation of group I mGlu receptors, expressed in human T cells, elicits intracellular Ca^{2+} responses sustained by both Ca^{2+} release from intracellular stores and Ca^{2+} entry from extracellular space. The transduction mechanisms leading to $[Ca^{2+}]_i$ increase seem to be linked to PLC activation and involve also $G_{i/o}$ protein. Lastly, the intracellular signals evoked by group I mGlu receptor stimulation modify immediate early gene (*c-fos* and *c-jun*) transcription.

In vitro pharmacological stimulation of $mGlu_1$ and/or $mGlu_5$ receptors, expressed on neuronal cells in either primary cell cultures [28,29] or brain slices [30,31] elicits Ins(1,4,5)P_3 formation and Ca^{2+} signals. Similar intracellular responses have been observed in non-neuronal cells, as recombinant cell systems (i.e., *Xenopus* oocytes or cell lines) [28,32,33], rat osteoblasts and bovine chromaffin cells [34,35]. These observations suggest that PLC/Ins(1,4,5)P_3/Ca^{2+} is the main intracellular signalling pathway functionally activated by group I mGlu receptor stimulation, and that it is used by different cell types, independently of their origin. The data demonstrate that human T cells (PBMC and Jurkat cells) also use this pathway as transduction signalling for group I mGlu receptor activation. (*S*)-3,5-DHPG, a selective group I mGlu receptor agonist, in fact, evoked intracellular Ca^{2+} responses and these were abolished by AIDA, a specific group I mGlu receptor antagonist, and by either U 73122, a selective PLC inhibitor or D609, a selective phosphatidylcholine-specific PLC inhibitor. These results are consistent with those observed by Storto et al., [8] in isolated rat thymocytes expressing both $mGlu_{1\alpha}$ and $mGlu_5$ receptors. In these T lymphocyte precursor cells, (1*S*,3*R*)-ACPD elicited intracellular Ins(1,4,5)P_3 formation, which may mediate the $[Ca^{2+}]_i$ increase. In human T cells, therefore, the PLC/Ins(1,4,5)P_3/Ca^{2+} intracellular pathway seems to be linked to group I mGlu receptors from the early stage of cell development to cell maturation (from thymocyte to lymphocyte or lymphoblast). These data differ from those published by Pacheco et al. [11], showing that agonist-induced group I mGlu receptor stimulation does not evoke Ca^{2+} responses in human PBMC and Jurkat cells. One possibility for these different findings may be related to the fact that Pacheco et al. compared cell responses of different amplitude: nanomolar for (*S*)-3,5-DHPG, and micromolar for anti-CD3 mAb. In these conditions, the group I mGlu receptor-induced $[Ca^{2+}]_i$ increase may be underlooked. In addition, differences in either cells (i.e., activation state, specific clone) or experimental conditions (i.e., basal $[Ca^{2+}]_i$, culture medium, fura-2 method) should be considered.

The pharmacological characterization of the group I mGlu receptor-mediated $[Ca^{2+}]_i$ increase we reported indicates the involvement of both $mGlu_1$ and $mGlu_5$ receptor subtypes, and provides some evidences for a predominant role for $mGlu_1$ relative to $mGlu_5$ receptors. In our system, indeed, LY 367385, a specific $mGlu_1$ receptor antagonist, resulted more effective than MPEP, a specific $mGlu_5$ receptor antagonist, in inhibiting agonist-mediated effects, suggesting the existence of a sort of functional "hierarchy" between these receptor subtypes in Jurkat cells. The functional prevalence of $mGlu_1$ receptors, we observed, may be due to: (i) its relative abundance; (ii) diversity of the Ca^{2+}

signals evoked by receptor stimulation. Firstly, the level of group I mGlu receptor expression may be quite different among various cell clones, and it changes during cell maturation and activation [8,11]. Neither the RT-PCR analysis of $mGlu_1$ and $mGlu_5$ mRNA nor the immunostaining of the proteins we performed did allow us to quantify the expression level of each receptor subtype in our cells (data not shown). Therefore, we cannot rule out the possibility that in our conditions the cells mainly express $mGlu_1$ receptor subtype. Secondly, in heterologous expression systems, the activation of rat $mGlu_1$ and $mGlu_5$ receptors gives rise to repetitive base-line separated Ca^{2+} oscillations with distinct frequencies [32,33,36], and it may be possible that in our experimental conditions $mGlu_1$ receptor-induced responses are better detectable. Furthermore, some differences in the Ca^{2+} buffering mechanisms (i.e., intracellular calcium-binding proteins), Ins(1,4,5)P_3 receptors, extracellular Ca^{2+} influx, SERCA and membrane Ca^{2+} pumps should be considered.

Using different experimental protocols, we have identified two phases in the above described Ca^{2+} responses: an initial phase (~2 min), mainly determined by Ca^{2+} release from intracellular stores (likely endoplasmic reticulum), and a second phase (~4–6 min) due to Ca^{2+} influx from extracellular space. When extracellular Ca^{2+} was omitted, the initial phase, was reduced ($36.3 \pm 2.7\%$), and the second phase was almost abolished. Moreover, when intracellular Ca^{2+} stores were emptied by pre-treatment with TG no $[Ca^{2+}]_i$ increase was observed, confirming that Ca^{2+} released from intracellular stores contributes to the initial phase of the cellular response. The dependence on extracellular Ca^{2+} of the functional responses to group I mGlu receptor stimulation may be due to: (i) a Ca^{2+} dependency of PLC activation; (ii) a quick activation of Ca^{2+} influx in Ca^{2+} containing buffer [37]; (iii) a Ca^{2+} modulation of the agonist-induced mGlu receptor activation [17,38]. The group I mGlu receptor-induced Ca^{2+} entry in T lymphocytes seems to be associated to the canonical transient receptor potential (TRPC) subfamily of transient receptor potential cation channels. Some of these channels, in fact, have properties [39,40] similar to those we showed. PLC inhibition, in fact, prevented both phases of cell response, and La^{3+}, but not VGCC blockers, significantly inhibited Ca^{2+} entry at low micromolar concentrations. Furthermore, the TRPC channel-group I mGlu receptor coupling has been recently demonstrated [41,42].

The analysis of cell responses showed that the effects of mGlu receptor agonists faded during the drug application: this could be a consequence of receptor desensitization due to phosphorylation of either the receptor itself or other molecules engaged in the signalling cascade [17]. Moreover, we cannot exclude that some autoregulatory mechanisms on Ca^{2+} influx may be activated by T cells upon agonist application and contribute to reduce the driving force for the ion entry [43,44].

$G_{q/11}$ protein-linked PLC/Ins(1,4,5)P_3/Ca^{2+} is the main transduction pathway associated to group I mGlu receptor activation. However, multiple (''promiscuous'') intracellular signalling cascades have been also described. They are the consequence of the linkage of these receptors with different intracellular G proteins (i.e., G_s and $G_{i/o}$) [17]. Patcheco et al. [11] reported that specific group I mGlu receptor agonists induce G_s protein-mediated intracellular cAMP increases in resting or activated human lymphocytes and Jurkat cells, whereas $G_{i/o}$ proteins seem to modulate PLC activation and receptor desensitization in transfected BHK cells expressing $mGlu_{1\alpha}$ receptors [25,26]. In our system, $G_{i/o}$-type proteins seem to be involved in the regulation of group I mGlu receptor signalling, since the inactivation of these proteins induced a significant increase of (*S*)-3,5-DHPG-mediated effects. The consequence of receptor-$G_{i/o}$ protein coupling may vary upon cell background, level of receptor expression, and presence of different splice variants [17]. We could speculate that this coupling is used for negative modulating L-glutamate effect on T lymphocyte.

A functional role of group I mGlu receptors in initiating stimulus-induced gene transcription has been described in the CNS [29]. The findings here reported extend this functional role of glutamate also in the immune system. The upregulation of *c-jun* and *c-fos* gene expression we detected following specific stimulation of these receptors in Jurkat cells suggests, in fact, that L-glutamate may modulate T lymphocyte function by activating multiple downstream signalling events that regulate cell proliferation and cytokine mRNA transcription [45,46].

The physiological significance of L-glutamate transmission in the immune system is still elusive, as for other peripheral tissues. The most intriguing question is how and when L-glutamate-mediated signalling contributes to the modulation of T cell functions. Theoretically, low L-glutamate concentrations are needed to allow the indispensable high signal-to-noise ratio and remove the tonic stimulation of peripheral glutamate receptors. Such conditions may be found by those lymphocytes migrating into the CNS, where extracellular L-glutamate concentrations are strictly maintained at a low level (~1 μM) [47]. Another possibility is the formation of ''immunological synapses'' between antigen-presenting cells (APC) (i.e., macrophages, dendritic cells, B lymphocytes, Langherans cells) and T cells in a manner similar to the CNS. The tight space within the synapse might create a favourable microenvironment for L-glutamate modulation of T cell function. Macrophages consistently express high-affinity L-glutamate transporters (namely excitatory amino acid transporters, EAAT), similar to those present in the CNS on astroglial cells and glutamatergic nerve endings, that are useful for reducing extracellular glutamate concentration [48].

In conclusion, the present data demonstrate that human PBMC and Jurkat cells posses an integral and functional

apparatus, similar to that present in neurons, able to generate Ca^{2+} signals in response to group I mGlu receptor stimulation. The activation of these receptors has a functional role in initiating early gene transcription.

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