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Group I Metabotropic Glutamate Receptors Mediate a Dual Role of Glutamate in T Cell Activation*

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Metabotropic glutamate receptors (mGluR) are present in cells of the nervous system, where they are activated by one of the main neurotransmitters, glutamate. They are also expressed in cells outside the nervous system. We identified and characterized two receptors belonging to group I mGluR, mGlu1R and mGlu5R, in human cell lines of lymphoid origin and in resting and activated lymphocytes from human peripheral blood. Both are highly expressed in the human Jurkat T cell line, whereas mGlu5R is expressed only in the human B cell line SKW6.4. In blood lymphocytes, mGlu5R is expressed constitutively, whereas mGlu1R is expressed only upon activation via the T cell receptor-CD3 complex. Group I receptors in the central nervous system are coupled to phospholipase C, whereas in blood lymphocytes, activation of mGlu5R does not trigger this signaling pathway, but instead activates adenylate cyclase. On the other hand, mGlu5R does not mediate ERK1/2 activation, whereas mGlu1R, which is coupled neither to phospholipase C nor to calcium channels and whose activation does not increase cAMP, activates the mitogen-activated protein kinase cascade. The differential expression of mGluR in resting and activated lymphocytes and the different signaling pathways that are triggered when mGlu1Rs or mGlu5Rs are activated point to a key role of glutamate in the regulation of T cell physiological function. The study of the signaling pathways (cAMP production and ERK1/2 phosphorylation) and the proliferative response obtained in the presence of glutamate analogs suggests that mGlu1R and mGlu5R have distinct functions. mGlu5R mediates the reported inhibition of cell proliferation evoked by glutamate, which is reverted by the activation of inducible mGlu1R. This is a novel non-inhibitory action mechanism for glutamate in lymphocyte activation. mGlu1R and mGlu5R thus mediate opposite glutamate effects in human lymphocytes.

There is increasing evidence that the activity of T cells in the central nervous system is regulated by neurotransmitters such as dopamine, gonadotropin-releasing hormones I and II, somatostatin, substance P, calcitonin-gene-related peptide, neuropeptide Y, and glutamate (1). The latter is the main excitatory neurotransmitter in the mammalian brain involved in learning and memory, as well as in neurotoxicity, and plays a critical role in the development and progression of diverse neurological disorders. T cells can also encounter glutamate outside the brain, in "glutamate-rich" peripheral organs such as liver, kidney, lung, muscle, and blood. It is unknown whether glutamate can be released by antigen-presenting cells, thus regulating T cell activation in the immunological synapse (2).

This amino acid acts at multiple receptor types, divided into two main groups: ionotropic glutamate receptors, which form ion channels and mediate fast excitatory glutamate responses, and metabotropic glutamate receptors (mGluR),[1] which are hepta-spanning membrane-receptors and belong to the superfamily of G protein-coupled receptors (3). So far, eight members of the mGlu receptor family have been identified and classified into three subgroups (I, II, and III) according to their sequence homology, agonist selectivity, and signal transduction machinery (4, 5). Group I contains mGlu1 and mGlu5 receptor subtypes, which are mainly coupled to phospholipase C, and quisqualic acid is their most potent agonist. Group II consists of mGlu2 and mGlu3 receptors, which couple negatively to adenylyl cyclase in transfected cells and for which L-2-(carboxycyclopropyl) glycine is the most potent agonist. Group III contains mGlu4, mGlu6, mGlu7, and mGlu8 receptors, which again couple negatively to adenylyl cyclase, and L-2-amino-4-phosphonobutyric acid is their most potent agonist.

Kostanyan *et al.* (6) performed pioneering studies on radiolabelled glutamate binding to human blood lymphocytes. This binding may be due to the presence of transporters and/or specific glutamate receptors on the cell surface. The low K_D value of glutamate binding and the inhibition by quisqualic acid strongly supports the presence of specific glutamate receptors of the metabotropic type (6). Among the limited number of reports on the characterization of metabotropic receptors in locations other than the nervous systems, Storto *et al.* (7) have reported the distribution of group I and II metabotropic receptors in murine thymocytes and thymic stromal cells. However, the distribution and physiological role of mGluR in human lymphocytes remain unknown.

In this paper, we provide evidence of the involvement of group I mGluR in the effects of glutamate on human lymphocytes. The presence of metabotropic glutamate receptor 1

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[1] The abbreviations used are: mGluR, metabotropic glutamate receptors; mGlu*n*R, metabotropic glutamate receptor *n*; PBS, phosphate-buffered saline; PE, phycoerythrin; DHPG, dihydroxyphenylglycol; ERK, extracellular signal-regulated kinases; PHA, phytohemagglutinin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

(mGlu1R) and/or 5 (mGlu5R) in human lymphocytes, resting or activated, and in human T and B cell lines was approached by immunocytochemistry and reverse transcription (RT)-PCR. Our results show that mGlu5Rs are constitutively expressed in blood lymphocytes, and functional assays indicate that they may contribute to the maintenance of the resting status. In contrast, mGlu1Rs are only expressed upon T cell activation and, unlike mGlu5Rs, are coupled to the mitogen-activated protein (MAP) kinase cascade.

EXPERIMENTAL PROCEDURES

Cells—The human T cell line Jurkat J-32 and the human B lymphoblast cell line SKW6.4 were grown in RPMI 1640 medium (Life Technologies, Inc., Paisley, UK) supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.) at 37 °C in a humid atmosphere of 5% CO_2. Human T cells were isolated from heparinized blood of healthy donors using the Ficoll gradient method described by Boyum (8). Lymphocytes were depleted of contaminating monocytes by adherence to a plastic plate incubated 2 h in XVIVO-15 medium (BioWhittaker, Walkersville, MD) supplemented with 1% autologous serum, 50 μg/ml gentamycin (Braun B., Melsungen, Germany) and 2.5 μg/ml Fungizone (Bristol-Myers Squibb, Munchen, Germany). Cells were cultured in XVIVO-10 medium at 37 °C in a humid atmosphere of 5% CO_2. Purified primary resting lymphocytes were activated by incubation (72 h) with 18 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich Company LTD, Dorset, UK) or 1 ng/ml OKT3, a monoclonal antibody directed against the T cell receptor/CD3 complex (9, 10).

Immunostaining—Cells were washed with phosphate-buffered saline (PBS), fixed in PBS containing 2% paraformaldehyde for 15 min and washed twice with PBS containing 20 mM glycine to quench the aldehyde groups. When necessary, cells were permeabilized by adding FACS™ permeabilizing solution (Becton Dickinson Bioscience, Erembodegem-Aalst, Belgium) for 10 min and washing twice with PBS. Cells were treated with PBS containing 1% bovine serum albumin, 20 mM glycine, and 0.1% NaN_3 (blocking buffer) and labeled with 3 μg/ml of affinity-purified rabbit polyclonal anti-group I mGluR antibody (F1-Ab) for 1 h at room temperature (11). Cells were washed 3× with blocking buffer and stained for flow cytometry analysis with the secondary antibody phycoerythrin (PE)-conjugated goat anti-rabbit (1:20; Sigma-Aldrich Company, Ltd) or AlexaFluor 488-conjugated goat anti-rabbit (1:1000; Molecular Probes, Leiden, The Netherlands) for confocal microscopy analysis during a 1-h period. Negative controls were performed without F1-Ab. Coverslips were mounted with ImmunoFluor mounting medium (ICN Biomedical Inc., Costa Mesa, CA). Confocal microscope observations were made with a Leica TCS-SP (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope. Flow cytometry analysis was done with an EPICS Profile flow cytometer (Coulter, Hialeah, FL). Cell populations were selected by forward and side-light scatter parameters.

Reverse Transcriptase-PCR—Total RNA was isolated from cells with a Quik-Prep™ total RNA extraction kit (Amersham Biosciences, Uppsala, Sweden). A 1-μg aliquot of total RNA and 1 μg of random hexamers (Invitrogen SA, Barcelona, Spain) as primers dissolved in 14 μl of RNase-free water were heated to 70 °C for 5 min and then cooled to 4 °C. 5 μl of RT buffer 5× Moloney murine leukemia virus (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol; Promega Corp, Madison, WI), 1.5 μl of 2′-deoxynucleoside 5′-triphosphate (mix, 10 mM each; Sigma), 1 μl of reverse-transcriptase enzyme (M-MLV RT (H−), Promega Corp.), 1 μl of RNase inhibitor (RNaseA OUT; Invitrogen SA), and 2.5 μl of RNase-free water were added to a final volume of 25 μl. The incubation was continued at 25 °C for 10 min and 42 °C for 52 min, and was terminated by 70 °C for 15 min. PCR was carried out for 50 cycles (1 min at 95 °C, 45-s annealing (see below), 2 min at 72 °C) in a final volume of 50 μl containing 25 μl of 2× PCR master mix (Promega Corp.), 5 μl of cDNA and primers, each at 0.6 μM. RT-PCR negative control was performed by loading diethyl pyrocarbonate water instead of cDNA. Primers were selected according to GenBank™ database resource, and annealing temperatures were as follows: mGlu1R, annealing at 60.5 °C; amplimer 453 bp; forward, 5′-ACC CGG TCC TCC TGC CCA ACA-3′; reverse, 5′-CGT CCA TTC CGC TCT CCC CAT AA-3′; mGlu5R, annealing at 58 °C; amplimer 661 bp; forward, 5′-TCC TGG CCA CCC TGT TTG TTA CTG-3′; reverse, 5′-GTG GCA CTG AGG CTG ACC GAG AAA-3′.

Intracellular Ca^{2+} Measurements—Cells (10^7 cell/ml) were loaded with 5 μM Fura-2/AM (Sigma-Aldrich Company Ltd) for 30 min at 37 °C in growing medium and subsequently resuspended (2×10^6 cell/ml) in calcium buffer (140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 20 mM HEPES, 1 mM $CaCl_2$, 10 mM glucose). Calcium peak induction was achieved by the addition of 5 μg/ml mouse monoclonal anti-CD3 antibody (secreted by 33–2A3 hybridoma, kindly provided by Dr. Jaume Martorell from Hospital Clinic, Barcelona, Spain) or 100 μM dihydroxyphenylglycol (DHPG) (Tocris, Bristol, UK). Intracellular calcium was determined at 37 °C in a dual-wavelength Shimadzu RF-5000 spectrofluorophotometer (Shimadzu Europe, Duisberg, Germany) by using the excitation wavelength ratio of 334/366 nm with emission cutoff at 500 nm. Free calcium concentration was calculated as described previously (12).

Intracellular cAMP Measurements—Jurkat cells and resting or activated T cells were incubated 16 h before the experiments with fetal bovine serum (for Jurkat cells) or phytohemagglutinin (PHA)-free (for activated T cells) medium. Cells (5×10^6 cell/3 ml) were washed twice and resuspended with HBSS buffer (1.25 mM $CaCl_2$, 5 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, 0.3 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, pH 7.4). Cells were incubated with 50 μM zardaverine (Tocris) for 10 min at 37 °C and treated without (control) or with 100 μM DHPG, 500 μM 2-chloro-5-hydroxyphenylglycine (CHPG; Tocris), or 200 nM CGS21680 (Sigma) as positive control for 25 min at 37 °C. The concentrations of agonists used in functional assays (cAMP, extracellular signal-regulated kinases (ERKs), and proliferation) were selected according to the reported K_D values of the binding of each agonist to its respective receptor. Cells were lysed, and levels of cAMP were measured using a cAMP Biotrak Enzymeimmunoassay system (Amersham Biosciences) according to the manufacturer's protocol without acetylation, using the lysed equivalent to 10^6 cells/well.

Phosphorylation of ERK—Jurkat cells and resting or activated T cells were incubated (16 h) with fetal bovine serum-free (for Jurkat cells) or PHA-free (for T cells) medium, washed twice, and resuspended in the same medium either containing or not the indicated inhibitors (GF 109203X, PD 98059, or H-89) for different time intervals. Cells were washed twice, resuspended with PBS (20×10^6 cell/ml for Jurkat or 10×10^6 cell/ml for T cells) and stimulated with the corresponding agonist. Cells were lysed with ice-cold lysis buffer 2× (2% Triton X-100, 100 mM Tris-HCl, pH 7.6, 80 mM β-glycerophosphate, 50 mM NaF, 2 mM Na_3VO_4, and protease inhibitor mixture (Sigma) containing 2.08 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.6 μM aprotinin, 42 μM leupeptin, 72 μM bestatin, 30 μM pepstatin A, and 28 μM E-64), and lysates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences), and phospho-ERK1/2 was detected by using a mouse monoclonal phosphor-specific ERK1/2 antibody (1:10,000, Sigma) and horseradish peroxidase-conjugated goat anti-mouse antibody (1:1000; Dako, Glostrup, Denmark). Immunodetection was done with SuperSignal™ West Pico chemiluminescent substrate (Pierce). Membrane was stripped and reprobed with rabbit polyclonal total ERK1/2-specific antibody (1:40,000, Sigma) and secondary antibody horseradish peroxidase-conjugated goat anti-rabbit (1:60,000; Chemicon International, Inc.) and detected as described previously. Densitometric analysis was performed by using the Scion Image program (integrated density was measured).

Proliferation Assays—Human primary T cells (2×10^5 cells/well) in 100 μl of XVIVO-10 medium were incubated with 50 μl of different compounds and immediately after with 50 μl of 4 ng/ml mouse monoclonal anti-CD3 antibody (OKT3) (9, 10). Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 for 96 h. After 78 h of incubation, cells were pulsed for the final 18 h with 1 μCi of thymidine ([^{3}H]methyl, 2 Ci/mmol; Moravek Biochemicals, Inc., Brea, CA) to measure [^{3}H]thymidine incorporation. Cells were fixed in 3.7% formaldehyde for 30 min, harvested onto filters, and tritium incorporation was determined by scintillation counting. Data are expressed as mean ± S.D. Differences between groups were evaluated using a Student's t test.

RESULTS AND DISCUSSION

To test whether human lymphocytes express group I metabotropic glutamate receptors in the plasma membrane, we attempted to detect mGlu1R and mGlu5R in a T cell line (Jurkat), in a B cell line (SKW6.4), and in resting and activated human blood lymphocytes by flow cytometry and immunocytochemistry. The antibody used, F1-Ab, is the only available antibody that recognizes an extracellular epitope; it cannot distinguish the two receptors, which share a high sequence homology. mGlu1R and/or mGlu5R were expressed with a high

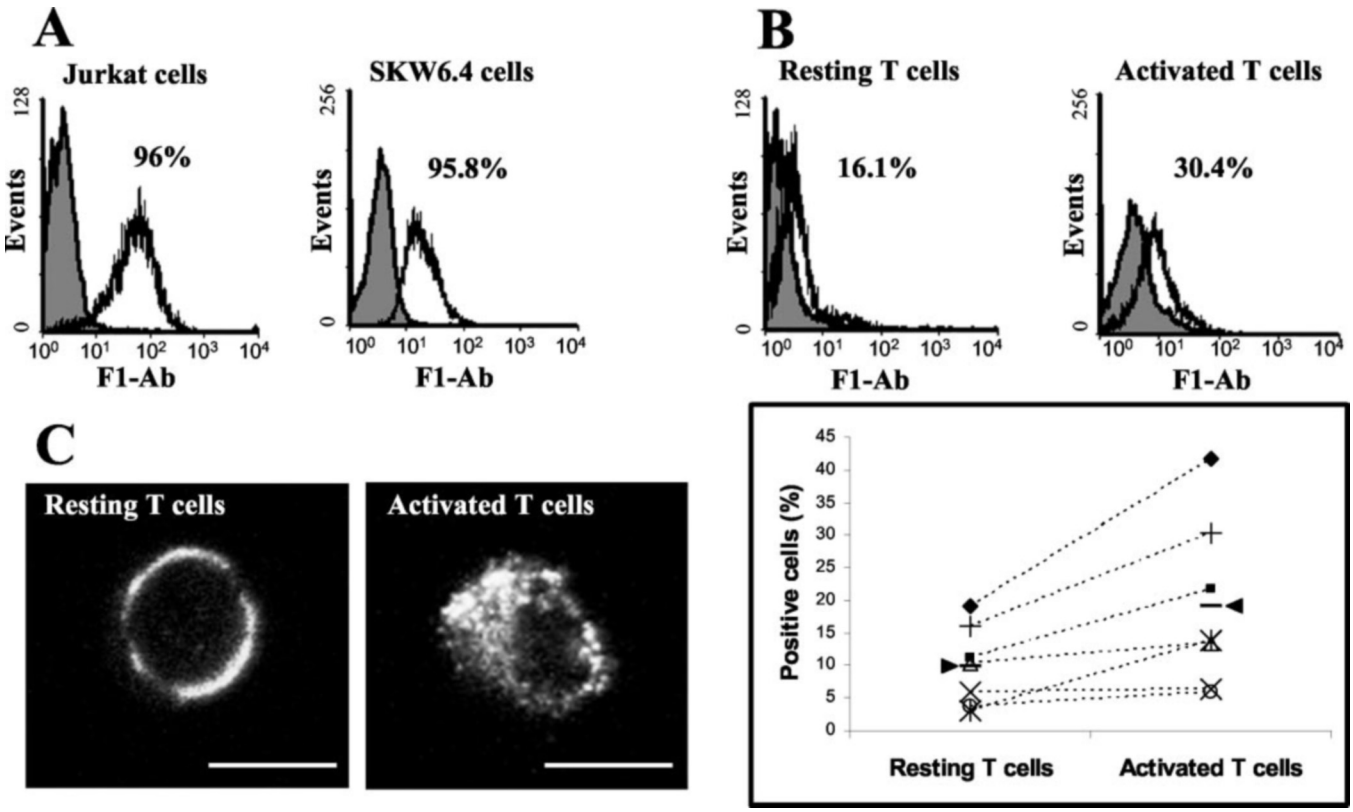

FIG. 1. **Group I mGluR expression in human lymphocytes.** Jurkat cells and SKW6.4 cells (*A*) and resting or activated peripheral blood T cells (*B*) were stained with anti-mGlu1/5R antibody (3 μg/ml F1-Ab) followed by a PE-conjugated goat anti-rabbit antibody (1/20) and analyzed by flow cytometry (numbers represent the percent of positive cells compared with the negative control indicated in *gray*). *B*, *upper panel*, a representative experiment using blood from a single healthy donor; *lower panel*, the percentage of positive cells of seven healthy donors (each symbol corresponds to a single healthy donor). The *black bars* (—) marked by the *arrowheads* represent the average. $p < 0.05$, significant mGlu1/5R expression in activated T cells *versus* resting T cells. *C*, resting and activated T cells were stained with anti-mGlu1/5R antibody (3 μg/ml F1-Ab) followed by AlexaFluor 488-conjugated goat anti-rabbit antibody (1/1000) and analyzed by confocal microscopy. *Bar*, 10 μm.

intensity of labeling in both cell lines, Jurkat and SKW6.4 (Fig. 1*A*); we found that more than 90% of Jurkat or SKW6.4 cells expressed the receptors. In resting human blood lymphocytes, the number of cells expressing mGlu1R and/or mGlu5R was low (16%), but upon activation, the number of cells expressing the targeted epitopes was markedly increased (30%) (Fig. 1*B*, *upper panel*). A variety of samples from several donors showed a similar pattern (Fig. 1*B*, *lower panel*). In activated cells, labeling intensity was much higher than in resting cells, and a clustered pattern of metabotropic glutamate receptor distribution was observed (Fig. 1*C*). Group I metabotropic glutamate receptors were expressed in both CD4+ and CD8+ populations (data not shown). To gain more insight into the subtype of receptor expressed in the cells, RT-PCR assays were performed after RNA isolation. mGlu5R transcripts are expressed in Jurkat cells, SKW6.4 cells, and in resting and activated blood lymphocytes (Fig. 2). In contrast, mGlu1R is expressed only in Jurkat T cells and in activated human blood lymphocytes. These results indicate that mGlu5R are constitutively expressed in blood lymphocytes, and mGlu1R are expressed only upon activation of the cells.

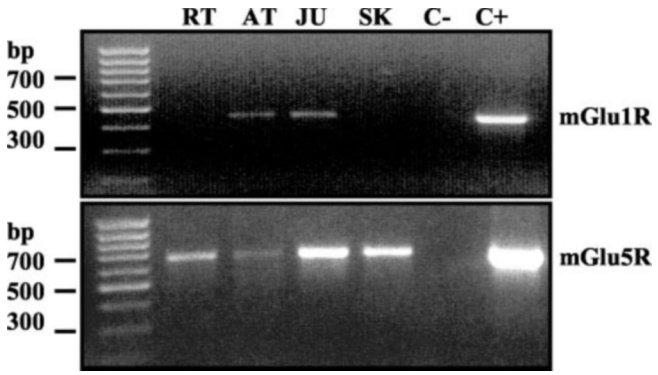

FIG. 2. **RT-PCR analysis of mGlu1R and mGlu5R in human lymphocytes.** mRNA from resting T cells (*RT*), activated T cells (AT), Jurkat cells (*JU*), and SKW6.4 cells (*SK*) was isolated, and RT-PCR analysis of mGlu1 (453 bp) and mGlu5 (661 bp) receptors was performed as described under "Experimental Procedures." Size markers are at the *left*. Extracts from human cerebral cortex were used as positive controls (+), and diethyl pyrocarbonate H_2O were used as negative controls (−). A representative experiment is shown.

It is well known that group I mGluRs are coupled to phospholipase C, thus increasing the levels of both inositol phosphates and intracellular calcium (3, 4). To identify the signaling pathways associated with the activation of the mGluRs expressed in the T cell line and in resting and activated lymphocytes, calcium mobilization was analyzed. 5 μg/ml of the antibody against the T cell receptor-CD3 complex substantially increased calcium levels in all cells assayed, whereas the non-selective group I mGluR agonist, DHPG, at concentrations up to 100 μM, failed to do so (Fig. 3). These results disagree with the well known coupling of group I mGluRs to phospholipase C in neuronal and glial cells (4). In heterologous expression systems, the activation of group I mGluRs increases cAMP (13). For this reason, cAMP production was examined. DHPG, the non-selective agonist, or CHPG, a highly selective mGlu5R agonist, significantly raised cAMP levels, and the effects of the two agonists on Jurkat cells and resting and activated lympho-

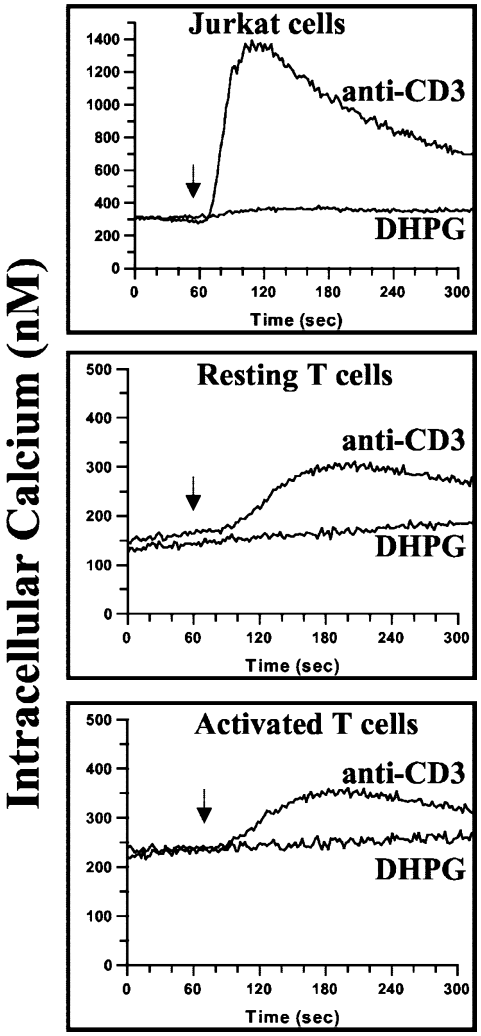

FIG. 3. **Effect of group I mGluR agonist on Ca^{2+} mobilization.** Jurkat cells, resting T cells, or activated T cells were loaded with Fura-2/AM and stimulated with 100 μM DHPG (*lower trace*) or with 5 μg/ml mouse monoclonal anti-CD3 antibody as a positive control (*upper trace*) where indicated by an *arrow*. Intracellular Ca^{2+} concentration was measured as described under "Experimental Procedures." Representative data of one of three independent experiments are shown.

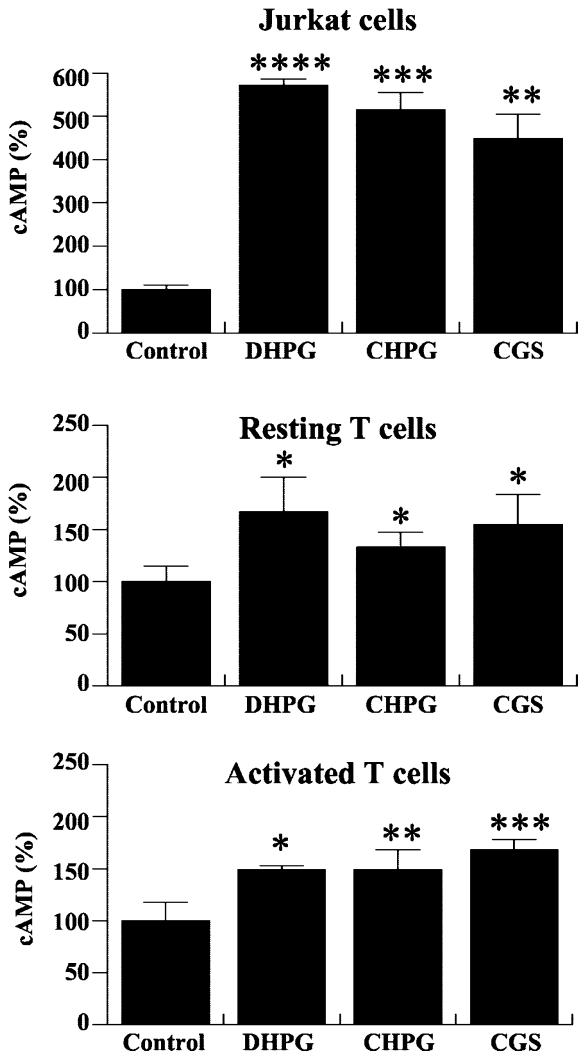

FIG. 4. **mGlu5R activation mediates cAMP increases.** Jurkat cells, resting T cells, and activated T cells were treated without (*Control*) or with 100 μM DHPG, 500 μM CHPG, or 200 nM CGS21680 (*CGS*) as a positive control; intracellular cAMP concentration was determined as described under "Experimental Procedures." Values are expressed as % of the control. Data are the mean ± S.D. of duplicates (Jurkat cells) or triplicates (resting and activated T cells). Representative data of one of three independent experiments are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0005$, compared with control.

cytes did not differ significantly (Fig. 4). This indicates that activation of mGlu5Rs elicits increases in cAMP levels. 1-Aminocyclopentane-1,3-dicarboxylate, a compound that activates metabotropic receptors, raises cAMP by an indirect mechanism that enhances the stimulation of adenylate cyclase by endogenous adenosine (14). The glutamate effects on cAMP levels in lymphocytes were not mediated by the extracellular adenosine present in the medium, because they were not prevented by the addition of adenosine deaminase, which degrades the endogenous extracellular nucleoside. There is controversy about the involvement of glutamate-mediated phospholipase C activation in the increase of cAMP levels after activation of these receptors (4). Our results clearly indicate that activation of phospholipase C and calcium mobilization are not a prerequisite for the increases in cAMP levels detected in lymphocytes when mGlu5Rs are activated. This suggests that in lymphocytes, mGlu5Rs, but not mGlu1Rs, are coupled to stimulatory Gs proteins. However, an indirect effect mediated by unidentified modulators whose receptors are coupled to Gs or the involvement of $\beta\gamma$-subunits in the activation of adenylate cyclase cannot be ruled out (4).

Jurkat cell treatment with DHPG induces ERK1/2 activation in a dose-dependent manner (Fig. 5). The kinetics of ERK1/2 phosphorylation was fast and peaked at 1 min of treatment with 100 μM DHPG. ERK1/2 activation was assayed in the presence of inhibitors of protein kinase C, protein kinase A, or MEK. ERK1/2 activation via mGluRs was abolished by PD 98059, the MEK inhibitor, in agreement with the role of MEK as a kinase that directly phosphorylates ERK1/2. GF 109203X, the inhibitor of protein kinase C, had no effect upon DHPG-induced ERK1/2 phosphorylation, thus indicating that for mGlu1R, protein kinase C is not upstream of MEK. These results agree with those presented in Fig. 3, which indicate that group I mGluRs cannot induce calcium mobilization in lymphocytes, which is the most common pathway associated with group I mGluR activation in neurons. H-89, the protein kinase A inhibitor, did not hinder ERK1/2 phosphorylation, indicating that mGluR1 does not mediate the activation of protein kinase A upstream of MEK. The higher ERK1/2 phosphorylation detected in the presence of H-89 may be due to the role of protein kinase A as an inhibitor of the MAP kinase pathway (15). To determine whether ERK1/2 activation also

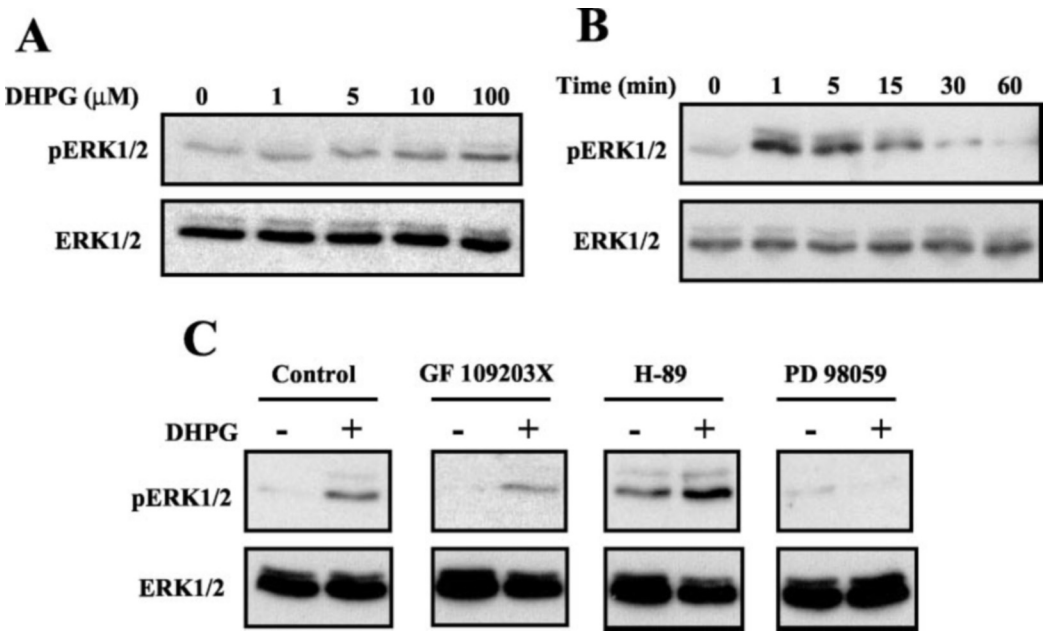

FIG. 5. **Effect of group I mGluR agonist on ERK1/2 phosphorylation in Jurkat cells.** Jurkat cells were stimulated with increasing DHPG concentrations for 5 min (*A*) or with 100 μM DHPG for various times (*B*). *C*, Jurkat cells were pretreated with 0.1% Me_2SO for 160 min (*Control*), 5 μM PKC inhibitor (*GF 109203X*) for 80 min, 10 μM protein kinase A inhibitor (*H-89*) for 160 min, or 25 μM MEK inhibitor (*PD 98059*) for 80 min, and then stimulated with 100 μM DHPG or PBS for 1 min. Cells were lysed, and ERK1/2 phosphorylation was analyzed by SDS-PAGE and Western blotting using specific anti-phospho-ERK1/2 antibody. Immunoblots were stripped and reprobed with ERK1/2-specific antibody, which recognizes total ERK1/2 to confirm equal loading. Representative data of one of three independent experiments are shown.

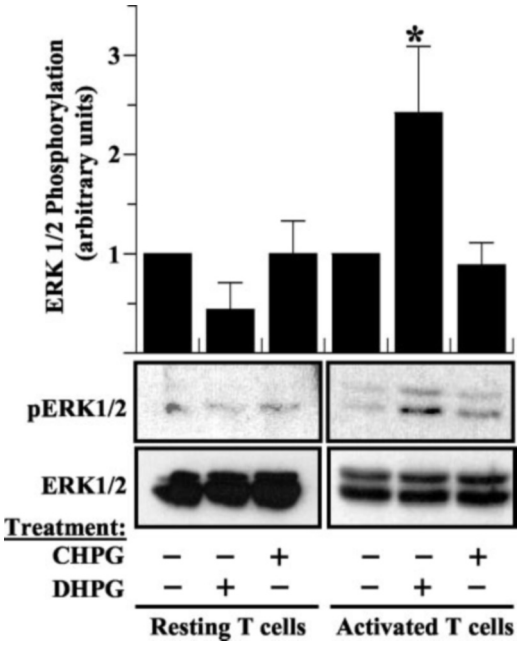

FIG. 6. **Phosphorylation of ERK1/2 in human T cells is mediated by mGlu1R but not by mGlu5R stimulation.** Resting T cells and activated T cells were treated (1 min) without or with 100 μM mGlu1/5R agonist (*DHPG*) or 500 μM mGlu5R-specific agonist (*CHPG*). Cells were lysed, and ERK1/2 phosphorylation was examined by SDS-PAGE and Western blotting using phospho-specific ERK1/2 antibody. Immunoblots were stripped and reprobed with ERK1/2-specific antibody, which recognizes total ERK1/2 to confirm equal loading. A representative experiment using blood from a single healthy donor is shown (*lower panel*). Western blots obtained from three healthy donors were analyzed by densitometry, normalized to total ERK1/2, and expressed in fold compared with the control (*upper panel*). *, $p < 0.005$.

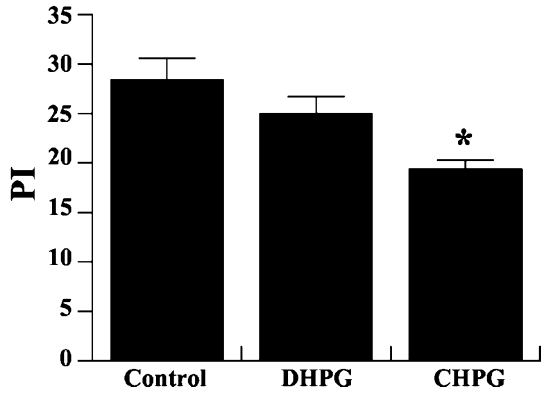

FIG. 7. **Agonists of mGlu5R inhibit the anti-CD3 antibody-induced proliferation, but non-selective agonists suppress the inhibition.** Human T cells were incubated with medium (*Control*), with 100 μM non-selective mGlu1/5R agonist (*DHPG*), or with 500 μM of the mGlu5R-selective agonist (*CHPG*) and were immediately treated with medium or 1 ng/ml mouse monoclonal anti-CD3 antibody. Cultures were incubated for 4 days, and proliferation was determined as [^{3}H]thymidine incorporation. Values are expressed as proliferation index (*PI*), determined as [^{3}H]thymidine incorporated in treated cells/[^{3}H]thymidine incorporated in cells treated only with medium. Data are the mean ± S.D. of triplicates. Representative data of one of three independent experiments are shown. *, $p < 0.005$, compared with control.

occurred in peripheral blood lymphocytes, similar experiments were performed in resting and activated lymphocytes, which were treated with either CHPG or DHPG. In resting lymphocytes, ERK1/2 activation was not triggered by either of the compounds used, whereas in activated cells, DHPG, but not CHPG, induced ERK1/2 phosphorylation (Fig. 6). Therefore, inducible mGlu1Rs, but not constitutively expressed mGlu5Rs, are coupled to the MAP kinase cascade in human peripheral T lymphocytes.

As is the case with receptors of other neurotransmitters, the metabotropic glutamate receptors in lymphocytes should be identified to understand the links and parallelisms between the immune and the nervous systems. Ganor *et al.* (1) have recently reported that glutamate directly activates, via ionotropic glutamate receptors, T cell function and triggers integrin-mediated adhesion and chemotactic migration. This al-

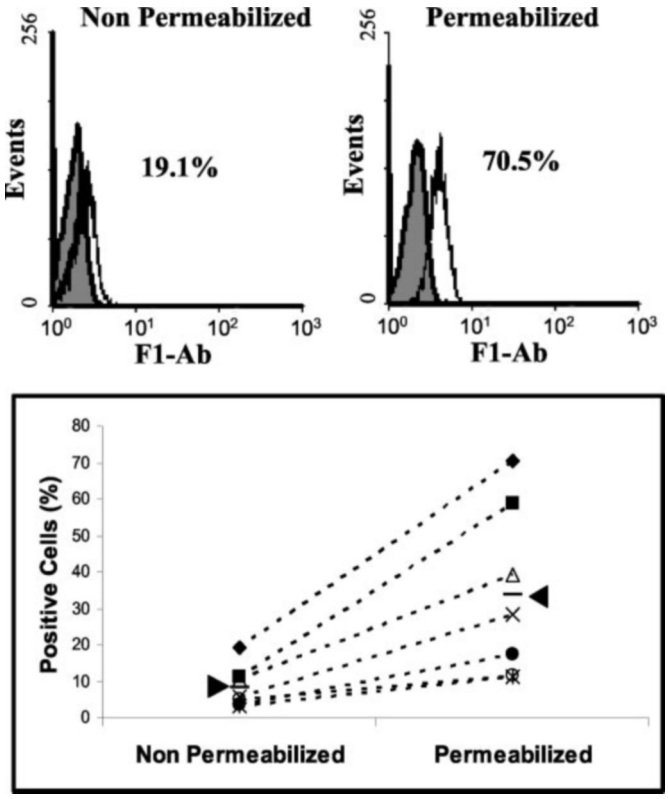

FIG. 8. **Intracellular expression of mGlu5R in human resting T cells.** Resting peripheral blood T cells permeabilized or not were stained with anti-mGlu1/5R antibody (3 μg/ml F1-Ab), followed by PE-conjugated goat anti-rabbit antibody (1/20) and analyzed by flow cytometry (numbers represent the percent of positive cells compared with the negative control indicated in *gray*). *Upper panel*, representative experiment using blood from a single healthy donor; *bottom panel*, percentage of positive cells of seven healthy donors (each symbol corresponds to a single healthy donor). The *bars* (—) marked by the *arrowheads* represent the average of all values. $p < 0.005$, significant expression in non-permeabilized *versus* permeabilized.

lows dialogues between the nervous and immune system in health and disease. The authors emphasize the relevance of these results in T cells transmigrating to the central nervous system and patrolling the brain, in T cell-mediated multiple sclerosis, and in autoimmune epilepsy. The activation of ionotropic receptors leads to T cell activation in the absence of an antigenic stimulus, whereas mGlu5Rs, like other receptors elevating cAMP levels, may behave as a strong inhibitor of T cell activity (16). To elucidate the effect of mGlu5R, which is positively coupled to adenylate cyclase, on cell proliferation, lymphocytes were treated with CHPG and then activated with the anti-T cell receptor-CD3 complex. Specific activation of mGlu5R by CHPG significantly inhibited cell proliferation, as measured by [^{3}H]thymidine incorporation (Fig. 7), which indicates that the reported inhibition of glutamate in lymphocytic proliferative response (17) is probably mediated by the constitutively expressed mGlu5R. This inhibitory effect was suppressed when activated cells were treated with the non-selective agonist DHPG. Glutamate may thus play a dual role in T cell function, because it inhibits cell proliferation via mGlu5R but reverts this inhibition when mGlu1R is expressed.

As K_D values for mGlu1R and mGlu5R are in the range 10–60 μM (4), they can be activated by the glutamate present in plasma, whose concentration varies between 30 and 60 μM (18, 19). Chronic activation of mGlu5R by glutamate in lymphocytes may lead to receptor desensitization and/or internalization. The few studies devoted to the desensitization of this receptor indicate that in heterologous systems, mGlu5R depends upon PKC activation (20). It is improbable that this mechanism operates in lymphocytes, because mGlu5R activation is not coupled to the PKC pathway. In heterologous cells, members of the GRK2 family are involved in receptor phosphorylation and internalization (21). The expression of active receptors on the surface of cells depends upon their traffic, which is influenced by internalization after receptor activation, and by the rate of receptor recycling, among others. Flow cytometry using permeabilized cells revealed that the percentage of cells expressing the receptor was much higher than in non-permeabilized cells. Also, the intensity of the labeling was higher in permeabilized cells. This indicates that a percentage of resting T lymphocytes express mGlu5R at the cell surface and in intracellular membranes, whereas another set of cells express the receptor-only intracellulary (Fig. 8). Intracellular mGlu5R pools may reflect down-regulation after the chronic exposure of lymphocytes to serum glutamate or else some kind of agonist-independent regulation of the cell-surface expression of the receptor. In fact, significant intracellular pools of G protein-coupled receptors are often found, even in the absence of agonist activation (22), but their role has not yet been completely elucidated. Because agonists increase the levels of the second messenger cAMP and inhibit T cell receptor-mediated cell proliferation, the mGlu5Rs expressed on the cell surface of resting blood T lymphocytes are functional.

Because of the presence of micromolar levels of glutamate in blood, it would be wise to assume that constitutively expressed mGlu5Rs on the surface of a population of blood T cells are tonically activated, leading to a tonic increase in cAMP levels. The increases in cAMP affect proliferation and cytokine expression (16), which may constitute a brake mechanism to prevent unspecific T cell activation. In contrast, the role of inducible mGlu1R may be crucial to the activation of both T cells in blood and T-lymphoblasts entering the central nervous system in brain pathologies and in immune-mediated illnesses.

In conclusion, resting lymphocytes express mGlu5R constitutively, whereas mGlu1R expression is induced upon activation. Neither receptor is coupled in lymphocytes to the calcium and phosphoinositide-signaling machinery operating in brain cells. Instead, mGlu1Rs are coupled to the MAP kinase pathway, and mGlu5Rs increase cAMP levels. In agreement with the complexity and variety of glutamate effects in the central nervous system, the variety of signaling mechanisms triggered by glutamate in lymphocytes by means of ionotropic and metabotropic receptors indicates that glutamate regulates lymphoid function and that further studies are required to fully understand its role. In principle, glutamate exerts a tonic inhibitory effect via constitutively expressed mGluR5, whereas it has a costimulatory role when mGluR1s are expressed upon T cell activation.

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