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Characterization of ionotropic glutamate receptors in human lymphocytes

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1 The effect of L-glutamate (Glu) on human lymphocyte function was studied by measuring anti-CD_3 monoclonal antibody (mAb) or phytohaemagglutinin (PHA)-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) rise (Fura-2 method), and cell proliferation (MTT assay).

2 Glu (0.001–100 μM) did not modify basal lymphocyte $[Ca^{2+}]_i$, but significantly potentiated the effects of anti-CD_3 mAb or PHA. Maximal $[Ca^{2+}]_i$ rises over resting cells were: 165 ± 8 and 247 ± 10 nM at 3.0×10^{-2} mg ml^{-1} anti-CD_3 mAb; 201 ± 4 and 266 ± 9 nM at 5.0×10^{-2} mg ml^{-1} PHA, in the absence or presence of 1 μM Glu, respectively.

3 The Glu effect showed a bell-shape concentration-dependent relationship, with a maximum ($+90 \pm 3$% for anti-CD_3 mAb and $+57 \pm 2$% for PHA over Glu-untreated cells) at 1 μM.

4 Non-NMDA receptor agonists (1 μM) showed a greater efficacy ($+76 \pm 2$% for (S)-AMPA; $+78 \pm 4$% for KA), if compared to NMDA ($+46 \pm 2$%), or Glu itself.

5 Ionotropic Glu receptor antagonists completely inhibited the effects of the corresponding specific receptor agonists (1 μM). The IC_{50} values calculated were: 0.9 μM for D-AP5; 0.6 μM for (+)-MK801; 0.3 μM for NBQX. Both NBQX and KYNA were able to abolish Glu effect. The IC_{50s} calculated were: 3.4 μM for NBQX; 0.4 μM for KYNA.

6 Glu (0.1–1 mM) did not change the resting cell proliferation, whereas Glu (1 mM) significant inhibited (-27 ± 4%) PHA (1.0×10^{-2} mg ml^{-1})-induced lymphocyte proliferation at 72 h.

7 In conclusion, human lymphocytes express ionotropic Glu receptors functionally operating as modulators of cell activation.

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Keywords: Glutamate; ionotropic glutamate receptors; human lymphocytes; immunomodulation; neuroinflammation

Abbreviations: (1S,3R)-ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; (S)-AMPA, (S)-α-amino-3-hydroxy-6-methyl-4-isoxazolepropionic acid; D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; KA, kainic acid; KYNA, kinurenic acid; (+)-MK801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide; NMDA, N-methyl-D-aspartic acid; $PBMNC_s$, peripheral blood mononuclear cells

Introduction

Early in the field of study of neural-immune interaction there was significant doubt that the central nervous system (CNS) and the immune system could mutually influence their respective functions (Ader *et al*., 1990). Within the past 10 years evidence of connections between the two systems has been clearly reported. Neuroinflammation, the inflammatory response in the CNS, plays a rôle in propagating cerebral ischemic injury (Morioka *et al*., 1991; Garcia *et al*., 1994; Sharkey & Butcher, 1994), contributes to the outcome of acquired immunodeficiency syndrome (AIDS) (Koyanagi *et al*., 1987; Spencer & Price, 1993), Alzheimer's disease (AD) (Dickson & Rogers, 1992; Akiyama *et al*., 2000; Lim *et al*., 2000), and it is involved in epilepsy (Li *et al*., 1997) and multiple sclerosis (Pitt *et al*., 2000; Smith *et al*., 2000). During neuroinflammation, peripheral infiltrated leukocytes and cells of the monocyte/macrophage lineage, together with microglia, the immune effector cells in the CNS, are activated and release various molecules, such as proteases, oxygen radicals (Lucchesi, 1993; Ren *et al*., 1994; Ding *et al*., 1988), neurotoxins (Heyes *et al*., 1998), cytokines (Licinio & Wong, 1997), nitric oxide (Merrill *et al*., 1993; Boje, 1996), eicosanoids (Lucchesi, 1993; Morris & Rodger, 1998), and autoantibodies (Rogers *et al*., 1994), which could be harmful to neurones.

On the other hand, the function of the immune system is regulated by various neurotransmitters and hormones. Human mononuclear leukocytes contain β_2-adrenoreceptors (Brodde *et al*., 1987), both neuronal-type nicotinic and muscarinic ACh receptors are present on the surface of human mononuclear leukocytes (Sato *et al*., 1999b), and $mRNA_s$ encoding for μ (Sedqi *et al*., 1995), δ (Chuang *et al*., 1994; Gaveriaux *et al*., 1995), and κ (Belkowski *et al*., 1995; Gaveriaux *et al*., 1995) opioid-like receptors were demonstrated in leukocytes and lymphocytes. Functional $GABA_A$ receptors have been described on T cells (Tian *et al*., 1999), and peripheral type benzodiazepine receptors on circulating blood cells as well (Parola *et al*., 1993). Moreover, human

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lymphocytes express substance P and neurokinin-1 receptors (Lai *et al*., 1998).

Concerning Glu, the principal mediator of excitatory transmission among vertebrate neurones, only binding experiments have been described in human blood T lymphocytes, showing that these cells express quisqualate-sensitive binding sites on their outer cell membranes (Kostanyan *et al*., 1997). The relevance of this observation could be substantial, because of the evidence that the excitatory amino acid neurotransmission is related to many pathological conditions characterized by impaired immune functions (AIDS, hepatic hencephalopathy, AD, chronic epilepsy, multiple sclerosis), and that high plasma Glu concentrations (commonly observed in patients with AIDS and neoplastic diseases) inhibit lymphocyte responses to mitogens, and macrophage cysteine release into the extracellular space (Droge *et al*., 1988; Eck *et al*., 1989). However, the possible relationship between radiolabelled binding sites and the existence of functional receptors has been object of debate. The ionic composition of the incubation medium/assay buffer and the specific experimental conditions have profound effects on the sites labelled by ^{3}H-L-Glu (Foster & Fagg, 1984; Monaghan *et al*., 1985), and often a 'physiological receptor' is not equivalent to a specific binding site (Robinson *et al*., 1985; Bridges *et al*., 1986; Fagg & Lanthorn, 1986).

These observations prompted us to investigate whether Glu may modulate human lymphocyte function by a receptor-mediated mechanism. The data we obtained demonstrate that human lymphocytes express ionotropic glutamate receptors, pharmacologically similar to neuronal receptors, able to modulate cellular function.

Methods

Cell isolation

Cells were obtained by standard procedures from healthy young volunteers on the day of the experiment. Briefly, heparinized blood was layered onto Ficoll-Hypaque separating media, according to the method of Boyum (1968). After centrifugation at 1200 r.p.m. for 10 min at room temperature the cells were collected and suspended in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (2 mM). The suspension of cells (PBMNC$_s$) consisted of lymphocytes (90$\pm$2%), monocytes (7$\pm$2%) and polymorphonuclear leukocytes (3$\pm$1%). For $[Ca^{2+}]_i$ measurements the monocytes were eliminated by 10% FBS plate adhesion at 37°C for 1 h. For proliferation assays, PBMNC$_s$ were cultured in 96-well plates (10^5 cells/0.1 ml/well).

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ concentrations were evaluated according to the procedure reported by Merritt *et al*. (1993). Briefly, lymphocytes were loaded with 0.5 μM Fura-2AM for 1 h at 37°C. After loading with the dye, the cells were washed, resuspended at 5×10^5 cells ml^{-1} in a buffered salt solution (BSS) pH 7.4, with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, glucose 11, supplemented with 2.5 mM probenecid to minimize Fura-2 leakage (Cobbold & Rink, 1987), and maintained at room temperature until assayed. In some experiments the nominal Ca^{2+} concentration of the buffer was altered by omitting $CaCl_2$ and by adding the extracellular calcium chelator EGTA, 1 mM (Andersson *et al*., 1986). Fura-2 fluorescence was monitored in a Jasco FP777 fluorimeter at 25°C with cells continuously stirred. Excitation and emission wavelengths (340 and 505 nm, respectively) were selected and $[Ca^{2+}]_i$ was calculated according to Damaj *et al*. (1996). The increase in $[Ca^{2+}]_i$ was measured as the difference between the basal level and the peak reached after drug addition and it was expressed as $\Delta[Ca^{2+}]_i$(nM). Each assay was performed in triplicate.

Cell proliferation assay

PBMCs were treated in 0.1 ml at 10^5 cells well^{-1} with varying concentrations of Glu in the absence or presence of PHA (1.0×10^{-2} mg ml^{-1}) and assayed at 72 h for proliferation using the MTT method (Mosmann, 1983).

Cell viability

Trypan blue exclusion test was performed to evaluate cell viability at the end of cell isolation and after drug treatment. Cell viability was greater than 95% in all experiments and it was not affected by drug treatments.

Materials

Ficoll-Hypaque (Histopaque®-1077), Fura-2AM, trypan blue, MTT, Glu, glutamine, KYNA, PHA, and probenecid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NMDA, D-AP5, (S)-AMPA, KA, (+)-MK801, (1S,3R)-ACPD were purchased from Tocris Neuramin (Bristol, U.K.). All other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Data analysis

Results are expressed as means$\pm$s.e.mean of n experiments. Maximum, EC_{50} and IC_{50} were calculated in each experiment on the basis of a semi-logarithmic concentration-response curve. Statistical significance in all experiments was evaluated by Student's t-test for paired varieties. Differences were considered statistically significant when $P<0.05$.

Results

Drug effects on intracellular calcium concentration

Basal $[Ca^{2+}]_{is}$ in human resting lymphocytes incubated *in vitro* was 88$\pm$16 nM ($n=102$, data not shown).

Figure 1 reports the concentration-response curves of the $[Ca^{2+}]_i$ increases obtained by stimulating lymphocytes with anti-CD$_3$ mAb (Figure 1a) or PHA (Figure 1b) (concentration range: $3.0\times10^{-4}-3.0\times10^{-2}$ mg ml^{-1}, and $1.0\times10^{-4}-5.0\times10^{-2}$ mg ml^{-1}, respectively) for 3 min. Maximal $[Ca^{2+}]_i$ increases over the basal level were obtained at 3.0×10^{-2} mg ml^{-1} for anti-CD$_3$ mAb, and at 5.0×10^{-2} mg ml^{-1} for PHA and were: 165$\pm$8 nM and 201$\pm$4 nM, respectively. The calculated EC_{50s} were:

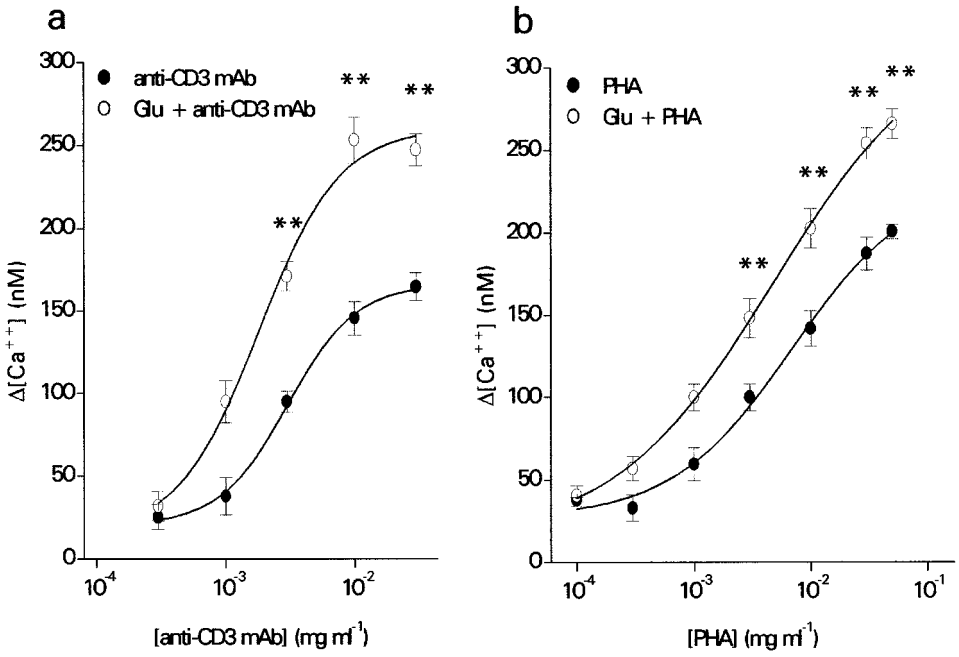

Figure 1 Concentration-response curves of $[Ca^{2+}]_i$ rise obtained by stimulating Glu-untreated or Glu-treated (1 μM, for 5 min) lymphocytes with anti-CD_3 mAb (a) and PHA (b) for 3 min. In the presence of Glu maximal potentiations were: $50\pm8\%$ for anti-CD_3 mAb and $32\pm2\%$ for PHA, at 3.0×10^{-2} mg ml^{-1} and 5.0×10^{-2} mg ml^{-1}, respectively. The EC_{50} values for anti-CD_3 mAb and PHA were: 3×10^{-3} mg ml^{-1}, and 6.9×10^{-3} mg ml^{-1} in Glu-untreated cells; and 1.8×10^{-3} mg ml^{-1} and 4.7×10^{-3} mg ml^{-1} in Glu-treated cells, respectively. Values are mean$\pm$s.e.mean of at least four experiments. $^{**}P<0.01$ vs Glu-untreated lymphocytes.

3.0×10^{-3} mg ml^{-1} ($n=4$) for anti-CD_3 mAb, and 6.9×10^{-3} mg ml^{-1} ($n=8$) for PHA. Chelation of extracellular Ca^{2+} by addition of 1 mM EGTA, immediately prior to the stimuli, completely abolished the effects of anti-CD_3 mAb or PHA ($n=4$; data not shown), indicating that the increase of $[Ca^{2+}]_i$ was predominantly due to ion influx rather than to intracellular mobilization, in accordance with reported data in human lymphocytes (Alcover *et al*., 1987; Gelfand *et al*., 1987; Linch *et al*., 1987).

Neither Glu nor the prototype agonists of Glu receptors modify, at any concentration tested (0.001 – 100 μM), basal $[Ca^{2+}]_i$ in resting lymphocytes ($n=5$ each; data not shown). On the contrary, when Glu was added prior to cell stimulation, it potentiated the $[Ca^{2+}]_i$ rise in response to either anti-CD_3 mAb or PHA alone. For our experiments we utilized the maximally effective Glu concentration (1 μM; see below) and 5 min Glu pre-exposure time. This time was selected according to time-course (1 – 30 min) studies previously performed in our laboratory (data not shown). Interestingly, Glu did not modify significantly the potency of either anti-CD_3 mAb or PHA (EC_{50s}: 1.8×10^{-3} mg ml^{-1} ($n=4$) for anti-CD3 mAb; 4.7×10^{-3} mg ml^{-1} ($n=8$) for PHA), but significantly ($P<0.05$) enhanced their efficacy (247 ± 10 nM for anti-CD_3 mAb, and 266 ± 9 nM for PHA) (Figure 1a, b), suggesting that Glu does not modify in a significant manner the affinity of anti-CD_3 mAb or PHA to their specific receptors.

Pharmacological characterization of Glu-induced potentiation of $[Ca^{2+}]_i$ rise

The Glu potentiating effect on anti-CD_3 mAb (3.0×10^{-3} mg ml^{-1}) or PHA (1.0×10^{-2} mg ml^{-1})-induced $[Ca^{2+}]_i$ rise in lymphocytes showed a bell-shape concentration-dependent relationship (Figure 2). Glu concentrations between 0.001 and 1 μM significantly ($P<0.01$) potentiated lymphocyte response, an effect which progressively declined between 3 and 100 μM. The maximum effects ($+90\pm3\%$ for anti-CD_3 mAb, and $+57\pm2\%$ for PHA over Glu-untreated cells) were obtained by treating the cells with 1 μM Glu.

To clarify if Glu-induced potentiation of anti-CD_3 mAb or PHA responses in lymphocytes is a receptor-mediated mechanism, we performed similar experiments by using the prototype Glu receptor agonists. Comparison of the concentration-response curves we obtained with anti-CD3 mAb or PHA in the presence of Glu clearly demonstrates a good correlation between antigen-induced, *via* CD3 antigen receptor, and PHA-induced, *via* CD2 antigen receptor (O'Flynn, *et al*., 1986), stimulation of the cells: therefore we decided to complete our studies by utilizing the lectin, an easy available vegetable derivative. NMDA, (S)-AMPA, and KA all produced a significant potentiation of $[Ca^{2+}]_i$ responses in activated lymphocytes similar to that observed with Glu: the concentration-response curves designed for these molecules exhibit the same bell shapes seen with Glu with the maximum effects obtained at 1 μM (Figure 3). From these curves the relative efficacy of each compound was then calculated by comparing the maximum effects they evoked with that of Glu (Figure 4). Non-NMDA receptor agonists showed a greater efficacy ($+76\pm2\%$ for (S)-AMPA; $+78\pm4\%$ for KA, over PHA-treated cells), if compared to NMDA receptor agonists ($+46\pm2\%$ for NMDA) or Glu itself ($+57\pm2\%$) ($n=5$ each). The addition of glycine (1 – 100 μM) to experimental buffer was unable to modify the NMDA response. On the contrary, (1S,3R)-ACPD, the prototype metabotropic Glu receptor agonist, was ineffective at all concentrations tested (0.001 – 100 μM).

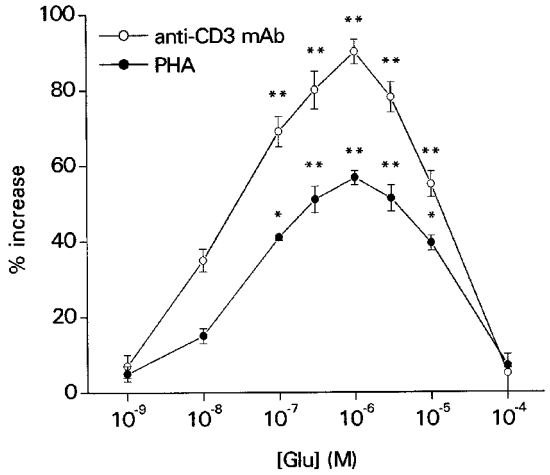

Figure 2 Concentration-response curves for the Glu potentiating effect on anti-CD_3 mAb (3.0×10^{-2} mg ml^{-1}) or PHA (1.0×10^{-2} mg ml^{-1})-induced (3 min) $[Ca^{2+}]_i$ rise in lymphocytes. Maximal $[Ca^{2+}]_i$ increases over Glu-untreated cells were: $+90\pm3\%$ for anti-CD_3 mAb, and $+57\pm2\%$ for PHA. Data are mean$\pm$s.e.mean of at least five experiments. $^{**}P<0.01$, $^{*}P<0.05$ vs Glu-untreated lymphocytes.

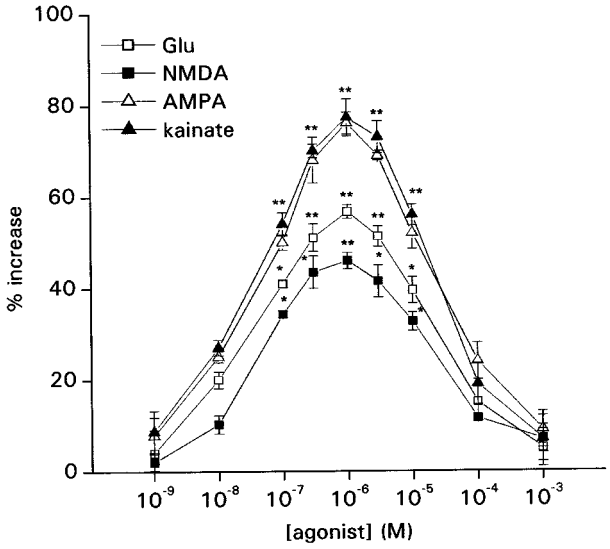

Figure 3 Concentration-response curves for the Glu or Glu receptor agonist potentiating effects on PHA (1.0×10^{-2} mg ml^{-1})-induced (3 min) $[Ca^{2+}]_i$ rise in lymphocytes. Data are mean$\pm$s.e.mean of at least four experiments. $^{**}P<0.01$, $^{*}P<0.05$ vs Glu-untreated lymphocytes.

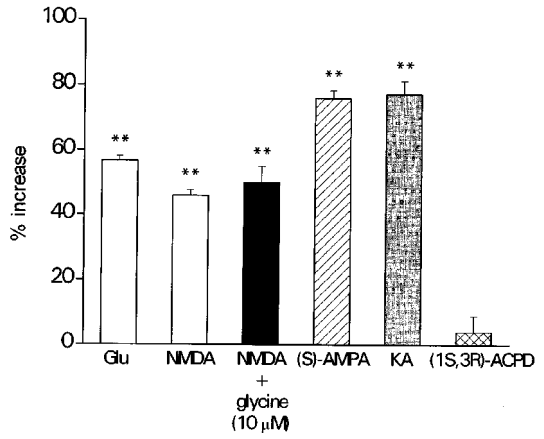

Figure 4 Maximal effects of the Glu or Glu receptor agonists (1 μM, for 5 min) on PHA-induced (1.0×10^{-2} mg ml^{-1}, 3 min) $[Ca^{2+}]_i$ rise in lymphocytes. Glycine (1–100 μM) was added immediately before NMDA treatment. Data are expressed as per cent increase of $[Ca^{2+}]_i$ rise over PHA-induced cell responses. Data are mean$\pm$s.e.mean of at leats five experiments. $^{**}P<0.01$ vs Glu-untreated cells.

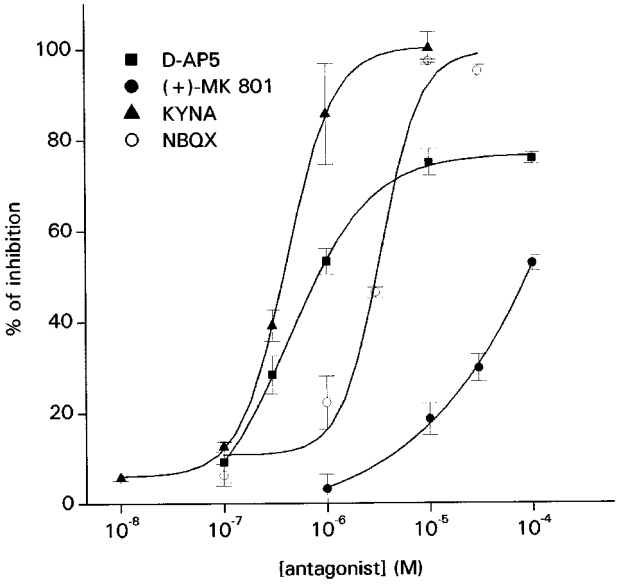

Figure 5 Inhibition of the Glu (1 μM, for 5 min) potentiating effect on PHA-induced (1.0×10^{-2} mg ml^{-1}, 3 min) $[Ca^{2+}]_i$ rise in lymphocytes by ionotropic Glu receptor antagonists. Cells were incubated for 5 min with the antagonist before the addition of Glu. The IC_{50} values for NBQX, and KYNA were 3.4 μM, and 0.4 μM, respectively. Values are mean$\pm$s.e.mean of at least four determinations.

Effects of competitive and non-competitive ionotropic Glu receptor antagonists on $[Ca^{2+}]_i$

None of the drugs utilized as selective ionotropic Glu receptor antagonists modified $[Ca^{2+}]_i$ in resting lymphocytes ($n=20$; data not shown). However, the exposure of cells to these molecules 5 min before the addition of 1 μM Glu inhibited the Glu potentiating effect in a concentration-dependent manner. Figure 5 shows the inhibition curves of D-AP5, a competitive NMDA receptor antagonist (Watkins *et al*., 1990), of (+)-MK801, the use-dependent NMDA ion channel blocker (Wong *et al*., 1986), of NBQX, a competitive antagonist of (S)-AMPA receptors (Sheardown *et al*., 1990), and of KYNA, a broad spectrum antagonist of both AMPA and NMDA receptors (Perkins & Stone, 1982), towards the Glu-induced potentiation of $[Ca^{2+}]_i$ rise in lymphocytes. NBQX (10 μM) and KYNA (3 μM) were able to abolish the effect of 1 μM Glu, whereas all the other compounds, even when tested at greater concentrations (up to 100 μM), did not antagonize completely Glu potentiation. The IC_{50} values were: 3.4 μM ($n=4$) for NBQX, and 0.4 μM ($n=5$) for KYNA.

When the cells were incubated with specific receptor antagonists, and then selectively stimulated by the maximally effective concentration of the corresponding specific receptor agonists, a complete inhibition was obtained (Figure 6a–c). The IC_{50} values calculated were: 0.9 μM ($n=5$) for D-AP5; 0.6 μM ($n=5$) for (+)-MK801, and 0.3 μM ($n=5$) for NBQX.

Cell proliferation studies

Since Ca^{2+} has been demonstrated to be a necessary, but not sufficient, signal for full cell cycle progression (Lu & Means, 1993; Means, 1994), we next evaluated the Glu effect on PHA-induced cell proliferation, as assayed by the MTT method. Resting or PHA (1.0×10^{-2} mg ml^{-1})-activated lymphocytes were incubated for 72 h in RPMI 1640 medium in the presence of 10% FBS and increasing Glu concentrations. The final Glu concentration present in each sample, as indicated in the Figure 7, was calculated by correcting for the standard cell culture medium Glu concentration (0.1 mM). Increasing Glu concentrations (0.1–1 mM) did not cause any change in the proliferation rate of resting lymphocytes, whereas a significant ($P<0.05$) concentration-dependent inhibition was observed in PHA-activated cells treated with Glu (1 mM) for 72 h (-27 ± 4%; $n=10$), if compared to PHA-treated cells, cultured in the standard medium.

Reduction of cell proliferation by Glu does not seem to be due to a toxic effect of the amino acid, as indicated by the lack of changes in cell viability assessed by the trypan blue exclusion test performed in the same cell cultures.

Discussion

The presence of peripheral Glu receptors has been shown in several tissues, including the guinea-pig myenteric plexus (Moroni *et al*., 1986), the unmyelinated sensory nerve terminals in the skin (Ault & Hildebrand, 1993; Carlton *et al*., 1995), the pancreatic islets of Langerhans (Bertrand *et al*., 1992; Inagaki *et al*., 1995; Weaver *et al*., 1998), the osteoclasts and osteoblasts in the bone (Chenu *et al*., 1998; Patton *et al*., 1998) and rat mast cells (Purcell *et al*., 1996), the cardiac ganglia in the heart (Gill *et al*., 1998) and the liver (Storto *et al*., 2000). Binding sites for Glu have been described on the outer membrane of human T lymphocytes

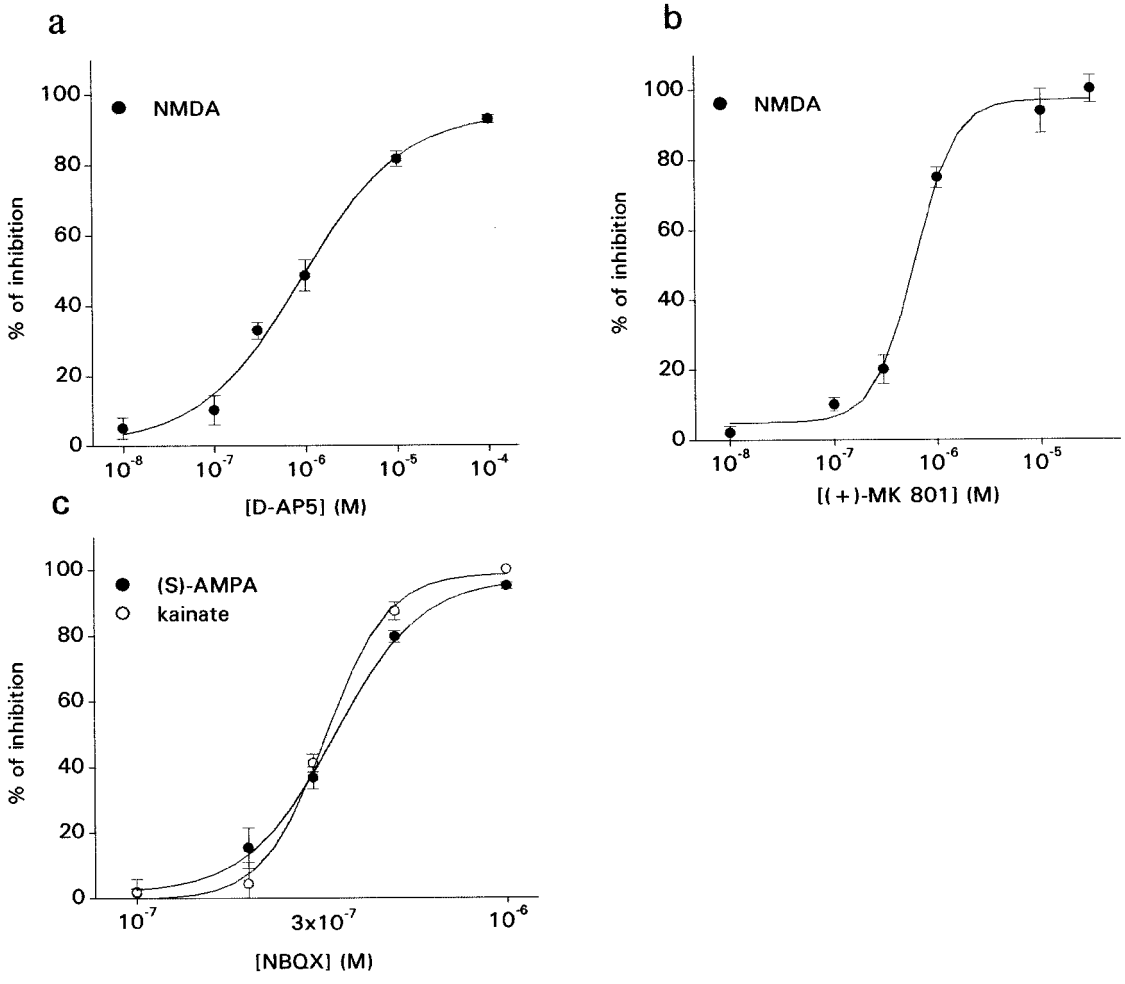

Figure 6 Inhibition of the NMDA, (S)-AMPA, or KA (1 μM, for 5 min) potentiating effect on PHA-induced (1.0×10^{-2} mg ml^{-1}, 3 min) $[Ca^{2+}]_i$ rise in lymphocytes by the corresponding specific receptor antagonists. Cells were incubated for 5 min with the antagonist before the addition of the corresponding agonist. The IC_{50} values for D-AP5, (+)-MK801, and NBQX were: 0.9 μM; 0.6 μM, and 0.3 μM, respectively. Values are mean$\pm$s.e.mean of at least five determinations.

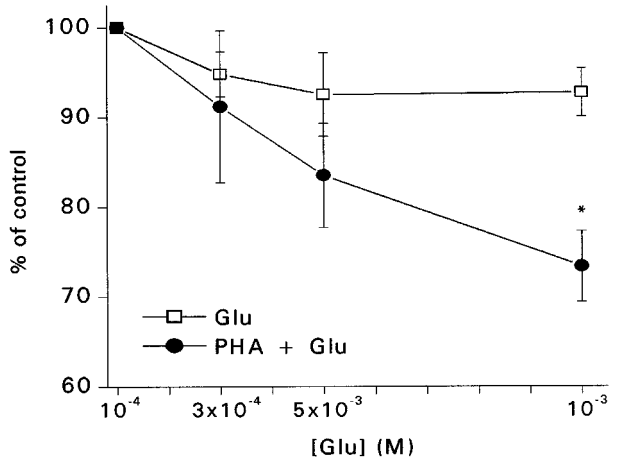

Figure 7 Inhibition of mitogenic responses by Glu. Resting or PHA (1.0×10^{-2} mg ml^{-1})-activated lymphocytes (10^5 cells 0.1 ml^{-1} well^{-1}) were incubated with increasing Glu concentrations and assayed at 72 h by MTT method. The final Glu concentration present in each sample, as indicated in Figure 6, are corrected for the standard cell culture medium concentration (0.1 mM). Controls (100% cell proliferation) were cells cultured in a standard medium. Values are mean ± s.e.mean of at least 10 determinations. $^*P < 0.05$ vs resting lymphocytes.

(Kostanyan *et al*., 1997), but their specific function(s) has not yet been established. Results reported here demonstrate that human peripheral blood lymphocytes express functional ionotropic Glu receptors.

In our studies, Glu in concentrations ranging from 0.001 μM to 1 mM did not modify either the $[Ca^{2+}]_i$ or the cell proliferation in resting lymphocytes. However, when cells were pre-exposed to Glu and then activated, significant modifications of cellular responses were obtained. Our experiments suggest that, during so called 'commitment period', in which continuous receptor occupancy is required to initiate morphological and functional changes (Milner, 1977), the presence of Glu induces molecular modifications, possibly capable of changing cellular functions.

The responses of lymphocytes to the plant lectin PHA (Tsien *et al*., 1982; Mills *et al*., 1985) or anti-CD_3 mAb (Davis *et al*., 1986) partially reproduce the antigen-T lymphocyte interaction: thus, we have experimentally used these compounds in place of specific antigens for stimulating cells. Two pathways by which human lymphocytes are activated have been demonstrated. The first, the antigen-dependent pathway, is composed by CD3 antigen receptor, the other one is linked to CD2 antigen receptor. PHA stimulates lymphocytes predominantly through this pathway (O'Flynn *et al*., 1985; 1986). The relationship between the two pathways suggests that activation *via* CD2 requires the presence of CD3 on the cell membrane, and in our conditions the good correlation between the results we obtained with CD3-mAb and PHA for both $[Ca^{2+}]_i$ variation and cell proliferation supports this hypothesis.

In our experimental conditions, the analysis of concentration-response curves of the $[Ca^{2+}]_i$ rise obtained by stimulating human lymphocytes with anti-CD_3 mAb or PHA, in the presence or absence of Glu, show that Glu does not modify in a significant manner their affinity to specific receptors, but significantly enhances their efficacy, suggesting the possibility of different tissue-specific responses.

The bell-shaped concentration-response curves for Glu or Glu agonists suggest the existence of two binding sites: a high affinity binding site, operating at low μM concentrations (0.001 – 1 μM) and positively modulating Ca^{2+} influx, and a low affinity binding site effective at greater Glu concentrations (3 – 100 μM) and possibly counteracting the ion entry. Many channel proteins implicated in the process of lymphocyte activation are expressed on lymphocyte surface, with properties in common with 'electrically excitable' cells as neurones or muscle cells (Gardner, 1990). At least three ion channels are postulated to participate in the activation process: a Ca^{2+} permeable channel, a voltage-activated K^+ channel and a Ca^{2+}-activated K^+ channel. Patch-clamp studies have confirmed the absence of voltage-gated Ca^{2+} channels in T-cell membranes (De Coursey *et al*., 1984; Matteson & Deutsch, 1984). A voltage-independent Ca^{2+} channel that may account for the enhanced transmembrane Ca^{2+} flux following stimulation of T lymphocytes by lectins or activating mAb has been described by Kuno *et al*. (1986). We can speculate that this channel protein contains two distinct Glu binding sites, or one binding site with two different conformations, characterized by high and low affinity, responsible for the stimulatory or inhibitory effect on Ca^{2+} entry.

Another possibility is the expression of Glu low affinity binding sites on other ion channels with different functions, such as voltage-activated K^+ channels, that are the predominant ion channels present on human lymphocytes (Matteson & Deutsch, 1984; Cahalan *et al*., 1985), and play an important role in setting the cell resting potential and in controlling lymphocyte activation. On the other hand, Ca^{2+} permeable channels inactivated by cytosolic free Ca^{2+} in an autoregulatory fashion have been described by Gardner (1989); thus the effect of Glu may depend on Glu-induced cytosolic Ca^{2+} fluctuation, not demonstrable by fluorimetric measurements.

Furthermore, exchange mechanisms by Ca^{2+} transporters have also been reported (Wacholtz *et al*., 1992), and a facilitatory or inhibitory Glu recognition site could be present on these proteins.

Finally, glycine has been shown to block agonist-induced $[Ca^{2+}]_i$ increase in Kupffer cells by activating a specific chloride channel in the cell membrane (Ikejima *et al*., 1997), and through an activation of this channel to cause hyperpolarization of the cell membrane and inhibition of lymphocyte proliferation (Stachlewitz *et al*., 2000). In lymphocytes, this chloride channel might be co-gated by glycine and Glu in a manner similar to the NMDA receptor channel in the CNS (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988).

Glu-induced potentiation showed pharmacological properties compatible with ionotropic Glu receptors present on neuronal cells (Watkins & Evans, 1981; Collingridge & Lester, 1989; Dingledine *et al*., 1999): the prototype antagonists acting on these receptors block the Glu-evoked effects on lymphocytes in a concentration-dependent manner. It is interesting to note that a complete antagonism of Glu effects was achieved only by incubating the cells with NBQX, a non-NMDA receptor antagonist, or with KYNA, a broad spectrum receptor antagonist, while competitive or non-

competitive NMDA receptor antagonists did not abolish Glu potentiation. Thus, we can hypothesize that binding sites for NMDA and non-NMDA agonists are present on the same cell surface protein, but it is sufficient to block non-NMDA receptors to antagonise completely Glu effects, suggesting a greater efficacy or a higher expression of this receptor type. In support of this possibility is the comparison of the maximum effects obtained by exposing the cells to NMDA or (S)-AMPA and KA, at the same concentrations. The smaller effects we observed with NMDA agonists are not due to the lack of glycine into the experimental buffer, as demonstrated by the same results obtained in the presence of glycine (1–100 μM).

The functional activation of resting blood lymphocytes is a multi-step process. Initially the cells are stimulated by antigen binding to specific membrane receptors (Goverman *et al*., 1986), subsequently transduction signals are generated that induce morphologic changes, proliferation and/or differentiation and the acquisition of immunological function (Crabtree, 1989). Increases in intracellular Ca^{2+} play a prominent role in the activation of resting lymphocytes and provides an excellent tool for cell functional studies. However, Ca^{2+} is a necessary, but not sufficient, signal for full cellular activation, and we considered to evaluate also the cell proliferation response to the mitogen PHA, as a conclusive index of lymphocyte activation. Preliminary studies in our laboratory have shown that, in a medium devoid of Glu or glutamine, lymphocytes completely lose their ability to proliferate. These cells need the presence of minimum 0.1 mM Glu in the culture medium for normal cell cycle progression. For this reason we could not use for these experiments Glu concentrations similar to those used for Ca^{2+} measurements, thus, it was necessary to consider the standard culture medium concentration (0.1 mM) as a basal value (see Methods). The physiological blood Glu concentration is elevated (0.5 mM), even if below the basal culture medium level, but it is subject to rapid changes in relation to diet or to many pathological conditions. Our results demonstrate that high Glu concentrations inhibit in a concentration-dependent manner lymphocyte proliferation in response to PHA. Evidence for a causal relationship between Glu concentrations and immunological reactivity has already been reported (Droge *et al*., 1988). However, this effect has been correlated with Glu inhibition of cystine transport into the cells (Bannai & Kitamura, 1980; Sato *et al*., 1999a). Taken together, our data support a receptor-mediated mechanism for Glu modulation of lymphocyte functions. The immunological relevance of this reduction remains to be clarified, but we can presume that either elevated plasma Glu concentrations (as measured in AIDS, neoplastic diseases, hepatic encephalopathy), or large Glu release in extracellular spaces of CNS (as present in multiple sclerosis, stroke, AD) (Olney, 1990; Lipton & Rosenberg, 1994), may impair lymphocyte functions and have important secondary immunological consequences.

In conclusion, our studies indicate the presence of ionotropic Glu receptors on human peripheral blood lymphocytes. These receptors seem to be pharmacologically similar to those expressed on neurones and are functionally operating as modulators of cell activation. Therefore, our data lend further support to the participation of Glu in a neural-immune signalling network.

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