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Rodent lymphocytes express functionally active glutamate receptors

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Abstract

RT-PCR demonstrated that ionotropic (iGluR NR1) and metabotropic (mGluR Group III) glutamate receptors are expressed in rodent lymphocytes. Flow cytometry showed that activation of iGluR NR1 by *N*-methyl-D-aspartate (NMDA) increased intracellular free calcium and reactive oxygen species (ROS) levels and activated caspase-3. The latter effect was attenuated by the NMDA antagonist, 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801), by the antioxidant *N*-acetylcysteine and by cyclosporin A. Treatment with L-2-amino-4-phosphonobutyric acid (L-AP4), an mGluR Group III agonist, increased lymphocyte ROS levels but to a lower extent than did NMDA. Activation of lymphocytes with both NMDA and L-AP4 caused a synergistic increase in ROS levels and induced necrotic cellular death without elevating the caspase-3 activation observed in the presence of NMDA alone. These results show that lymphocyte iGluR NR1 and mGluR Group III receptors may be involved in controlling rodent lymphocyte functions and longevity as they regulate events in cell proliferation, maturation, and death.

Keywords: Ionotropic glutamate receptors; Metabotropic glutamate receptors; Rodent lymphocytes; *N*-Methyl-D-aspartate; Reactive oxygen species; Caspase-3; Calcium; *N*-Acetylcysteine

Glutamate plays a key role in neurotransmission across the fast excitatory synapses in brain [1]. The role of glutamate in neurotransmission is complex as neurons can express a number of membrane receptors, which can bind glutamate and modulate a variety of intracellular events. Currently, two principal groups of glutamate receptors are recognized, which are designated as either ionotropic [1,2] or metabotropic [3,4]. The ionotropic glutamate receptors (iGluR) behave as ion-channels and are involved in Na^+ (and Ca^{2+}) movement across the cell membrane, and these have been subdivided into the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptor sub-types, based on the ability of these compounds to act as agonists for physiological effects of these various receptors that can be triggered by glutamate binding [1]. The metabotropic glutamate receptors (mGluR) are members of the G-protein coupled receptor family, and glutamate binding causes G-protein recruitment [4]. This event causes changes in intracellular metabolism by subsequent activation of different signal transduction pathways involving synthesis of IP_3 and elevation of intracellular calcium ion levels (mGluR Group I) or decreases in

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cAMP levels (mGluR Groups II and III) [3,5]. Furthermore, recent evidence indicates that the iGluR and mGluR receptors may function either synergistically or antagonistically in neurons as one type of metabotropic receptor (mGlu Group III) enhances the excitotoxicity induced by activation of NMDA-activated iGluR receptors whereas another (mGluR Group I) attenuates the excitotoxic effect of NMDA [4,6,7].

In addition to the existence of glutamate receptors in neurons, it has become clear during the last decade that a variety of non-neuronal cells also contain iGluR and/or mGluR receptors [8,9]. In the case of lymphocytes, which are critical cells involved in the immune response, initial findings were published in 1997 [10], showing that human lymphocytes can bind glutamate with a very high affinity ($K_a = 2.36 \times 10^{-7}$ M) and that the mGluR antagonist, quisqualate, prevented such binding. Later on, it was found that exposure of lymphocytes to NMDA resulted in intracellular concentration increases in calcium ions [11] and reactive oxygen species (ROS) [12], but no information was obtained concerning the functional role of these events. Additionally, during preparation of this paper, a publication appeared [13] reporting the presence and possible functions of inducible, and constitutive mGluR Group I sub-types (mGlu1R and mGlu5R, respectively) in human lymphocytes.

In order to further define the expression and function of glutamate receptors in lymphocytes, we have investigated the properties of expressed glutamate receptors in rodent lymphocytes. The results of this work show that rodent lymphocytes express only the NMDA-activated iGluR1 and a single sub-type (Group III) of mGluR receptors. The functionality of the iGluR1 receptor was demonstrated as its activation resulted in increases in intracellular calcium and ROS levels and in activation of caspase-3 activity. Stimulation of the mGluR Group III alone did not affect calcium levels and increased ROS levels not as pronouncedly as NMDA did, but simultaneous action of NMDA and L-2-amino-4-phosphonobutyric acid (L-AP4), an mGluR Group III agonist [3], had a synergistic excitotoxic effect, with increases in both ROS levels, and necrotic cell death without activation of caspase-3 activity.

Materials and methods

Cell preparation. Lymphocytes were prepared by a standard method [14] from freshly obtained heparinized (50 U/ml) blood from Wistar rats, grey Shinshilla rabbits, and ICR mice by centrifugation (30 min at 600*g*) on LymphoSep medium. The opalescent lymphocyte ring was carefully collected by capillary pipette and diluted with Hanks' solution to a cell concentration corresponding to 5×10^6 cells/ml. Suspensions of the cells were then used immediately for experiments.

RT-PCR procedures. The published procedure for the RT-PCR protocol [15] was modified as follows. Total mRNA isolation from lymphocytes was performed with the Qiagen RNeasy Mini kit (Qiagen GmbH, Germany) with an on-column DNA digestion step, and the resultant mRNA was diluted in 40 μl RNAse-free water. The RNA concentration was determined at 260 nm using an Ultrospec 1100 Pro spectrophotometer. First strand cDNA was prepared with the Promega M-MLV RT kit (Promega, USA). For the RT, 1 μg of total mRNA was mixed with oligo(dT)$_{15}$ primer (1 μg), dNTPs, RNAse inhibitor RNAsin (34 U), M-MLV-RT (200 U), and master buffer and incubated for 1 h at 42 °C. The cDNA concentration was measured at 260 nm.

PCR was performed in Tercyc amplifier (DNA Corporation, Russia) with MBI Fermentas (Lithuania) PCR reagents. The PCR solution contained 25 mM $MgCl_2$ (2 μl), 10× Buffer $(NH_4)_2SO_4$

Table 1
PCR primers used for detection of glutamate receptor mRNAs in lymphocytes

Agonist	Receptor type	GenBank Accession No.	Base numbers	Sequence (5′–3′)	Amplimer size (bp)
NMDA	iGluR NRI	X63255	3332–3352	CCACTGATATCACGGGCCCGC	483
			3791–3815	TCAAGTTGCAGAGAGCAGGCGCTGG	
	iGluR NR2	M91561	2161–2185	GCCAACCTGGCTGCCTTCATGATCC	546
			2683–2707	CCATGGCTGCAGCCAGCATGTAGAA	
	iGluR NR3	AF073379	2470–2494	TTGGAGCCTTCATGTGGCCACTCCA	485
			2931–2955	CATCAGGGGTGGCTGGCACGTTGTA	
Kainate	iGluR KA1	Z11715	2035–2060	GCTAACCTAGCCGCCTTTCTGACTGT	532
			2543–2567	CCGGCTGCCAGGACAATGAAGATAC	
	iGluR KA2	NM_012572	2369–2394	CTGGCCATTCTCCAGCTGCAGGAGAA	493
			2843–2862	TGGAAGCGGCGGCACTCCGG	
AMPA	iGluR A	X17184	2214–2242	TTCAGGAGATCTAAAATCGCTGTGTTTGA	475
			2662–2689	TTGTAGCAGAACTCGATTAAGGCAACCA	
LAP-4	mGlu RI	X57569	2446–2469	GAAGCCCAGATTCATGAGCGCTTG	710
			3131–3156	AGGGGTTTGATTACGGCTGTTTGGTT	
	mGlu RII	XM_343470	2145–2167	ACCAAGACCAATCGCATTGCTCG	521
			2641–2666	CACATTCTTCTGTGGCTGGAAAAGGA	
	mGlu RIII	U47331	2702–2719	CATCGTCAAGGCCTCGGGC	467
			3144–3169	ATGGCGTACACAGTACACGTGACCAT	

The upper primer sequence is complementary to the target sequence at the 5′ end of the mRNA and the lower sequence is complementary to the target sequence at the 3′ end.

(2.5 μl), 10 mM dNTPs (0.5 μl), 5 μM primer (20 pmol of each), 1 U/μl *Taq* polymerase (2 μl), and 0.12 μg/μl cDNA template (2 μl). The PCR was started with an initial denaturation (5 min at 95 °C), followed by amplification for 36 cycles using a three-step protocol: 30 s at 94 °C (denaturation), 1 min at 62 or 67 °C (annealing), and 1 min at 72 °C (elongation).

The oligonucleotide primers, purchased from Roth (Germany), were designed to amplify conservative regions of all glutamate receptor gene mRNAs within a single receptor sub-type in order to detect sub-type mRNAs in rat, rabbit, and mouse lymphocytes (Table 1). A maximum of four base mismatches was allowed between primer and target sequences in the three species. The primer sequences were checked with a BLAST search and found to be specific for glutamate receptor gene sequences. Rat GAPDH primers were used as the positive control for all experiments, and negative controls were performed in which the cDNA was omitted and replaced with RNAse-free water or eluted RNA.

Agarose gel electrophoresis. After PCR amplification, 6× Loading Dye Buffer (MBI Fermentas, Lithuania) was added to the samples, and electrophoresis in 1.5% agarose was performed using 20 μl aliquots of samples. Gels were then stained with ethidium bromide, and DNA bands were visualized under UV light and photographed with a Samsung CCD camera.

Dye-coupled flow cytometry. Immediately after preparation, lymphocyte suspensions were loaded, if necessary, with one of the following dyes: CDCF (5-(and 6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate, Molecular Probes, USA) for measurement of ROS [16], Fluo-3M (Molecular Probes, USA) for measurement of intracellular calcium [17], or the synthetic fluorogenic caspase-3 substrate PhiPhiLux (OncoImmunin, USA) for measurement of caspase-3 activity [18]. After incubation of the cells with the dyes for 30–60 min at 37 °C, 0.1 ml aliquots were diluted with 0.9 ml Hanks' solution and then selectively incubated with 3-hydroxyphenylglycine (DHPG; Tocris, USA), L-AP4 (Tocris, USA), 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801; Sigma, USA), and NMDA (Sigma, USA) as indicated in the text. At the end of the incubation period, 10 μl of 1 mM propidium iodide (PI, Sigma, USA) was added for measurement of the proportion of necrotic cells in the population [17], and after 5 min, flow cytometric measurements were made using a Beckman Coulter Epics Altra flow cytometer.

Results

Expression of iGluR and mGluR receptors mRNAs in lymphocytes

Fig. 1 shows the results of RT-PCR analysis of rat lymphocyte mRNA using specific primers for all six sub-types of iGluR and all the three sub-types of mGluR. These results indicate that only the mRNAs for the NMDA-activated iGluR NR1 and the mGluR Group III are transcribed, and in identical analyses (results not shown) performed on lymphocyte RNA from two other rodent species, mouse and rabbit, the same pattern of expression and non-expression of mGluR and iGluR sub-types was seen. In rat and rabbit, the relative amounts of the transcribed iGluR and mGluR mRNAs were found to be similar (respective ratios of 1.3:1 and 0.8:1), but in mouse, there was substantially more of the iGluR in comparison to the mGluR mRNA (relative ratio 3.7:1).

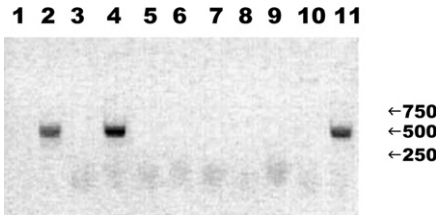

Fig. 1. Detection of glutamate-receptor mRNAs in rat lymphocytes. RT-PCR of total RNA from adult rat lymphocytes was performed as described under Materials and methods using primers for different glutamate receptor types. Anticipated PCR products (~500 bp) were then separated by electrophoresis in a 1.5% agarose gel and visualized by staining with ethidium bromide. Lane identities: 1, negative control; 2, positive control (GAPDH); 3, iGluR NR3; 4, iGluR NR1; 5, iGluR NR2; 6, iGluR KA1; 7, iGluR KA2; 8, iGluR A; 9, mGluR I; 10, mGluR II; and 11, mGluR III. Arrows show the positions of standard DNA markers, with the numbers indicating the base pair size.

These results indicate that only the mRNAs for NMDA-activated iGluR NR1 and mGluR Group III are present in these cells and suggest that only those glutamate receptor proteins are expressed in these cell types, which are translated from these mRNAs. In order to determine if such putative proteins were functionally similar to the same protein receptors expressed in other cell types, further experiments were performed as described below.

Effects of NMDA and L-AP4 on intracellular free calcium levels

NMDA activates ionotropic glutamate receptors and would be expected to increase intracellular calcium ion concentrations in cells. This effect and excitotoxicity (evidenced by the increase in the percentage of necrotic cells in the population) were observed as shown in Table 2, in agreement with other recent results on human T-cells [11]. In contrast, L-AP4 would not be expected to elevate intracellular calcium ion concentrations, and such a result was observed as shown in Table 2, along with the lack of an excitotoxic effect. However, when cells were treated with NMDA and L-AP4 simulta-

Table 2
Effects of NMDA and L-AP4 on lymphocyte intracellular free calcium ion concentration and necrotic cell death

Condition	Fluo-3M fluorescence intensity	% Necrotic cells
Control	2.2 ± 0.2	1.4 ± 0.4
NMDA (0.5 mM)	10.5 ± 0.5	44.7 ± 3.7
L-AP4 (10 μM)	2.4 ± 0.3	1.8 ± 0.2
NMDA + L-AP4	11.1 ± 0.3	59.1 ± 5.2

All measurements were performed using flow cytometry after 30 min incubation with the ligands. Free calcium ion concentrations were measured by the fluorescence intensity of Fluo-3M (expressed in arbitrary fluorescence intensity units), and necrotic cells in the samples were detected by staining with propidium iodide. In the experiment with both NMDA and L-AP4 present, respective concentrations of 0.5 mM, and 10 μM were used.

neously, although there was no appreciable increase in intracellular calcium concentration in comparison to that observed with NMDA alone, there was a significantly higher excitotoxic effect (an increased percentage of PI-staining necrotic cells), suggesting that occupancy of both types of ionotropic and metabotropic receptors had a synergistic effect on excitotoxicity, which however was not paralleled by increases in intracellular calcium concentration.

Effects of NMDA and L-AP4 on lymphocyte ROS levels

NMDA elevated ROS levels in lymphocytes in a dose-dependent manner and maximal activation was found to be at approximately 500 μM, similar to that for neurons [1]. Fig. 2 shows the effect of 100 μM NMDA on the mean fluorescence of CDCF pre-loaded cells after activation with NMDA. It is seen that from a 40% to more than a 110% increase in ROS levels occurred in cells from the three different rodent species. Rabbit lymphocytes had the lowest response, and rat cells had the highest response to NMDA. At this concentration of NMDA, there was no appreciable increase in the proportion of PI-stained (necrotic) cells in all three species. Increases in NMDA concentration resulted in progressive accumulation of PI sensitive cells.

When the cells were exposed to 10 μM L-AP4, a concentration sufficient to activate mGluR Group III receptors in brain [3], stimulation of ROS generation was statistically significant but did not reach the level which was found in the case of NMDA-exposed cells (Fig. 3). Surprisingly, simultaneous presence of NMDA and L-AP4 in the incubation medium caused synergistic stimulation of ROS production and pronounced increases in the proportion of PI-stained cells (see Table 2). In other experiments (data not shown), a specific agonist for mGluR Group I receptors, DHPG, had no effect on intracellular ROS levels in these cells in the presence or absence of NMDA when the DHPG concentration used (10 μM) was up to 10 times that of the IC50 for its receptors mGlu1 and mGlu5 [3]. This result is in agreement with the PCR data which indicated that the mRNA for mGluR Group I receptors was not transcribed in rodent lymphocytes.

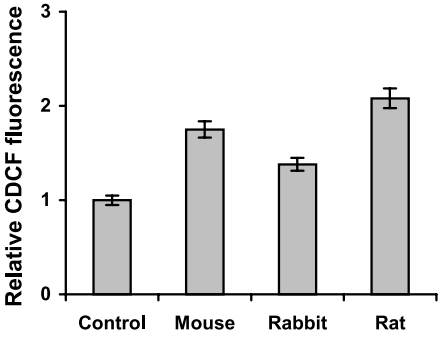

Fig. 2. Effects of NMDA on rodent lymphocyte ROS levels measured. Samples were incubated with 100 μM NMDA for 30 min. The data bars for the species show relative mean CDCF fluorescence intensities based on control cells (not treated with NMDA) having an arbitrary fluorescence intensity of 1.0 U in control samples.

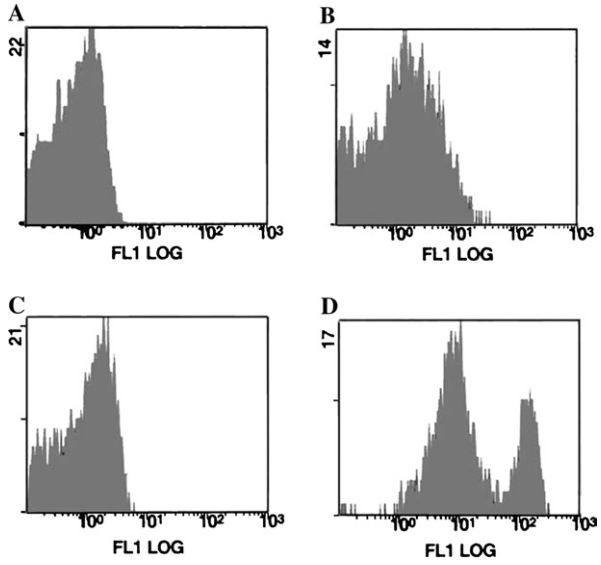

Fig. 3. Effects of NMDA and L-AP4 on ROS production by mouse lymphocytes. The cells were treated for 30 min with NMDA (250 μM) or L-AP4 (10 μM) alone or in combination. A typical experiment is shown with CDCF fluorescence values (FL1 Log) versus cells counted (ordinate). The mean relative CDCF fluorescence values for cells, based on the peak of the control cells (graph A) having an arbitrary fluorescence intensity of 1.0 U, are 3.8 (graph B, NMDA), 1.4 (graph C, L-AP4), and 39.0 (graph D, NMDA plus L-AP4).

Caspase-3 activity in surviving lymphocytes activated by NMDA

Caspase-3 is one of the most important enzymes in cell function as it is involved in stimulation of apoptotic programmed cell death, plasticity, and cell maturation [18–20]. Fig. 4 shows that incubation of mice lymphocytes with 10 μM staurosporine, a compound which leads to caspase-3 activation [21,22], resulted in a significant shift of the cell population along the abscissa to higher fluorescence values, reflecting accumulation of the fluorescent product derived from PhiPhiLux by caspase-3 activity. A similar and even more pronounced effect was found when the cells were incubated with NMDA. Both the staurosporine and NMDA activating effects resulted in movement of the whole cell population along the fluorescence axis, which means that all the cells possessed a similar ability to activate caspase-3 in response to agonist binding at the NMDA receptor. The activating effect of NMDA was inhibited by up to 70% by 1 μM cyclosporin A, a compound which prevents caspase-3 activation by inhibition of cytochrome *c* release from mitochondria [22]. These results demonstrate the activation of this enzyme through spe-

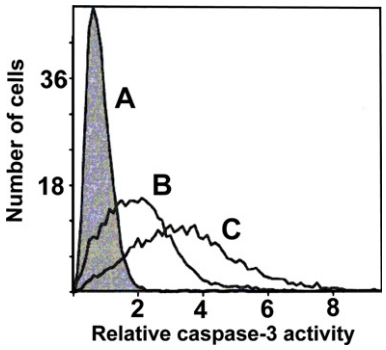

Fig. 4. Activation of caspase-3 activity in mouse lymphocytes by staurosporine and NMDA. Samples were incubated for 4 h in the absence of staurosporine and NMDA (A), in the presence of 10 μM staurosporine (B), and in the presence of 0.5 mM NMDA (C). The figure shows relative fluorescence intensity data for cells based on the peak of the control cells having an arbitrary fluorescence intensity of 1.0 U.

cific glutamate receptors belonging to the iGluR NR1 sub-type.

Specificity of the NMDA effect on caspase-3 activity

As widely recognized in the literature, ROS can function as intracellular messengers whose accumulation in the cell results in the activation of caspase-3 [23]. Accordingly, it was important to determine how specific activation of NMDA receptors related to ROS generation and caspase-3 activation. As seen in Fig. 5, there were comparable relative increases in ROS levels and caspase-3 activity following NMDA activation of the lymphocytes. Similarly, comparable decreases in these levels occurred in the presence of *N*-acetylcysteine, a membrane permeable ROS scavenger [24]. Fig. 5 also shows that simultaneous exposure of cells to NMDA and MK-801, a specific NMDA receptor antagonist [25], suppressed increases in both ROS levels and caspase-3 activation. In other experiments, 10 μM L-AP4 alone had no effect on caspase activity, and the combination of 10 μM L-AP4 and 500 μM NMDA did not exacerbate the activation of caspase-3 caused by 500 μM NMDA alone (results not shown).

Discussion

Our results clearly show that in lymphocytes from three different rodent species, only two different subtypes of glutamate receptors are expressed and functional. One of these is the NMDA-activated ionotropic iGluR NR1 sub-type and the other is the metabotropic mGluR Group III sub-type. Recent studies have established that in human lymphocytes both iGluR [15] and mGluR [13] sub-types were expressed. Different proteins of mGluR Group I (mGlu1R and mGlu5R, respectively) were expressed in constitutive and inducible fashions, although the mGluR Group III sub-type was not expressed [13]. It is possible that such variations in lymphocyte glutamate receptor expression and consequent signaling processes may affect patterns of immunological responsiveness, and it will therefore be important to examine patterns of glutamate receptor expression in lymphocytes of other species.

Our results show that binding of NMDA to the rodent iGluR NR1 receptor elevates intracellular calcium ion levels, and that this can lead to increases in intracellular ROS levels, and activation of intracellular caspase-3. Although we have not identified the sources of calcium in these events, our previous studies on neurons have implicated either intra- or extracellular sources of calcium depending on the mode of neuronal activation [17], and it will be interesting to determine if such a similarity of calcium supplies exists in lymphocytes. Increases in intracellular calcium in other cell types are known to affect ROS levels through effects on a number of enzymes such as mitochondrial monoamine oxidases [17] and cytoplasmic NADPH oxidases [26]. Thus, identification of lymphocyte enzymes which are responsible

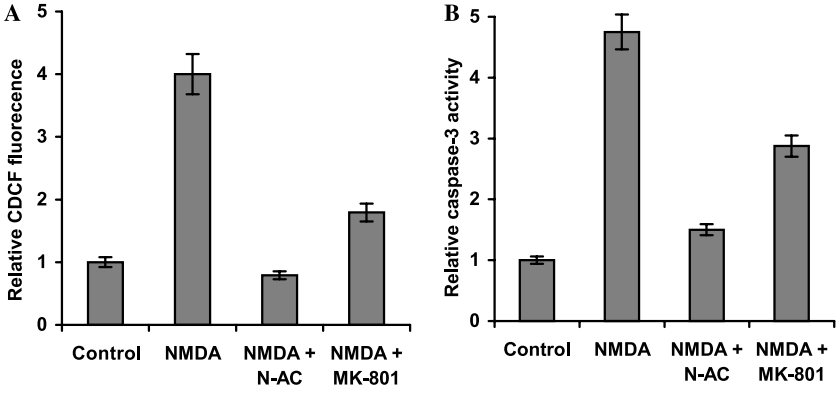

Fig. 5. Comparison of the effects of NMDA, *N*-acetylcysteine, and MK-801 on ROS production (A) and caspase-3 activity (B) in mouse lymphocytes. The ligands were used at the following concentrations: NMDA, 0.5 mM; *N*-acetylcysteine (N-AC), 5 mM; and MK-801, 10 μM. The charts show relative fluorescence intensity data for cells based on the peak of the control cells having an arbitrary fluorescence intensity of 1.0 U.

for increases in ROS levels and ultimately in caspase-3 activation will be important. Because activation of caspase-3 can induce not only apoptosis, but also non-apoptotic cellular events such as proliferation and maturation [19,20,27], agonist binding to lymphocyte glutamate receptors may have quite complex effects on lymphocyte function and longevity.

In the case of the expressed mGluR Group III receptor, binding of its agonist L-AP4 did not cause an increase in intracellular calcium levels or in caspase-3 activity, although it did cause an increase in ROS levels. This means that there must be other, as yet unidentified, mechanisms which trigger increases in ROS levels in response to glutamate binding and cause oxidative damage which leads to necrotic cell death independent of caspase-3 activation. Thus, ROS generated by agonist binding at iGluR or mGluR receptors may induce either apoptotic or necrotic cell death through effects such as cellular compartmentalization, given that ROS can be generated in different intracellular compartments [17,26]. Such compartmentalization may explain why the simultaneous presence of agonists for both iGluR and mGluR receptors causes amplification of ROS-producing reactions, with ROS levels in some cells reaching sufficiently high levels (Fig. 3D) which can lead to necrotic transformation (Table 2). The existence of some lymphocytes within the total cell population which have a greater sensitivity to the effects of the mGluR Group III agonist, even though they all respond similarly to NMDA alone in terms of caspase-3 activation (Fig. 4), may have a number of possible explanations. These include differences within sub-populations of cells in terms of the expression or involvement of different glutamate receptors in intracellular signaling processes.

In conclusion, we have demonstrated the existence of selective expression of glutamate receptors in rodent lymphocytes which can trigger different cellular events involving changes in intracellular calcium ion and ROS levels and caspase-3 activity. This indicates that glutamate may have multiple effects on lymphocyte properties in vivo, and these effects are suggestive of cell-to-cell signaling. Our results therefore offer support for previous ideas concerning the possibility of glutamate receptors involvement in signaling between the neural and immune systems [13,28].

Acknowledgments

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