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NMDA Receptors as a Possible Component of Store-Operated Ca^{2+} Entry in Human T-Lymphocytes

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Abstract—Elevation of intracellular Ca^{2+} in T-lymphocytes as a consequence of T cell antigen receptor activation triggers transcriptional programs resulting in effector cytokine secretion and immune response coordination. Increase of Ca^{2+} concentration in T-lymphocytes follows both the Ins(1,4,5)P_3-dependent release from an intracellular store and subsequent influx from extracellular milieu. Flow cytometry and the fluorescent dye Fluo-4AM have been used to demonstrate that noncompetitive NMDA receptor antagonist (+)-MK801 inhibits Ca^{2+} influx in T cells induced by thapsigargin. Combination of thapsigargin and (+)-MK801 with following incubation does not affect Ca^{2+} mobilization from intracellular stores, while decreased Ca^{2+} entry was observed. Overall data indicate that the ion channel blocker (+)-MK801 is able to inhibit the Ca^{2+} influx and confirm our suggestion about involvement of NMDA receptor in the store-operated Ca^{2+} entry mechanisms in human T-lymphocytes. To identify the signal transduction pathways associated with NMDA receptors in mitogen-stimulated T-lymphocytes, the cells were incubated with (+)-MK801, then activity of key phosphorylated protein kinases of MAP-activated (pERK1/2, pSAPK/JNK, p-p38), Ca^{2+}-dependent (pCaMKII), PI3/Akt-dependent (pGSK-3β), and PKC-activated (pPKCθ) pathways were detected. The data we obtained demonstrate that (+)-MK801 treatment leads to more prominent decrease in Ras-activated protein kinases pERK1/2 and Rac-activated proteins p-p38 and pSAPK/JNK, as compared to DAG-dependent pPKCθ and Ca^{2+}-dependent pCaMKII. These results show that NMDA receptors are mainly involved in regulation of Ras/Rac-dependent signaling in T-lymphocytes.

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Glutamate is the major excitatory neurotransmitter of the central nervous system, which is implicated in many fundamental processes in neurons, such as regulation of neuronal differentiation in development, sustaining survival of mature cells, formation of memory and learning mechanisms, motor coordination, and pathogenesis of neurological and neurodegenerative disorders [1]. At present, glutamate receptors are well characterized and subdivided into three classes of ionotropic (iGluR) and eight classes of metabotropic (mGluR) receptors [2]. Among ionotropic glutamate receptors, the NMDA (*N*-methyl-D-aspartate) subtype is the best studied because of its crucial importance in neuronal function, both in healthy individuals and in pathology. Structurally, the NMDA receptor is a tetramer composed of two subunit types, NR1 and NR2, which form an ion channel that is highly permeable for K^+, Na^+, and Ca^{2+} [3].

In recent years data have appeared on involvement of glutamate and other neurotransmitters in regulation of immune system functions, which is thought to serve the communication between the immune and nervous systems. By analogy with central nervous system, one can suppose that transmitters, when bound with their receptors on the periphery, in particular, with receptors of circulating T cells, are implicated in regulation of their functions, contributing to both local and systemic reactions mediated by T-lymphocytes [4, 5]. To date, expression of both metabotropic and ionotropic receptors is proved on the surface of human and rodent T cells, and the role of these receptors in regulation of key functions of immunocompetent cells is found out. In particular, glutamate receptors are involved in regulation of cytokine

Abbreviations: APC/MHC, antigen-presenting cell association with the main histocompatibility complex; BSS, buffered saline solution; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, diacylglycerol; mAB, monoclonal antibodies; MAP, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; TCR, T cell antigen receptor.

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secretion, polarization of T helper subpopulation, control of cell cycle, proliferation, and apoptosis, change of cell transmembrane potential, modulation of ion channel activities, genes expression, induction of reactive oxygen species production, and integrin-mediated adhesion to glycoproteins of extracellular matrix [6-14]. In our opinion, it is notable that some data are indicative of implication of iGluR and mGluR in regulation of calcium homeostasis in activated T cells.

Engagement of the T cell antigen receptor (TCR) is known to result in Ins(1,4,5)P_3-mediated Ca^{2+} release from intracellular stores followed by its influx from extracellular milieu across calcium channels in the plasma membrane [15]. Among the store-operated pathways of calcium influx into the cell, the most studied to date is highly selective calcium flow via CRAC (calcium release-activated calcium) channels composed of a pore-forming subunit (a member of the Orai transmembrane protein family) and endoplasmic reticulum calcium sensor STIM [16]. Besides, it is shown that in activated T-lymphocytes voltage-gated calcium and potassium channels (Ca_V and $K_V1.3$, respectively) as well as calcium-activated potassium channels (KCa3.1) and non-selective cationic channels TRPM7 (transient receptor potential channels) are also implicated in store-operated calcium entry (SOCE) into T-lymphocytes [17].

Despite obvious progress in decoding the mechanisms of Ca^{2+} entry during TCR-mediated T-lymphocyte activation, identity of other channels involved in controlling of calcium influx into T cells remains unclear. The route of Ca^{2+} entry into T-lymphocytes appears to be important since the channel through which Ca^{2+} enters can distinguish the Ca^{2+} signal (i.e. amplitude and duration of Ca^{2+} rise). This, in turn, determines downstream intracellular events including the activation of certain signaling pathways, distinct transcription factors and hence, gene expression resulting in specificity of cell response [18, 19].

By analogy with neurons, in which NMDA receptors, along with voltage-gated L-type calcium channels, mainly contribute to Ca^{2+} entry into the cells, we suppose the NMDA receptors to be probable candidates mediating SOCE in T-lymphocytes. All these considerations stimulate us to evaluate experimentally the possible role of NMDA receptors in mechanisms of store-operated Ca^{2+} entry in human T-lymphocytes. Another interest is to elucidate the signaling pathways are associated with NMDA receptors in these cells upon TCR-mediated activation.

MATERIALS AND METHODS

Chemicals. The following chemicals, buffers, media, and antibodies were used: (+)-MK801 ((5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) (Tocris, USA); thapsigargin and BSA (Sigma, USA); Fluo-4AM, pluronic acid, HEPES, and Alamar Blue (Invitrogen, USA); Ficoll and RPMI-1640 (PanEco, Russia); SDS and Immun-Star™ Chemiluminescent Protein Detection Systems (BioRad, USA); CD45-ECD, anti-CD3 mABs (clone OCT3) (eBioscience, USA); anti-CD28 mAB (clone CD28.2) (BD Pharmingen, USA); pERK44/42, pSAPK/JNK, p-p38, pPKCθ, pCaMKII, pGSK-3β, and β-tubulin (Cell Signaling, USA); anti-mouse or anti-rabbit IgG secondary antibody conjugates with Alexa Fluor 488 (Invitrogen). The chemicals used for preparation of buffers were of extra or chemical purity grade.

Isolation of lymphocytes. Lymphocytes were isolated from the peripheral blood of healthy volunteers. All donors gave informed consent to the study. The study was approved by the Local Ethics Committee at the Institute of Biochemistry and Genetics of Ufa Research Center, Russian Academy of Sciences. Mononuclear fraction was isolated by the standard method of gradient centrifugation [20]. Fractions of granulocytes and monocytes were eliminated by adhesion on 48-well plates containing 10% BSA for 1 h at 37°C. The fraction of non-leukocytic cells was determined from parameters of side scattering of light (SS) and fluorescence of conjugated CD45-ECD antibodies by flow cytometry. This fraction of cells did not exceed 5 ± 2%.

Lymphocyte culture. Isolated mononuclear cells were washed twice (10 min, 110*g*) with serum-free RPMI-1640 containing 0.3 mg/ml of L-glutamine and 50 μg/ml of gentamycin sulfate and then incubated for 2 h in 48-well plates (Costar, USA) (37°C, 5% CO_2) in complete RPMI-1640 medium supplemented with 10% fetal calf serum. The culture density was $2 \cdot 10^6$ cells/ml. The monoclonal antibodies (mAB) against CD3 receptor (aCD3 mABs, 2.5 μg/ml) and monoclonal antibodies against CD28 (aCD28 mABs, 1.25 μg/ml) were used as mitogens.

Calcium flux study. Freshly isolated lymphocytes were washed in calcium-free buffered saline (BSS), pH 7.4, containing (mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $NaHPO_4$ (24.9), glucose (11), and Hepes (5). Then the cell suspension was diluted up to $2 \cdot 10^6$ cells/ml in buffer containing 2 μM of Ca^{2+}-indicator Fluo-4AM and 0.02% pluronic acid and incubated for 20 min at 30°C. Then cells were washed twice in buffer without dye, resuspended in 450 μl of BSS, and incubated for 20 min in the dark at room temperature. The fluorescence background of the dye (from 0 to 200 arbitrary units) and its change with addition of Ca^{2+} were scanned using the Cytomics FC 500 flow cytometer (Beckman Coulter, USA) on the FL1 channel (505-545 nm) in linear scale. The rate of measurement was 600-700 events per second. Collected data were analyzed using kinetic module of FCS Express 4 software (*De novo* software, USA) to obtain the mean values of fluorescence intensity at indicated time intervals. Statistical data analysis was carried out using the Wilcoxon *t*-test (n = 5-6, $p < 0.05$).

The level of intracellular protein kinases was estimated by flow cytometry following immunocytochemical staining. After incubation with NMDA receptor antagonist and/or mitogens lymphocytes ($2 \cdot 10^6$ cells/ml) were fixed with 4% paraformaldehyde in phosphate buffer for 10 min at 37°C, permeabilized with 90% ethanol for 30 min on ice, blocked for 10 min in buffer containing 0.5% BSA, and incubated for 30 min at room temperature with primary antibodies against pERK44/42 (1 : 200), pSAPK/JNK (1 : 200), p-p38 (1 : 200), pPKCθ (1 : 200), pCaMKII (1 : 200), or pGSK-3β (1 : 200). Then the cells were washed and stained with secondary antibody IgG-Alexa Fluor 488. In each experiment, an isotype control specific to the certain proteins was used. The data were processed using the FCS Express 4 software (*De novo* Software). Statistical analysis was performed using the Wilcoxon *t*-test ($n = 5$, $p < 0.05$).

Immunoblotting. Protein extracts were subjected to 6-16% gradient SDS-PAGE. Immunoblotting and hybridization with antibodies against pSAPK/JNK (Thr183/Tyr182) and β-tubulin was carried out following the manufacturer's protocol. Specific signals were detected using Immun-Star™ Chemiluminescent Protein Detection System Kit. Densitometry and quantification were performed using TotalLab.2.0 software (USA). The paired Student's *t*-test ($n = 3$, $p < 0.05$) was used for statistical analysis.

RESULTS AND DISCUSSION

Earlier it has been demonstrated that glutamate and its synthetic analogs – selective agonists of ionotropic and metabotropic receptors – stimulate Ca^{2+} level elevation upon the mitogen stimulation of T cells, whereas iGluR and mGluR antagonists inhibit this effect [6, 9, 11-13]. In particular, NMDA considerably (by 50%) stimulated the increase of $[Ca^{2+}]_i$ in phytohemagglutinin-activated human T-lymphocytes in presence of glutamate, whereas both competitive (D-AP5) and noncompetitive ((+)-MK801) antagonists of NMDA receptors inhibited the increase of $[Ca^{2+}]_i$ under the same conditions [12]. It is worth noting that the investigation of the role of NMDA receptors in maintenance of calcium homeostasis in T cells was not continued. However, the question seems important whether the NMDA receptors are implicated in the mechanisms of store-operated entry of Ca^{2+} (SOCE) in this cell type. To test this hypothesis, several series of experiments were carried out. T-lymphocytes were incubated in calcium-free conditions in presence of thapsigargin (inhibitor of SERCA Ca^{2+}-ATPases), then the medium was replaced with one containing Ca^{2+}, and changes in the intracellular Ca^{2+} level were monitored by flow cytometry. As follows from Fig. 1a, a characteristic biphasic signal is observed in the presence of thapsigargin: transient and insignificant elevation of $[Ca^{2+}]_i$ reproducing Ca^{2+} mobilization from the stores is followed by a much longer phase reflecting Ca^{2+} entry from the extracellular space. To clarify the possible involvement of NMDA receptors in mechanisms of store operated Ca^{2+} entry, NMDA receptor channels blocker (+)-MK801 (100 μM) was added 5 min before the replacement with Ca^{2+}-containing medium (1 mM). One can see from Fig. 1b that preincubation with (+)-MK801 reduces the thapsigargin-induced Ca^{2+} entry into lymphocytes by 19 ± 6% (table). Treatment of cells with (+)-MK801 in presence of Ca^{2+} also suppresses the phase of Ca^{2+} entry into lymphocytes (Fig. 1c). In case of (+)-MK801 incubation with thapsigargin (Fig. 1d) the antagonist has no effect on the phase of Ca^{2+} mobilization from intracellular stores, and Ca^{2+} entry from extracellular space is inhibited by 23 ± 5%. Taken together, these data suggest that (+)-MK801 has blocking effect on Ca^{2+}entry and support our hypothesis on implication of NMDA receptors in mech-

Change in intracellular Ca^{2+} level in T-lymphocytes

Experimental conditions	Group	Fluorescence maximum (MFLI), arbitrary units	Ca^{2+} level*
Pretreatment (5 min), Fig. 1b ($n = 6$)	without TG	108.92 ± 9.66	1.55 ± 0.14
	TG + BSS	259.05 ± 19.25	3.92 ± 0.29
	TG + (+)-MK801	214.54 ± 14.71	3.19 ± 0.22**
Simultaneous addition, Fig. 1c ($n = 5$)	without TG	90.92 ± 8.02	1.35 ± 0.11
	TG + BSS	301.55 ± 15.3	3.48 ± 0.16
	TG + (+)-MK801	276.12 ± 14.38	3.07 ± 0.17**
Co-incubation with TG, Fig. 1d ($n = 6$)	without TG	123.94 ± 10.32	1.48 ± 0.13
	TG + BSS	453.24 ± 22.71	5.47 ± 0.29
	TG + (+)-MK801	370.77 ± 19.03	4.20 ± 0.22**

Note: MFLI, mean fluorescence linear intensity; TG, thapsigargin; BSS, buffered saline solution.

* Maximum MLFI/background MFLI ratio (mean ± standard deviation).

** Significance of the difference was estimated using the Wilcoxon *t*-test ($n = 5$-6, $p < 0.05$).

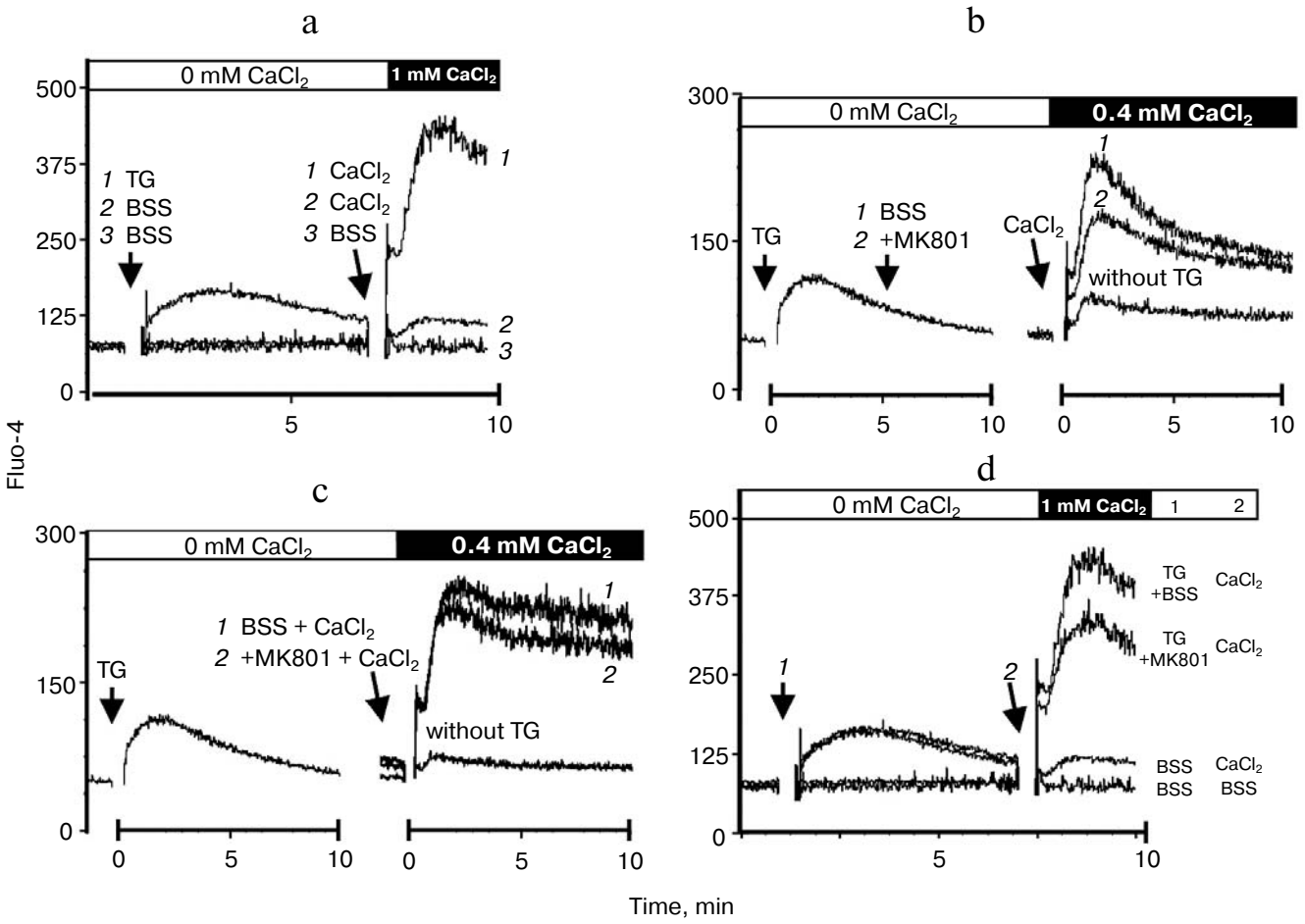

Fig. 1. NMDA receptors mediate the store-operated Ca^{2+} entry in human T-lymphocytes. Arrows indicate the time of addition of stimuli. a) Increase in the level of intracellular Ca^{2+} was initiated by pretreatment with (*1*) thapsigargin (TG) (0.4 μM, 5 min) in calcium-free medium followed by its replacement with medium containing 1 mM Ca^{2+}; (*2*, *3*) TG-free and both TG-free and calcium-free controls, respectively. b, c) (+)-MK801 suppresses the TG-induced Ca^{2+} entry into human T-lymphocytes. b) Cells were stimulated with TG (0.4 μM, 5 min) and, following incubation with (+)-MK801 (100 μM, 5 min), the background fluorescence level was detected (30 sec) and 0.4 mM Ca^{2+} was added (*2*). In controls an equal volume of BSS was added instead of antagonist (*1*) ($n = 6$). c) Cells were stimulated with TG (0.4 μM, 10 min), and then (+)-MK801 (100 μM) was added simultaneously with 0.4 mM Ca^{2+}(*2*). In controls only medium containing 0.2 mM Ca^{2+} was added (*1*) ($n = 5$). d) Cells were stained in calcium-free conditions, then the background fluorescence was detected (30 sec), followed by addition of TG (0.4 μM) and (+)-MK801 (100 μM) and – after 5 min – 1 mM Ca^{2+}. In controls an equal volume of buffer was added instead of antagonist ($n = 6$). TG-free and both TG- and calcium-free controls are also shown.

anisms of store-dependent Ca^{2+} entry in human T-lymphocytes. It is likely that less expressed decrease of Ca^{2+} level in the presence of antagonist takes place because NMDA receptors only mediate some portion of calcium influx induced by Ca^{2+} mobilization from intracellular stores. It could be supposed this ionotropic glutamate receptor subtype is selectively activated in distinct T cell subpopulations.

It is worth noting that no data are presently known about glutamate receptors' implication in mechanisms of store-operated calcium entry in non-excitable cells, particularly lymphocytes. At present there are a few studies elucidating the participation of glutamate receptors in SOCE in other cell's type. It has been shown that activation of NMDA receptors stimulates mobilization of Ca^{2+} from endoplasmic reticulum, and calcium entry through the NMDA receptors contributes to refill the intracellular calcium stores in neurons [21]. Moreover, the NMDA receptor-dependent activation of calcium flow via store-operated channels (SOC) required for continued maintenance of $[Ca^{2+}]_i$ was found in a culture of pyramidal hippocampal cells [22]. Also, there are data about implication of metabotropic glutamate receptors in store-operated Ca^{2+} entry in neurons [23]. However, mechanisms of NMDA receptor involvement in processes of store-dependent Ca^{2+} entry in different cell types remain unknown.

Generally, signaling mechanisms determining activation of calcium-dependent ion channels of plasma membrane in response to depletion of intracellular Ca^{2+}

stores remain poorly studied, both for excitable and non-excitable cells. Recently, the proteins of STIM family (STIM1 and STIM2), which are calcium sensors in endoplasmic reticulum, and proteins of the Orai family (Orai1, Orai2, and Orai3), which are pore-forming subunits of store-operated calcium channels, were found in T-lymphocytes and other cell types. When stores are depleting, STIM1 undergoes homooligomerization to form small clusters, which are transported to the plasma membrane. This localization is thought to allow oligomerized STIM1 proteins to interact and thus activate various ion channels involved in SOCE [24]. It is proved that in T-lymphocytes STIM1 regulates activity of CRAC channels via interaction between coiled-coil domains of Orai1 and STIM1 [25]. Perhaps members of the STIM protein family can also contribute to signal transduction from depleted calcium stores to NMDA receptors of T cells.

NMDA receptor activation upon TCR stimulation can result from phosphorylation by serine/threonine and tyrosine protein kinases. Wang and Salter have demonstrated that phosphorylation of NMDA receptor subunits NR2A and NR2B by tyrosine kinases of the Src family, particularly Fyn, leads to activation of the ion channel, as

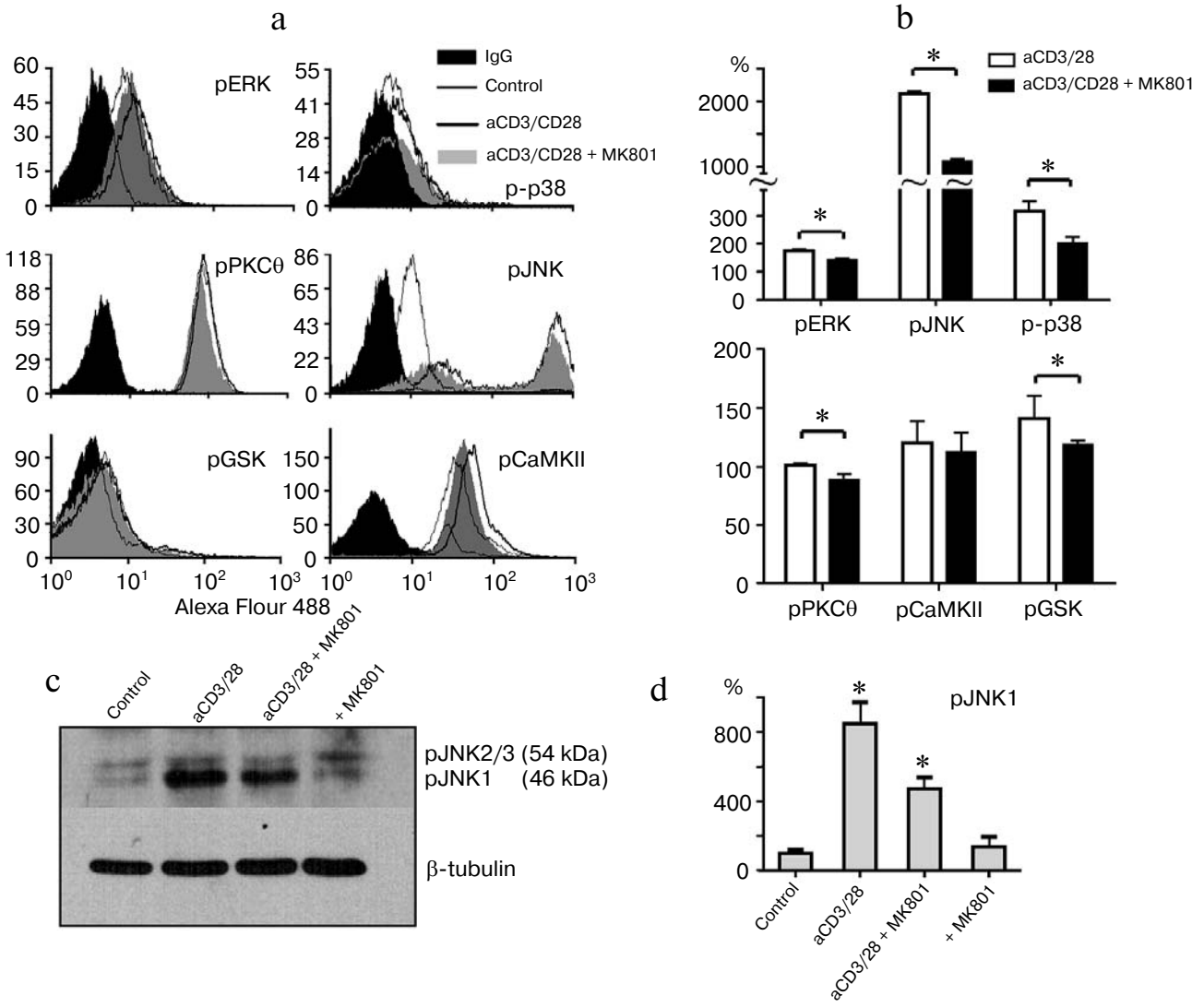

Fig. 2. Effect of NMDA receptor selective antagonist on the activity of key protein kinases participating in Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways in human T-lymphocytes. a) Fluorescence level of corresponding protein kinases was determined by flow cytometry. Freshly isolated T-lymphocytes were incubated with (+)-MK801 for 5 min and activated by aCD3/CD28 (2.5-1.25 μg/ml) during various time intervals. b) Levels of protein kinases under NMDA receptor blockade were analyzed by the fluorescence median. The plots represent ratios of fluorescence mean levels in mitogen-stimulated groups to control ones (mean ± standard deviation). c) Western-blot analysis of pSAPK/JNK level (Thr183/Tyr185) under NMDA receptor blockade in the mitogen-stimulated T cells. T-Lymphocytes were incubated with (+)-MK801 for 5 min and activated with aCD3/CD28 (2.5-1.25 μg/ml) for 2 h. d) Quantification of the pJNK1 level. Normalization performed by β-tubulin. * $p < 0.05$.

is evident from increase in amplitude and duration of Ca^{2+} signal in cultured hippocampal neurons [26]. Moreover, association between neuronal NMDA receptor complex and tyrosine kinase Fyn via the anchor postsynaptic density protein 95 (PSD-95) has been established [27]. Since the Src family tyrosine kinases are involved in signal transduction pathway upon TCR activation by APC/MHC, one can suppose, by the analogy with neurons, participation of these tyrosine kinases in regulation of functional state of the NMDA receptors in T cells under their stimulation with antigen.

As mentioned above, activation of peripheral T cells through the engagement of TCR with MHC-peptide complexes on antigen presenting cells leads to the transcriptional activation of a number of genes that are involved in the coordination of immune response. According to some authors, activation of various calcium channel types is an important mechanism of fine regulation supporting Ca^{2+} at effectively high levels (duration of calcium signal), which is necessary for realization of T cell functions such as proliferation, T helper polarization, cytokine differential secretion, etc. [17]. Moreover, it is ascertained that routes of Ca^{2+} influx and signal cascades associated with distinct ion channels are the crucial factors determining selectivity and specificity of immunocompetent cell response [28]. Note that it remains unknown what signal transduction pathways are coupled with NMDA receptors in lymphocytes, and evidences on the role of NMDA receptors in regulation of T cell functions are very limited and contradictory.

To identify the signal transduction pathways are associated with NMDA receptors in mitogen-activated T-lymphocytes, cells were incubated with antagonist (+)-MK801 then stimulated with aCD3/aCD28 mABs and stained with antibodies against phosphorylated forms of key protein kinases involved in MAP-activated (pERK1/2, pSAPK/JNK, p-p38), Ca^{2+}-dependent (pCaMKII), PI3/Akt-dependent (pGSK-3β), or PKC-activated (pPKCθ) signal pathways. As is evident from the data shown in Fig. 2 (a and b), the pretreatment of mitogen-stimulated cells with the NMDA receptor selective antagonist leads to the decrease in level of phosphorylated forms of all studied protein kinases. We have detected a slight inhibitory effect of (+)-MK801 in mitogen-stimulated lymphocytes on the levels of pPKCθ (by 12%) and pCaMKII (by 7%) and more significant inhibitory effect on the levels of pERK1/2, p-p38, and pGSK-3β (by 34, 114, and 22%, respectively). A prominent (almost twofold) decrease in levels of pJNK/SAPK proteins under the blockade of NMDA receptors is noteworthy. Western-blot analysis (Fig. 2, c and d) has confirmed the obtained data and detected differences in sensitivity of pJNK1 (46 kDa) and pJNK2/3 (54 kDa) to (+)-MK801 treatment. As is evident from Fig. 2 (c and d), the most significant decrease in level of pJNK1 compared to pJNK2/3 likely suggests NMDA-dependent JNK1 preferential activation in lymphocytes. As follows from our data, when (+)-MK801 is applied the decrease in diacylglycerol (DAG)-dependent pPKCθ and Ca^{2+}-dependent protein kinase pCaMKII is not so prominent as compared with Ras-activated protein kinase pERK1/2 and Rac-activated proteins p-p38 and pSAPK/JNK, thus possibly reflecting preferential regulation of Ras-/Rac-dependent signaling pathway by NMDA receptors in T-lymphocytes. Besides, participation of the key protein kinase PI3/Akt-dependent GSK-3β signaling pathway in mechanisms of signal transduction from NMDA receptors in T cells is noteworthy.

As we mentioned above, signaling pathways and mechanisms of their coupling with glutamate receptors in T-lymphocytes are largely uncharacterized at present. Several works have demonstrated participation of mGluR1 in activation of the PLC/Ins(1,4,5)P_3/Ca^{2+} signaling pathway and ERK1/2 kinases in human lymphocytes and in Jurkat cell line, while mGluR5 are involved in stimulation of cAMP-dependent protein kinase A [11, 29]. Activation of neuronal NMDA receptors is known to be accompanied by the induction of Ca^{2+}-, MAP-, and PI3/Akt-dependent signal cascades controlling survival, neuronal plasticity, and neuronal apoptosis [30]. Comparative analysis of literature data and our results suggests that NMDA receptor stimulation is accompanied by the stimulation of signaling pathways that are also activated under the stimulation of T cell receptor in T-lymphocytes. Detailed mechanisms of interaction between ionotropic glutamate NMDA receptors and T cell receptor and the role of NMDA receptors in regulation of immunocompetent cell functions are the subjects of further work.

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