N-methyl-D-aspartate receptors in human erythroid precursor cells and in circulating red blood cells contribute to the intracellular calcium regulation

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Makhro A, Hänggi P, Goede JS, Wang J, Brüggemann A, Gassmann M, Schmugge M, Kaestner L, Speer O, Bogdanova A. N-methyl-D-aspartate receptors in human erythroid precursor cells and in circulating red blood cells contribute to the intracellular calcium regulation. Am J Physiol Cell Physiol 305: C1123–C1138, 2013. First published September 18, 2013; doi:10.1152/ajpcell.00031.2013.—The presence of N-methyl-D-aspartate receptor (NMDAR) was previously shown in rat red blood cells (RBCs) and in a UT-7/Epo human myeloid cell line differentiating into erythroid lineage. Here we have characterized the subunit composition of the NMDAR and monitored its function during human erythropoiesis and in circulating RBCs. Expression of the NMDARs subunits was assessed in erythroid progenitors during ex vivo erythropoiesis and in circulating human RBCs using quantitative PCR and flow cytometry. Receptor activity was monitored using a radiolabeled antagonist binding assay, live imaging of Ca2+ uptake, patch clamp, and monitoring of cell volume changes. The receptor tetramers in erythroid precursor cells are composed of the NR1, NR2A, 2C, 2D, NR3A, and 3B subunits of which the glycine-binding NR3A and 3B and glutamate-binding NR2C and 2D subunits prevailed. Functional receptor is required for survival of erythroid precursors. Circulating RBCs retain a low number of the receptor copies that is higher in young cells compared with mature and senescent RBC populations. In circulating RBCs the receptor activity is controlled by plasma glutamate and glycine. Modulation of the NMDAR activity in RBCs by agonists or antagonists is associated with the alterations in whole cell ion currents. Activation of the receptor results in the transient Ca2+ accumulation, cell shrinkage, and alteration in the intracellular pH, which is associated with the change in hemoglobin oxygen affinity. Thus functional NMDARs are present in erythroid precursor cells and in circulating RBCs. These receptors contribute to intracellular Ca2+ homeostasis and modulate oxygen delivery to peripheral tissues.

NMDA receptors; calcium; red blood cells; erythroid precursors

INTRACELLULAR CALCIUM IS A REGULATOR OF MULTIPLE PROCESSES IN MAMMALIAN RED BLOOD CELLS (RBCs; Ref. 6). Functions of key glycolytic enzymes are calcium sensitive (e.g., Refs. 2, 36, 75). Increase in the intracellular calcium to 1 μM and more causes activation of Ca2+-dependent K+ channels (Gardos channels), loss of KCl from the cells, cell shrinkage, and dehydration (37). Along with the changes in cell volume and cation content, accumulation of Ca2+ was shown to facilitate intercellular adhesion (60). Upregulation of the intraerythrocytic Ca2+ results in activation of calpain and promotes cleavage of cytoskeletal proteins (actin, spectrin, ankyrin, and band 4.1 protein) as well band 3 protein and the plasma membrane Ca2+ pump (PMCA; Ref. 6). Thus the cell membrane becomes destabilized in RBCs upon Ca2+ overload. The changes in rheology associated with cell dehydration and proteolysis of cytoskeletal proteins are followed by the alteration of hemodynamics. Uncontrolled Ca2+ accumulation upon treatment of RBCs with Ca2+ ionophore A23187 was reported to cause phosphatidylserine (PS) exposure to the outer membrane leaflet (16). This process may contribute to enhanced RBC clearance by macrophages in patients with a number of diseases. Apart of PS exposure, high intracellular Ca2+ may promote oxidative stress due to the activation of NADPH oxidase and nitric oxide synthase in its uncoupled mode (20, 51). Whereas extracellular Ca2+ concentration in the plasma reaches 1.8 mM, the intracellular free Ca2+ level is precisely controlled and does not exceed 50–100 nM in healthy human RBCs (64). This impressive transmembrane gradient is maintained by an active Ca2+ extrusion via the PMCA and low passive permeability of the RBC plasma membrane for Ca2+ which is mainly mediated by ion channels (28). PMCA in RBCs has been well characterized, whereas our knowledge on the ion transport systems mediating Ca2+ uptake in human RBCs is rather limited. Among the transport systems contributing to Ca2+ uptake into human RBCs are several classes of cation channels (28). An increase in Ca2+ “leak” and the concomitant elevation of intracellular Ca2+ concentrations are typical for RBCs of patients with phosphofructokinase deficiency (75), thalassemia (7), and sickle cell disease (7, 70). The importance of electrogenic Ca2+ uptake pathway (P_sickle) in promoting sickle cell transformation upon deoxygenation has been acknowledged (8, 70, 72). The molecular identity of the nons elective cation channels, including those mediating P_sickle, remains unknown.

Recently, we have reported the presence of N-methyl-D-aspartate receptors (NMDARs) in the plasma membrane of rat RBCs and in the human erythroid precursor cell (EPC) line UT-7/Epo (38). These receptors are ligand-gated, nonslectric cation channels showing a 10-fold preference for Ca2+ over Na+ or K+ (12). These receptors are comprised of four subunits of which eight different isoforms coded by independent genes have been described (68). Subunits NR1 and NR3A and B are binding glycine or α-serine whereas NR2A, B, C, and D interact with glutamate and its homologues homocysteine, homocysteic acid.
intracellular Ca²⁺ exposure of the cells to antagonists on the contrary reduced the

**DESIGN AND METHODS**

**Human subjects.** Our subject pool consisted of 36 adult subjects (Caucasians, both genders, median age 32.5; range 25–49) with no history of hematological disorders. Five to ten milliliters of venous heparinized blood were obtained after informed, written consent, in accordance with the Declaration of Helsinki, at the Divisions of Hematology of the University Hospital and the Children’s Hospital Zurich as well as at the Medical Faculty, Saarland University, Homburg/Saar, Germany and processed immediately. The Institutional Ethics Board of the University Hospital in Zurich and that of the Canton of Zurich as well as the Ethics committee of the medical association of the Saarland approved the study protocol.

**RBC purification for flow cytometry and quantitative real-time PCR.** Blood was washed three times with 10 ml of PBS containing 2 mM EDTA and separated in a Ficoll-Hypaque gradient (GE Healthcare, Glattbrug, Switzerland) to remove mononuclear cells and platelets. Reticulocytes were then filtered through a leuco-depletion filter (Purecell Neo; Pall, Basel, Switzerland). The leuco-depleted reticulocytes were washed and resuspended in PBS/EDTA. The purity grade of reticulocytes was assessed by an automated blood cell analyzer (Sysmex Digita, Horgen, Switzerland), as well as by flow cytometry for surface expression of CD45 with fluorescence-labeled antibodies (Becton Dickinson, Rotkreuz, Switzerland). In addition, depletion of leukocytes and platelets and enrichment of late EPCs and reticulocytes were analyzed by quantitative real-time PCR (qPCR). The pan-leukocyte marker CD45 was used as a white blood cell (WBC) marker, and mRNAs encoding hemoglobin subunits were chosen to detect late EPCs and reticulocytes.

**Ex vivo hematopoiesis.** EPCs were cultured using a two-phase liquid system as described elsewhere (41, 42). At phase I mononuclear cells were isolated from peripheral blood according to the protocol provided by the GE Healthcare producing Ficoll-Paque PLUS and seeded in StemSpan SFEM Medium supplemented with a cytokine mix supplements CC100 StemSpan (StemCell Technologies, Grenoble, France) and penicillin/streptomycin. Cells were maintained in phase I medium at a density of 0.1–1 × 10⁶ cells/ml for 5 days. Thereafter, nonadherent cells were reseeded in StemSpan SFEM Medium with 2% penicillin/streptomycin, 20 ng/ml SCF, 1 U/ml Epo, 5 ng/ml IL-3, 2 μM dexamethasone, and 1 μM β-estradiol and maintained in it for 6, 12, or 18 days.

**mRNA isolation and qPCR.** Total RNA was purified from cells by the TRIZol/glycogen/osopropanol. GRIN1, GRIN2A, GRIN2C, GRIN2D, GRIN3A, GRIN3B, and CD45 mRNAs, as well as control GAPDH mRNA, were quantified using the TaqMan Gene Expression Kits acquired from Applied Biosystems (Rotkreuz, Switzerland). The following primers were used: Hs02758991_g1 Gapdh: TTCACATCCAACTCAGCCCCCA; Hs00690557_m1 Grin1: TACTTCACCGCCAAGGTCTACCC; Hs00168219_m1 Grin2a: GGAATTGGAAAAAGTGCGGAG; Hs00168220_m1 Grin2b: TGGGAAGGTTGGGAATGAGGAAG; Hs01016626_m1 Grin2c: TGGCCTTTCAAGGGGCTGCTACTACAG; Hs00367969_g1 Grin2d: CAGCTCAAGGCAGGGAGTGCCAGC; Hs00370290_m1 Grin3a: ACATGACCCCAATTTACATCTC; and Hs03687969_m1 Grin3b: GCCTCTIGGCACCTGCTTGCACC.

**Flow cytometry for detection of NMDA abundance and distribution in EPCs.** Staining of EPCs for the NMDAR subunit-specific antibodies was performed as follows. Cells were washed once with working solution (PBS supplemented with 0.1% BSA) and centrifuged for 30 s at 3,000 rpm. The supernatant was discarded and the cells resuspended in working solution and fixed with 0.05% glutaraldehyde solution prepared in PBS for 9 min. Cells were washed twice, supernatant was discarded, and cells were resuspended in Tween solution (working solution supplemented with 0.1% of NaN₃ and 0.1% of Tween20) to permeabilize them. Before the primary antibody was added, the EPCs were washed once and resuspended in working solution.

After incubation with primary antibodies for 15 min in the darkness at room temperature, the cells were washed once and exposed to the allopophocyanin-conjugated secondary antibodies for 15 min in the darkness. The following antibodies were used: IgG1 mouse anti human Anti-CD45 PerCP, clone 2D1 (BD, San Jose, CA) as a marker of WBCs; IgG2a mouse anti-human CD71, clone M-A712 FITC-conjugated (BD) as a marker of reticulocytes; anti-NMDAR3 (NR2C), goat polyclonal IgG, no. sc-1478221; anti-NMDAR4 (NR2D), mouse monoclonal IgG, no. sc-17822; IgG no. sc-51160; and anti-NR3B, goat polyclonal IgG, no. sc-55731. All primary antibodies against the NMDAR subunits were provided by Santa Cruz Biotechnology (Dallas, TX) and used at a final concentration of 0.2 μg/ml. Allopophocyanin-conjugated AffiniPure F(ab’2) Fragment Donkey Anti-Goat IgG, Code 705–136–147, and allopophocyanin-conjugated AffiniPure F(ab’2) Fragment Goat Anti-Mouse IgG, Code 155–136–146 were used as secondary antibodies at 1:625 and 1:2500 dilutions, respectively.

Stained cells were washed once with the working solution supplemented with 1% formaldehyde and resuspended in an appropriated volume of the working solution, and fluorescence intensity was measured using the BD Biosciences FACSCalibur flow cytometer (BD Biosciences). Cells stained with the secondary antibodies alone were used as a negative control. Data were analyzed by using FCS express software (De Novo Software, Los Angeles, CA). All chemicals were provided by Sigma-Aldrich (St. Louis, MO).

**Fractionation of EPCs on Percoll density gradient.** Fractionation of EPCs into light (L), medium (M), and dense (D) fractions was performed in 90% isotonic Percoll solution prepared by mixing a 90-ml aliquot of sterile Percoll (GE Healthcare; density 1.130 g/ml) with 10 ml × 10 PBS (Sigma-Aldrich) and 11 ml × 1 PBS (Sigma-Aldrich). Blood or RBC suspension was filtered on cellulose filter to remove WBCs, platelets were as described elsewhere (5), and 1.0–1.5 ml was overlaid then over 12.5 ml isotonic Percoll and centrifuged using Sorvall RC 5C plus centrifuge equipped with a SM-24 rotor at 4°C for 30 min at 45,000 g. The obtained L, M, and D fractions of RBCs were collected and washed three times with the

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incubation medium containing the following (mM): 145 NaCl, 4 KCl, 10 glucose, 10 Tris HCl, 0.1 EDTA, and 0.1% BSA (pH 7.4 at room temperature). Therefore, the obtained RBCs were used for detection of the number of \(^{3} \text{H}\)MK-801 binding sites or for fluorescence live imaging. The amount of reticulocytes in L, M, and D fractions was 18.1 ± 2.2, 1.5 ± 4, and 0.89 ± 0.08% respectively, with mean reticulocyte counts of 1.9 ± 0.6%. Mean cell volume (MCV) of cells in fractions was 103.6 ± 1.9, 90.9 ± 0.7, and 82.6 ± 1.5 fl for the L, M, and D fraction, respectively, mean MCV being 86.5 ± 1.4 fl

Percoll solutions of lower density (82–86% Percoll) were used to monitor rapid changes in volume occurring within the M fraction containing the majority of mature RBCs in response to the NMDAR stimulation. Final ion composition in Percoll solution was adjusted to 145 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.4 at room temperature), 10 mM glucose, and 2 mM CaCl\(_2\). Control samples were agonists free. Stimulation of the NMDARs was performed by supplementation of Percoll solution with 200 \(\mu\)M of 1:1 NMDA-glycine mixture. Washed RBCs (0.5 ml packed cells per 12 mM of Percoll solution) were added to the Percoll solution, mixed with it and centrifuged for 2 min at 48,000 g at room temperature. Due to the interindividual variability in basal cell density and in the amplitude of responses, Percoll density was slightly adjusted for each of five healthy subjects tested to achieve better separation of the M fraction.

In four out of five donors, the agonists-induced changes in RBC density developed within 2 min and were reversed already after 5 min of stimulation. In one subject the volume changes prevailed even 15 min after the onset of exposure to the agonists.

**NMDAR activity measurements in circulating RBCs.** Changes in the intracellular Ca\(^{2+}\) levels in RBCs caused by exposure to the NMDAR agonists or antagonists were monitored in L, M, and D fractions by means of microfluorescent live imaging using fluo-4 AM (Molecular Probes, Eugene, OR) as a marker (31). Loading of RBCs with the fluorescent dye (1–5 \(\mu\)M) was performed for 30 min at room temperature in the darkness. During loading, the cells were resuspended in the incubation medium supplemented with 0.1% BSA and 2 mM CaCl\(_2\) to a density of ~4 \(\times\) 10\(^{6}\) cells/ml. Thereafter, the cells were placed in an imaging chamber and allowed to settle for 10 min. In experiments in which kinetics of responses of individual cells to the NMDA agonists or the antagonists was monitored over time, the coverslip serving as a bottom of the imaging chamber was coated with polylysine to immobilize the cells. A series of images (bright field and fluorescent images excited at center wavelength of 480 nm with a pump blocker sodium orthovanadate (4 mM). These cells were addressed as to calcium-clamped cells.

Analysis of the obtained images was performed using CellFinder program developed by Dr. M. Makhinya (Computer Vision Lab; ETH Zurich). This software was designed to detect the cell projection area (an indicator of cell volume, the average fluorescent intensity within this area corrected for the background fluorescence, and anisotropy (longest to shortest diameter ratio).

Radiolabeled antagonist binding assay was used as an alternative approach to assess the number of active receptors per cell. \(^{3} \text{H}\)MK-801 is a specific NMDAR antagonist interacting with the channel pore of active receptors exclusively (18). The number of \(^{3} \text{H}\)MK-801 binding sites per cell in the absence or in the presence of a 200-fold excess of nonlabeled MK-801 was assessed for evaluation of specific and nonspecific binding.

Washed RBCs were incubated with \(^{3} \text{H}\)MK-801 (20 Ci/mmol; American Radiolabeled Chemicals) at a concentration of 5 \(\times\) 10\(^{-7}\) mmol/ml (1 h, room temperature) in the presence of 300 \(\mu\)M NMDA and 100 \(\mu\)M Gly. Thereafter the cells were fractioned according to their density on 90% Percoll gradient (45,000 g, 30 min). L, M, and D fractions were collected, washed three times in buffer 1 containing the following (in mM): 145 NaCl, 5 KCl, 10 glucose, 0.1 EDTA, 10 Tris-HCl (pH 7.4 room temperature) supplemented with 0.1% BSA, and lysed. One part of packed RBCs was mixed with 30 parts of lysis buffer containing 25 mM NaH\(_{2}\)PO\(_{4}\) and 1 mM EDTA (pH 7.0 at 0°C) and incubated on ice for 30 min. Thereafter, RBC membranes were precipitated (45,000 g for 30 min at 4°C) and the amount of \(^{3} \text{H}\)MK-801 bound to the membranes was assessed using Packard 1600 TR liquid scintillation analyzer. The number of \(^{3} \text{H}\)MK-801 binding sites per cell was then calculated using the following equation:

\[
N_{\text{NR}} = \frac{N_{\text{cells}}}{A_{\text{sp}}(MK - 801)}
\]

\(N_{\text{NR}}\) is a number of NMDARs per cell, \(A_{\text{sp}}\) denoted activity of bound \(^{3} \text{H}\)MK-801 after nonspecific binding is subtracted [Bq] and \(A_{\text{sp}}\) stands for specific activity of the \(^{3} \text{H}\)MK-801 in [Bq/mmol], \(N_{\text{cells}}\) is the Avogadro constant (6.022 \(\times\) 10\(^{23}\) mol\(^{-1}\)), and \(N_{\text{cells}}\) are the amount of cells in the sample.

Whole cell currents were measured using a NPC-16 Patchliner (Nanion, Munich, Germany). The “pipette” solution contained the following (in mM): 50 KCl, 10 NaCl, 60 KF, 20 EGTA, and 10 HEPES (pH adjusted to 7.2 by KOH), while the bath solution contained the following (in mM): 140 NaCl, 4 KCl, 1 MgCl\(_{2}\), 2 CaCl\(_{2}\), 5 glucose, and 10 HEPES (pH adjusted to 7.4 by NaOH). NMDA, glycine, and MK-801 were added as indicated in the experiments. The pipette resistance was in the range of 3–5 M\(\Omega\), and the seal resistance was between 800 M\(\Omega\) and 5 G\(\Omega\) at a holding potential of \(-40\) mV. Measurements were performed with fresh RBC samples from three healthy donors within 2–3 h after blood withdrawal.

**pH measurements in RBCs and in RBC lysates.** Shifts in the intracellular pH were monitored over 10 min using microfluorescence live imaging. RBCs were washed three times in isotonic buffer containing the following (in mM): 15 NaCl, 5 KCl, 10 glucose, 20 HEPES-imidazole buffer, 0.1 EDTA, and 0.1% BSA. The cells were centrifuged and resuspended in the same buffer supplemented with 2 mM CaCl\(_{2}\) and 2 \(\mu\)M of pH-sensitive fluorescent dye BCECF-AM and incubated for 45 min at 37°C in the darkness to achieve optimal dye loading. Some samples were supplemented with 50 \(\mu\)M of MIA during loading with the dye. The kinetics of the changes in BCECF fluorescence in response to the treatment with 300 \(\mu\)M of 1:1 NMDA-glycine mixture were recorded at 488-nm excitation and 535-nm emission wavelengths using Axiovert 200M fluorescent microscope (Carl Zeiss, Jena, Germany). At least five images were taken before the addition of agonists or antagonists and used to calculate baseline levels of fluorescence. Solvent (incubation medium) addition was performed in control samples.

Calcium was performed using cells resuspended in calcium buffer containing 20–200 nM free Ca\(^{2+}\) (a set of CaCl\(_{2}\)-EGTA mixtures) in the presence of Ca\(^{2+}\) ionophore A23187 (10 \(\mu\)M) and the Ca\(^{2+}\) pump blocker sodium orthovanadate (4 mM). These cells were addressed as to calcium-clamped cells.

Analysis of the obtained images was performed using CellFinder program developed by Dr. M. Makhinya (Computer Vision Lab; ETH Zurich). This software was designed to detect the cell projection area as an indicator of cell volume, the average fluorescent intensity within this area corrected for the background fluorescence, and anisotropy (longest to shortest diameter ratio).

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Results

Expression of the NMDAR subunits in erythroid progenitors during erythropoiesis. The number of functional NMDAR heterotetramers in the immortalized human erythroid cell line UT-7/Epo was estimated to be \(~350,000\) copies per cell \((38)\).

We have then performed characterization of the expression pattern of NMDARs in human EPCs during their differentiation monitored in the course of transformation from pluripotent precursor \((\text{day } 0)\) cells through the proerythroblast \((\text{day } 6)\) and basophilic and polychromatic erythroblast \((\text{day } 12)\) stages to normoblasts and enucleated reticulocytes \((\text{day } 18)\). Differentiation stages were verified morphologically (Fig. 1, A–C) as well as by expression of CD71 (Fig. 2). We have detected transcripts of the genes...
Fig. 2. Changes of protein levels of NMDAR subunits during erythropoiesis. Peripheral blood-derived erythroid cultures were analyzed by flow cytometry on day 6 (d6), 12 (d12), and 18 (d18) after seeding. NMDAR subunit expression at various EPC differentiation states was assessed. Surface expression of CD71 (CD71 FITC) was used as a marker of EPCs in the presented dot blots. The number of cells expressing NMDAR subunits are shown as histograms. Grey plots show the signal obtained from staining with secondary APC-conjugated antibodies alone, and black plots show specific anti-NR-APC signal. The geometric mean fluorescence intensity (MFI) and the fraction of cells showing specific anti-NR-APC signal were analyzed from 60,000 cells each measurement and represented as box plots for the subunits NR2C (A), NR2D (B), NR3A (C), and NR3B (D). Values are represented as box plots of 9 erythroid cultures, each started with cells from a different donor (n = 9).
Table 1. Expression of NMDAR subunits in WBCs

<table>
<thead>
<tr>
<th>Gene</th>
<th>ID*</th>
<th>Protein</th>
<th>ΔCt</th>
<th>ΔΔCt (fold mRNA expression compared with EPCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIN1</td>
<td>2902</td>
<td>NR1</td>
<td>19.8 ± 1.6</td>
<td>7.0</td>
</tr>
<tr>
<td>GRIN2A</td>
<td>2903</td>
<td>NR2A</td>
<td>15.8 ± 1.9</td>
<td>11.2</td>
</tr>
<tr>
<td>GRIN2B</td>
<td>2904</td>
<td>NR2B</td>
<td>18.8 ± 1.4</td>
<td>(Not expressed in EPCs)</td>
</tr>
<tr>
<td>GRIN2C</td>
<td>2905</td>
<td>NR2C</td>
<td>15.6 ± 1.8</td>
<td>0.7</td>
</tr>
<tr>
<td>GRIN2D</td>
<td>2906</td>
<td>NR2D</td>
<td>12.0 ± 0.9</td>
<td>11.0</td>
</tr>
<tr>
<td>GRIN3A</td>
<td>116443</td>
<td>NR3A</td>
<td>18.3 ± 2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>GRIN3B</td>
<td>116444</td>
<td>NR3B</td>
<td>17.3 ± 1.6</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Values are ± SE for 3 cultures, each started with cells from a different donor (n = 3). EPC, erythroid precursor cell. Relative amounts of transcripts of N-methyl-D-aspartate receptor (NMDAR) subunits were detected by TaqMan quantitative PCR. White blood cells (WBCs; predominantly monocytes) were obtained from mononuclear cells in culture and separated from the hematopoietic stem cells (HSC) at day 3 of culturing as "adherent cell fraction." Individual values were normalized to GAPDH (ΔCt), and the individual fold change compared with the median ΔCt values of erythropoiesis at day 6 was calculated (expΔCt, fold change in mRNA expression). Comparison with the values for mRNA expression in HSCs at day 3 was not possible as too few cells were available at that time. GRIN2B expression in HSCs was fixed to 40 cycles. *NCBI gene ID.

GRIN1, GRIN2A, GRIN2C, GRIN2D, GRIN3A, and GRIN3B encoding NMDAR subunits NR1, NR2A, 2C, and 2D and NR3A and 3B in EPC at various stages of erythropoiesis (Fig. 1; Table 1). Relative expression of GRIN1 and GRIN2C mRNA increased substantially from day 6 to day 18 in culture (P < 0.05; Fig. 1E). A tendency to increased expression of GRIN2A, GRIN3A, and GRIN3B mRNA over 18 days in culture was detected but was not statistically significant (Fig. 1E). Maximal expression of GRIN2D mRNA was found in cells after 12 days in culture, whereas in 18-day-old cells the expression levels dropped to the values similar to those detected at day 6 (Fig. 1G). No transcripts of GRIN2B were detected at any differentiation stage. In peripheral RBCs we were not able to detect any GRIN transcripts, indicating that those were eliminated during erythrocyte maturation (data not shown).

Protein levels of subunits NR2C, 2D, 3A, and 3B were measured using flow cytometry (Fig. 2). The number of erythroid cells in culture expressing NMDAR subunits varied between 10 and 30%. The number of erythroid cells expressing NR2C and NR2D increased during the basophilic and polychromatic cell stages and decreased again once cells matured towards normoblasts and reticulocytes (Fig. 2, A and B). However, the number of copies for these subunits was rather stable throughout the culture period as follows from the mean fluorescence intensity levels (Fig. 2, A and B). The subunit NR3A appeared to be expressed by an increasing proportion of cells throughout erythropoiesis; however, its expression decreased slightly (Fig. 2C). The number of cells expressing NR3B remained stable during the erythropoietic culture, whereas its expression increased slightly (Fig. 2D). Specifically during ex vivo differentiation, the GRIN2D levels of the median subunit per cell remained relatively unchanged from day 6 (proerythroblast) with 186,000 (41,000–320,000) to day 12 (basophilic erythroblast) with 140,000 (18,300–540,000) subunits per cell. Towards days 18–24 in culture (polychromatic- and normoblast, and also reticulocytes), expression of the NR2D subunit dropped to 120,000 (4,100–240,000) subunits per cell. Thus the stable expression of GRIN2D mRNA during differentiation from myeloid progenitor towards the basophilic/polychromatic erythroblast was mirrored by a stable expression in GRIN2D protein levels. During differentiation towards erythrocytes the transcripts appear to become degraded, as they are not detected anymore, whereas NR2D protein is retained although at much lower levels (data not shown).

The importance of functional NMDARs in EPCs in the course of differentiation was explored. The kinetics of changes in the intracellular Ca²⁺ levels in EPCs in response to stimulation of the NMDARs with a mixture of NMDA and glycine (300 μM each) in the absence or presence of 50 μM MK-801 were monitored using fluorescence microscopy. Administration of NMDA/glycine to the cells at day 12 resulted in acute increase in the intracellular Ca²⁺ levels as shown in Fig. 3A.

![Fig. 3. NMDAR function in EPCs. A: cells on day 12 were loaded with 3 μM fluo-4 AM for 45 min at 37°C in cell culture medium and thereafter fluorescence recordings were initiated using Axiovert 200M microscope in time-lapse mode at a frequency of one frame in 7 s. Cells were stimulated with 300 μM of NMDA/glycine 1 min after the onset of recordings and the changes in fluorescence followed for 10 min. Some cells were pretreated with 50 μM of MK-801 for 45 min before the imaging procedure. The antagonist was present in the cell culture medium during the imaging. The number of cells analyzed was 542 for control nontreated group (closed squares), 511 for the NMDA/Glycine-stimulated group (grey triangles), and 494 for the cells pretreated with MK-801 before the stimulation with antagonists (open circles). B: cells at day 12 (closed circles) or at day 18 (open circles) were exposed to various concentrations of MK-801 for 24 h and cellular viability was assessed thereafter using flow cytometry (FacsCalibur; BD). Nuclear staining of propidium iodide was used as a marker of cell death.](http://ajpcell.physiology.org/)
Whenever MK-801 was present in the incubation medium before the receptor activation, the response was biphasic. A transient and incomplete Ca\(^{2+}\) accumulation during the first 30 s upon NMDA/glycine addition was followed by a decrease in the intracellular Ca\(^{2+}\) significantly below the levels observed in control nontreated cells. On day 18, EPCs lost their sensitivity to NMDA/glycine and stimulation of the receptors did not result in any acute significant changes in the intracellular calcium along with higher intercellular variability in the intracellular Ca\(^{2+}\) levels and responsiveness to the stimulation (data not shown). The effects of long-term exposure on the EPCs to different concentrations of the pore blocking NMDAR antagonist MK-801 were explored. In line with the data on NMDA/Gly-sensitivity of the intracellular Ca\(^{2+}\) levels, exposure of EPCs at day 12 to 50 \(\mu\text{M}\) MK-801 for 24 h resulted in significant increase of mortality whereas the cells at day 18 were antagonist resistant (Fig. 3B).

NMDAR expression pattern in WBCs. To evaluate the impact of potential contaminations of myeloid progenitors in our culture systems and that of WBCs in our RBCs preparations, we used CD45 as a marker of myeloid and WBCs (45). We were unable to detect myeloid cells in our culture system after day 6. No WBCs were detected in purified RBCs, as shown previously (3). We have assessed the expression GRIN transcripts in adhesive WBCs separated at day 2 from our erythroid culture. These cell preparations contained predominantly monocytes. Expression of GRIN1 in this myeloid cell population exceeded that in erythroid cells at day 6 of the erythropoietic culture by 4.4-fold, that of GRIN2A by 6.5-fold, and that of GRIN2B by 9.8-fold whereas GRIN2C was 50% lower in WBCs than in erythroid progenitors (Table 1). Compared with erythropoietic cultures at day 18, WBCs expressed 0.6-fold less GRIN1, 3.8-fold more GRIN2A, 0.06-fold less GRIN2C, and 10.8-fold more GRIN2D compared with the levels in basophilic and polychromat erythroblasts (Table 1).

These findings provided further evidence for the absence of myeloid cells or WBCs in erythroid cell culture. Expression profiles for NMDAR subunits GRIN1, GRIN2C, and GRIN3A in differentiated erythroid cells differed substantially from those in WBCs. Furthermore, WBCs appeared to express GRIN2B subunit whereas in erythroid cells the GRIN2B transcripts remained below detection level at any time in culture (Table 1).

NMDAR activity and intracellular Ca\(^{2+}\) concentration. In the following set of experiments, the activity of NMDAR was assessed in circulating RBCs. Changes in the intracellular calcium levels in RBCs upon treatment with agonists NMDA, glutamate, and HCA are shown in Fig. 4A. Increase in the intracellular Ca\(^{2+}\) levels, which was induced within minutes of exposure to the agonists, could be blocked by the pore-blocking antagonist interacting with the open receptor channel MK-801 (Fig. 4, A and B). Responses of individual cells to the NMDAR stimulation varied substantially (Fig. 4C). However, despite pronounced intercellular variability, the majority of cells responded to the treatment with the NMDAR agonists with acute Ca\(^{2+}\) uptake.

NMDAR-induced whole cell currents in RBCs. Calcium uptake following activation of the NMDAR was associated with an increase in whole cell currents across the RBC plasma membranes (Fig. 5). Changes in membrane conductance were assessed using automated patch clamp platform Patchliner (Nanion). Similar to the observations obtained using microfluorescence live imaging (Fig. 4C), sensitivity of the cells to agonists and antagonists varied markedly between individual cells. An increase in currents associated with exposure to the NMDA-glycine mixture was observed in 29.4% of cells. Currents induced by the treatment of cells with agonists could be blocked by the treatment of cells with MK-801. Examples of the NMDA-sensitive currents are shown in Fig. 5, A and C, and statistical analysis of the findings in responding cells is given in Fig. 5E. The appearance of the current-voltage (I-V) curves changes when the NMDAR is activated (green line in Fig. 5D) or when it is blocked (blue line in Fig. 5D). To relate the current traces to the other data in the paper the slope of the I-V curves was analyzed in the physiological relevant range between −30 and 0 mV (Fig. 5D). At stimulated NMDAR, the whole cell conductance is significantly increased compared with naïve RBCs (green column vs. red column in Fig. 5E). When the RBCs with activated NMDARs are treated with MK-801 to block NMDAR activity, the whole cell conductance is significantly decreased (green column vs. blue column in Fig. 5E).

NMDAR abundance in RBC fractions of high medium and low density. In the next sets of experiments RBCs were separated into fractions according to their density using Percoll density gradient centrifugation. D, M, and L fractions were isolated as shown in Fig. 6A. The basal activity of the receptor and total number of the functional NMDAR units was measured in each fraction using two independent approaches: radioactive antagonist binding assay and agonists/antagonists-induced changes in the intracellular calcium levels. Basal receptor activity was evaluated as the cells were allowed to interact with \([\text{H}]\text{MK-801}\) in plasma and then fractioned on Percoll gradient. The number of binding sites for radiolabeled antagonist per cell obtained that way reflected the number of NMDARs active in plasma-borne RBCs. The values for the \([\text{H}]\text{MK-801}\) binding to “activated” cells were obtained in the presence of saturating concentrations of NMDA in plasma and hence represented the total number of receptors. As shown in the Fig. 6B, the number of active receptors interacting with \([\text{H}]\text{MK-801}\) was maximal in the L fraction enriched with reticulocytes and young cells (see DESIGN AND METHODS). In this fraction, no further activation of the receptor could be caused by glutamate/NMDA supplementation of plasma. In contrast to that, the receptors in M and D fractions, although significantly lower in number, could be further activated by addition of agonist to plasma (Fig. 6B).

These experimental settings could give rise to artefacts due to the WBCs contamination (average of 5.6, 4.9, and 1.4 per 1,000 cells in the L, M, and D fractions; Ref. 45). To avoid the overestimation of the receptor activity in RBCs, we have used an alternative approach monitoring the agonist-induced Ca\(^{2+}\) uptake in individual RBCs of L, M, and D fractions isolated from blood of three healthy individuals by microfluorescence live imaging. Receptor activity in RBCs of these donors was assessed by \([\text{H}]\text{MK-801}\) binding and presented in the right panel of Fig. 6 (in red for donor 1, in green for donor 2, and in blue for donor 3). The observations obtained by means of microfluorescence imaging were in agreement with the ones generated using radiolabeled antagonist binding assay. Maximal amount of “responding” RBCs of each individual donor was present in the L fraction (Fig. 7A). Maximal amplitude of NMDA-induced Ca\(^{2+}\) uptake was recorded in the least dense
Fig. 4. Changes in the intracellular Ca\(^{2+}\) levels in red blood cells (RBCs) exposed to agonists and antagonist of NMDARs. A: an increase in the intracellular Ca\(^{2+}\) in response to administration of 100 \(\mu\)M NMDA, 100 \(\mu\)M NMDA + 100 \(\mu\)M glycine, 100 \(\mu\)M glutamate, or 50 \(\mu\)M homocysteic acid (HCA) was observed. Data are means of 34–80 single cell recordings ± SE. The orange bar on top of each panel schematically indicates the duration of exposure to the agonists. B: sensitivity of the HCA-induced Ca\(^{2+}\) uptake by RBCs to the NMDAR antagonist MK-801 (50 \(\mu\)M). Pretreatment with antagonist for 10 min abolished the effects of subsequent exposure to HCA. The green trace represents the average Ca\(^{2+}\)-sensitive fluorescent signal recordings for 444 unstimulated control HBA RBCs in Tyrode’s solution. The red trace shows the F/Fo ratio for RBCs stimulated with NMDAR agonist HCA (100 \(\mu\)M) 1 min after the start of the recordings averaged from 776 single cell recordings. Preincubation of the cells with NMDAR antagonist MK-801 (100 \(\mu\)M) administered 10 min before the onset of the recordings did not affect the basal intracellular Ca\(^{2+}\) levels during 20 min of exposure (blue trace, average of 775 individual cell recordings), but effectively inhibited the HCA-sensitive Ca\(^{2+}\) influx, as depicted by the brown trace (average of 742 individual cell recordings). Each curve shows data from 9 independent measurements of 3 healthy donors ± SE. (C) A selection of traces from individual cells. The images above the diagram exemplify the original fluorescent images of cells at the time points indicated by the dotted lines. The false color coding scale is shown next to the original tracks of the changes in fluorescence intensity over time. The colors of traces correspond to those of the circles marking the individual cells in the leftmost image. The tremendous cell-to-cell variation is obvious in both the images and the traces.
fraction of RBCs of donor 2. Transient Ca\(^{2+}\)/H\(^{11001}\) accumulation in RBCs was associated with a transient shrinkage of cells that could be caused by the activation of Gardos channel. Medium density fraction of the donor 2 was also rich in responding RBCs and showing a transient reduction in cell volume in response to the NMDAR stimulation (Fig. 7B, middle).

We used Percoll density gradient to assess the amount of cells changing their volume in response to the NMDAR stimulation in the M fraction to which majority of RBCs belong (Fig. 6A). The density of Percoll solution used in these experiments was adjusted to resolve the changes in the M fraction. As shown in Fig. 7D, most of the cells forming M fraction migrated downwards in Percoll gradient 2 min after the onset of stimulation with 300 \(\mu\)M of 1:1 NMDA/glycine mixture. This increase in RBC density was transient and could not be detected 5 min after the onset of exposure to the NMDAR agonists in four out of five donors. Cells of the fifth donor remained dehydrated even after 15 min of exposure to the agonists. No volume changes were observed in RBCs of all donors suspended in nominally Ca\(^{2+}\)/H\(^{11001}\)-free Percoll solution containing 0.1 mM EDTA supplemented with NMDA and glycine (data not shown).

In fraction D (Fig. 7C), the cells remained largely insensitive to the agonist treatment and showing modest transient increase in the intracellular Ca\(^{2+}\). The obtained data revealed vast interindividual and intercellular variation in the number of functional receptor units and the amplitude of responses to the NMDAR agonists. Among typical morphological characteristics of responding cells are big volume (cell surface projection) and low density as L fraction is enriched with responding cells. Medium fraction also contains some of the responders, whereas D fraction is largely deprived of them.

The impact of NMDAR activation state on Gardos channel function. The following experiments were performed to assess the possible influence of NMDA-sensitive Ca\(^{2+}\) uptake on K\(^{+}\) transport across the RBC membrane. Oubain-resistant chloride-independent K\(^{+}\) (86Rb) influx was assessed at room temperature, as was the case for Ca\(^{2+}\) imaging presented in Fig. 7.
No density fractioning was performed before flux measurements. As shown in Table 2, treatment of RBCs suspended in Ca$^{2+}$-containing but not in nominally Ca$^{2+}$-free incubation medium with 50 μM memantine caused reduction in unidirectional K$^+$ influx. Administration of 100 μM NMDA triggered the activation of clotrimazol-sensitive Ca$^{2+}$-dependent K$^+$ influx. In nonactivated cells, Gardos channels remained quiescent.

Effect of the NMDAR activation on hemoglobin oxygen affinity. The effect of the intracellular Ca$^{2+}$ on oxygen binding to hemoglobin was tested. Intracellular free Ca$^{2+}$ concentration was clamped as cells were treated with Ca$^{2+}$-ionophore A23187, and extracellular concentration of free calcium ions was adjusted to 10–200 nM by using an EGTA-CaCl$_2$ buffer solution. Oxygen dissociation curves were then measured and P$_{O_2}$ required to achieve hemoglobin oxygen saturation of 50%, P$_{S_0}$, was assessed for the calcium-clamped RBCs. Calcium levels in clamped RBCs were assessed using microfluorescence imaging. As shown in Fig. 8, the fluorescent signal intensity of fluo-4 follows linearly the changes in the intracellular Ca$^{2+}$ within the 10- to 200-nM interval (Fig. 8A). Intr erythrocytic Ca$^{2+}$ levels in intact and NMDA-stimulated cells lies within this concentration range (triangle symbols). Oxygen affinity of Ca$^{2+}$-clamped cells drops (P$_{S_0}$ increases from 2.28 ± 0.01 to 4.1 ± 0.27 kPa) as intracellular Ca$^{2+}$ increased from 20 to 200 nM (Fig. 8C). Exposure of RBCs in suspension to 300 μM NMDA-100 μM glycine mixture was also associated with an increase in P$_{S_0}$ (Fig. 8, B and D).

Acute transient RBC shrinkage triggered by exposure to the NMDAR agonists results in transient increase in the intracellular levels of 2,3-DPG and ATP due to the cytosolic water loss. Additional experiments were performed to explore if this factor contributed to a decrease in hemoglobin oxygen affinity. Oxygen dissociation curves were monitored in RBCs suspended in the medium containing 100 mM KCl/50 mM NaCl medium or in the presence of clotrimazol. The effect of NMDA/glycine on P$_{S_0}$ was less pronounced but still preserved in both conditions, (3.04 ± 0.65 kPa in control vs. 3.16 ± 0.04 kPa in depolarizing high KCl medium, P = 0.011).

The effect of NMDAR agonists on the intracellular pH was assessed microscopically using proton-sensitive fluorescent dye BCECF-DA. Treatment of RBCs with NMDA/glycine resulted in a modest transient decrease in pH-dependent BCECF fluorescence indicating transient intracellular acidification (Fig. 8F). The signal we reported was underestimated as shrinkage following the NMDAR activation caused concentration of the fluorescent dye. This acidification reaction monitored in bicarbonate-free medium was insensitive to the inhibitor of Na$^+/H^+$ exchanger methyl isobutyl amiloride applied at the concentration of 50 μM. The ability of Ca$^{2+}$ to cause the proton release from hemoglobin was explored as RBC lysates (Hb concentration of 0.25 μM, equilibrated with ambient air) were exposed to various concentrations of Ca$^{2+}$. Cell lysates prepared in 150 mM NaCl solution showed no changes in pH in response to Ca$^{2+}$ administration. However, dose-dependent acidification was observed if cell lysates were exposed to Ca$^{2+}$.
Fig. 7. Kinetics of Ca\textsuperscript{2+} uptake and volume changes in response to NMDAR activation in L, M, and D fractions. Intracellular calcium levels were assessed in RBCs forming L (A), M (B), and D (C) fractions after their separation on Percoll density gradient using fluorescent live cell imaging. The experiments were performed using blood of subjects 1 (red), 2 (green), and 3 (blue). The values of [\textsuperscript{3}H]MK-801 binding obtained for RBCs of these subjects are highlighted in corresponding colors in Fig. 6. Presented in the left panels are quantification of Fluo-4 fluorescence intensity F upon stimulation with glutamate (300 μM) and glycine (100 μM) normalized to that at time zero in agonist-free medium (F₀). Middle: changes in cell projection area in the bright field images over the period of stimulation with the NMDAR agonists. The numbers of cells used for analysis varied from 13 to 35. Data are presented as mean ± SE. Right: representative fluorescent images at 3 points in time (before, 1, and 6 min after the stimulation) for the cells in L, M, and D fractions obtained from blood of subject 2 (green plots). Kruskal-Wallis test was performed to follow the changes in Ca\textsuperscript{2+} levels and cell surface (a marker of volume) after the receptor activation. *P < 0.05, ** and ***P < 0.01 and ****P < 0.001 compared with time zero. D: shift of RBCs within the M fraction down the Percoll density gradient in response to 2 min of stimulation with 300 μM of 1:1 NMDA/glycine mixture in donors 1, 2, and 3.
in the presence of 2 mM 2,3-DPG (Fig. 9). Exposure of 2,3-DPG alone to Ca\(^{2+}\) did not result in the changes in pH (Fig. 9). Oxygen binding to hemoglobin within RBC lysates was tested in the presence of 2,3-DPG and various concentrations of Ca\(^{2+}\). As shown in Fig. 9 Ca\(^{2+}\)-induced proton release was associated with a dose-dependent rightward shift in oxygen binding curve, the effect observed in Ca\(^{2+}\)-loaded or NMDA-glycine stimulated RBCs (Fig. 8). Similar to that of pH, oxygen binding to hemoglobin in lysates remained calcium-insensitive in the absence of 2,3-DPG (data not shown).

### Table 2. Effects of NMDA and memantine on the unidirectional ouabain-insensitive Cl\(^{-}\)-independent K\(^{+}\) (\(^{86}\)Rb\(^{+}\)) influx in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1.8 mM Ca(^{2+}) (mmol·L(^{-1})·h(^{-1}))</th>
<th>Ca(^{2+})-free (mmol·L(^{-1})·h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0134 ± 0.0017</td>
<td>0.0176 ± 0.0029</td>
</tr>
<tr>
<td>Memantine</td>
<td>0.0056 ± 0.0012*</td>
<td>0.0162 ± 0.0015</td>
</tr>
<tr>
<td>Clotrimazol</td>
<td>0.0158 ± 0.0016</td>
<td>NA</td>
</tr>
<tr>
<td>NMDA</td>
<td>0.0201 ± 0.0017*</td>
<td>0.0160 ± 0.0008</td>
</tr>
<tr>
<td>NMDA-memantine</td>
<td>0.0102 ± 0.0059</td>
<td>0.0168 ± 0.0028</td>
</tr>
<tr>
<td>NMDA-clotrimazol</td>
<td>0.0126 ± 0.0023</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, in one-way ANOVA analysis with Dunnett multiple comparison posttest; NA, not assessed.

### DISCUSSION

Following up on our earlier observations on the presence of NMDARs in rat RBCs and in human myeloid cell line differentiating into erythroid progeny, UT-7/EPO (38), we have demonstrated that NMDAR subunits are expressed in human EPCs. Circulating human RBCs retain a limited number of functional receptor units compared with the EPCs. Mammalian RBCs may therefore be added to the list of cells possessing NMDARs along with neurons and a number of nonneuronal cells and tissues (22, 57). Expression of the GRIN transcripts coding for the NMDAR subunits was changing dynamically during erythropoiesis, as did the protein abundance. NMDARs appear to be essential for survival, particularly at the early stages of differentiation of precursor cells during erythropoiesis. Subunit composition of the erythroid receptors differed substantially between the erythroid progeny and WBCs.

Calcium uptake was reported to be of key importance for promoting differentiation and proliferation of EPCs at the stages of burst-forming units erythroid (BFU-E) colony-forming units erythroid (CFU-E; Refs. 43, 56). Increase in the intracellular Ca\(^{2+}\) is an integral part of signaling pathway activated by binding of erythropoietin to its receptor (44). Our data suggest that glutamate released from megakaryocytes (63)
may contribute to the regulation of Ca\(^{2+}\) levels in EPCs at day 12 corresponding to the stage of basophilic and polychromatoy erythroblasts (Fig. 3A). The number of EPCs possessing NR2C, 2D, and 3A subunits is maximal at this stage (Fig. 2). Accordingly, the cells display high sensitivity to treatment with NMDAR agonists in our ex vivo erythropoietin system (Fig. 3A).

Ca\(^{2+}\) uptake is inhibited and differentiation and survival of EPCs is compromised in calcium-free medium (43, 56). Similar to that, inhibition of Ca\(^{2+}\) uptake by MK-801 in our ex vivo erythropoiesis system resulted in 45.5 ± 12.8% mortality of cells at the stage of basophilic and polychromatoy erythroblasts (Fig. 3B). Cells at later differentiation stages (normoblasts at day 18 and enucleated RBCs) are less or not at all damaged by MK-801.

Transient receptor potential channels TRPC2, 3, and 6 were suggested to mediate Ca\(^{2+}\) currents in EPCs (23, 67). However, observations on the activation of the TRPC channels by the downstream targets of erythropoietin [e.g., phospholipase C (PLC), and protein kinase C (PKC)] were exclusively reported in nonerythroid channel expressing systems (CHO and HEK cells). Similar to TRPCs, NMDARs were shown to be sensitive to the activation of PLC-inositol-3-phosphate kinase signaling cascade and targets of regulatory PKC-induced phosphorylation (39, 73). Thus both types of ligand-gated channels may coexist in membranes of EPCs (63).

Subunit composition of the erythroid NMDARs. From the data shown in this study, we conclude that human erythroid NMDARs are hetero-tetramers composed of NR1, NR2A, 2C, and 2D and NR3A and 3B subunits. Subunit composition of the erythroid NMDARs differs strikingly from that of the neuronal receptors. As follows from our observations, NR2D and NR2C are most abundant NMDAR subunits in EPCs and, most likely, in circulating RBCs. In the mammalian brain, this subunit shows particularly restricted temporal and spatial expression pattern. Very high levels of expression of NR2D in some areas of embryonic rat brain (precocious midline diencephalic structures and medial geniculate, anterovernuclear nucleus, and periaqueductal grey zones) drop down dramatically during postnatal period (49). In adult rodent brain, NR2D is only detected in cerebellum and Golgi structures (9, 47) and is highly expressed in motoneurons of the spinal cord (66). In bone marrow and in megakaryoblasts derived from CD34\(^+\) precursor cells, the NR2D subunit was found along with NR1 and NR2A (19, 24). Among the characteristic features reported for the NR2D/NR1 heterodimers are reduced Mg\(^{2+}\) block, lower ion conductance and slow inactivation (15, 54). However, based on the available data obtained in transfected cells by other groups, one can suggest that the erythroid-specific receptor channels share at least some of these features, such as slow inactivation, since glutamate-binding NR2D subunit is highly abundant in these cells. Calcium accumulations observed upon activation of the receptor activity with glutamate, NMDA, and homocysteic acid that could be blocked by MK-801 (Fig. 4) are in line with this hypothesis (33, 46, 48). However, in erythroid precursors the glycine-binding NR3A and B subunits were highly expressed, whereas the expression of a common NR1 subunit was modest compared with that in the brain (Figs. 1 and 2). These subunits are known as “inhibitory subunits” in the brain as they are glutamate-insensitive but excited by glycine only (35). Currents induced by glycine in NR1/NR3 diheteromers are insensitive to MK-801 or m Randone (58). This is not the case for the erythroid NMDARs, as the receptors respond to the pore-targeting antagonists in both EPCs and circulating RBCs. Thus the receptors are either NR1/NR2 heterodimers or are built up of all three subunit subtypes (NR1/NR2C, D/NR3 heterotetramers), although the existence of the latter has been questioned (69). Any speculations on the electrophysiological characteristics of erythroid NMDARs require verification upon detailed investigation.

Receptor function in circulating RBCs of healthy human subjects. NMDARs retained in circulating RBCs remain functional and keep responding to stimulation with glutamate, HCA, or NMDA as well as to the inhibition by MK-801 (Figs. 3–6). Calcium transport through the receptor is electrically, putting NMDARs in line with other calcium-transporting ion channels (Fig. 4). The number of such ion channels in RBC is rather limited and their molecular identity often remains unknown (28). Among the TRP channels, only TRPC6 is described in RBC (17). Furthermore, there is biochemical and functional evidence for a C\(_{\text{av}}\)2.1 channel (1, 71). There are numerous electrophysiological reports of Ca\(^{2+}\)-permeable channels that can be grouped in two categories: nonselective voltage activated cation channels (NSVAC; e.g., Refs. 4, 29) and receptor-mediated channels (e.g., Refs. 13, 25). Although the \(J-V\) curve for NSVAC in whole cell conductance mode (55) differs from that shown in Fig. 4D, NMDARs and NSVACs have one common property. Both channels share the hysteresis of whole cell current-voltage curves as their opening probability depends on the direction in which membrane potential changes occur (50).

The receptor-activated channels previously described in RBCs share some conductance properties with the channel described in Fig. 5 (13, 25). However, it is premature to make any conclusions on the molecular identity of these channels. Our findings strongly suggest that the currents presented in Fig. 5 are NMDAR mediated. Stimulation of RBCs with NMDA induced an increase in conductance whereas administration of the pore-blocking antagonist suppressed it. Asymmetrical alterations in conductance in response to NMDA treatment...
suggest that it is may be channel mediated. The current measured is a superposition of NMDAR-channel current, currents mediated by other ion channels (e.g., those reported elsewhere; Refs. 11, 62), and a leak current. Channel-mediated components show a slight outward rectification and a positive reversal potential (Fig. 5D, blue I-V curve). Considering the composition of the bath and pipette solutions this could well be chloride channel activity (61). The contribution of the NMDAR channel can be estimated as the difference between putative fully activated channels (Fig. 5D, green trace) and the corresponding inhibited channels (Fig. 5D, blue trace). In the physiological relevant range between -30 and 0 mV, this difference amounts to a conductance of -930 pS (Fig. 5E). The decreased current at negative membrane potentials in the presence of Mg²⁺ in the bath solution is another indication for the NMDAR identity of the channel (15).

So far channel-mediated Ca²⁺ uptake by RBC was always associated with pathophysiological conditions, such as clot formation (30, 74) or Ca²⁺-induced clearance (34). To our knowledge, our study is the first one revealing possible physiological role of controlled transient Ca²⁺ uptake following the activation of NMDARs, namely the regulation of oxygen affinity of hemoglobin (Figs. 8 and 9).

Among endogenous regulators of the erythroid NMDAR activity are plasma-borne glutamate, glycine, d-serine, homocysteine, and HCA. The rodent NMDAR EC₅₀ for glutamate is 96 ± 20 μM (26), being ~10 μM for HCA (53), and ~40 μM for glycine (38, 58). These data were obtained for the receptors in neurons and in artificial expression systems. These values are close to the EC₅₀ for glutamate (88.2 μM) and homocysteic acid (21 μM) reported for the NMDARs in rat RBCs (38). Glutamate concentrations in plasma of healthy human subjects vary substantially depending on physical condition, exercises, gender, diet, and age, making up from 7 ± 1 μM (14) to 255 ± 26 μM (32). Local glutamate and glycine availability in vicinity of RBC membranes may exceed the bulk plasma levels due to high abundance of these amino acids in the cytosol (32). Homocysteine and HCA also contribute to the activation of erythroid NMDARs. Plasma total homocysteine levels ranges from 4.2 to 17.2 μM in healthy subjects up to 470 μM in patients with hyper-homocysteinemia (59).

Transient activation of the NMDARs by glutamate released from the exercising muscle (14) or obtained with meal (27) might also contribute to a transient increase in the intracellular Ca²⁺ promoting thereby oxygen release from hemoglobin (Fig. 8). The exact mechanism of a cross talk between calcium levels in RBCs and hemoglobin oxygen affinity remains to be studied in details.

Shrinkage (Fig. 8; Table 2) results in a rapid transient increase in 2,3-DPG levels in the cytosol, which in turn could stabilize hemoglobin in T state and promote oxygen release (Fig. 7, B and D). However, a shift in oxygen affinity of hemoglobin caused by the stimulation of the NMDARs was preserved in the presence of clotrimazol or in high KCl medium, conditions preventing Ca²⁺-induced KCl and water loss from RBCs. The obtained data reveal that changes in the intracellular pH result from the shifts in protonation of hemoglobin (Fig. 8). The latter result in the 2,3-DPG-dependent alterations in P₅₀ (Figs. 8 and 9). These observations are consistent with earlier reports on an increase in free Ca²⁺ concentration in the cytosol of RBCs undergoing deoxygenation in which hemoglobin gets protonated (65). Conformational changes responsible for these Ca²⁺-induced changes in oxygen affinity of hemoglobin await further characterization.

Taken together, the obtained findings reveal the presence of functional NMDARs in EPCs and, at much lower levels, in circulating RBCs. The abundance and activity of these receptors in plasma membrane of RBCs and their progenitors are proportional to the intracellular Ca²⁺ levels. Among the downstream targets of Ca²⁺ accumulation are cell volume and hemoglobin oxygen affinity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


