Functional NMDA receptors in rat erythrocytes

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¹Institute of Veterinary Physiology and the Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland; ²Institute for Molecular Cell Biology, Medical Faculty, Saarland University, Homburg/Saar, Germany; and ³International Biotechnology Centre, Department of Biochemistry, Moscow State University, Moscow, Russia

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Makhro A, Wang J, Vogel J, Boldyrev AA, Gassmann M, Kaestner L, Bogdanova A. Functional NMDA receptors in rat erythrocytes. Am J Physiol Cell Physiol 298: C1315–C1325, 2010. First published March 31, 2010; doi:10.1152/ajpcell.00407.2009.—N-methyl-D-aspartate (NMDA) receptors are ligand-gated nonselective cation channels mediating fast neuronal transmission and long-term potentiation in the central nervous system. These channels have a 10-fold higher permeability for Ca²⁺ compared with Na⁺ or K⁺ and binding of the agonists (glutamate, homocysteine, homocysteic acid, NMDA) triggers Ca²⁺ uptake. The present study demonstrates the presence of NMDA receptors in rat erythrocytes. The receptors are most abundant in both erythroid precursor cells and immature red blood cells, reticulocytes. Treatment of erythrocytes with NMDA receptor agonists leads to a rapid increase in intracellular Ca²⁺ resulting in a transient shrinkage via Gardos channel activation. Additionally, the exposure of erythrocytes to NMDA receptor agonists causes activation of the nitric oxide (NO) synthase facilitating either NO production in l-arginine-containing medium or superoxide anion (O₂⁻) generation in the absence of l-arginine. Conversely, treatment with an NMDA receptor antagonist MK-80, or the removal of Ca²⁺ from the incubation medium causes suppression of Ca²⁺ accumulation and prevents attendant changes in cell volume and NO/O₂⁻ production. These results suggest that the NMDA receptor activity in circulating erythrocytes is regulated by the plasma concentrations of homocysteine and homocysteic acid. Moreover, receptor hyperactivation may contribute to an increased incidence of thrombosis during hyperhomocysteinemia.

NMDA RECEPTORS (NRs) are ligand-gated nonselective cation channels that mediate fast neuronal transmission and long-term potentiation in the central nervous system (CNS) (4). Glutamate is a primary regulator of activity of the NRs in the brain. This specific class of glutamate receptors plays an essential role in regard to memory, cognition, sensation, and motor control. Itako contributes to the glutamate excitotoxicity that is involved with numerous neurological disorders including stroke, schizophrenia, Alzheimer’s disease, and Parkinson’s disease (39). Recently, NR expression has been reported to exist also outside the CNS in a number of peripheral tissues such as the lungs, heart, kidney, liver, spleen, bone, vascular endothelium, and lymphoid cells (7, 11, 18, 19, 29, 32). The physiological role of NRs in nonneuronal tissues remains largely unclear.

The nature of NR agonist(s) in control of receptor function in nonneuronal tissues is a matter of debate. Glutamate concentration in the synaptic cleft of the brain reaches millimolar levels, whereas the circulating plasma concentrations of glutamate in healthy human subjects ranges from 14 to 70 μM (13, 47). These concentrations are below the 100 μM threshold recognized as the IC50 for the receptor activation in neurons (26). Glutamate, thus, is most likely not a major contributor to the regulation of the NRs in blood cells in healthy humans. In addition to glutamate, homocysteine (HC), and homocysteic acid (HCA) have also been shown to function as NR agonists in mammals. Total plasma HC concentration in healthy subjects is ~7–10 μM, which can increase to 22.3 ± 12.6 μM in response to normal aging (42). Pathological increases of HC have also been observed as a result of either a hereditary predisposition affecting methionine metabolism or conditions of folate or vitamin B12 deficiency (16). For instance, in hyperhomocysteinemic patients HC has been observed to reach as high as 476 μM (43). Both HC and HCA possess a high affinity to bind to NRs such that even a modest increase in plasma concentrations may result in activation of the receptors (6).

As previously mentioned, NRs are nonselective ligand-gated cation channels; however, the selectivity of the NR channels for Ca²⁺ exceeds that for Na⁺ 10-fold and thus ligand-mediated activation of the receptors typically results in an induction of transient inward Ca²⁺ current. Consequently, both activation and inactivation of NRs, irrespective of host tissue, are linked to the local changes in intracellular Ca²⁺ levels. Such changes in intracellular Ca²⁺ have been reported to function in assisting the proliferation of the vascular smooth muscle cells and aortic endothelia cells in response to HC- or HCA-mediated NR activation (11, 46). Megakaryoblasts initiate differentiation and reduction in pro-platelet formation in response to NMDA exposure, whereas proliferation rate and survival remain unaltered (25).

Lymphocytes and thrombocytes are among the blood cells expressing NRs (7, 17). To the best of our knowledge no studies have been performed to assess the presence of functional NRs in erythrocytes, although interaction of noncompetitive and competitive NR antagonists [³H]MK-801 and [³H]CGS-19755, respectively, with red blood cells (RBCs) has previously been reported (36). Furthermore, dehydration of erythrocytes was reported in patents with hyperhomocysteinemia (34). In this study, with the use of rat erythrocytes, we have demonstrated the presence of functional NRs in RBCs and investigated their distribution pattern in addition to their role in regulation of cell volume, osmotic resistance, endothelial nitric oxide synthase (eNOS, NOS3) activity, and cellular redox state.

MATERIALS AND METHODS

Cell isolation and handling. For all experiments we used RBCs from adult male Wistar rats (200–300 g). The animals were purchased

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from Eleveage Janvier (Le-Genest-Saint-Ile, France) and kept on commercial rodent chow in the sterile laboratory animal facilities at the Institute of Veterinary Physiology. Animal handling and experimentation were reviewed, approved, and carried out in accordance with the Swiss animal protection laws and institutional guidelines. Blood samples were withdrawn from the caudal vena cava into heparinized syringes, and erythrocytes were isolated via centrifugation at 1,000 g for 5 min. The buffy coat and plasma were discarded while the remaining RBCs were washed three times with an incubation medium of the following composition (in mM): 145 NaCl, 5 KCl, 1 CaCl2, 0.15 MgCl2, 15 glucose, and 10 Tris-HCl, pH 7.4. Packed cells were then resuspended in the incubation medium supplemented with 0.1% BSA to a hematocrit of 40%.

UT-7/Epo cells were cultured in α-MEM supplemented with 20% fetal calf serum and 3 U/ml human recombinant erythropoietin (Eprex, Janssen-Cilag, Neuss, Germany). Bone marrow cells were harvested from the femur bone. Cells were suspended, filtered through a 200-nm mesh, concentrated by centrifugation at 2,000 g for 5 min, and used for either [3H]MK-801 binding experiments or immunoblotting.

Cerebellar tissue was isolated from rat pups at postnatal day 10 (n = 5) and used for immunoblotting. Cerebellar granule cells were isolated from rat pups at postnatal day 10 as described elsewhere (40) and used for [3H]MK-801 binding experiments.

Erythrocyte cytoskeleton-free membrane preparation. Packed RBCs (about 500 μl) were hemolyzed in 2 ml of ice-cold lysis buffer containing (in mM) 10 Tris-HCl (pH 7.4), 1 EDTA, 0.1 PMSF, 10 Na2HPO4, 10 NaF, plus 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Membranes were pelleted at 47,000 g for 20 min at 4°C. To enrich the membrane fraction for the NR protein, the obtained ghosts were deprived of most of the cytoskeletal proteins as described elsewhere (23). Protein concentration in the obtained smooth membrane samples was determined by using BCA Protein Assay (Pierce; Rockford, IL) with BSA as standard.

Radiolabeling with MK-801. A radioactivelabeling assay was used to detect the number of putative NRs in erythrocytes, UT-7/Epo cells, and cerebellar granule cells. The respective cells were washed in PBS and incubated with 5 nM [3H]MK-801 (20 Ci/mmol American Radiolabeled Chemicals) per 10^6 cells. The unspecific binding was thereafter using the following equation:

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

where \( N_{NR} \) is a number of NRs, \( A_{cells} \) is activity of the MK-801 bound to the cells in [Bq], \( A_{sp} \) is a specific activity of the [3H]MK-801 in [Bq/mmol], \( N_A \) is the Avogadro number (6.022 \times 10^{23} \text{ mol}^{-1}) and \( N_{cells} \) are the number of cells in the sample.

Western blot analysis. For Western blot analysis cells were washed two times in PBS (2,000 g, 5 min) and lysed in Laemmli buffer. Membrane proteins were first separated on the 7.5% SDS-PAGE (500 μg protein/lane) and then transferred to Protran BA83 nitrocellulose membranes. Even protein transfer was controlled by Poncova red staining. Membranes were then blocked for 1 h at room temperature followed by an overnight incubation at 4°C with primary antibodies against the NR1 subunit of NRs (dilution 1:1,000; I–564 fragment, Novus Biologicals) dissolved in TBS containing 5% milk. The membranes were then washed three times in TBST and incubated for 1 h at room temperature with suitable horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution; antimouse, Jackson Immunoresearch Laboratories, West Grove, PA). The enhanced chemiluminescent detection Western blotting system (FujiFilm LAS-3000 System, FUJIFILM Life Science) was used for detection and quantification of protein. Actin served as a loading control.

Immunohistochemistry and flow cytometry. To assess the NR levels in the membranes of reticulocytes and mature erythrocytes, staining for both the NR1 and NR2 subunits of the NR and for the transferrin receptor (TrR) as a marker of reticulocytes was performed. For fluorescent imaging the cells were suspended in the incubation medium at hematocrit of 0.4% and incubated for 60 min on ice with a cocktail of the following primary antibodies: mouse monoclonal anti-NMDA NR1 Pan antibody (1:100 dilution, Novus Biologicals), rabbit polyclonal anti-NMDA NR2 antibody (1:100 dilution; Affinity BioReagents), and rat monoclonal anti-transferrin receptor antibodies (1:100 dilution; Abcam). Thereafter, the cells were washed from the primary antibodies (centrifugation for 5 min at 500 g at 10°C) and incubated for an additional 60 min on ice with the corresponding secondary antibodies: anti-rabbit Cy3-conjugated, and anti-mouse Cy5-conjugated, and anti-rat Cy2-conjugated, all in dilution 1:50. After one wash of the secondary antibodies was completed, the specific staining was assessed microscopically (Zeiss Axiorex 200M). Flow cytometry was used to assess the NR expression in RBC population. Erythrocytes in suspension were treated with the mouse monoclonal anti-NR1 antibody (dilution 1:50; Novus Biologicals) for 60 min on ice and thereafter with the secondary anti-mouse Cy-5 conjugated antibody (1:50 dilution) and fluo-4 (final concentration 10 μM) for further 30 min at room temperature. Cells treated with only secondary antibodies was used as a negative control.

Unidirectional K+ (86Rb) influx measurements. Unidirectional influx rates for K+ were assessed by using 86Rb as a tracer. Unidirectional residual (ouabain-resistant Cl−-independent) K+ influx was detected in erythrocytes incubated at hematocrit of 5–8% in the chloride-free medium containing (mM) 145 NaCH3SO4, 5 KCH3SO4, 0.15 MgSO4, 1 Ca-glucuronate (when not Ca2+ free), 10 glucose, 10 sucrose, and 10 HEPES-Tris, pH 7.4 at room temperature. Incubation medium was always supplemented with 1 mM ouabain to block active K+ influx and with 100 μM l-arginine. Various amounts of NMDA, HCA or glutamate, 1 μM clorotimazole, or 50 μmol MK-801 were added to some of the samples. Cells were preincubated for 15 min to achieve full inhibition of the Na-K-ATPase and allow agonists and inhibitors of the NRs and the blocker of calcium channels, clorotimazole, to bind to their targets. Influx was then initiated and measured by addition of 86Rb. Aliquots of the RBC suspension (400 μl) were collected 10, 20, 30, 45, and 60 min after the onset of incubation with the tracer and washed from the extracellular 86Rb with ice-cold washing medium containing 100 mM Mg(NO3)2 and 10 mM imidazole buffered with HNO3 to the pH 7.4 when on ice. After washing was completed, the cell pellet was lysed in 5% trichloroacetic acid (TCA), and the amount of 86Rb accumulated in erythrocytes was assessed in deproteinized supernatant and normalized to the amount of the radioactive tracer in the incubation medium. Unidirectional K+ influx was then calculated from the slope of the linear part of the radioactive tracer uptake plot.

Intracellular calcium content monitoring. Live cell imaging was performed to monitor intracellular Ca2+ kinetics in individual cells treated with NMDA. Cells were loaded with fluo-4 AM (Molecular Probes) at a concentration of 5 μM for 1 h at 37°C. Then cells were washed in Tyrode solution containing (mM) 135 NaCl, 5.4 KCl, 1.5 CaCl2, and 10 HEPES. The pH was adjusted to 7.35 by using NaOH. Cells were plated on poly-L-lysine (Sigma)-coated coverslips in Tyrode solution, and then the cell sedimentation and dye de-esterification were allowed to occur. Fluorescence was finally measured on an inverted microscope (TE2000, Nikon) equipped with a ×100 Plan Apo 1.4 objective. Attached to the microscope was a video imaging setup (TIll Photonics) consisting of a monochromator (Polychrome IV), a camera (Imago), and the control hard- and software (TILL-vision). Cells were observed at an excitation of 480 nm and images were collected every 5 s. A 505-nm long-pass dichroic mirror separated the emission light from the
excitation light, and a 535/40 bandpass filter was used to further improve the image quality. A local perfusion system was utilized to quickly exchange solutions in the field of view and to apply the agonist NMDA at a concentration of 100 μM. Images were processed in ImageJ (Wayne Rasband, National Institute of Mental Health) and traces handled by Igor Pro software (WaveMetrics).

Osmotic resistance and hemolysis. The effect of agonists and antagonists of the NR on osmotic resistance was assessed acutely by preincubated cells with 1 mM of NMDA or HCA for 1 min at room temperature in the incubation medium. Thereafter, 3 μl of packed cells were added to the cuvette equipped with a magnetic stirrer containing 2 ml of 0.9% NaCl solution (final cell number ~10^7 cells/2 ml). A bolus of distilled water was added to reduce the osmolarity from 300 to 130 mosM. Optical density was monitored at 630 nm every 0.25 s after the initiation of hypoosmotic stress to assess hemolysis. A detailed description of this method may be found elsewhere (48).

Cellular fragility was determined by monitoring shear stress-induced hemolysis. The cells were suspended in either Ca2+-containing or Ca2+-free medium at hematocrit of 40% and incubated for 1 h at 37°C in an Eppendorff Thermomixer (900 rpm shaking speed) in the presence and absence of 100 μM NMDA and/or 100 μM MK-801. Hemolysis was assessed at the end of the incubation as the amount of hemoglobin released into the medium. After centrifugation (5,000 g for 10 min at 4°C), supernatant was collected and hemoglobin concentration was determined spectrophotometrically using Drabkin reagent.

Morphological changes of RBCs exposed to the NR agonist. Erythrocytes were suspended in the incubation medium at a ratio 1:200, allowed to settle on the imaging chamber bottom, and pretreated with 10 μM clotrimazole or 5 mM Na3VO4 for 15 min at room temperature. Thereafter, HCA at a final concentration of 1 mM was added just before the measurement, and time course of the morphological alterations was followed.

Reduced and oxidized glutathione measurement. Cellular reduced (GSH) and oxidized (GSSG) glutathione content was determined by using Ellman’s reagent as described elsewhere (44). After incubation in the presence or absence of 100 μM NMDA, 100 μM HCA and/or 50 μM MK-801 in 1-arginine-free or 1-arginine-containing medium for 60 min, 100-μl aliquots of erythrocytes suspension (40% hematocrit) were mixed with 900 μl of 5% TCA, and the obtained lysates were centrifuged for 10 min at 16,000 g to pellet denatured proteins. Formation of the colored complex of GSH with the Ellman’s reagent was assessed spectrophotometrically at 412 nm.

Nitrite and nitrate assessment. Erythrocytes in suspension (40% hematocrit) or whole blood were incubated with 100 μM of NMDA, or HCA, or 50 μM MK-801 for 60 min at 25°C in presence or in absence of 200 μM L-arginine or NO3-nitro-L-arginine methyl ester (L-NAME). After the incubation nitrite levels were assessed in the incubation medium or plasma by using a chemiluminescence detector CLD 88 (Eco Medics AG, Switzerland) as described elsewhere (33).

Statistical analysis. All data are mean values of at least 5 independent experiments and are presented as means ± SE when not stated specially. The comparison between the experimental groups was performed by using a normality test followed by the two-tailed Student’s t-test for paired or unpaired samples (GraphPad InStat.V3.05). The level of statistical significance was set at P < 0.05, P < 0.01, or p < 0.001. Fitting of the data obtained for the dose dependence of the HCA and glutamate effects on the residual K+ influx was performed by using SigmaPlot 8.0 nonlinear regression module (100 iteration steps). The equation chosen for fitting was y = Y0 + ax/(b + x), assuming pseudo-single binding site kinetics for the agonist-receptor interaction.

RESULTS

Presence of NMDA receptor in mature RBCs and bone marrow. The presence of NRs in rat erythrocytes and in erythroid precursor cells has been addressed by using several independent techniques including: immunoblotting, immunohistochemistry, flow cytometry, and radiolabeled antagonist binding assay. Double staining of the bone marrow-derived precursor cells with the antibodies against CD36, a marker of erythroid lineage, and against the NR1 subunit of the NRs revealed the presence of cells expressing both CD36 and the NR (Fig. 1A). These observations were confirmed via immunoblotting (Fig. 1B). NRs were expressed in rat bone marrow and UT-7/Epo human erythroleukemic cell line and could also be detected in the erythrocyte membrane fraction. The size of NR1 subunit (~120 kDa) in erythroid cells was similar to that in rat cerebellum (Fig. 1B). The number of receptor copies per cell was assessed from the binding of the radiolabeled NR antagonist [3H]MK-801 covalently interacting with the receptor unit (a tetramer composed of two NR1 and two NR2 subunits) at a 1:1 stoichiometry. Binding studies revealed the presence of 3.5 × 10^5 copies/cell in UT-7/Epo cells and 8 × 10^6 copies/cell in dispersed cerebellar granule cells, whereas 8 ± 1.4 binding sites are retained in circulating erythrocytes when equal binding of the antagonist to all cells in the population was assumed. This assumption proved to be false when the distribution of NR (NR1 and NR2 subunits) was assessed in the RBC population with immunocytochemistry and flow cytometry. As depicted in Fig. 1C, very few cells contained a high number of NR1 and NR2 subunits. Most of these receptor-possessing cells showed positive staining for the reticulocytes marker TrR. Some cells retained high number of NRs, whereas TrRs was already lost. Figure 2 depicts the scatter plots for RBCs stained with only secondary Cy5-conjugated anti-mouse antibody used as a negative control (Fig. 2A), and the cells were loaded with fluo-4 (Y-axis) and exposed to the monoclonal mouse anti-NR1 antibodies (X-axis) followed by the above-mentioned secondary antibodies (Fig. 2B). As can be seen from the Fig. 2B a low number (2.37 ± 0.32%, N = 5) of cells appearing in the G1 quadrant are characterized by high numbers of the NR copies and high intracellular Ca2+ levels. The amount of these receptor-enriched cells could be underestimated as they were found to be more susceptible to hemolysis under conditions of shear stress and could have been lost during centrifugation and the passage through the cytomter (see below). It varied from 0.5 and 4% in different animals. Many more cells in the population residing in G2 quadrant at the scatter plot contained significantly less copies of the receptor and low intracellular Ca2+ levels (Fig. 2B).

Function of the NRs. NRs are nonselective cation channels that are more sensitive to transport Ca2+ into neuronal cells upon activation. High intracellular Ca2+ levels in RBCs containing the highest number of NR copies, as indicated in Fig. 2B, suggests that the NRs in erythrocyte membrane are functional and mediate basal Ca2+ influx into the cells even in the absence of the agonists in the incubation medium. Treatment of the cells with NMDA dramatically facilitated Ca2+ accumulation in some but not all cells. Kinetics of the NMDA-induced Ca2+ entry into single rat erythrocytes was assessed with live imaging. In agreement with flow cytometry data (Fig. 2B), live imaging revealed presence of two subpopulation of RBCs: I) “responders,” in which the addition of 100 μM NMDA

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Nonspecific increase in the residual $K^+$ influx (IC50) was estimated by fitting the respective curves and determined about 30% of maximal specific activation was already observed at 50 and $10^{-6}$ M without an effect (Fig. 4A). Although the exact estimation of the IC50 value for HCA and 88.2 ± 0.1 μM glutamate (Fig. 4A). Only the exact estimation of the IC50 value for NMDA could not have possibly been determined, since it was observed in the whole erythrocyte population treated with NMDA, although the separation of cells into “responders” and “nonresponders” subpopulations revealed that changes in $Ca^{2+}$ were much more pronounced and restricted to the limited number of cells.

Treatment of the cells with HCA, glutamate, or NMDA caused a dose-dependent increase in a unidirectional, residual (ouabain-resistant, chloride-independent) $K^+$ influx, which was measured by using $^{86}$Rb as a tracer for $K^+$ influx. The NMDA-sensitivity of the $K^+$ influx was lost in the presence of MK-801, an antagonist of NRs (Fig. 5A). NMDA-mediated upregulation in $K^+$ influx was clearly secondary to the $Ca^{2+}$ uptake as it was suppressed in $Ca^{2+}$-free medium (Fig. 5B). This finding along with sensitivity of NMDA-induced fluxes to clotrimazole, a Gardos channel blocker, suggests that entry of $Ca^{2+}$ into the cells through the NR facilitates the opening of the Gardos channel.

Cell volume control, osmotic resistance, and morphological alterations. Activation of Gardos channels is known to have a profound effect on cell volume. The $Ca^{2+}$ uptake kinetics (Fig. 3) suggests that NMDA-induced cellular shrinkage occurs within the first several minutes of treatment. The corresponding alterations in cellular morphology were observed in response to HCA treatment in some cells within the first 80 s of exposure to the NR agonist; however, this effect was not ubiquitous among all cells (Fig. 6A). The respective alterations in morphology were completely reversed within 180 s of cellular exposure to HCA alone but not during the simultaneous treatment of HCA and the $Ca^{2+}$ pump inhibitor $Na_3VO_4$. Vanadate treatment did, however, increase the number of responding cells suggesting that the low $Ca^{2+}$ levels in erythrocytes with few NRs (Fig. 2B) was maintained due to the efficiency of the $Ca^{2+}$ pump. Activation of the Gardos channels was involved in the temporary morphological changes as treatment of the cells with clotrimazole almost entirely suppressed the HCA-induced cupping and echinocytosis (Fig. 6A).
An acute osmotic resistance test also indicated that both HCA and NMDA result in RBC shrinkage thus making the cells more resistant to osmotic hemolysis (Fig. 6B). Hemolysis rate was assessed via the observation of hemoglobin liberation into the medium during the first 60 s of cells exposure to hypoosmotic shock.

Cellular shrinkage is known to trigger a regulatory volume increase response (RVI) mediated by the volume-sensitive Na/H exchanger or Na-K-2Cl cotransporter in rat erythrocytes (37). We have also observed the late RVI response in rat erythrocytes treated with NMDA or HCA. Swelling of cells treated with NR agonists for 1 h was monitored and water content was seen to increase from 65.8 ± 1.0% in control to 66.7 ± 0.6% in cells exposed to 100 µM HCA and 67.2 ± 1.7% after treatment with 100 µM NMDA at 37°C. The fact that long-term treatment with the NR agonists made the cells more susceptible to the shear stress-induced hemolysis (Fig. 6C) could also be explained as a result of secondary swelling. This agonist-induced hemolysis could be completely abolished by treating erythrocytes with MK-801 and significantly reduced through the use of a nominally Ca²⁺-free medium.

NO synthase activation and glutathione oxidation. Function of the eNOS present in the red cell membrane of rodents and humans (27, 33) is Ca²⁺ dependent (45). Treatment of the whole blood with MK-801 resulted in a dose-dependent inhibition of NO₂⁻/NO₃⁻ accumulation in plasma, whereas exposure to NMDA facilitated de novo NO production (Fig 7A). These observations were confirmed using erythrocytes suspension...
The NRs were not homogeneously distributed in the erythrocyte population and were most prevalent in reticulocytes (Fig. 1C). Although the number of receptor copies per reticulocyte were high compared with mature and senescent red cells, they were 3500-fold lower than that in UT-7(Epo) cell line suggesting that receptor levels are dramatically reduced during the late stages of differentiation of the erythroid precursors. Moreover, reduction in numbers has also been shown for other receptor types, some of which (e.g., TrR) are used as a marker of reticulocyte count. Although not all cells lacking TrR were also deprived of NR (Fig. 1C) suggesting that the NR is not present solely in reticulocytes and thus cannot be used as marker for them. Existence of erythrocytes with particularly high or low levels of NRs may also be observed by using logical inhibitors that are more selective and well-characterized. In erythrocytes MK-801 irreversibly inhibits the receptor-mediated ion transport and downstream processes at micromolar concentrations used in electrophysiological studies on brain slices (e.g., 49).

The physiological role of the NR receptor may only be fully understood once we know the abundance of the receptor present in erythrocytes as well as the mechanisms regulating the function of the receptor as well as the nature of agonists and antagonists. Whereas some speculations may be made on the nature of agonists, mechanisms in control over the NRs abundance in red cell membrane remains unknown. Plasma glutamate concentration in healthy subjects (14 to 47 μM; 3, 13, 47) are significantly lower than that measured in the synaptic cleft where it reaches several millimoles (14). Our data indicate that glutamate binding to the NRs in rat brain and erythrocytes share similar IC50 of 96 ± 2 μM (26) and 88.2 ± 0.01 μM (Fig. 4A), respectively. Plasma HC and HCA concentrations reported in healthy subjects range between 8 and 15 μM (24). These values may rise substantially (up to 400 μM) as a result of folate or vitamin B12 deficiency, pregnancy, aging, or as a result of a hereditary methionine metabolism-related disorder (41). Values reported for the NR’s IC50 in the brain for HC and HCA are 14 ± 4 μM (51) and 14 μM (51), respectively, whereas it is 21.1 ± 0.78 μM for erythrocytes (Fig. 4A). These data suggest that in healthy subjects these two agonists predominantly control NR activity in circulating RBCs, whereas glutamate will only be of importance in pathological cases such as stroke when plasma glutamate concentration may rise up to 200 μM (10). Magnesium is one of the physiologically relevant inhibitors of NRs in the brain. In the present study we only tested responses of NRs present in erythrocytes to pharmacological inhibitors that are more selective and well-characterized. In erythrocytes MK-801 irreversibly inhibits the receptor-mediated ion transport and downstream processes at micromolar concentrations used in electrophysiological studies on brain slices (e.g., 49).
Fig. 6. Secondary changes in erythrocyte morphology, osmotic resistance, and resistance to shear stress caused by NR agonists. A: acute morphological alterations triggered by a high dose (1 mM) of NR agonist homocysteic acid (HCA) in the absence or in the presence of 10 μM clotrimazole or 5 mM Na₃VO₄. Images are taken from the cells before and 100 or 180 s after the addition of the HCA. Cells highlighted with arrows show characteristic reversible shape changes when exposed to HCA alone and or in the presence of clotrimazole. Inhibition of the Ca²⁺ pump with Na₃VO₄ facilitated the rate of echinocytic transformation and the number of responding cells compared with that in the presence of HCA alone as well as made these changes irreversible. B: osmotic resistance of rat erythrocytes treated with 1 mM HCA or NMDA. Osmotic resistance was assessed as a rate of hemolysis in response to acute hypoosmotic stress. Exposure of cells to agonists of the NR for 1 min caused a significant decrease in hemolytic rates calculated from the linear slopes of the plots of hemoglobin release. Shown are the representative hemoglobin liberation kinetics plots. Inset: statistical analysis of the rate of osmotic hemolysis for nine independent experiments; **P < 0.01 compared with the nontreated control. C: shear stress-induced hemolysis in cells after a long-term exposure to NMDA. Accumulation of hemoglobin in the incubation medium during 1 h at 37°C in an Eppendorf Thermomixer (900 rpm shaking speed) was assessed in control cells and in the cells exposed to 100 μM NMDA ± 100 μM MK-801 in the presence or absence of 1 mM Ca²⁺ in the incubation medium. Data are means of seven independent experiments ± SE; **P < 0.01.
when Epo receptors are dormant, which is known as neocytolysis (2, 12). In contrast to the common opinion that Ca$^{2+}$ levels increase with red cell maturation and senescence (15), our data indicates that reticulocytes contain more Ca$^{2+}$ than the either adult or senescent cells (Fig. 2). Thus Ca$^{2+}$-induced processes may control the rapid decrease in blood cell mass, whereas clearance of senescent cells is regulated by other factors (31). Assessing the effects that Epo has on the activation state of NR and intracellular Ca$^{2+}$ levels may shed light on the mechanism(s) involved in selective clearance of reticulocytes and young erythrocytes during conditions of Epo deficiency and the potential effects of Epo on the “non-selective cation channels” described my Myssina et al. (35).

Accumulation of Ca$^{2+}$ in the cells following treatment with NR agonists is a common mechanism demonstrating NMDA/HCA cytotoxicity in erythrocytes and neurons (28) (Figs. 2 and 3). Exposure of erythrocytes to NMDA or HCA gives rise to numerous Ca$^{2+}$-dependent responses. Downstream targets of the Ca$^{2+}$-sensitive signaling cascade have different Ca$^{2+}$ sensitivity thresholds. Gardos channel activation requires 1–3 μM Ca$^{2+}$ to reach half-activation (20). These channels remain closed under most physiological conditions and are only activated by maneuvers that result in intracellular Ca$^{2+}$ accumulation (5). Activation of the Ca$^{2+}$-dependent, clotrimazole-sensitive K$^{+}$ influx can be observed in the presence of 50–100 μM NMDA, whereas treatment of the cells with MK-801 or clotrimazole shows no effect (Fig. 5). Changes in the intracellular Ca$^{2+}$ trigger characteristic alterations in erythrocyte morphology (21, 22) resembling those we have observed in NMDA-treated cells (Fig. 6B). These reversible alterations in cell shape, which can be blocked by clotrimazole, reveal the result of Gardos channel activation.

De novo NO production by RBC-eNOS is Ca$^{2+}$ dependent. Half-activation of eNOS requires 50–300 nM Ca$^{2+}$ depending on the phosphorylation state of the enzyme (45). Thus threshold levels of intracellular Ca$^{2+}$ for eNOS activation are lower than those for Gardos channel activation (1–3 μM). Inhibition of Ca$^{2+}$ uptake via NRs by MK-801 compromises eNOS function as Ca$^{2+}$ levels drop below 50–100 nM due to its removal from the cytosol by the Ca$^{2+}$ pump (Fig. 7A). On the other hand, treatment of the cells with NMDA further stimulates NO production in the presence of l-arginine, which suggests that steady-state Ca$^{2+}$ levels are insufficient to support function of the eNOS at maximal rates. Changes in the NO production by erythrocytes in turn are reported to affect deformability, redox state, and oxygen-carrying capacity of RBCs (33, 38).

In rat erythrocytes, alterations in activity of RBC-eNOS had an immediate effect on the intracellular redox balance. As shown before in mouse erythrocytes (33), activation of eNOS in rat RBCs by NR agonists was pro-oxidative under conditions of l-arginine deprivation as eNOS was forced into the O$_2^-$-generating mode (Fig. 6C). Similar effects were observed in mouse erythrocytes upon treatment with Epo (33). Oxidative stress triggered by NR activation may possibly contribute to an increased susceptibility of erythrocytes to shear-stress-induced hemolysis after a long-term exposure to NMDA or HCA (Fig. 5C).

Taken together our findings indicate that an increase in the number or activity of NRs may cause oxidative stress, abnor-
mal volume regulation, and hemolysis. These events may contribute to the increased incidence of thrombosis and anemia that has been reported in patients with high concentration of HC, which can reach 200–300 μM (1, 8). Macrocytosis and abnormal RBC morphology are reported in patients with hyperhomocysteinemia (1) in which similar mechanisms as those described here may actually occur in vivo. So far the effect of HC on platelets has been suggested to be the only cause of thrombotic complications (34). If our observations on rat erythrocytes reflect the conditions in human red cells, these cells would also contribute to HC-induced thrombus formation and hemolytic anemia in patients with vitamin B12 and folate deficiency (50). The final impact of HC/HCA on the survival prognosis or mortality risk in hemodialysis patients was shown to increase by 3% per each additional 1 μM of HC in the plasma (9). The next step therefore would be to assess the levels of NRs in erythrocytes of healthy subjects and patients with various forms of anemia. This work is currently in progress.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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