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Full-length article

Phorbol-induced surface expression of NR2A subunit homologues in HEK293 cells[1]

Chan-ying ZHENG[2], Xiu-juan YANG[3], Zhan-yan FU[4], Jian-hong LUO[2,5]

[2]Department of Neurobiology, Zhejiang University School of Medicine, Hangzhou 310031, China; [3]Institute of Neuroscience, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai 200031, China; [4]Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington 20057, USA

Key words

NMDA receptor; NR2A subunit; PMA; surface expression; HEK293 cells

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[5] Correspondence Prof Jian-hong LUO.
Phn 86-571-8721-7010.
Fax 86-571-8721-7044.
E-mail luojianhong@zju.edu.cn

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Abstract

Aim: *N*-methyl-*D*-aspartate receptors (NMDAR) are heteromeric complexes primarily assembled from NR1 and NR2 subunits. In normal conditions, NR2 subunits assemble into homodimers in the endoplasmic reticulum (ER). These homodimers remain in the ER until they coassemble with NR1 dimers and are trafficked to the cell surface. However, it still remains unclear whether functional homomeric NMDAR exist in physiological or pathological conditions. **Methods:** We transfected GFP-NR2A alone into HEK293 cells, treated the cells with PKC activator 12-myristate-13 acetate (PMA), and then detected surface NR2A subunits with a live cell immunostaining method. We also used a series of NR2A mutants with a partial deletion of its C-terminus to identify the regions that are involved in the PMA-mediated surface expression of NR2A subunits. **Results:** NR2A subunits were expressed on the cell membrane after incubation with PMA (200 nmol/L, 30 min), although no functional NMDA channels were detected after PMA-induced membrane trafficking. Immunostaining with an ER marker also revealed that NR2A subunits were exported from the ER after PMA treatment. Furthermore, the deletion of amino acids between 1149–1347 or 1354–1464 of NR2A inhibited PMA-induced surface expression of NR2A subunits. **Conclusion:** First, our data suggests that PMA treatment can induce the surface expression of homomeric NR2A subunits. Furthermore, this process is probably mediated by the NR2A C-terminal region between positions 1149 and 1464.

Introduction

Three classes of subunits have been identified in the family of *N*-methyl-*D*-aspartate receptors (NMDAR): NR1, NR2 and NR3. The NR1 subunit is essential to the structure of NMDAR, whereas different NR2 subunits endow the receptor complexes with different characteristics[1]. The majority of functional NMDA receptors are generally thought to be tetrameric complexes composed of both NR1 and NR2 subunits[2]. Their structure is thought to be a dimer of dimers[3]. It was reported that expressing NR1-1a alone in *Xenopus* oocytes produces a glutamate and glycine-activated current, although the amplitude was only 1/10 compared with that in the NR1/NR2 coexpressed cells[4–6]. This evidence indicates that the NR1 subunit assembles to form functional homomeric receptors in oocytes. On the other hand, we have proven that NR2 subunits were assembled in HEK293 cells using fluorescent resonance energy transfer (FRET) technology. When we expressed NR2A or NR2B alone in HEK293 cells, FRET occurred in each transfection[7]. Previous studies also suggested that NR2 subunits were retained in the endoplasmic reticulum (ER) until they assembled with NR1 subunits[8]. However, there is no evidence indicating that NR2 subunits can express on the plasma membrane or form functional channels before assembling with NR1 subunits[1,9,10].

The surface expression of NMDAR has been shown to be regulated by the phosphorylation of the intracellular C-terminal regions of their composing subunits[11–14]. For

example, the activation of protein kinase C (PKC) leads to the up-regulation of surface NMDAR through the effect of Src family kinases. Activation of the cAMP-dependent protein kinase (PKA) sites in the C1 cassette of NR1 subunits increases the synaptic targeting of NMDAR, while CaMKII can inactive synaptic Ras-GTPase activating protein and then downregulate synaptic NMDAR. 12-Myristate-13 acetate (PMA) is an activator of PKC, which are classified into 3 groups and 11 isozymes based on their structure[15]. PMA is thought to be a tumor promoter, playing roles in cell cycling, cell transformation, vesicle trafficking and gene expression. It has been shown that the application of PMA increases both the number and the open probability of the surface NMDA receptors composed of NR1 and NR2 subunits[16]. Biochemical studies have identified several PKC phosphorylation sites in the C-terminus of the NR2 subunits[17]. Many potential phosphorylation sites in NR2A C-terminus are thought to be important for PMA-based potentiation of NR2A in heteromeric NMDAR. Residues S1291, S1308 and S1312 are essential for PKC-mediated insulin potentiation of NR1/NR2A receptors[17]. Residues Y1105, Y1267 and Y1387 were utilized in Src-potentiation of whole cell NR1/NR2A currents in zinc sensitivity[18]. Also, the amino acids between 1400–1406 of NR2A are involved in PMA-potentiation of NR1/NR2A receptors[13]. However, since PMA-induced potentiation could hardly be blocked by site mutation[13], several indirect pathways may be involved in the processes. These regulatory mechanisms are largely uncertain.

In this study, we found that NR2A subunits were expressed on the cell surface after the cells were incubated with PMA. Interestingly, the surface expression does not require the coexpression of NR1 subunits. The NR2A C-terminal region required for the PMA-induced surface expression was further identified by a series of NR2A mutants with partial deletion of its C-terminus.

Materials and methods

Molecular biology The generation of expression vectors for the green fluorescence protein (GFP) N-terminally tagged NR2A (GFP-NR2A) and a variety of GFP-NR2A mutants with C-terminal deletions used in this study have been described previously[19,20] (Figure 2A).

Transfection of heterologous cells HEK293 cells were cultured and plated on polylysine-coated coverslips (8 mm× 8 mm) in 35 mm dishes 1 d before transfection. The plasmids (1.5 μg in amount) for the NR2 subunits were transfected alone or with NR1-1a at a molar ratio of 1:1 using Lipofectamine 2000 (Invitrogen, Los Angeles, CA, USA) according to the manufacturer's instruction. After transfection, cells were grown in the presence of 0.5 mmol/L ketamine and 1 mmol/L kynurenic acid (Sigma-Aldrich, St Louis, MO, USA). The transfected cells were used for electrophysiological recording and immunocytochemical staining 24–48 h after transfection.

Immunofluorescent staining For live cell surface staining, GFP-tagged NMDAR on the cell surface were labeled using immunofluorescent staining in live cells as described previously[19]. The transfected HEK293 cells grown on coverslips were incubated with rabbit anti-GFP antibody for 7 min, rinsed 3 times, and then incubated with Cy3-conjugated goat anti-rabbit secondary antibody (Chemicon, Los Angeles, CA, USA) for another 7 min. After brief washing and fixation, the cells were observed and imaged under a confocal microscope. The imaging analysis software used was FluoView (FV500, Olympus, Tokyo, JAPAN). All procedures were performed at room temperature. The surface expression of GFP-tagged NMDA receptors in HEK293 cells was measured by counting the number of surface-labeled cells in the population of GFP-positive cells.

For permealized labeling, HEK293 cells were washed with PBS, fixed on ice with 4% paraformaldehyde for 20 min, washed 3 times with PBS, and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After 3 washes with PBS, the cells were blocked with 10% bovine serum albumin (BSA) in PBS for 1 h and then incubated in primary antibody for 15 h at 4 °C. After another 3 washes with PBS, the cells were incubated with the appropriate secondary antibodies for 1 h at room temperature. The cells were then observed and imaged using a confocal microscopy.

Results

PMA-induced surface expression of NR2A in HEK293 cells We cotransfected HEK293 cells with NR1-1a and GFP-NR2A by Lipofectamine 2000 and then measured surface expressed receptors 24–48 h after transfection using a live surface staining method. GFP-tagged NMDAR were expressed on the cell membrane, with N-terminal GFP tags exposed extracellularly. The exposed GFP tags were stained using polyclonal antibodies. Positive labeling was indicated by numerous red puncta on the cell surface, indicating the surface expression of the NMDAR (Figure 1A,1B). After quantification, we found that 49%±4% of transfected HEK293 cells were surface labeled (Figure 1G).

We then transfected HEK293 cells with GFP-NR2A alone and measured surface expressed receptors 24 h after transfection. No surface staining was detected in these HEK293 cells expressing GFP-NR2A alone (Figure 1C, 1D, 1G).

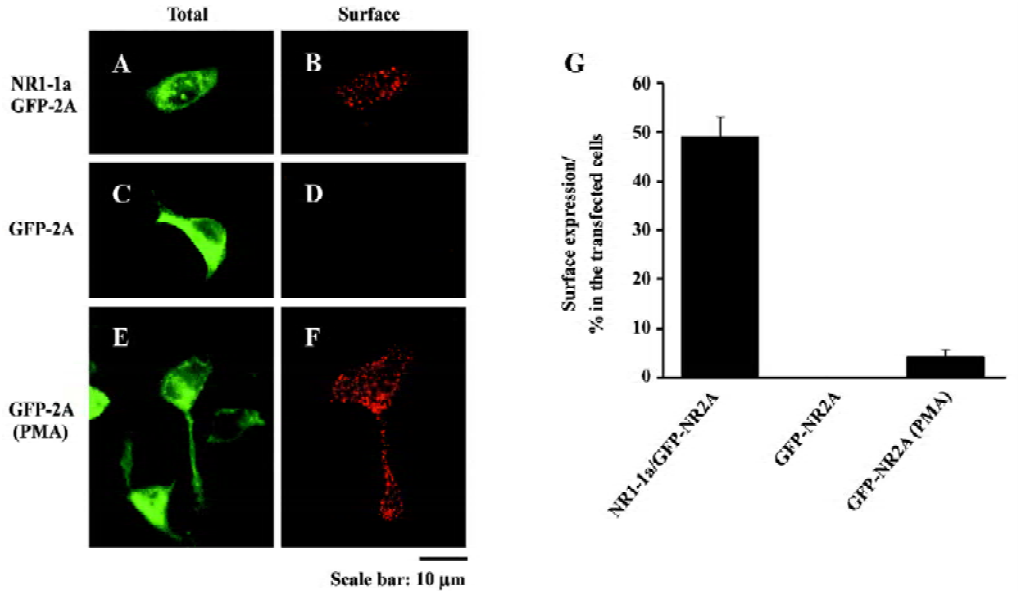

Figure 1. Surface expression of transfected GFP-NR2A in HEK293 cells after incubating with PMA. GFP-NR2A was transfected into HEK 293 cells alone or with NR1-1a using Lipofectamine 2000. Twenty-four hours after transfection, HEK 293 cells were surface stained. (A, B) surface labeling was identified in cells coexpressing NR1-1a and GFP-NR2A; (C, D) No surface labeling was detected in GFP-NR2A expressing HEK 293 cells without PMA incubation; (E, F) After PMA treatment, typical surface labeling was found in HEK 293 cells transfected with GFP-NR2A alone. HEK293 cells were incubated with FBS-free medium plus 200 nmol/L PMA for 30 min, rinsed 3 times, and then put back in PMA-free culture medium for another 2 h. Scale bar, 10 μm. (G), surface expression was quantified by calculating the percentage of cells with positive surface labeling from total transfected cells. The data were from 3 separate transfections, n=100–200 per transfection and expressed as Mean±SE. $^{c}P<0.01$ *vs* control group (two-tailed, unpaired Student's *t*-test).

Twenty-four hours after transfection, HEK293 cells transfected with GFP-NR2A alone were incubated with fetal bovine serum (FBS)-free culture medium plus 200 nmol/L PMA (Chemicon, Los Angeles, CA, USA) for 30 min. After briefly washing with PBS, the cells were incubated with PMA-free culture medium for 2 h. Finally, surface expressed GFP-NR2A subunits were measured by live surface staining (Figure 1E, 1F). We surprisingly found that 4.1%±1.5% of GFP expressing cells exhibited positive labeling (Figure 1G). These results indicate that some of the transfected cells could deliver NR2A subunits to the cell surface after incubating with PMA.

Segment between 1149D and 1464V was necessary for PMA mediated surface expression of homomeric NR2A subunits It has been reported that the region between amino acids 1105–1400 or 1267–1458 of NR2A is sufficient for PKC-mediated potentiation of NR1/NR2A heteromers[13,21]. We used a similar method to study whether this region also plays a role in the PMA-induced potentiation of NR2A subunits.

We generated a series of GFP-NR2A truncations: 2AΔ897–1017, 2AΔ1024–1142, 2AΔ1149–1347, and 2AΔ1354–1464[20]; the numbers following 2AΔ indicating the first and last residues of the deleted segments, respectively. Each mutation contained a GFP tag in the extracellular *N*-termini (Figure 2A). HEK293 cells were transfected with these mutants. PMA effects were assessed by incubation of parallel transfection with or without PMA (200 nmol/L, 30 min). We did not find any surface stained cells in any transfection without PMA incubation (Figure 2B). However, surface expression was found in cells expressing 2AΔ897–1017 or 2AΔ1024–1142 after incubating with PMA. In contrast, HEK293 cells expressing 2AΔ1149–1347 or 2AΔ1354–1464 did not show surface labeling after incubating with PMA (Figure 2B). After incubating with PMA, the percentage of surface-labeled cells from transfected cells was normalized to 100%. The relative percentage of surface-labeled cells from transfected cells expressing 2AΔ897–1017, 2AΔ1024–1142, 2AΔ1149–1347, or 2AΔ1354–1464 was 36.3%±37.4%, 60.1%±39.7%, 0%±0%, or 0%±0 %, respectively (Figure 2B).

PMA-induced NR2A subunits exporting from the ER Previous studies have provided evidence that NR1 dimers and NR2 dimers are retained in the ER until they coassemble[7,22]. However, whether NR2 subunits export from the ER or not after incubating with PMA remains unknown. Our immunocytochemical staining of HEK293 cells transfected with NR2A

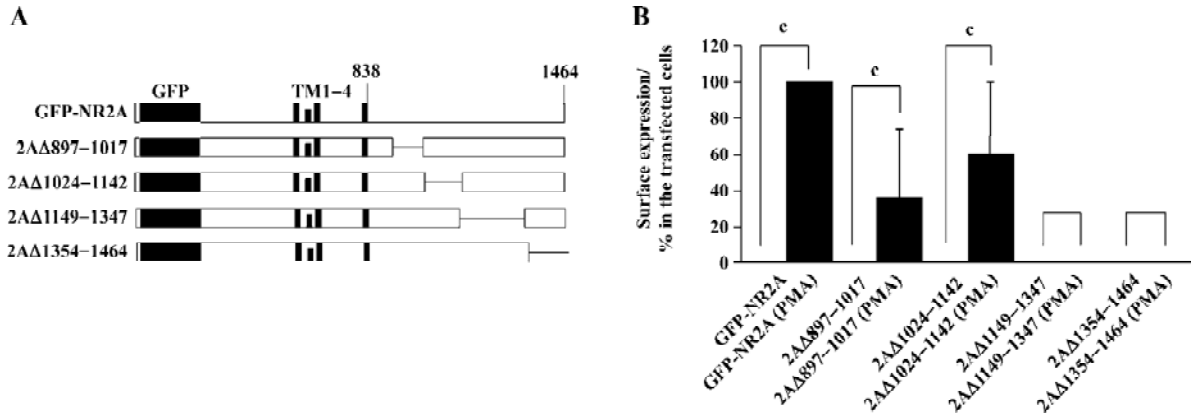

Figure 2. Surface expressions of transfected GFP-NR2A mutants in HEK293 cells with or without PMA incubation (200 nmol/L, 30 min). (A) A schematic representation of GFP-NR2A and their mutants with partial C-terminal deletions. Gray boxes indicate the GFP tags. Black boxes indicate the transmembrane domains. Lines indicate the deleted region. Mutants were named 2AΔ897–1017, 2AΔ1024–1142, 2AΔ1149–1347, and 2AΔ1354–1464. The numbers following 2AΔ indicated the first and last residues of the deleted segments. (B) Without PMA incubation, no cell surface staining was detectable in any transfection. After incubation with PMA, cells expressing 2AΔ1149–1347 or 2AΔ1354–1464 did not show surface labeling, while 2AΔ897–1017 and 2AΔ1024–1142 had detectable surface expression. The percentage of surface-labeled cells from GFP-positive cells was normalized to 100%. All of the data, expressed as Mean±SD, are from 3 separate transfections. n=100–200 per transfection. $^cP<0.01$ *vs* control group (two-tailed, unpaired Student's *t*-test).

alone showed extensive colocalization of NR2A subunits with an ER marker (Figure 3). We also fixed the cells 2 h after PMA treatment (200 nmol/L, 30 min) and then performed immunocytochemical staining. Our results showed that many NR2A puncta did not colocalize with an ER marker (Figure 3). These results suggest that PMA induces NR2A subunit export from the ER position.

Discussion

Although the NR1 subunit homologues have been studied in Xenopus oocytes and mammalian cells[23], it still remains unclear whether there are functional homomeric NMDAR existing in physiological or pathological conditions. Previous studies have shown that transiently expressing NR1-1a in COS cells did not produce functional NR1-1a homomeric receptors[24]. In agreement with these reports, we did not detect surface expressed NR2A after transfecting HEK293 cells with GFP-NR2A alone, even after transfecting as much as 5 μg of GFP-NR2A plasmid into HEK293 cells. However, using the live surface staining method, some of the cells were found to be surface labeled with GFP-NR2A subunits after incubating with PMA (200 nmol/L, 30 min).

The molecular mechanisms underlying the PMA medi-

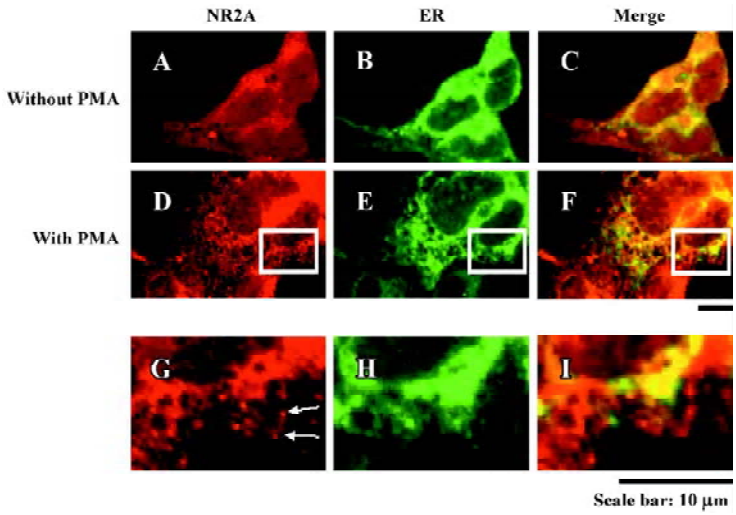

Figure 3. Direct immunofluorescence of HEK293 cells expressing NR2A. Twenty-four hours after transfecting with NR2A, the HEK293 cells were incubated with FBS-free medium with or without PMA (200 nmol/L) for 30 min. The cells on coverslips were fixed immediately after incubation, and then colabeled with FITC-conjugated anti-ER antibody and CY3-conjugated anti-NR2A antibody. (A, B, C) In the cells that were not incubated with PMA, NR2A was closely colocalized with an ER marker; (D, E, F) in the cells exposed to PMA, some NR2A puncta were identified in the area that was not stained by the ER marker; (G, H, I) zoom in of the area inside the white frame in panels D, E and F. Red puncta that did not colocalize with green ER marker (arrows) indicated NR2A subunits which did not colocalize with the ER antibody.

ated potentiation of NR2 subunits remains unclear. Phorbol PMA is an activator of PKC. Previous studies have provided evidence that PMA induces the phosphorylation of several serine and tyrosine sites[17,18]. It is possible that some phosphorylation sites act in concert to mediate the potentiation of surface NR2 subunits. This may explain why the PMA-potentiation of NR2A subunits could not be induced by deleting the amino acids between 1149–1347 or between 1354–1464. Our results suggest that the surface expression of NR2A subunits was mediated by the simultaneous phosphorylation of several residues between 1149–1464.

We detected surface NR2 subunits in HEK293 cells by immunochemical methods. However, it seems difficult to identify whether or not the surface NR2 subunits form ion channels in HEK293 cells. Voltage clamp is one of the most direct ways to identify ion channels. In NR1/NR2B coexpressing HEK293 cells, our data showed that the current amplitudes were reduced significantly after surface staining (Figure 4), suggesting that surface labeling reduced the sensitivity of the whole cell recording. After PMA incubation, we could not produce significant NMDA currents in surface-labeled HEK293 cells expressing NR2 alone. There are 2 possible explanations for this: First, although some of the NR2A subunits export from the ER by PMA-based induction, they fail to assemble an NMDA channel. There may be monomeric, dimeric, trimeric or some other formation of homomeric NR2A complexes that express on the cell surface. However, they do not have normal NMDAR function and can not be activated by glutamate. Second, the NR2A subunits may assemble into homomeric receptors, but the current is far more less than normal NMDA currents. So after surface staining, they could hardly be recorded.

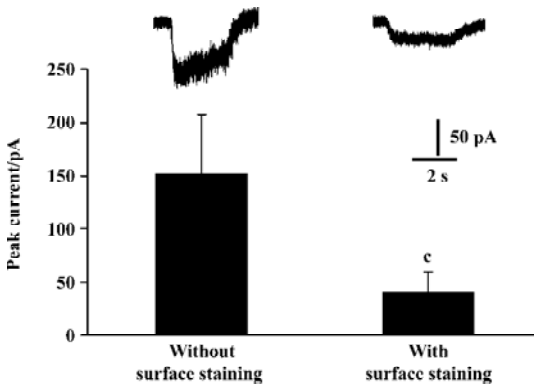

Figure 4. Typical whole-cell currents in HEK293 cells coexpressing NR1-1a and GFP-NR2B before and after surface labeling. Two waveform representative examples of whole cell recording currents (holding potential, -60 mV). Responses were evoked by 100 μmol/L glutamate and 20 μmol/L glycine. Mean±SD. $n>4$ for each kind of recording. $^cP<0.01$ *vs* control group (one-tailed, unpaired Student's *t*-test).

In summary, our experiments demonstrate that in a small fraction of transfected cells, NR2A subunits can traffic to the cell membrane after PMA incubation. This process is mediated by the NR2A C-terminal region between amino acids 1149 and 1464. Thus, our work has provided further evidence that the NR2A C-terminus plays an important role in PMA-induced potentiation of NR2A-containing NMDAR. In addition, it would be interesting to explore whether NR2A subunits express on the surface of neurons under proper conditions *in vivo* in the future.

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