Free Radical Biology and Medicine 97 (2016) 520–530

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Molecular basis of reactive oxygen species-induced inactivation of α4β2 nicotinic acetylcholine receptors

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ARTICLE INFO

Article history:
Received 12 March 2016
Received in revised form
3 July 2016
Accepted 16 July 2016
Available online 18 July 2016

Keywords:
Neuronal nicotinic acetylcholine receptors
Reactive oxygen species
Parkinson's disease

ABSTRACT

The α4β2 neuronal nicotinic acetylcholine receptors (nAChRs) are the most widespread heteromeric nAChR subtype in the brain, mediating fast synaptic transmission. Previous studies showed that α4β2 nAChRs could be inactivated by reactive oxygen species (ROS), but the underlying mechanism is still obscure. We found that H_2O_2 induced the rundown of ACh-evoked currents in human α4β2 nAChRs and the replacement of the conserved cysteine in the M1–M2 linker of either α4 Cys245 or β2 Cys237 with an alanine residue could prevent the current rundown. Structurally, α4 Cys245 and β2 Cys237 are hypothesized to be in close proximity when the receptor is activated. Western blotting results showed that α4 and β2 subunits were cross-linked when the agonist-bound receptor encountered H_2O_2, which could be prevented by the substitution of the conserved cysteine in the M1–M2 linker to an alanine. Thus, when agonist bound to the receptor, α4 Cys245 and β2 Cys237 came close to each other and ROS oxidized these conserved cysteines, leading subunits to be cross-linked and trapping α4β2 nAChRs into the inactivation state. In addition, we mimicked an experimental Parkinson's disease (PD) model in PC12 cells and found that ROS, generated by 6-hydroxydopamine (6-OHDA), could cause the current rundown in α4β2 nAChRs, which may play a role in PD.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) shape electrical signals between neurons. nAChRs are made up of five subunits and each subunit has a topology with a large extracellular N-terminal domain, four trans-membrane segments (M1–M4), and a large intracellular loop between M3 and M4 [1,2]. The α4β2 subtype is the most widespread heteromeric nAChR subtype in the central nervous system (CNS), involved in memory, drug addiction and excitement [3]. Dysfunction of brain nAChRs has been implicated in the origin of a variety of neurological and psychiatric disorders, such as Parkinson's disease (PD), Tourette's syndrome and schizophrenia [4,5].

Oxidative stress is widely associated with brain disorders including PD, schizophrenia and multiple sclerosis [6,7]. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radical, are byproducts of mitochondrial metabolism and many other processes [8]. ROS have important physiological functions in regulating cell growth, survival and neurotransmitter release [9,10]. Under pathological states, ROS are elevated and surpass the cellular antioxidant capacity, resulting in oxidative damage and oxidative modification. In many neurodegenerative disorders such as PD and Alzheimer's disease, oxidative modifications of nucleic acids, lipids and proteins were observed in cerebrospinal fluid and postmortem brain tissue of patients [11,12]. The oxidative modification of proteins can profoundly alter their structure, function and antigenicity with pathophysiological consequences [13]. Particularly, H_2O_2, which possesses the ability to cross the cell membrane, can directly target the cysteine and methionine in proteins, altering enzyme activity and functionality

Abbreviations: nAChRs, neuronal nicotinic acetylcholine receptors; ROS, reactive oxygen species; PD, Parkinson's disease; CNS, central nervous system; H_2O_2, hydrogen peroxide; α3* nAChRs, α3-containing nAChRs; WT, wild type; SIN-1, 3-morpholinosydnonimine; DTT, dithiothreitol; GSH, glutathione; 6-OHDA, 6-hydroxydopamine; CAT, catalase; CA, catecholamine

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http://dx.doi.org/10.1016/j.freeradbiomed.2016.07.012
0891-5849/

of ion channels [14,15].

ROS were reported to induce neuronal nAChRs to an inactivated state. Previous study showed that H_2O_2 and hydroxyl radical from Fenton reaction caused a long-lasting, use-dependent rundown of α3* nAChRs (α3-containing nAChRs) on neonatal rodent sympathetic neurons [16,17]. Further experiments showed that hyperglycemia could also inactivate nAChRs on autonomic neurons by increasing hydrogen peroxide (H_2O_2), superoxide and hydroxyl radical [18], leading to a depression of ganglionic synaptic transmission which is involved in pathology of diabetes [19,20]. Similar findings were observed in nAChR α4β2-expressing oocytes induced by antimycin-A which initially generated mitochondrial superoxide [16]. A highly conserved cysteine residue, located at the short intracellular linker between transmembrane segments M1 and M2 (in nAChR), was demonstrated to be an important determinant of receptor inactivation by ROS, in that the substitution of the cysteine to an alanine in α3 subunit prevented the receptor inactivation [19,21]. Despite intensive studies in the past, the molecular mechanism by which ROS induce nAChRs inactivation has not been clarified.

In this study, we attempted to gain insight into the molecular basis of inactivation of α4β2 nAChRs by ROS. We demonstrated that ROS could suppress the currents of α4β2 nAChRs detected by whole-cell voltage clamp. Further, ROS could oxidize the conserved cysteine in the M1–M2 linker of α4 and β2 subunits, leading the subunits of α4β2 nAChRs to be cross-linked and trapping the receptor into the non-conducting state.

2. Materials and methods

2.1. Molecular biology

The cDNA encoding the wild-type human α4 and β2 nAChR subunit was cloned into the pcDNA3.1 vector. The highly conserved Cys residue, in the M1–M2 linker of α4 and β2 subunits was mutated to an alanine. The mutations were performed using the PCR-based QuikChange method of site-directed mutagenesis with PfuUltra DNA polymerase (Agilent Technologies, Hercules, CA). The mutations were confirmed by sequencing the entire coding region.

2.2. Cell culture and transfections

The HEK 293T cells were grown in DMEM with 10% fetal bovine serum at 37 °C under 5% CO_2. PC12 cells received DMEM with 5% fetal bovine serum and 10% heat-inactivated horse serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HEK293T and PC12 cells were transfected by Hitrans reagent (Jpson Biosciences). Equimolar amounts of human α4 and β2 plasmids were used in both cases. Experiments were performed after transfection for 48 h.

2.3. Whole-cell patch clamp recording

Recording experiments were performed at room temperature. The electrode solution contained 150 mM KCl, 2.5 mM $MgCl_2$, 10 mM HEPES, 0.2 mM EGTA, 2 mM ATP, pH = 7.4, and the bath solution contained 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM Glucose, pH = 7.4. We separately introduced 10 μM, 100 μM, 1000 μM H_2O_2 in electrode solution into HEK293T cells through the recording electrodes. The recording was conducted with HEKA EPC-10 patch-clamp amplifier with associated software (PULSE and PatchMaster, HEKA Electronic Inc., Germany). A microperfusion device (MPS-2; INBIO, Wuhan, China) with a fast exchange time (< 100 ms) among eight channels was used to puff ACh locally to the cell in the study. The 10 μM or 60 μM ACh ($EC50_1$ = 0.95 μM, $EC50_2$ = 83 μM) [22] was dissolved in bath solution and applied for 2 s at 30 s interval in transfected HEK293T or PC12 cells. Data were analyzed with Igor (WaveMetrics, Inc., Portland, OR) software [16]. The peak current amplitude in response to the xth application was represented by "I_x", "x" represented the number of ACh application.

2.4. Western blotting

The dishes of HEK293T cells or PC12 cells were washed with PBS and incubated with 10 μM or 60 μM ACh dissolved by PBS for 10 min at room temperature. All dishes were washed with PBS and then incubated with 10 mM NEM dissolved by ice cold PBS for 10 min. To extract membrane proteins, we collected cells and homogenized them in ice-cold lysis buffer (15 mM Tris, 0.25 M sucrose, 2 mM EDTA, 1 mM EGTA, 10 mM Na3VO4, 25 mM NaF, 10 mM sodium pyrophosphate, and protease inhibitor, pH 7.6). After centrifugation at 800g for 5 min, the remaining supernatant was subjected to 10,000g centrifugation for 10 min. The supernatant was collected for further experiment. The pellet was suspended in lysis buffer containing 1% Triton X-100 and 300 mM NaCl, and centrifuged at 16,000g for 15 min to obtain the Triton soluble fraction (P1) and Triton insoluble fraction (P2) which mainly included the membrane-associated proteins [23]. The insoluble fraction was dissolved in 1% SDS. The samples under reducing or non-reducing conditions were separated on 8% SDS-PAGE gel and transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membranes were probed with rabbit anti-α4 (1:500, Santa Cruz, CA, USA), anti-β2 (1:1000, Abcam, Cambridge, UK; 1:500, Santa Cruz, CA, USA). Anti-pan Cadherin (1:5000, Abcam, Cambridge, UK) and anti-Na^+/K^+-ATPase (1:500, Santa Cruz, CA, USA) were used as membrane protein-loading control. Anti-GAPDH (1:5000, Sigma, St. Louis, MO, USA) was used as cytosolic protein-loading control. Primary antibodies were revealed by infrared-conjugated IgG IRDye 680RD or 800CW (Li-COR Bioscience). Then blots were scanned with the Odyssey Infrared Imaging System (Li-COR Bioscience), and bands intensity was determined with software ImageJ (imagej.nih.gov/ij/).

2.5. Confocal microscopy

The PC12 cells were treated with 50 μM 6-hydroxydopamine (6-OHDA) (Sigma, St. Louis, MO, USA) for 6 h at 37 °C [24]. The 450 U/mL catalase (CAT) was co-incubated with 6-OHDA in PC12 cells to suppress ROS induced by 6-OHDA. After the treatment for 6 h, PC12 cells were incubated for 30 min at 37 °C with fresh medium containing 10 μM H_2DCF-DA (Sigma) and 5 μg/mL Hoechst 33342, and then were washed with PBS. Intracellular ROS production was detected by H_2DCF-DA under a Leica confocal microscope. Fluorescence was read using an excitation wavelength of 480 nm and an emission wavelength of 530 nm. For Hoechst 33342, which was used to stain nucleus, fluorescence was read using an excitation wavelength of 350 nm and an emission wavelength of 461 nm.

2.6. Homology modeling

The dimensional model of the human nicotinic receptor α4 and β2 subunits were made using the Discovery Studio 1.7 software (Molsoft LLC, San Diego, CA) running in a T5500 Dell Precision computer (Dell, Austin, TX). The amino acid sequences of human nicotinic receptor α4 and β2 subunits were downloaded from the NCBI website with the following accession IDs: CHRNA4: NM_000744 and CHRNB2: NM_000748. The crystal structure of the homologous human $GABA_A$ receptor (PDB ID of 4COF) was used as the template. The homology model was built using the