Neuropeptides 57 (2016) 59–64

Contents lists available at ScienceDirect

Neuropeptides

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

Effect of nicotinic acetylcholine receptor alpha 1 (nAChRα1) peptides on rabies virus infection in neuronal cells

Basavaraj Sajjanar [a], Shikha Saxena [a], Deepika Bisht [a], Arvind Kumar Singh [a], G.B. Manjunatha Reddy [b], Rajendra Singh [c], R.P. Singh [d], Satish Kumar [a,*]

[a] Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Bareilly 243122, Uttar Pradesh, India
[b] National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru 560064, Karnataka, India
[c] Division of Veterinary Pathology, Indian Veterinary Research Institute, Bareilly 243122, Uttar Pradesh, India
[d] Division of Biological Products, Indian Veterinary Research Institute, Bareilly 243122, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 22 September 2015
Received in revised form 3 November 2015
Accepted 26 November 2015
Available online 30 November 2015

Keywords:
Rabies virus
Receptor subunits
Peptides
Neuroblastoma cells

ABSTRACT

Rabies virus (RABV) is neurotropic and causes acute progressive encephalitis. Herein, we report the interaction of nAChRα1-subunit peptides with RABV and the effect of these peptides on RABV infection in cultured neuronal cells. Peptide sequences derived from torpedo, bovine, human and rats were synthesized and studied for their interactions with RABV using virus capture ELISA and peptide immunofluorescence. The results showed specific binding of the nAChRα1-subunit peptides to the RABV. In the virus adsorption assay, these peptides were found to inhibit the attachment of the RABV to the neuronal cells. The nAChRα1-subunit peptides inhibited the RABV infection and reduced viral gene expression in the cultured neuroblastoma (N2A) cells. Torpedo peptide sequence (T-32) had highest antiviral effect ($IC_{50} = 14 \pm 3.01$ μM) compared to the other peptides studied. The results of the study indicated that nAChRα1-subunit peptides may act as receptor decoy molecules and inhibit the binding of virus to the native host cell receptors and hence may reduce viral infection.

1. Introduction

Rabies encephalitis remains one of the important public health problems in Asian and African countries (Huang et al., 2015; Kabeta et al., 2015). The burden of rabies is highest among all the neglected tropical diseases with 1.91 million disability adjusted life years and the projected annual loss of US$ 6 billion (WHO, 2013). In the rabies bites, only prompt post-exposure prophylaxis (PEP) can protect the patients and fatalities occur in almost 100% of cases following the onset of clinical disease (Bourhy et al., 2010). Presently no pragmatic treatment methods are available and induction of coma is considered only as a part of supportive care in case of clinical rabies (Wilde and Hemachudha, 2015). RABV infections can be prevented by interfering at any of the steps in the viral life cycle. Attachment to the receptors and entry of the virus into the host cells is the initial critical step in the virus life cycle. Rabies virus has receptors in the nervous system and the neuronal receptor peptides may act as potential antiviral molecules by blocking the attachment and entry of the RABV into the host cells.

RABV is a prototype neurotropic virus and belongs to the genus *Lyssavirus* in the *Rhabdoviridae* family (King et al., 2012). It has small negative sense single stranded RNA genome of 12 kb which codes for five different proteins, namely the nucleoprotein, the phosphoprotein, the matrix protein, the glycoprotein and the large RNA dependent RNA polymerase (Finke and Conzelmann, 2005). Rabies virus glycoprotein (RVG), organized as trimers is the only protein exposed on the virus particle. It interacts with the host cell, mediates pH dependent fusion and promotes viral entry from the peripheral site to the nervous system (Roche and Gaudin, 2004). Further RVG is the major contributor to the pathogenicity and is involved in trans-synaptic spread of RABV within the CNS (Pulmanausahakul et al., 2008; Klingen et al., 2008). It is less clear about the host molecule to which the RVG binds. In vivo, RABV was found to multiply in neurons, muscle fibers and salivary gland cells however the virus can be adapted and propagated in various continuous cell types in vitro (Seganti et al., 1990). RABV restricted cell tropism in vivo indicates the presence of unique host cell receptors. Nicotinic acetylcholine receptor (nAChR) was the first identified receptor for RABV (Lentz et al., 1982). Other potential host cell receptors include neuronal cell adhesion molecule (NCAM) (Lafon, 2005) and low-affinity nerve growth factor ($p75^{NTR}$) (Tuffereau et al., 1998). However, RABV was able to infect mice which were deficient in either NCAM or p75NTR similar to wild-type mice (Tuffereau et al., 2007). This indicates that although these molecules may have role in infection process, they are not essential for entry of RABV. Earlier the interaction of purified nAChR with the RABV was confirmed in different experiments (Bracci et al., 1988; Gustka et al., 1996). The toxin loop identified within the RVG has homologous sequence to other neurotoxins and is

* Corresponding author.
E-mail address: drsatishkumar_ivri@yahoo.co.in (S. Kumar).

http://dx.doi.org/10.1016/j.npep.2015.11.090
0143-4179/

responsible for binding to the nAChRs (Lentz, 1990). Conformational studies identified an internal tetra-peptide of RVG toxin loop (RVG-29mer) as an essential part of the binding site to nAChR (Rustici et al., 1993). Besides being present in the muscle membranes at synaptic junctions, nAChRs are present in the central and peripheral nervous system with similar acetyl choline binding properties (Albuquerque et al., 2009). nAChRs may be involved in the entry of RABV at post-synaptic junctions and also spread within the nervous system (Schnell et al., 2010). Harnessing the binding ability of RVG to the nAChR, recent studies have shown the successful use of RVG-29mer peptide to deliver therapeutic molecules to the brain (Son et al., 2011; Kim et al., 2013; Gao et al., 2014). Considering the wide spread presence of nAChRs in the nervous system and the binding of RVG to these receptors, nAChR peptides may act as receptor decoy molecules for RABV. The binding of receptor peptides may prevent the attachment and entry of the RABV into the host neuronal cells which is the essential step in viral infectivity cycle.

In the present study, nAChRα1-subunit peptide variant sequences from different species were synthesized and tested for their interactions with RABV. The interacting peptides were evaluated against RABV infection in cultured neuroblastoma cells.

2. Experiments

2.1. Virus propagation in N2A cells and titration

N2A cells were obtained from the National Centre for Cell Science, Pune, India and grown at 37 °C under 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (Hyclone). The mouse-brain-adapted rabies challenge virus standard 18 (RABV-CVS18) strain was propagated in the N2A cells. After three passages, the virus titer in the culture supernatants was determined by fluorescent focus unit (FFU) assay following standard method prescribed by world organization for animal health (OIE) (Smith et al., 1977). The culture supernatant had an infectious potency of 3 × 10 FFU/ml. This stock of virus was made into aliquots and used for further experiments. For virus attachment studies, concentrated virus preparations were obtained by mixing the culture supernatants with polyethylene glycol (8%), NaCl (2.2%) and phenyl methyl-sulfonyl fluoride (0.01%). The resulting solution was incubated overnight at 4 °C with gentle stirring followed by centrifugation at 15,000 ×*g* for 90 min and finally resuspended in the tissue culture medium to use in further experiments.

2.2. Peptide synthesis

Solid phase methodology with Fmoc chemistry was used to synthesize sequences of native nAChRα1-subunit peptides derived from torpedo, bovine, human and rat. These peptide sequences characteristically fall between 174 aa to 203 aa region of α1 subunit of the receptor. Phage display derived α-bungarotoxin binding peptides discovered earlier were also included in the present study (Moshe et al., 1997). Briefly after 1 h swelling of rink amide MBHA resin (Nova Biochem), 20% piperidine treatment was done to remove the protecting Fmoc group. The first Fmoc-amino acid (5 equivalent to the loading capacity of the resin) was activated with equivalent amount of 1-(Bis (dimethylamino) methylene)-1 H-benzotrizoliumhexaflurophosphate (1-) 3 oxide (HBTU) and 1-hydroxybenzotriazol (HOBT) and made to react with the resin in the presence of di-isopropyl ethylamine (DIEA). Coupling was allowed for 2 h followed by end capping with acetic anhydride. Loading/coupling efficiency was monitored at each steps of synthesis using Kaiser test (Kaiser et al., 1970). Subsequent deprotection, coupling and end capping were repeated till the completion of synthesis. Peptides were deprotected and cleaved from resin beads using a treatment of trifluoroacetic acid/phenol/thioanisol/1dodecanethiol/water (82.5:5:5:2.5:5 v/v) mixture for 4 h and precipitated in chilled dry diethyl ether.

2.3. Purification and characterization of peptides

Precipitated peptides in crude forms were purified by reversed-phase chromatography (RP-HPLC) on a C-18 semi-preparative column (7 × 300 mm; 10 μ particle size) using UFLC pump system (Shimadzu, Tokyo, Japan) fitted with photo diode array (PDA) detector. The binary gradient of water/acetonitrile having 0.1% TFA (v/v) was used for purification of peptides. The flow rate was kept at 1 ml/min and the major peaks were collected. The collected elutions were dried in Speed Vac concentrator (Eppendorf, Germany) and resuspended in HPLC grade water. Peptides were further analyzed for purity on analytical C-18 column (4 × 150 mm; 5 μ particle size). The identity and purity of the final products were confirmed by Ettan™ MALDI–TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS (Amersham Biosciences) (Supporting materials). The sequences of the synthesized peptides and their physico-chemical properties were determined by using ProtParam algorithm at ExPASy (the Expert Protein Analysis System) provided by the Swiss Institute of Bioinformatics (SIB) (Table 1).

2.4. Virus capture ELISA

Microtitre plates were coated with 100 μg/ml peptides for 24 h at 4 °C. Plates were blocked with 1% BSA in Tris buffered saline (TBS) for 1 h at room temperature, followed by 2 h at room temperature with 10 μg per well of β-propiolactone (BPL) inactivated RABV. After extensive washing, plates were probed with 1:1000 dilution of monoclonal mouse anti-rabies IgG (Rab sc-57994, Santa Cruz) for 1 h at 37 °C. Plates were washed with PBS and a 1:2000 dilutions of chicken anti-mouse HRP conjugate (sc-2954, Santa Cruz) was added to each well, incubated for 1 h at 37 °C. The wells were washed with PBS and developed by using TMB substrate (Amresco) at room temperature for 20 min. Plates were read at 450 nm after addition of stopping solution (1 M H_2SO_4). The binding was calculated by determining the change in virus binding compared to the wells coated with BSA alone.

2.5. Cytotoxicity test for peptides in N2A cells

Cytotoxicity test for nAChRα1-subunit peptides was performed using the MTT method (Mosmann, 1983). Briefly equal numbers of cells (2.5 × 10 were seeded in each well of 96 wells culture plates. 50 μl of different concentration of peptides (6.25, 12.5, 25, 50, 100 & 150 μM) diluted in DMEM were added to the cells. 20% DMSO and DMEM alone were included as the positive and negative control groups respectively. Plates were incubated for 24 h at 37 °C, under a humidified 5% CO_2 atmosphere. The medium was removed and 50 μl of MTT solution was added to each well and incubated for 4 h. 100 μl of DMSO was added to the wells and gently rocked to solubilize formazan. Absorbances were measured using spectrophotometer at 540 nm. The CC_{50} was calculated as the concentration of the peptides that reduced the absorbance of treated wells to 50% when compared to the control wells.

2.6. Virus inhibition assay

Virus inhibition assay was performed using NIH test protocol, modified rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973). N2A cells were seeded in 24 well plates on the previous day to reach 80–90% confluence. The cells were inoculated with peptide pretreated (0 to 50 μM of peptide) and untreated RABV-CVS18 at an MOI of 0.1 for 1 h at 37 °C. Following the adsorption, inoculum was removed and cells were incubated in DMEM containing 2% fetal bovine serum (FBS). The supernatants were collected at after 48 h (hpi), and stored at −80 °C for viral RNA isolation. The infected cells monolayers were fixed with 80% acetone to detect RABV in infected cells by direct immunofluorescence test using FITC-labeled antibody against RABV nucleocapsid protein (BioRad). Cell nuclei were counterstained with Prolong gold anti-fade reagent with DAPI (Invitrogen). The viral foci were counted in 6 random