Neuropeptides xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Neuropeptides

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

PK2β ligand, a splice variant of prokineticin 2, is able to modulate and drive signaling through PKR1 receptor

Roberta Lattanzi[a], Daniela Maftei[a], Lucia Negri[a], Ilaria Fusco[a], Rossella Miele[b,*]

[a] Dipartimento di Fisiologia e Farmacologia "Vittorio Erspamer", Sapienza Università di Roma, Piazzale Aldo Moro 5, I-00185 Rome, Italy
[b] Dipartimento di Scienze Biochimiche A. Rossi Fanelli, CNR Istituto di Biologia e Patologia Molecolare, Sapienza Università di Roma, Piazzale Aldo Moro 5, I-00185 Rome, Italy

ARTICLE INFO

Keywords:
Prokineticin 2β
Prokineticin receptors
G protein coupled receptor
Yeast-STAT3
Thermal hyperalgesia
Tactile allodynia

ABSTRACT

Prokineticin-2 (PK2) is a secreted bioactive peptide that signals through two GPCRs, the prokineticin receptors (PKRs), and regulates a variety of biological processes including angiogenesis, immunity and nociception. The PK2 primary transcript has two alternative splice variants, PK2 and PK2L (a Long form) which is cleaved in an active peptide, named PK2β that preferentially binds to PKR1 receptor.

The aim of this study was to characterize the PK2β.

Using different *Saccharomyces cerevisiae* strains, we examined the specificity of PKR1 and PKR2 G-protein coupling following PK2β binding. Data obtained in yeast confirmed that PK2 binds both receptors, inducing a comparable response throughout a promiscuous coupling of G protein subtypes. Conversely, we demonstrated, for the first time, that PK2β preferentially binding to PKR1, activates a signaling cascade that not depends on $G\alpha_{i/o}$ coupling. The binding specificity of PK2β for PKR1 was evaluated by the analysis of PKR mutant in yeast and GST pull-down experiments, suggesting an important role of PKR1 amino-terminal region. We also evaluated the ability of PK2β to differentially activate PKR1 and/or PKR2 by *in vivo* nociceptive experiments and we showed that PK2β induces intense sensitization of peripheral nociceptors to painful stimuli through the activation of PKR1. To analyze PK2β-induced signal transduction, we demonstrated the inability of PK2β to induce STAT3 protein phosphorylation in organotypic primary explants from mice Dorsal Root Ganglion (DRG), an important pain station. The control of the concentration ratio between PK2β and PK2 could be one of the keys to allow the specificity of the cell response of prokineticin signaling pathway.

1. Introduction

Prokineticin-2 (PK2) is a secreted bioactive peptide that is highly conserved across species. The PK2 primary transcript has two alternative splice variants, PK2 and PK2 Long (PK2L). PK2L presents an insertion of 21 basic amino acids sequence containing a site recognized by Furin, which generates the smaller active form of PK2 protein, called PK2β (Chen et al., 2005; Giannini et al., 2009; LeCouter et al., 2003; Martucci et al., 2006; Negri et al., 2006; Wechselberger et al., 1999). The binding of PK2 to their cognate GPCRs, prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2), activates $G\alpha_{q/11}$ signaling leading to the mobilization of intracellular calcium (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). PK2-induced ERK phosphorylation and chemotaxis of human monocytes, mainly expressing PKR2, are inhibited by pertussis toxin (Lecouter et al., 2004; Lin et al., 2002b; Martucci et al., 2006), suggesting the involvement of the $G\alpha_i$ proteins. However, PKRs can also activate other pathways, including the modulation of intracellular cAMP levels *via* $G\alpha_s$ (Chen et al., 2005). Recently, it was demonstrated that PKR2 may also couple $G\alpha_{12}$ in coronary endothelial cells (Guilini et al., 2010). Receptor activation has been shown to mediate different biological processes including nociception, feeding behavior, the control of physiological and neoplastic angiogenesis, the regulation of circadian rhythms and the modulation of cell functions, growth and survival in the immune and central nervous system (Gordon et al., 2016; Negri and Ferrara, 2018; Severini et al., 2015). We already demonstrated that in rodents, the activation of PKR1, widely distributed at peripheral level, and PKR2, highly expressed in the central nervous system by Bv8 (the amphibian homologue of PK2), induces intense sensitization to thermal and mechanical stimuli (Negri et al., 2002; Negri and Lattanzi, 2012; Negri and Maftei,

Abbreviations: GPCR, G Protein-Coupled Receptor; PK, Prokineticin; PKR, Prokineticin Receptor; PK2β, Prokineticin 2β; ERK, Extracellular signal-Regulated Kinase; DRG, Dorsal Root Ganglion; STAT3, Signal Transducer and Activator of Transcription 3; MAPK, Mitogen-Activated Protein Kinase; GST, Glutathione S-Transferase; WT, Wild Type; KO, Knock Out

* Corresponding author.
E-mail address: rossella.miele@uniroma1.it (R. Miele).

https://doi.org/10.1016/j.npep.2018.06.005
Received 1 February 2018; Received in revised form 27 June 2018; Accepted 27 June 2018

2017). It was demonstrated that in PKR1 or PKR2 transfected cells, PK2β preferentially binds to PKR1 and induces cAMP accumulation and calcium mobilization by $G\alpha_s$ and $G\alpha_q$ coupling, respectively (Chen et al., 2005).

In this study, we expressed PK2β in *Pichia pastoris* allowing us to obtain the functional protein that we used for all *in vitro* and *in vivo* assays.

The heterologous expression of the prokineticin system in different *Saccharomyces cerevisiae* strains (Brown et al., 2000; Dowell and Brown, 2009), each one expressing a different type of G protein chimera, is a powerful tool that allowed us to evidence differences in PKRs binding selectivity and activation efficacy of PK2β respect to PK2. To dissect the binding selectivity, we studied the interaction mechanisms between PK2β and the prokineticin receptors by analysis of PKR mutant in yeast and by GST pull-down experiments. To evidence a potential physiological role induced by PK2β we also evaluated its effect on nociception and we analyzed the specific roles of the two receptors using mice lacking the pkr1 or pkr2 gene. Finally, to confirm data obtained in yeast, on G-coupling specificity, we analyzed STAT3 activation using organotypic primary explants from mice Dorsal Root Ganglion (DRG), an important pain station.

2. Materials and methods

2.1. Drugs and reagents

3-Amino-1,2,4-triazole (AT), glass beads (425–600 µm, acid-washed), yeast nitrogen base, fluorescein di-β-D-galactopyranoside, heparin, amino acids, Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media, bovine fetal serum, pertussis toxin (PTX), phosphatases inhibitor cocktails, penicillin and streptomycin were purchased from Sigma Aldrich. All enzymes used for molecular cloning and an enhanced chemiluminescence detection kit were from Roche Molecular Biochemicals. All other chemicals used for SDS-polyacrylamide gel electrophoresis and Western blotting were purchased from Bio-Rad. A goat polyclonal antibody (sc-48,070) against the PK2 amino-terminal region encompassing residues 1–18 was from Santa Cruz Biotechnology, anti-HIS monoclonal antibody horseradish peroxidase conjugate was from Roche Molecular Biochemicals, mouse anti-STAT3 antibody and mouse anti-pSTAT3 antibody were from Cell Signaling Technology, and anti-β-actin antibody was from Abcam. The anti-goat IgG antibody conjugated to horseradish peroxidase and nitrocellulose membranes (0.45 µm; Hybond-C Extra) were from Amersham Pharmacia Biotech.

2.2. Expression of PK2 and PK2L in Pichia pastoris

Expression of PK2 and PK2L in the yeast *Pichia pastoris* strain GS115 was performed as described in our previous article (Miele et al., 2010). Human PK2 and PK2L cDNA was amplified using as template human brain Marathon-Ready cDNA (Clontech) with oligonucleotides PK2kex2XhoI and PK2EcoRIdw (Table 1) and inserted in the *Pichia pastoris* integrative vector pPIC9K (Invitrogen) fused to the α-factor sequence. As the integrity of the amino-terminal region is essential for the function of PK2, this cDNA was inserted, in the proper reading frame, to avoid the inclusion of additional residues between the α-factor signal peptide and the start codon of the protein, as described for Bv8. Expression of PK2L was carried out in the same conditions as for Bv8. Briefly, induction of PK2L synthesis was carried out in BMMY for 120 h, with daily supplementation of 1% methanol. The recombinant proteins were purified from crude culture supernatants by CM-Sephadex, followed by reverse-phase HPLC on a Vydac C-18 column, as described (Miele et al., 2010). Protein concentration was measured. Integrating the area of two peaks 33–35 min. From C-18 reverse column and comparing with a natural Bv8 standard.

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequence	T(°C)
RDwHISEcoRI	5′-TTG AAT TC TAC GTG GTGGTGGTGGTGGTG C TT C AG C CT G AT ACA G TC C-3′	72.4
PK2 kex2XhoI	5′-TTA TCT CGA GAA AAG AGC TGT TAT CAC TGG CGC CTG-3′	62.1
PK2EcoRIdw	5′-ATA GAA TTC TCA TTT CCG GGC CAA GCA AAT AAA CCG G- 3′	70.5
PKR1up BamHI	5′-CCG GAT CCA TGG AGA CCA CCA TGG GG-3′	65.0
PKR2 p BamHI	5′-ATA GGA TCC ATG GCA GCC CAG AAT GG-3′	65.0
DN up BamHI	5′- TAG GAT CCA TGA CCA TGC CTC CTC T-3′	58.0
GST-NR1dw	5′-TAG GAT CCG GAA TTG GTC ACA TCC TC-3′	65,0
GST-NR2dw	5′-TAG GAT CCTGGAATTGGTCACATCCTC-3′	65.0

2.3. Animals

Experiments were carried out in male C57BL/6 J WT, PKR1- or PKR2-null mice generated by Lexicon Genetics (The Woodlands, TX) weighing 25–30 g. Mice were housed in individual plastic cages, in a temperature-controlled environment (22 ± 2 °C), under a 12-h light/12-h dark cycle (07:00–19:00 h lights on), with food and water *ad libitum*.

2.4. Ethics statement

All procedures involving animal care or treatments (behavioral test and sacrifice by CO_2) were approved by the Animal Care and Use Committee of the Italian Ministry of Health (number: 116/2015-PR) and in compliance with the IASP and European Community (E.C.L. 358/118/12/86) guidelines. Every effort was made to minimize animal suffering and to reduce the number of animals used.

2.5. Drugs administration

PK2 and PK2β were injected at the appropriate dilution into the plantar region (i.pl.) of the right hind paw of mice in a volume of 20 µL. Control mice were injected with an equal volume of sterile saline solution.

2.6. Nociceptive behavioral tests

The nociceptive threshold to thermal stimuli was measured with *Hot-Plate Test,* while nociceptive threshold to tactile stimuli was measured with *von Frey filaments.*

Thermal hyperalgesia*:* mice were placed on a surface heated to 48 °C, surrounded by a Plexiglas cylinder (Hot Plate, Ugo Basile, Varese, Italy). The latencies to licking or flinching of the injected paw or to jumping in order to escape were recorded.

Tactile allodynia: was assessed using calibrated von Frey filaments (2 Biological Instruments, Italy) and an up-down paradigm (Chaplan et al., 1994). Animals were placed in individual Plexiglas boxes on a raised metal mesh surface and allowed to acclimatize for 30 min before test. Testing was initiated with a medium-sized filament, which was applied for 7 s to the plantar area, until the filament bent slightly. If the mouse withdrew or lifted the paw the response was considered positive and a filament one size smaller was tried. Conversely, if no response was observed, a filament one size larger was tried. The protocol was repeated until five changes in behavior had been observed. The 50% withdrawal threshold was determined according to the following equation: Xf + kD, where Xf is the value of the last von Frey filament used, k is the Dixon value for the positive/negative pattern, and D is the logarithmic difference between stimuli (Dixon, 1980).

The effect of the drug tested was calculated as the percentage change in nociceptive threshold from the baseline threshold (%ΔNT) according to the following equation: $(\%\Delta NT) = 100 \times (NT_{TS} - NT_B)/$