Biochimica et Biophysica Acta 1858 (2016) 1278–1287

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Interaction of lipids with the neurotensin receptor 1

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ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form 2 February 2016
Accepted 24 February 2016
Available online 21 March 2016

Keywords:
Neurotensin
GPCR
Lipids
Oligomerization
ESR

ABSTRACT

Information about lipid–protein interactions for G protein-coupled receptors (GPCRs) is scarce. Here, we use electron spin resonance (ESR) and spin-labelled lipids to study lipid interactions with the rat neurotensin receptor 1 (NTS1). A fusion protein containing rat NTS1 fully able to bind its ligand neurotensin was reconstituted into phosphatidylcholine (PC) bilayers at specific lipid:protein molar ratios. The fraction of motionally restricted lipids in the range of 40:1 to 80:1 lipids per receptor suggested an oligomeric state of the protein, and the result was unaffected by increasing the hydrophobic thickness of the lipid bilayer from C-18 to C-20 or C-22 chain length PC membranes. Comparison of the ESR spectra of different spin-labelled lipids allowed direct measurement of lipid binding constants relative to PC (K_r), with spin-labelled phosphatidylethanolamine (PESL), phosphatidylserine (PSSL), stearic acid (SASL), and a spin labelled cholesterol analogue (CSL) K_r values of 1.05 ± 0.05, 1.92 ± 0.08, 5.20 ± 0.51 and 0.91 ± 0.19, respectively. The results contrast with those from rhodopsin, the only other GPCR studied this way, which has no selectivity for the lipids analysed here. Molecular dynamics simulations of NTS1 in bilayers are in agreement with the ESR data, and point to sites in the receptor where PS could interact with higher affinity. Lipid selectivity could be necessary for regulation of ligand binding, oligomerisation and/or G protein activation processes. Our results provide insight into the potential modulatory mechanisms that lipids can exert on GPCRs.

1. Introduction

Several studies have shown that GPCR function is affected by the lipid environment (reviewed in ref. [1–4]), with most reports focusing on the role of cholesterol and lipid microdomains influencing receptor activity, localisation, and G protein coupling in celulla. However, the molecular mechanisms that govern these interactions are poorly understood, and there is little information on how specific lipid species interact with GPCRs in membranes. This is due to the technical challenges associated with expression [5,6], purification [7–9] and reconstitution [10–16] of GPCRs into model bilayers that allow controlled, specific analysis of lipid–protein interactions, and then comparison with functional assays. This type of detailed information is essentially limited to rhodopsin [17–20], which functions in the very specific membrane environment of the outer rod segment. The purpose here is to extend this knowledge for another prototype GPCR of pharmacological relevance, the neurotensin receptor 1 (NTS1).

Neurotensin is a tridecapeptide that functions as a neurotransmitter or neuromodulator in the central nervous system and as a local hormone in peripheral tissues. Three different receptors have been identified for neurotensin, namely NTS1, NTS2 and NTS3, of which NTS1 is the principal mediator of the known effects of neurotensin. The neurotensin receptor 1 belongs to the group A of GPCRs and homologues have been described in mammals (mouse, rat, humans), amphibians, fish and birds [21,22]. In rat, NTS1 is found in distinctive areas of the brain [23–26] and spinal cord [27] as well as in the heart, duodenum, small intestine, large intestine, and liver [23]. A wide distribution is also seen in humans [28] and other species [21]. Through its roles in central nervous system NTS1 has been associated with several neurological disorders [29]. It has also been found to play a role in cell proliferation and cancer, and is a potential biomarker [30] and target [31] in cancer therapy.

Abbreviations: CHS, cholesterol hemisuccinate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CSL, 4′,4′-dimethylspiro(5α-cholestane-3,2′-oxazolidin)-3′-yloxyl; C14:0PC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; C15:0PC, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine; C16:0–C18:1PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; C16:0–C18:1PE, (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine); C16:0–C18:1PS, (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine); C18:1PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; C20:1PC, 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine; C22:1PC, 1,2-dierucoyl-*sn*-glycero-3-phosphocholine; DDM, n-dodecyl-β-D-maltoside; ESR, electron spin resonance; GPCR, G protein-coupled receptor; MBP, maltose binding protein; NTS1, neurotensin receptor 1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; rPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulphonyl); SA, stearic acid; TM, transmembrane helix; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl) stearoyl]-*sn*-glycero-3-phosphocholine; 14-PESL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoethanolamine; 14-PSSL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoserine; 14-SASL, 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid.

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http://dx.doi.org/10.1016/j.bbamem.2016.02.032
0005-2736/

The wide tissue distribution of NTS1 and its involvement in numerous physiological and pathological processes highlight the extreme complexity of this receptor and anticipate an exquisite regulation of its functions at the molecular level. NTS1 is able to activate a range of signalling pathways through interaction with $G_{q/11}$ at its intracellular loop 3 (IC3) [32], and through interaction with $G_{i/o}$ and G_s at the first half of its C-terminal portion [33,34] and a recent study shows that this is complicated by the possible $G_{\alpha\beta\gamma}$ heterotrimers that can interact with NTS1 [35]. Some transduction pathways can be suppressed by mutation or deletion of particular intracellular domains while leaving unrelated pathways intact, suggesting that pathways can be individually regulated by direct interactions with the receptor [36]. Further complexity is added when considering the potential functional consequences from receptor homo- [10,11] or heterodimerisation [37,38], and modifications such as phosphorylation, acylation and glycosylation [39]. Finally, when considering the various lipid environments that NTS1 can experience in different cell types, the possibility arises that receptor function could be fine-tuned to serve particular cell type requirements according to cell type membrane composition, adding an extra dimension of complexity to receptor modulation.

Recently, we showed that binding of neurotensin to NTS1, receptor homodimerisation, and coupling to $G_{\alpha i1}$ are greatly affected by the specific lipid composition of the bilayer [40,41], and Grisshammer and co-workers showed that the same holds for G_q protein activation [12]. Here, we now analyse how major lipid species of the mammalian plasma membrane interact with the transmembranous region of rat NTS1 using a fusion protein termed NTS1-B. We present a protocol for reconstituting NTS1-B at low lipid to protein molar ratios in a controlled manner which allows us to measure directly interactions of spin-labelled lipids with the receptor using electron spin resonance (ESR).

2. Materials and methods

2.1. Materials

The phospholipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (C18:1PC), 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (C20:1PC), 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (C22:1PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulphonyl) (rPE) were obtained from Avanti Polar Lipids. The spin-labelled phospholipids 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine (14-PCSL), 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoethanolamine (14-PESL), and 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoserine (14-PSSL), and the spin-labelled fatty acid 14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearic acid (14-SASL) were synthesized as described by Marsh and Watts [42]. The spin-labelled cholesterol analogue 4′,4′-dimethylspiro(5α-cholestane-3,2′-oxazolidin)-3′-yloxyl (CSL), cholesterol hemisuccinate (CHS) and sodium cholate were obtained from Sigma-Aldrich. The detergents, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and n-dodecyl-β-D-maltoside (DDM) were from Melford Laboratories.

2.2. Protein production

The plasmid with the rat NTS1-B construct (MBP-N10-Tev-rT43NTR-CH2-N5G3S-G3S-TrxA-H10) was a gift of Dr. Grisshammer and is described in ref. [7]. The construct consists of the *E. coli* maltose binding protein (MBP, residues Lys^1 to Thr^{366}), a GSN_{10}ENLYFQSGS linker including a tobacco etch virus (Tev) cleaving recognition site (underlined), the rat NTS1 (residues Thr^{43} to Tyr^{424}), a $FQSN_5G_3SG_3SEF$ linker containing part of a second Tev cleaving recognition site which starts in the C-terminus of NTS1, followed by the *E. coli* thioredoxin (Trx, residues Ser^2 to Ala^{109}), and a GT link to a ten histidine tag. Expression and purification protocols have been extensively described [5,8]. NTS1-B was expressed in *E. coli* BL21 (DE3) cells. All steps were performed at 4 °C or on ice and when required between days along the protocol the sample was frozen in liquid N_2 and stored −80 °C. Cell pellets were solubilised with buffer A [50 mM Tris pH 8 (4 °C), 30% glycerol (*v*/*v*)] containing 200 mM NaCl, 0.1% CHS, 0.5% CHAPS, and 1% DDM (*w*/*v* in all cases for the detergents) and in the presence of protease inhibitors, lysozyme and DNAase. Insolubilised material was discarded after centrifugation (60 min; 65,000 *g*) and NTS1-B was purified first through a 5 ml Ni-NTA column eluting in buffer A with 350 mM imidazole, 200 mM NaCl, 0.1% CHS, 0.5% CHAPS, and 0.1% DDM. The eluate was diluted with buffer A containing 0.1% DDM and 0.01% CHS to reach 50 mM NaCl and then further purified through a neurotensin ligand affinity column. Pure NTS1-B was eluted in buffer A with 1 M NaCl, 0.1% DDM and 0.01% CHS. Fractions were frozen in liquid N_2 and stored at −80 °C.

2.3. Reconstitution into bilayers

The required amount of lipid from chloroform stocks was dried in glass vials as a thin film under a stream of N_2, then under vacuum ($<10^{-5}$ Torr) overnight. The lipid film was resuspended in buffer B [50 mM Tris pH 8 (4 °C), 50 mM NaCl] with 1 mM EDTA and the necessary concentration of sodium cholate to achieve 20 mM final concentration after addition of the receptor to the solubilised lipid. The resuspended lipid was fully solubilised to clarity in a bath sonicator (9 min; Langford Sonomatic Ultrasonics 475H). Purified NTS1-B was first concentrated by binding it to a 1 ml Ni-NTA column at a slow flow rate (rebinding the flow through once more markedly increased the amount of protein bound). The bound protein was washed with 50 ml of buffer B containing 0.1% DDM (*w*/*v*) to eliminate CHS. The receptor was eluted with 350 mM imidazole and the concentration determined by absorbance at 280 nm using a molar extinction coefficient of 138,785 cm^{-1} M^{-1} as calculated [43], using the ExPasy ProtParam Tool [44]. Subsequently, the appropriate amounts of solubilised lipid and protein were mixed to achieve the desired lipid:protein molar ratio. At this point, 2 mol% spin-labelled lipid (with respect to total lipid) was added to the mixture from ethanol stocks (except in the case of 14-SASL, which must be added later as it is otherwise lost in posterior dilution/dialysis), the final ethanol concentration in the mixture being lower than 1% (*v*/*v*), as described previously [17]. In the case of the samples for determination of relative lipid binding constants, a large sample was first prepared by mixing lipid and protein, and only then was the sample split into identical aliquots to which the required spin-labelled lipids were added (only 14-SASL was added later). This was to ensure the lipid:protein molar ratio in each of these samples was identical. Reconstitution was performed by 100 fold dilution with buffer B with 1 mM EDTA taking the detergents below their critical micelle concentration. Any remaining detergent was eliminated by extensive dialysis against 100× the sample volume (24 h; 4 °C) replacing the buffer after approximately 3 h and 7 h. Note that generally only 0.5–1 mol% spin-labelled lipid is necessary, but during dialysis about 50% becomes reduced so that it is necessary initially to add an excess. Reconstituted proteoliposomes were collected by ultracentrifugation (100,000 *g*; 2 h; 4 °C) and resuspended in about 1.5 ml of buffer B with 1 mM EDTA. A small sample was taken for determination of the final lipid:protein molar ratio, and 0.5 mol% 14-SASL was added to the corresponding sample from an ethanol stock ensuring that the final ethanol concentration was below 1%. Proteoliposomes were pelleted in a bench top centrifuge, loaded into 50 μl glass capillaries for ESR measurements, snap frozen in liquid N_2 and kept at −80 °C until the day of measurement.

2.4. Determination of lipid:protein molar ratio by absorbance spectroscopy

For determination of the final lipid:protein molar ratio in each sample, rPE was initially mixed with the lipids in chloroform prior to