Development of a CCK1R-membrane nanoparticle as a fish-out tool for bioactive peptides

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Abstract

The cholecystokinin receptor type 1 (CCK1R) is a G protein-coupled receptor (GPCR) that is involved in several biological processes including the regulation of the secretion of digestive enzymes. The peptide hormone cholecystokinin (CCK) binds to CCK1R, which is an important pharmacological target for several diseases, including obesity. Interestingly, nutritional dietary peptides also appear to activate CCK1R, and may play a role in CCK1R signaling in the gut. In this study, a novel technique to screen for CCK1R ligands based on affinity-selection is described. Functional expressed CCK1R is reconstituted into membrane nanoparticles called NABBs (nanoscale apo-lipoprotein bound bilayers). NABBs are native-like bilayer membrane systems for incorporation of GPCRs. CCK1R-NABBs were characterized using a fluorescently-labeled CCK analog and can be used as a cutting-edge technology to screen for CCK1R ligands using affinity-selection mass spectrometry.

Keywords:

Cholecystokinin receptor type 1, cholecystokinin, NABBs, GPCR, affinity-selection

1. Introduction

The cholecystokinin receptor type 1 (CCK1R) is a well-known G protein-coupled receptor (GPCR) that is involved in the regulation of several physiological functions including satiation induction, gall bladder emptying, and gut motility. The CCK1R is located in the stomach, gall bladder, small intestine and on vagal afferents in the gut. The CCK1R exerts its function upon binding with its natural ligand, the peptide hormone cholecystokinin (CCK). When food is ingested, CCK is produced by enteroendocrine I-cells of the small intestine and released at the basolateral side and into the blood where it subsequently binds to its receptor [7, 13, 16].

Previous studies have shown that certain protein hydrolysates, such as soy protein hydrolysate, contain *in vitro* potential to activate the CCK1R [19]. However, until now it was not possible to determine precisely which peptides are responsible for the observed CCK1R-activity. From this unresolved question, the motivation to develop a convenient and highly-selective identification tool for CCK1R-activating peptides arose. Moreover, the CCK signaling system is conserved throughout the bilaterians, with sulfakinin receptors (SKRs) in insects being homologous to CCKRs [26]. Therefore, the identification tool will also benefit the study of SKRs in insects and the evolutionary perspective of CCK signaling system in Bilateria in the animal kingdom.

NABB (nanoscale apo-lipoprotein bound bilayer) particles are a unique native-like bilayer membrane system for incorporation of GPCRs. They have a diameter of about 12 nm and consist of a discoid bilayer of phospholipids, held together by a protein belt structure from engineered zebrafish apo-lipoproteins (zap1) [2]. The advantage of NABBs over sol-gel entrapped proteins and proteins immobilized in vesicle membranes [3, 17] is that the GPCR can be incorporated rapidly into the NABBs and then remains in a more native conformation

while providing accessibility to both topological surfaces of the receptor simultaneously [2]. NABBs containing the CCK1R (CCK1R-NABBs) could be used as a highly innovative affinity selection mass spectrometry technique to fish out CCK1R-binding peptides from complex hydrolysate mixtures.

This study elaborates the first step to develop such a technique for the formation of CCK1R-NABBs. As shown in Fig. 1, to form CCK1R-NABBs, it was necessary to obtain the CCK1R and the belt protein, zap1, in a purified form. CCK1R was heterologously expressed in HEK-293T cells and CCK-dependent calcium flux was measured as was done previously for CHO-CCK1R cells [17]. Receptor internalization after ligand binding was also assessed. Receptor internalization is a cellular mechanism to desensitize the cell for circulating hormones that induce a biological response in the cell by reducing the number of cell surface receptors [22]. In addition, during these experiments, the functionality of a fluorescently labeled CCK peptide (FCKK) was evaluated. The CCK1R was purified using immunoprecipitation from the lysed HEK-293T cells. Purification of CCK1R was performed both with and without devazepide, a low MW CCK1R antagonist reported to stabilize the receptor [5]. Also a small amount of cholesterol detergent was added to facilitate CCK1R function [1, 15]. Zap1 protein was heterologously expressed in *Escherichia coli* and purified by affinity and subsequent size exclusion chromatography. In the final step, these proteins were combined with lipids and detergents in the presence of devazepide, which then self-assembled into NABBs. This is the first report on the incorporation of functional CCK1R into NABBs.

2. Materials and methods

2.1. Cells, plasmids and reagents

HEK-293T cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown at 37 °C and 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose and 2 mM glutamine), obtained from Life Technologies (Paisley, UK) and supplemented with 10% fetal bovine serum (FBS), obtained from Atlanta Biologicals (Lawrenceville, GA). pcDNA3.1(+), Lipofectamine PLUS, PLUS reagent, Lipofectamine 2000, OptiMEM medium, gels for SDS-PAGE and Dynabeads Protein G were also obtained from Life Technologies. CCK-8S was purchased from Bachem (Bubendorf, Switzerland). Black with clear bottom 384-well plates were purchased from Corning Life Sciences (Tewksbury, MA). Thirty-five mm² glass dishes were purchased from IBIDI (μ Dish high, Planegg, Germany). A FLIPR calcium 4 assay kit was purchased from Molecular Devices (St. Grégoire cedex, France). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), were obtained from Avanti Polar Lipids (Alabaster, AL). Dodecyl β-D-maltoside (DM), cholesteryl-hemasuccinate (CHS) and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Anatrace, Inc. (Maumee, OH). Sepharose 2B, poly-D-lysine-hydro-bromide (PDL), phenylmethanesulfonyl fluoride solution (PMSF) and devazepide (DVZ) were obtained from Sigma-Aldrich. Detergents were obtained from Cisbio (Bedford, MA). A Micro-Spin column was obtained from Pierce (Rockford, IL). An Amicon Ultra 10 kDa cutoff centrifugal filter device and Immobilon, a polyvinylidene difluoride (PVDF) membrane, were obtained from Millipore (Billerica, MA). 1D4 monoclonal antibody was couple to sepharose 2B resin using standard procedures. 1D5-peptide (TETSQVAPA) was purchased from Biobasic Canada Inc. (Markham, ON, Canada). Primary antibodies against the engineered 1D4 epitope were obtained from the National Cell Culture Center. Horseradish peroxidase (HRP)- conjugated α-mouse antibody, an α-His₆ tag monoclonal antibody conjugated with HRP-α-mouse, reagents for enhanced

chemiluminescence, Terrific Broth medium and Sephadex G-50 Superfine were purchased from GE Healthcare (Amersham, Freiburg, Germany). A HyBlot CL autoradiography film was purchased from Denville Scientific, Inc. (South Plainfield, NJ). His60 Ni resin was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Pierce detergent removal resin and BupH phosphate buffered saline packs were purchased from Thermo Scientific (Rockford, IL). A fluorescent labeled CCK analog (FCCK, ATTO647N -AEEAc-Gly-Asp-Tyr(SO₃H)-Nle-Gly-Trp-Nle-Asp-Phe-amide, AEEAc: Amino-Ethoxy-Ethoxy-Acetic acid) designed after the analog described by Harikumar et al. [9] with slight modifications, was purchased from Bachem.

2.2. Construction of CCK1R cDNA

The rat CCK1R cDNA (GenBank: M88096), with a C-terminal 1D4 epitope of 18 amino acids (DEASTTVSKTETSQVAPA) and a N-terminal serotonin 5HT3 membrane import sequence was constructed by Genewiz. The CCK1R and serotonin 5HT3 membrane import sequence were N-terminally preceded by a Kozak sequence (CACC) for better expression. A DYV linker was C-terminally incorporated after the 5HT3 membrane import sequence. Restriction enzymes used were EcoRI, BamHI and NotI. The rat CCK1R sequence was codon-optimized for expression in mammalian cells by Genewiz. An overview of the construct can be found in Supplementary Fig. 1. The construct was subcloned into pcDNA3.1(+) using either EcoRI and NotI (further referred to as E/N) or BamHI and NotI (further referred to as B/N). In the former case, the serotonin 5HT3 membrane import sequence was included in the construct, but in the latter it was not.

2.3. Transfection of HEK-293T cells.

2.3.1. For receptor purification

HEK-293T cells were transfected with the plasmid DNA using Lipofectamine PLUS as previously described for the rhodopsin receptor [23]. In brief, 3.5 µg of plasmid DNA and 10 µl of PLUS reagent were dissolved in 0.75 ml DMEM and incubated for 15 min at room temperature. Next, this mixture was added to 0.5 ml DMEM containing 17 µl of Lipofectamine PLUS and incubated for another 15 min after which the total volume was brought to 4 ml with DMEM. Subsequently, this solution was added to a 10 cm diameter cell culture dish containing HEK-293T cells at 70-80% confluence. After 4 h, 4 ml of DMEM with 20% FBS was added to the cells. The transfected cells were incubated at 37 °C with 5% CO₂ and harvested after 48h with PBS containing 1% PMSF. The cell pellets were stored at -20 °C until further use.

2.3.2. For fluorescent measurements

For fluorescent measurements using a plate reader, HEK-293T were transfected while replated in 384-well plates (black with clear bottom) with either the E/N or B/N construct. The plates were pretreated with 10 µl of 0.1 mg/mL of PDL per well, washed three times with double distilled water and air dried. An amount of 10 ng DNA was mixed with 0.075 µl of Lipofectamine 2000 and 10 µl DMEM per well and incubated at room temperature for 20 min. This mixture was added to 10 µl per well of DMEM supplemented with 20% FBS, containing 800,000 cells/ml and subsequently plated in the 384-well plates. Control cells were transfected with the same amount of empty vector DNA (pcDNA3.1(+)). Cells were incubated for 48 h at 37 °C with a 5% CO₂ atmosphere.

For fluorescent measurements using fluorescence microscopy, HEK-293T cells were cotransfected with CCK1R B/N and green fluorescent protein (GFP, pEGFP-C1, Genbank: U55763) while replated in 35 mm² glass dishes. The glass dishes were pretreated with 700 µl of 50 µg/mL PDL in 2 mM HEPES for 1h and then washed three times with nuclease free water and dried overnight. For one dish, 0.8 µg of CCK1R B/N and 0.8 µg of GFP were mixed in 100 µl of Optimem medium and 4 µl of Lipofectamine was added to 100 µl of Optimem medium in a separate tube. These two mixtures were incubated for 5 min. at room temperature and then combined and further incubated for an additional 20 min. After that, 400 µl of DMEM with 10% FBS was added to the DNA-Lipofectamine mixture. Meanwhile, HEK-293T cells were diluted to 333,000 cells/ml in DMEM with 10% FBS. Subsequently, 600 µl of the DMEM containing the DNA-Lipofectamine mixture was mixed with 600 µl of the diluted cell suspension and seeded in a glass dish. For control cells, empty vector DNA instead of CCK1R B/N was used. Cells were incubated for 48 h at 37 °C with 5% CO₂.

2.4. Calcium-flux measurement in HEK-293T cells expressing the CCK1R construct

Calcium-flux in HEK-293T cells transfected with the CCK1R B/N construct or the empty vector after addition of CCK-8S or FCCK-8S was measured in a 384-well plate using a FlexStation II 384 Plate Reader (Molecular Devices). Two days after transfection, cells were incubated for 1 h at 37 °C with 20 µl dye (FLIPR Calcium 4 Assay Kit) per well. The dye was solved in HBSS-H (Hank's Balanced Salt Solution, with 20mM HEPES buffer, pH 7.4), supplemented with 0.4% BSA according to the manufacturer. Subsequently the plate was allowed to stand for 15 min before the start of the measurement in the plate reader preheated to 37 °C. The ligands were diluted in HBSS-H supplemented with 0.4% BSA. The fluorescence was monitored during 90 s, with a 2.5 s interval and 10 µl of the ligands were

1 added *in fluxo* 20 s after the start of the measurement. The excitation and emission
2 wavelengths were respectively set to 488 nm and 530 nm. Each sample concentration was
3 measured in three wells (technical replicates) for both transfections and every experiment was
4 repeated three-four times (biological replicates). The difference between the maximum
5 fluorescence signal and the baseline ($\Delta\text{max-min}$) was calculated for each cell type and
6 subsequently that of the control cells was subtracted from that of the cells transfected with
7 CCK1R to correct for the background fluorescence of the fluorescent labeled ligand. From
8 these results, sigmoid dose-response curves for $(\Delta\text{max-min})_{\text{CCK1R}} - (\Delta\text{max-min})_{\text{control}}$ versus
9 sample concentration were derived with GraphPad Prism (La Jolla, CA) and EC_{50} values were
10 calculated. The EC_{50} values are the mean of at least three independently repeated dose-
11 response curves (biological replicates), which are based on triplicates (technical replicates) for
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31 **2.5. Receptor internalization**

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37 Visualization of receptor internalization was performed on HEK-293T cells in glass
38 dishes cotransfected with GFP and the CCK1R B/N construct or the empty vector using a
39 Zeiss AxioVert 200M inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).
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44 Two days after transfection, the medium in the glass dishes was exchanged with the assay
45 medium (DMEM-F12 containing 15 mM HEPES and supplemented with 10% FBS and 1%
46 L-glutamine). The dishes were kept at room temperature. Subsequently, half of the dishes
47 were incubated for 15 min in assay medium supplemented with 1 μM of devazepide (DVZ), a
48 CCK1R antagonist. The ligands were dissolved in DMSO and diluted in the assay buffer in
49 such a way that the concentration of DMSO in the assay medium was 1% (v/v). The medium
50 was removed from the dishes and replaced with 1 ml of assay medium containing FCCK for
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30 s. In the case of pretreatment with devazepide, assay medium containing FCCCK and 1 μ M devazepide was used. Then the medium was removed and after 45 s, the dishes were washed with 1 ml of assay medium. The washing step was repeated two more times. The dishes were kept at 4 °C and re-equilibrated to room temperature for 20 min before imaging. Per dish, 7x7 regions were imaged using a motorized x,y-stage with CRISP aligned to the glass/media interface using an X-Cite 120 lamp (Lumen Dynamics, Mississauga, Canada) with an intensity of 4 to 5. Three types of images were acquired for each position: 500 ms of brightfield exposure, 5 ms of epifluorescence with a 18 nm bandpass centered at 482 nm to visualize the GFP, and 2500 ms of epifluorescence with a 14 nm bandpass centered at 640 nm to visualize FCCCK, using 20 x gain on an electron multiplying-charge coupled device (EM-CCD), eVolve 512 (Photometrics, Tucson, AZ). The emission filter is a quadruple bandpass (centered at 446, 523, 600, and 677 nm, Semrock, Rochester, NY).

2.6. Solubilization and purification of heterologously expressed CCK1R

The cell pellets of the transfected HEK-293T cells were lysed with 1 ml of lysis buffer (DPBS containing 19.6 mM DM, 1.96 mM CHS-Tris, 50 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM CaCl₂ and protease inhibitors) per 10 cm diameter dish for 1 h on a nutator and subsequently centrifuged at 1,000 g for 15 min at 4 °C to remove the cell debris. Next, 100 μ l of 1D4-sepharose resin was added to the cleared lysate and incubated on a nutator for 16 h at 4 °C. The resin was spun down at 1,000 g for 10 min and the supernatant was removed. It was subsequently washed three times for 15 min with wash buffer (DPBS containing 1.96 mM DM, 0.196 mM CHS-Tris). Then the resin was put into a Micro-Spin column and the receptor was eluted by incubating the beads with 100 μ l of 1D5-peptide solution (0.36 mg/ml in wash buffer) per plate on ice for 30 min and subsequent centrifugation for 5 min at 10,000 g. The

1 elution was repeated twice and analyzed with sodium-dodecyl-sulfate-polyacrylamide gel
2 electrophoresis (SDS-PAGE) followed by western blot. Experiments were performed both
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4 with and without 5 μ M devazepide.
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9 **2.7. Western blot**

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14 Western blot analysis was performed as described before [10]. First, samples were
15 resolved by SDS-PAGE (NuPage Novex 4-12% Bis-Tris Gel) and subsequently transferred
16 onto a PVDF membrane using a semi-dry transfer cell (Biorad). To detect the CCK1R, a
17 primary antibody against the 1D4 epitope (1:5,000) and HRP (horseradish peroxidase)-
18 conjugated anti-mouse (1:10,000) as secondary antibody were used. Zap1 was probed with an
19 α -His₆ tag monoclonal antibody conjugated with HRP- α -mouse. The membrane was treated
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36 **2.8. Production and purification of zap1**

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41 Recombinant zebrafish apo-lipoprotein (zap1) was produced and purified as described
42 previously by Banerjee et al. [2] with slight modifications. BL21-DE3 cells, a *rosetta2* strain
43 of *E. coli*, were transformed with pE28-zap1. A starter culture was grown overnight in LB
44 medium supplemented with 50 μ g/ml kanamycin and chloramphenicol. The next day, this
45 starter culture was diluted 1:100 in 1 liter of Terrific Broth medium. When the OD₆₀₀ (optical
46 density at 600 nm) reached 0.8, expression of zap1 (see Supplementary Fig. 2. for protein
47 sequence) was induced with 1 mM isopropyl- β -D-thiogalactopyranoside.
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58 After 2 h of incubation, the cells were harvested by centrifugation and the cell pellet
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was resuspended in 50 ml storage buffer (40 mM Tris pH 8, 0.3 M NaCl, 5 mM 2-mercaptoethanol, 1x aprotinin, complete EDTA-free protease inhibitor tablets (1 tablet per 50 ml), 2 mM PMSF) and stored at -80 °C. The resuspended cells (50 ml) were thawed on ice and 500 µl of 100% Triton and 50 µl of DNase were added to the cells and incubated on ice for 30 min. Then, 25 mL of the sample was mixed with 10 ml of Tris-buffer (25 mM Tris pH 8, 0.3 M NaCl) and sonicated for 15 min with intervals of 5 s (5 s “on”, 5 s “off”) with an output level of 24 W or greater. Next, the zap1 protein was purified using affinity chromatography. Two ml of His60 Ni Superflow Resin was equilibrated by adding 20 ml of equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 7.4) on ice. Then, the resin was spun down (1,500 g, 5 min, 4 °C) and the supernatant was removed. Next, 25 ml of the sample was applied to the resin and put on a nutator for 1 h at 4 °C. The resin was spun down (1,500 g, 5 min, 4 °C) again and the supernatant containing the cell debris was removed. Next, 20 ml of equilibration buffer and 20 ml of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole, pH 7.4) were applied to the column (resin was spun down and supernatant was removed after each step). Then, 10 ml of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 7.4) was added to the resin to elute the recombinant zap 1. Again the resin was spun down and the supernatant containing the zap1 was concentrated using a 10 kDa MWCO centrifugal filter. The buffer was exchanged with Tris-HCl pH 8 and 1 mM EDTA buffer to get rid of the imidazole. This sample was applied to a Microcon-MC 0.45 µm microporous membrane spin filter unit to remove protein precipitate. The sample was further purified by size exclusion chromatography (SEC) using a Superdex 200 10/30 GL column connected to an Äkta purifier at a flow rate of 0.5 ml/min buffer G (10 mM Tris pH 8, 0.15 M NaCl, 0.5 mM TCEP). Fractions of 0.5 ml were collected. The fractions containing the sample were combined and concentrated using a 10 kDa MWCO centrifugal filter. The quality of the purification of each

step was analyzed with SDS-PAGE.

2.9. Incorporation of CCK1R into NABBs

The method as described by [10] with slight modifications was followed to make NABBs. A ratio of 1/75 belt protein zap1/lipids was used. For NABBs containing the CCK1R (CCK1R-NABBs), 50 μ l of the CCK1R elution was combined with 2.5 nmol of zap1, 187.5 nmol of POPC (14.25 μ l of 1% (w/v) POPC/CHAPS), 0.33% DM-CHS buffer (196 mM DM, 19.6 mM CHS-Tris), 1.5% sodium cholate and brought to 150 μ l with buffer S (20 mM Tris-HCl (pH 7), 100 mM $(\text{NH}_4)_2\text{SO}_4$ and 10% glycerol) and incubated on ice for 30 min. Subsequently, this mixture was put on 1 ml of Pierce Detergent Removal resin which was pre-equilibrated with two column volumes (CV) of buffer S. The elution was done under gravity flow by addition of buffer S. Five fractions of 150 μ l were collected and the protein content was determined by monitoring the absorbance at 280 nm. To make empty NABBs (NABBs without receptor), the same recipe was followed but the 50 μ l of CCK1R elution was replaced by 50 μ l of PBS without Ca^{2+} and Mg^{2+} supplemented with 0.1% of DM-CHS buffer. Experiments were performed with and without 5 μ M devazepide.

2.10. Binding of FCCK to CCK1R-NABBs

CCK1R-NABBs were further purified using a Sephadex G-50 Superfine SEC column with a CV of 2 ml to remove the 1D5-peptide from receptor purification. To prepare the column, the Sephadex G-50 resin was first blocked with 5 CV of 10 mg/ml BSA in BupH (0.1 M Na_3PO_4 , 0.15 M NaCl, pH 7.2) and subsequently washed twice with 5 CV of BupH. Then, 300 μ l of CCK1R-NABBs from the combined third and fourth elution obtained after

detergent removal was applied to the column and five fractions of 300 μ l were collected on ice by addition of buffer S on the column. In the meantime, Dynabeads labeled with 1D4-antibody were prepared as follows: 25 μ l of Dynabeads Protein G were washed twice in 100 μ l of BupH and subsequently incubated with 100 μ l BupH supplemented with 1 mg/ml 1D4-antibody for 10 min at room temperature. Next, the labeled beads were washed in 100 μ l Buffer S and incubated for 1 h on ice with 200 μ l of CCK1R-NABBs from the third fraction resulting from the Sephadex G-50 SEC, to capture the CCK1R-NABBs on the beads via the 1D4-tag in the CCK1R. The unbound fraction was washed away with 100 μ l of buffer S. Then, a solution of 1 nM FCCCK and a solution of 1 nM FCCCK and 10 μ M CCK-8S were prepared in buffer S. Next, 20 μ l of each solution together with 2 μ l of the CCK1R-NABBs captured on 1D4-beads was added to a 384-well plate and fluorescence was acquired with a Zeiss AxioVert 200M inverted microscope using similar settings as described in 2.5. 3.

3. Results

In preliminary experiments, the expression levels of both two different constructs, i.e. CCK1R E/N and CCK1R B/N, after transfection in HEK-293T cells were compared via Western blot. The HEK-293T cells transfected with CCK1R B/N construct yielded higher receptor expression as well as higher calcium flux (data not shown). Therefore, it was decided that all further experiments would be performed using the CCK1R B/N construct.

3.1. Functionality of heterologously expressed CCK1R and fluorescently labeled CCK peptide (FCCCK)

The functionality of the CCK1R after heterologous expression in HEK-293T cells after

transfection with CCK1R cDNA, was evaluated with two different assays: 1) measuring the calcium flux after activation of the receptor, and 2) assessing receptor internalization after ligand binding. The calcium flux was measured for both the natural ligand CCK-8S and FCCCK (as depicted in Fig. 2**Error! Reference source not found.**).

3.2. Calcium-flux measurement

The calcium flux induced by FCCCK in HEK-293T expressing the CCK1R was compared to the one induced by the natural ligand CCK-8S. Ligand concentrations ranging from 0.002 to 1000 nM were tested, and HEK-293T cells transfected with the empty vector were used as a negative control. The control cells exhibited no increase in fluorescence, except for the highest concentrations of FCCCK, which is probably due to the background fluorescence of the ligand itself. Therefore it was decided to subtract the fluorescence increase of the control cells from that of CCK1R-expressing cells. Sigmoid dose-response curves were generated for the corrected calcium flux $(\Delta\text{max-min})_{\text{CCK1R}} - (\Delta\text{max-min})$ versus sample concentration, and then EC_{50} values were calculated (Fig. 2**Error! Reference source not found.**). The EC_{50} values were 7.0 ± 4.5 nM (mean $\pm$ SEM, $n=3$) and 69 ± 41 pM (mean $\pm$ SEM, $n=3$) for FCCCK and CCK-8S, respectively. The lower EC_{50} value of FCCCK compared to that of CCK-8S suggests a lower affinity of FCCCK towards the CCK1R, which can arise from steric hindrance of the dye. However, the full agonism of FCCCK seemed to be retained as the highest calcium flux that can be obtained is similar for both ligands. Furthermore, the EC_{50} value found for CCK-8S in this experiment for HEK-293T cells is not significantly different from the results obtained for CHO-cells heterogeneously expressing the CCK1R (24 ± 4 pM) as obtained in an earlier study [18].

3.3. Receptor internalization

HEK-293T cells were co-transfected with GFP and the CCK1R cDNA at a ratio of 1:1. The cells were incubated for 30 s with FCCK, with or without pre-incubation with devazepide. Subsequently, receptor internalization was visualized by monitoring the fluorescence of FCCK. Three different concentrations (10 nM, 100 nM and 1000 nM) of FCCK were tested, but only the highest concentration enabled visualization of the internalization of FCCK-bound CCK1R (Fig. 3**Error! Reference source not found.**). To confirm that FCCK binds specifically to the CCK1R and that the observed internalization of FCCK is not due to non-specific endocytosis, the experiment was repeated in the presence of 1 μ M devazepide, an antagonist. When treated with devazepide, no more fluorescence from FCCK could be seen. Also the control cells that were transfected with GFP and the empty vector only showed fluorescence for GFP and no fluorescence for FCCK. These experiments demonstrate specific binding of FCCK to the CCK1R.

From the calcium flux assay and the receptor internalization experiment, it can be concluded that the CCK1R is functionally expressed in the CCK1R and that FCCK is functional as a full agonist, based on the equal amount of calcium that can be released by CCK-8S and FCCK, for this receptor.

3.4. Solubilization and purification of recombinant CCK1R

HEK-293T cells heterologously expressing the CCK1R, were lysed and the CCK1R was captured via its 1D4-tag on 1D4-sepharose resin. It was subsequently eluted using 1D5-peptide. On the Coomassie-stained gel after SDS-PAGE, the cell lysate contained a wide range of proteins that cannot be detected anymore in the elutions containing the purified

receptor (Fig. 4). The CCK1R including the 1D4-tag had a MW of 51.3 kDa. On the Western blot (Fig. 4), the CCK1R was detected as a band around 60 kDa, which is higher than the calculated MW, but this is probably due to glycosylation of the receptor. Treatment of the sample with PNGaseF to remove the N-glycans resulted in a decrease in MW (data not shown). The band around 60 kDa was clearly enriched for the CCK1R elutions in comparison to the cell lysate, showing that the purification of the CCK1R was successful. The band around 20 kDa seen in the CCK1R elution lane can be 1D4-antibody that came off the 1D4-beads. For the cell lysate, also a band around 150 kDa and 37 kDa appeared; the former can be a dimerized receptor and the latter is probably the truncated receptor, which was removed during purification as they interact with the 1D4-beads. As a reference, 10 ng of purified rod-outer-segment (ROS) rhodopsin was loaded onto the gel. Approximately 50 ng of CCK1R was present in 6.5 μ l of CCK1R elution loaded on the gel. This means that about 3 μ g of purified CCK1R can be obtained per 10 cm diameter culture dish of HEK-293T cells transfected with 3.5 μ g of pcDNA.

3.5. Production and purification of recombinant zap1

The zap1 protein was expressed in *E. coli* BL21-DE3 and purified using affinity chromatography (AC) and subsequent SEC (Supplementary Fig. 3**Error! Reference source not found.**). From the Coomassie-stained gel obtained with SDS-PAGE in Supplementary Fig. 3a, it can be seen that after elution of the His60 Ni Superflow Resin for AC, the band of zap1 (MW 30 kDa) was enriched but impurities were still present. Therefore this sample was applied on a Superdex 200 10/30 column for further purification using SEC (Supplementary Fig. 3b).

Fractions of 0.5 ml were collected and the purity was assessed with SDS-PAGE

(Supplementary Fig. 3a). A clear band around 30 kDa can be seen with less impurities compared to before SEC. Fractions 22 to 36 were combined and concentrated using a 10 kDa MWCO centrifugal filter. In total, 800 µl of purified recombinant zap1 having a concentration of 9.48 g/l, as determined with UV-VIS, was obtained. This means that the yield for this purification method was 18 mg zap1 for 1 liter of cell culture.

3.6. Formation of NABBs

NABBs with and without the CCK1R were made, by combining the belt protein and the lipids in the presence of detergent. Measurement of the protein concentration in the different fractions obtained after detergent removal using the Pierce detergent removal resin, indicated that most of the protein was present in fraction 3 and 4. These fractions were combined and analyzed with western blot against the 1D4- and His₆-tag (Fig. 5) of the CCK1R and zap1 protein, respectively. For empty NABBs, only a band for zap1 could be seen. However, for CCK1R-NABBs a band for zap1 and for the CCK1R was present, indicating the formation of CCK1R-NABBs.

3.7. Binding of FCCCK to CCK1R-NABBs

This experiment was performed without the presence of devazepide as a stabilizing agent for the receptor, as no benefit of adding devazepide could be proven in preliminary experiments. To evaluate whether the CCK1R incorporated into the NABBs was functional, an attempt was made to visualize binding to the CCK1R in NABBs with FCCCK. Therefore, 1 nM of FCCCK was added to the CCK1R-NABBs with or without co-incubation of 10 µM of CCK-8S as a competitor. It was expected to see less fluorescence on the beads when the

sample was co-incubated with CCK-8S. However, the opposite occurred. The reaction where no CCK-8S was used showed no fluorescence, whereas the incubation with CCK-8S did show fluorescence and even single molecules could be observed (Fig. 6). It indicated that the FCCCK was unable to bind to the CCK1R in NABBs and that the addition of CCK-8S facilitated the binding of FCCCK to CCK1R in NABBs.

4. Discussion

From the calcium flux and receptor internalization assays, it could be confirmed that the CCK1R is functionally expressed in the HEK-293T cells. The necessary concentration to visualize receptor internalization, namely 1 μ M of FCCCK, was very high in comparison with the EC_{50} of the calcium flux (7 nM) of this ligand. However, this is in agreement with a study of Wu et al. [22] where a concentration of 1 μ M CCK-8S was used to visualize internalization of labeled CCK1R receptor, while the EC_{50} value for calcium flux was around 0.3 nM. As FCCCK appeared to be able to fully activate the CCK1R with only a 100-fold lower affinity than CCK-8S, this ligand can thus be used in future experiments for the examination of ligand-receptor binding.

Furthermore, the purified zap1 protein and the CCK1R were sufficiently clean to be further incorporated into NABBs. The Western blot showing bands of both CCK1R and zap1 for CCK1R-NABBs, and only a band of zap1 for empty NABBs indicates the formation of CCK1R-NABBs. In a study of Szecowka et al. [20] on the purification of the CCK1R from pancreas, it is reported that after solubilization, the CCK1R does retain its affinity and specificity for its ligands. The current study indicated that the CCK1R in the prepared NABBs could bind with FCCCK only when CCK-8S was also present. Therefore, it can be suggested that CCK-8S acts as a primer for the activation of CCK1R, which phenomenon may lead to

cooperativity [6]. Hence, it has been reported that GPCRs have multiple signaling states [14]. The binding of CCK-8S to CCKR1 may induce certain functionally distinct conformational state of CCK1R, which FCCCK can bind to efficiently. However, this remains to be illustrated in future experiments due to the lack of experimental evidence to date.

The CCK1R is an important regulator in weight maintenance and satiety induction. Previous anti-obesity studies focusing on this receptor have shown that protein hydrolysates can activate the CCK1R [8, 19]. However, as these protein hydrolysates are complex in composition, it remained impossible to identify the peptide (or peptides) that was (were) responsible for the activity. In insects, the homolog system of the CCK pathway consists of the membrane-bound SKR and sulfakinin (SK) ligand that also regulate insect feeding. Treatment with SK ligand could reduce food intake in different insects, including important pests [11, 12, 21, 24, 25].

We believe that the technique as described here can be used as a “fishout” tool to identify peptides that bind to the CCK1R, and can give inspiration for a similar strategy also to find ligands for the SKR or other membrane-bound protein receptors. The CCK1R-NABBs can be incubated with the peptide mixture and unbound peptides can be washed away. Consequently the bound peptides will be eluted using high-affinity CCK1R ligands, such as CCK-8S, and can be identified in the eluents by mass spectroscopy (MS). This procedure will need to be optimized in terms of ligands and buffers used for elution. CCK is very well suited to elute the bound peptides, however it might interfere with MS identification. Therefore, small molecule ligands are the preferred choice for elution, as they are structurally distinct from peptides and will greatly simplify MS identification. Nevertheless, it should also be taken into account that these might have allosteric binding sites and therefore might not be able to elute the peptides. Also, if the peptides are rather hydrophobic, they might stick to the NABBs themselves. Finally, it can be mentioned that a concept similar to the CCK1R-

1 NABBs as a “fish-out” tool, has been previously described by Borch et al. [4] to identify
2 proteins that interact with membrane glycolipids. This technique therefore cannot only be
3
4 used to screen for bioactive peptides binding to the CCK1R, but can also be applied to other
5
6 (pharmaceutical) components.
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10 11 **Acknowledgement**

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Figure legends

Fig. 1. Schematic view of the formation of CCK1R-NABB (CCK1R-nanoscale apo-lipoprotein bound bilayer) particles. They have a diameter of about 12 nm and consist of a discoid bilayer of phospholipids and recombinantly produced CCK1R, and are held together by a protein belt structure from engineered zebrafish apo-lipoproteins (zap1).

Fig. 2. Representative dose-dependent CCK1R-mediated calcium fluxes in HEK-293T cells for increasing concentrations of CCK -8S and FCCCK (0.002 – 1000 nM) based on 3 experiments (biological replicates) in which the measurements for each concentration were repeated 3 times (technical replicates).

Fig. 3. Image of HEK-293T cells transfected with either GFP and CCK1R or GFP and the empty vector after incubation with 1 μ M FCCCK, with or without treatment with 1 μ M devazepide (transmission). For each image (82 μ m squares) the fluorescence of GFP (excitation/emission wavelength 488/507 nm) and of FCCCK (excitation/emission wavelength 488/507 nm) are shown.

Fig. 4. Coomassie stained gel and Western blot against 1D4 for recombinant CCK1R purification. 6.5 μ l of cell lysate and combined CCK1R elutions were loaded on the gel.

Fig. 5. Western blots indicating formation of NABBs with and without receptor. Western blots were performed against the 1D4 tag of the CCK1R and the His-tag of zap1. Twenty microliters of NABBs were loaded on the gel; samples were analyzed after affinity

1 chromatography (AC) and size exclusion chromatography (SEC). As a reference, 1 μ l of the
2 CCK1R elution and 25 ng of zap1 were also loaded on the gel.
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10 **Fig. 6.** Dynabeads that hold CCK1R-NABBs in presence of 1 nM FCCK with or without co-
11 incubation with 10 μ M of CCK-8S.
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Figure 1

Figures

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

1 nM FCCK

1 nM FCCK + 10 μ M CCK-8S