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Agonist potency differentiates G protein activation and Ca^{2+} signalling by the orexin receptor type 1

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$[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration
CMV, cytomegalovirus promoter
EGFP, enhanced green fluorescent protein
OX_1R, orexin receptor type 1
MAPK, mitogen-activated protein kinase

ABSTRACT

The G protein coupling characteristics of a flag epitope-tagged orexin receptor type 1 (OX_1R) was investigated in HEK293 cells. Immunoprecipitation of the OX_1R and immunoblotting revealed interactions with G_q/G_{11} proteins as well as with G_s and G_i proteins. Stimulation with orexin-A did not affect the ability of the OX_1R to coprecipitate G_q/G_{11} proteins, but it robustly elevated the intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$. No changes in cAMP levels could be detected upon receptor stimulation. To get further insight into the functional correlation of G protein activation and Ca^{2+} signalling, we used baculovirus transduction to express chimeric G proteins, containing the $G\alpha_s$ protein backbone with various $G\alpha$ donor sequences ($G\alpha_{s/x}$) at the N and C termini, and measured cAMP as functional output. The $G\alpha_{s/x}$ chimeric proteins with $G\alpha_{11}$($G\alpha_q$) and $G\alpha_{16}$ structure in the C terminus were stimulated by the OX_1R. Concentration–response curves with $G\alpha_{s/16}$ revealed an agonist potency correlation between G protein activation and the elevation of $[Ca^{2+}]_i$ via discharge of intracellular Ca^{2+} stores, a feature also recognized for the muscarinic M_3 receptor. However, in contrast to the M_3 receptor, the OX_1R elevated $[Ca^{2+}]_i$ via influx from extracellular space at about 30-fold lower agonist concentration. The results suggest that the OX_1R is linked to influx of Ca^{2+} through a signal pathway independent of G_q/G_{11} protein activation.

1. Introduction

Orexins, also named hypocretins, are hypothalamic peptides known to activate two distinct membrane receptors, orexin receptor type 1 (OX_1R) and type 2 (OX_2R) [1,2]. In vivo the orexins are involved in the regulation of food intake and energy metabolism as well as vigilance and sleep/wake status [3–5]. At the neuronal level, orexins display a multitude of excitatory actions, such as increased synaptic activity, depolarisation and Ca^{2+} entry [2,6–11]. OXRs appear to couple

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to several different signalling pathways. Initial studies in heterologous expression systems indicated that both receptor subtypes couple to robust Ca^{2+} elevations [1,12]. This action was suggested to be due to activation of the G_q/G_{11} proteins–phospholipase C–$InsP_3$ pathway. A feature characteristic of G_q/G_{11} coupling is a primary Ca^{2+} discharge from internal stores followed by a capacitative Ca^{2+} entry from outside the cell. Others and we have found that the Ca^{2+} elevation at low concentrations of orexins is strictly dependent on extracellular Ca^{2+} [12,13] and membrane potential [13]. This suggests that OXRs activate a distinct pathway for Ca^{2+} entry. In addition, evidence for a role of transient receptor potential canonical channels in orexin-activated Ca^{2+} entry has been presented [14].

In certain neuroendocrine tissues and cells, orexins promote secretion [15–18]. In the adrenals, where the signalling mechanisms have been studied in more detail, the OXRs appear to activate G proteins associated with adenylate cyclase regulation, i.e. G_i/G_o and G_s proteins [17,19,20]. In a recombinant cell model, second messenger measurements have indicated a highly potent activation of pertussis toxin-sensitive G proteins by the OX_2R [21]. When heterologously expressed in Chinese hamster ovary cells, also the OX_1R shows interactions with the adenylate cyclase system via both pertussis toxin-sensitive and -insensitive mechanisms [22].

The versatility of orexin-mediated signalling pathways creates a need to study interactions between the OXRs and the G proteins directly and how these interactions relate to the cellular responses observed upon receptor stimulation. In this study we have studied how the G protein interactions of the OX_1R relate to Ca^{2+} signalling using immunoprecipitation of receptor–G protein complexes and functional G protein activation output of chimeric $G\alpha_s$-based G proteins.

2. Materials and methods

2.1. Materials

Orexin-A was from Bachem (St. Helens, UK). The OX_1R antagonists [^{3}H]-SB-674042 (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((*S*)-2-(5-phenyl-(1,3,4)oxadiazol-2-yl-methyl)-pyrrolidin-1-yl)-methanone) (27 Ci/mmol) [23] and SB-334867 (1-[2-methylbenzoxazol-6-yl]-3-(1,5)naphthyridin-4-yl-urea hydrochloride) [24] were kind gifts from Drs. Neil Upton and Christopher Langmead (Neurology CEDD, GlaxoSmithKline Pharmaceuticals, Harlow, UK). Dynazyme II™ was from Finnzymes (Espoo, Finland), FuGENE6™ from Roche (Espoo, Finland) and fura-2 acetoxymethyl ester from Molecular Probes (Eugene, OR, USA). The anti-FLAG M2 affinity gel and mouse anti-FLAG M2 antibody were from Sigma–Aldrich, Finland (Helsinki, Finland). Rabbit anti-OX_1R antibody was from Alpha Diagnostic International (San Antonio, TX, USA), rabbit anti-$G\alpha_q/G\alpha_{11}$ and rabbit anti-$G\alpha_o$ from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-$G\alpha_{i1/2}$ and rabbit anti-$G\alpha_s$ from Calbiochem (San Diego, CA, USA). Digitonin, Forskolin, IBMX (3-isobutyl-1-methyl-xanthine) and carbachol (carbamylcholine chloride) were from Sigma–Aldrich, Finland (Helsinki, Finland).

2.2. Gene transfer vectors

The human OX_1R cDNA in pcDNA3 (Invitrogen, Carlsbad, CA, USA) was a gift from Dr. M. Detheux (Euroscreen s.a., Bruxelles, Belgium). For expression of the epitope-tagged receptor, the ORF of OX_1R was amplified by PCR using the sense primer 5′-ccatggagccctcagccacc, the antisense primer 5′-aggcctcgagcgtcaccacagtgc (stop codon removed and replaced by an *Xho*I site) and Dynazyme II™. A PCR fragment of correct size was inserted into pGEM-T Easy (Promega, Madison, WI, USA) to make pGEM-OX_1R. To generate a fusion protein the 99 bp *Mbi*I–*Xho*I 3′-end fragment was cut out from pGEM-OX_1R and ligated together with the 1.1 kbp *Eco*RI–*Mbi*I 5′-end fragment of the original OX_1R cDNA into pIRES-hrGFP-1a (Stratagene, La Jolla, CA, USA). DNA sequencing confirmed that the OX_1R ORF was in frame with the triple flag sequence.

The cDNA for bovine $G\alpha_s$ [25] in pVL1392 was a kind gift from Dr. T. Haga (University of Tokyo, Tokyo, Japan). The cDNA was excised from pVL1392 with *Xba*I and *Hin*dIII and subcloned into a pBluescript vector (Stratagene). To generate chimeric G proteins, a *Pst*I–*Hin*dIII fragment of the $G\alpha_s$ cDNA from pBluescript, including cDNA sequence encoding the carboxyl terminus domain of $G\alpha_s$, was further subcloned into pBluescript. Overlapping oligonucleotide linkers, encoding various G protein C-terminal sequences, were ligated into an *Sph*I (nt + 1157)–*Hin*dIII gap in the resulting plasmid. The whole coding sequences of the different constructs were subsequently regenerated. The $G\alpha_{s/11NC}$ was generated by PCR with sense primer 5′-aaatctagaccatgactctggagtccatgatgggctgtctcg-gaaacagc, antisense primer M13 reverse, pBluescript-$G\alpha_s$ as template and Dynazyme II™. The N-terminal sequence of the chimeric PCR product was subcloned into $G\alpha_{s/11}$ with *Xba*I and *Eco*RI and verified by sequencing.

For mammalian cell transduction, an *Ase*I (blunted)–*Not*I fragment from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA), including the cytomegalovirus (CMV) promoter and the gene for enhanced green fluorescent protein (EGFP), was subcloned into a *Sna*BI–*Not*I gap in pFastBac1, removing the polyhedrin promoter. This vector construct was further used to subclone $G\alpha_s$, the chimeric $G\alpha_{s/x}$ constructs and the M_3 receptor cDNA [26] behind the CMV promoter. Recombinant baculoviruses for all constructs in the pFastBac-CMV vector were obtained using the Bac-to-Bac expression system (Invitrogen).

2.3. Cell culture and gene transfer

Human embryonic kidney (HEK) 293 cells were grown in standard DMEM cell culture medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin–streptomycin (Invitrogen). To generate the stable cell line HEK-OX_1R-FLAG, the pIRES-hrGFP-1a-OX_1R vector was cotransfected with pcDNA3.1/hygro (Invitrogen) at a ratio of 1/20 using FuGENE6 according to manufacturer's recommendations. One day after transfection, 0.4 mg hygromycin (Invitrogen)/ml was added to the medium. After 7 days, cells surviving selection were reseeded and grown for 3 weeks with regular change of medium (0.1 mg hygromycin/ml). Single colonies were isolated, expanded and tested for responsiveness to orexin-A.

Transient expression using the baculovirus vector was done by seeding HEK-OX_1R-FLAG cells at 20% confluency in six-well plates (Greiner Bio-One, Frickenhausen, Germany) for cAMP measurements or tissue culture dishes (Greiner Bio-One, 94 mm diameter) for suspension measurements of intracellular [Ca^{2+}]. After 2 days, half of the medium was replaced with a high titer virus stock (10^7 pfu/ml) originating from Sf9 cell infection. The transduction was allowed to proceed for 3–4 h whereafter the medium was exchanged with fresh medium. Plates were incubated for 24–28 h prior to experimental use. The transduction efficiency of EGFP-transduced cells was regularly checked by fluorescence recordings on an Olympus IX70 microscope equipped with a CCD camera linked to the microCCD 3.02 software (Apogee Instrument, Auburn, CA, USA).

2.4. Ligand binding

HEK-OX_1R-FLAG cells in culture flasks (Nunc, Roskilde, Denmark) were harvested in PBS containing 0.5 mM EDTA, centrifuged 1500 × *g* and stored as pellets at −70 °C until use. For binding experiments the cells were resuspended in binding buffer (100 mM NaCl, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$; pH 7.4) and homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). About 15–20 μg of protein of the homogenate was incubated with different concentrations of [^{3}H]-SB-674042 in a volume of 150 μl for 60 min at room temperature. SB-334867 (10 μM) was used to determine nonspecific binding. Reactions were terminated by rapid filtration through prewashed GF/B filters (Packard Instrument, Meriden, CT, USA) followed by five washes with cold buffer containing 180 mM NaCl, 25 mM $MgCl_2$, and 20 mM HEPES, pH 7.4, in a microplate harvester (Packard Instrument). The GF/B filters were pretreated with 0.3% polyethyleneimine to reduce binding to filter membranes. Radioactivity on filters was determined in a microplate scintillation counter (Packard Instrument).

2.5. Measurement of intracellular [Ca^{2+}]

For single cell imaging, HEK-OX_1R-FLAG cells were seeded on glass coverslips at 10% confluency 1 day before the experiments. For measurements, the cells were loaded with 4 μM fura-2 acetoxymethyl ester in a HEPES-buffered medium (HBM: 137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 20 mM HEPES, 10 mM glucose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) supplemented with 0.5 mM probenecid and incubated at 37 °C for 20 min. The coverslip was attached to the bottom of a thermostatted (37 °C) perfusion chamber and cells were stimulated by perfusion with orexin-A diluted in the HBM. The cells were excited by alternating 340 and 380 nm UV light with a filter exchanger in the control of an InCyt2™ system (Intracellular Imaging, Cincinnati, OH, USA) and emission was measured through a dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. A new ratio image (340/380 nm) was collected every second.

For suspension culture measurements of $[Ca^{2+}]_i$, cells were detached with PBS containing 0.5 mM EDTA, washed in the HBM and loaded with fura-2 acetoxymethyl ester as above. After loading, the cells were diluted with HBM without $CaCl_2$ to a final concentration of 0.3 mM $CaCl_2$ and stored at room temperature in suspension until use. Using this protocol the responses to receptor stimulation were stable over several hours. For fluorescence recordings an appropriate volume of the cell suspension was spun down, washed twice in HBM without $CaCl_2$, resuspended in HBM with or without $CaCl_2$ and placed in a thermostatted (37 °C) cuvette with magnetic stirring in a QuantaMaster™ fluorescence spectrophotometer (Photon Technology International, Ford, UK). The fluorescence was monitored at 340/380 nm (excitation) and 510 nm (emission). Experiments were calibrated with 60 μg digitonin/ml (F_{max}) and 10 mM EGTA (F_{min}). The $[Ca^{2+}]_i$ was calculated from the fluorescence (F) obtained at 340 nm using the equation $[Ca^{2+}]_i = (F - F_{min})/(F_{max} - F) \times 224$ nM (K_d for fura-2) in which the extracellular fura-2 fluorescence is subtracted from F values.

2.6. Immunoprecipitation

Cells on tissue culture plates (Greiner Bio-One, 94 mm diameter) were washed twice with PBS and scraped off in ice-cold PBS. The cell pellets were frozen and stored at −70 °C. For whole cell lysate preparations, pellets of approximately 5×10^6 cells were resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.4% digitonin, protease inhibitor mixture (Roche)) and incubated for 30 min at 4 °C with rotation. Lysates were centrifuged for 10 min at 12 000 × g and the supernatant collected and mixed with equal amount of lysis buffer without digitonin. In the case of FLAG immunoprecipitation, the lysate was added to the anti-FLAG M2 affinity gel and incubated at 4 °C for 2 h. The precipitated samples were centrifuged for 15 s at maximum speed in a microcentrifuge and washed four times with washing buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, protease inhibitor mixture). Beads were eluted in sample buffer (62.5 mM Tris–HCl pH 7.4, 25% glycerol, 2% SDS, 0.01% bromophenol blue), or more gently by 3×FLAG peptide 150 ng/μl (Sigma–Aldrich) in the wash buffer for 30 min at 4 °C and mixed with the equal amount of sample buffer. 5% (v/v) β-Mercaptoethanol was added to the elution supernatants.

2.7. Immunoblotting

Samples were incubated at 96 °C for 3 min before loading onto a polyacrylamide gel. Proteins were separated at constant voltage and transferred electrophoretically from polyacrylamide gels to PVDF membrane (Bio-Rad, Hercules, CA, USA) in 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were blocked for nonspecific binding by incubating in 5% milk, 0.1% Tween 20 in PBS and incubated overnight with the primary antibody, anti-FLAG M2, anti-OX_1R, anti-$G\alpha_q/G\alpha_{11}$, anti-$G\alpha_o$, anti-$G\alpha_{i1/2}$ or anti-$G\alpha_s$. Membranes were washed and incubated with secondary antibody (goat anti-rabbit or goat anti-mouse IgG coupled horseradish peroxidase (Santa Cruz Biotechnology)). Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL plus (Amersham Biosciences Finland)). Immunoreactive bands were visualized following exposure of the membranes to Hyperfilm (Amersham Biosciences Finland).

2.8. Measurement of cAMP

Cyclic AMP was measured using a CatchPoint™ Cyclic-AMP Fluorescent Assay Kit (Molecular Devices, Sunnyvale, CA, USA). HEK-OX_1R-FLAG cells were detached from the six-well plates in PBS containing 0.5 mM EDTA, washed once with the HBM and finally suspended in an appropriate volume of the HBM containing 0.5 mM IBMX. After 10 min preincubation with IBMX at 37 °C, the reactions were started by adding cells to a 96-well plate containing premade solutions of desired drugs in a total volume of 150 μl/well. The reactions were stopped after 10 min by adding 50 μl stop solution included in the kit. Twenty microliters from each well were analysed according to manufacturer's instructions and fluorescence recorded in a FLUOstar™ plate reader (BMG LabTechnologies, Offenburg, Germany) using a 544 nm excitation filter and a 590 nm emission filter.

3. Results

In order to investigate protein interactions of the orexin receptor type 1 (OX_1R), we expressed an epitope-tagged OX_1R with a triple FLAG peptide fused to its C-terminus (OX_1R-FLAG) in HEK293 cells. Saturation binding on homogenates of the stably transfected cell line, HEK-OX_1R-FLAG, with the OX_1R antagonist [^{3}H]-SB-674042 gave a K_d value of 1.35 ± 0.18 nM and a B_{MAX} value of 5.5 ± 0.2 pmol/mg protein (Fig. 1A). This indicated an expression level high enough to enable detectable immunoprecipitation of the receptor. Saturation binding of HEK293 cells transfected to express an unmodified OX_1R gave a K_d value of 1.11 ± 0.17 nM (data not shown), indicating that the addition of the triple FLAG epitope of OX1R-FLAG did not significantly affect the ligand-binding structure of the receptor.

The functionality of the OX_1R-FLAG was tested by single cell fura-2 fluorescence imaging (Fig. 1B). The increase in fura-2 fluorescence ratio upon orexin-A application ensured a functional construct localised in the plasma membrane. Non-transfected HEK293 cells did not respond to orexin-A (data not shown).

Immunoprecipitation of the receptor was then performed using the anti-FLAG affinity purification method. The OX_1R-FLAG was immunoprecipitated with the FLAG antibody and detected by the anti-FLAG antibody as well as by the OX_1R antibody (Fig. 2). No specific binding of the FLAG antibody or of the OX_1R antibody was detected in non-transfected control cells (Fig. 2).

To identify interacting G proteins, we performed immunoprecipitation of OX_1R-FLAG and blotted against different G protein α subunits (Fig. 3A). The $G\alpha_q/G\alpha_{11}$ proteins co-immunoprecipitated with OX_1R in all experiments. This interaction was detected in solubilisation buffer containing up to 1.2% digitonin, with 0.4% being the optimum. The interaction was not affected by the presence of orexin-A (1 or 100 nM) or Ca^{2+} in the incubation medium prior to cell lysis (Fig. 3B).

The OX_1R-FLAG also showed interactions with $G\alpha_i$ and $G\alpha_s$, whereas binding of $G\alpha_o$ was negative (Fig. 3A). The $G\alpha_i$ and $G\alpha_s$ interactions appeared weaker than binding of $G\alpha_q/G\alpha_{11}$ and were not detected in all experiments with our protocol. No nonspecific binding to the antibody or resin was detected when immunoprecipitation was performed on non-transfected HEK293 cells.

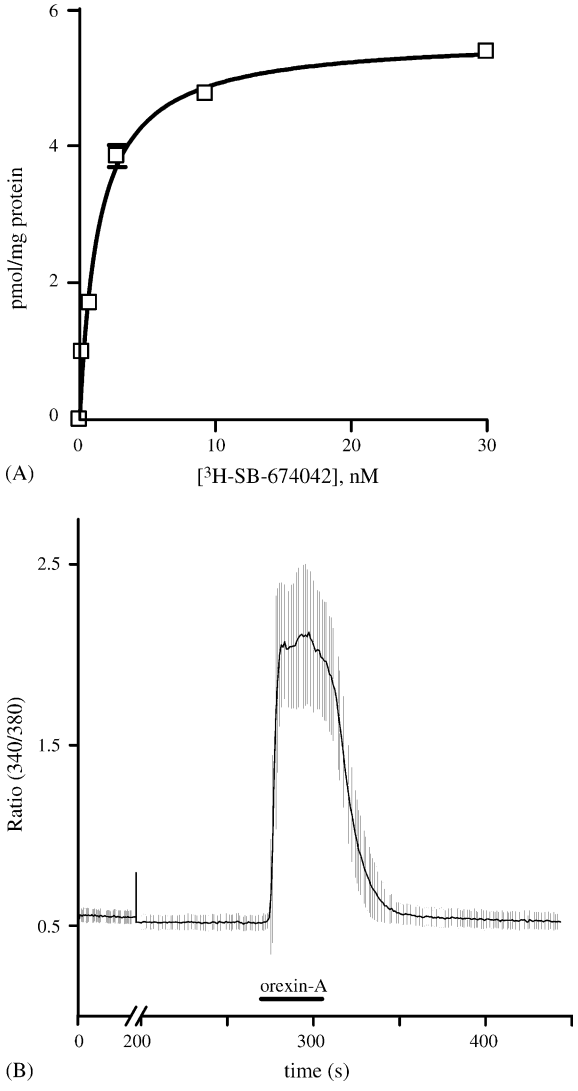

Fig. 1 – Characterisation of OX_1R-FLAG. (A) Saturation binding of homogenates of HEK-OX_1R-FLAG cells with [^{3}H]-SB-674042. Data points are means ± S.D., $N = 3$. (B) HEK-OX_1R-FLAG cells were analysed for orexin-A-stimulated increases in intracellular [Ca^{2+}] using fura-2 at the single cell level. 10 nM orexin-A (bar) was perfused and subsequently washed away. The trace with scatter represents the mean values ± S.D. of 47 cells.

Since the OX_1R-FLAG co-immunoprecipitated $G\alpha_i$ and $G\alpha_s$ proteins we performed measurements of cAMP levels in HEK-OX_1R-FLAG cells. No significant change in the basal cAMP level was detected upon stimulation with orexin-A (Table 1). To detect inhibition of adenylate cyclase activity through G_i proteins, it is often necessary to preactivate the enzyme. Forskolin, a direct activator of adenylate cyclase, was used to stimulate the production of cAMP. However, no inhibition of this response in the presence of orexin-A was detected (Table 1).

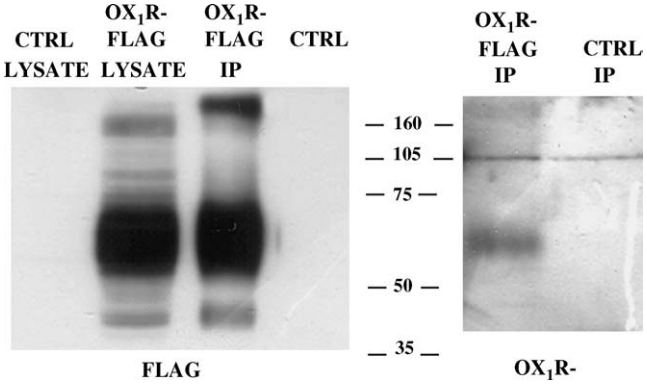

Fig. 2 – Immunoprecipitation of OX_1R-FLAG. HEK-OX_1R-FLAG cells and HEK293 control cells (CTRL) were subjected to immunoprecipitation with the anti-FLAG antibody and both cell lysates and precipitated proteins were separated on SDS-PAGE and blotted onto PVDF membranes. OX_1R-FLAG was detected using the anti-FLAG antibody (left panel) or the OX_1R antibody (right panel). Molecular weight markers in kDa are indicated between panels.

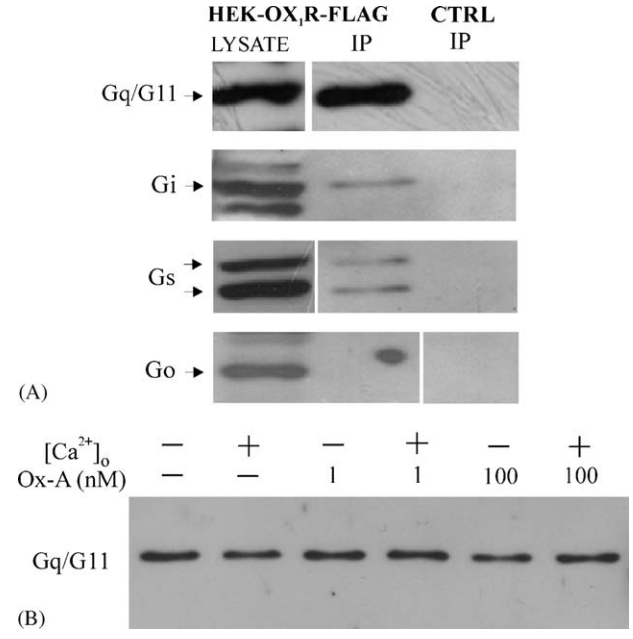

Fig. 3 – Co-immunoprecipitation of G proteins. Co-immunoprecipitation of G protein α subunits using the anti-FLAG antibody to precipitate OX_1R-FLAG. (A) Control immunoprecipitations were done with non-transfected HEK293 cells (CTRL). $G\alpha_q/G_{11}$ was detected in 15 out of 15 experiments, $G\alpha_i$ in 6/11, $G\alpha_s$ in 2/3, $G\alpha_o$ in 0/5. (B) HEK-OX_1R-FLAG cells were treated with low (1 nM) or high (100 nM) concentrations of orexin-A (Ox-A) for 30 s before lysis. The influence of extracellular Ca^{2+} $[Ca^{2+}]_o$ was also tested. Additional experiments using different times (up to 10 min) of treatment with orexin-A gave qualitatively similar results.

Table 1 – Cyclic AMP responses in HEK-OX_1R-FLAG cells

Condition	cAMP (pmol/well) mean ± S.D.
Basal	0.39 ± 0.10
Ox-A	0.53 ± 0.19
Forskolin	10.83 ± 1.90
Forskolin + Ox-A	11.39 ± 0.78

Cells were stimulated with 0.1 μM orexin-A (Ox-A) or 5 μM forskolin. Data values are from one representative experiment. Qualitatively similar results were obtained in a total of four experiments performed in triplicate.

As the OX_1R-FLAG did not alter the basal cAMP level, we constructed chimeric $G\alpha_{s/x}$ proteins that would stimulate adenylate cyclase if activated by the receptor (Table 2). A C-terminal switch of sequences of Gα proteins has been used rather extensively to direct G protein activation to a particular effector output [27,28]. The $G\alpha_{s/11NC}$, in which a proposed receptor-interacting N-terminal extension of $G\alpha_{11}$ was added to the $G\alpha_{s/11}$ [29], was constructed at a later stage of the study to try to improve the activation of the $G\alpha_{s/11}$ chimera by the OX_1R-FLAG. $G\alpha_{s/o}$ was chosen as a negative control, since we did not detect interactions with $G\alpha_o$ in co-immunoprecipitation experiments. A fairly strong functional coupling of this construct to the G_i/G_o protein-coupled muscarinic M_4 receptor has been detected when coexpressed in Sf9 insect cells (Näsman, unpublished observations). This suggests this construct structurally compatible with G_i/G_o-coupled receptors and functional at the adenylate cyclase level.

To express the chimeric G proteins we employed the novel technology of gene transfer mediated by baculovirus transduction of mammalian cells [30,31]. To test the efficiency of this technique, we generated a recombinant baculovirus with the CMV promoter driving the expression of EGFP. Transduction of HEK293 cells with this construct resulted in GFP fluorescence in more than 90% of cells at 1 day post-transduction (Fig. 4).

To assess the coupling specificity of the OX_1R we transduced HEK-OX_1R-FLAG cells with baculovirus harbouring the chimeric G protein constructs under the CMV promoter. Stimulation of OX_1R-FLAG resulted in significant increases in cAMP production in cells expressing $G\alpha_{s/11}$, $G\alpha_{s/11NC}$ or $G\alpha_{s/16}$, but not in cells expressing $G\alpha_s$, $G\alpha_{s/o}$ or EGFP, which was used as a control for the general effect of challenging cells with virus (Fig. 5).

The stimulation of the $G\alpha_{s/11}$ by the OX_1R-FLAG was unexpectedly poor. To find out whether our chimeric G proteins were weak coupling partners of G_q/G_{11} protein-

Table 2 – Structure of chimeric G proteins

G protein	Sequence	Donor protein
	386	
$G\alpha_s$	MHLRQYELL	None
$G\alpha_{s/o}$	NLRGCGLY	$G\alpha_{o1}$ ($G\alpha_{o2}$)
$G\alpha_{s/11}$	NLKEYNLV	$G\alpha_{11}$ ($G\alpha_q$)
	1	
$G\alpha_{s/11NC}$	MTLESM-$G\alpha_s$ 1-386-NLKEYNLV	$G\alpha_{11}$
$G\alpha_{s/16}$	YLDEINLL	$G\alpha_{16}$

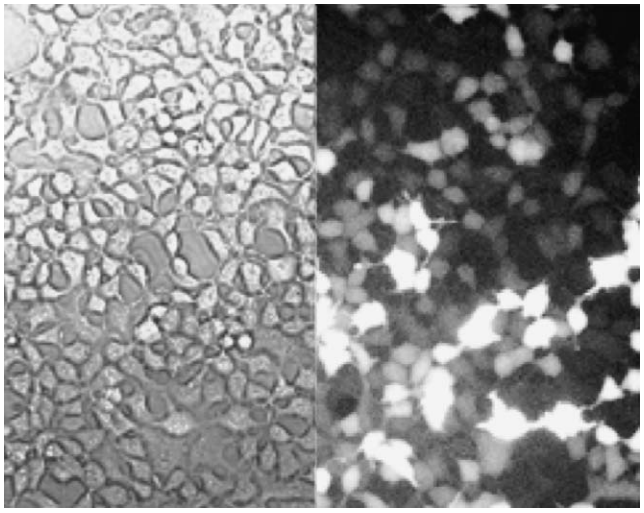

Fig. 4 – Baculovirus-mediated gene expression in HEK293 cells. HEK293 cells were transduced with baculovirus carrying the gene for EGFP under the transcriptional control of the CMV promoter. One-day post-transduction, brightfield contrast images (left) and fluorescence images (right) were collected.

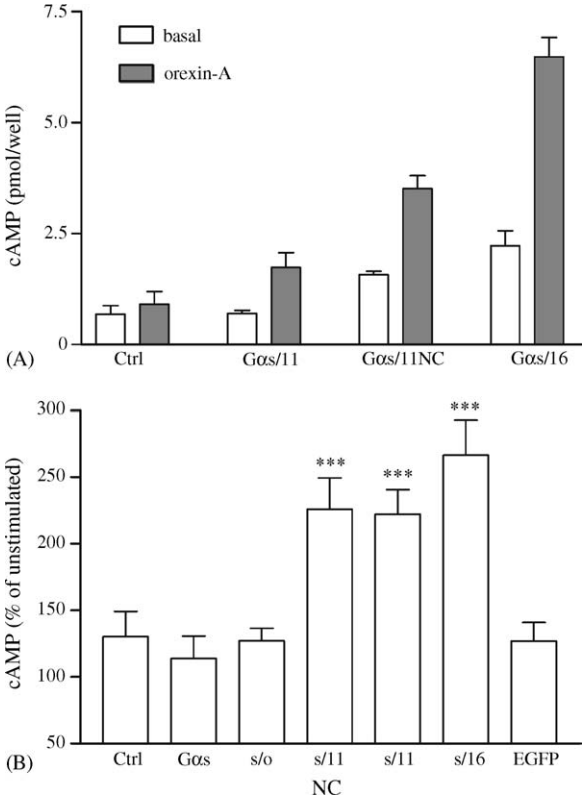

Fig. 5 – Stimulation of cAMP production via chimeric G proteins. HEK-OX_1R-FLAG cells were transduced with baculovirus for expression of different $G\alpha_{s/x}$ constructs. Measurement of cAMP production was performed 24–28 h post-transduction. (A) Data (mean ± S.D.) from representative experiment performed in triplicate showing absolute levels of cAMP. Basal levels were obtained from cells given no drug and stimulated levels from cells challenged with 100 nM orexin-A. (B) Compiled data (mean ± S.D.) from three to five independent experiments given as percent stimulation over basal. Statistically significant changes compared to control are indicated by * ($p < 0.001$), as determined with Student's two-tailed *t*-test.**

coupled receptors or if the OX_1R is a poor stimulator of G_q/G_{11} type of G proteins, we measured the responses of an expressed muscarinic M_3R. The HEK-OX_1R-FLAG cell line expresses endogenous muscarinic receptors (MRs) of unknown molecular identity. Of the $G\alpha_{s/q}$ type chimeras expressed, only the $G\alpha_{s/16}$ protein was stimulated (1.3-fold above basal level) by the endogenous MRs with 100 μM carbachol. The transduced M_3R exhibited a similar activation profile of the chimeric G proteins as the OX_1R-FLAG except that the activation of $G\alpha_{s/16}$ was much more pronounced (Fig. 6).

To compare the Ca^{2+} signalling of the two receptors, we performed intracellular [Ca^{2+}] measurements on suspensions of HEK-OX_1R-FLAG cells. Since the cAMP measurements were also conducted with suspended cells we thought this a reliable comparison. Despite the inefficient activation of $G\alpha_{s/16}$ compared to the M_3R, the OX_1R-FLAG showed Ca^{2+} elevations superior to the M_3R, but with an absolute requirement for extracellular Ca^{2+} at submaximal agonist concentrations (Fig. 7). A calcium elevation was detected at 0.1 nM orexin-A in the presence of extracellular Ca^{2+}, whereas 3 nM orexin-A was the limit of detection in the absence of extracellular Ca^{2+}. Similar experiments in the cAMP assay with $G\alpha_{s/16}$ showed a correlation between the G protein activation and the ability to release Ca^{2+} from intracellular stores (Fig. 8). At a concentration of orexin-A (3 nM) needed to almost fully elevate the intracellular [Ca^{2+}] via the OX_1R-FLAG, a $G\alpha_{s/16}$ activation was barely detectable.

4. Discussion

Two different approaches, immunoprecipitation and functional output measurements, were used to determine the interaction of the OX_1R with G proteins in HEK293 cells. Immunoprecipitation of the epitope-tagged OX_1R revealed a stable association of G_q/G_{11} proteins with this receptor subtype, in accordance with the ability of OX_1R to activate phospholipase C. Treatment with receptor agonist did not affect this association. Thus, it was not feasible to study the functional activation using this approach. Similar experiments with the beta adrenergic receptor have revealed a stable association with its cognate G protein under quite harsh experimental conditions [32], suggesting that the diffusion of G proteins away from receptors is very limited or that recycling of deactivated G proteins is very fast. Nevertheless, the results demonstrated a selective and stable physical connection between the OX_1R and G_q/G_{11} proteins.

The approach to demonstrate functional G protein coupling, using N- and C-terminally modified $G\alpha_s$ proteins, enabled measurements of the coupling to different G proteins with the same readout. At 100 nM orexin-A, the OX_1R-FLAG activated all $G\alpha_{s/x}$ constructs harbouring $G_{11/q/16}$ sequences in

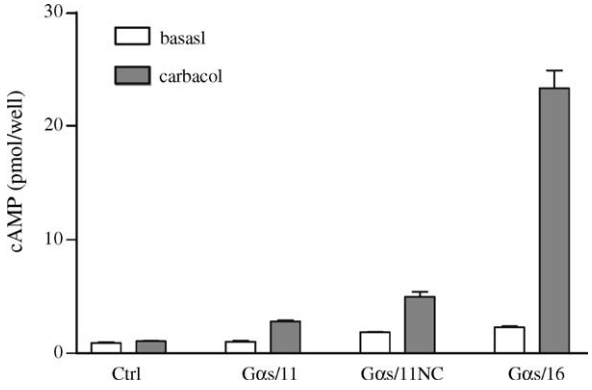

Fig. 6 – M_3R stimulation of chimeric G proteins. HEK-OX_1R-FLAG cells were transduced with baculovirus for expression of the M_3R and $G\alpha_{s/x}$ constructs. Measurement of cAMP production was performed 24–28 h post transduction. Cells were stimulated with 100 μM carbachol. Data (mean ± S.D.) is from one representative experiment performed in triplicate. Two more experiments gave qualitatively similar results.

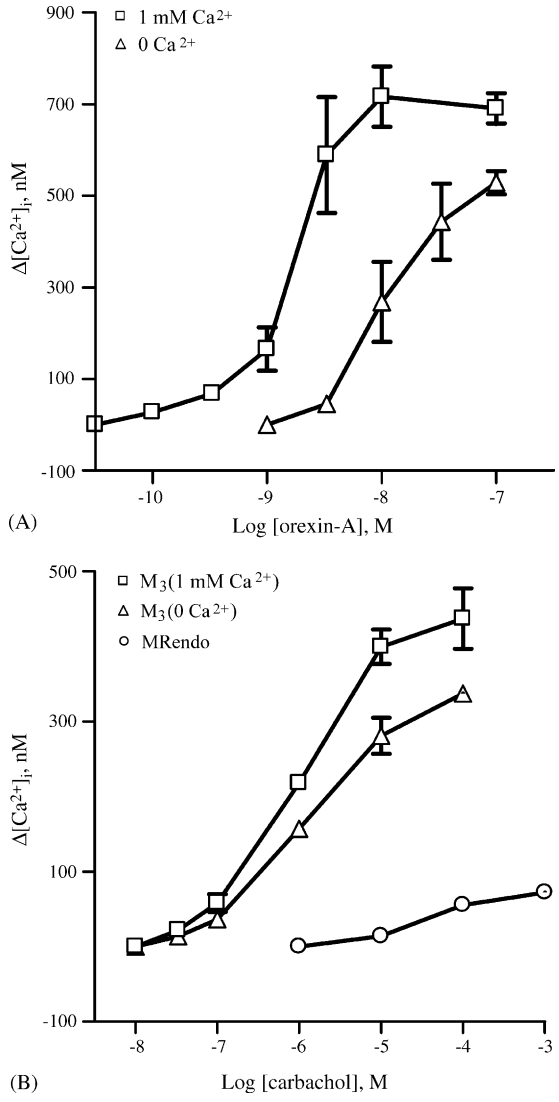

Fig. 7 – Suspension measurements of changes in intracellular $[Ca^{2+}]$. HEK-OX_1R-FLAG cells were loaded with fura-2 and receptor-stimulated changes in fluorescence converted to changes in $[Ca^{2+}]$. (A) Orexin-A dose–response relations in the presence (squares) or absence (triangles) of extracellular Ca^{2+}. Data points are means ± S.D. of three to five measurements. (B) Carbachol dose–response relations for the transduced M_3R in the presence (squares) or absence (triangles) of extracellular Ca^{2+} and for the endogenous muscarinic receptor (MRendo; circles) in the presence of extracellular Ca^{2+}. Data points are means ± S.D. of three measurements for M_3R. Single measurements are shown for MRendo.

their C termini. An orexin-A dose–response determination revealed that the G protein activation did not correlate with the Ca^{2+} elevating response. The G protein activation correlated instead with the ability of the receptor to release Ca^{2+} from intracellular stores, indicating that G_q/G_{11} protein activation is not necessary for the primary influx response. $InsP_3$ release is generally regarded as evidence for receptor coupling to the G_q protein family. A survey of the literature on OXR signalling indicates that the $InsP_3$ levels reach significance over basal levels at orexin-A concentrations ≥10 nM, and this seems independent of tissues or cell lines studied [13,20,33,34]. Thus, even if the cAMP response with $G\alpha_{s/16}$ appeared relatively weak, as compared to the M_3R, it was seen at concentrations of orexin-A generally leading to $InsP_3$ release. The difference between the OX_1R and M_3R in their efficiency to activate $G\alpha_{s/16}$ is unclear at present. When comparing the signalling at the Ca^{2+} level, the OX_1R produced bigger releases from intracellular stores at the respective relevant agonist concentration, suggesting an efficient coupling to G_q/G_{11} proteins for this receptor. Since the M_3R was also expressed in the HEK-OX_1R-FLAG cell line, both receptors should have availability to the same pool of G proteins. There may be differences between supposedly similar receptors in their ability to activate chimeric G proteins. This has been documented in the case of a $G\alpha_{s/q}$ construct, which was suitable as coupling partner for a bombesin receptor, but not for the oxytocin receptor [35]. It has also been suggested that the potency of agonist-stimulated receptors to activate chimeric G proteins does not necessarily follow the potency of activation of the preferred natural G protein [36], although the measurements of natural G protein activation, i.e. microphysiometry, were very indirect in the cited study. Because of these premises, it is impossible to fully exclude a G_q/G_{11} protein-coupling at low concentrations of orexin-A. However, the obvious difference between the OX_1R and the M_3R in signalling related to extracellular requirement for Ca^{2+} is an additional strong indication in this direction. Our hypothesis is that activation of the OX_1R leads to a primary signal cascade opening Ca^{2+} permeable ion channels in the plasma membrane, a secondary response being G_q/G_{11} protein activation. Results from an earlier study indicate that G_q/G_{11} protein activation may function as a negative feed-back control to shut off ion channels through concomitant protein kinase C activation [14], enabling rapid Ca^{2+} elevations combined with protection against harmful prolonged Ca^{2+} influx.

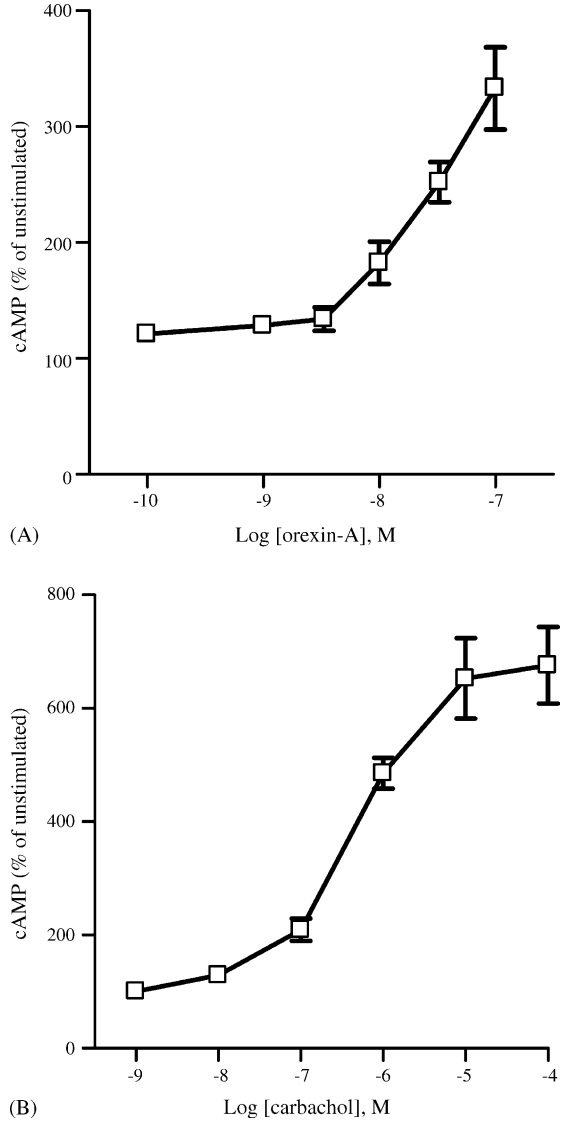

Fig. 8 – Cyclic AMP dose–response relations with $G\alpha_{s/16}$. HEK-OX_1R-FLAG cells transduced to express $G\alpha_{s/16}$ (A) or M_3R and $G\alpha_{s/16}$ (B) were assayed for agonist-dependent stimulation of cAMP production. Data is given as percent change compared to basal level and represents means ± S.E.M. of three experiments.

Why would the OX_1R possess two separate pathways for increasing $[Ca^{2+}]_i$? An interesting hypothesis is that the primary membrane cation influx need not be Ca^{2+} selective, indicating that this mechanism could also serve important depolarizing effects in excitable cells. A functional G_q/G_{11} protein-coupling, on the other hand, may of course be needed for other signalling pathways engaged by the OX_1R. For example, MAPK phosphorylation has been shown in response to OX_1R stimulation [4,37]. The agonist concentration-dependence of this response appears to correlate with the production of inositol phosphates [37], as well as with the G_q/G_{11} protein-coupling capability described in this study. An interesting finding from the study of Hilairet et al. [37] is the cross-talk between the OX_1R and the cannabinoid CB_1 receptor, i.e. in cells expressing the OX_1R, the potency for orexin-A-mediated MAPK activation is increased by about 100-fold when the CB_1R is coexpressed and basally active. The sensitised OX_1R response is thereby detected at subnanomolar concentrations. Keeping in mind that the $[Ca^{2+}]_i$ elevations in most cell types tested are also detected at the same concentrations [13,21,38], this could mean that if a primed MAPK is dependent on an influx of Ca^{2+}, or other potential players engaged in the influx pathway, then this primary action of the OX_1R would fulfill an important cellular signalling role. The importance of Ca^{2+} influx for MAPK activation has indeed recently been demonstrated in Chinese hamster ovary cells [39].

The co-immunoprecipitation of G_s and G_i proteins was not unexpected since several reports have suggested OXR interactions with these proteins [17,19–22,40]. The co-immunoprecipitation of G_i and G_s was not as consistently detected on blots as that of G_q/G_{11} proteins and we did not observe any significant action at the adenylate cyclase level. It has been reported that the G protein interactions of OXRs may be different at different stages of development as well as at different states of energy demand [41]. In such cases, one would expect that some cofactors are involved in the signalling unit or that some covalent modifications, such as phosphorylation, of the receptor could alter the signalling capability. In agreement with this is the finding that the stimulatory effect of OX_1R on adenylate cyclase requires an interplay of PKC and G_s proteins in Chinese hamster ovary cells [22]. It should also be noted that the stimulation of cAMP production occurs at orexin-A concentrations two orders of magnitude higher than increases in $[Ca^{2+}]_i$ in these cells [22]. Overall, it is evident that the coupling of OXRs to G_s and G_i proteins is inconsistent between cell types and may involve mechanisms not yet characterized.

In conclusion, we have demonstrated in this study that the human OX_1R is indeed both physically and functionally coupled to G_q/G_{11} proteins. The functional coupling correlated with the ability of the receptor to provoke discharge of intracellular Ca^{2+} stores, similar to the muscarinic M_3 receptor. However, agonist concentrations clearly elevating the intracellular $[Ca^{2+}]$, through an influx of Ca^{2+} from extracellular space, were far lower than those activating G_q/G_{11} family proteins, suggesting that the influx pathway is gated by another G protein or, more likely, other protein–protein interactions. These findings are important for the understanding of receptor function in general for two reasons. Firstly, receptor-operated Ca^{2+} influx may function independently of G protein activation and could thus involve signalling cascades with novel therapeutic targets. Secondly, the higher agonist potency for the Ca^{2+} influx pathway compared to G protein activation suggests that G protein-independent signalling could be the primary biological function of some receptors.

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