Novel function of β-arrestin2 in the nucleus of mature spermatozoa

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Summary

A growing number of proteins originally found in endocytic structures of the plasma membrane appear to be able to traffic into the nucleus, but the cellular function of this translocation remains unclear. We have found that β-arrestin2, which typically shows a cytoplasmic localization owing to constitutive nuclear export, appears in the nucleus after stimulation of the G-protein-coupled odorant receptor hOR17-4. In the nucleus, β-arrestin2 was involved in transcriptional regulation as shown by a Gal4-based transactivation assay. Moreover, we discovered that β-arrestin2 and hOR17-4, a receptor known to have a role in sperm-egg communication, colocalize in the midpiece of mature human spermatozoa. Stimulation of hOR17-4 in spermatozoa induced PKA-dependent translocation of β-arrestin2 to the nucleus and nuclear accumulation of phosphorylated MAPKs. Analysis of the interaction partners of β-arrestin2 indicates that odorant receptor signaling in spermatozoa may be important for the regulation of gene expression during the early processes of fertilization.

Key words: Olfactory receptor, Fertilization, Spermatozoa, β-arrestin2

Introduction

β-Arrestins are regulatory proteins that uncouple agonist-activated, phosphorylated seven-transmembrane receptors from heterotrimeric G proteins. Moreover, they have been shown to mediate the endocytosis of most G-protein-coupled receptors (GPCRs), activate the cytoplasmic protein tyrosine kinase Src upon GPCR activation and serve as scaffolding proteins for the MAP kinases ERK1/2 and JNK3 (Luttrell, 2003; Lefkowitz and Shenoy, 2005). β-arrestin1 is found in the cytoplasm and in the nucleus, whereas β-arrestin2 shows a predominant cytoplasmic localization as a result of constitutive nuclear export by a NES-dependent pathway (Scott et al., 2002; Wang et al., 2003a). The constitutive nucleocytoplasmic shuttling of β-arrestin2 was shown to control the subcellular distribution of JNK3 by retaining JNK3 within the cytoplasm after GPCR activation (McDonald et al., 2000). Also Mdm2, which is normally found in the nucleus complexed with p53, is relocalized in the cytoplasm of β-arrestin2-expressing cells and is recruited to the plasma membrane when receptors are activated (Wang et al., 2003b). Although not yet associated with specific functions, nuclear accumulation after inhibition of nuclear export has recently been reported for some endocytic proteins such as eps15, epsin1, the clathrin assembly lymphoid myeloid leukemia (CALM) and α-adaptin (Vecchi et al., 2001; Poupon et al., 2002). Nuclear Eps15 and CALM may be involved in transcriptional regulation, as they were shown to act as modulators of transcription in a reporter gene assay (Vecchi et al., 2001; Poupon et al., 2002). Recently it was shown in a heterologous cell system, that β-arrestin1 moves to the nucleus in response to GPCR stimulation, where it regulates gene expression by facilitating histone acetylation at specific gene promoters (Kang et al., 2005). The physiological relevance of the observed nuclear function remains unclear.

There have been multiple studies on the roles of β-arrestin2 in the regulation of G-protein-coupled receptors, but little is known about the biggest subfamily of GPCRs, the odorant receptors (ORs) (Malnic et al., 2004; Godfrey et al., 2004). Chemosensory ORs are expressed in the knobs and cilia of neurons in olfactory epithelium where they detect volatile substances. β-arrestin2 is localized in the same parts of the olfactory receptor neurons, and was shown to be involved in agonist-dependent desensitization in vertebrate olfaction (Dawson et al., 1993). In addition to their expression in the olfactory epithelium, ORs are expressed in the testis (Vanderhaeghen et al., 1993; Vanderhaeghen et al., 1997). At least some testicular ORs reside in mature spermatozoa and were shown to function in chemotaxis of human (Spehr et al., 2003) and mouse (Fukuda et al., 2004) sperm. ORs and β-arrestin2 may colocalize in the same subcellular compartment (Walensky et al., 1995), suggesting that β-arrestin2 could regulate chemoreceptor responses in mature spermatozoa.

In the present work we show for the first time that β-arrestin2 can be translocated to the nucleus upon GPCR stimulation and is involved in the regulation of transcriptional activities after translocation. This nuclear translocation takes place in mature human spermatozoa after stimulation of the testicular OR hOR17-4, and could be important for the initiation or modulation of early transcriptional events during fertilization.

Results

Activation of hOR17-4 causes nuclear translocation of β-arrestin2

We started our investigation with a recently identified human testicular OR (hOR17-4) that is activated by bourgeonal and can be functionally expressed in the heterologous HEK293 cell system (Spehr et al., 2003). To investigate whether odorant receptor activation leads to the recruitment of β-arrestin2 to the receptor protein, we tagged β-arrestin2 with GFP and visualized its redistribution upon activation of hOR17-4 with bourgeonal in HEK293 cells. Using confocal microscopy, we observed an unexpected redistribution of the cytosolic β-arrestin2 from the cytoplasm to the nucleus after odorant stimulation (Fig. 1A). About 2% of the cells cotransfected with hOR17-4 showed nuclear accumulation of β-arrestin2-GFP within 20 minutes after ligand application, which is similar to the number of cells responding to bourgeonal in ratiofluorometric Ca^{2+} imaging experiments (Spehr et al., 2003). Quantification of the fluorescence intensities in the nucleus relative to the cytosol revealed a ~fourfold increase in the amount of nuclear β-arrestin2 in the responding cells (Fig. 1B). The translocation was specific to the activation of hOR17-4, because it occurred neither after application of odorants, which do not activate this special receptor, nor after bourgeonal application to HEK293 cells that do not express hOR17-4 (data not shown). Moreover, activation of other transfected odorant receptors tested did not cause nuclear translocation of β-arrestin2 in HEK293 cells (data not shown). Translocation of β-arrestin2 in HEK293 cells could be blocked with concanavalin A, an inhibitor of clathrin-mediated endocytosis (Fig. 1C), other inhibitors of clathrin-mediated endocytosis (phenylarsineoxide and monodansylcadaverine) showed the same effect. Disruption of caveolae with methyl-cyclodextrin, which sequesters membrane cholesterol, did not affect nuclear

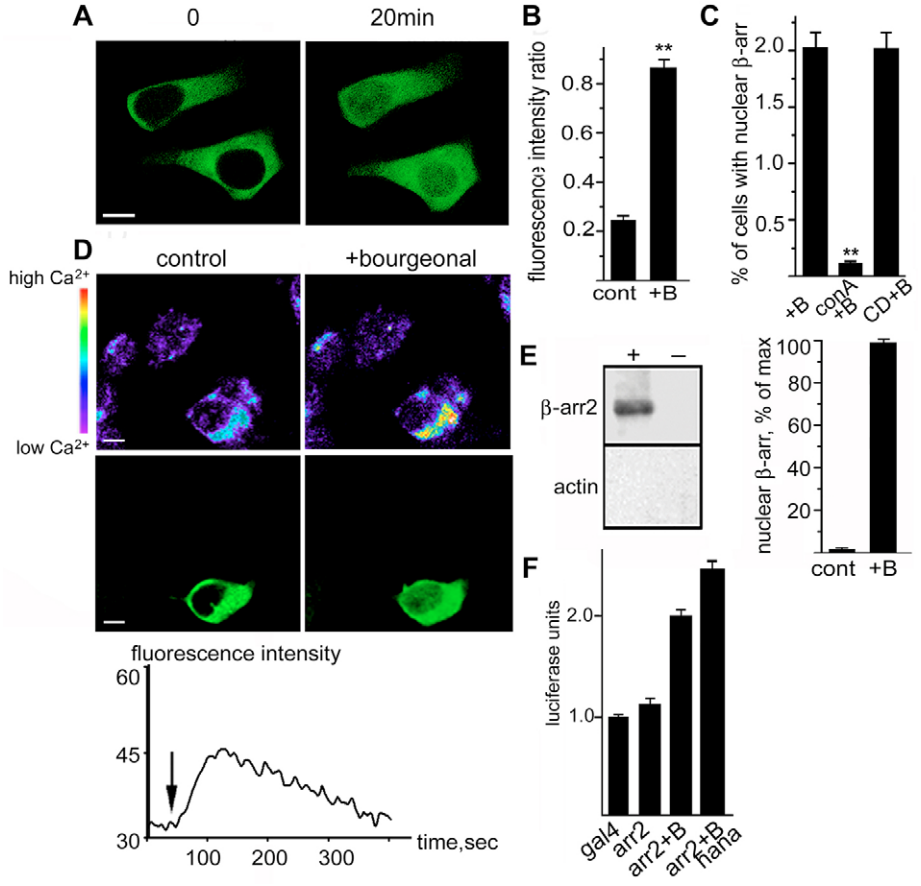

Fig. 1. β-arrestin2 accumulates in the nucleus and acquires a modulatory function in a GAL4-based transcription assay upon odorant receptor stimulation. (A) Confocal microscopy of HEK293 cells cotransfected with hOR17-4 and β-arrestin2-GFP before (0) and after (20 min) bourgeonal stimulation. Bourgeonal treatment causes nuclear translocation of β-arrestin2-GFP. (B) Quantification of the fluorescence intensities in the nucleus relative to intensities in the cytosol measured in single confocal sections. The averages of at least 100 HEK293 cells demonstrate a ~fourfold increase in the amount of nuclear β-arrestin2 in responding cells. Error bars represent s.d. (**P<0.01 compared with levels in the control). (C) Pre-treatment of cells with concanavalin A (+conA) as inhibitor of clathrin-mediated endocytosis blocks β-arrestin2-GFP nuclear translocation, pre-treatment with β-cyclodextrin (+CD) as inhibitor of caveolae-mediated endocytosis has no effect. At least 5000 cells were investigated and the number of cells showing nuclear accumulation of β-arrestin2 was counted and given as percentage of the total number of cells investigated. Error bars represent SD (**P<0.01). (D) Nuclear translocation of β-arrestin2-GFP is specific to hOR17-4 activation. The upper panel shows changes in the Ca^{2+} concentration in Calcium-Orange-loaded individual cells that were cotransfected with hOR17-4 and β-arrestin2-GFP in pseudocolors; the lower panel shows β-arrestin2-GFP localization. Bourgeonal (5 μM) was present during the whole experiment. The β-arrestin2-GFP expressing cell which show nuclear translocation of the β-arrestin2 also showed an increase in the intracellular Ca^{2+} concentration in response bourgeonal (5 μm). The curve shows the mean change in the fluorescence intensities in response to Borgeonal application as a function of time for ten Calcium Orange loaded cells cotransfected with β-arrestin2-GFP and hOR17-4. (E) Western blot analysis of fractionated HEK293 cells cotransfected with β-arrestin2 and hOR17-4, nonstimulated (–) and stimulated (+) with bourgeonal for 20 minutes. The purity of the nuclear fraction was verified by staining with anti-β-actin antibodies. Nuclear β-arrestin2 signals were quantified by densitometry; the graph depicts the average from three independent experiments, error bars represent s.e.m. (F) HEK293 cells and Hana3A were cotransfected with a GAL4-regulated luciferase reporter construct and chimeric constructs encoding the GAL4 DNA-binding domain alone (gal4), or fused to β-arrestin2. Luciferase activity was measured 48 hours after transfection using equal amounts of total cellular lysates from untreated cells (β-arr2) and from HEK293 (β-arr2+B) and Hana3A (β-arr2+B hana) cells that were treated with bourgeonal for 6 hours. Luciferase activity significantly increased upon hOR17-4 stimulation. For each panel, results of five independent experiments performed in triplicate were averaged and plotted, error bars represent s.e.m. Bars, 10 μm.

translocation of β-arrestin2 (Fig. 1C). To confirm that the nuclear translocation of β-arrestin2 is specific to the activation of hOR17-4, we monitored the bourgeonal-induced rise in $Ca^{2+}$and the translocation of β-arrestin2-GFP in the same cell. We used Calcium Orange to monitor the ligand-induced Ca^{2+} rise and β-arrestin2-GFP translocation simultaneously in confocal sections. Nuclear translocation of β-arrestin2-GFP occurred in 90% of the cells that specifically responded to bourgeonal with an increase in the fluorescence emission of the Calcium Orange, indicating a rise in the intracellular Ca^{2+} concentration (Fig. 1D).

β-Arrestin2 redistribution was also observed after cell fractionation and western blotting, where the usually cytosolic protein can be detected in the nuclear fraction only after odorant receptor stimulation (Fig. 1E).

β-Arrestin2 acts as a transcriptional regulator in a GAL4-based transactivation assay

To determine whether nuclear β-arrestin2 mediates transcriptional activation, β-arrestin2 was fused to the DNA-binding domain of GAL4 and transiently expressed in HEK293 cells with a reporter plasmid encoding the luciferase gene under the transcriptional control of a GAL4-responsive promoter. This assay is commonly used to identify whether a protein of interest is capable of conferring transactivation. The GAL4 DNA-binding domain anchors the protein on the DNA, and if the protein has an intrinsic transactivation domain, increased reporter gene activity will result. To investigate possible functions of β-arrestin2 in the nucleus, we used the transactivation assay on cells cotransfected with the fusion protein between β-arrestin2-GAL4 DNA-binding domain, the reporter plasmid and the receptor hOR17-4. We performed the transactivation assay with the GAL4-β-arrestin2 fusion construct in HEK293 cells and observed only minor transactivation over the basal value obtained in the presence of GAL4 alone (Fig. 1F). We then stimulated the cotransfected receptor with bourgeonal to induce nuclear translocation of β-arrestin2, and observed a marked increase in the basal transactivation activity (Fig. 1F). Owing to the fact that ORs typically show low expression levels, it is likely that the observed degree of transactivation is underestimated. We therefore tried to improve the expression of hOR17-4 by using Hana3A cells – HEK293T cells stably expressing the specific chaperones RTP1/2 and REEP1 to promote export of ORs to the plasma membrane (Saito et al., 2004) – and observed about 20% increase in the amount of transactivation (Fig. 1F).

Odorant-induced nuclear translocation of β-arrestin2 in spermatozoa

Similar to the findings in the heterologous cell system, we also observed translocation of β-arrestin2 to the nucleus of human spermatozoa after stimulation of the odorant receptor hOR17-4. Immunohistochemical staining of β-arrestin2 showed that it translocates from the midpiece to the head of mature spermatozoa upon bourgeonal treatment (Fig. 2A). The translocation occurred in ~80% of the cells and could be blocked by undecanal, a specific antagonist for hOR17-4 (Spehr et al., 2003). To confirm the specificity of the labeling, we preincubated the antibody with a blocking peptide and performed labeling using only the secondary antibody as a control (Fig. 2B). Translocation was also detected by western blotting of fractionated human spermatozoa. In untreated spermatozoa, β-arrestin2 was mainly detected in the cytosolic fraction, but only after stimulation of hOR17-4 with bourgeonal it can be found in the nuclear preparation (Fig. 2C,D). To confirm the specificity of the labeling also at the western blot level, we preincubated the antibodies with a blocking peptide and showed labeling of recombinant β-arrestin2 as a positive control (Fig. 2C,E).

OR activation entails targeting of activated MAP kinases to the nucleus of spermatozoa

Mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) mediate the intracellular response to a variety of extracellular signals. The MAPK cascade has been shown to be involved in spermatogenesis, spermatozoon motility and capacitation in different species (Sun et al., 1999). Activation of most members of the MAPK family typically occurs in the cytoplasm and is followed by translocation to the nucleus and contribution to a mitogenic response. As the mechanism of MAP kinase activation has a substantial impact on MAP kinase function, we investigated activation of these proteins in spermatozoa upon bourgeonal treatment. We found that activation of hOR17-4 in spermatozoa leads to phosphorylation of ERK1/2 and p38 MAPK (Fig. 3A-C). Although it is generally believed that MAPK activation via GPCRs and β-arrestins primarily increases cytosolic kinase activity (Lefkowitz and Shenoy, 2005), we detected phosphorylated MAPK in purified nuclei of bourgeonal-treated spermatozoa by western blotting (Fig. 3D,E).

Redistribution of hOR17-4 in human spermatozoa upon ligand binding

Interestingly, not only β-arrestin2 but also the hOR17-4 protein itself is translocated into the head region of spermatozoa upon bourgeonal treatment. To be able to localize the OR protein we generated an antibody against hOR17-4 by a genetic immunization technique (Chambers and Johnston, 2003), which detects the heterologously expressed protein in HEK293 cells (Fig. 4C). Using this antibody, we found that the receptor is localized in the midpiece and in the equatorial segment in non-treated spermatozoa (Fig. 4A), which confirms former ratiofluorometric Ca^{2+} imaging data where the rise in intracellular Ca^{2+} started in the midpiece (Spehr et al., 2004). After long-term exposure (20 minutes) of the cells to bourgeonal, the receptor protein is depleted from the midpiece and can only be detected in the head region (Fig. 4A). This depletion of the receptor protein was blocked by preincubation with the hOR17-4 antagonist undecanal. We were unable to detect the receptor in a nuclear preparation of bourgeonal-treated spermatozoa by western blotting (Fig. 4B).

hOR17-4 desensitization in human spermatozoa is mediated by PKA

Tyrosine phosphorylation of proteins plays an important role in capacitation, acrosome reaction and spermatozoa penetration (Breitbart, 2003). It is unclear whether specific ligands induce the signal transduction cascades leading to tyrosine phophorylation during these different processes. In immunohistochemical analysis we detected localization of the second-messenger-activated protein kinase A (PKA) in the midpiece of human spermatozoa (Fig. 5A), where hOR17-4

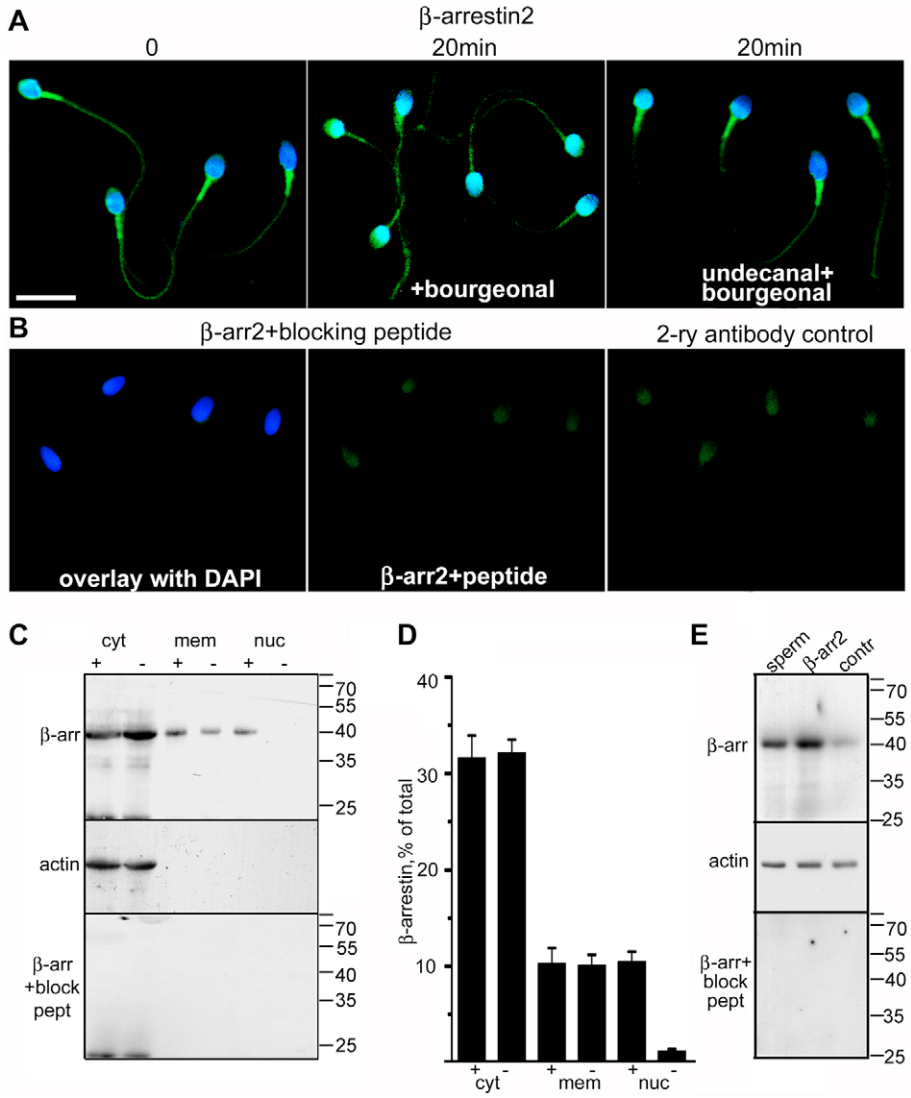

Fig. 2. Redistribution of β-arrestin2 in human spermatozoa upon bourgeonal application. Subcellular localization of β-arrestin2 is changed upon bourgeonal stimulation of human spermatozoa. (A) Immunostaining of human sperm using anti-β-arrestin2 antibodies (green). Before treatment (0) β-arrestin2 is localized to the midpiece, with some minor staining in the head. After bourgeonal stimulation (20 min), β-arrestin2 is translocated to the head, as indicated by increasing colocalization with DAPI (blue). Pretreatment with the hOR17-4 antagonist undecanal (+undecanal) blocks β-arrestin2 redistribution in response to bourgeonal stimulation. Bar, 10 μm. (B) Specificity of the β-arrestin2 antibody was confirmed by pre-incubation with a specific blocking peptide. Midpiece staining is completely abolished, and barely detectable staining in the head is observed (shown as overly with DAPI and without DAPI staining). The staining was similar to the background, which is present when only secondary antibodies are applied (2-ry antibody control). (C) Western blot analysis of fractionated human sperm showing that β-arrestin2 is present in the nucleus after bourgeonal stimulation. Nonstimulated (–) and bourgeonal stimulated (+) human sperm were fractionated as described in the Materials and Methods, the purity of nuclear fraction (nuc) was verified with anti-β-actin antibodies. Specificity of the β-arrestin2 antibody was confirmed by preincubation with a blocking peptide, no β-arrestin2 specific band (~44 kDa) was detected under this condition. cyt, cytoplasmic fraction; mem, membrane fraction. (D) β-arrestin2 signals in the different sperm fractions were quantified by densitometry. Averages from three independent experiments are shown, error bars represent s.e.m. (E) The β-arrestin2 antibody detects endogenous β-arrestin2 in HEK293 cells (contr), in human sperm lysate (sperm) and recombinant β-arrestin2 in HEK293 cells transfected with β-arrestin2 (β-arr2). Anti-actin antibodies were used as a loading control. Preincubation with a blocking peptide confirms the specificity of the antibody labeling.

and β-arrestin2 were also localized. Moreover, we found that treatment of spermatozoa with bourgeonal induced agonist-promoted phosphorylation. Stimulation of hOR17-4 with bourgeonal activated PKA and induced phosphorylation of R-x-x-S/T motif containing proteins. The strongest signal was observed at ~40 kDa (Fig. 5B), but we also observed phosphorylation of proteins with a molecular mass of ~80 kDa, which has been reported to accompany spermatozoa capacitation (O'Flaherty et al., 2004). The detected 40 kDa band could include the phosphorylated odorant receptor. This is supported by the fact that the PKA inhibitor H-89 prevents proper desensitization of the hOR17-4 response in sperm cells. The increase in the intracellular Ca^{2+} concentration in spermatozoa caused by a short exposure to bourgeonal decreased in 20-30 seconds, whereas increasing concentrations of H-89 caused a strong delay in the desensitization kinetics (Fig. 5C).

Translocation of β-arrestin2 to the nucleus depends on receptor phosphorylation

We next investigated whether PKA-mediated phosphorylation events also affect receptor-mediated β-arrestin2 translocation. Odorant-induced translocation of β-arrestin2 into the nucleus of human spermatozoa was inhibited by the PKA inhibitor H-89, and the inhibitor concentrations needed for this inhibition were similar to the ones that caused the delayed desensitization of hOR17-4 (Fig. 6A). The percentage of spermatozoa that exhibited a clear nuclear localization of β-arrestin2 decreased with increasing H-89 concentrations. Furthermore, we performed site-directed mutagenesis experiments to delete potential PKA phosphorylation sites within the third intracellular loop and the C-terminus of hOR17-4. We found that both of these domains could be involved in β-arrestin2 binding and receptor desensitization, because both receptor constructs failed to induce nuclear translocation of β-arrestin2-

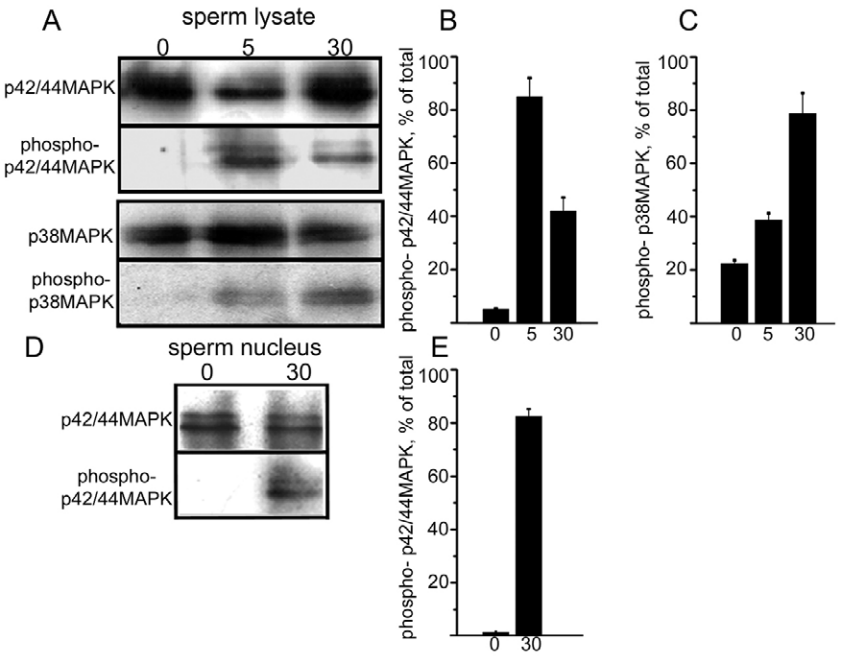

Fig. 3. Odorant receptor stimulation results in phosphorylation and nuclear translocation of MAPK in human spermatozoa. (A) p42/44 and p38 MAPK are phosphorylated in response to bourgeonal stimulation of human sperm. Western blot analysis of total cell lysate of untreated human sperm cells (0) and cells that were treated with bourgeonal for 5 and 30 minutes. Lysates were probed for the presence of phosphorylated p44/42 MAPK and phosphorylated p38 MAPK using antibodies that specifically recognize the phosphorylated form of the proteins. Detection of the total amounts of p42/44 and p38 are shown as control. (B,C) p42/44 MAPK and p38 MAPK signals and signal from the unphosphorylated forms were quantified by densitometry. The amount of each phosphorylated MAPK was normalized to the total MAPK level in three independent experiments. Error bars represent s.e.m. (D) Phosphorylated p42/44 MAPK is present in the nuclei of bourgeonal-treated human sperm; detection of the total amount of p42/44 in the nuclear fraction is shown as a control. (E) The signal from phosphorylated p42/44 MAPK in the nuclear fraction was quantified by densitometry and normalized to a total amount p42/44 MAPK levels in two independent experiments. Error bars represent s.e.m.

GFP in HEK293 cells (Fig. 6B). Similar effects were observed after pretreatment of the cells with H-89 (Fig. 6B). The lack of β-arrestin2 interaction was not due to complete lack of functionality of the mutant receptors, because the percentage of transfected cells that responded to bourgeonal in Ca^{2+}-imaging experiments was similar for the mutant and wild-type receptor constructs (data not shown). However, the duration of the response from the onset of the stimulus to the decrease to the basal level was ~fivefold longer with mutated receptor constructs (Fig. 6C), indicating that receptor phosphorylation is important for OR desensitization.

Nuclear interaction partners of β-arrestin2

The unexpected finding of nuclear translocation of β-arrestin2 raises the question of the physiological meaning of this event. β-arrestin2 seems to participate in signaling events between plasma membrane receptors and the nucleus in spermatozoa, but the nature of the putative nuclear signals is not clear. Any clues as to the potential function of β-arrestin2 in the nucleus might be revealed by examining its nuclear partners. Thus, we performed an immunoprecipitation with antibodies against β-arrestin2 in nuclear extracts of bourgeonal-treated human spermatozoa and analysed the β-arrestin2-associated nuclear proteins using LC-ESI MS/MS (Table 1). Under these conditions, we did not find any protein if the immunoprecipitation was carried out without stimulation of the spermatozoa with bourgeonal. Interestingly, most of the β-arrestin2-associated proteins found were

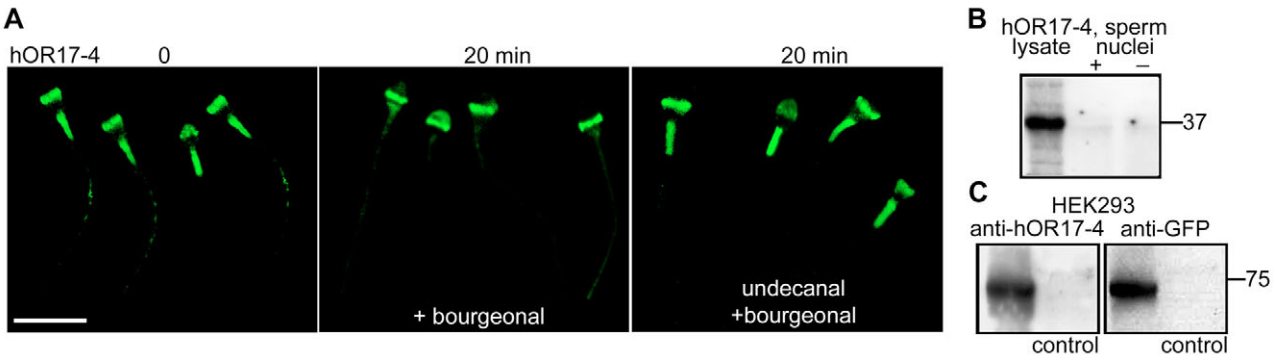

Fig. 4. Redistribution of hOR17-4 in human spermatozoa upon bourgeonal application. (A) Subcellular localization of hOR17-4 is changed upon bourgeonal stimulation of human spermatozoa. An antibody against hOR17-4 raised in mouse labeled the midpiece of non-treated human spermatozoa (0), some cells show a ring-like staining around the head; after bourgeonal stimulation (20 min) staining is strongly decreased in the midpiece. Treatment with undecanal blocks hOR17-4 redistribution, similar to β-arrestin2. Bar, 10 μm. (B) Western blot analysis of human sperm with the anti-hOR17-4 antibody shows that the hOR17-4 protein can be detected at ~40 kDa in the total cell lysate, but does not translocate to the nucleus in response to bourgeonal stimulation. (C) Control western blots showing the specificity of the antibody. Equal amounts of hOR17-4-GFP-transfected HEK293 cells and mock-transfected cells were probed with anti-hOR17-4 and anti-GFP antibody.

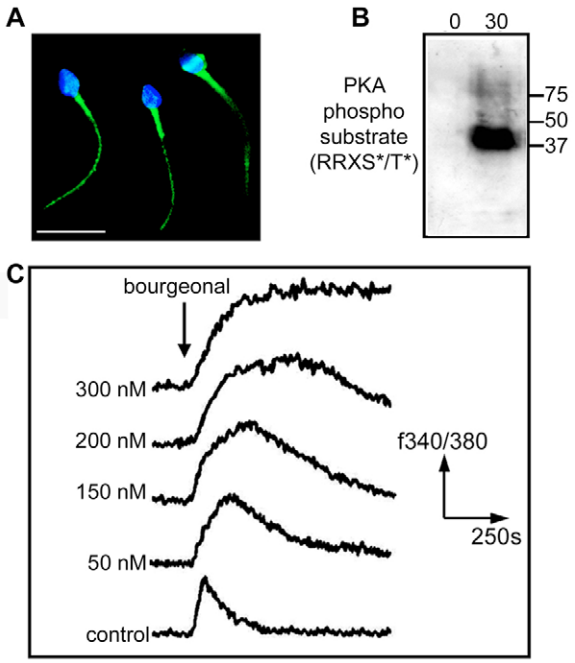

Fig. 5. PKA is involved in hOR17-4 desensitization. (A) Immunostaining of human sperm using anti-PKA antibodies (green) shows, that PKA is localized to the midpiece and the tail in varying amounts, some cells also show ring-like staining around the head; nuclei are counterstained with DAPI. Bar, 10 μm. (B) Western blot analysis of human sperm lysate with anti-PKA phospho-substrate antibodies. Whereas non-stimulated cells (0) showed no staining with this antibody, cells that were treated with bourgeonal for 30 minutes showed labeling of a strong band of ~40 kDa. (C) Representative ratiofluorometric recordings of the head and midpiece of individual human spermatozoa showing that PKA might be involved in hOR17-4 desensitization. The cytosolic Ca^{2+} level of Fura-2-loaded cells is depicted as the integrated fluorescence ratio (f_{340}/f_{380}) and viewed as a function of time. Cells were pre-incubated with PKA inhibitor H-89 in varying concentrations, bourgeonal was applied for 5 seconds in each case.

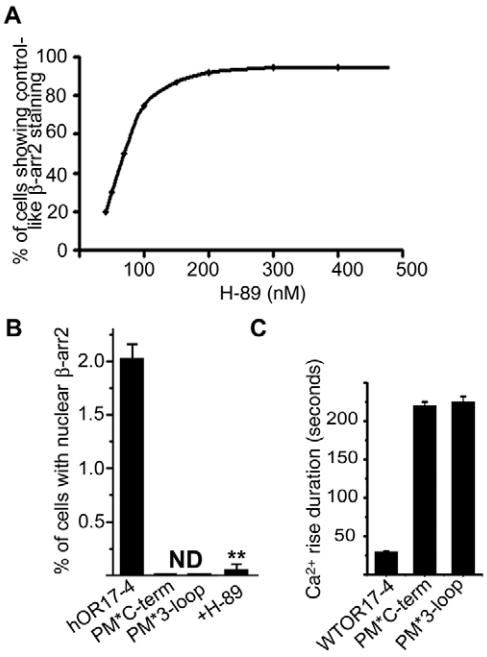

Fig. 6. PKA phosphorylation is important for nuclear translocation of β-arrestin2. (A) Treatment with PKA inhibitor H-89 blocks β-arrestin2 redistribution in human sperm in response to bourgeonal treatment. Human sperm were pretreated with different concentrations of H-89 inhibitor as described and stimulated with bourgeonal for 20 minutes. Cells still showing control-like mid-piece staining with the anti-β-arrestin2 antibody after bourgeonal stimulation were counted and plotted against the H-89 concentration. (B) Site-directed mutagenesis experiments were performed to delete the potential PKA phosphorylation sites within the third intracellular loop (positions S230A, S232A, S239A; PM*3-loop) and the C-terminus (position T312A; PM*C-term) of hOR17-4 as described in the Materials and Methods. Both mutant receptors failed to induce nuclear translocation of β-arrestin2-GFP in HEK293 cells upon bourgeonal treatment. A similar failure to induce nuclear translocation of β-arrestin2 was observed with the wild-type receptor after inhibition of PKA with H-89. At least 5000 cells were investigated; the number of cells showing nuclear β-arrestin2 accumulation was counted and given as percentage of total cells investigated. Error bars represent s.d. (**P<0.01 compared with untreated cells). ND, no cells with nuclear β-arr2 detected. (C) Mutation of the potential PKA phosphorylation sites leads to the increased response duration in Ca^{2+}-imaging experiments in transfected HEK293 cells. Duration of the Ca^{2+} rise was measured from the onset of the stimulus to the return to basal levels.

previously seen to localize to nuclease-hypersensitive chromatin fractions of spermatozoa, indicating that they may be involved in the initiation of the genetic activities that take place in the male pronucleus of the zygote soon after sperm penetration in the oocyte.

Discussion

Although it has been known for some time that ORs reside in spermatozoa (Vanderhaeghen et al., 1997), the function of these receptors is still in part unknown. Activation of the recently described human testicular OR hOR17-4 was shown to promote chemotaxis of human spermatozoa (Spehr et al., 2003). hOR17-4 may therefore be involved in the chemical communication between sperm and egg during the fertilization process. We demonstrate here, that activation of this OR leads to translocation of the cytosolic protein β-arrestin2 to the nucleus. Nuclear translocation was specific to the activation of hOR17-4 and was dependent on receptor phosphorylation and endocytic events; in addition, it was shown to regulate transcriptional events.

GPCR signaling through traditional G-protein-dependent second messenger cascades was previously shown to regulate gene expression (Mayr and Montminy, 2001). Very recently, a completely new way by which GPCRs regulate gene transcription was introduced by showing opioid receptor-driven nuclear translocation of β-arrestin1 in a heterologous cell system (Kang et al., 2005). As a result of the longer time

Table 1. β-arrestin2-associated nuclear proteins

Q14562	ATP-dependent helicase DHX8
O14556	GAPDH-2, testis-specific
P16104	Histone H2A.X
Q15058	Kinesin-like protein KIF14
P18754	RCC-1

β-arrestin-dependent GPCR signaling lasts compared with G-protein dependent signaling events (Beaulieu et al., 2005), the involvement of β-arrestins in GPCR-stimulated transcription may be required in physiological situations when sustained signaling is needed. Our findings indicate that nuclear translocation of arrestins is not restricted to β-arrestin1, and moreover is also used by other GPCRs, as we found the mechanism being used by certain ORs. Moreover, we showed in our study that nuclear translocation of β-arrestin occurs under physiologically relevant conditions, although more detailed studies will be required to clearly establish the role of β-arrestin2 in vivo.

β-arrestin2 is well known to function as a scaffold for some MAP kinase modules thereby contributing to the activation of mitogen-activated protein kinases by GPCRs. We investigated activation of MAPKs in spermatozoa upon bourgeonal treatment and found that activation of hOR17-4 leads to phosphorylation of ERK1/2 and p38 MAPK. Interestingly, activated ERK1/2 showed nuclear localization. MAPK activation by GPCR-bound arrestin is generally thought to target the MAPK to specific extranuclear locations (Luttrell, 2003; Lefkowitz and Shenoy, 2005). In spite of this, it was recently shown that β-arrestin2 promotes a subset of ERK1/2-mediated transcriptional responses to lysophosphatidic acid receptor activation (Gesty-Palmer et al., 2005) and β2-adrenergic receptor-mediated nuclear translocation of ERK (Kobayashi et al., 2005). Whether the observed nuclear translocation of β-arrestin2 upon hOR17-4 activation in spermatozoa causes the nuclear translocation of ERK1/2, as in the cases of activated GPCRs mentioned above, or whether the activated receptor stimulates MAPK phosphorylation through transactivation of RTKs culminating in sequential activation of the Ras-Raf-MAPK cascade (Wetzker and Bohmer, 2003) remains to be investigated in future. Nevertheless, it is tempting to speculate that the general belief that MAPK phosphorylation upon EGF receptor activation leads to nuclear translocation of the kinase, whereas MAPK activation via GPCR-bound arrestin primarily increases cytosolic kinase activity does not hold true for every case. Moreover, it indicates that nuclear β-arrestin2 may act as a scaffold for the phosphorylation of nuclear proteins, which adds a new function for nuclear β-arrestins to the described modulation of histone acetylation by β-arrestin1 (Kang et al., 2005).

We could not detect the receptor hOR17-4 in the nuclei of spermatozoa upon odorant stimulation, although it was clearly depleted from the midpiece. This may be due to the fact that hOR17-4 is not part of the nuclear complex, raising the possibility that similar to β-arrestin1 (Kang et al., 2005), β-arrestin2 might act as molecular scaffold in the absence of the receptor. Another interesting aspect could be, that only parts of the receptor enter the nucleus after cleavage of the receptor protein in the cytosol, which has recently been shown for synaptic Dfrizzled2 receptors (Mathew et al., 2005).

We found that stimulation of the nuclear translocation of β-arrestin2 by hOR17-4 has a positive modulatory effect on transcription in a GAL4-based transactivation assay, thus raising the possibility of a role of β-arrestin2 in transcriptional regulation. Other endocytic proteins like eps15 and epsin also show transactivation activity, but do so without a preceding nuclear accumulation process (Vecchi et al., 2001; Poupon et al., 2002). Which genes are regulated by nuclear arrestins, and whether arrestin mediates widespread or more localized changes in gene expression needs to be investigated in future experiments.

An important question concerns the mechanism by which the activated receptor induces β-arrestin2 translocation to the nucleus. Activation-dependent GPCR endocytosis is known not only to terminate the initial signaling process through the heterotrimeric G-protein, but also to be involved in other signaling functions, serving as an important pathway to transmit signals from the cell surface to the cytoplasm and the nucleus (Benmerah, 2004). Some receptor downstream cascades are preferentially activated after internalization of receptor-ligand complexes into endosomal compartments (Miaczynska et al., 2004a). A protein that is involved in this transmission process is the endocytic protein Rab5, which translocates APPL1 from the membrane to the nucleus, where it interacts with regulators of chromatin structure and gene expression (Miaczynska et al., 2004b). β-Arrestins are mediators of endocytosis of seven membrane-spanning receptors, which bind to the receptor after ligand-induced phosphorylation. Phosphorylation of hOR17-4 contributes to receptor desensitization, indicating a failure in uncoupling of the receptor from the G-protein owing to a lack of β-arrestin2 recruitment. We found that bourgeonal-induced hOR17-4 phosphorylation is required for nuclear translocation of β-arrestin2. Interestingly, also in the case of receptor-induced nuclear translocation of β-arrestin1 a role of GPCR phosphorylation has been proposed (Kang et al., 2005). Typically, arrestins promote receptor internalization by recruiting the endocytic machinery, which also seems to occur in the case of hOR17-4. Binding of β-arrestin2 to the phosphorylated receptor and subsequent internalization of the receptor-arrestin complex seems to be required for nuclear translocation in the heterologous expression system. Alltogether, the potential role of β-arrestin2-mediated endocytosis of hOR17-4 for the translocation process has to await future investigation, especially in spermatozoa.

It is tempting to speculate that β-arrestin2 functions in the nucleus by providing scaffolding structures, such as those in endocytic pathways, which modify nuclear localization of specific transcription factors. The finding that β-arrestin2 mediates transactivation upon hOR17-4 stimulation suggests participation in nuclear functions in mammalian spermatozoa, despite the fact that these cells have a highly condensed, inactive chromatin structure. Interestingly, a growing number of studies indicate that the nucleus of mature spermatozoa contains potentially active chromatin domains (Gardiner-Garden et al., 1998; Pittoggi et al., 2001).

Based on our results, it is proposed that β-arrestin2 participates in the initiation of transcription during fertilization or early embryogenesis by localizing transcription factors. To examine the function of β-arrestin2 in the nucleus, formed nuclear complexes were subjected to a preliminary proteomic characterization. Interestingly, some of the nuclear β-arrestin2-associated proteins found were previously seen to localize to nuclease-hypersensitive chromatin fractions of spermatozoa. These nuclease-hypersensitive chromatin domains in sperm chromatin were shown to contain histones assembled with the DNA in a typical nucleosomal organization, despite the fact that the bulk chromatin in mature spermatozoa is highly condensed protamine-bound DNA (Pittoggi et al., 2001;

Wykes and Krawetz, 2003). The histone-bound nuclease-hypersensitive DNA domains contain clusters of potential sites for transcription factors and are indeed associated with transcription factors in sperm nuclei. This indicates that they represent active chromatin foci capable of activities typically associated with somatic cell chromatin (Pittoggi et al., 2001; Wykes and Krawetz, 2003). It has been postulated that the sequence-specific packaging of human sperm chromatin by histones and protamines may serve to designate a specific subset of early embryonic-expressed genes that may become transcriptionally competent soon after fertilization and possibly even before the conversion of the sperm nucleus in a male zygotic pronucleus (Wykes and Krawetz, 2003).

H2A.X, a member of the histone H2A family, is a chromatin component that is expressed at high levels in germ cells, but also in small amounts in somatic cells of other tissues (Tadokoro et al., 2003; Churikov et al., 2004). Association of RCC1 with chromatin is crucial for the control of nucleocytoplasmic transport by Ran guanosine triphosphatase and could provide a mechanism by which GPCRs regulate β-arrestin translocation to the nucleus and gene expression. RCC1 was shown to bind directly to mononucleosomes and to histones H2A and H2B, thereby establishing the polarity of the Ran-GTP gradient that drives nuclear transport and other nuclear events (Nemergut et al., 2001). The nuclear Ran and RCC1 proteins have been found to be associated with the hypersensitive chromatin fraction in sperm nuclei (Pittoggi et al., 1999). Another known nuclear interaction partner of RCC-1 is GAPDH, which we also identified in the β-arrestin2-associated complexes. GAPDH is normally localized in the cytoplasm of noncycling cells, but can be translocated to the nucleus, where it is important for the transcriptional regulation of histone gene expression, has a role in nuclear membrane fusion and modulates telomere structure (Sirover, 2005). KIF14 is expressed at low levels in all adult tissues and probably acts as a chromokinesin (Corson et al., 2005), which are known to act in various steps of mitosis and cytokinesis and help to maintain genome stability. Kif14 (a member of the kinesin-3 family) is required for chromosome congression and alignment (Zhu et al., 2005). It contains a forkhead-associated (FHA) domain that can act as a phosphorylation-dependent protein-protein interaction module and is typically found in nuclear proteins, and an ERM (ezrin-radixin-moesin) domain. Interestingly, ERM proteins have recently been implicated in linking the plasma membrane with nuclear events (Batchelor et al., 2004). Moreover, improper localization or absence of chromokinesins is one of the reasons for poor reproductive success in nuclear transfer embryos in primates (Simerly et al., 2003).

In conclusion, it appears that β-arrestin2, as well as other proteins from plasma-membrane-associated structures, is able to shuttle in and out of the nucleus in a receptor-regulated manner. This shuttling might be important in regulating gene expression, and β-arrestin2 may be involved in the regulation of a limited and specific set of genes implicated in particular processes in sperm fertilization and postfertilization events. In principle, these interactions are indicative of new mechanisms of signaling propagation from G-protein-coupled receptors, offering supporting evidence that β-arrestin2 may recruit cytosolic proteins following ligand-receptor engagement and relocate to the nucleus to implement or regulate transcription. This behaviour is reminiscent of other proteins involved in the regulation of transcription, which are held outside of the nucleus until particular signaling events trigger their nuclear translocation. If β-arrestin2 and phosphorylated MAPK are localized in the sperm nucleus, they are probably internalized within the oocyte after sperm-egg binding and sperm-egg fusion. This may be important for the regulation of initiation of the genetic activities that take place in the male pronucleus of the zygote soon after spermatozoon penetration of the oocyte. It is tempting to speculate, that the nuclear translocation of β-arrestin2 helps to identify preset functional compartments of sperm nuclei to support genetic activities during fertilization and/or in early steps of embryonic development.

Materials and Methods

HEK293 and sperm cell fractionation

Human sperm were freshly obtained from young healthy donors. Percoll density gradient centrifugation was performed to isolate mature and motile sperm as described (Spehr et al., 2004). Spermatozoa were resuspended at 4°C in standard Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose) with protease inhibitors (Roche Complete® protease inhibitor mixture). Cells were lysed by homogenization with a tight-fitting pestle (60-70 strokes) with subsequent sonification twice for 10 seconds at 40 watts (Sonifier B12, Branson Sonic Power Co.). HEK293 cells were harvested, pelleted and homogenized with a Dounce homogenizer. Nuclei were obtained by centrifugation (750 ***g***, 5 minutes); supernatants were centrifuged for 2 hours at 40,000 ***g*** to obtain membrane pellets. Cytosolic proteins in the supernatant were pelleted by centrifugation (100,000 ***g***, 4 hours). The membrane pellet was solubilized using 2% Chapso (Sigma); protein concentrations were determined using the Bradford microassay (Bio-Rad).

Antibodies

The following primary antibodies were used: (1) mouse polyclonal antibody against PKA (UTSW CBI Antibody Core, Southwestern Medical Center, University of Texas); (2) mouse monoclonal antibody against β-arrestin2 (H-9) from Santa Cruz Biotechnology (sc-13140) raised against the N-terminus of β-arrestin2 of human origin and recommended for detection of β-arrestin2 of mouse, rat and human origin by WB, IP and IF. The specificity of this antibodies was confirmed by preincubation with a blocking peptide (Santa Cruz, sc-13140P) for 24 hours at –4°C; (3) mouse monoclonal antibody against β-actin (Sigma); (4) rabbit polyclonal antibodies against p44/42 MAPK and against phosphorylated p44/42 MAPK (New England Biolabs); (5) rabbit polyclonal antibodies against p38 MAPK and phosphorylated p38 MAPK (New England Biolabs); (6) rabbit polyclonal antibody against the phospho-(Ser/Thr) PKA substrate recognizing the R-x-x-S/T-x-x motif (New England Biolabs); (7) mouse polyclonal antibody against hOR17-4 which was raised against the C-terminal sequence (SLRNKDMHGALGRLLDKHFK) using a genetic immunization technique provided at the University of Texas-Southwestern Medical Center (Chambers and Johnston, 2003). Secondary goat anti-rabbit or goat anti-mouse antibodies conjugated to Alexa Fluor 546 (Molecular Probes) or to HRP (Bio-Rad) were used.

DNA constructs and plasmids

Human β-arrestin2 was amplified from RNA obtained from human testis tissue by RT-PCR using specific primers that amplify the complete open reading frame and contain restriction sites for further subcloning. The pcDNA3-β-arrestin2-GFP plasmid was generated by cloning the PCR product into a linearized GFP-containing plasmid with restriction sites for the generation of N-terminal fusion constructs (Neuhaus et al., 2005).

A pcDNA3 plasmid that encodes AA1-147 from Gal4-DNA binding domain was generated by cloning of a PCR-amplified Gal4-DNA binding domain, the pcDNA3-Gal4 DNA-binding domain-β-arrestin2 fusion construct was generated by subcloning β-arrestin2 into the linearized Gal4 DNA binding domain-containing plasmid.

The receptor construct for hOR17-4 was obtained by PCR of human genomic DNA and cloning of the full-length receptor into pcDNA3 (Invitrogen). Phosphorylation site mutations were introduced at the C-terminus (position T312A) and in the third intracellular loop (positions S230A, S232A, S239A).

The C-terminus mutant of hOR17-4 was obtained by PCR from the pcDNA3 vector containing the full-length receptor using specific downstream primer with a T312A mutation. The intracellular loop 3 mutant (positions S230A, S232A, S239A) was generated using an ‘overlap extension mutagenesis via PCR’ strategy. Two separate PCR reactions were performed, each with mutated primer mut-loop-5′ (GCT AAG AAA TAC AAA GCC TTC GCC GTC TGT GCC GCC CAT TTG TT)

or mut-loop-3′ (CGG CAC AGA CGG CGA AGG CTT TGT ATT TCT TAG CGA CTG AG) and the complement primers T7 or SP6, respectively. PCR products that had a length of 750 bp in case of moot-loop-3′/T7 and 400 bp in the case of mut-loop-5′/Sp6 primer pairs were mixed and used in the next PCR reaction to amplify the complete mutated hOR17.4 using specific primers for hOR17.4 with restriction sites for subcloning into the pcDNA3 vector. All generated plasmids were verified by sequencing.

Immunocytochemistry

Percoll-purified spermatozoa were incubated with standard Ringer solution with and without bourgeonal (500 μM) and plated on poly-L-lysine-coated coverslips (80–100 μm thick; Menzel Gläser, Germany). Coverslips were washed in Ringer solution and fixed in 3% paraformaldehyde in Ringer solution containing 10 mM glucose (30 min at room temperature). Cells were permeabilized with 0.1% Triton X-100 in PBS containing 1% cold-water fish skin gelatin (Sigma) and incubated with primary antibodies in PBS-gelatin-Triton X-100. After washing, cells were incubated with fluorescently labeled secondary antibodies; nuclei were counterstained with DAPI (Molecular Probes) and coverslips were mounted in ProLong Antifade (Molecular Probes). All fluorescence images were obtained with a confocal microscope (LSM510 Meta; Zeiss), quantification of the intensities of the arrestin staining in the nucleus and cytosol was performed with the original data. Images were further processed with Photoshop (Adobe Systems, San Jose, CA).

Western blotting

Sample aliquots of fractionated spermatozoa were mixed with Laemmli buffer (30% glycerol, 3% SDS, 125 mM Tris-HCl, pH 6.8), resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (Protran; Schleicher & Schuell). The nitrocellulose membranes were stained with Ponceau S (Sigma), blocked with TBST (150 mM NaCl, 50 mM Tris-HCl, Tween20, pH 7.4) containing 5% non-fat dried milk (Bio-Rad) and incubated with primary antibodies diluted in 3% dried milk in TBST. After washing and incubation with HRP-coupled secondary antibodies, detection was performed with ECL plus or ECL Advance (Amersham) on Hyperfilm ECL (Amersham). p44/42 MAPK, p38 MAPK and β-arrestin2 signals were quantified by densitometry with ChemImager v5.5 (Alpha Innotech Corporation).

Single-cell Ca^{2+} imaging

Cell density of Percoll-purified sperm was photometrically adjusted to an extinction of E_{260nm}=0.035. Sperm were incubated (45 minutes at 35.5°C) in Ringer solution containing 7.5 μM Fura-2-AM (Molecular Probes) and 0.1% Pluronic F-127 (Sigma). After removal of extracellular Fura-2, cells were transferred to concanavalin A (Sigma)-coated dishes and were treated by the indicated concentrations of water-soluble PKA inhibitor H-89 (Calbiochem) for 30 minutes. Ratiofluometric Ca^{2+} imaging was performed as described (Spehr et al., 2003) using a Zeiss inverted microscope equipped for ratiometric imaging. Images were acquired from up to 15 spermatozoa in a randomly selected field of view, and integrated fluorescence ratios (f_{340}/f_{380}) were measured. Exposure to bourgeonal® (Quest, Naarden, Netherlands) was accomplished using a specialized microcapillary application system. Only spermatozoa with heads and midpiece attached and tails beating were included in the analysis.

To measure the responses of HEK293 cells transfected with mutated hOR17-4 constructs, cells were incubated (45 minutes) in Ringer solution containing 7.5 μM Fura-2-AM at room temperature and measured after exposure to either 100 μM bourgeonal (Spehr et al., 2004) as described above.

To measure β-arrestin2-GFP translocation and Ca^{2+} influx simultaneously HEK293 cells were incubated for 30 minutes at room temperature in Ringer Solution containing 5 μM Calcium Orange (Molecular Probes). After washing, Ca^{2+} imaging was performed on a confocal microscope (LSM510 Meta; Zeiss) using 543 nm excitation and 565-615 nm emission filters. Images were collected every 5 seconds for 10-15 minutes; bourgeonal (5 μM) was applied at the 50-second time point and was present continuously throughout the whole experiment.

Cell culture, transfection and microscopy

Hana3A cells (Saito et al., 2004) were kindly provided by Dr Hiroaki Matsunami (Department of Molecular Genetics and Microbiology, Duke University Medical Center). HEK293 and Hana3A cells were maintained under standard conditions in MEM supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, and 2 mM L-glutamine. Transfections were performed using a standard calcium phosphate precipitation technique. Cells were transfected with a 5:1 ratio of the different hOR17-4 constructs and β-arrestin2-GFP. Two days after transfection growth media were removed and replaced with standard Ringer solution; bourgeonal and inhibitors were added at room temperature at concentrations of 500 μM bourgeonal, 0.25 mg/ml concanavalinA (Sigma), 10 μM phenylarsine oxide (Sigma), 5 μg/ml filipin III (Sigma) or 10 mM β-cyclodextrin (Sigma); inhibitors were applied 30 to 60 min before bourgeonal application. Images were collected at 8 frames/minute for 30 minutes using 488 nm excitation and a 505-nm long-pass filter on a Zeiss laser-scanning microscope (LSM510 Meta; Zeiss). Quantification of the intensities of the arrestin-GFP in the nucleus and cytosol was performed with the original data using the LSM510 version 3.2SP2 program. The patch was outlined using the freehand drawing tool available as part of the software package, and the average pixel intensity of the resulting region of interest was obtained for each cell. The mean pixel intensity is the average of values from at least 50 different cells in each condition. This quantification is representative of at least of five independent experiments. Images were adjusted for contrast with Photoshop (Adobe Systems, San Jose, CA).

Luciferase reporter assays

HEK293 or Hana3A cells grown in 35mm dishes were transiently transfected with 0.15 μg of the GAL4-TK-luciferase reporter (kindly provided by L. Klein-Hitpass, IFZ Essen, Germany), 0.45 μg of the Gal4DBD or Gal4DBD-β-arrestin2 fusion construct and 2.4 μg of hOR17-4 using ExGen 500 transfection reagent (Fermentas). 48 hours after transfection, luciferase activity was measured using equal amounts of lysates from control HEK293 cells coexpressing the receptor and Gal4 DNA-binding domain alone (gal4) and cells coexpressing hOR17-4 and Gal4DBD-β-arrestin2 fusion construct before (β-arr) and after treatment with bourgeonal for 6 hours (β-arr+B) using a commercial kit (Promega).

LC-ESI MS/MS and protein identification

A dual gradient system HPLC pump (Dionex, Amsterdam) including a Famos auto sampler and Switchos was connected to a Finnigan LTQ ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA). The LTQ was operated using the Instrument Method files of Xcalibur to acquire a full MS scan between 350 and 2000 m/z followed by full MS/MS scans of the three most intensive ions from the preceding MS scan. Samples were loaded onto a 15 cm fused silica column as described elsewhere (Gatlin et al., 1998). The column flow rate was set to 0.15-0.25 μl/minute and a spray voltage of 1.8 kV was used. The buffer solutions used for the chromatography were 5% acetonitrile (ACN); 0.012% heptafluorobutyric anhydride (HFBA); 0.5% acetic acid (buffer A) and 80% ACN; 0.012% HFBA; 0.5% acetic acid (buffer B). After equilibration for 5 minutes with buffer A, a linear gradient was generated within 60 minutes. The SEQUEST algorithm was used to interpret MS/MS spectra as described previously (Spehr et al., 2004). We considered only those proteins that were unambiguously identified by a minimum of two peptides in two independent experiments.

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