G protein-coupled receptor kinase 2 and β-arrestins are recruited to FSH receptor in stimulated rat primary Sertoli cells

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Abstract

FSH-receptor (FSH-R) signaling is regulated by agonist-induced desensitization and internalization. It has been shown, in a variety of overexpression systems, that G protein-coupled receptor kinases (GRKs) phosphorylate the activated FSH-R, promote β-arrestin recruitment and ultimately lead to internalization. The accuracy of this mechanism has not yet been demonstrated in cells expressing these different molecules at physiological levels. Using sucrose gradient fractionation, we show that FSH induces the recruitment of the endogenous GRK 2 and β-arrestin 1/2 from the cytoplasm to the plasma membrane of rat primary Sertoli cells. As assessed by ligand binding, the FSH-R was found expressed in the fractions where GRK 2 and β-arrestins were recruited upon FSH treatment. In addition, the endogenous β-arrestin 1 was found dephosphorylated in an agonist-dependent manner. Finally, a significant FSH-binding activity was co-immunoprecipitated with the endogenous β-arrestins from agonist-stimulated but not from untreated Sertoli cell extracts. This FSH-R interaction with β-arrestins was sustained for up to 30 min. In conclusion, our data strongly suggest that the GRK/β-arrestin machinery plays a physiologically relevant role in the regulation of the FSH signaling.

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Introduction

The follicle-stimulating hormone receptor (FSH-R), expressed by granulosa in female and Sertoli cells in male, is pivotal for gonadal development and gamete production during the fertile phase of life (Simoni *et al.* 1997, Crepieux *et al.* 2004). The FSH-R, together with the lutropin and thyrotropin receptors, belongs to the glycoprotein hormone receptor subfamily of seven membrane-spanning receptors (7MSR) (Simoni *et al.* 1997). Following coupling of the FSH-R to Gs (heterotrimeric GTP-binding protein alpha subunit), cAMP and protein kinase A have long been shown to mediate FSH-specific intracellular signaling events ultimately leading to gene expression. Amongst the multiple mechanisms that regulate 7MSR signaling is their phosphorylation by the G protein-coupled receptor kinases (GRKs). These serine–threonine kinases are able to phosphorylate agonist-occupied receptors and promote their desensitization. The association of β-arrestins with GRK-phosphorylated receptors uncouples them from their effector G proteins (Kohout & Lefkowitz 2003). The β-arrestins also function as adaptor proteins that target the desensitized receptor to components of the endocytic machinery such as AP-2 and clathrin (Claing *et al.* 2002). In addition to their role in desensitization and internalization, β-arrestins are also able to scaffold various signaling proteins, including the extracellular signal-regulated kinase 1/2 (ERK) mitogen activated protein kinase (MAPK) components (Luttrell & Lefkowitz 2002, Lefkowitz & Shenoy 2005). As a prerequisite to their activation, GRK 2/3 and β-arrestin 1/2 are rapidly recruited, upon agonist stimulation, from cytoplasm to βγ G protein subunits and to GRK-phosphorylated receptor respectively (Touhara *et al.* 1994, Barak *et al.* 1997, Lodowski *et al.* 2003). GRK2 associates to microsomial membranes in endogenous conditions (Garcia-Higuera & Mayor 1994, Garcia-Higuera *et al.* 1994, Murga *et al.* 1996), in contrast, GRK 5 and 6, the two other ubiquitous GRK, are mainly associated to the PM due to their polybasic and palmitoylated C-terminal ends respectively (Kohout & Lefkowitz 2003). It has recently been shown that GRK5 and 6 were also able to undergo some intracellular traffic upon agonist stimulation, but the functional consequences of these relocalization events are much less documented (Marion *et al.* 2002, Johnson *et al.* 2004).

Like many other 7MSR, the FSH-R becomes functionally uncoupled from Gs upon continuous stimulation (Verhoeven *et al.* 1980, Simoni *et al.* 1997). When overexpressed, different GRKs phosphorylate the FSH-R in an agonist-dependent manner and desensitize cAMP production (Lazari *et al.* 1999, Troispoux *et al.* 1999, Reiter *et al.* 2001, Kishi *et al.* 2002, Marion *et al.* 2002, Krishnamurthy *et al.* 2003*a*, 2003*b*). The interaction of the phosphorylated FSH-R with β-arrestins is essential for its functional uncoupling (Lazari *et al.* 1999,

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Troispoux *et al.* 1999). Moreover, activation of the FSH-R results in the internalization of the agonist–receptor complex via clathrin-coated pits by a pathway that requires both dynamin and arrestins (Nakamura *et al.* 1998, Lazari *et al.* 1999). Notably, in the studies published to date, the different partners (i.e. the GRK, the β-arrestin or the FSH-R) were heterogenously overexpressed. We have shown that GRKs and β-arrestins were able to uncouple the FSH-induced cAMP response in rat primary Sertoli cells, but the GRK and β-arrestins were transfected (Marion *et al.* 2002). After overexpression, the stoichiometry within the signaling complexes is modified; the compartmentalization of the transfected factors is also questionable.

In the present study, we tested the hypothesis that the 'recruitment' of GRKs and β-arrestins to the activated FSH receptor that has been observed in cells overexpressing the participating proteins also occur at physiological levels of the partners. To this purpose, we used the well-characterized relocalization of GRK 2 and β-arrestin 1/2 upon agonist stimulation as a read-out of their recruitment to the FSH-R in rat primary Sertoli cells. We also took advantage of the agonist-induced dephosphorylation of β-arrestin 1 to look at the FSH effects in our model (Lin *et al.* 1997).

Materials and Methods

Materials

Leibovitz's (L 15) medium and Dulbecco's minimum essential medium (DMEM) were purchased from Gibco-BRL Life Technologies. Penicillin, streptomycin, trypsin, trypsin inhibitor (Soybean), DNase type-I, and Human Transferrin were obtained from Sigma Chemical Co. Porcine FSH (pFSH) preparations were kindly donated by Y Combarnous (Nouzilly, France) and J Closset (Liège, Belgium).

The β-arrestin monoclonal antibody F4C1 was kindly provided by Dr L A Donoso (Thomas Jefferson University, PA, USA). The A1CT β-arrestin polyclonal antibody was a generous gift from Dr R J Lefkowitz (Durham, NC, USA) (Attramadal *et al.* 1992). The GRK 2/3 monoclonal antibody C5/1 was purchased from Upstate Biotechnology (Waltham, MA, USA). Anti-E-cadherin, -Rab 4, and -Rab 11 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). The rabbit polyclonal anti-Rab 7 antibody was a kind gift of Dr S R Pfeffer (Stanford University, CA, USA). The anti-Golgi p58-K was from Sigma. Phospho-β-arrestin1 antibody was from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Sertoli cells were prepared from testes of 12-day-old rats (Wistar, Janvier, France), according to a previously described method (Crepieux *et al.* 2001). The study was performed according to the European legislative, administrative and statutory measures concerning the protection of animals used for experimental or other scientific purposes (86/609/EEC). Our Sertoli cell preparations contained less than 10% of germ cells and 2% of myoid cells (Troispoux *et al.* 1998). Sertoli cells were plated at a density of 30×10^6 cells in 100-mm dishes. Cultures were performed in DMEM supplemented with 100 U/ml penicillin, 2·5 μg/ml amphotericin B, 100 μg/ml streptomycin, 5 μg/ml human transferrin, 2 mM glutamine, 200 ng/ml vitamin E and 50 ng/ml vitamin A (retinol) at 34 °C in a humidified atmosphere of 5% CO_2.

Subcellular fractionation

The day following their initial seeding, Sertoli cells were stimulated or not for 4 or 30 min with pFSH (100 ng/ml). Plates were ice-chilled and the cells were washed with ice-cold PBS. Cells were scraped into ice-cold buffer A (20 mM Hepes pH 7·5, 0·15 M NaCl, 250 mM sucrose) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptine, 5 mM EDTA, 3 mM EGTA, 50 mM β-glycerophosphate, 10 mM sodium fluoride, 0·1 mM orthovanadate, and 100 nM okadaic acid) and 1 mg/ml of cycloheximide and a postnuclear supernatant was prepared as previously described (Pasquali *et al.* 1999). The fractionation procedure was carried out on a 10–40% continuous sucrose gradient as described by Fialka *et al.* (1997). Then, 23 fractions were collected on ice from the bottom in 200 μl aliquots under constant volume delivery and stored at −20 °C. Fractions were numbered from the bottom of the gradient.

Immunoblotting

Equal volumes of each fraction (gradient samples) or equal amount of total cell lysates (*p*-β-arrestin1 experiments) were boiled for 5 min in reducing conditions (125 mM Tris–HCl, pH 6·8, 20% glycerol, 4% β-mercaptoethanol, 2% SDS, and 0·02% bromophenol blue), prior loading on SDS-PAGE (10% acrylamide). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, immunoblotted with specific antibodies and revealed using enhanced chemiluminescence autoradiography (NEN Life Science Products, Boston, MA, USA). The films as well as their corresponding PVDF membranes stained with Coomassie Blue were quantified using the NIH Image software (Bethesola, MD, USA). Signal intensities corresponding to the proteins specifically immunodetected in the different fractions were normalized using the total amounts of proteins loaded on each lane as determined by Bradford assays.

Enzymatic assays

The 5′-nucleotidase activity (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5, 5′-NT) has been measured in gradient fractions as described (Ames & Durbin 1960). Aryl sulfatase activity was assayed on each fraction according to Chang *et al.* (1981). Specific activities were expressed as percentage of

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maximal response. To label the early endosomes (EE), 30×10^6 Sertoli cells were incubated with 5 mg/ml of horseradish peroxidase (HRP) in DMEM for 4 min at 34 °C and washed three times with BSA (1%) in cold PBS. Then, the subcellular fractionation was carried out at 4 °C as described above. To label the late endocytic compartments, Sertoli cells were incubated with 5 mg/ml of HRP in DMEM for 4 min at 34 °C and then chased for 20 or 40 min at 34 °C in BSA (1%) DMEM. HRP activity of 30 µl of each gradient fraction was detected with 100 µl/well of a substrate mixture containing 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) at 0·04 M in 0·05 M glycine/citric acid buffer pH 4·0, and 0·01% H_2O_2 (Innamorati *et al.* 2001). After incubation for 10 min at room temperature, absorbance at 405 nm was measured with an automated microtiter plate reader Multiskan MKII (Labsystems, Helsinki, Finland). The background of endogenous HRP activity measured in unlabeled cells tested in the same conditions was subtracted and the results were expressed as percentage of total absorbance at 405 nm.

Binding assay

The assay was carried out in 50 mM Tris–HCl pH 7·4 containing 0·1% BSA and 6 mM $CaCl_2$. Aliquots of the gradient fractions were incubated for 2 h at 37 °C in the presence of [^{125}I]-pFSH labeled as previously described (Vandalem *et al.* 1979). The nonspecific binding was measured by incubating separate tubes with an excess of unlabeled pFSH. The reaction was stopped by the addition of ice-cold buffer containing 15% of polyethylene glycol 6000. Tubes were subsequently centrifuged, washed, and counted.

Co-immunoprecipitation

Sertoli cells plated in 150 mm dishes were stimulated for the indicated time with 100 ng/ml of pFSH. At the end of the stimulation, cells were washed with ice-cold PBS and lysed in 1 ml of RIPA buffer (Tris-Cl 50 mM, pH 7·5, NaCl 300 mM, EDTA 5 mM, Triton X-100 and protease inhibitors). The lysates were centrifuged and the supernatants were precleared with 25 µl of protein A-sepharose (Sigma) for 1 h at 4 °C. Lysates were then incubated at 4 °C for 16 h in the presence of 10 µg of A1CT anti-β-arrestin antibody (Attramadal *et al.* 1992). A control series of samples was incubated with normal rabbit serum instead of A1CT. In order to immunoprecipitate the immune complexes, 25 µl of protein A-sepharose were added to the samples for 1 h at 4 °C. The protein A sepharose beads were centrifuged and washed five times with RIPA buffer. The beads were subsequently resuspended in binding buffer and processed for binding assay as described above. The number of counts measured in the control series (i.e. no A1CT antibody) was subtracted from the values obtained in the other series.

Statistical analysis

Statistical significance was determined by using one-way ANOVA (Bonferoni's multiple comparison tests; Prism, GraphPad Software Inc., San Diego, CA, USA).

Results

We wanted to determine whether or not the activated FSH-R was able to recruit GRK 2 and β-arrestins. For this purpose, postnuclear supernatants of primary rat Sertoli cells were subjected to continuous 10–40% sucrose gradient fractionation. A typical distribution of total proteins after fractionation is shown (Fig. 1A). To determine the distribution pattern of intracellular organelles along the gradient fractions, we used established organelle marker proteins (Fig. 1B). E-Cadherin immunoreactivity denoted the presence of baso-lateral membranes in dense fractions 1–9 (Chen *et al.* 1999). p58K, a trans-Golgi network (TGN) protein was also immunodetected in those fractions. Rab 4, which is involved in the recycling events from the EE to the PM, was immunodetected in dense fractions 5–9, likely anchored to the PM and recycling endosomes (RE), and in lighter fractions 15–19. Rab 7 protein is implicated in downstream endocytic traffic from the EE to the late endosomes (LE) (Chavrier *et al.* 1990) and in the continuous fusion events occurring between the EE and the lysosomes (Feng *et al.* 1995). In fractionated Sertoli cells, Rab 7 was immunodetected both in fractions 5–9 and 14–18. Rab 11, a specific marker of recycling compartments, was found detected in fractions 1–9 and 15–21. Similar Rab 4, 7, and 11 detection in two distinct regions of a gradient has already been reported in other cells (Fialka *et al.* 1997). The labeling observed in the dense part of the gradient could be due to their anchoring to the PM and lysosomes which are known to be arrival compartments for these Rab proteins.

To further characterize our gradient, HRP was internalized as fluid phase marker (Leighton *et al.* 1968) (Fig. 1C). After 4 and 20 min of internalization, HRP was found in fractions 15–21. Then, HRP activity shifted to fractions 1–4 after 40 min of chase. This distribution of HRP confirms that EE and LE are enriched in fractions 15–21, while lysosomes are mainly located in the heaviest fractions (1–4).

Aryl sulfatase and 5′-nucleotidase activities were also measured to further confirm the localization of the lysosomal fractions and of the apical PMs respectively (Fig. 1D). The major peak of aryl sulfatase activity was observed in fractions 1–2. In contrast, the 5′-nucleotidase activity was found in the lightest fractions (21–23). Together these data show that our fractionation procedure was able to separate the apical PM (fractions 21–23) from the baso-lateral PM (fractions 1–9) and EE/LE (fractions 15–21).

The next step in the characterization of our rat primary Sertoli cell gradients was to localize the FSH-R enriched fractions. Since there is no FSH-R specific antibody able to detect the endogenous level of this receptor, we assayed each

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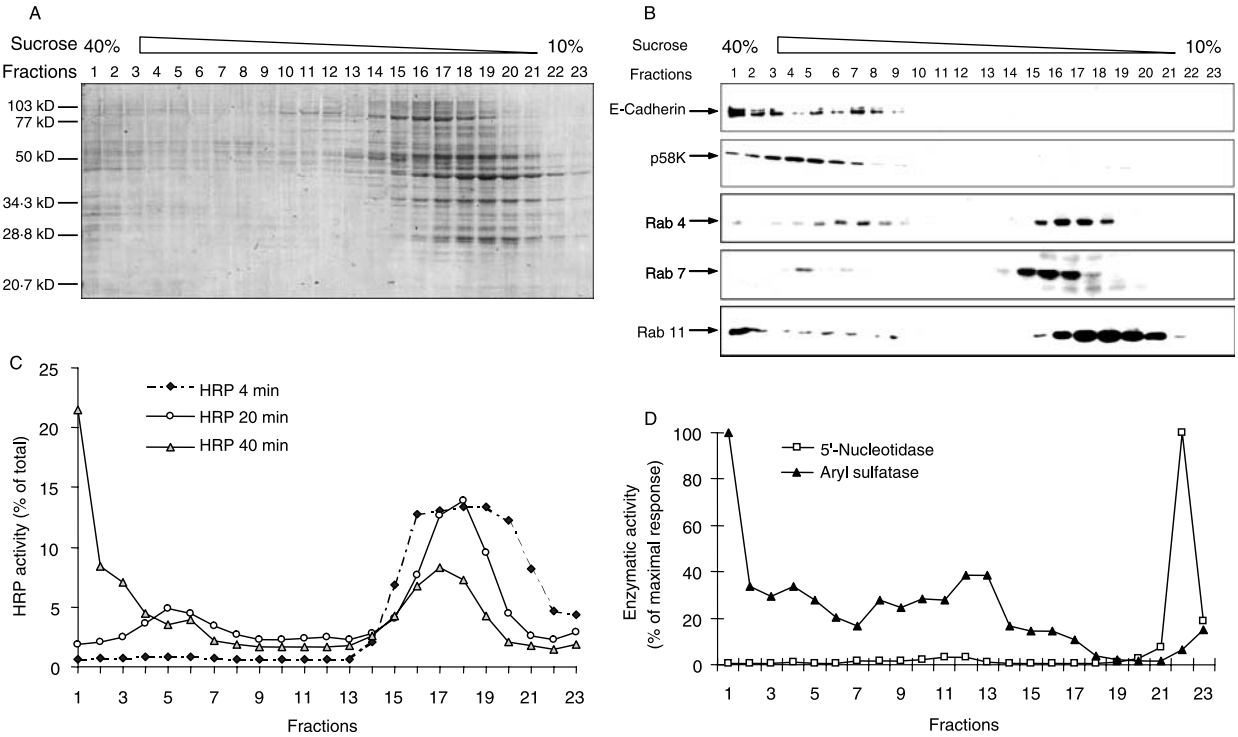

Figure 1 Characterization of Sertoli cells subcellular particles in 10–40% sucrose gradient fractions. Cell lysates were prepared from rat primary Sertoli cells and were subsequently fractionated upon a continuous 10–40% sucrose gradient. Equal volumes of each fraction were loaded on 10% SDS-PAGE. (A) A representative profile of major proteins after gradient fractionation is shown. Total proteins were stained with Coomassie Blue after electrotransfer onto PVDF membrane. (B) A panel of compartment-specific antibodies was used for subcellular particles characterization: E-cadherin (baso-lateral membranes); p58K (TGN); Rab 4 (EE–PM–RE); Rab 7 (EE–LE–lysosomes); Rab 11 (recycling compartments). (C) Fluid-phase internalization of HRP. (D) Distribution of organelle-specific enzymatic activities after continuous sucrose gradient fractionation. Representative results are shown. All experiments have been independently repeated at least three times.

fraction for binding activity to [^{125}I]-FSH (Fig. 2). The FSH binding sites were clearly located in the baso-lateral PM (fractions 1–9) and apical PM (fractions 21–23) enriched regions of the gradient. This result is consistent with the notion that the FSH-R mainly stays in the PMs in the absence of FSH.

We reasoned that the agonist-triggered translocation of GRK 2 from the cytoplasm to the PM, if it happened in primary Sertoli cells upon FSH stimulation, should be visualized in our gradient system. Thus, the subcellular localization of the endogenously expressed GRK 2 was determined in rat primary Sertoli cells stimulated with FSH for 0, 4, or 30 min. A representative experiment is shown in Fig. 3A,B. In the absence of FSH, GRK 2 was predominantly detected in light EE/LE/RE-rich fractions 14–21 (Fig. 3A,B). After 4 min stimulation, a significant increase of GRK 2 was detected in fractions 1–5 containing the baso-lateral PM. Interestingly, a very significant accumulation was also observed in fractions 22–23 containing the apical PM.

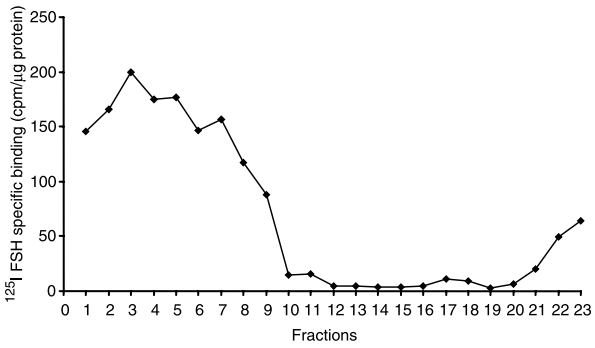

Figure 2 Distribution of the endogenous FSH-R in rat primary Sertoli cells fractionated on continuous 10–40% sucrose. Equal volumes of each fraction of the gradient were assayed for specific binding to [^{125}I]-FSH. Specific binding values were normalized with respect to the total protein content present in each fraction. A representative experiment is shown. Similar repartitions of the FSH binding activities have been independently observed in three separate experiments.

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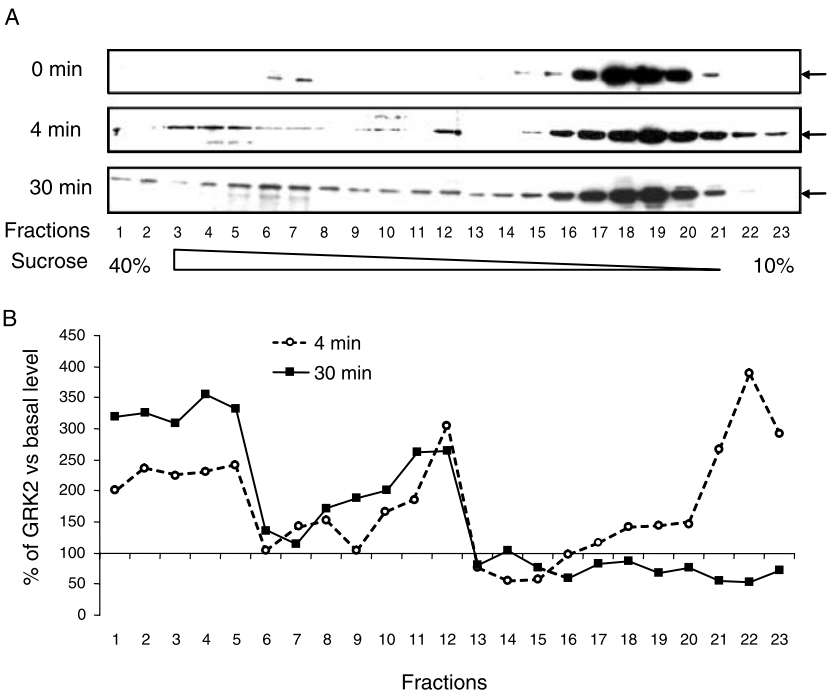

Figure 3 Relocalization of the endogenous GRK 2 upon FSH stimulation in rat primary Sertoli cells. (A) Sertoli cells stimulated for 0, 4, or 30 min were fractionated on a continuous 10–40% sucrose gradient. Equal volumes of each fraction were immunoblotted using a mouse monoclonal antibody detecting both GRK 2 and 3. (B) Relative amounts of GRK 2 were normalized with respect to the total protein content present in each fraction. The level of GRK 2 present in each fraction in the absence of FSH has been arbitrarily chosen as 100%. A representative experiment is shown. Similar results have been independently observed in at least three separate experiments.

After 30 min of stimulation, the GRK 2 translocation in fractions 1–6 and 9–12 was reinforced. Inversely, the GRK 2 accumulation, which was consistently observed in fractions 22–23 after 4 min, was abolished after 30 min of FSH stimulation. The sensitivity of GRK 3 detection was unfortunately insufficient in the gradient to achieve reliable quantification and analyses.

Next, we investigated whether FSH could also induce the translocation of endogenous β-arrestins in our model system (Fig. 4A,B). In the absence of FSH, endogenous β-arrestins were predominantly detected in EE/LE-rich fractions 14–21. After 4 min stimulation, a significant increase of both β-arrestin 1 and 2 was detected in fractions 1–6 containing the baso-lateral PM. A significant accumulation was also consistently observed in fractions 22–23 containing the apical PM. After 30 min of stimulation, the β-arrestins were more abundant in fractions 1–6 than in controls and 4 min stimulated cells. Interestingly, β-arrestin 2 was found preferentially recruited in fraction 1–6 upon both 4 and 30 min of FSH stimulation. However, the accumulation of β-arrestins observed after 4 min stimulation in fractions 22–23 was abolished after 30 min of agonist treatment.

It is well established that β-arrestin 1, which is phosphorylated on Ser-412 in the absence of agonist stimulation, undergoes rapid dephosporylation upon receptor activation (Lin *et al.* 1997). Based on the data presented in Fig. 4 of this paper, we hypothesized that FSH should induce β-arrestin 1 dephosphorylation in rat primary Sertoli cells. To test this hypothesis, Sertoli cells lysates were analyzed using an antibody specific for the phosphorylated form of β-arrestin 1. The results presented in Fig. 5A,B show 28 and 64% of β-arrestin 1 dephosphorylation after 4 and 30 min of FSH stimulation respectively. Further, this finding confirms the ability of the FSH-R to trigger the recruitment of β-arrestins.

That FSH-triggered GRK 2 and β-arrestin relocalization in FSH-R enriched cellular compartments strongly suggested the involvement of the GRK/β-arrestin system in the regulation of this receptor signaling. In order to provide direct evidence, we immunoprecipitated the endogenous β-arrestins and measured the FSH-binding activity in the precipitates from 0, 4, or 30 min FSH-treated Sertoli cells (Fig. 6). No significant specific binding was measured in the unstimulated cells. However, specific FSH-binding activity was consistently co-immunoprecipitated with the endogenous β-arrestins upon FSH stimulation for 4 and 30 min.

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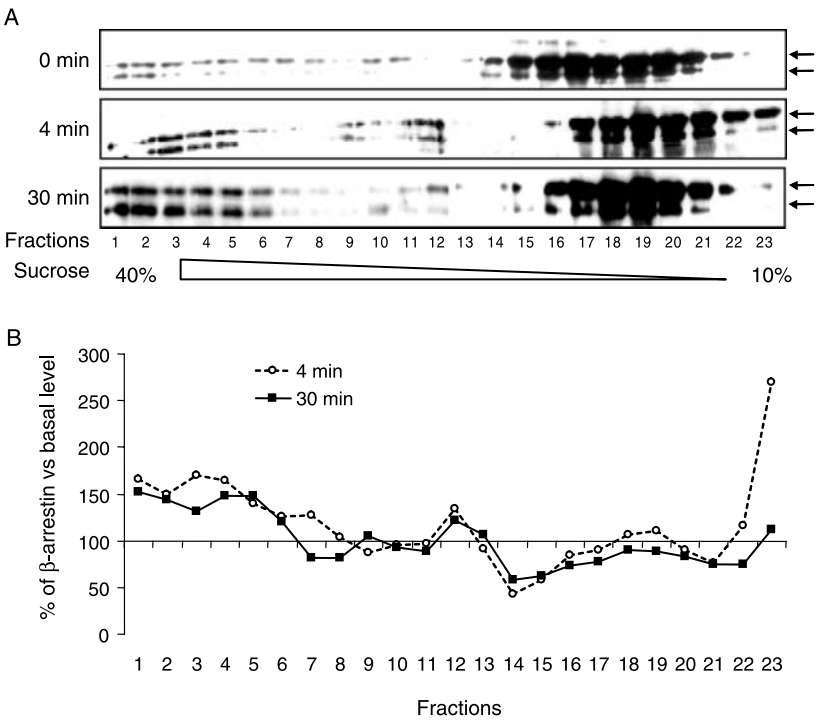

Figure 4 Relocalization of the endogenous β-arrestins upon FSH stimulation in rat primary Sertoli cells. (A) Immunodetection of β-arrestin 1 and 2 in gradient fractions. Immunoblotting was carried out using the F4C1 mouse monoclonal antibody detecting both β-arrestin 1 and 2. Sertoli cells stimulated for 0, 4, or 30 min were fractionated on a continuous 10–40% sucrose gradient as described. (B) Quantification of β-arrestin 1 level in gradient fractions. Relative amounts of β-arrestin 1 were normalized with respect to the total protein content present in each fraction. β-arrestin 1 levels in the nonstimulated controls were arbitrarily chosen as 100%. A representative experiment is shown. Similar results have been independently observed in at least three separate experiments.

These data show, in a physiologically relevant model, that FSH induces GRK 2 and β-arrestins recruitment and triggers the formation of a stable complex between the activated FSH-R and the β-arrestins.

Discussion

To date, the available data concerning the regulation of the FSH-R function by GRKs and β-arrestins have been accumulated using overexpression of at least one of the protein in heterologous or homologous cellular systems. Using this type of approaches, it has been demonstrated that different GRKs and β-arrestins are involved in regulating the internalization and signaling of the FSH-R (Lazari *et al.* 1999, Troispoux *et al.* 1999, Reiter *et al.* 2001, Kishi *et al.* 2002, Marion *et al.* 2002, Krishnamurthy *et al.* 2003*a*, 2003*b*). However, when expressed above the physiological level, the stoichiometry of these factors within signaling complexes is altered and this might ultimately modify GRK and/or β-arrestin specificity. In the present study, using nontransfected rat primary Sertoli cells as a model, we provide strong evidence that endogenous GRK 2 and β-arrestin are recruited to the FSH-R in an agonist-dependent manner.

Upon agonist stimulation, the FSH-R is internalized, whereas the hallmark of GRK 2/3 and β-arrestin 1/2 activation is their rapid relocalization from the cytoplasm to the PM (Kohout & Lefkowitz 2003). Thus, we sought to determine whether FSH treatment was able to trigger comparable trafficking processes. However, primary Sertoli cells do not allow the direct detection of the FSH-R using immunochemical techniques because of the unavailability of antibodies that are able to detect the minute amounts of endogenously expressed receptors (about 1000/cell) (Simoni *et al.* 1997). Moreover, we were not able to unambiguously localize the endogenous GRK 2/3 and β-arrestins in our primary Sertoli cell cultures by immunocytochemistry. These problems are usually overcome using epitope-tagged or GFP-fused versions of the proteins to detect these different proteins. However, this implies heterologous expression of the modified proteins and was not compatible with the aim of the present study. As an alternative, we have developed and

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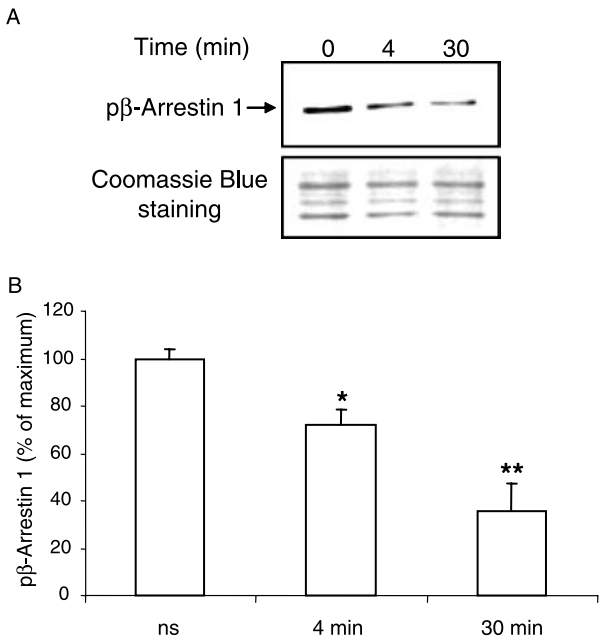

Figure 5 Dephosphorylation of β-arrestin 1 upon FSH stimulation in rat primary Sertoli cells. (A) Immunodetection of phospho-β-arrestin 1 (pβ-arrestin 1) in rat primary Sertoli cells treated with FSH for 0, 4, or 30 min. Immunoblotting was carried out using a monoclonal antibody recognizing the phosphorylated form of β-arrestin 1. Total proteins were stained on the same membrane and were used to monitor the accuracy of the loading and of the transfer. (B) Relative amounts of pβ-arrestin 1 were normalized with respect to the total protein content present in each fraction. pβ-arrestin 1 levels in the nonstimulated controls were arbitrarily chosen as 100%. Each data point represents the mean ± S.E.M. from three independent experiments. $*P<0.05$, $**P<0.01$ compared to the control values.

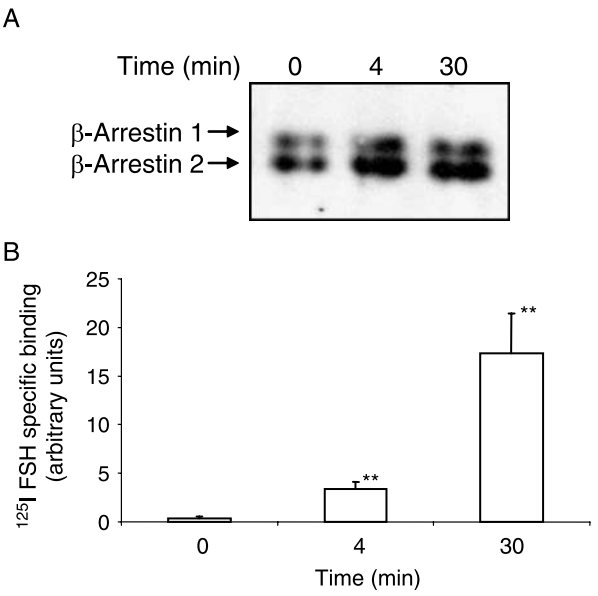

Figure 6 FSH-induced association of β-arrestins and FSH-R endogenously expressed in rat primary Sertoli cells. Endogenous β-arrestins were immunoprecipitated from rat primary Sertoli cells stimulated for 0, 4, or 30 min with FSH. The complexes interacting with the anti-β-arrestin antibody (A1CT) was then precipitated using protein A-sepharose. (A) Immunoblotting was carried out using the F4C1 mouse monoclonal antibody in order to monitor β-arrestin 1 and 2 immunoprecipitation. (B) The protein A-sepharose beads were washed and assayed for [^{125}I]-FSH-binding activity, as described in Materials and Methods. We used normal serum instead of the A1CT antibody to measure non-specific binding. Binding activity obtained with the nonspecific control reactions were subtracted from the other values (i.e. 0, 4, and 30 min of FSH stimulation). Each data point represents the mean ± S.E.M. from three independent experiments. $**P<0.01$ compared to the control values.

characterized a subcellular fractionation procedure for the primary rat Sertoli cells. This method, combined with GRK 2/3 or β-arrestin 1/2 immunoblotting and with FSH binding, allowed us to reveal FSH-induced translocations and co-localizations of the different partners in primary Sertoli cells.

Our results clearly show that FSH induces a rapid relocalization of endogenous GRK 2 in three distinct regions of the gradient. The translocation measured for GRK 2 increased between 4 and 30 min of FSH stimulation in the dense part of the gradient (fractions 1–5), whereas it was clearly transient in the lightest fractions (21–23). These two regions of the gradient were enriched with PM: the dense fractions were positive for E-cadherin, a basolateral PM marker; the light fractions were positive for 5′ nucleotidase activity, an apical PM marker. Interestingly, in addition to E-cadherin, fractions 1–5 were also positive for p58K, Rab 11, and aryl sulfatase activity, which are markers of the TGN, the recycling compartment and the lysosomes respectively. Oppositely, fractions 21–23 were found positive only for the apical PM marker. Together, these data strongly suggest that after 4 min of FSH stimulation, GRK 2 was recruited to the PM, most likely via its interaction with the pleckshin homology domain of the free Gβγ (Touhara *et al.* 1994, Lodowski *et al.* 2003). After 30 min of FSH treatment, GRK 2 might be co-localized together with the FSH-R and the β-arrestins inside the cytoplasm, in TGN and/or the recycling compartment. This hypothesis is consistent with published data showing GRK 2 in endocytic vesicles also containing a receptor and β-arrestin (Ruiz-Gomez & Mayor 1997). Moreover, the TGN is well known to play an important role in the sorting of endocytosed receptors between the recycling and the degradation pathways (Vandenbulcke *et al.* 2000). The same fractions also contained most of the lysosomes supporting this explanation. In the absence of agonist stimulation, the FSH-binding sites were located in fractions 1–9 and 21–23 of the gradient. Thus, this repartition nicely overlaps the GRK 2 recruitment sites and strongly supports our model. GRK 2 was also re-localized in a third region of the gradient (fractions 9–13) at both 4 and 30 min of FSH stimulation. However, none of our specific

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organelle markers were enriched in these fractions and we currently have no explanation for this result.

Phosphorylation of the agonist-occupied receptor by GRKs is a prerequisite to β-arrestin recruitment and to subsequent β-arrestin-dependent functional uncoupling, internalization, and signaling (Claing *et al.* 2002, Luttrell & Lefkowitz 2002, Kohout & Lefkowitz 2003, Shenoy & Lefkowitz 2003). Thus, GRK 2 is recruited to PM and FSH-R enriched regions of our gradient upon FSH stimulation suggesting that, as described in heterologous overexpression models (Lazari *et al.* 1999), GRK 2 could phosphorylate the FSH-R in primary Sertoli cells. In agreement with this hypothesis, endogenous β-arrestins were relocalized in the gradient upon FSH stimulation. The pattern and kinetics of recruitment were remarkably similar to those determined for GRK 2. The formation of a complex between the receptor, GRK 2 and β-arrestin 1/2 within the cytoplasm is further substantiated by this observation. We also observed that β-arrestin 2 was preferentially recruited in fraction 1–6 of the gradients. The meaning of this preferential recruitment is not understood, but this finding supports the emerging notion that β-arrestin 1 and 2 do not have entirely overlapping functions (Reiter & Lefkowitz 2006). As a result of the very low FSH-R number expressed at the PM of Sertoli cells, one can speculate that the observed changes in GRK2 and β-arrestins subcellular distribution triggered by FSH stimulation might not only be the consequence of multimolecular complex formation with FSH-R, but also reflect other FSH signaling-mediated modifications in GRK2 and β-arrestins trafficking. Further investigations will be necessary to test this hypothesis.

In addition, we demonstrated that β-arrestin 1 was dephosphorylated on Ser-412 upon FSH stimulation. The cytoplasmic form of β-arrestin 1 is known to be constitutively phosphorylated on this C-terminal Ser and to be recruited to the PM by agonist stimulation of the receptors. At the PM, β-arrestin 1 has been shown to undergo rapid dephosphorylation, a process that is required for its clathrin binding and receptor endocytosis, but not for receptor binding and desensitization (Lin *et al.* 1997). Therefore, this result strongly suggests that the GRK/β-arrestin regulatory system is involved in the FSH-induced internalization of the FSH-R in primary Sertoli cells.

We also provide evidence for the FSH-induced formation of a complex between the FSH-R and β-arrestins. To achieve this at endogenous level of expression of the untagged partners, we have immunoprecipitated the β-arrestins using the A1CT polyclonal antibody. This antibody has been well characterized and used successfully to immunoprecipitate endogenous β-arrestins (McDonald *et al.* 2000). We have used the high specificity and sensitivity of the FSH ligand binding to detect the co-immunoprecipitated FSH-R. This method has been proved to be reproducible and specific since virtually no interaction was found in the absence of FSH stimulation in agreement with many reports of β-arrestin recruitment to different 7MSR using a variety of other methods (Azzi *et al.* 2003, Tohgo *et al.* 2003).

Based on their internalization properties, 7MSRs have been recently divided into two classes, A and B (Oakley *et al.* 2000). In the case of class A receptors, such as the β_2-adrenergic receptor, β-arrestin 2 translocates to the receptor more readily than β-arrestin 1. The receptor and β-arrestin 2 co-localize in clathrin-coated pits at or near the cell surface, and rapidly dissociate before the receptor travels towards the endosomes. In the case of class B receptors, such as the AT_{1A} angiotensin or the V2 vasopressin receptors, equivalent quantities of β-arrestin 1 or 2 translocate to the receptor. Moreover, the receptor and β-arrestin co-localize throughout the internalization processes, and can be found together in the endosomes. Interestingly, we found that the interaction between the FSH-R and the β-arrestins was sustained for up to 30 min of FSH stimulation. This result suggests that the FSH-R belongs to the class B 7MSRs.

In conclusion, the present study provides some original data concerning the spatio-temporal pattern of agonist-induced relocalization of the FSH-R, GRK 2, and β-arrestins in a physiologically relevant model. These findings shed light on the cellular mechanisms governing the availability of the FSH-R to the cell surface, its responsiveness and possibly its signaling specificity.

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References

Ames BH & Durbin DJ 1960 The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *Journal of Biological Chemistry* **235** 769–775.

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG & Lefkowitz RJ 1992 Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *Journal of Biological Chemistry* **267** 17882–17890.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M & Pineyro G 2003 Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *PNAS* **100** 11406–11411.

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Barak LS, Ferguson SS, Zhang J & Caron MG 1997 A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *Journal of Biological Chemistry* **272** 27497–27500.

Chang PL, Rosa NE & Davidson RG 1981 Differential assay of arylsulfatase A and B activities: a sensitive method for cultured human cells. *Analytical Biochemistry* **117** 382–389.

Chavrier P, Parton RG, Hauri HP, Simons K & Zerial M 1990 Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* **62** 317–329.

Chen YT, Stewart DB & Nelson WJ 1999 Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-cadherin in polarized MDCK cells. *Journal of Cell Biology* **144** 687–699.

Claing A, Laporte SA, Caron MG & Lefkowitz RJ 2002 Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Progress in Neurobiology* **66** 61–79.

Crepieux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, Guillou F & Reiter E 2001 The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. *Oncogene* **20** 4696–4709.

Crepieux P, Lecureuil C, Marion S, Kara E, Piketty V, Martinat N, Guillou F, Royere D & Reiter E 2004 A mechanistic overview on male infertility and germ cell cancers. *Current Pharmaceutical Design* **10** 449–469.

Feng Y, Press B & Wandinger-Ness A 1995 Rab 7: an important regulator of late endocytic membrane traffic. *Journal of Cell Biology* **131** 1435–1452.

Fialka I, Pasquali C, Lottspeich F, Ahorn H & Huber LA 1997 Subcellular fractionation of polarized epithelial cells and identification of organelle-specific proteins by two-dimensional gel electrophoresis. *Electrophoresis* **18** 2582–2590.

Garcia-Higuera I & Mayor F Jr 1994 Rapid desensitization of neonatal rat liver beta-adrenergic receptors. A role for beta-adrenergic receptor kinase. *Journal of Clinical Investigation* **93** 937–943.

Garcia-Higuera I, Penela P, Murga C, Egea G, Bonay P, Benovic JL & Mayor F Jr 1994 Association of the regulatory beta-adrenergic receptor kinase with rat liver microsomal membranes. *Journal of Biological Chemistry* **269** 1348–1355.

Innamorati G, Le Gouill C, Balamotis M & Birnbaumer M 2001 The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. *Journal of Biological Chemistry* **276** 13096–13103.

Johnson LR, Scott MG & Pitcher JA 2004 G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. *Molecular and Cellular Biology* **24** 10169–10179.

Kishi H, Krishnamurthy H, Galet C, Bhaskaran RS & Ascoli M 2002 Identification of a short linear sequence present in the C-terminal tail of the rat follitropin receptor that modulates arrestin-3 binding in a phosphorylation-independent fashion. *Journal of Biological Chemistry* **277** 21939–21946.

Kohout TA & Lefkowitz RJ 2003 Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Molecular Pharmacology* **63** 9–18.

Krishnamurthy H, Galet C & Ascoli M 2003*a* The association of arrestin-3 with the follitropin receptor depends on receptor activation and phosphorylation. *Molecular and Cellular Endocrinology* **204** 127–140.

Krishnamurthy H, Kishi H, Shi M, Galet C, Bhaskaran RS, Hirakawa T & Ascoli M 2003*b* Postendocytotic trafficking of the follicle-stimulating hormone (FSH)–FSH receptor complex. *Molecular Endocrinology* **17** 2162–2176.

Lazari MF, Liu X, Nakamura K, Benovic JL & Ascoli M 1999 Role of G protein-coupled receptor kinases on the agonist-induced phosphorylation and internalization of the follitropin receptor. *Molecular Endocrinology* **13** 866–878.

Lefkowitz RJ & Shenoy SK 2005 Transduction of receptor signals by beta-arrestins. *Science* **308** 512–517.

Leighton F, Poole B, Beaufay H, Baudhuin P, Coffey JW, Fowler S & De Duve C 1968 The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with triton WR-1339. Improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. *Cell Biology* **37** 482–513.

Lin FT, Krueger KM, Kendall HE, Daaka Y, Fredericks ZL, Pitcher JA & Lefkowitz RJ 1997 Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1. *Journal of Biological Chemistry* **272** 31051–31057.

Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ & Tesmer JJ 2003 Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gβγ. *Science* **300** 1256–1262.

Luttrell LM & Lefkowitz RJ 2002 The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of Cell Science* **115** 455–465.

Marion S, Robert F, Crepieux P, Martinat N, Troispoux C, Guillou F & Reiter E 2002 G protein-coupled receptor kinases and beta arrestins are relocalized and attenuate cyclic 3′,5′-adenosine monophosphate response to follicle-stimulating hormone in rat primary Sertoli cells. *Biology of Reproduction* **66** 70–76.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ & Lefkowitz RJ 2000 Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290** 1574–1577.

Murga C, Ruiz-Gomez A, Garcia-Higuera I, Kim CM, Benovic JL & Mayor F Jr 1996 High affinity binding of beta-adrenergic receptor kinase to microsomal membranes. Modulation of the activity of bound kinase by heterotrimeric G protein activation. *Journal of Biological Chemistry* **271** 985–994.

Nakamura K, Krupnick JG, Benovic JL & Ascoli M 1998 Signaling and phosphorylation-impaired mutants of the rat follitropin receptor reveal an activation- and phosphorylation-independent but arrestin-dependent pathway for internalization. *Journal of Biological Chemistry* **273** 24346–24354.

Oakley RH, Laporte SA, Holt JA, Caron MG & Barak LS 2000 Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *Journal of Biological Chemistry* **275** 17201–17210.

Pasquali C, Fialka I & Huber LA 1999 Subcellular fractionation, electromigration analysis and mapping of organelles. *Journal of Chromatography B, Biomedical Sciences and Applications* **722** 89–102.

Reiter E & Lefkowitz RJ 2006 GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in Endocrinology and Metabolism* **17** 159–165.

Reiter E, Marion S, Robert F, Troispoux C, Boulay F, Guillou F & Crepieux P 2001 Kinase-inactive G-protein-coupled receptor kinases are able to attenuate follicle-stimulating hormone-induced signaling. *Biochemical and Biophysical Research Communications* **282** 71–78.

Ruiz-Gomez A & Mayor F Jr 1997 Beta-adrenergic receptor kinase (GRK2) colocalizes with beta-adrenergic receptors during agonist-induced receptor internalization. *Journal of Biological Chemistry* **272** 9601–9604.

Shenoy SK & Lefkowitz RJ 2003 Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochemical Journal* **375** 503–515.

Simoni M, Gromoll J & Nieschlag E 1997 The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocrine Reviews* **18** 739–773.

Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ & Luttrell LM 2003 The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *Journal of Biological Chemistry* **278** 6258–6267.

Touhara K, Inglese J, Pitcher JA, Shaw G & Lefkowitz RJ 1994 Binding of G protein beta gamma-subunits to pleckstrin homology domains. *Journal of Biological Chemistry* **269** 10217–10220.

Troispoux C, Reiter E, Combarnous Y & Guillou F 1998 Beta2 adrenergic receptors mediate cAMP, tissue-type plasminogen activator and transferrin production in rat Sertoli cells. *Molecular and Cellular Endocrinology* **142** 75–86.

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Troispoux C, Guillou F, Elalouf JM, Firsov D, Iacovelli L, De Blasi A, Combarnous Y & Reiter E 1999 Involvement of G protein-coupled receptor kinases and arrestins in desensitization to follicle-stimulating hormone action. *Molecular Endocrinology* **13** 1599–1614.

Vandalem JL, Bodart C, Pirens G, Closset J & Hennen G 1979 Development and application of homologous radioimmunoassays for porcine gonadotrophins. *Journal of Endocrinology* **81** 1–10.

Vandenbulcke F, Nouel D, Vincent JP, Mazella J & Beaudet A 2000 Ligand-induced internalization of neurotensin in transfected COS-7 cells: differential intracellular trafficking of ligand and receptor. *Journal of Cell Science* **113** 2963–2975.

Verhoeven G, Cailleau J & de Moor P 1980 Desensitization of cultured rat Sertoli cells by follicle-stimulating hormone and by L-isoproterenol. *Molecular and Cellular Endocrinology* **20** 113–126.

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