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Regulatory mechanisms underlying GKR2 levels in U937 cells: Evidence for GRK3 involvement

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ABSTRACT

G protein-coupled receptors represent the most diverse group of proteins involved in transmembrane signalling, that participate in the regulation of a wide range of physicochemical messengers through the interaction with heterotrimeric G proteins. In addition, GPCRs stimulation also triggers a negative feedback mechanism, known as desensitization that prevents the potentially harmful effects caused by persistent receptor stimulation. In this adaptative response, G protein-coupled receptor kinases (GRKs) play a key role and alterations in their function are related to diverse pathophysiological situations. Based on the scarce knowledge about the regulation of GRK2 by other kinases of the same family, the aim of the present work was to investigate the regulation of GRK2 levels in systems where other GRKs are diminished by antisense technique. Present findings show that in U937 cells GRK2 levels are regulated by GRK3 and not by GRK6 through a mechanism involving InsP upregulation. This work reports a novel GRK3-mediated GRK2 regulatory mechanism and further suggests that GRK2 may also act as a compensatory kinase tending to counterbalance the reduction in GRK3 levels. This study provides the first evidence for the existence of GRKs cross-regulation.

1. Introduction

For the survival of any living organism the proper communication between individual cells is crucial, and this is mainly achieved by surface receptors that recognize and respond to extracellular stimuli. G protein-coupled receptors (GPCRs) represent the most diverse group of protein involved in transmembrane signalling, whose members interact with a wide range of physicochemical messengers [1]. By ways of interaction with heterotrimeric G proteins, receptor stimulation leads to the modulation of the activity of effector proteins (adenylyl cyclase, phospholipases, phosphodiesterases, and ion channels) that in turn triggers changes in second messenger levels or ionic composition. Moreover, it has been recently reported that these seven transmembrane receptors can also modulate kinase cascades independently of G protein coupling [2].

By initiation of these multiple intracellular signalling pathways, GPCRs regulate an extensive range of physiological

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Abbreviations: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; G-418, geneticin; IBMX, isobutylmethylxanthine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; InsP, inositol phosphate; amthamine, 2-amino-4-methylthiazole-5-ethanamine; PDE, phosphodiesterase; RPMI, Roswell Park Memorial Institute

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processes including, but not limited to, cell metabolism, differentiation, proliferation, migration and survival [3].

Agonist GPCR stimulation not only induces the activation of G proteins but it also triggers a negative feedback mechanism, known as desensitization, which prevents the potentially harmful effects caused by persistent receptor stimulation. In this adaptative response, G protein-coupled receptor kinases (GRKs) play a key role by mediating the arrest of G protein signalling [4,5].

Seven mammalian genes encoding these serine-threonine kinases (GRK1–7) have been cloned up to date. Based on structural and functional similarities, these kinases have been divided into three different sub-families: (1) rhodopsin kinases (GRK1 and GRK7), (2) β-adrenergic receptor kinases (GRK2 and GKR3) and (3) GRK4 subfamily (GRK4–GRK6). With the exception of GRK1, 4 and 7, which are expressed almost exclusively in specific organs, the other GRKs are ubiquitously expressed, though their levels differ among the different tissues and cell types [6,7].

As GPCRs are the largest family of cell surface receptors known, alterations in GRK function have been associated with diverse pathophysiological situations. A great body of evidence supports the participation of most if not all the members of this family of kinases in a growing number of pathologies, including psychiatric disorders (schizophrenia and bipolar disorder), cancer and Oguchi disease [8]. Furthermore, the role of GRK2 has been mainly associated with cardiovascular related disorders such as heart failure and hypertension as well as thyroid and ovarian carcinoma, depression and cystic fibrosis [8,9].

Nowadays, a great therapeutic interest exists in manipulating (either enhancing or suppressing) GPCR signalling. Although most current strategies are restricted to ligand activation or receptor blockade, kinase regulation may provide a novel way to manipulate GPCR signalling for therapeutic purposes. It is noteworthy that protein kinases have now become the second most important group of drug target, after GPCRs [10].

Due to the importance of GRKs in cell signalling, these kinases are tightly regulated. Several mechanisms regarding the modulation of the localization, activity, transcription and degradation of GRKs have been reported. The mechanisms governing GRK2 functionality and expression involve the regulation by Gβγ subunits, phorbol esters, proinflammatory cytokines, PKA and PKC mediated phosphorylation, as well as c-Src and ERK1/2 induced degradation [6,7,11,12].

Based on the scarce knowledge regarding the regulation of GRK2 by other kinases of the same family and the impact of GRK modulation on cell signalling, the aim of the present work was to investigate the regulation of GRK2 levels in systems with diminished GRK3 or GRK6.

Present findings show that GRK2 levels are regulated by GRK3 but not by GRK6 expression. The mechanism involves the upregulation of the InsP cascade activity related to the downregulation of GRK3 in U937 cells transfected with an antisense-sequence for GRK3. These findings provide the first evidence for the existence of a GRKs cross-regulation mechanism. This work shows a novel GRK3-mediated GRK2 regulatory mechanism and further suggests that GRK2 may also act as a compensatory kinase to counterbalance the reduction in GRK3 levels.

2. Material and methods

2.1. Materials

Cell culture medium, antibiotics, isobutylmethylxanthine (IBMX), cAMP, ATP, phenylephrine, U73122 and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO). Fetal calf serum was purchased from Natocor (Argentina). Amthamine, tiotidine were from Tocris Cookson Inc. (Ballwin, MO). [^{3}H]cAMP, myo-[^{3}H]inositol and [^{3}H]tiotidine were purchased from Perkin-Elmer Life Sciences (Boston, MA). All other chemicals used were of analytical grade. Trifluoromethylphenyl histamine was a kind gift of Dr. W. Schunack (Freie Universitat Berlin, Institut fur Pharmazie, Berlin, Germany). Bovine GRK3 or human GRK6 cloned in the expression vector pBlueScript were a kind gift of Dr. J. Benovic (Thomas Jefferson University, Microbiology and Immunology Department, Kimmel Cancer Center, Philadelphia, USA).

2.2. Plasmid constructions

To prepare the GRK3 antisense construct, GRK3-pBluescript [13] was used to isolate the *Eco*RI/*Spe*I fragment containing the GRK3 bovine cDNA. The eukaryotic expression vector pCEFL [14] was cut with restriction endonucleases *Spe*I and *Eco*RI. The larger *Spe*I/*Eco*RI fragment from the plasmid was isolated and ligated to the fragment containing the GRK3 bovine cDNA. The resulting plasmid contained the GRK3 cDNA in reverse orientation with respect to the pCEFL promoter (pCEFL-antiGRK3).

To prepare the GRK6 antisense construct, GRK6-pBluescript [15] was used to isolate the *Hind*III/*Xba*I fragment containing the GRK6 human cDNA. This fragment was then subcloned in the *Hind*III/*Xba*I sites of the pCEFL vector as described above. The resulting plasmid contained the GRK6 cDNA in reverse orientation with respect to the pCEFL promoter (pCEFL-antiGRK6). Plasmid purification was performed using reagents from QIAGEN (Valencia, CA) following the manufacturer's instructions.

2.3. Cell culture and transfection

U937 cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin.

For stable transfection, U937 cells were harvested by centrifugation from cultures in exponential growth phase, washed in phosphate-buffered saline (PBS), and resuspended at a density of 2×10^7 cells/ml in fresh RPMI medium on ice. pCEFL-antiGRK3 or 6 (10 μg), were linearized with *Sal*I. They were then added to the cell suspension (250 μl) and kept on ice for 10 min. Cells and DNA were then subjected to a pulse of 200 V at a capacitance of 950 μF using a Gene Pulser (Bio-Rad, Hercules, CA), returned to ice for 10 min and incubated in a non-selective medium overnight (ON). Cells were then plated in a 48-well culture plate in 0.5 ml/well RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin containing 0.8 mg/ml G-418. After 2–3 weeks, the surviving clones were amplified.

2.4. [^{3}H]inositol phosphate production

For total inositol phosphate production measurement, cells were seeded in 48-well culture dishes and incubated overnight in 0.2 ml RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin containing myo-[^{3}H]inositol (2 Ci/ml). Thereafter, cells were treated with 10 mM LiCl for 20 min and then exposed for 20 min to 10 μM ATP, 100 μM phenylephrine, 10 μM trifluoromethylphenyl histamine (histamine receptor type I agonist) or 5 μM U73122 (PLC inhibitor). The incubation was stopped by the addition of 700 μl PBS and centrifugation at 1500 × *g* for 10 min. Then 2 ml of cold chloroform:methanol (1:2 v/v, freshly prepared) was added, and the phases were separated by the addition of 1 ml of water and 0.6 ml of chloroform. The mixture was then centrifuged at 1500 × *g* for 10 min, and the total water-soluble inositol phosphate fraction was purified in an anion exchange chromatography column. The radioactivity present in the eluted fractions was measured using a Wallac 1410 liquid scintillation counter. Results were normalized to total [^{3}H]inositol radioactivity recovered from the initial water wash of the columns [16].

2.5. cAMP assays

For dose-response assays, cells were incubated 3 min in RPMI 1640 medium supplemented, with 1 mM IBMX at 37 °C, followed by 9 min exposure to different concentrations of amthamine.

For time-course cAMP accumulation studies, cells were resuspended in RPMI 1640 medium in the absence of IBMX, at a density of 10^6 cells/ml, and exposed to 10 μM amthamine at different periods of time.

For the desensitization assays, pretreatment of cells with 10 μM amthamine was performed in RPMI 1640 medium at 37 °C in a 5% CO_2 humidified atmosphere for periods ranging from 1 min to 4 h, in the absence of IBMX. Cells at a density of 10^6 cells/ml were washed and resuspended in RPMI 1640 medium containing 1 mM IBMX, and exposed to 10 μM amthamine for 9 min, to evaluate whether the system was able to generate cAMP.

In all experiments, the reaction was stopped by ethanol addition followed by centrifugation at 2000 × *g* for 5 min. The ethanol phase was then dried and resuspended in 50 mM Tris–HCl, pH 7.4, 0.1% BSA. cAMP content was determined by competition of [^{3}H]cAMP for PKA, as previously described [17].

2.6. Radioligand binding assay

Triplicate assays were performed in polyethylene tubes in 50 mM Tris–HCl, pH 7.4. For saturation studies, 10^6 cells/tube were incubated for 40 min at 4 °C with increasing concentrations of [^{3}H]tiotidine, ranging from 0.4 to 240 nM in the absence or in the presence of 1 μM tiotidine, in a total volume of 200 μl. The incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris–HCl, pH 7.4, and rapid filtration under reduced pressure onto Whatman GF/B glass-fibers filters, followed by three washes with 3 ml ice-cold buffer [18]. Experiments on intact cells were carried out at 4 °C to avoid ligand internalization. The kinetic studies performed with 2 nM [^{3}H]tiotidine at 4 °C showed that the equilibrium was reached at 30 min and persisted for 4 h (data not shown).

2.7. Western blots

Cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 8, 5 mM EDTA, 1% triton X-100, 0.1% dithiothreitol, 1 mM phenylmethylsulfonyl, 5 μM aprotinin, 10 μM leupeptin, 5 μM pepstatin, 1 mM sodium vanadate). Samples were then incubated 2 min in liquid nitrogen and 3 min at 37 °C with vigorous agitation for three times. The total amount of proteins was quantified as described by Bradford [19]. Sample buffer 5× (250 mM Tris–HCl, pH 6.8, 10% SDS, 500 mM 2-mercaptoethanol, 50% glycerol, and 0.25% bromphenol blue) and enough water to obtain a 2 μg/μl final protein concentration were added to the samples. They were then boiled 5 min, and aliquots subjected to electrophoresis in 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% non-fat powdered milk in PBS-Tween (PBS containing 0.05% Tween-20), and membranes were incubated with 1 μg/ml of rabbit anti-GRK2, 3, 6 or anti-actin antibody (Santa Cruz Biotechnology, CA), in PBS-Tween, followed by subsequent washes with the same buffer. Reactivity was developed using an anti-rabbit polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham, Bukinghamshire, England). Densitometry analyses of bands were performed by Scion Image (Scion Corporation, Frederick, MD).

2.8. Statistical analysis

Binding data and sigmoidal dose-response fittings were performed with GraphPad Prism 3.00 for Windows, GraphPad Software (San Diego, CA). One-way ANOVA with Dunnett's post-test was performed using GraphPad InStat version 3.01, GraphPad Software (San Diego, CA). Specific binding was calculated by subtraction of non-specific binding from total binding.

3. Results

3.1. Generation of cell lines stably expressing GRK3-antisense sequence

To evaluate the effect of GRK3 reduction on GRK2, U937 cells were stably transfected with the GRK3-antisense cDNA construct. Two clones resistant to G418 termed B1 and B3 were obtained. GRK3 protein levels, assessed by Western blot, were reduced by 50% and 40% in B1 and B3 clones, respectively, compared to U937 cells (Fig. 1). It is worth mentioning that B1 and B3 phenotype was similar to U937 naïve cells and there were not observed any modifications in the maturation state of these cell lines, for example CD88, c-myc and c-fos expression levels (data not shown).

To determine whether the reduction in GRK3 actually modifies cell signalling, we evaluated the response of a receptor that it is known to be desensitized by GRK3 like

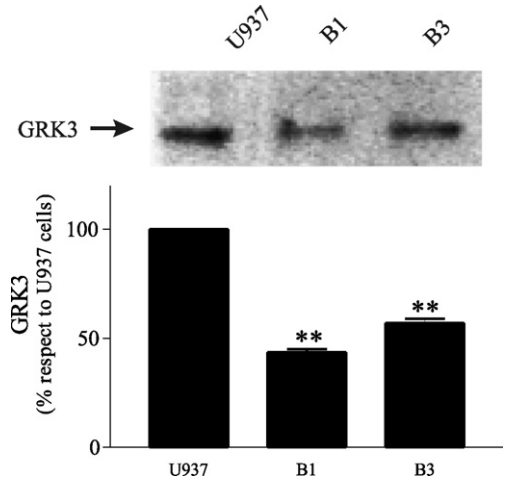

Fig. 1 – Effect of GRK3 antisense sequence on GRK3 levels. Top, whole-cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK3. Bottom, densitometric analysis obtained with the Scion Image program. Data were calculated as the mean ± S.E.M. ($n = 5$). $^{**}p < 0.01$ compared to U937 cells.

the α adrenergic receptor (αAR) [20]. Therefore InsP response to phenylephrine was assessed in the clones with lower levels of GRK3. Fig. 2 illustrates that InsP maximal response is increased by 200% and 170% in the B1 and B3 clones, respectively. It is worth noting that both GRK3 levels and InsP maximal response to epinephrine differ significantly between B1 and B3 clones ($p < 0.05$ and $p < 0.001$, respectively, one-way ANOVA, Bonferroni post-test) suggesting that the lower the level of GRK3, the higher the InsP response to phenylephrine. Accordingly, the response to phenylephrine in B1 clones that exhibited a reduction of 50% in GRK3 levels was higher than that of B3 clones that showed a reduction of 40% in GRK3 content. These results support that the reduction of GRK3 levels achieved in B1 and B3 clones results in functional consequences on cell signalling.

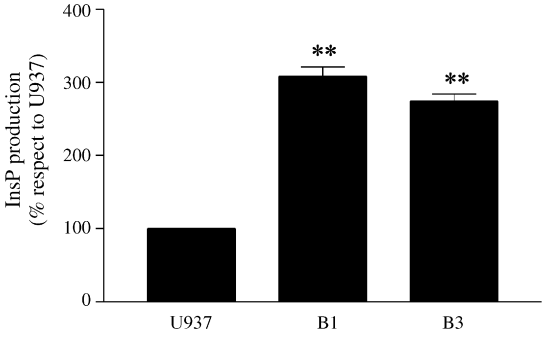

Fig. 2 – Effect of GRK3 antisense expression on phenylephrine-induced InsP production. U937 and anti GRK3 clones were treated with 10^{-7} M phenylephrine and InsP production measured as described under Section 2. Values are the mean ± S.E.M. ($n = 3$), calculated as % (phenylephrine response-basal values) respect to U937 cells. $^{**}p < 0.01$ respect to U937.

3.2. *Regulation of GRK2 levels in GRK3-antisense clones*

In order to study the effect of GRK3 reduction on GRK2, we evaluated GRK2 protein levels by Western blot, GRK2 levels increased by 75% and 50% in B1 and B3 clones, respectively. Again, the degree of increase in GRK2 protein levels relates with the extent of the reduction in GRK3 ($p < 0.05$ between B1 and B3 GRK2 protein levels, one-way ANOVA, Bonferroni) (Fig. 3). These results strongly suggest the existence of a regulatory mechanism tending to compensate the reduction in GRK3 by increasing the protein levels of GRK2, another kinase belonging to the same subfamily that shares similar structural and functional characteristics. In an attempt to assess the functional consequences of GRK2 upregulation we studied histamine H2 receptor (H2r) signalling, a GPCR that, as we have previously described, is regulated in U937 cells by GRK2-mediated desensitization [21]. In concentration-response assays to a selective H2 agonist (amthamine) in the presence of IBMX (PDE inhibitor) we observed that the maximal response achieved in B1 and B3 clones was lower than that of U937 cells ($MaxR_{U937} = 618 \pm 16$; $MaxR_{B1} = 127 \pm 6$; $MaxR_{B3} = 206 \pm 7$ pmol/5×10^5 cells; $n = 3$) (Fig. 4A). However, in the three systems assessed the EC50 values were similar ($pEC50_{U937} = 5.86 \pm 0.08$; $pEC50_B = 5.99 \pm 0.09$; $pEC50_{U937} = 6.01 \pm 0.05$; $n = 3$). To determine cAMP levels resulting from the intracellular production-degradation balance, time course assays were carried out in the absence of IBMX. Kinetic experiments showed that B1 and B3 clones exhibited lower cAMP levels than U937 cells in most of the times evaluated. However, the residual response following 1 h stimulation was similar for the three cell lines (about 3 pmol/5×10^5 cells; $n = 3$) (Fig. 4B). We have previously reported that a reduction in GRK2 levels by cDNA antisense constructs determines a higher and sustained cAMP response mediated by H2r, due to a lower receptor desensitization [21]. Therefore, it is likely to assume that the increase in GRK2 protein levels may shorten the receptor response. To evaluate this hypothesis, U937 cells as well as B1 and B3 clones were exposed to 10 μM amthamine (maximal response) at different periods of time in the absence of IBMX, washed and re-stimulated to determine whether cells were able to generate cAMP. U937 desensitization curve, in accordance with our previous report, showed a half-maximal desensitization time of 10 ± 2 min (mean ± S.E.M.) [22]. In the present study, B1 and B3 clones exhibited a faster desensitization with a half-maximal desensitization time of 4.9 ± 0.1 min and 5.2 ± 1.1 min (mean ± S.E.M.; $n = 3$), respectively (Fig. 4C). To further support the hypothesis that the reduction in H2r cAMP response observed in B1 and B3 clones resulted from the upregulation of GRK2 and not from a reduction in the receptor number, [^{3}H]tiotidine binding experiments were carried out. Conversely, saturation analysis performed in intact cells revealed that B1 and B3 clones had a significantly higher receptor number (B1: $Bmax_1 = 3125 \pm 148$, $Bmax_2 = 32{,}510 \pm 1500$; B3: $Bmax_1 = 3200 \pm 180$, $Bmax_2 = 29{,}248 \pm 1360$ sites/cell; $n = 3$) than naïve cells ($Bmax_1 = 2550 \pm 200$, $Bmax_2 = 22{,}930 \pm 2500$ sites/cell; $p < 0.05$; $n = 6$) (Fig. 4D). Furthermore, there were no significant differences among Kd values (B1: $Kd_1 = 2.1 \pm 1.1$, $Kd_2 = 19 \pm 4$; B3: $Kd_1 = 1.9 \pm 0.8$, $Kd_2 = 20 \pm 3$ and U937: $Kd_1 = 2.3 \pm 0.8$, $Kd_2 = 20 \pm 3$ nM), that were also in accordance with values previously reported by our group [18]. These findings strongly support that the reduced H2r

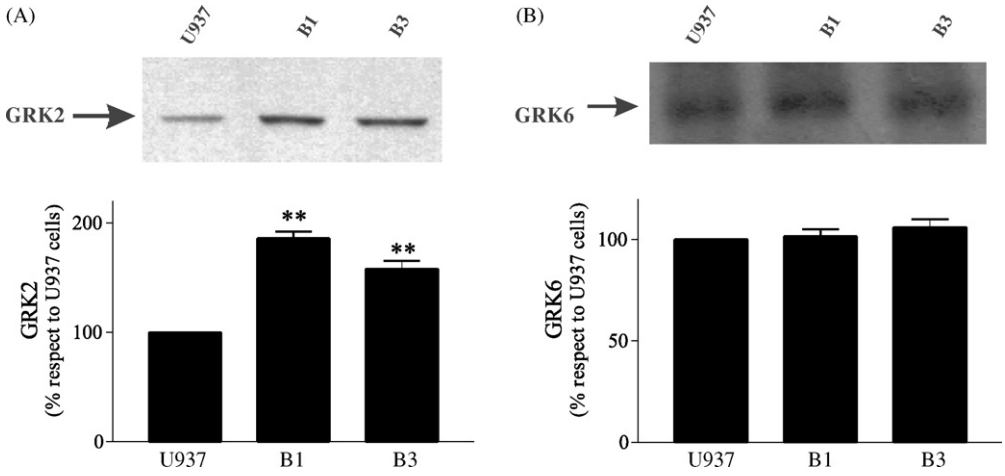

Fig. 3 – Effect of GRK3 antisense expression on GRK2 and GRK6 levels. Top, whole-cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2 (A) or GRK6 (B). Bottom, densitometric analysis obtained with the Scion Image program. Data were calculated as the mean ± S.E.M. ($n = 5$). $^{**}p < 0.01$ compared to U937 cells.

signalling observed in anti GRK3 clones is due to an increase in GRK2 levels and not to a reduction in the number of H2r sites. Furthermore, if GRK3 were responsible for H2r desensitization, H2r response in anti GRK3 clones should have been higher and more sustained than in U937 cells. However, as we observed a profile of H2r response consistent with GRK2 upregulation it can be inferred that the participation of GRK3 in H2r desensitization is at least less relevant than the role of GRK2.

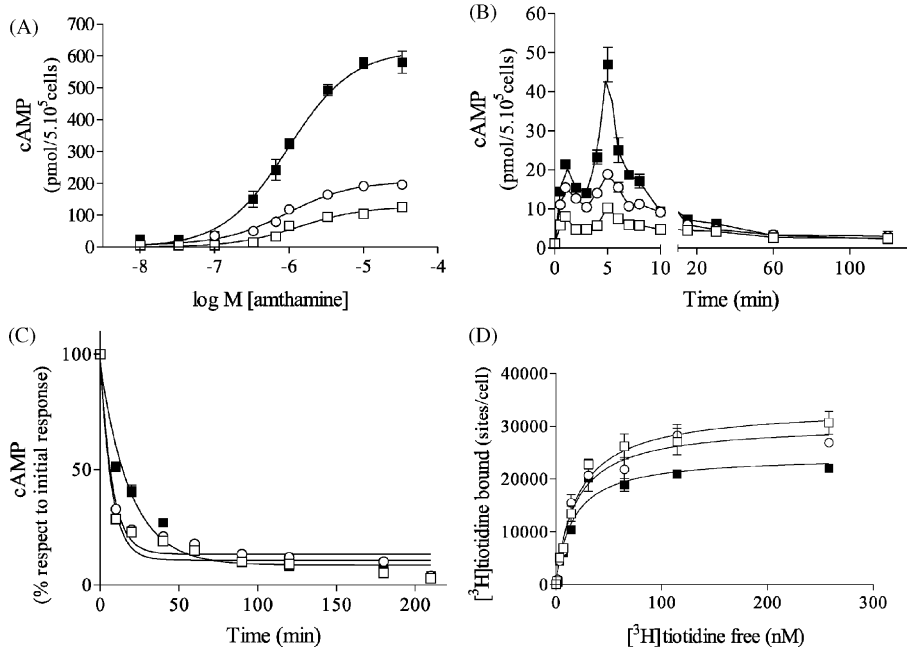

Fig. 4 – Effect of GRK3 antisense expression on the H2r response. (A) Concentration-cAMP response curves to amthamine. U937 (■), B1 (□) and B3 (○) cells were incubated for 9 min with increasing concentrations of amthamine at 37 °C in the presence of 1 mM IBMX, and cAMP levels were determined. (B) Time course of cAMP levels in the absence of IBMX. U937 (■), B1 (□) and B3 (○) cells were incubated at different periods of time with 10 μM amthamine at 37 °C, and cAMP levels were determined. (C) Desensitization kinetics. U937 (■), B1 (□) and B3 (○) cells were preincubated for different periods of time with 10 μM amthamine, washed, and re-stimulated with 10 μM amthamine in the presence of 1 mM IBMX, and cAMP levels were determined. (D) [^{3}H]tiotidine binding assay. Saturation assays for [^{3}H]tiotidine in U937 (■), B1 (□) and B3 (○) cells. Kd (nM) and Bmax (sites/cell) values were calculated by the equation for two binding sites. In (A), (B), (C) and (D) data were calculated as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

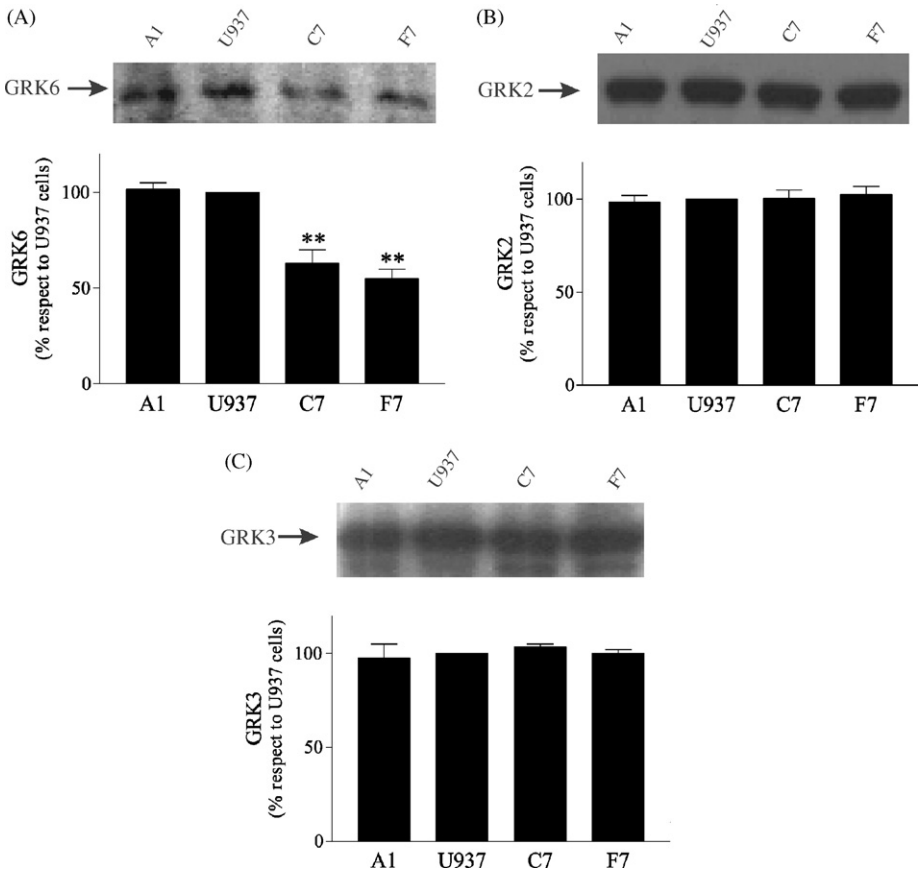

Fig. 5 – Effect of GRK6 antisense expression on GRK2, 3 and 6 levels. Top, whole-cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK6 (A), GRK2 (B) and GRK3 (C). Bottom, densitometric analysis obtained with the Scion Image program. Data were calculated as the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$ compared to U937 cells.

3.3. *GRK2 levels in GRK6-antisense clones*

As U937 cells only express GRK2, 3 and 6 [23], we transfected these cells with an antisense cDNA construct for GRK6 in order to evaluate whether the increase in GRK2 was a general mechanism triggered by the reduction of GRKs levels. Three clones resistant to G418, A1, C7 and F7 were obtained. However, when GRK6 protein levels were assessed by Western blot only two of the three clones showed reduced protein levels of this kinase. GRK6 levels were reduced by 40% and 48% in C7 and F7 clones, respectively compared to U937 cells (Fig. 5A). However the protein levels of GRK2 and GRK3 were not affected (Fig. 5B and C). Consistently, cAMP response evoked by H2r remained unchanged in the clones transfected

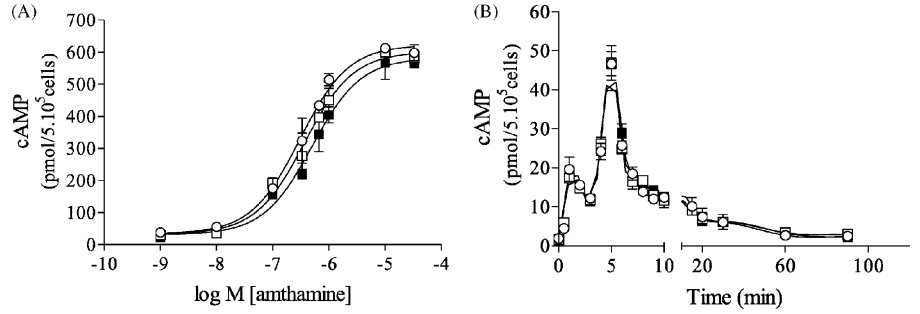

Fig. 6 – Effect of GRK6 antisense expression on the H2r response. (A) Concentration-cAMP response curves to amthamine. U937 (■), F7 (□) and C7 (○) cells were incubated for 9 min with increasing concentrations of amthamine at 37 °C in the presence of 1 mM IBMX, and cAMP levels were determined. (B) Time course of cAMP levels in the absence of IBMX. U937 (■), F7 (□) and C7 (○) cells were incubated for different periods of time with 10 μM amthamine at 37 °C, and cAMP levels were determined. In (A) and (B) data were calculated as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

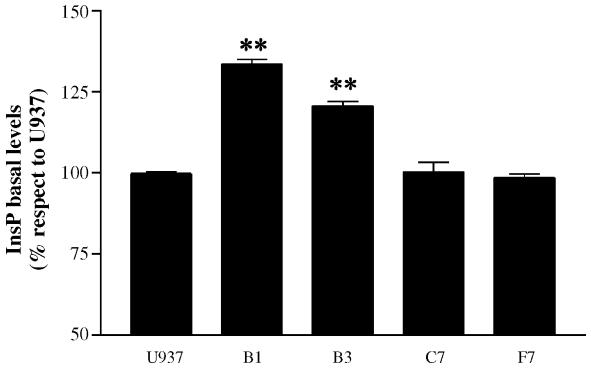

Fig. 7 – Effect of GRK3 and GRK6 antisense expression on InsP basal levels. U937 and anti GRK3 (B1 and B3) and GRK6 (C7 and F7) clones were treated for 20 min with 10^{-2} M LiCl and InsP production measured as described under Section 2. Values are the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$ respect to U937.

with GRK6-antisense sequence as compared with U937 cells (Fig. 6A and B). These findings support that the upregulation of GRK2 does not result from a general reduction in GRKs levels but it represents a specific GRK3-mediated regulatory mechanism.

3.4. *InsP involvement in GRK2 upregulation in anti GRK3 clones*

It has been previously reported that the activity of the GRK2 promoter is stimulated by the activation of the α_q/protein kinase C (PKC) signalling pathway [24]. Therefore, InsP levels were measured in U937 cells as well as in the obtained clones. Results showed that InsP basal levels were increased by 35% and 22% in the B1 and B3 clones, respectively (Fig. 7). On the other hand, anti GRK6 clones failed to modulate this second messenger levels consistent with the observation that no regulation of GRK2 was observed in these clones. Moreover, when PLC was inhibited by 5 μM U73122 (PLC specific inhibitor), anti GRK3 clones and U937 cells showed a reduction in both InsP and GRK2 levels (Fig. 8A and B). These findings support that InsP are involved in the increase of GRK2 expression. To further study the regulation of GRK2 by InsP, U937 cells were stimulated at different times with phenylephrine. The treatment induced a time dependent increase in GRK2 levels up to 24 h (Fig. 9A). This effect proved to be non-specific, since other InsP stimulating agents as histamine type 1 receptor selective agonist and ATP (Fig. 9B) also induced GRK2 upregulation after 24 h treatment (Fig. 9C). In summary, the upregulation of GRK2 only observed in anti-GRK3 clones can be ascribed to the increased InsP levels present in these cells.

4. Discussion

The major finding of the present work is the existence of a regulatory mechanism for GRK2 levels mediated by GRK3 modulation. Although GRK2 is involved in a variety of pathophysiologial situations, very little is known about the mechanisms that regulate this kinase levels. In the present study we showed that the downregulation of GRK3 and not GRK6 by antisense specific sequence leads to an increase in GRK2 levels. This upregulation of GRK2 correlates with an increase in InsP basal levels present in anti-GRK3 clones.

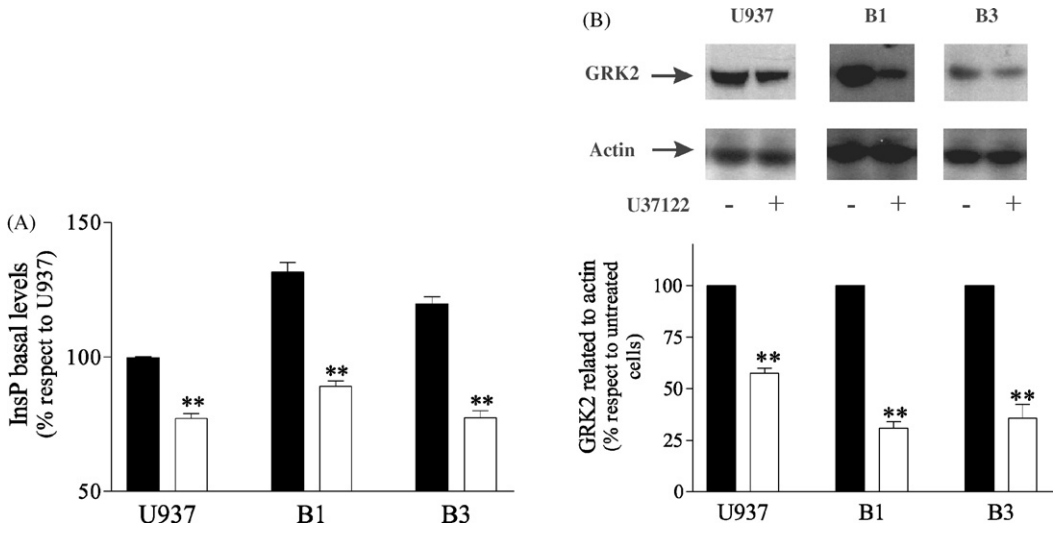

Fig. 8 – Effect of InsP basal levels reduction on GRK2 expression. (A) Effect of U73122 (PLC inhibitor) on InsP basal levels. U937 cells and anti GRK3 clones were incubated for 20 min in the presence (□) or in the absence of 5×10^{-6} U73122 (■), and InsP production was measured as described under Section 2. Values are the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$ respect to untreated cells. (B) Effect of U73122 (PLC inhibitor) on GRK2 expression. Top, U937 and anti GRK3 clones were treated before harvesting, for 24 h in the presence (□) or in the absence of 5×10^{-6} U73122 (■), and then lysed as described under Section 2. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2. Bottom, densitometric analysis obtained whit the Scion Image program. Data were calculated as the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$ respect to basal levels.

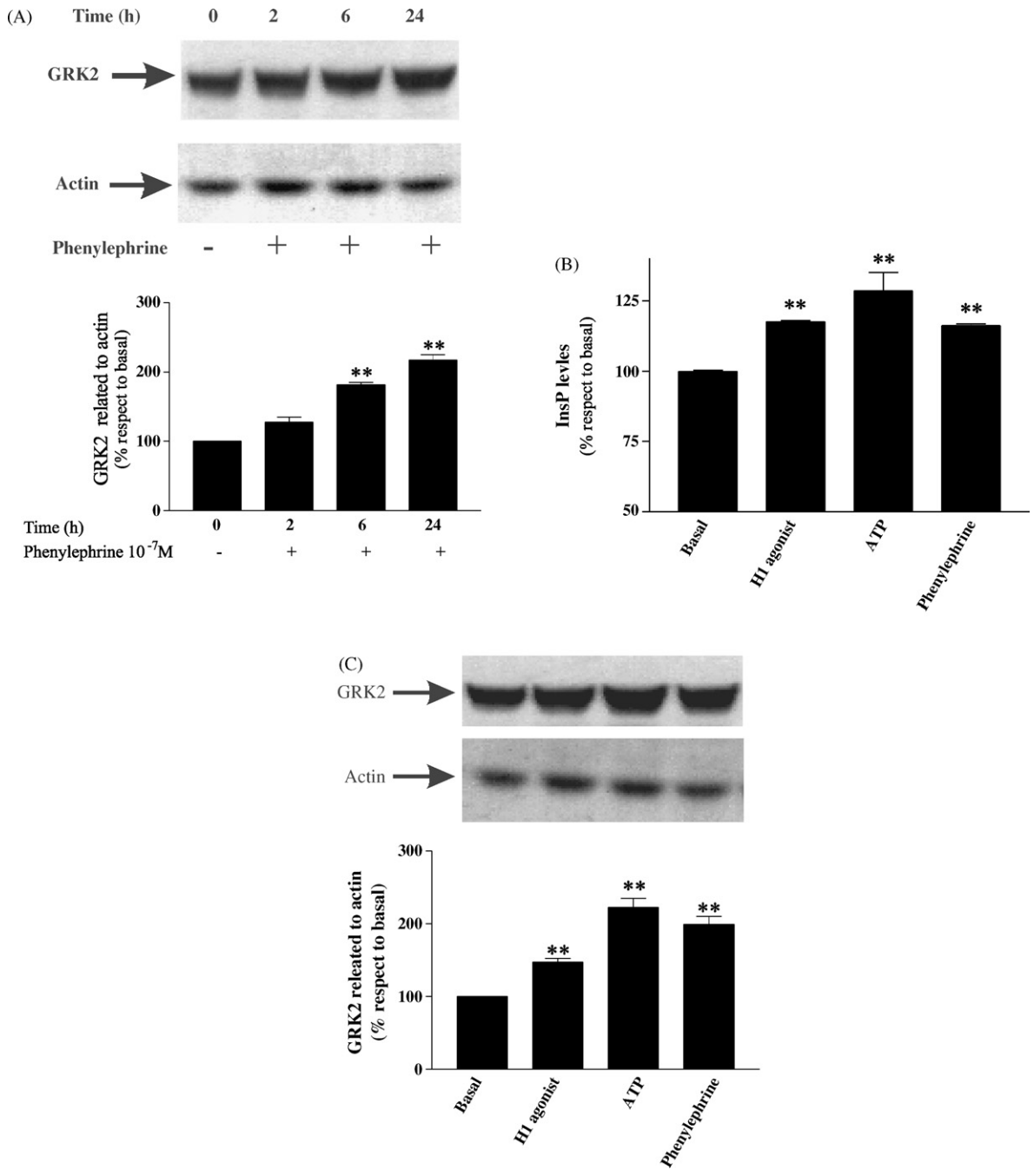

Fig. 9 – Effect of InsP increase on GRK2 expression in U937 cells. (A) Kinetic effect of phenylephrine on GRK2 expression. Top, cells were incubated with 10^{-7} M phenylephrine at the indicated times. Bottom, densitometric analysis. Data were calculated as the mean ± S.E.M. (n = 3). (B) InsP production. U937 cells were treated with 10^{-5} M trifluoromethylphenyl histamine (H1 agonist); 10^{-5} M ATP or 10^{-7} M phenylephrine and InsP production was measured as described under Section 2. Values are the mean ± S.E.M. (n = 3). H1 agonist: 121 ± 2; ATP: 128 ± 6 and phenylephrine: 122 ± 3% respect to basal levels. $^{**}p < 0.01$ respect to basal. (C) Effect of different InsP stimulating agents on GRK2 expression in U937 cells. Top, cells were incubated with 10^{-5} M trifluoromethylphenyl histamine (H1 agonist); 10^{-5} M ATP or 10^{-7} M phenylephrine for 24 h. Bottom, densitometric analysis. Data were calculated as the mean ± S.E.M. (n = 3). In (A) and (C) cells were harvested, and lysed as describe under Section 2. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2. Densitometric analysis obtained with the Scion Image program.

The increase in GRK2 expression might result either from a specific reduction of GRK3 or from a more general mechanism to compensate the reduction of GRKs activity.

Previous studies have shown that InsP signalling pathway may play a pivotal role in GRK2 regulation [24]. These studies show that prolonged stimulation of A10 cells (aortic smooth muscle cells) with phorbol esters leads to an induction of the GRK2 promoter activity. In addition, overexpression of a constitutive active form of Gαq protein significantly increases GRK2 expression. Based on these observations the authors hypothesized that a prolonged response of Gαq coupled receptors results in an increased expression of GRK2.

Consistent with this hypothesis, the present study shows that the stimulation with different agents that signal through the Gαq pathway leads to an increase in GRK2 levels in U937 cells. Hence, it is likely that the increment of GRK2 levels in systems where GRK3 is downregulated may result from a higher basal activity of those GPCRs coupled to Gαq that are regulated by GRK3. This is consistent with the increased response to phenylephrine shown by anti GRK3 clones and the observation that a PLC inhibitor induces a reduction in both InsP and GRK2 levels.

On the other hand, GRK2 upregulation in anti GRK3 clones may result from either, an increase of GRK2 gene transcription or from a lower GRK2 degradation. However, as GRK2 regulation was observed following a prolonged treatment with Gαq stimulating agents, it is likely to assume that this modulation may occur at the transcriptional level, in accordance with the findings reported by Ramos-Ruiz et al. [24]. However, it is worth noting that these authors observed that the stimulation with phorbol esters showed tissue specificity, since GRK2 levels were not affected in other cell types such as U87 o HEK293. Nevertheless the present study provides strong evidence that this GRK2 regulation by InsP is not only restricted to aortic cells, since it also occurs in the human leukemic U937 cells.

To our knowledge, this is the first report to provide evidence for the existence of a cross-regulatory mechanism between two GRKs, where GRK3 modulation induces intracellular signalling alterations (namely Gαq pathway activation) that in turn modifies the expression of a related kinase such as GRK2.

Compensatory regulation of closely related proteins is becoming an increasingly common phenomenon given the availability of strategies to acutely up-regulate or down-regulate specific proteins. In this way, it may be assumed that this regulatory mechanism between GRK2 and GRK3 occurs reciprocally, in other words, that the modulation of GRK2 levels may lead to a modification in GRK3, as a compensatory mechanism tending to restore GRKs functionality. Nevertheless, we have previously reported that transfection of U937 cells with an antisense sequence for GRK2 leads to a reduction in GRK2 levels without modifying the expression levels of other GRKs including those of GRK3 [21]. These results support that GRK2 reduction is not compensated by modifications in other kinases levels, as it was observed for GRK3 down-regulation in the present study. Similarly, GRK6 levels were not modified in either GRK2 [21] or GRK3 antisense clones. Indicating that this compensatory regulation between isoenzymes of GRKs family is not reciprocal. The findings of the present work are in accordance with the observation performed by Jaber et al. [25]. The authors reported that GRK2$^{-/-}$ knockout mice do not survive beyond gestational day 15.5, whereas GRK3 deletion in GRK3$^{-/-}$ knockout mice allows normal embryonic and postnatal development, pointing to GRK2 as an essential kinase that no other GRK may compensate for its loss.

The present study shows that GRK2 upregulation induced functional cellular alterations. Both in amthamine concentration-response and time course assays we observed a decreased cAMP response in anti GRK3 clones respect to naïve cells. These findings indicate that in anti GRK3 clones, H2r signalling is more attenuated than in naïve U937 cells. In this respect, H2r desensitization has been extensively studied [21,23,26,27]. In previous studies, by overexpression of the different GRKs in COS7 cells, we reported that both GRK2 and GRK3 were able to desensitize and phosphorylate the H2r [27]. In experiments carried out in U937 cells we observed that a reduction in GRK2 levels by antisense technology led to an H2r higher and more sustained cAMP response [21]. In the present study the H2r signalling profile is consistent with GRK2 upregulation but not with GRK3 downregulation. These results suggest that if GRK3 were involved in H2r desensitization, its participation would be less crucial than that of GRK2.

The discrepancy observed between the results obtained in the present work in U937 cells and the previous results in COS7 cells, can be assigned to the same differences generally observed between naive and overexpression systems. In overexpression systems, as COS7 cells, where one or more components of the signalling machinery are augmented, promiscuous effects are often observed as a consequence of higher levels of proteins artificially achieved. In consequence, the interactions observed in overexpression systems do not necessarily reflect what may occur in naïve systems.

Similar results were obtained with α and β adrenergic receptors, where transient overexpression of either GRK2 or GRK3 in cultured cells results in an agonist-stimulated increase in both α1b and β adrenergic receptor phosphorylation and desensitization [28,29]. Interestingly, experiments carried out in transgenic mice showed that GRK3 is the relevant GRK for the desensitization of the α1b adrenergic receptor in vivo. Moreover, in vivo overexpression of GRK2 leads to attenuation of the β adrenergic receptor signalling, but in vivo overexpression of GRK3 results in normal β adrenergic receptor signalling [6]. These results indicate that these GRKs show substrate specificity in vivo even though this cannot be evidenced in experiments with cultured cells.

In summary, present findings show that GRK3 reduction induced by anti GRK3 sequences leads to an increase of GRK2 protein levels mediated by the InsP signalling pathway, supporting a novel cross-regulation mechanism between these kinases. Taking into account that alterations in the levels of GRK2 have been reported for a variety of pathophysiological situations [8], it would be relevant to deepen the knowledge about the mechanisms underlying the regulation of this kinase. The present work sheds new light on the regulation of GRK2 levels, showing a novel GRK3-mediated mechanism.

A more detailed knowledge about GRKs synthesis and degradation may be eventually beneficial for the design of

novel therapeutic and diagnostic protocols involving GRKs modulation.

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