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Taxol normalizes the impaired agonist-induced β_2-adrenoceptor internalization in splenocytes from GRK2+/− mice

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Abstract

G protein-coupled receptor kinase 2 (GRK2) is involved in the agonist-induced desensitization of β_2-adrenoceptors. In addition, GRK2 is capable of binding and phosphorylating tubulin. Interestingly, microtubule dynamics profoundly affect agonist-induced internalization of β_2-adrenoceptors. Here, we analyzed agonist-induced β_2-adrenoceptor internalization and signaling in splenocytes from GRK2+/− mice that have a ~50% lower level of GRK2 protein compared to wild type (WT) mice. In addition, we investigated the role of microtubule stability in these processes. Splenocytes from GRK2+/− mice express ~50% less β_2-adrenoceptors on the cell surface and show impaired agonist-induced β_2-adrenoceptor internalization. Disruption of microtubules using colchicine reduces agonist-induced β_2-adrenoceptor internalization in cells from WT, but not in cells from GRK2+/− mice. Importantly, increasing tubulin stability by taxol almost completely restores the defective agonist-induced β_2-adrenoceptor internalization in cells from GRK2+/− animals, without affecting WT cells. Despite lower surface receptor numbers, cells of GRK2+/− mice show normal β_2-adrenoceptor agonist-induced cAMP responses. Although interfering with microtubule stability has major effects on agonist-induced receptor internalization in GRK2+/− cells, microtubule dynamics do not influence cAMP responses. Our data suggest that cells with low GRK2 adapt to the lower GRK2 level by decreasing the number of β_2-adrenoceptors on the cell surface. In addition, the cellular GRK2 level determines the extent of agonist-induced β_2-adrenoceptor internalization via a mechanism involving microtubule stability. Importantly, however, normalization of agonist-induced receptor internalization by taxol is not sufficient to alter receptor signaling.
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Keywords: β_2-adrenoceptor; G protein-coupled receptor kinase 2; Tubulin; Receptor internalization; cAMP; Microtubules

1. Introduction

G protein-coupled receptors are important regulators of many vital cellular functions. The function of G protein-coupled receptors is tightly regulated by a number of mechanisms, allowing receptor signaling to adapt to a constantly changing environment. One of the early regulatory mechanisms that is activated after stimulation of a G protein-coupled receptor is the rapid loss of receptor responsiveness, also referred to as homologous receptor desensitization (Chuang et al., 1996). A family of specific G protein-coupled receptor kinases (GRKs) plays a central role in this process. To date, seven distinct GRKs (GRK1–7) have been identified (Penn et al., 2000). GRK2 (also known as β-adrenergic receptor kinase, βARK) is a ubiquitous member of the GRK family that has been shown to phosphorylate a variety of G protein-coupled receptors including the β_2-adrenoceptor (Pitcher et al., 1998a). GRK-mediated phosphorylation of agonist-activated receptors initiates rapid uncoupling of the receptor from the G protein (Pitcher et al., 1998a; Ferguson, 2001). Furthermore, receptor phosphorylation by GRK2 facilitates binding of β-arrestins, which target the phosphorylated receptor to clathrin-coated pits, thereby triggering receptor internalization into endocytic vesicles (Carman and Benovic, 1998; Pierce and Lefkowitz, 2001).

Notably, increasing evidence indicates that GRKs may have additional roles beyond their effects on G protein-coupled receptor coupling. For instance, a tyrosine kinase-induced

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interaction of GRK2 with the (non-G protein-coupled receptor) epidermal growth factor receptor was recently documented by Gao et al. (2005). In addition, GRK2 has been shown to phosphorylate a number of non-receptor substrates, including synuclein (Pronin et al., 2000), phosducin (Ruiz-Gomez et al., 2000) and tubulin (Haga et al., 1998; Carman et al., 1998; Pitcher et al., 1998b). More specifically, GRK2 was shown to interact with microtubules and with soluble tubulin, both in cellular extracts and in living cells. Agonist occupancy of a G protein-coupled receptor promotes GRK2-tubulin complex formation and tubulin phosphorylation (Pitcher et al., 1998b). Interestingly, the kinetics of tubulin phosphorylation by GRK2 mirrors those of GRK2-mediated phosphorylation of other GRK2 substrates including the β_2-adrenoceptor. These observations may suggest a potential role for GRK2 in tubulin-mediated processes.

Microtubule dynamics play an important role in regulating motility and trafficking of β_2-adrenoceptors. Colchicine, a microtubule-disrupting agent, was shown to relieve the motional constraint of receptors within the plasma membrane and increase receptor mobility (Cherksey et al., 1980). In addition, isoproterenol-induced redistribution of cardiac β-adrenoceptors from membrane to cytosol is dependent on microtubules, since it can be prevented by microtubule disruptors (Limas and Limas, 1983; Shumay et al., 2004). Taken together, these observations suggest that GRK2 may link G protein-coupled receptor activation with a cytoskeletal component, although the exact role of GRK2-tubulin interactions in regulating receptor internalization is still unclear (Pitcher et al., 1998b). Importantly, most studies on the role of GRK2 in the regulation of receptor expression and function use cell lines transfected with (relatively high levels of) GRK2 and the substrate receptor. Little is known, however, about the consequences of changes in GRK2 level for receptor distribution in a physiological system.

In the present study we aimed at investigating the consequences of a reduction (by ~50%) of intracellular GRK2 levels for β_2-adrenoceptor internalization by using splenocytes from GRK2+/− mice. In addition, we investigated whether modulation of microtubule stability using drugs like colchicine and taxol, can affect agonist-stimulated β_2-adrenoceptor internalization in splenocytes from GRK2+/− mice. Finally, we investigated the consequences of a low GRK2 level and changes in microtubule stability for β_2-adrenoceptor signaling.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, penicillin/streptomycin, glutamine and fetal bovine serum were obtained from Life Technologies. Colchicine, (−) isoproterenol, (−) propranolol, isobutyl-methylxanthine (IBMX), superoxide dismutase, catalase and taxol (Paclitaxel) were purchased from Sigma Chemical Co. (St. Louis, MO). CGP12177 (4-[3-*t*-butylamino-2-hydroxypropoxy] benzimidazol-2-1) was purchased from Tocris Cookson Ltd. (Bristol, U.K.). [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) and cAMP[^{125}I] scintillation proximity assay (SPA) system kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

2.2. Experimental animals

The gene-targeted mice used for this study were heterozygous for targeted disruption of the GRK2 (βARK1) gene (Jaber et al., 1996) (a gift from Dr. M G. Caron, Duke University, Durham, NC). Offspring were genotyped by Polymerase Chain Reaction (PCR) analysis on DNA extracted from tail biopsies. Male mice of 6–8 week of age were used and compared with their wild type (WT) littermates. All animal experiments were approved by the institutional ethics committee.

2.3. Cell isolation

Mice were sacrificed by cervical dislocation and spleens were collected and dispersed through filter chambers. Splenocytes were then washed once in ice-cold RPMI supplemented with 5% fetal bovine serum and 50 μM β-mercaptoethanol. Erythrocytes were removed by lysis in hypotonic buffer for 2 min. Subsequently, splenocytes were washed twice in RPMI.

2.4. β_2-adrenoceptor binding assays and receptor internalization

β_2-adrenoceptors were quantified using the ligand [^{125}I] iodocyanopindolol ([^{125}I]-ICYP) (Amersham, U.K.). The assay was carried out in triplicate. The incubations were performed in a total volume of 250 μl of binding buffer (RPMI + 20 mM Hepes pH 7.4, in the presence of 0.5% bovine serum albumin (BSA). Total binding was determined by incubating splenocytes (0.5×10^6 cells/tube) with 8 concentrations [^{125}I]-ICYP (range 6.25–175 pM) at 4 °C for 15–18 h. The total amount bound never exceeded 10% of the radioactivity added. Non-specific binding was determined under the same conditions, in the presence of 1 μM (−) propranolol. Incubations were stopped by the addition of 5 ml ice-cold PBS, followed by rapid filtration through Whatman GF/C glass fiber filters under vacuum and two additional 5 ml washes.

For agonist-induced β_2-adrenoceptor sequestration, splenocytes were resuspended at a density of 1.5×10^6 cells/ml and incubated in the presence or absence of 10 μM colchicine or 10 μM taxol for 1 h at 37 °C. Cells were then exposed to 1 μM (−) isoproterenol or medium for 10 min at 37 °C and washed twice with ice-cold PBS. Isoproterenol-induced desensitization/internalization is rapidly reversed at 37 °C, but it is stable for many hours at 4 °C. Therefore, all subsequent procedures were performed at 4 °C (Madden et al., 1994).

Cells were resuspended in binding buffer at a density of 3.75×10^6 cells/ml. Total binding was determined in a final volume of 250 μl containing 0.75×10^6 cells by using 175 pM of [^{125}I]-ICYP alone. The number of internalized receptors was determined by using 175 pM of [^{125}I]-ICYP plus 1 μM of the hydrophilic antagonist CGP12177, and non-specific binding was determined by using 175 pM of [^{125}I]-ICYP plus 1 μM (−)

propranolol (Inglese et al., 1993). β_2-adrenoceptor sequestration was calculated as the ratio of (specific receptor binding of [^{125}I]-ICYP in the presence of CGP12177)/(specific receptor binding of [^{125}I]-ICYP in the absence of CGP12177). The radioactivity of the filters was determined in a gamma counter.

2.5. CD3 internalization

Splenocytes were resuspended in RPMI (+5% FBS) at 4×10^6 cells/ml and incubated in the presence or absence of 10 μM colchicine or 10 μM taxol for 1 h at 37 °C. Cells were then exposed to (a saturating concentration of) 10 μg/ml anti-mouse CD3 (Pharmingen/BD) for 30 or 60 min at 37 °C. Control samples were incubated with 10 μg/ml anti-CD3 on ice, in the presence of 0.1% sodium azide. After subsequent washing with ice-cold PBS containing 1% BSA and 0.01% sodium azide, cells were incubated with R-Phycoerythrin-conjugated anti-IgG (Pharmingen/BD). CD3 fluorescence intensity was determined by flow cytometric analysis and internalization of CD3 was calculated as follows: % internalization = 100 − (CD3 fluorescence at 37 °C / CD3 fluorescence on ice × 100).

2.6. Tubulin phosphorylation

Splenocytes were metabolically labeled with [$^{32}P_i$] (Amersham Int.; 40 μCi/ml) in phosphate-free medium (custom-made by GIBCO) containing 5% FBS (phosphate-free) for 3 h at 37 °C. Subsequently, cells were stimulated with 10 μM isoproterenol for 10 min. After treatment, cells were lysed in lysis buffer (20 mM Tris–HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 0.5% triton, 0.5% CHAPS) with protease- and phosphatase inhibitors. Tubulin was immunoprecipitated using anti-tubulin antibody (Sigma) and proteinA/G sepharose. Immunoprecipitates were dissociated and resolved by sodium dodecyl sulfate-polyacrylamine gel electrophoresis. Radioactively labeled receptors were visualized by autoradiography.

2.7. cAMP accumulation

Splenocytes were resuspended in RPMI containing 20 mM HEPES (pH 7.4) and 5% fetal bovine serum at a density of 1.5×10^6 cells/ml. In all samples cellular viability, assessed by trypan blue exclusion, was higher than 90%. Cells were incubated in the presence or absence of 10 μM colchicine or 10 μM taxol for 1 h at 37 °C in 95% air, 5% CO_2. cAMP accumulation was induced by adding 0.5 ml of cell suspension to 0.5 ml RPMI, pre-warmed at 37 °C, containing 1 mM isobutyl-methylxanthine (IBMX, to inhibit phosphodiesterase activity) with or without 1 μM isoproterenol (Sigma, Chemical Co., St. Louis, MO) for 5 min. at 37 °C. Isoproterenol was protected against oxidation in these incubations by adding 20 μg/ml of superoxide dismutase and catalase (Leiber et al., 1993). The reactions were terminated by centrifugation for 30 s. The supernatant was quickly removed and the pellet was resuspended in ice-cold Tris–EDTA (50 mM Tris, 4 mM EDTA) buffer at pH 7.5. The samples were placed in a boiling water bath for 5 min, after which they were stored at −20 °C. After thawing, samples were sonicated and the protein flocculate was pelleted by centrifugation. 50 μl aliquots of the resulting supernatants were assayed using a cAMP[^{125}I] scintillation proximity assay (SPA) system kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) following manufacturer's instructions.

2.8. Statistical analysis

Data are expressed as a mean value ± S.E.M. All results were confirmed in at least three separate experiments. Specific measurements were compared using Student's *t* test or ANOVA followed by Bonferroni's analysis. Two-tailed $P<0.05$ was considered statistically significant.

3. Results

3.1. β_2-adrenoceptor expression in splenocytes from GRK2+/− and WT mice

First, we determined the characteristics of β_2-adrenoceptors on splenocytes from GRK2+/− and WT mice, that express a 50% reduction in GRK2 protein level (Vroon et al., 2004). Saturation binding experiments were performed using [^{125}I]-ICYP. Since previous studies demonstrated that splenocytes do not express β_1-adrenoceptors, we refer to our results as measurement of β_2-adrenoceptor numbers (Sanders et al., 2003). The total number of splenocyte β_2-adrenoceptors from GRK2+/− mice was significantly reduced compared to WT cells (Fig. 1 and Table 1). However, affinity for binding of [^{125}I]-ICYP to WT and GRK2+/− cells was similar (Table 1).

3.2. Agonist-induced β_2-adrenoceptor internalization in GRK2+/− splenocytes

Overexpression studies clearly demonstrated the involvement of GRK2 in agonist-induced β_2-adrenoceptor internalization (Menard et al., 1997). To get more insight in the role of the endogenous level of GRK2 in determining the efficiency of

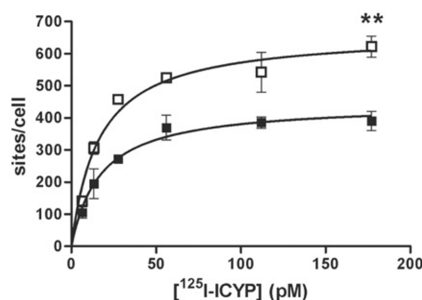

Fig. 1. β_2-adrenoceptor binding in splenocytes from WT (□) and GRK2+/− (■) mice. Saturation binding experiments were preformed by using [^{125}I]-ICYP. Non-specific binding was determined under the same conditions in the presence of 1 μM (−) propranolol. Data represent molecules radiolabeled β_2-adrenoceptors bound per cell. Results are the means ± S.E.M. of one representative experiment ($n=4$). $^{**}P<0.01$.

Table 1
Characteristics of β_2-adrenoceptor expression in splenocytes from GRK2+/− and WT mice

	WT	GRK2+/−	
Bmax	707±40	469±11	$P<0.01$
Kd	19.8±4.7	22±4.3	NS

Competitive binding assays were performed, using [^{125}I]-ICYP. Non-specific binding was determined under the same conditions in the presence of 1 μM (−) propranolol. Receptor number (Bmax) and Kd were calculated using GraphPad Prism Software. Results are the means±S.E.M. of two independent experiments ($n=4$).

agonist-induced β_2-adrenoceptor internalization, we investigated the consequences of ~50% decrease of GRK2 in splenocytes from GRK2+/− mice for agonist-induced receptor sequestration. Receptor internalization was analyzed by [^{125}I]-ICYP binding in the presence or absence of the hydrophilic ligand CGP-12177. Internalized receptors were defined as those binding sites to which the hydrophilic CGP12177 did not have access. (see "Materials and methods")(De Blasi et al., 1985; Ferguson et al., 1998).

In line with previous data (De Blasi et al., 1985), initial experiments showed that isoproterenol-induced β_2-adrenoceptor sequestration reached a maximum after 5–10 min (data not shown). Therefore, we used an incubation period of 10 min with isoproterenol in subsequent experiments. Surprisingly, the number of intracellular receptors in GRK2+/− mice vs. WT mice was significantly different in unstimulated conditions (Fig. 2A). In splenocytes from GRK2+/− mice ~32% of the β_2-adrenoceptors are intracellular, versus ~12.5% in cells of WT mice ($P<0.01$, $n=9$). Furthermore, the isoproterenol-induced increase in intracellular β_2-adrenoceptors was significantly impaired in GRK2+/− mice, showing a ~1.4 fold increase as compared to a ~3.7 fold increase in cells of WT mice ($P<0.0001$, $n=9$) (Figs. 2B and 3). Ten minutes incubation with isoproterenol did not change the total number of β_2-adrenoceptors in cells from WT or GRK2+/− animals (data not shown).

Importantly, GRK2+/− spleens display no alterations in cellular composition (Vroon et al., 2004) that could have explained the observed changes in β_2-adrenoceptor number and internalization.

3.3. *Effect of colchicine and taxol pretreatment on agonist stimulated β_2-adrenoceptor internalization*

Isoproterenol-induced redistribution of β-adrenoceptors from membrane to cytosol is dependent on an intact microtubule network (Limas and Limas, 1983; Shumay et al., 2004). Interestingly, GRK2 binds and phosphorylates tubulin. Although the potential physiological significance of GRK2-mediated tubulin binding and phosphorylation is still unclear (Pitcher et al., 1998b), it is of interest that tubulin phosphorylation promotes the assembly of tubulin and the stability of microtubules (Serrano et al., 1989; Khan and Luduena, 1996). These data led us to hypothesize that a difference in (local) microtubule stability might be responsible for the reduced agonist-induced β_2-adrenoceptor internalization in GRK2+/− compared to WT

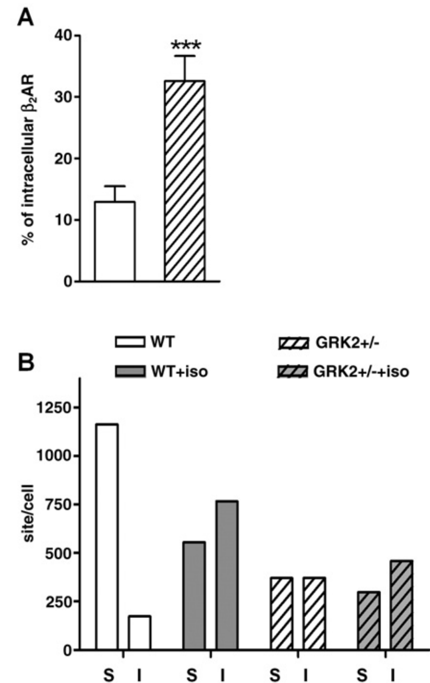

Fig. 2. β_2-adrenoceptor internalization in splenocytes from GRK2+/− (hatched bars) and WT mice (white bars). (A) β_2-adrenoceptor internalization was analyzed by [^{125}I]-ICYP binding in the presence or absence of the hydrophilic ligand CGP-12177. Internalized receptors were defined as those binding sites to which the hydrophilic CGP-12177 did not have access. (see "Material and methods"). Results are the means±S.E.M. of three independent experiments ($n=9$). ***$P<0.001$ (B) β_2-adrenoceptor internalization in response to 10 min incubation with 1 μM isoproterenol. Three independent experiments were performed ($n=9$). Representative example depicting numbers of surface (s) and intracellular (i) receptors before and after agonist treatment.

mice. Colchicine and taxol are widely used to modulate microtubule dynamics, and their specificity and mechanism of action are well documented (Correia and Lobert, 2001). If our

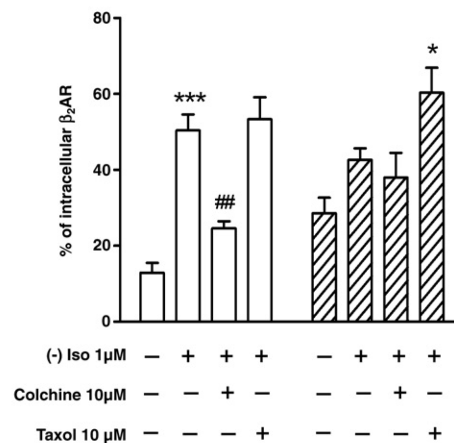

Fig. 3. Effect of colchicine and taxol pretreatment on agonist stimulated β_2-adrenoceptor internalization. β_2-adrenoceptor internalization was determined in splenocytes ($n=9$; hatched bars: GRK2+/−, white bars: WT) incubated with 1 μM isoproterenol for 10 min with or without 1 h pre-incubation with 10 μM colchicine or 10 μM taxol. Results are the means±S.E.M. of three independent experiments. ***$P<0.001$ (versus control), ##$P<0.01$ (versus isoproterenol alone), *$P<0.05$ (versus control).

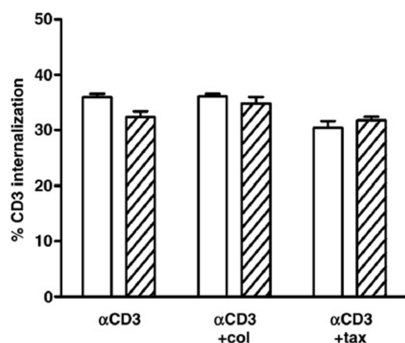

Fig. 4. Effect of colchicine and taxol pretreatment on internalization of CD3. CD3 internalization was determined in splenocytes ($n=4$; hatched bars: GRK2+/−, white bars: WT) incubated with 10 μg/ml anti-CD3 for 60 min with or without 1 h pre-incubation with 10 μM colchicine (col) or 10 μM taxol (tax). The data represent the percentage of anti-CD3 induced internalization of CD3, determined as percent change in the mean fluorescence intensity of CD3 positive cells. Data represent means ± S.E.M.

hypothesis is correct, treatment of WT splenocytes with colchicine, which destabilizes microtubules, should mirror the situation in cells of GRK2+/− mice by reduced agonist-induced receptor internalization. Conversely, treatment of GRK2+/− splenocytes with taxol, which stabilizes microtubules, should "rescue" the defective agonist-induced receptor internalization.

Cells were pretreated for 1 h with 10 μM colchicine or 10 μM taxol or control medium followed by incubation with 1 μM isoproterenol for 10 min. The results summarized in Fig. 3 clearly show that pretreatment of splenocytes from WT mice with colchicine markedly reduced agonist-induced β_2-adrenoceptor internalization by 50% ($P<0.01$ vs. isoproterenol-induced receptor internalization in untreated cells). No significant changes were induced by taxol pretreatment of WT cells ($P>0.05$). Conversely, colchicine pretreatment of splenocytes from GRK2+/− did not have any significant effect on agonist-induced β_2-adrenoceptor internalization ($P>0.05$ vs. untreated cells stimulated by isoproterenol). However, taxol pretreatment restored agonist-induced β_2-adrenoceptor internalization in splenocytes from GRK2+/− animals ($P<0.05$). Neither colchicine nor taxol pretreatment significantly changed basal levels of intracellular β_2-adrenoceptors in splenocytes from WT and GRK2+/− mice ($P>0.05$, data not shown).

To determine whether the effects of taxol and colchicine on receptor internalization in GRK2+/− cells are specific for GRK2 substrate receptors, we also analyzed the effect of these drugs on GRK2-independent receptor internalization. Treatment of splenocytes with anti-CD3 induced internalization of CD3 to a similar extent in WT and GRK2+/− cells. More importantly, CD3 internalization was not affected by pretreatment with taxol or colchicine in either WT or GRK2+/− splenocytes (Fig. 4), suggesting that modulation of microtubule stability does not affect GRK-independent internalization mechanisms.

3.4. *Agonist-induced tubulin phosphorylation in GRK2+/− splenocytes*

To investigate whether reduced GRK2 has consequences for agonist-induced tubulin phosphorylation, we labeled splenocytes metabolically with [$^{32}P_i$], stimulated the cells with isoproterenol, and immunoprecipitated tubulin. The basal level of phosphorylated tubulin did not differ in lysates from WT and GRK2+/− splenocytes. Unfortunately, isoproterenol-induced tubulin phosphorylation was not reproducibly detectable. Nonetheless, an isoproterenol-induced increase in phosphorylated tubulin was observed in 4 out of 7 WT cell lysates. However, only 2 out of 7 GRK2+/− lysates displayed an enhanced level of phosphorylated tubulin after agonist treatment (data not shown). These data suggest that agonist-induced tubulin phosphorylation may be impaired in GRK2+/− splenocytes.

3.5. *Isoproterenol-stimulated cAMP accumulation in splenocytes from GRK2+/−*

We next addressed the question whether the reduction in β_2-adrenoceptor number on the surface of splenocytes from GRK2+/− mice results in a reduced isoproterenol-induced cAMPresponse. As shown in Fig. 5, basal levels of cAMP, expressed as pmol/10^6 cells, did not differ between GRK2+/− and WT cells. Moreover, the isoproterenol-stimulated cAMP accumulation was similar in GRK2+/− and WT cells, despite lower numbers of β_2-adrenoceptors in GRK2+/− cells. To investigate whether changes in adenylyl cyclase activity could explain the normal cAMP response despite lower receptor numbers, we assessed forskolin-stimulated cAMP accumulation, as a measure of adenylyl cyclase activity independent of β_2-adrenoceptor stimulation. However, we did not observe significant differences in this response between cells from GRK2+/− and WT mice (WT: 19.1 ± 2.7, GRK2+/−: 20.2 ± 2.8 pmol/10^6 cells, $n=6$ per group).

It could be possible that the normal cAMP response in cells from GRK2+/− mice results from the impaired agonist-induced receptor internalization in these cells. To address this question, we investigated the effect of colchicine or taxol treatment on

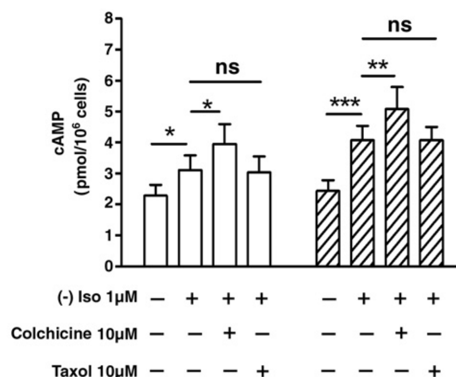

Fig. 5. (A) Isoproterenol-stimulated cAMP accumulation in splenocytes from GRK2+/− (hatched bars) and WT mice (white bars). Splenocytes ($n=8$) were stimulated for 10 min at 37 °C with 1 μM isoproterenol in the presence of 1 mM IBMX to inhibit phosphodiesterase activity. Effect of colchicine and taxol treatment on isoproterenol-induced cAMP responses was analyzed after pre-incubation for 1 h with 10 μM colchicine or 10 μM taxol. Data are expressed as pmol/10^6 cells. Results are the means ± S.E.M. of two independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

isoproterenol-induced cAMP responses. Cells were pre-incubated for 1 h with 10 μM colchicine or 10 μM taxol and stimulated with 1 μM isoproterenol for 10 min. The results summarized in Fig. 5 show that in the same condition in which colchicine was able to reduce agonist-induced β_2-adrenoceptor internalization in WT cells, it did not change agonist-induced cAMP production. Similarly taxol pretreatment of GRK2+/−, which rescues β_2-adrenoceptor internalization in these cells, did not normalize the cAMP response in these cells. Colchicine or taxol pretreatment did not significantly alter basal cAMP levels.

4. Discussion

In this study we demonstrate that a 50% reduction of GRK2 results in a higher basal level of intracellular β_2-adrenoceptors and an impaired agonist-induced receptor internalization. Moreover, we show that the impaired agonist-induced β_2-adrenoceptor internalization in splenocytes from GRK2+/− mice is almost completely "rescued" by pretreatment with taxol, a drug that has been shown to enhance microtubule polymerization and to "stabilize" microtubules (Jordan et al., 1998).

By using fluorescently labeled β_2-adrenoceptors, it has been shown that receptors are motionally constrained within the plasma membrane. Apparently, the interaction between receptors and microtubules plays an important role in this motional constraining, since colchicine, a microtubule-disrupting agent, relieves the constraint and increases receptor mobility (Cherksey et al., 1980). Furthermore, previous studies have shown that agonist-induced β-adrenoceptor internalization is dependent on microtubules and that microtubule-disrupting agents are capable of preventing agonist-induced β-adrenoceptor internalization (Limas and Limas, 1983; Shumay et al., 2004). We confirmed these observations by showing that colchicine pretreatment of splenocytes from WT mice is indeed capable of reducing agonist-stimulated β_2-adrenoceptor internalization by 50%. However, disrupting microtubules does not affect agonist-induced β_2-adrenoceptor internalization in splenocytes from GRK2+/− animals. These data suggest that the remaining low level of agonist-induced receptor internalization in the cells from GRK2+/− animals is independent of (local) microtubule stability. Interestingly, taxol pretreatment, which stabilizes microtubules, results in increased agonist-induced β_2-adrenoceptor internalization only in cells from GRK2+/− mice, but not in cells from WT animals. Importantly, stabilizing microtubules only increases GRK-dependent internalization mechanisms, as internalization of CD3 in WT and GRK2+/− cells was not affected by taxol or colchicine. In addition, the observation that neither colchicine nor taxol pretreatment significantly changed basal levels of intracellular β_2-adrenoceptors in splenocytes from WT and GRK2+/− mice, suggests that the state of microtubule assembly is relevant only for the agonist-induced β_2-adrenoceptor internalization in these animals.

The kinetics of β_2-adrenoceptor and tubulin phosphorylation by GRK2 are very similar (Pitcher et al., 1998b). It is known that agonist occupancy of β_2-adrenoceptor induces translocation of GRK2 to the plasma membrane, which in turn promotes β_2-adrenoceptor phosphorylation (and desensitization). In view of the current findings, it may well be possible that GRK2 activity also mediates local changes in microtubule dynamics, and thereby support agonist-induced receptor internalization. GRK2 binds and phosphorylates tubulin in vitro (Haga et al., 1998; Pitcher et al., 1998b) and colocalizes with microtubules in COS1 cells (Carman et al., 1998). Recently, the phoshorylation sites for GRK2 in beta-tubulin were determined to be Thr409, Ser420 and Ser444, located on the outer surface of microtubules (Yoshida et al., 2003). Although it is technically difficult to show directly in vitro that GRK2 phosphorylation affects local tubulin assembly (Carman et al., 1998), indirect evidence for this comes from the observation that phosphorylation of βIII tubulin promotes assembly and stability of microtubules (Khan and Luduena, 1996). Interestingly, under similar phosphorylation conditions, βIII tubulin is phosphorylated by GRK2 (Carman et al., 1998). The existence of a direct relation between GRK2 levels and activity and microtubule assembly is further supported by a study showing that microtubule assembly is increased 8 weeks after induction of cardiac hypertrophy in rats (Ishibashi et al., 1996). In a similar model, GRK2 activity and expression was shown to be increased by ~3-fold (Choi et al., 1997).

Using HEK-293 cells overexpressing GRK2 and β-adrenoceptors, Pitcher et al. demonstrated an increased physical interaction of GRK2 and tubulin following β-adrenoceptor activation, which was associated with increased tubulin phosphorylation. Our data may suggest that isoproterenol-induced tubulin phosphorylation in GRK2+/− splenocytes is indeed impaired. However, as agonist-induced changes in tubulin phosphorylation in splenocytes (with physiological expression levels of β_2-adrenoceptors and GRK) were not reproducibly detectable, we can not provide strong evidence for this conclusion. Possibly, impaired phosphorylation of all tubulin phosphorylation sites contributes to the technical difficulties in detecting phosphorylated tubulin in GRK2+/− splenocytes. Alternatively, physiological levels of β_2-adrenoceptors and GRK2 only locally affect tubulin phosphorylation, resulting in local changes in microtubule dynamics that support agonist-induced receptor internalization. In addition, we have to take into account the possibility that altered β_2-adrenoceptor internalization in GRK2+/− cells reflects complex functional interactions of GRK2 and tubulin/microtubules that are independent of kinase activity. In conclusion, we propose that the 50% reduction in GRK2 in GRK2+/− mice results in local changes of (agonist-induced) microtubule dynamics that affect agonist-induced β_2-adrenoceptor internalization.

The basal level of intracellular β_2-adrenoceptors is significantly increased in GRK2+/− splenocytes compared to WT cells. The molecular mechanism responsible is, at present, unclear. It is not likely that the state of microtubule assembly in unstimulated GRK2+/− cells affects receptor redistribution, since neither colchicine nor taxol treatment changes the number of intracellular receptors in cells from GRK2+/− or WT mice. Possibly, the increased number of intracellular β_2-adrenoceptors at baseline represents defective targeting of newly formed receptors to the plasma membrane and/or impaired recycling of

receptors that have been exposed to the agonist in vivo. The overall decrease in total number of receptors in GRK2+/− mice is likely specific for the β_2-adrenoceptor since Bmax values of another GRK2 substrate receptor, CCR5, are not altered in these animals (Vroon et al., 2004). Whether the reduction in receptor number is associated with a lower β_2-adrenoceptor mRNA expression in splenocytes of GRK2+/− mice remains to be elucidated. However, it is of interest to note that the state of microtubule assembly is capable of affecting β_2-adrenoceptor mRNA levels in rat C6 glioma cells (Hough et al., 1994).

Although the number of β_2-adrenoceptors on the surface of GRK2+/− cells was 50% lower than on WT cells, the cAMP response/cell was similar in GRK2+/− and WT animals. These data imply that the cAMP response per receptor is markedly increased in GRK2+/− cells. Increased responses to specific agonists have been described before under conditions of low GRK2 expression. For example, GRK2+/− mice display an enhanced sensitivity of chemokine receptor CCR5, at the level of chemotaxis as well as signaling (Vroon et al., 2004). Moreover, isoproterenol-induced myocardial contractile function is enhanced in GRK2+/− mice (Rockman et al., 1998b). Interestingly, targeted overexpression of βARKct (a peptide inhibitor of GRK2) in GRK2+/− mice, leading to a further decrease in GRK2 activity, resulted in an in vivo phenotype of both enhanced basal and isoproterenol-stimulated contractility (Rockman et al., 1998a). These authors suggested that impaired receptor desensitization and/or internalization, resulting in prolonged activation of the receptor and an increased cellular response, could explain the increased response to the agonist. Therefore, we hypothesized that the impaired agonist-induced β_2-adrenoceptor internalization might have been responsible for the increased cAMP response per receptor in cells from GRK2+/− animals. However, this explanation does not seem valid since in the same time frame during which colchicine or taxol exerts its effect on β_2-adrenoceptor internalization, these drugs do not influence the cAMP response. In addition, neither the adenylyl cyclase activity differs between WT and GRK2+/− mice, since forskolin-induced cAMP production was not altered in GRK2+/− cells.

The high cAMP response/receptor in GRK2+/− cells may reflect a reduced initial desensitization of the receptor, due to impaired GRK2-induced uncoupling of the receptor from the G protein. This would mean that receptor internalization is not needed for termination of signaling in this time frame. In line with this hypothesis, CCR5-induced calcium mobilization was significantly increased in GRK2+/− cells, despite a normal refractoriness to restimulation by the agonist (Vroon et al., 2004). In addition, preliminary evidence suggests that also the response to prolonged agonist exposure is normal in GRK2+/− cells. For example, β_2-adrenoceptor-induced inhibition of IFN-γ production is similar in GRK2+/− and WT cells, despite the 50% lower receptor number on the surface of GRK2+/− cells (unpublished observations). In light of these observations, we hypothesize that the reduced number of β_2-adrenoceptors and the higher basal level of intracellular receptors in splenocytes might well reflect an adaptive mechanism for the cells to counteract the "chronic" reduced level of GRK2.

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