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G Protein-coupled Receptor Kinase 3 (GRK3) Gene Disruption Leads to Loss of Odorant Receptor Desensitization*

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Karsten Peppel‡, Ingrid Boekhoff§, Patricia McDonald‡, Heinz Breer§, Marc G. Caron¶, and Robert J. Lefkowitz‡‖

From the Department of Medicine, ‡Biochemistry, and ¶Cell Biology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710 and the §Department of Physiology, Universität Hohenheim, D-70593 Stuttgart, Germany

G protein-coupled receptor kinases (GRKs) 2 and 3 (β-adrenergic receptor kinases 1 and 2 (βARK1 and -2)) mediate the agonist-dependent phosphorylation and uncoupling of many G protein-coupled receptors. These two members of the GRK family share a high degree of sequence homology and show overlapping patterns of substrate specificity *in vitro*. To define their physiological roles *in vivo* we have generated mice that carry targeted disruption of these genes. In contrast to GRK2-deficient mice, which die *in utero* (Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross, J. JR., Lefkowitz, R. J. Caron, M. G., and Giros, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 12974–12979), GRK3 deletion allows for normal embryonic and postnatal development. GRK3 is expressed to a high degree in the olfactory epithelium, where GRK2 is absent. Here we report that cilia preparations derived from GRK3-deficient mice lack the fast agonist-induced desensitization normally seen after odorant stimulation. Moreover, total second messenger (cAMP) generation in these cilia preparations following odorant stimulation is markedly reduced when compared with preparations from wild-type littermates. This reduction in the ability to generate cAMP is evident even in the presence of nonodorant receptor stimuli (GTPγS and forskolin), suggesting a compensatory dampening of the G protein-adenylyl cyclase system in the GRK3 (−/−) mice in the olfactory epithelium. These findings demonstrate the requirement of GRK3 for odorant-induced desensitization of cAMP responses.

Many G protein-coupled receptors (GPCRs)[1] show diminished ability to signal and couple to G proteins after prolonged or repeated agonist stimulation. This phenomenon, referred to as agonist-mediated desensitization, occurs very rapidly and is initiated via receptor phosphorylation by G protein-coupled receptor kinases (GRKs) that serve to uncouple the receptor from its G protein (1).

Whereas the function of these proteins has been mostly studied *in vitro* and in tissue culture the physiological relevance of the mechanisms initiated by them have just begun to be explored. While there is some evidence of substrate specificity among the different members of the GRK family, most show activity toward a wide variety of agonist-occupied receptors *in vitro*. This, in addition to their ubiquitous tissue expression, has made it difficult to precisely determine the role of the GRKs *in vivo*. To clarify the physiological role of the individual members of this family, we have generated mice that carry targeted disruptions of the GRK2 or GRK3 (βARK2) genes. GRK2 deletion is embryonically lethal as homozygous mice die *in utero* before gestational day 15.5 of severe cardiac malformations (2). Whereas GRKs 2 and 3 show 81% amino acid identity (3) and an overlapping pattern of tissue expression (4), GRK3 apparently is not able to compensate for the loss of GRK2 in embryogenesis. In most tissues examined GRK2 is the predominant form. However, in the olfactory epithelium GRK2 is virtually absent, and GRK3, as well as the second messenger-dependent protein kinases PKA and PKC, is thought to be responsible for the desensitization of olfactory receptors (5), which are members of the GPCR superfamily (6).

Here we report that ablation of the GRK3 gene by homologous recombination indeed leads to a phenotypic abnormality of the olfactory system. Unlike GRK2-deficient mice, the GRK3-disrupted mice develop and breed normally but show a loss of odorant receptor-mediated desensitization. In addition, the marked down-regulation in odorant receptor-mediated cAMP generation in olfactory epithelia of these mice suggests that major adaptive changes in the second messenger generating system have occurred in response to the lack of GRK3.

EXPERIMENTAL PROCEDURES

Materials—The odorants citralva (3,7-dimethyl-2,6-octadiennitrile), hedione (3-oxo-2-pentyl cyclopentaneacetic acid methyl ester), eugenol (2-methoxy-4-(2-propenyl)phenol), geraniol (3,7-dimethyl-2,6-octadien-1-ol), and menthone (5-methyl-2-(1-methylethyl)cyclohexanone) were provided by DROM (Baierbrunn, Germany). GTPγS, forskolin, 3-isobutyl-1-methylxanthine (IBMX), and the goat-anti-rabbit IgG conjugate (horseradish peroxidase) were obtained from Sigma (Deisenhofen, Germany). Radioligand assay kits for cAMP as well as the enhanced chemiluminescence systems (ECL) for Western blots were provided by Amersham (Braunschweig, Germany). RNAzol reagent was from Tel-Test, and the Nytran membranes and turboblotter kits used for nucleic acid transfer were from Schleicher & Schuell. All primers were from Genosys. The Moloney murine leukemia virus reverse transcriptase was from Life Technologies, Inc. Unless otherwise specified, all reagents were from Sigma. All chemicals were of the highest purity available.

GRK3 Gene Disruption—A bacteriophage λ library of mouse 129SVJ genomic DNA (Stratagene) was screened with a cDNA for rat GRK3 (3). Positive phages were identified and analyzed by restriction digest. A 2.4-kb *Bam*HI fragment was shown to contain two exons encoding part of the catalytic subdomain I. The second exon located in this fragment contains the consensus G*X*G*XX*G motif of protein kinases (7). The targeting vector was assembled by subcloning a 1.9-kb *Sal*I-*Bam*HI fragment, located immediately 5′ to the exon *a* and *b* containing *Bam*HI fragment into plasmid pBS SK (Stratagene). A 3.9-kb *Bam*HI-*Not*I fragment containing exon *c* (3′ of the 2.4-kb fragment containing exons *a* and *b*) was then added. Finally this construct was digested with

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‖ To whom correspondence should be addressed. Tel.: 919-684-2974; Fax: 919-684-8875; E-mail: lefko001@mc.duke.edu.

[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; RT-PCR, reverse transcription-polymerase chain reaction; IBMX, isobutylmethylxanthine; kb, kilobase(s); bp, base pair(s); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

*Bam*HI, and a 1.9-kb cassette containing the neomycin resistance gene under control of the human glycerol phosphate kinase promoter (from plasmid pD383, R. Hen, Columbia University, New York) was inserted in antisense orientation. This cassette contains a single *Eco*RI site which was used to analyze homologous recombinants. The entire targeting vector was sequenced. This targeting vector was digested with *Sal*I, gel-purified, and electroporated into mouse embryonic stem cells. Electroporation, cell culture, and G418 selection and generation of chimeric animals were as described (8). After electroporation, 54 G418 resistant clones were analyzed by Southern blotting of *Eco*RI-restricted DNA. Two clones positive for one event of homologous recombination were found. One clone, termed EP 149-7, was karyotyped and used for blastocyst injection. It generated two chimeric male animals, one of which transmitted the stem cell character to its offspring. All mice were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (25).

Southern Blot Analysis—Mouse tail DNA was isolated following overnight digestion with proteinase K in 1% SDS at 65 °C. Approximately 10 μg of DNA was digested overnight with *Eco*RI, separated on a 0.8% agarose gel, and transferred to Nytran membranes (Schleicher & Schuell). The 5′ probe was a 189-bp *Eco*RI-*Hin*dIII fragment located 5′ to the targeting vector. The 3′ probe was a 200-bp DNA fragment that was PCR-amplified with primers 5′-TATAGTGCACACCAGCTC-3′ and 5′-CACTGAGGTGGCTGAGAG-3′. All DNAs used as probes were gel-purified and labeled with [α-^{32}P]dCTP using Stratagene's random primer labeling kit. Prehybridization and hybridization were done at 55 °C in 4 × SSC, 25% formamide, 1% SDS, 50 μg/ml tRNA, and 10% dextran sulfate. Following overnight hybridization the filters were washed initially in 2 × SSC, 1% SDS at 65 °C. The final wash was in 0.2 × SSC, 0.2% SDS at 70 °C. The filters were exposed to X-R Kodak film for up to 3 days.

RNA Isolation and RT-PCR—Total brain RNA was isolated using the RNAzol reagent according to the manufacturer's instructions (Tel-Test). The reverse transcriptase reaction was carried out with antisense primer 5′-atatctagaCAGTGCTAACGAGAGACAGCATGATCC-3′ (position 719–745 of the rat GRK3 cDNA) for 1 h in 1 × superscript buffer (Life Technologies, Inc.) including 4 units of RNAsin (Promega) at 45 °C using 5 μg of total mouse brain RNA and 2 units of Moloney murine leukemia virus reverse transcriptase in a total volume of 20 μl. This reaction was then used for a PCR reaction in a total volume of 100 μl, containing 1 × Thermo Buffer, 200 μM of each dNTP, and 500 nM of each oligonucleotide primer. PCR was carried out using the same primer as was used for the RT reaction and sense primer 5′-attgaattcCGGCAGATGTACGACGCCTACATCATG-3′ (position 316–342 of the rat GRK3 cDNA) under standard conditions (9). Reactions were assembled on ice without polymerase and heated to 95 °C for 5 min. 2.5 units of *Taq* DNA polymerase (Promega) were added. Reactions were cycled 35 times for 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C with a final extension time of 5 min at 72 °C. Following gel electrophoresis on an 1% agarose gel the PCR products were denatured and transferred to nylon membrane and hybridized with the end-labeled sense primer 5′-CCAAGAAGCAGGTGACGC-3′ (position 413–430 of the rat GRK3 cDNA). The deletion introduced into the GRK3 gene spans nucleotides 506–650 of the GRK3 cDNA. In addition, PCR products were digested with *Eco*RI and *Xba*I (underlined in sequence above), subcloned into pBluescript (Stratagene), and sequenced.

Northern Blot Analysis—20 μg of total RNA was denatured with glyoxal, separated on a 0.8% agarose gel, and transferred to Nytran membrane using Schleicher & Schuell's turboblotter kit for 2 h in 10 mM NaOH, 3 × SSC. Following neutralization and UV crosslinking the membranes were prehybridized, hybridized, and washed as described above for the Southern blots. The probes used were rat cDNAs for GRK2, GRK3, and actin.

Isolation of Olfactory Cilia—Partially purified preparations of chemosensory cilia from mouse olfactory epithelium were isolated with the calcium shock method (10).

Determination of Odor-induced Second Messenger Responses—A rapid kinetic system was used to determine odorant-induced changes of second messenger concentrations in the subsecond time range. Stimulation experiments were performed at 37 °C as described previously (11).

For stimulation experiments requiring IBMX, cilia preparations were pretreated on ice for 5 min with 1 mM IBMX and subsequently incubated with the stimulation buffer for 2 min in a shaking water bath at 37 °C before the reaction was terminated. The stimulation buffer, cilia preparation, and stop solution were the same as in the subsecond time range stimulation experiments, except that 1 mM IBMX was applied in the stimulation buffer. Quenched samples were stored on ice for 20 min and then analyzed for second messenger concentrations as described previously (12).

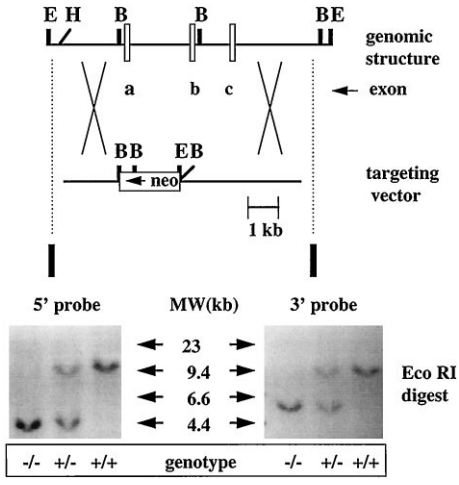

FIG. 1. **Partial structure of the GRK3 gene.** *Upper panel*, organization of the 8.2-kb genomic fragment containing three exons (*open boxes*). Exon *b* contains the catalytic subdomain I, which is part of the ATP binding site. *Middle panel*, map of the targeting vector used for homologous recombination. Transcription of the neomycin cassette is opposite to that of the GRK3 gene. *Lower panel*, Southern blot analysis of mouse tail DNA isolated from homozygous knockout (−/−), heterozygous (+/−), and wild-type (+/+) mice. Tail DNA was restricted with *Eco*RI, transferred to nylon, and hybridized to a probe located either 5′ or 3′ of the targeting vector (*solid boxes*, *stippled lines* indicate location of probes). Location of molecular size standards is indicated. *E*, *Eco*RI; *B*, *Bam*HI; *H*, *Hin*dIII.

The concentration of free Ca^{2+} was calculated by the method described by Pershadsingh and McDonald (13); magnesium and calcium present in the tissue were not included in the calculation.

Western Blot Analysis—Different tissues from normal and GRK3-knockout mice were prepared for SDS-polyacrylamide gel electrophoresis as described previously (14). Western blots were stained with Ponceau S, dried, and stored at 4 °C until use. For analysis, nonspecific binding sites were blocked with 5% non-fat dry milk (Naturaflor) in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST). The blots were incubated overnight at 4 °C with specific antibodies against GRK3 (1:5000 in TBST, containing 3% nonfat dry milk). After three washes with TBST, a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 in TBST, 3% nonfat dry milk) was applied, and the ECL system was used to monitor bound antibodies.

RESULTS

A 129SVJ mouse genomic library in bacteriophage λ (Stratagene) was screened with a rat cDNA probe to GRK3. 12 positive clones were identified. Restriction analysis indicated that they were overlapping, and primer hybridization and DNA sequencing confirmed the location of three exons (Fig. 1). The intron-exon boundaries between GRKs 2 and 3 are conserved for the exons examined; however the average intron size for the GRK3 gene is considerably larger than that of the GRK2 gene and they share no sequence homology (data not shown). Exon *b* contains the GRGGFGEV motif of the catalytic subdomain I known to be involved in ATP binding and essential for enzyme activity (7). This exon is homologous to exon 8 of the GRK2 gene (2) given the conservation of the exon boundaries between these two genes. A *Bam*HI fragment containing exons *a* and *b* was removed by homologous recombination and replaced with the neomycin resistance marker. Of 54 G418-resistant colonies two were found to have undergone a single homologous replacement event. One of the clones (designation 149-7) was karyotyped and used for injection into blastocysts. The resultant chimeric animals passed the stem cell character on to their offspring, and sibling mating between heterozygous animals generated homozygous GRK3 (−/−) mice with the expected frequency. Gross pathological examination of GRK3 gene-de-

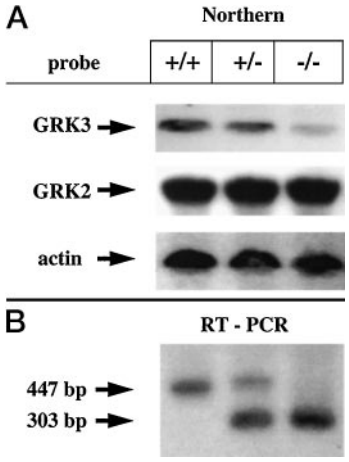

FIG. 2. **RNA analysis of GRK3-knockout mice.** *A*, Northern blot analysis of total RNA isolated from brain of GRK3 homozygous knockout (−/−), heterozygous (+/−), and wild-type (+/+) mice. *B*, RT-PCR analysis of total RNA. The PCR primers flank the 144-bp deletion introduced by the homologous recombination event. PCR products were transferred to nylon membrane and hybridized to a third primer that is located between the PCR primers but outside of the deletion. Expected size of the PCR products is indicated.

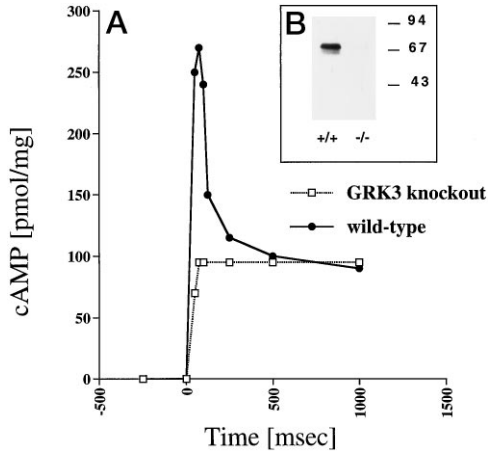

FIG. 3. **GRK3 expression and time course of odor-induced cAMP formation in olfactory cilia from GRK3 (−/−) and control mice.** *A*, olfactory cilia isolated from GRK3-knockout (−/−; *open squares*) and wild-type (+/+; *solid circles*) mice were stimulated with an odorant mixture (citralva, hedione, eugenol, each 1 μM), and the concentration of cAMP was determined at various time intervals. The results are expressed as odorant-induced changes in the concentration of cAMP (pmol/mg). Data are the means of three independent experiments. *Inset*, *B*, fractions of isolated olfactory cilia were assayed for GRK3 expression using GRK3-specific antibodies. The blots were incubated with the polyclonal GRK3-antiserum (1:5000) overnight at 4 °C; immunoreactive bands were visualized using the ECL system. The positions and molecular sizes (kDa) of standard proteins are indicated.

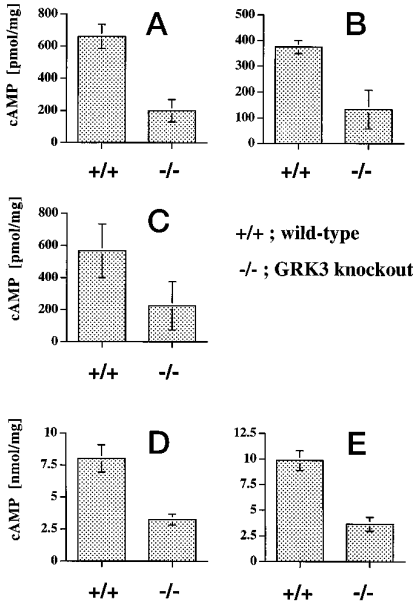

FIG. 4. **cAMP formation in isolated olfactory cilia from control and GRK3 (−/−) mice.** Isolated olfactory cilia from normal (+/+) and GRK3-knockout (−/−) mice were pretreated with IBMX and subsequently stimulated for 2 min at 37 °C. After stopping the samples with PCA, the concentration of cAMP was determined. The results are expressed as odor-induced changes in the concentration of cAMP. Data are the means of 3 experiments ± S.D. *A*, stimulation with a mixture of three odorants (citralva, hedione, eugenol) each at a concentration of 10 μM. *B*, stimulation with the floral odorant geraniol (10 μM). *C*, stimulation with the minty odorant menthone (10 μM). *D*, stimulation with 20 μM GTPγS. *E*, stimulation with 10 μM forskolin.

leted animals was unremarkable (data not shown). This indicates that GRK3 deletion is compatible with normal development. This is in stark contrast to the deletion of the highly homologous GRK2 gene, which results in embryonic heart failure and death *in utero* (2).

Analysis of RNA extracted from brain of GRK3 (−/−) mice demonstrated that the gene was still transcribed and GRK3 mRNA still present, albeit at a somewhat lower level (Fig. 2*A*). Therefore we used RT-PCR analysis to confirm the absence of the ATP binding motif located in the catalytic subdomain I (Fig. 2*B*). Western blot analysis of nasal epithelium, where GRK3 is most highly expressed, and cerebral cortex demonstrated the absence of the GRK3 protein in the GRK3 (−/−) mice, indicating that the residual transcribed RNA for GRK3 does not give rise to functional protein (Fig. 3*B*).

GRK3 is normally highly expressed in the olfactory epithelium (5). In addition, at least one other member of the GRK 4–6 subfamily is expressed there, as determined by Western blotting (data not shown). Using polyclonal anti-GRK2 or -3 antibodies in permeabilized rat olfactory cilia preparations the involvement of GRK3 (but not GRK2) as well as cAMP-dependent protein kinase in the desensitization of the odorant-induced cAMP generation has been demonstrated (5). We, therefore, isolated olfactory cilia from GRK3 (−/−) and wild-type mice and examined second messenger generation over time following stimulation with an odorant mixture. Whereas stimulation of cilia preparations isolated from wild-type mice generates the typical biphasic response indicative of rapid agonist-induced desensitization, cilia preparations from GRK3 (−/−) mice do not show any evidence of desensitization. However, the maximal rise in cAMP generated in these cilia preparations is markedly reduced when compared with preparations isolated from wild-type mice (Fig. 3*A*). This is evident even in the presence of the phophodiesterase inhibitor IBMX, suggesting that up-regulation of this enzyme is not responsible for the decreased cAMP-generating capacity of these cilia preparations (Fig. 4*A*). Furthermore, this reduced ability to generate cAMP was independent of the type of odorant stimulus (geraniol or menthone), and hence odorant receptor, used (Fig. 4, *B* and *C*).

The dampening of the second messenger generating capacity observed in GRK3-deleted epithelia preparations might be attributed to a down-regulation of the odorant receptors. However, because of the multitude of odorant receptor subtypes present in olfactory cilia (6) and the lack of procedures to quantitate these receptors, this issue cannot easily be addressed. Alternatively, in an attempt to adapt to the lack of GRK3, reduction in the G protein or in the level of adenylyl cyclase type III could account for the diminished cAMP response. To address some of these questions we treated cilia

preparations from wild-type or GRK3 (−/−) mice with GTPγS to directly stimulate the G protein or with forskolin, a stimulator of adenylyl cyclase. As can be seen in Fig. 4, *D* and *E*, treatment with GTPγS as well as forskolin leads to a significantly reduced cAMP accumulation in cilia preparations from GRK3 (−/−) mice. The level of reduction is virtually the same as that seen following odorant stimulation (compare with Fig. 4*A*). This strongly suggests reduced activity of the odorant receptor-associated G protein-adenylyl cyclase complex. This dampening of the cAMP generating capacity however is restricted to the olfactory epithelium and was not seen in membrane preparations from brain, heart, kidney, or testis of GRK3 (−/−) mice treated with GTPγS or forskolin (data not shown).

DISCUSSION

Repeated or prolonged agonist stimulation of many GPCRs, including odorant receptors, rapidly leads to a loss of further signal transduction, a process termed desensitization, that is initiated by the phosphorylation of the activated receptor (15). Both GRKs and/or second messenger-dependent kinases can be involved in this event. GRKs are a family of kinases that specifically phoshorylate only the active conformation of the receptor leading to homologous desensitization. In the visual system, rhodopsin kinase (GRK1) initiates quenching of signal transduction from activated rhodopsin by phosphorylation of the receptor. This in turn leads to the binding of a cytosolic protein, termed arrestin, to rhodopsin, which sterically interdicts its further coupling to the G protein transducin (16). An analogous situation exists for the β_2-adrenergic receptor and other GPCRs (15, 17, 18).

Recent evidence is beginning to suggest that alteration in GPCR desensitization mechanisms may have profound biochemical and physiological consequences. Mutations in both the rhodopsin kinase (GRK1) gene (19) and the retinal arrestin (20) gene lead to Oguchi disease, a form of stationary night blindness caused by the prolonged activity of photoactivated rhodopsin. In the *Drosophila* system, inactivation of arrestin leads to the degeneration of the photoreceptor cells in a light-dependent manner (21), and in the well studied β-adrenergic system, overexpression of an inhibitor of GRKs 2 and 3 (β-adrenergic receptor kinases 1 and 2) in a heart-specific manner, leads to an increased contractile response to β-agonist stimulation in transgenic mice (22). In addition, gene inactivation of GRK2 causes embryonic lethality in mice via severe cardiac malformation and heart failure (2). Furthermore, the up-regulation of GRK2 seen in patients with heart failure might contribute to the etiology of this condition (23, 24).

In light of these findings it is somewhat surprising that gene inactivation of GRK3 has no immediately obvious phenotype. GRK3 (−/−) mice develop and breed normally. This may be due to the compensation of GRK3 activity by the highly homologous GRK2. In most tissues GRKs 2 and 3 are co-localized, with GRK2 usually being the predominant isoform. We have previously shown the involvement of GRK3 in the desensitization of olfactory signal transduction (5). In olfactory epithelium GRK2 is virtually absent and GRK3 is highly expressed. Consistent with this finding we show here that GRK3 gene ablated mice completely lack the fast odorant-induced desensitization of second messenger generation (Fig. 3*A*). However, a previously unappreciated counterregulatory feature of this system was also seen. Total cAMP generation in isolated cilia preparations past 500 ms, *i.e.* following desensitization in wild-type mice, is almost indistinguishable between GRK3 (−/−) and GRK3 (+/+) mice. This dampened response is likely due to a compensatory down-regulation of G protein or adenylyl cyclase (or both) because direct stimulation of the signal transduction pathway, via GTPγS or forskolin, leads to the same reduced second messenger generation in olfactory epithelium.

In conclusion, gene deletion *in vivo* can serve as a valuable tool to differentiate the physiological functions of highly homologous members of the GRK family that cannot be immediately appreciated through *in vitro* assays. In addition, adaptive mechanisms can compensate somewhat for the loss of GRK3, but not GRK2, activity.

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