Communication

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

(Received for publication, July 29, 1997)

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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 (ARK2) 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-octadienitrile), hedione (3-oxo-2-pentyl cyclopentanenitrile), methyleugenol (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). GTPβS, 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 Generantis. 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 BamHI fragment was shown to contain two exons encoding part of the catalytic subdomain I. The second exon located in this fragment contains the consensus GXGXXG motif of protein kinases (7). The targeting vector was assembled by subcloning a 1.9-kb Sall-BamHI fragment, located immediately 5’ to the exon a and b containing BamHI fragment into plasmid pBBS SK (Stratagene). A 3.9-kb BamHI-NotI 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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BamHI, 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 EcoRI site which was used to analyze homologous recombinants. The entire targeting vector was digested with SaI, 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 EcoRI-restricted DNA. Two clones positive for one event of homologous recombination were selected. One clone was termed EP 149-7 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 EcoRI, separated on a 0.8% agarose gel, and transferred to Nytran membranes (Schleicher & Schuell). The 5′ probe was a 189-bp EcoRI-HindIII fragment located 5′ to the targeting vector. The 3′ probe was a 200-bp DNA fragment that was PCR-amplified with primers 5′-TATATGCAACAGCACGTAC-3′ and 5′-CAGCTAGGTGCTGAGAAG-3′. All DNAs used as probes were gel-purified I and labeled with [32P]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 RNA, 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 up to for 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′-atattagataCAGTGTAACGAGACGAGCATGATCC-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. cDNA (1 μg) was amplified using primers 5′-CCAAGAAGCAGGTGACGC-3′ and 5′-TATAGTGCACACCAGCTC-3′ (9). Reaction products were separated on a 0.8% agarose gel, separated on a 0.8% agarose gel, and transferred to 3MM nitrocellulose. Following hybridization and UV crosslinking the filter was probed with a specific probe specific to the targeted gene. Prehybridization and hybridization were done at 55°C in 4× SSC, 25% formamide, 1% SDS, 50 μg/ml RNA, 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 up to for 3 days.

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 exon-intron boundaries between GRK2s 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 (2) given the conservation of the exon boundaries between these two genes. A BamHI 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-
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. 2A). Therefore we used RT-PCR analysis to confirm the absence of the ATP binding motif located in the catalytic subdomain I (Fig. 2B). 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. 3B).

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. 3A). This is evident even in the presence of the phosphodiesterase inhibitor IBMX, suggesting that up-regulation of this enzyme is not responsible for the decreased cAMP-generating capacity of these cilia preparations (Fig. 4A). 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. 4A). 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 phosphorylate 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 interferes with the receptor's ability to bind G protein and adenyl cyclase, thus dampening the second messenger generation in olfactory epithelium (16). An analogous situation exists for the β2-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. 3A). 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 (+/+). 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.

**Acknowledgments**—We thank Dr. B. H. Koller for the generation of homologous recombinant murine stem cells and chimeric mice, Dr. Richard Premont for critical reading of the manuscript, and Mary Holben and Donna Addision for expert secretarial assistance.

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