nents present only in immature thymocytes play a role in transportation of the TCRβ-CD3γδε complex to the cell surface. An alternative is that such partial complexes were degraded more rapidly in mature T cells. Our results also suggest that the TCRα subunit is important for the association of CD3ζ subunits with a TCR. In T cell hybridomas lacking CD3ζ/η, a small amount of TCRαβ-CD3γδε surface expression has been observed (*19*). Transfection of CD3ζ, η, or both up-regulated surface expression of the TCR in these mutants, demonstrating the importance of CD3ζ/η subunits in transportation of the TCR complex (*20*).

The potential function of the TCRβ chain surface complex in early T cell development remains to be determined. In precursor B cells, it has been proposed that a surface complex that includes the Ig heavy chain in association with surrogate light chains participates in signaling the allelic exclusion of Ig heavy chain and onset of the assembly of Ig light chain V genes (*21*). It is possible that the TCRβ-CD3γδε complex identified by the immunoprecipitation studies herein could function in signaling such regulatory events. Finally, the ability of the TCRβ transgene to promote the phenotypic conversion of DN to DP cells and expand the pool of thymocytes in RAG-$2^{-/-}$ mice likely reflects an important normal physiological function of this TCR subunit that should now be readily amenable to further analysis.

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12 The TCR transgenic RAG-$2^{-/-}$ mice were originally generated by breeding RAG-$2^{-/+}$ mice (129, *H-2*^{b}) with TCR transgenic mice (*H-2*^{b} or *H-2*^{d}) and identified by a combination of PCR assays to detect these TCR transgenes and the RAG-2 mutation using the following PCR primers: V_αDO1 (5′-TGCAGCTGGATGGGATGAGC-CAAGG-3′) and J_αDO1 (5′-TGGCTCTACAGT-GAGTTTGGTGCCA-3′) for the TCRα chain gene, V_βDO2 (5′-ATGTACTGGTATCGGCAGGA-CACGG-3′) and J_βDO2 (5′-CAACTGTGAGTCTG-GTTCCTTTACCA-3′) for the TCRβ chain gene, and 5EX-RG2 (5′-AAGAGTATTTCACATCCAC-3′) and RG2-0 (5′-GCTTTTCCCTCGACTACAC-CACG-3′) for the RAG-2 mutation. All of these mice were maintained in nonbarrier facilities and analyzed at approximately 3 to 4 weeks of age. In total, 42 mice were analyzed.
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β-Adrenergic Receptor Kinase–2 and β-Arrestin–2 as Mediators of Odorant-Induced Desensitization

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β-Adrenergic receptor kinase (βARK) and β-arrestin function in the homologous or agonist-activated desensitization of G protein–coupled receptors. The isoforms βARK-2 and β-arrestin–2 are highly enriched in and localized to the dendritic knobs and cilia of the olfactory receptor neurons where the initial events of olfactory signal transduction occur. Odorants induce a rapid and transient elevation of adenosine 3′,5′-monophosphate (cAMP), which activates a nonspecific cation channel and produces membrane depolarization. Preincubation of rat olfactory cilia with antibodies raised against βARK-2 and β-arrestin–2 increased the odorant-induced elevation of cAMP and attenuated desensitization. These results suggest that βARK-2 and β-arrestin–2 mediate agonist-dependent desensitization in olfaction.

The primary events of olfactory signal transduction occur in the cilia of olfactory receptor neurons and lead to the generation of the intracellular second messengers cAMP and inositol 1,4,5-trisphosphate (IP_3) (*1–3*). Isoforms of several components of these transduction pathways are highly enriched in olfactory neuronal cilia and include a type III adenylyl cyclase (*4*), a cyclic nucleotide–gated nonspecific cation channel (*5*), and an α subunit of a stimulatory G protein, G_{olf} (*6*). The membrane receptors presumed to transduce the odorant stimulus are members of a large family of G protein–linked receptors (*7*) and are localized in the olfactory neuron too (*8*).

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As with other responses mediated by G protein–coupled receptors, odorant-stimulated signals attenuate rapidly even in the continual presence of stimulus, a phenomenon termed desensitization. Although the initial events of olfactory signal transduction have been extensively explored, the mechanisms of desensitization to odorants are not known.

Desensitization of signal transduction may occur through a variety of processes, including receptor internalization or receptor uncoupling mediated by receptor phosphorylation (*9*). Homologous or agonist-induced desensitization of the β_2-adrenergic receptor occurs through agonist-activated receptor phosphorylation that is catalyzed by a specific receptor kinase called β-adrenergic receptor kinase (βARK) (*10, 11*). Further quenching of signal transduction requires the binding of the protein β-arrestin to phosphorylated receptors (*12*). Three receptor kinases—rhodopsin kinase (*13*), βARK-1 (*12*), and βARK-2 (*14*)—have been identified and three arrestins are known—arrestin (*15*), β-arrestin–1 (*12*), and β-arrestin–2 (*16*). Localiza-

tion studies reveal a broad tissue distribution for these proteins and suggest that they may have broad receptor specificity, regulating several G protein–coupled receptors (*16*, *17*). Because the putative odorant receptors (*7*) are members of the G protein–coupled receptor family, we investigated the possibility that isoforms of βARK and β-arrestin might be expressed in the olfactory neuroepithelium. Our results show that βARK-2 and β-arrestin–2 are enriched in the dendritic knobs and cilia of olfactory receptor neurons in the neuroepithelium and that βARK-1 and β-arrestin–1 are absent from these processes. Antibodies to βARK-2 and β-arrestin–2 prevented desensitization to odorants in isolated cilia, thus suggesting that βARK-2 and β-arrestin–2 function in olfactory desensitization.

βARK and β-arrestin isoforms were identified in the olfactory neuroepithelium with affinity-purified rabbit antibodies raised against glutathione-S-transferase fusion proteins containing the COOH-terminus of rat βARK-1, βARK-2, β-arrestin–1, or β-arrestin–2 (*16*, *17*). βARK-2 immunoreactivity was evident in the olfactory neuroepithelium (Fig. 1A), whereas βARK-1 immunoreactivity was completely absent (Fig. 1E), which stands in contrast to our previous studies (*16*, *17*) in which βARK-1 was consistently more abundant than βARK-2. βARK-2 was localized to the apical surfaces of the olfactory neuroepithelium and appeared to be enriched in the dendritic knobs and cilia. Localization of βARK-2 to olfactory cilia was confirmed by confocal microscopy. βARK-2 was also evident, but less prominent, in the outer third of the epithelium, a region containing sustentacular cells as well as dendrites of the olfactory receptor neurons. This pattern of immunoreactivity is similar to those of G_{olf} and the IP_3 receptor (*18*), which have been implicated in olfactory signal transduction (*6*), and type III adenylyl cyclase, which is highly enriched in olfactory neuronal cilia (*4*). Putative odorant receptors (*7*) have also been localized to olfactory neurons and their cilia (*8*). βARK-2 was absent from the region of the adjacent respiratory epithelium (Fig. 1B). βARK-1 immunoreactivity was absent from both olfactory neuroepithelium and respiratory epithelium (Fig. 1, E and F).

To confirm the localization of βARK-2 to the distal processes of the olfactory receptor neuron, we performed unilateral bulbectomies. Olfactory receptor neurons in rats degenerate approximately 1 week after their target, the olfactory bulb, is removed (*19*). The residual olfactory neuroepithelium consists primarily of sustentacular cells; after bulbectomy, βARK-2 immunoreactivity within the olfactory neuroepithelium was reduced (Fig. 1C). Several dendritic processes were still present and were immunoreactive for βARK-2. In addition, primary cultures of neonatal rat olfactory receptor neurons (*20*) were examined for βARK-1 and βARK-2 immunoreactivity. Whereas βARK-1 immunoreactivity was absent, βARK-2 immunoreactivity was present throughout the cell body and processes as well as in the region of the dendritic knob of all of the neurons in culture (Fig. 1D).

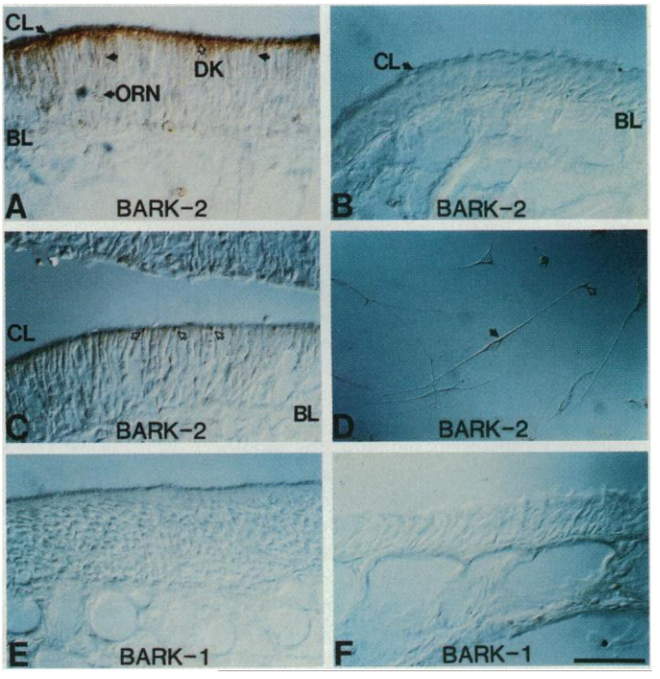

Fig. 1. Nomarski color photomicrographs illustrating the localization of βARK-2 to olfactory cilia. (**A**) Section of olfactory mucosa stained with affinity-purified antibody to βARK-2. CL, olfactory cilia; DK, dendritic knobs; arrows, olfactory dendrites; ORN, olfactory receptor neurons; and BL, basal lamina. Preadsorption with purified βARK-2 fusion protein completely eliminated staining. (**B**) Section of olfactory tissue containing respiratory epithelium. (**C**) βARK-2 immunoreactivity after unilateral bulbectomy. Open arrows indicate residual dendritic knobs. (**D**) βARK-2 immunoreactivity in primary cultures of olfactory receptor neurons. The open arrow indicates the dendritic knob, and the closed arrow indicates the cell body. (**E**) βARK-1 immunoreactivity in olfactory epithelium. (**F**) βARK-1 immunoreactivity in respiratory epithelium. Immunohistochemistry was performed as described (*25*). Bar = 50 μm.

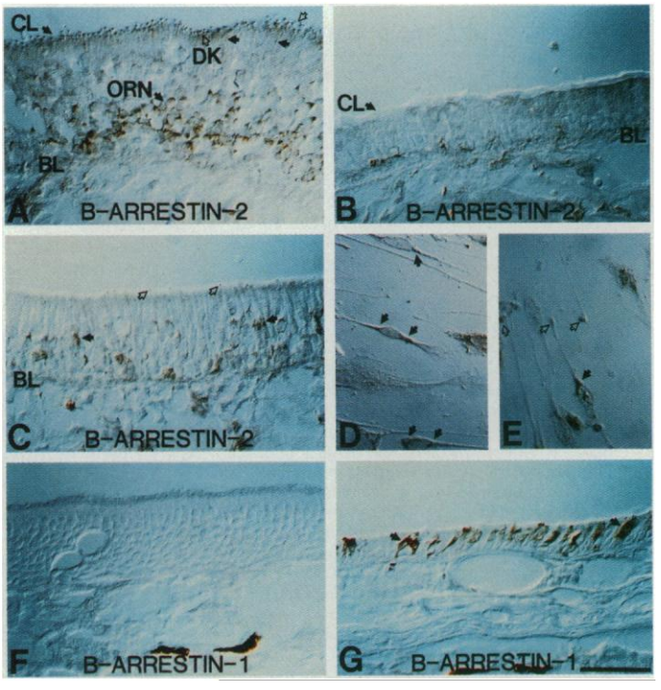

Fig. 2. β-arrestin immunostaining of olfactory epithelium. (**A**) Olfactory mucosa stained with affinity-purified antibody to β-arrestin–2. DK (open arrows), olfactory dendritic knobs; closed arrows, dendrites; ORN, olfactory receptor neurons; CL, cilia; and BL, basal lamina. Preadsorption with purified β-Arrestin–2 fusion protein completely eliminated staining. (**B**) β-Arrestin–2 immunostaining of respiratory epithelium. (**C**) Bulbectomy (*19*) eliminates β-Arrestin–2 immunoreactivity from olfactory dendritic knobs. Olfactory receptor neurons (closed arrows) and dendritic knobs (open arrows) are indicated. (**D** and **E**) β-Arrestin–2 immunoreactivity in primary cultures of olfactory receptor neurons (*20*); the open arrows indicate putative dendritic knobs, and the closed arrows indicate cell bodies. (**F**) β-Arrestin–1 immunoreactivity in olfactory epithelium. (**G**) β-Arrestin–1 immunoreactivity in respiratory epithelium. Immunohistochemistry was performed as described (*25*). Bar = 50 μm.

Because further quenching of the activity of G protein–coupled receptors requires the presence of an arrestin-like protein (*10, 12*), we also examined the distribution of β-arrestin–1 and β-arrestin–2. β-Arrestin–2 was localized within the apical dendritic knobs of olfactory receptor neurons as well as within the cell bodies that express a greater amount of protein (Fig. 2A). This may be a result of a favorable disposition of the epitopes while in the endoplasmic reticulum. The distribution of β-arrestin–2 differed slightly from that of βARK-2. Whereas β-arrestin–2 was most concentrated in dendritic knobs, βARK-2 was most prominent in both the dendritic knobs and the ciliary layer, consistent with previous localization studies (*16, 17*) in which β-arrestin and βARK were localized to the same set of neurons although in different subcellular compartments. It may be that the cell maintains a repository of β-arrestin, which is seen as dendritic staining. We confirmed the presence of β-arrestin–2 in olfactory dendritic knobs with confocal microscopy. Immunoreactivity for β-arrestin–1 was completely absent from olfactory neuroepithelium (Fig. 2F). After bulbectomy, β-arrestin–2 immunoreactivity was markedly attenuated within the layers that contained the apical dendritic knobs and olfactory receptor neurons, which thus indicates a specific localization to olfactory receptor neuronal processes. Whereas β-arrestin–1 was absent from cultures of olfactory receptor neurons (*20*), β-arrestin–2 was present in the cell processes in a pattern similar to that seen for βARK-2 (Fig. 2D). Within respiratory epithelium, β-arrestin–2 was completely absent; β-arrestin–1, however, was present.

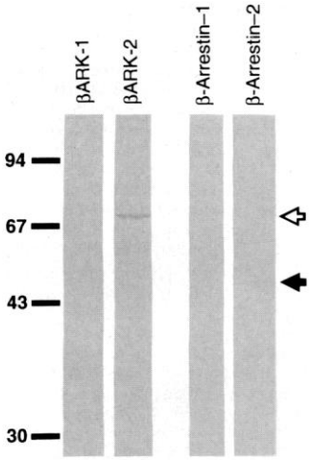

Fig. 3. Protein immunoblot analysis (*26*) of antibodies to isoforms of βARK and β-arrestin. The positions of molecular size standards in kilodaltons are indicated. The open arrow indicates βARK-2, and the closed arrow indicates β-arrestin–2.

To demonstrate that the antibodies used for immunohistochemistry and immunocytochemistry are specific for βARK-1, βARK-2, β-arrestin–1, and β-arrestin–2, we conducted immunoblot analysis. In olfactory cilia, βARK-2 antiserum interacted with a single band of 77 kD (*17*) and β-arrestin–2 interacted with a single band of 45 kD (*16*) (Fig. 3), the same sizes as expressed by βARK-2 and β-arrestin–2, respectively, in transfected COS cells (*16, 17*); these results thus confirm the presence of both βARK-2 and β-arrestin–2 in olfactory cilia. Therefore, although the most prominent staining for β-arrestin–2 was seen in the dendritic knobs, some must have also been present in cilia. Immunoblot analysis failed to detect βARK-1 or β-arrestin–1 in cilia. Primary olfactory receptor neurons in culture gave a similar but less prominent staining pattern (*21*).

Recently, a large G protein–coupled receptor gene family has been identified from an olfactory cDNA library (*7*), and some of these gene products were also identified in germ cells (*22*). To further investigate the potential role of βARK and β-arrestin in signal desensitization, we examined the distribution of these proteins in the rat testis. Both βARK-2 and β-arrestin–2 were enriched within mature spermatids (Fig. 4, A and C), whereas βARK-1 (Fig. 4B) and β-arrestin–1 (Fig. 4D) were absent. Immunoblot analysis of rat testes demonstrated that βARK-2 antiserum labeled a single band of 77 kD and β-arrestin–2 antiserum labeled a single band of 45 kD, whereas both βARK-1 and β-arrestin–1 were not detected.

Odorants activate adenylyl cyclase (*1, 2*), and the increase in cAMP initiates the electrophysiologic response by activating a nonspecific cation channel in the sensory cilia (*5*). In the presence of odorants, adenylyl cyclase is rapidly and only transiently activated, which suggests that active desensitization uncouples the receptor–G protein–adenylyl cyclase pathway. To assess βARK-2 and β-arrestin–2 function, we used a rapid quench system (*23*) to monitor cAMP levels in response to odorants in the presence or absence of neutralizing antibodies.

As previously reported (*23*), the odorant citralva causes a rapid elevation of cAMP levels within rat olfactory cilia isolated by calcium shock (*2*) and cAMP levels decline rapidly thereafter, which suggests active desensitization (Fig. 5). In the absence of stimulation by citralva, antibodies to βARK and β-arrestin did not alter cAMP amounts. Thirty-minute pretreatment of cilia with antibodies to β-arrestin–2 (25 μg/ml) resulted in an elevation of peak cAMP amounts in the presence of 100 μM citralva. Preincubation with antibodies to βARK-2 (25 μg/ml) resulted in an even higher peak cAMP response in the presence of citralva. Preincubation with a combination of antibodies to βARK-2 and to β-arrestin–2 resulted in additive effects. Presumably, the slower decline of cAMP levels in the presence of antibodies to βARK-2 or to β-arrestin–2 reflects blockade of desensitization. Preincubation of antibodies to βARK-1 (25 μg/ml) resulted in some elevation of the peak cAMP levels, which did not decline at subsequent times. Similar results were obtained by preincubating the cilia with antibodies to β-arrestin–1 (25 μg/ml). At the concentration of antibody used in these experiments, both antibodies to βARK-1 and to β-arrestin–1 probably cross-react with the endogenous isoforms of βARK and β-arrestin within olfactory cilia (*16, 17*), thereby accounting for the modest effect on the amounts of cAMP. In contrast, preincubation of cilia with rabbit immunoglobulin G at comparable concentrations did not affect cAMP amounts in

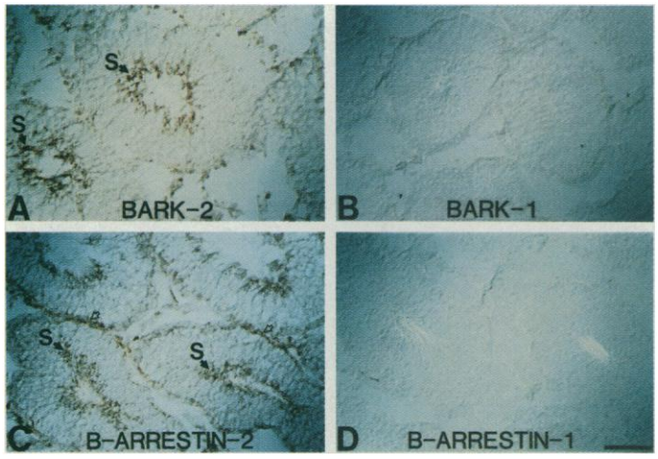

Fig. 4. βARK-2 and β-arrestin–2 immunoreactivity in spermatozoa (S) of testes. (**A**) βARK-2 immunoreactivity in a cross section of rat testes. (**B**) βARK-1 immunoreactivity in rat testes. (**C**) β-Arrestin–2 immunoreactivity in a cross section of rat testes. Open arrows indicate labeling of basement membrane, which contains specialized smooth muscle. (**D**) β-Arrestin–1 immunoreactivity in rat testes. Immunohistochemistry was performed as described (*24*). Bar = 50 μm.

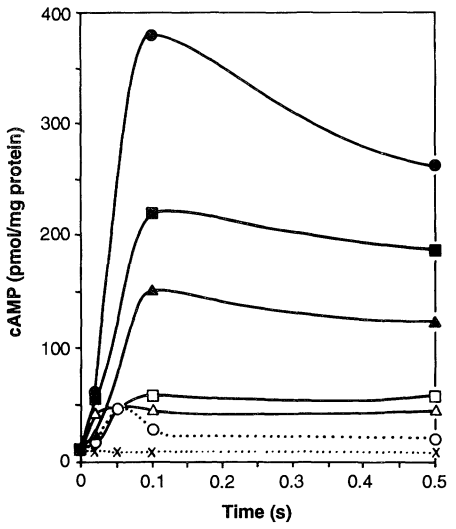

Fig. 5. Effect of neutralizing antibodies on odorant-induced elevation of cAMP amounts in isolated rat olfactory cilia. Cilia previously equilibrated in buffer were mixed with reaction mix (*23*) with or without citralva at a concentration of 100 μM. Cilia and odorants in the reaction mix were allowed to incubate from 0 to 500 ms, and the samples were then quenched with trichloroacetic acid. X, nonimmune rabbit immunoglobulin G in absence of odorants; ○, 100 μM citralva; □, 100 μM citralva plus antibodies to βARK-1; △, 100 μM citralva plus antibodies to β-arrestin–1; ■, 100 μM citralva plus antibodies to βARK-2; ▲, 100 μM citralva plus antibodies to β-arrestin–2; ●, 100 μM citralva plus antibodies to both βARK-2 and β-arrestin–2. Samples were collected, and cAMP was assayed as described (*27*).

the presence of citralva. Several members of the G protein–coupled receptor family have been shown by in vitro studies (*10*) to be affected by receptor kinases of the βARK family as well as by β-arrestin (*12*). Our data show that neutralization of these two proteins results in the attenuation of desensitization. Whether the isoforms of βARK and β-arrestin localized to the olfactory neuroepithelium are βARK-2 and β-arrestin–2 is unclear. Although they react with antibodies that specifically recognize these two isoforms, they may be new members of this group of proteins.

The ability of neutralizing antibodies to βARK-2 and β-arrestin–2 to inhibit the decline in the amount of cAMP in isolated olfactory cilia strongly supports a role for βARK and β-arrestin in olfactory desensitization. Olfactory desensitization may proceed by a combination of several mechanisms, as has been demonstrated in the β-adrenergic receptor system (9). Other studies with the rapid quench system have shown that protein kinase A and protein kinase C may be involved in desensitization to odorants (*24*). Thus, the regulation of odorant signaling may involve many of the same components involved in the regulation of neurotransmitter and hormonal signaling systems that operate through guanine nucleotide regulatory proteins.

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19. Bulbectomies cause retrograde degeneration of olfactory receptor neurons in the olfactory mucosa [F. L Margolis, N Roberts, D Ferriero, J. Feldman, *Brain Res* **81**, 469 (1974)] We performed them unilaterally, which allowed comparison of normal and denervated sides Adult male Sprague-Dawley rats were anesthetized intraperitoneally with chloral hydrate (0.5 g per kilogram of body weight) and placed in a stereotaxic head holder. The olfactory bulb was identified on the right side of all animals and subsequently aspirated with a glass pipette After bulbectomy, the surgical site was packed with gel foam, and the skin was sutured with surgical staples The animals were allowed to recover and were subsequently prepared for immunohistochemistry as described (*25*).

20 Primary cultures of olfactory receptor neurons were prepared on two-chamber Labtek (Nunc, Niles, IL) tissue culture slides as described [G V. Ronnett, L D. Hester, S. H. Snyder, *J. Neurosci.* **11**, 1243 (1991)]. After 5 to 7 days in culture, the slides were rinsed three times with phosphate-buffered saline (PBS, pH 7 4) and placed in methanol at −20°C for 15 to 20 min Immunohistochemistry was performed as described (*25*).

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25. Adult male Sprague-Dawley rats were used for the immunohistochemical studies After administration of pentobarbital (100 mg/kg) anesthesia, the rats were perfused transcardially with PBS followed by 4% freshly depolymerized paraformaldehyde in 0 1 M phosphate buffer. Their testes and olfactory turbinates and bulbs were removed en bloc and fixed again for 1 hour with 4% paraformaldehyde in PBS followed by cryoprotection in 20% (volume) sucrose in PBS Sections (10 μm) through the olfactory cilia and sections containing testes were cut on a cryostat (Microm, Hanover, MD) and thaw-mounted, dried, and stored at −70°C The slide-mounted sections were then brought to room temperature and subsequently permeabilized with 0 2% Triton X-100 in tris-buffered saline [TBS, 50 mM tris-HCl (pH 7.4) and 1.5% NaCl] We next blocked the sections for 1 hour in 4% normal goat serum (NGS) and 0.1% Triton X-100 in TBS. The sections were then incubated overnight at 4°C in TBS that contained 2% NGS, 0 1% Triton X-100, and affinity-purified fusion protein antibodies to βARK-1 (1 5 dilution), to βARK-2 (1 50 to 1 500) (*17*), to β-arrestin–1 (1·50 to 1 100), and to β-arrestin–2 (1·25 to 1 100) (*16*) Dilutions depended on the batch of antibodies used The sections were then stained with an avidin-biotin-peroxidase system (Vector Laboratories) with diaminobenzidine as a chromogen We performed blocking experiments by preadsorbing the antibodies to βARK-1, to βARK-2, to β-arrestin–1, and to β-arrestin–2 with 100 μg of their respective purified fusion protein antigen. Confocal microscopy was performed as described (*18*), and sections were labeled with antibodies as described above, except that goat-antirabbit conjugated with rhodamine (1 100) (Jackson ImmunoResearch Laboratories) was used as the secondary antibody for visualization

26 Cytosolic extracts were prepared from rat olfactory cilia, olfactory turbinates, primary olfactory cultures, and testes The olfactory tissues were homogenized with a Brinkmann Polytron in 2 ml of 50 mM tris (pH 7 5), 5 mM EDTA, 0 1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin (5 μg/ml), and benzamidine (10 μg/ml) and centrifuged at 300,000*g* for 20 min at 4°C, and then the pellets were discarded The testes were homogenized with a Brinkmann Polytron in 50 mM tris (pH 7.4) that contained 1 mM EGTA, 1 mM 2-mercaptoethanol, 0 4 mM PMSF, antipain (4 8 μg/ml), and leupeptin A (9.6 μg/ml) (10 volumes of buffer per gram of tissue wet weight) Testicular homogenates were centrifuged for 30 min at 45,000*g*, and the pellets were discarded The supernatants were assayed for protein with the Coomassie blue assay from Pierce or by the Bradford method [M M Bradford, *Anal Biochem* **72**, 248 (1976)] Protein samples (100 to 150 μg per lane) were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels for olfactory tissues and gradient (4 to 14%) gels for testes, and the separated proteins were transferred to nitrocellulose All samples for the antibodies to β-arrestin were treated with 0 5 M iodoacetamide for 30 min in the dark before SDS-PAGE. The blots were then blocked with 3% bovine serum albumin (BSA) in TBS and then incubated with affinity-purified antibodies at dilutions of 1·30 to 1·2000 Dilutions varied depending on the batch of the primary antibody. Blots were developed with a goat-antirabbit alkaline phosphatase kit (Bio-Rad) according to the manufacturer's protocol except for the use of 3% BSA instead of gelatin in all antibody buffers

27 Cilia were prepared from male rats according to the procedure of Sklar *et al.* (*2*) A rapid quench device, designed by Update Instrument, was used with three syringes to measure cAMP amounts at the millisecond range The first syringe contained cilia suspended in buffer that consisted of 10 mM tris-HCl, 3 mM $MgCl_2$, and 2 mM EDTA (pH 8 0). The second syringe contained the reaction mix consisting of 200 mM

NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM $MgCl_2$, 1 0 mM dithiothreitol, 1 0 mM sodium adenosine triphosphate, 1 0 μM guanosine triphosphate, 0 05% sodium cholate, and citralva at appropriate concentrations (pH 7 4) The free Ca^{2+} in this solution was 45 nM as measured by a Ca^{2+} electrode (Orion, Boston, MA) The third syringe contained 13 5% trichloroacetic acid A time course with intervals ranging from 20 ms to 500 ms was used, and the quenched samples were collected on ice and spun 10 min in a microfuge at 4°C The supernatant was collected, and the cAMP was assayed with the Amersham ^{125}I-labeled cAMP assay system The protein concentration in the first syringe averaged 150 μg per milliliter of cilia buffer, as determined by the method of Lowry [O H Lowry, N J Rosebrough, A L Farr, R J Randall, *J Biol. Chem* 2, 265 (1951)]

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Role of the Acylated Amino Terminus of Recoverin in Ca^{2+}-Dependent Membrane Interaction

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Recoverin, a calcium ion (Ca^{2+})–binding protein of vertebrate photoreceptors, binds to photoreceptor membranes when the Ca^{2+} concentration is greater than 1 micromolar. This interaction requires a fatty acyl residue covalently linked to the recoverin amino (NH_2)-terminus. Removal of the acyl residue, either by proteolytic cleavage of the NH_2-terminus or by production of nonacylated recoverin, prevented recoverin from binding to membranes. The acylated recoverin NH_2-terminus could be cleaved by trypsin only when Ca^{2+} was bound to recoverin. These results suggest that the hydrophobic NH_2-terminus is constrained in Ca^{2+}-free recoverin and liberated by Ca^{2+} binding. The hydrophobic acyl moiety of recoverin may interact with the membrane only when recoverin binds Ca^{2+}.

A variety of proteins with diverse biological functions have their NH_2-termini linked to short-chain fatty acids such as myristic acid (*1–11*). Membrane association of $p60^{v\text{-}src}$, which is necessary for $p60^{v\text{-}src}$ oncogenic activity (*2*) and for its interaction with a plasma membrane receptor (*3*), requires that its NH_2-terminus be myristoylated. Intracellular membrane transport may be mediated through a guanosine triphosphate (GTP)– and myristic acid–dependent interaction of adenosine diphosphate (ADP) ribosylation factor (ARF) with membranes (*4*). The NH_2-termini of two vertebrate photoreceptor proteins, transducin and recoverin, are heterogeneously acylated at their NH_2-termini with one of four types of fatty acyl residues, including myristoyl (C14:0, a fatty acyl residue with 14 carbons and no double bonds), C14:1, C14:2, and C12:0 moieties (*9–11*).

Recoverin is a 23-kD Ca^{2+}-binding protein that is soluble and may influence the recovery phase that follows photoexcitation of vertebrate photoreceptors (*12, 13*). During photoexcitation, guanosine 3′,5′-monophosphate (cGMP) is hydrolyzed by means of a guanine nucleotide–binding protein (G protein)–mediated cascade of reactions (*14, 15*). Because Ca^{2+} enters photoreceptors primarily through cGMP-gated channels, cGMP hydrolysis disrupts Ca^{2+} influx. The consequent net loss of intracellular Ca^{2+} by means of a plasma membrane Na^+-Ca^{2+} exchanger stimulates resynthesis of cGMP by activating a Ca^{2+}-sensitive guanylate cyclase. Preparations of recoverin have been reported to activate this guanylate cyclase in vitro only when the free concentration of Ca^{2+} is below ~400 nM (*12, 13*). The mechanism by which recoverin participated in guanylate cyclase regulation in those experiments remains unknown. Another possible role of recoverin may be to regulate the rate of inactivation of photoreceptor cGMP phosphodiesterase (*16*).

We investigated the possible role of NH_2-terminal acylation in recoverin function. NH_2-terminal fatty acyl residues may enhance protein-protein (*11*) or protein-membrane interactions (*17*). Alternatively, they may function as covalently bound allosteric regulators of enzyme activity. The studies described here show that the NH_2-terminal fatty acyl residue of recoverin participates in Ca^{2+}-dependent membrane interaction.

Ca^{2+}-dependent interaction of recoverin with rod outer segment (ROS) membranes was initially observed in an experiment designed to identify Ca^{2+}-dependent membrane-binding proteins from bovine photoreceptors. We washed photoreceptor membranes several times with buffer containing Ca^{2+} (0.5 mM) to remove proteins that were loosely bound in the presence of Ca^{2+}. Subsequent washes with buffer containing EGTA (1 mM) continued to elute loosely bound proteins. However, they also specifically eluted six additional proteins that had resisted elution in the presence of Ca^{2+} (Fig. 1). One was a 26-kD protein that reacts with affinity-purified antibody to bovine recoverin (*12*). The remaining five proteins of 20, 21, 31, 70, and 80 kD have not yet been identified.

Ca^{2+}-dependent binding of recoverin to membranes was enhanced by high concentrations of protein and membrane. Most of the recoverin in ROS membranes could be eluted even without EGTA if highly diluted membranes were washed many times (*18*). We used this method to strip ROS membranes of endogenous recoverin for the studies described here. Others have also observed Ca^{2+}-dependent membrane binding of recoverin (*19*) and of S-modulin (*16, 20*), a frog photoreceptor protein similar to bovine recoverin.

We characterized the interaction of recoverin with the membrane by reconstituting purified bovine retinal recoverin with photoreceptor membranes stripped of endogenous recoverin. The stripped membranes bound recoverin in the presence of Ca^{2+} but not in its absence (Fig. 2A). Recoverin binding to the membranes was saturated in these experiments at ~40% of the total recoverin as the concentration of Ca^{2+} was raised to 10 μM (Fig. 2B). The partial binding of retinal recoverin to membranes appears to reflect a weak affinity for membranes, rather than an effect of acyl group heterogeneity. Myristoylated recoverin binds to membranes with approximately the same efficiency as heterogeneously acylated retinal recoverin (Fig. 3A).

Ca^{2+}-dependent binding of recoverin to membranes does not appear to be a general divalent cation effect because the Mg^{2+} (5 mM) present in all of these assays did not promote binding. Experiments suggest that recoverin may bind to the phospholipid component of the membranes because Ca^{2+} also promotes recoverin binding to liposomes made from a 1:1 mixture of phosphatidylserine and phosphatidylcholine (*18, 19, 21*). However, our experiments do not rule out the possibility that recoverin also interacts with a protein in ROS membranes.

To determine if the NH_2-terminal acyl residue participates in the interaction of recoverin with ROS membranes, we measured membrane binding of acylated and nonacylated recoverin produced in *Esche-*

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