Transduction Proteins of Olfactory Receptor Cells: Identification of Guanine Nucleotide Binding Proteins and Protein Kinase C†

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ABSTRACT: We have analyzed guanine nucleotide binding proteins (G-proteins) in the olfactory epithelium of *Rana catesbeiana* using subunit-specific antisera. The olfactory epithelium contained the α subunits of three G-proteins, migrating on polyacrylamide gels in SDS with apparent molecular weights of 45 000, 42 000, and 40 000, corresponding to G_s, G_i, and G_o, respectively. A single β subunit with an apparent molecular weight of 36 000 was detected. An antiserum against the α subunit of retinal transducin failed to detect immunoreactive proteins in olfactory cilia detached from the epithelium. The olfactory cilia appeared to be enriched in immunoreactive $G_{s\alpha}$ relative to $G_{i\alpha}$ and $G_{o\alpha}$ when compared to membranes prepared from the olfactory epithelium after detachment of the cilia. α subunits of G-proteins were not detected in cilia detached from the nonchemosensory respiratory epithelium of the palate. Immunohistochemical studies using an antiserum against the β subunit of G-proteins revealed intense staining of the ciliary surface of the olfactory epithelium and of the axon bundles in the lamina propria. In contrast, an antiserum against a common sequence of the α subunits preferentially stained the cell membranes of the olfactory receptor cells and the acinar cells of Bowman's glands and the deep submucosal glands. Prolonged incubation periods with these antisera tended to obliterate these differences in staining patterns, giving rise to staining by both antisera of the ciliary surface, the olfactory receptor cell membranes, the axon bundles, and the acinar cells of the glands. In addition to G-proteins, we have identified protein kinase C in olfactory cilia via a protein kinase C specific antiserum and via phorbol ester binding. However, in contrast to the G-proteins, protein kinase C occurred also in cilia isolated from respiratory epithelium.

Olfactory reception is mediated via chemosensory neurons located in the olfactory epithelium. Differential activation of these neurons by different odorants generates distinct patterns of afferent neuronal activity, which are relayed to the central nervous system (Getchell et al., 1984; Lancet, 1984, 1986). The primary recognition and transduction events that lead to stimulation of the appropriate neurons occur at cilia that project from the dendritic tips of the olfactory receptor cells into the mucus that lines the nasal mucosa (Rhein & Cagan, 1980; Mair et al., 1982; Adamek et al., 1984; Pace et al., 1985; Sklar et al., 1986). In the frog these cilia can be readily detached from the olfactory epithelium by a calcium shock, providing a partially purified preparation of chemosensory membranes amenable to biochemical studies of olfactory reception in vitro (Chen & Lancet, 1984; Anholt et al., 1986; Sklar et al., 1986).

Recently, an odorant-sensitive adenylate cyclase has been identified in olfactory cilia (Pace et al., 1985). This enzyme is stimulated in a dose-dependent manner primarily by odorants that belong to fruity, floral, herbaceous, or minty odor classes that tend to be hydrophobic but not by more polar putrid odorants or odorous chemical solvents (Sklar et al., 1986). Stimulation of the olfactory adenylate cyclase by odorants is GTP-dependent (Pace et al., 1985; Sklar et al., 1986). A 42-kDa[1] substrate for ADP-ribosylation by *Vibrio cholerae* toxin has been identified in cilia from the olfactory epithelium of *Rana ridibunda* and may represent the α subunit of the regulatory G-protein "G_s", which usually mediates stimulation of adenylate cyclase (Pace et al., 1985). In addition, substrates for ADP-ribosylation by *Bordetella pertussis* toxin occur both in olfactory cilia and in membranes prepared from the olfactory epithelium after removal of the cilia (Pace et al., 1985). A doublet of polypeptide bands with apparent molecular weights of 39 000–41 000 was resolved, and the heavier band was tentatively designated as the α subunit of "G_i", a G-protein generally associated with inhibition of

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[1] Abbreviations: G-proteins, guanine nucleotide binding regulatory proteins; G_s and G_i, G-proteins that mediate stimulation and inhibition, respectively, of adenylate cyclase; G_o, related G-protein of unknown function; T, transducin (the major G-protein of the retinal rod outer segment); Ringer's solution, 2 mM HEPES, 112 mM NaCl, 3.4 mM KCl, 2.4 mM $NaHCO_3$, pH 7.4; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; PDBu, phorbol 12,13-dibutyrate; PDA, phorbol 12,13-diacetate; PMA, phorbol 12-myristate 13-acetate; kDa, kilodalton(s).

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adenylate cyclase [Pace et al., 1985; for reviews, see Gilman (1984), Schramm and Selinger (1984), and Stryer and Bourne (1986)].

We have examined transduction proteins in the olfactory epithelium of the bullfrog *Rana catesbeiana*, using subunit-specific antisera. Here, we describe three distinct G-proteins, G_s, G_i, and G_o. Olfactory cilia isolated from the epithelium appear to be enriched in G_s relative to G_i and G_o. No proteins immunoreactive with an antiserum against the α subunit of retinal transducin are detectable. We have also identified protein kinase C in olfactory cilia. However, in contrast to the G-proteins, protein kinase C also occurs in cilia isolated from nonchemosensory respiratory epithelium.

Materials and Methods

Preparation of Olfactory Cilia. R. catesbeiana (20–25 cm) were supplied by Amphibians of North America (Nashville, TN) or Acadian Biological (Rayne, LA). Frogs were killed by decapitation, and the ventral and dorsal sheets of olfactory epithelia were removed from both nasal chambers. The palate was dissected for the preparation of respiratory cilia. Olfactory and respiratory cilia were prepared as previously described (Anholt et al., 1986). Briefly, the epithelia are bathed in Ringer's solution supplemented with 2 mM EDTA to remove the mucus, and the cilia are detached via a calcium shock (10 mM $CaCl_2$ in Ringer's solution). The deciliated epithelia are sedimented in a serological centrifuge, and the supernatant containing the detached cilia is layered on top of a 45% (w/w) sucrose cushion. After centrifugation for 30 min at 350000*g* at 4 °C, the band of partially purified cilia is collected. The cilia are diluted with Ringer's solution, pelleted by centrifugation at 350000*g* for 15 min at 4 °C, and resuspended in a small volume of Ringer's solution.

Deciliated epithelial membranes were prepared from the olfactory epithelia after removal of the cilia by homogenization for 3 × 15 s in a Polytron in ice-cold Ringer's solution, followed by filtration of the homogenate through two layers of surgical gauze and centrifugation at 10000*g* for 15 min at 4 °C. The pellet was resuspended in Ringer's solution, layered on top of a 45% (w/w) sucrose cushion, and centrifuged for 30 min at 350000*g* at 4 °C to obtain a white band of deciliated epithelial membranes free of pigmented debris, which was diluted in Ringer's solution and collected by centrifugation. Brain membranes were obtained by a similar procedure, unless indicated otherwise.

Retinal rod outer segments from the bullfrog were obtained after dissection of the retinas by gently agitating the tissue for 10 min on an end-over-end shaker at 4 °C in Ringer's solution. The detached retinal rod outer segments were then collected and processed in the same way as olfactory or respiratory cilia.

Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Antisera. The rabbit antisera raised against synthetic peptides of bovine G-proteins used in this study have been described previously (Mumby et al., 1986). The antisera that are reactive with a sequence common to the α subunits or β subunits of G_s, G_i, G_o, and transducin have previously been described under the designations A-569 and U-49, respectively (Mumby et al., 1986). The antisera that react specifically with the α subunits of G_o and transducin have been described as B-770 (similar to S-214) and U-42, respectively (Mumby et al., 1986). A polyclonal antiserum raised in rabbit against protein kinase C purified from pig brain has also been described previously (Girard et al., 1985). Monoclonal anti-α-tubulin was obtained from Amersham (Arlington Heights, IL).

Immunoblotting. Polyacrylamide gel electrophoresis in SDS was performed after treatment of the samples with 2-mercaptoethanol on 10% slab gels in the discontinuous buffer system of Laemmli (1970). Phosphorylase *b* (92.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa), all from Bio-Rad (Richmond, CA), served as molecular weight standards.

For immunoblotting with antisera against G-proteins, electrophoretic transfer onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) was performed at 10 °C for 16–18 h at 60 V in a TE50 Transphor unit (Hoefer Scientific Instruments, San Francisco, CA) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3. Molecular weight markers were visualized by staining with amido black. Prior to immunoblotting, the membrane was incubated for 1 h at ambient temperature in 50 mM Tris·HCl, 1 mM EDTA, 0.1% gelatin, and 0.1% Triton X-100, pH 7.7. Incubation with the primary antibody at the appropriate dilution was at 4 °C for 16–18 h in 10 mM sodium phosphate buffer, 0.9% NaCl, and 0.05% Triton X-100, pH 7.5. Bound antibody was visualized either by incubation with ^{125}I protein A (60000 cpm/mL; New England Nuclear, Boston, MA) followed by autoradiography (exposure periods ranged beween 4 and 24 h) or by the formation of complexes with biotinylated secondary antibody (goat anti-rabbit), avidin, and biotinylated horseradish peroxidase with the Vectastain kit from Vector Laboratories (Burlingame, CA). The complexes were visualized in 10 mM sodium phosphate buffer and 0.9% NaCl, pH 7.5, containing 0.5 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.015% hydrogen peroxide.

For immunoblotting with polyclonal antibodies against protein kinase C, the gel pattern was electrophoretically transferred onto a Zeta-Probe blotting membrane (Bio-Rad, Richmond, CA) and bound antibody was detected by autoradiography after incubation with ^{125}I protein A, as described previously (Girard et al., 1986).

Immunohistochemistry. Immunohistochemical localization of G-proteins was performed on 6 μm thick coronal sections of formalin-fixed decalcified nasal sacs of *R. catesbeiana* embedded in paraffin and mounted on chromalum–gelatin-coated microscope slides. The sections were deparaffinized in xylene and rehydrated through a series of graded alcohols from absolute ethanol to water. The rehydrated sections were preincubated for 30 min at ambient temperature in 10 mM sodium phosphate buffer and 0.9% NaCl, pH 7.4, containing 0.5% normal goat serum. Incubation with the primary antibody was in the same buffer at 4 °C for 2 h or 16–18 h, as indicated. Bound antibody was visualized with the Vectastain kit by using 3,3′-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide as substrates, followed by fixation of the reaction product in OsO_4 vapors. To evaluate nonspecific background staining, appropriate controls, described under Results, were processed along with the sections of interest. Control sections were washed and stained together with the sections of interest in the identical solutions for the same period of time.

Phorbol Ester Binding Assays. Binding of [^{3}H]phorbol 12,13-dibutyrate (PDBu; specific activity 30 Ci/mmol; New England Nuclear, Boston, MA) was assayed in triplicate in plastic tubes (Sarstedt, Nuembrecht, West Germany) in 50 mM Tris·HCl buffer and 1 mM $CaCl_2$, pH 7.7, in a total volume of 0.5 mL. An excess of unlabeled PDBu (10 μM) was used to determine the extent of nonspecific binding. The total binding of [^{3}H]PDBu was at least twice the nonspecific binding. Unlabeled PDBu, phorbol 12-myristate 13-acetate (PMA), and phorbol 12,13-diacetate (PDA) were obtained

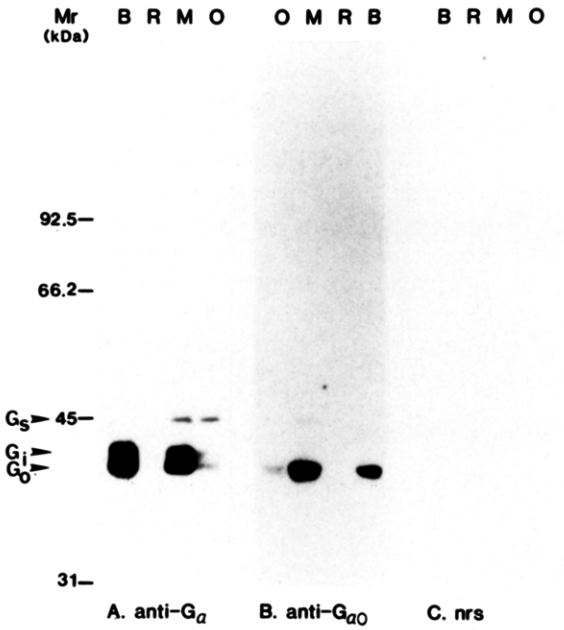

FIGURE 1: Immunochemical identification of the α subunits of G-proteins after polyacrylamide gel electrophoresis in SDS and electrophoretic transfer. The lanes were loaded with 100 μg of protein of brain membranes (B), respiratory cilia (R), deciliated olfactory epithelial membranes (M), or olfactory cilia (O). (A) Immunoblot using a 2500-fold dilution of an antiserum reactive with a common region of the α subunits of all G-proteins (A-569). (B) Immunoblot using a 500-fold dilution of an antiserum specific for the α subunits of G_o (B-770). (C) Immunoblot using a 500-fold dilution of normal rabbit serum (nrs). Bound antibody was visualized by autoradiography following incubation with ^{125}I protein A.

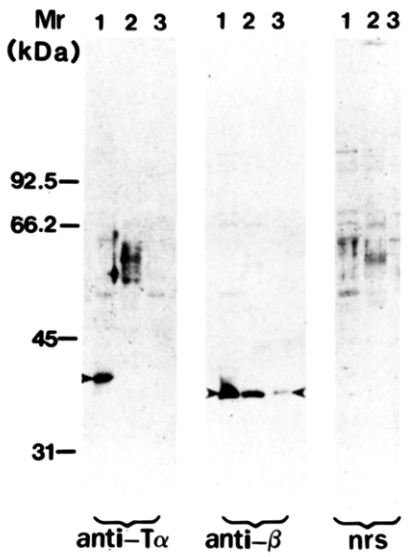

FIGURE 2: Immunochemical identification of transduction proteins in retinal rod outer segments, olfactory cilia, and respiratory cilia with an antiserum against the α subunits of transducin and with an antiserum reactive with the β subunits of all G-proteins. The lanes were loaded with 30 μg of protein of retinal rod outer segments (lane 1), olfactory cilia (lane 2), or respiratory cilia (lane 3), and immunoblotting was performed with a 200-fold dilution of an antiserum monospecific for the α subunit of transducin (U-42), a 10 000-fold dilution of an antiserum reactive with the β subunit of G-proteins (U-49), or a 200-fold dilution of normal rabbit serum (nrs). Bound antibody was visualized with a biotinylated goat anti-rabbit antibody complexed with avidin and biotinylated horseradish peroxidase with the Vectastain kit as described under Materials and Methods. The T_α band in the left-hand panel and the β bands in the middle panel are indicated by arrowheads. Note the nonspecific staining in the 55–66-kDa region, which is apparent on the blot probed with anti-T_α serum and is also evident on the blot probed with the same dilution of normal rabbit serum. The detection limit of the anti-T_α serum for the α subunit of transducin on Western blots is approximately 10 ng. Lane 1 of the left panel is estimated to contain 1–2 μg of transducin.

from LC services Corp. (Boston, MA). After incubation of the assay mixtures for 15 min at ambient temperature, bound [^{3}H]PDBu was separated from unbound ligand via rapid vacuum filtration through poly(ethylenimine)-coated glass fiber filters (No. 32, Schleicher and Schuell, Keene, NH). The filters were washed with 3 × 2 mL of ice-cold 50 mM Tris·HCl buffer and 1 mM $CaCl_2$, pH 7.7, extracted in Formula 963 scintillation cocktail (New England Nuclear, Boston, MA), and counted in a scintillation counter at 48% efficiency.

RESULTS

Identification of G-Proteins. We have analyzed G-proteins in olfactory cilia as well as in brain membranes, respiratory cilia, and deciliated olfactory epithelial membranes of *R. catesbeiana* by immunoblotting with an antiserum reactive with a common sequence of the α subunits of G_s, G_i, G_o, and transducin. The antiserum reveals three immunoreactive polypeptides in isolated olfactory cilia and in deciliated olfactory epithelial membranes with apparent molecular weights of 40 000, 42 000, and 45 000, corresponding to the relative mobilities of the α subunits of G_o, G_i, and G_s, respectively (Figure 1A) (Gilman, 1984; Schramm & Selinger, 1984; Stryer & Bourne, 1986; Mumby et al., 1986). No immunoreactive species are detected in respiratory cilia. In brain membranes this antiserum reveals only the 40- and 42-kDa bands, while the 45-kDa species is not discernible under these experimental conditions, in agreement with previous observations (Mumby et al., 1986) (Figure 1A). On a per milligram protein basis the 45-kDa $G_{s\alpha}$ species is present in olfactory cilia in the same amount as in deciliated olfactory epithelial membranes and appears in olfactory cilia to be enriched relative to $G_{o\alpha}$ and $G_{i\alpha}$. Similar results were obtained with the $G_{s\alpha}$ specific antiserum A-572 (Mumby et al., 1986). Detection with this antiserum was more difficult due to species differences between frog tissues analyzed and bovine antigen used to generate this antiserum (data not shown). In addition to $G_{s\alpha}$, a significant amount of the 40-kDa immunoreactive polypeptide is detected in the olfactory cilia preparation (Figure 1A,B). However, unlike $G_{s\alpha}$ the concentration of this component in olfactory cilia represents only a small fraction of that found in deciliated olfactory epithelial membranes. This 40-kDa polypeptide is reactive with an antiserum specific for $G_{o\alpha}$ (Figure 1B).

To investigate whether, in addition to $G_{o\alpha}$, the 40-kDa band might contain a transducin-like G-protein, we have used an antiserum specific for the α subunit of retinal transducin. Although this antiserum reveals a prominent 40-kDa polypeptide band in retinal rod outer segments from *R. catesbeiana*, no immunoreactive species are detected in either olfactory or respiratory cilia even on immunoblots subjected to a 100-fold higher concentration of antiserum than is necessary for unambiguous detection of a 40-kDa band in retinal rod outer segments (Figure 2). Immunoblotting with an antiserum reactive with the β subunits of G-proteins reveals a single immunoreactive band at 36 kDa, both in the case of retinal rod outer segments and olfactory cilia. Only a faint 36-kDa band is observed with this antiserum in respiratory cilia (Figure 2).

Immunohistochemical Localization of G-Proteins in Olfactory Epithelium. Immunohistochemical studies using the common anti-G_α and anti-G_β antisera reveal unexpected differences in the apparent distribution of the α and β subunits as visualized by deposits of brown reaction product. The

anti-G_α serum stains primarily the membranes of the olfactory receptor cells and the acinar cells of Bowman's glands and of the submucosal glands in the lamina propria (Figure 3A). In contrast, the anti-G_β serum stains exclusively and intensely the ciliary surface of the epithelium and the axon bundles in the lamina propria (Figure 3B). Staining by each of these antisera can be prevented by preincubation of the serum with only the appropriate synthetic peptide used to generate the antiserum (Figure 3C,D). The synthetic peptide used to generate the anti-G_β serum cannot prevent staining by the anti-G_α serum and vice versa (data not shown). In addition, no staining is apparent with preimmune sera obtained from the same rabbits (Figure 3E,F). The differences in staining patterns between the anti-G_α and anti-G_β sera become less apparent when the incubation period with these antisera is extended. After prolonged incubation staining of the ciliary surface of the epithelium can also be observed with the anti-G_α antiserum and some staining of the glands and receptor cells becomes evident with the anti-G_β serum (Figure 4D,F). The cartilage, connective tissue, and a layer directly below the ciliary surface, primarily representing the sustentacular cells, are not stained (Figure 4). In addition, no staining is observed along the ciliary surface of the small patch of respiratory epithelium that separates the dorsal sheet from the ventral sheet of olfactory epithelium (data not shown).

Unfortunately, antibodies specific to $G_{o\alpha}$ did not bind to paraffin-embedded sections of formalin-fixed, decalcified tissue. In addition, no binding of this antiserum could be obtained in frozen sections of isolated olfactory epithelium either unfixed or fixed under different conditions with various fixatives. We attribute the failure of these antibodies to bind to the sections to inaccessibility rather than to denaturation of the antigenic determinants, since they bind with high affinity to the SDS-denatured proteins after gel electrophoresis and electrophoretic transfer (Figure 1B). Further support for this notion is provided by the observation that the anti-G_β antiserum only binds to sections after pretreatment of the sections with 0.1% SDS to unmask determinants of the β subunits.

Identification of Protein Kinase C. Proteolysis of protein kinase C generates fragments that retain enzymatic activity but no longer display the characteristic calcium and phospholipid dependence (Girard et al., 1986). This and the presence of other kinases in our preparation made it difficult to quantitate the extent of protein kinase C mediated phosphorylation. We have identified protein kinase C in olfactory cilia via a protein kinase C specific polyclonal antiserum and via the binding of phorbol esters.

Immunoblotting with a protein kinase C specific antiserum reveals in frog brain two major immunoreactive polypeptide bands migrating at 70 and 58 kDa and two minor bands migrating at 84 and 52 kDa (Figure 5, lane 1). The 70-, 58-, and 52-kDa bands may represent proteolytic fragments of the native 84-kDa species (Girard et al., 1986). In olfactory and respiratory cilia a major immunoreactive species of 72 and 73 kDa, respectively, is detected (Figure 5, lanes 2 and 3). In addition, a faint immunoreactive band migrating at 86 kDa can be discerned in olfactory cilia along with some faint bands at apparent molecular weights of 69 000, 54 000, and 45 000. Unfortunately, the low cross-reactivity of this antiserum with frog tissue precluded its use for immunohistochemistry.

Due to the extreme sensitivity of protein kinase C to proteolysis and the relatively low cross-reactivity of our antiserum with frog tissue, identification of immunoreactive species as proteolytic fragments of protein kinase C had to be considered tentative. Therefore, we utilized binding of tritiated phorbol ester as an additional independent technique to demonstrate the presence of protein kinase C in olfactory cilia. Binding of [^{3}H]PDBu to isolated cilia reveals an appreciable amount of phorbol ester binding sites (Figure 6). We performed drug displacement studies to evaluate the pharmacological specificity of this binding. The potencies of PMA, PDBu, and PDA in competing with [^{3}H]PDBu for the phorbol ester binding site were assessed in the presence of a concentration of [^{3}H]PDBu well below its K_D. The more hydrophobic ligand, PMA, is a more potent displacer than PDBu itself, whereas the more hydrophilic ligand, PDA, is less potent, a profile characteristic of a classical protein kinase C linked phorbol ester binding site (Niedel et al., 1983; Blumberg et al., 1984; Nishizuka, 1984). A similar pharmacological profile was obtained for the binding of [^{3}H]PDBu to respiratory cilia (data not shown). Scatchard analysis shows that in both olfactory and respiratory cilia binding of [^{3}H]PDBu is saturable with a single component (Figure 7). The binding affinities for both preparations are similar (K_D = 24 nM). The B_{max} values for olfactory and respiratory cilia are about 4.8 and 3.5 pmol of PDBu binding sites/mg of ciliary protein, respectively. Hence, if we assume a molecular weight of 84 000 for the native protein (Niedel et al., 1983; Nishizuka, 1984; Blumberg et al., 1984; Girard et al., 1985, 1986), it would represent approximately 0.03–0.05% of the total ciliary protein. For comparison, frog brain membranes contain 10.2 pmol of PDBu binding sites/mg of protein with a somewhat lower binding affinity (K_D = 80 nM; Figure 7).

Discussion

We have used a set of subunit specific antisera to characterize the G-proteins present in olfactory epithelium. On the basis of semiquantitative comparisons of the reactivity of these antisera with G-proteins from frog and their reactivity with G-proteins from bovine brain, we estimate that the total amount of guanine nucleotide binding proteins in olfactory cilia may represent as much as 1–5% of the total ciliary protein. In particular, G_s appears to be enriched in the cilia relative to G_o and G_i, as might be expected from the high activity of odorant-stimulated GTP-regulated adenylate cyclase observed in this preparation (Pace et al., 1985; Sklar et al., 1986).

In addition to G_s, isolated olfactory cilia appear to contain a minor amount of G_i but a substantial amount of G_o, migrating as a 40-kDa band. Pace et al. (1985) have identified a 40-kDa substrate for ADP-ribosylation by *B. pertussis* toxin in olfactory cilia from *R. ridibunda* that was resolved as a doublet. Our observations suggest that the *B. pertussis* toxin substrates observed by these investigators may have represented $G_{o\alpha}$ and $G_{i\alpha}$. Similarly, the 40-kDa band that we previously identified using a different antiserum against a 39-kDa subunit of a G-protein from bovine cortex can now also be designated as $G_{o\alpha}$ (Anholt et al., 1986). The 45-kDa polypeptide, immunoreactive with the anti-G_α antiserum, which we have designated as $G_{s\alpha}$, appears to be a substrate for ADP-ribosylation by cholera toxin (R. Anholt, unpublished observations) and most likely corresponds to the 42-kDa cholera toxin substrate described by Pace et al. (1985).

We were able to perform immunohistochemical studies with the antisera against the common α region and the β subunits of G-proteins. Interestingly, the anti-G_α antiserum stained primarily the receptor cells and the glands whereas the anti-G_β antiserum displayed a preference for the ciliary surface and the axon bundles. The most plausible explanation for this unexpected difference in staining patterns between the anti-G_α and anti-G_β antisera is that it may reflect differences in accessibility of the antisera to their respective binding sites within

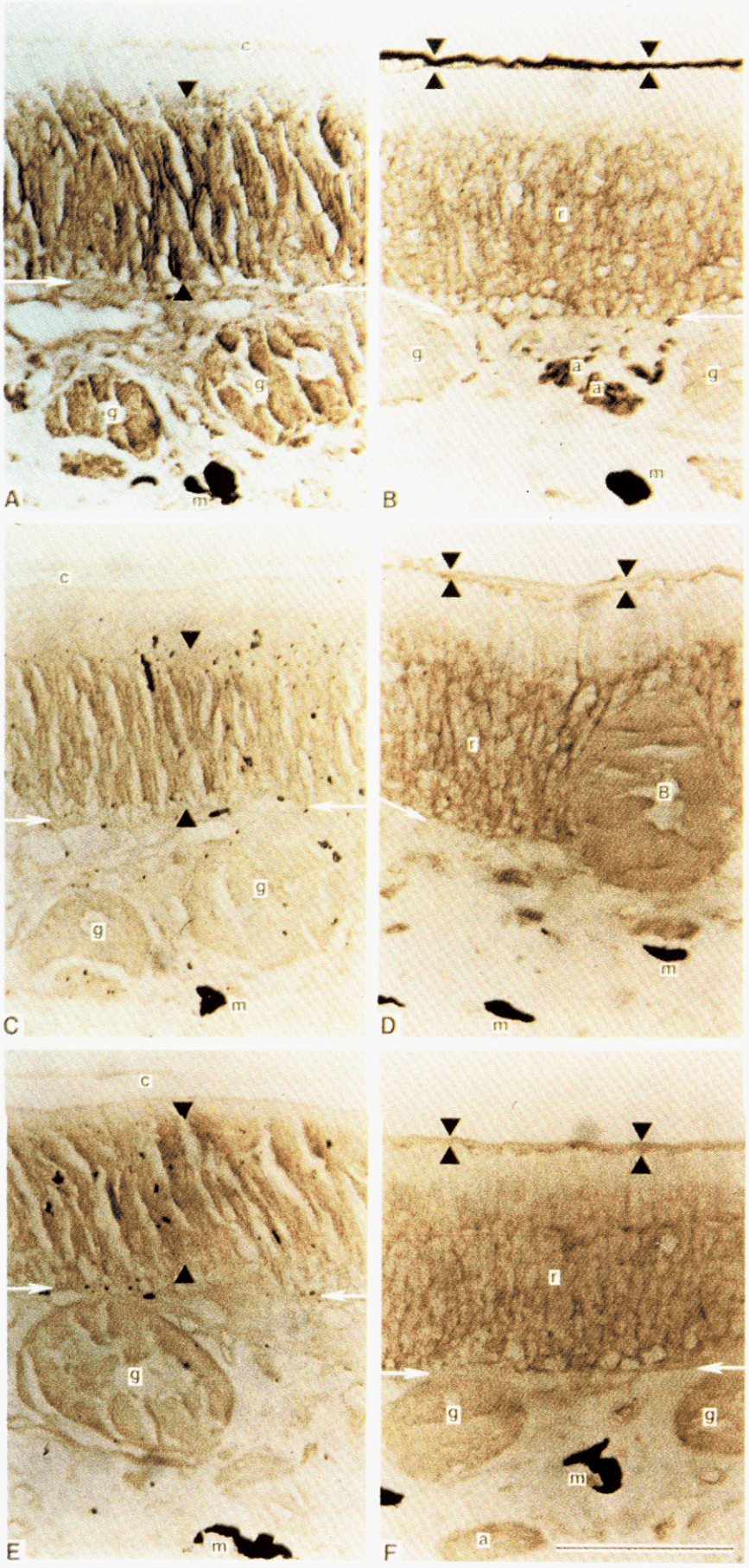

FIGURE 3: Immunohistochemical localization of G-proteins in the olfactory epithelium of *R. catesbeiana*. (A) Immunohistochemical staining with the common anti-G_α antiserum (A-569). Notice the staining of the olfactory receptor cells (between the arrowheads) and of the submucosal glands (g) and the virtual absence of staining over the ciliary surface (c). The position of the basement membrane is indicated by the horizontal arrows. m designates a melanocyte. (B) Immunohistochemical staining with the anti-G_β antiserum (U-49). Notice the intense staining of the ciliary surface of the olfactory epithelium (indicated by the arrowheads). Axon bundles (a) in the lamina propria are also stained. Notice the virtual absence of staining in the layer of olfactory receptor cells (r) and the submucosal glands (g). The position of the basement membrane is indicated by the horizontal arrows. m designates a melanocyte. (C) Immunohistochemical staining with the common anti-G_α antiserum (A-569) after 18 h of preincubation at 4 °C of the antiserum with 5 mg/mL synthetic peptide against which it was generated. The amino acid sequence of this peptide in single letter code is CGAGESGKSTIVKQMK. Labels in the figure are the same as in panel A. (D) Immunohistochemical staining with the anti-G_β antiserum (U-49) after preincubation at 4 °C of the antiserum with 3 mg/mL synthetic peptide against which it was generated. The amino acid sequence of this peptide in single letter code is CEGNVRVSRELAGHTGY. Labels in the figure are the same as in panel B. B designates a Bowman gland. (E) Immunohistochemical staining with preimmune serum obtained from the rabbit in which the anti-G_α antiserum (A-569) was raised. Labels in the figure are the same as in panel A. (F) Immunohistochemical staining with preimmune serum obtained from the rabbit in which the anti-G_β antiserum (U-49) was raised. Labels in the figure are the same as in panel B. The sections were pretreated with 0.1% SDS before immunohistochemistry. All sera were used at 1000-fold dilution. Incubation with the primary antibody was for 2 h at ambient temperature. The bar represents 100 μm.

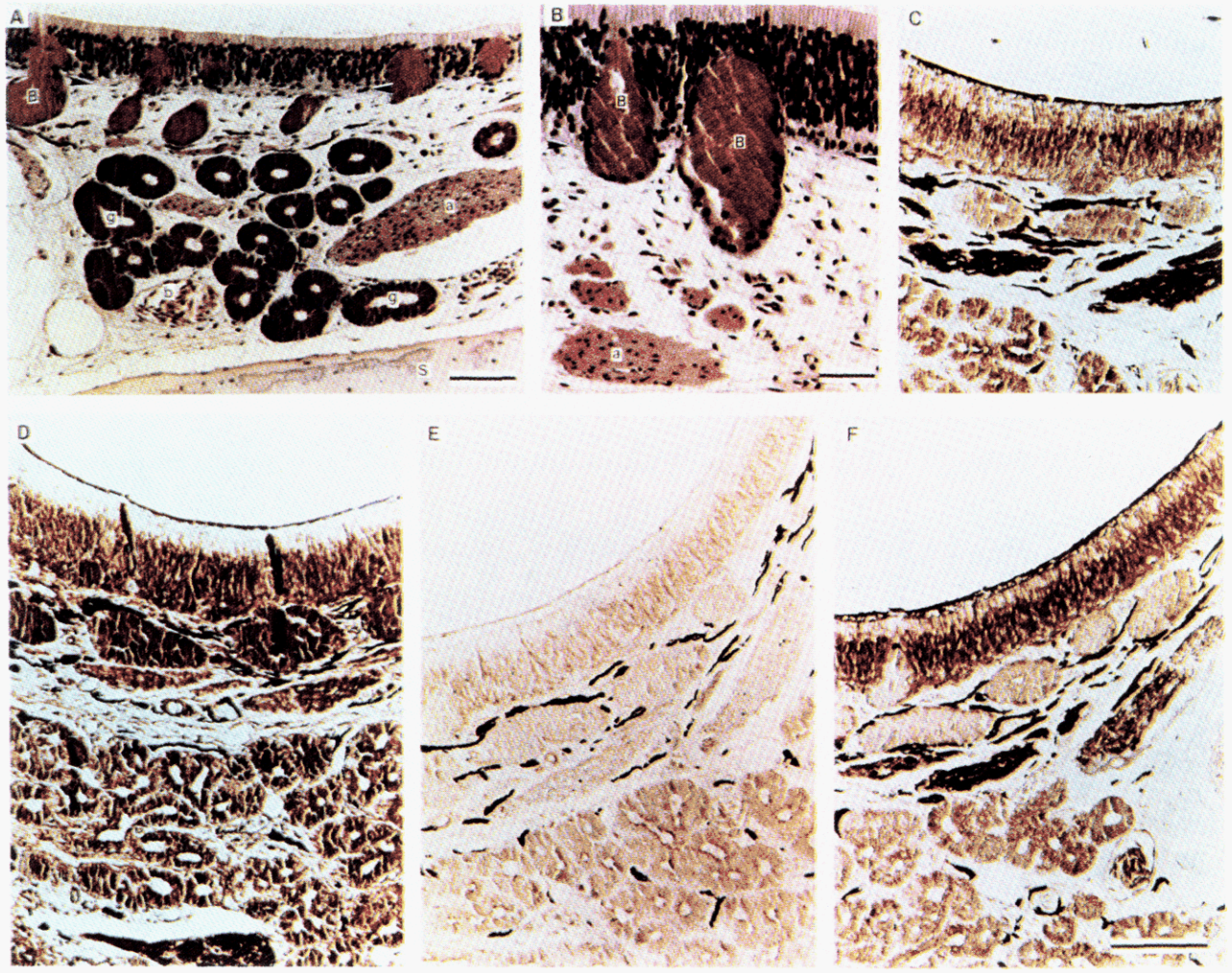

FIGURE 4: Immunohistochemical localization of G-proteins in the olfactory epithelium of *R. catesbeiana* after prolonged incubation (16–18 h) with the primary antibody. (A, B) Hematoxylin and eosin stain of the olfactory epithelium of *R. catesbeiana*. The top row of nuclei represent the sustentacular cells. Cuboidal cells near the base of the neuroepithelium on top of the basement membrane (arrows) may represent neurogenic basal cells. Note the axon bundles (a), submucosal glands (g), and blood vessels (b) in the lamina propria. Bowman's glands are designated B and the septum is indicated as S. The bars in panels A and B represent 250 and 100 μm, respectively. (C) Immunohistochemical staining with monoclonal anti-α-tubulin to visualize the intensely stained layer of cilia at the surface of the olfactory epithelium. Bound antibody is detected via the horseradish peroxidase reaction and is visualized as brown deposits of reaction product. (D) Immunohistochemical localization of G-proteins using a 1000-fold dilution of an antiserum reactive with the α subunits of all G-proteins (A-569). Note the staining of the ciliary surface and the staining of the receptor cell membranes, the axon bundles in the submucosa, and the acinar cells of Bowman's glands and the submucosal glands. (E) Immunohistochemical staining with a 1000-fold dilution of normal rabbit serum. Pigmented melanocytes appearing as black heteromorphous bodies in the lamina propria are particularly prominent in this panel. (F) Immunohistochemical staining with a 1000-fold dilution of an antiserum reactive with the β subunit of G-proteins (U-49) after preincubation of the section in 0.1% SDS. Note the similarity between this pattern of staining and that in panel D. Staining of the ciliary surface is here particularly intense. The bar in panel F represents 250 μm and applies to panels C–F.

different G-proteins. Thus, the anti-G_β antibodies may bind preferentially to the β subunit of G_s on the cilia rather than to the β subunits of G_i or G_o in the glands, while the anti-G_α antibodies may have better access to the α subunits of G-proteins in the glands than to those on the cilia. Alternatively, an excess of free β subunit may exist in the ciliary surface and axon bundles. One additional possibility is that a substantial fraction of the β subunit on the cilia is present in association with an as yet unidentified, novel G-protein, which is recognized poorly by our anti-G_α antiserum.

Unfortunately, our antibodies specific for $G_{o\alpha}$ did not bind to tissue sections, probably due to inaccessibility of the antigenic determinants, as outlined above, precluding a differential localization of the α subunits of the different G-proteins by immunohistochemistry. In addition, two antisera raised against synthetic peptides of bovine G_s and G_o, previously described as A-572 and U-46 (Mumby et al., 1986), on blots were only weakly cross-reactive with the 45- and 40-kDa subunits, respectively, of frog G-proteins and, therefore, were not useful for immunohistochemistry. Thus, in the absence of a monospecific antiserum against $G_{o\alpha}$ that can be used for immunohistochemistry, we cannot localize G_o definitively to olfactory cilia. Therefore, we cannot fully exclude the possibility that some or all of the G_o observed in the isolated olfactory cilia may occur in contaminating membranes from other sources, such as the mucosal glands. It seems unlikely that the $G_{o\alpha}$ observed in the olfactory cilia preparation is glandular in origin, since no immunoreactivity is detected at all in the cilia isolated by a similar procedure from the highly glandular respiratory epithelium of the palate. Since neuronal tissue is notoriously rich in G_o (Sternweis & Robishaw, 1984; Huff et al., 1985), it seems likely that the G_o in the olfactory cilia preparation occurs at least to some extent on the olfactory neurons.

On the basis of structural and electrophysiological parallels between the olfactory system and the retina, we considered

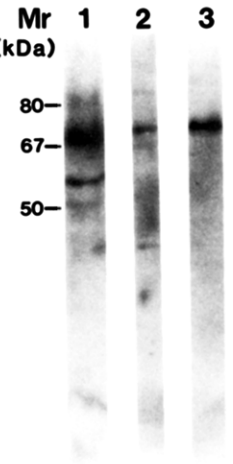

FIGURE 5: Autoradiographic identification of protein kinase C immunoreactive polypeptides in isolated olfactory and respiratory cilia after polyacrylamide gel electrophoresis and electrophoretic transfer. Lane 1: Brain homogenate. Frog brains were homogenized with a glass–Teflon homogenizer in 2% boiling SDS in 10 mM Tris·HCl and 10 mM EGTA, pH 7.7. Lane 2: Olfactory cilia. Lane 3: Respiratory cilia. All lanes contained 150 μg of protein. The positions of the 80-kDa protein kinase C band and its 67- and 50-kDa immunoreactive fragments are indicated by using rat brain homogenate as a standard (Girard et al., 1986). The antiserum was used at a 400-fold dilution.

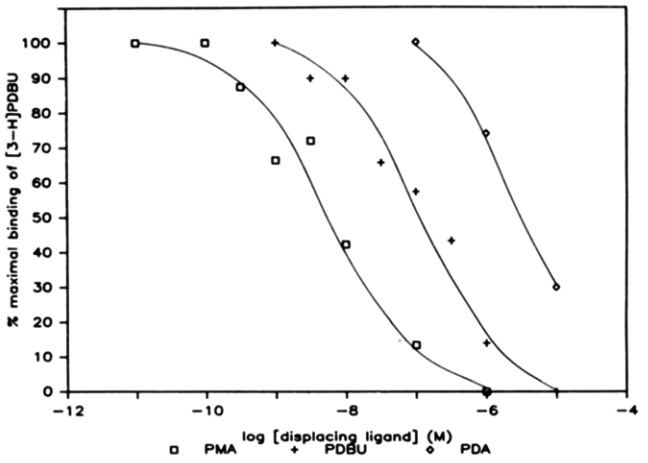

FIGURE 6: Pharmacological characterization of [^{3}H]PDBu binding sites in isolated olfactory cilia. Binding assays were performed with 8 nM [^{3}H]PDBu in the presence of the indicated concentrations of displacing ligand. Data points are the average of two independent experiments, each of which consisted of triplicate measurements. The average amplitude of maximal specific binding of [^{3}H]PDBu was 1800 cpm. The protein concentration of the olfactory cilia was 30 μg/mL. Standard errors are within 15%.

it of interest to investigate whether a transducin-like G-protein might be present on olfactory cilia. We were not able to detect a protein species immunoreactive with a monospecific antiserum against the α subunit of transducin under conditions in which a prominent 40-kDa polypeptide can be readily identified in retinal rod outer segments. Since under our conditions the detection limit for the α subunit of transducin is ~10 ng (see Figure 2 legend), we estimate that any putative transducin-like protein would represent less than 3% of the total G-protein present in olfactory cilia. This suggests that olfaction involves transduction processes that are more similar to hormonal transduction mechanisms [reviewed in Gilman (1984) and Schramm and Selinger (1984)] than to the retinal phototransduction process [reviewed in Stryer (1983) and Stryer and Bourne (1986)].

The identification of an odorant-sensitive GTP-dependent adenylate cyclase in the olfactory epithelium (Pace et al., 1985;

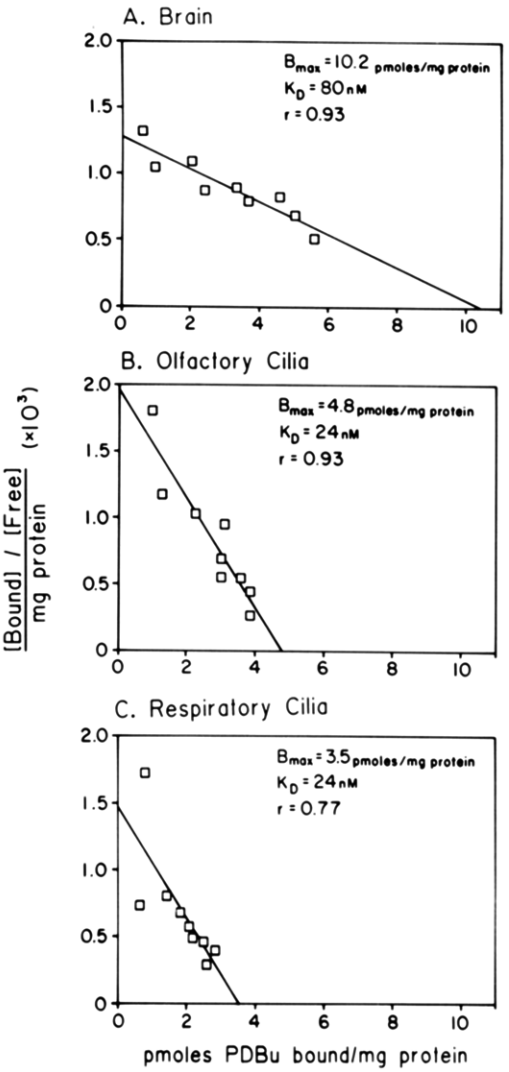

FIGURE 7: Scatchard analysis of the binding of phorbol esters to isolated olfactory and respiratory cilia. Regression lines were drawn through the points after least-squares fit analysis. Protein concentrations of brain, olfactory cilia, and respiratory cilia were 280, 30, and 16 μg/mL, respectively. Brain membranes were prepared by homogenization of frog brains in a glass–Teflon homogenizer, followed by centrifugation of the homogenate for 10 min at 10000g and resuspension of the pellet in Ringer's solution containing 1 mM $CaCl_2$.

Sklar et al., 1986) along with the localization of G_s to the olfactory cilia suggests a role for this GTP-regulated adenylate cyclase in olfaction. However, the observation that not all odorants stimulate this enzyme suggests the involvement of additional transduction mechanisms (Sklar et al., 1986). It is tempting to speculate that such mechanisms could involve G_o. The precise function of G_o is not yet clear, but a possible linkage with phosphatidylinositol turnover (Cockcroft & Gomperts, 1985; Joseph, 1985) or with potassium channels (Pfaffinger et al., 1985) has been suggested.

As a first step toward the identification of additional transduction elements in the olfactory epithelium, we have identified protein kinase C in olfactory cilia via a protein kinase C specific antiserum and via the binding of phorbol esters. However, in contrast to the G-proteins, protein kinase C is also readily detectable in respiratory cilia. Whereas most of the protein in respiratory cilia is axonemal, olfactory cilia are highly enriched in membrane protein (Menco, 1980; Chen & Lancet, 1984; Anholt et al., 1986). Since the B_{max} values for the binding of [^{3}H]PDBu in the olfactory and respiratory cilia preparations are comparable, the density of protein kinase C as a fraction of the total membrane-associated protein is likely

to be higher in respiratory than in olfactory cilia. In addition, microtubule-associated proteins have been identified as natural substrates for protein kinase C (Takai et al., 1984). Thus, we cannot exclude the possibility that protein kinase C in olfactory cilia may have a general ciliary function rather than a specific role in chemosensory transduction.

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REFERENCES

Adamek, G. D., Gesteland, R. C., Mair, R. J., & Oakley, B. (1984) *Brain Res. 310*, 87–97.

Anholt, R. R. H., Aebi, U., & Snyder, S. H. (1986) *J. Neurosci. 6*, 1962–1969.

Blumberg, P. M., Jaken, S., Konig, B., Sharkey, N. A., Leach, K. L., Jeng, A. Y., & Yeh, E. (1984) *Biochem. Pharmacol. 33*, 933–940.

Chen, Z., & Lancet, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1859–1863.

Cockcroft, S., & Gomperts, B. D. (1985) *Nature (London) 314*, 534–536.

Getchell, T. V., Margolis, F. L., & Getchell, M. L. (1984) *Prog. Neurobiol. (Oxford) 23*, 317–345.

Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.

Girard, P. G., Mazzei, G. J., Wood, J. G., & Kuo, J. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3030–3034.

Girard, P. R., Mazzei, G. J., & Kuo, J. F. (1986) *J. Biol. Chem. 261*, 370–375.

Huff, R. M., Axton, J. M., & Neer, E. J. (1985) *J. Biol. Chem. 260*, 10864–10871.

Joseph, S. K. (1985) *Trends Biochem. Sci. (Pers. Ed.) 10*, 297–298.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lancet, D. (1984) *Trends NeuroSci. (Pers. Ed.) 7*, 35–36.

Lancet, D. (1986) *Annu. Rev. Neurosci. 9*, 329–355.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mair, R. G., Gesteland, R. C., & Blank, D. L. (1982) *Neuroscience (Oxford) 7*, 3091–3103.

Menco, B. Ph. M. (1980) *Cell Tissue Res. 211*, 5–29.

Mumby, S. M., Kahn, R. A., Manning, D. R., & Gilman, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 265–269.

Niedel, J. E., Kuhn, L. J., & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 36–40.

Nishizuka, Y. (1984) *Nature (London) 308*, 693–698.

Pace, U., Hanski, E., Salomon, Y., & Lancet, D. (1985) *Nature (London) 316*, 255–258.

Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M., & Hille, B. (1985) *Nature (London) 317*, 536–538.

Rhein, L. D., & Cagan, R. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4412–4416.

Schramm, M., & Selinger, Z. (1984) *Science (Washington, D.C.) 225*, 1350–1356.

Sklar, P. B., Anholt, R. R. H., & Snyder, S. H. (1986) *J. Biol. Chem. 261*, 15538–15543.

Sternweis, P. C., & Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806–13813.

Stryer, L. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 841–852.

Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391–419.

Takai, Y., Kikkawa, U., Kaibuchi, K., & Nishizuka, Y. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 18*, 119–158.