Inhibition and enhancement of odorant-induced cAMP accumulation in rat olfactory cilia by antibodies directed against $G_{\alpha s/olf}$- and $G_{\alpha i}$-protein subunits

Srikumar Sinnarajah[a], Patrick I. Ezeh[b], Suram Pathirana[c], Anthony G. Moss[d], Edward E. Morrison[a], Vitaly Vodyanoy[a,*]

[a] *Department of Anatomy, Physiology and Pharmacology, 109 Greene Hall, Auburn University, Auburn, AL 36849, USA*
[b] *Department of Neurobiology and Physiology, 2153 N. Campus Dr., Northwestern University, Evanston, IL 60208, USA*
[c] *Institute for Biological Detection Systems, 410 Green Hall Annex, Auburn University, Auburn, AL 36849, USA*
[d] *Department of Zoology and Wildlife Sciences, 331 Funchess, Auburn, AL 36849, USA*

Received 12 January 1998; revised version received 17 March 1998

Abstract The odorant-induced accumulation of cAMP can be inhibited by antibodies directed against $G_{\alpha s/olf}$. In contrast, antibodies raised against $G_{\alpha i}$-subunits caused a strong enhancement of the odorant-induced cAMP accumulation. Western blotting and immunoelectron microscopy revealed the presence of both $G_{\alpha S/olf}$- and $G_{\alpha i}$-subunits in rat cilia preparations. The existence of both stimulatory and inhibitory odorant-induced regulation of adenylyl cyclase activity in olfactory cilia may indicate that an initial integration of different odorant stimuli begins at the level of primary reactions in the same effector enzyme.

Key words: Olfaction; Olfactory receptor neuron; Odorant response; Olfactory cilia; G-protein; Adenylyl cyclase

1. Introduction

The primary events of olfactory transduction occur at the cilia of olfactory receptor neurons and involve the binding of odorants to receptor proteins [1] followed by activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins) [2,3], and effector enzymes [4–6]. Two different effector enzymes, G-protein-coupled adenylyl cyclase and phospholipase C, were proposed to control primary responses of olfactory neurons to odor stimulation in invertebrates and vertebrates [7,8]. Although the excitatory reactions involving cAMP and IP_3 pathways are well explored, the inhibitory primary responses to odor stimulation are not very well understood. Down-regulation of any step in the olfactory signal transduction cascade could contribute to termination of odorant-evoked responses. Therefore, receptor phosphorylation [9], inactivation of G-protein [10], reduction of adenylyl cyclase activity [5], and activation of phosphodiesterase [11] could cause a decrease in intracellular cAMP concentrations. In addition, the cyclic nucleotide gated channels are subjected to modulation and adaptation [12–14].

Here we report evidence that a single effector enzyme, adenylyl cyclase, in cilia from rat olfactory epithelium, can be coupled with both activator and inhibitor G-proteins.

2. Materials and methods

2.1. Olfactory cilia preparation

Olfactory cilia were prepared by a modification of published procedures [15,16]. Rat olfactory epithelium from four adult male Sprague-Dawley (200–225 g, Blue Spruce Farms) were rapidly dissected, pooled in ice-cold EDTA-Ringer's solution containing 2 mM HEPES, pH 7.4, 112 mM NaCl, 3.4 mM KCl, 2.4 mM $NaHCO_3$, 2 mM EDTA and then subjected to sonication for 2 min in a Microson tip sonicator (Heat Systems, NY) at 10 W, 23 kHz, at 7°C under nitrogen gas. The deciliated epithelia were sedimented for 10 min on ice and then the supernatant containing the detached cilia was layered on top of a 45% (w/w) sucrose cushion. After centrifugation for 50 min at $120\,000 \times g$, 4°C the cilia were collected from the interface on top of the sucrose cushion, diluted with an equal volume of EDTA-Ringer's solution, then centrifuged for 25 min at $120\,000 \times g$, 4°C. The pellet was resuspended in 500 μl of Ringer's solution and used for experiments. Non-sensory cilia from respiratory epithelium was identically treated and used as control. All steps of the deciliation and partial purification were monitored for structural and biochemical properties using high resolution dark-field and electron microscopy immunohistochemical methods. The physical and biochemical characteristics of the cilia preparation agree well with those described by Anholt et al. [2].

2.2. Adenylyl cyclase assays

Adenylyl cyclase assays were performed in triplicate and contained 50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM ATP, 20 mM creatine phosphate, 50 U/ml of creatine kinase, 10 μM GTP, and 2 mg/ml of BSA. Odorant mixture containing equal parts of ethyl butyrate, eugenol, and (+)- and (−)-carvones were included at total concentrations of 10, 25, 100, 400 and 1600 μM in a final assay volume of 100 μl. 1 μg of ciliary protein, as determined by the BCA protein assay (Pierce, Rockford, IL), was included in each reaction assay. Reactions were terminated after 5 min at 30°C by addition of 250 μl of ice-cold 50 mM Tris-HCl and 4 mM EDTA (pH 7.4). Accumulation of intracellular cAMP was assessed by a cAMP [^{125}I] radioimmunoassay (PerSeptive BioSystems, Cambridge, MA). Effects of antibodies on the odorant-induced cAMP accumulation were examined by using commercial affinity-purified rabbit polyclonal IgG antibodies directed against $G_{\alpha S/olf}$- and $G_{\alpha i}$-subunits. Anti-$G_{\alpha S/olf}$ was prepared by injection of a rabbit with a $G_{\alpha S}$ peptide corresponding to amino acids 377–394 of rat origin; anti-$G_{\alpha i}$ was raised against a peptide corresponding to amino acids 93–112 of rat origin, and was predominantly specific against $G_{\alpha i1}$-subunit (Santa Cruz Biotechnology, Santa Cruz, CA). A rabbit preimmune IgG was a kind gift of Dr. R. Kemppainen (Auburn University). Specific inhibitor of Ca^{2+}-calmodulin, 1-(*n*,*o*,-bis-[5-isoquinoline-sulfonyl]-*n*-methyl-L-tyrosyl)-4-phenyl-piperazine (KN-62), and specific phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX), were obtained from Sigma Chemicals (St. Louis, MO).

2.3. Western blotting

200 μg of ciliary proteins were separated by SDS-PAGE on an 8% curtain gel (14 cm × 16 cm, 1.5 mm thick; SE 600 apparatus, Hoefer Scientific, San Francisco, CA). The gel was blotted onto nitrocellulose

*Corresponding author. Fax: (1) (334) 844-5388.
E-mail: vodyavi@vetmed.auburn.edu

PII S0014-5793(98)00368-8

membrane (Nitropure, Micron Separations, Milford, MA; Transblot, Hoefer Scientific) at 0.9 A overnight, as described for ciliary and flagellar proteins [17].

Protein immunostaining was carried out using the same antibodies directed against $G_{\alpha S/olf}$- and $G_{\alpha i}$-subunits and was carried out in a staining manifold ('Surf-blot', Idea Scientific, Minneapolis, MN) to provide a reliable register of the lanes. Molecular mass of polypeptides was estimated by a spline curve fit to the molecular standards data (molecular weight standards: Sigma, SDS 6H).

2.4. Immunoelectron microscopy

Olfactory epithelium for immunoelectron microscopy was obtained from the septum region of three (150–200 g) Sprague-Dawley rats and processed for post-embedded immunoelectron microscopy [18]. Animals were deeply anesthetized with sodium pentobarbital (0.648 mg/ml per 100 g body weight) and 0.1 ml heparin sodium sulfate (1000 UPS units/ml) injected intraperitoneally, then transcardially perfused with 50 ml saline (0.9% NaCl) followed by 250 ml of cold 0.6% paraformaldehyde and 2% glutaraldehyde in buffered sodium cacodylate (pH 7.4). The olfactory epithelium was removed from the underlying septum, buffer (0.01 M sodium cacodylate pH 7.4) rinsed for 20 min then post-fixed 1 h with 1% osmium tetroxide, buffer rinsed (1 h) then dehydrated in graded ethanol and flat-embedded in Ducupan resin. Thick (1 μm) sections were stained with toluidine blue for light microscopy examination and orientation. Ultrathin sections with gold and purple interface were collected on naked nickel grids (100–200 mesh thin bar hexagonal). All procedures related to antibody binding were carried out on both sides of the grids. Grids were etched for 5 min with 5% H_2O_2 then quenched (1 M ammonium chloride) 1 h, blocked with 1% BSA+10% normal goat serum (30 min), then incubated overnight at 4°C with primary anti-$G_{\alpha i}$ (30 μg/ml, Santa Cruz, CA). Following a PBS buffer rinse primary antibody binding was determined with goat anti-rabbit IgG (1:15 Sigma G-7402) conjugated to 10 nm gold particles. Normal goat sera in place of primary antibody served as negative controls for immunoreactivity. After washing the grids in distilled water the sections were dried, then stained with 2% uranyl acetate and 0.4% lead citrate, viewed and photographed with a Phillips 301 transmission microscope at 60 kV accelerating voltage.

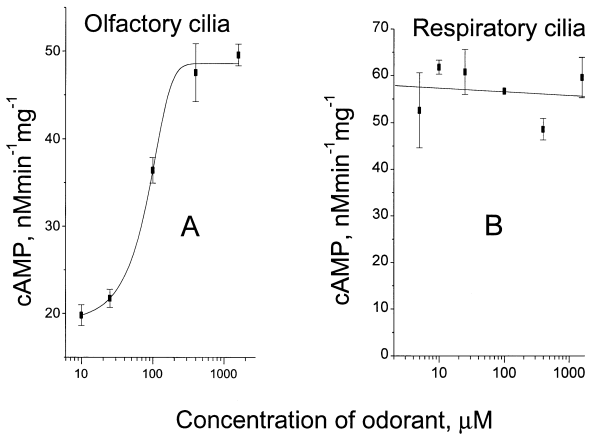

Fig. 1. Dose-response curve of odorant-induced cAMP accumulation in rat cilia preparations. A: Cilia isolated from rat olfactory epithelium. Experimental conditions are given in the text. The figure points depict mean experimental values ± S.D.; the line is a cubic spline interpolation. The dose-response curve fits well the Hill equation ($r = 0.98$, $P < 0.003$) with the apparent Hill coefficient and EC_{50} equal to 1.06 ± 0.12 and 25.0 ± 4.9 μM, respectively. B: Cilia isolated from rat respiratory epithelium were used as control and also assayed with the same odorant mixture. Points are mean experimental values ± S.D., and the line is a linear regression.

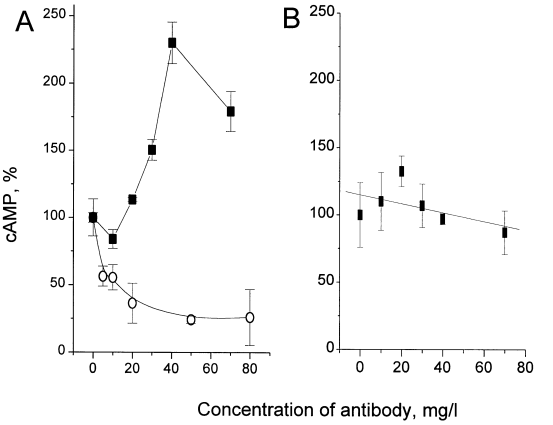

Fig. 2. Antibodies against $G_{\alpha S/olf}$ and $G_{\alpha i}$ modulate production of cAMP. A: Dose-response curves for stimulation of the olfactory cilia preparations by odorant mixture in the presence of antibodies. The antibodies against $G_{\alpha S/olf}$ (circles) and $G_{\alpha i}$ (squares) at given concentrations were preincubated with cilia preparations for 10 min and subsequently assay mixtures were activated by 10 μM of odorant mixture. The amplitude of the response to the odorant mixture in the absence of antibodies was corrected for the basal activity and relative values of cAMP accumulation were plotted as function of antibody concentrations. The inhibition curve (circles) fits well ($r = 0.98$, $P < 0.02$) the Lineweaver-Burk double reciprocal plot with the apparent dissociation constant and maximal inhibition by antibodies against $G_{\alpha s/olf}$ equal to 9.4 ± 2.0 mg/l and 88 ± 7%, respectively. B: Controls utilized preimmune affinity-purified rabbit IgG. Other experimental conditions are given in the text.

3. Results

3.1. Odorant-induced excitation and inhibition of adenylyl cyclase

Odorant mixture applied to olfactory cilia preparations at concentrations from 10 to 1600 μM induced a dose-dependent accumulation of cAMP (Fig. 1A) only in the presence of 10 μM guanosine triphosphate (GTP) and 2 mM adenosine triphosphate (ATP). The dose-response curve indicates that olfactory cilia preparations respond to odorants up to an apparent saturation value of about 300 μM with a basal adenylyl cyclase activity of 15.7 ± 13.2 nM/min/mg. Control preparations of rat respiratory cilia did not respond to odor (Fig. 1B).

Antibodies directed against $G_{\alpha s/olf}$ inhibited adenylyl cyclase activity elicited by odorants (Fig. 2A, open circles). In contrast, antibodies raised against $G_{\alpha i}$-subunits caused a strong enhancement of the odorant-induced cAMP accumulation (Fig. 2A, filled squares). At a $G_{\alpha i}$-antibody concentration of 40 mg/l the odorant-induced cAMP accumulation was more than twice as high compared to one induced by the odorant alone. Prior incubation of the $G_{\alpha i}$-antibody with an excess of $G_{\alpha i}$-peptide corresponding to amino acids 93–112 eliminated the enhancement of the odorant-induced cAMP accumulation. When IgG was substituted for antibodies, the odorant-induced adenylyl cyclase activity was unchanged within experimental accuracy (Fig. 2B). $G_{\alpha i}$-antibody alone produced no significant effects up to 70 mg/l. When 25 μM of KN-62, a specific inhibitor of Ca^{2+}-calmodulin [19], or 20 μM of IBMX, a specific phosphodiesterase inhibitor [20], was added together with odorants, the same effect produced by odorants alone was observed.

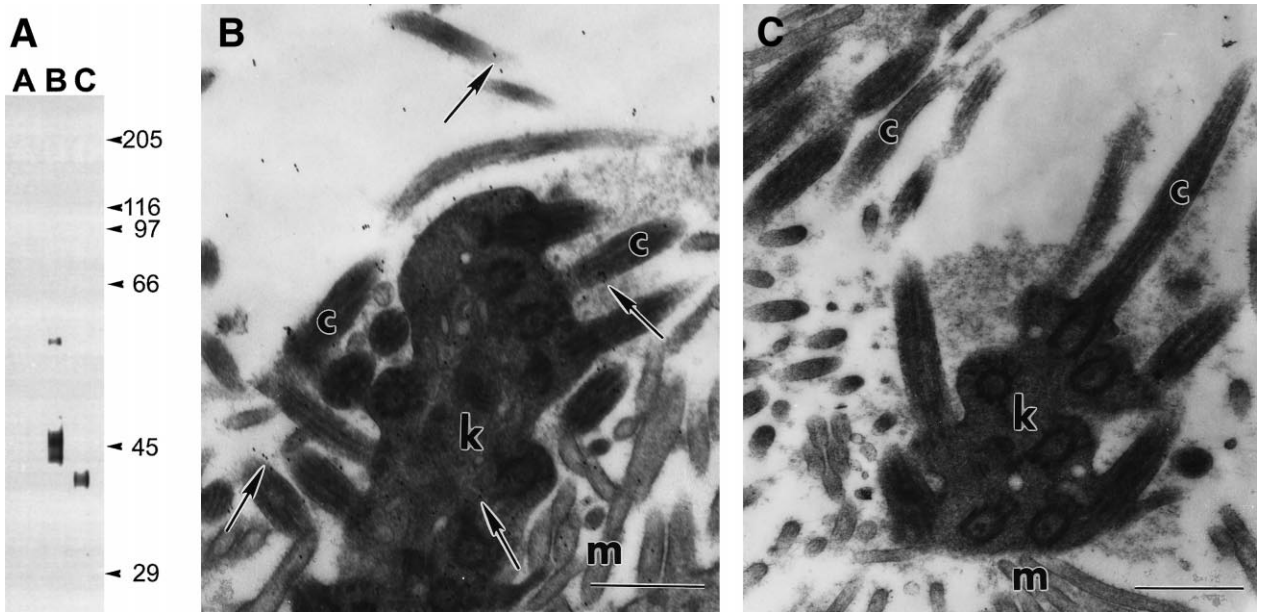

Fig. 3. A: $G_{\alpha S/olf}$ and $G_{\alpha i}$ are present in olfactory cilia. Western blot analysis reveals both $G_{\alpha S/olf}$ and $G_{\alpha i}$ are present in olfactory cilia. Lane A was probed with a rabbit preimmune control IgG antibody; lane B indicates ciliary proteins revealed by a primary antibody against $G_{\alpha S/olf}$; lane C shows proteins labeled with a primary antibody directed against $G_{\alpha i}$. B: High magnification electron micrograph showing olfactory dendritic knob (k), sensory cilia (c), and supporting cell microvilli (m). The olfactory epithelium was labeled with polyclonal antibodies against $G_{\alpha i}$ and visualized with goat anti-rabbit secondary antibody conjugated to 10 nm gold particles as secondary probes. Olfactory cilia and dendritic knobs were labeled with gold particles (arrows), with supporting cell microvilli showing occasional label. C: Control olfactory epithelium was incubated with the secondary antibody conjugated to gold particles, without primary antibody. No gold particle label was observed. Bar, 0.5 μm.

3.2. Western blotting analysis

Western blotting (Fig. 3A) revealed the presence of both $G_{\alpha S/olf}$- and $G_{\alpha i}$-subunits in cilia preparations. As expected [21] prominent bands at 52 and 45 kDa were revealed by antibodies raised against $G_{\alpha S/olf}$ subunit peptides (lane B). A single band at 41–42 kDa [22] was revealed by anti-$G_{\alpha i}$-subunit (lane C). No bands were present in lane A, which was probed with preimmune IgG.

3.3. Electron microscopy

Electron microscopy was used to identify $G_{\alpha i}$-antibody binding to cellular structures of the olfactory epithelium. Antibody binding of gold particles was localized along the ciliary processes, dendritic knob and occasionally observed in adjacent supporting cell microvilli (Fig. 3B). No immunoreactivity was observed in negative control sections (Fig. 3C).

4. Discussion

The properties of adenylyl cyclase observed in our experiments correspond well to those obtained with cilia-enriched preparations from frog and rat olfactory neuroepithelia [4,5]. Odorants induced production of cAMP in a dose-dependent manner (Fig. 1A). These responses were dependent upon the presence of GTP indicating that they were mediated by G-proteins. Indeed, antibodies directed against $G_{\alpha s/olf}$- and $G_{\alpha i}$-subunits strongly modulated the adenylyl cyclase activity (Fig. 2). Antibodies directed against $G_{\alpha s/olf}$ effectively inhibited odorant-induced adenylyl cyclase activity. This result is consistent with the presence of a specialized type III adenylyl cyclase found in olfactory cilia [6], and suggested to be activated by G_{olf} [3]. The result is also in a good agreement with the finding of activation of rat olfactory adenylyl cyclase by exogenous $G_{\alpha s}$ [23]. That the odorant-induced adenylyl cyclase activity was enhanced in a dose-dependent manner by both odorants and antibodies against $G_{\alpha i}$-subunits (Figs. 1 and 2) suggests involvement of G_i-protein in the odorant-mediated inhibition of adenylyl cyclase in our olfactory cilia preparations.

If odorants act through G_i-proteins to inhibit adenylyl cyclase during initial stages of the odorant-mediated cascade in olfactory cilia, then one must be able to demonstrate certain criteria to establish their physiological role. These criteria are similar to those suggested for justifying claims that a given effector produces its effect as a result of the stimulation of adenylyl cyclase [24–26]. The main criteria are as follows: (i) the olfactory receptor neurons should contain G_i-proteins, (ii) the activity of the type III adenylyl cyclase (which is the predominant form in olfactory cilia) can be modulated by G_i-proteins, and (iii) the adenylyl cyclase inhibition should not occur in response to odorants when G_i-proteins are blocked or inhibited.

Criterion (i) is supported by immunohistochemical observations in rat and other species. The presence of G_i-proteins has been reported in the lobster olfactory organ [27], in chemosensory membranes from the channel catfish [28], and in the nasal epithelium of the frog [4,2] and rat [29,21,22]. G_i-proteins are present in olfactory cilia [4]. Although all three $G_{\alpha i}$-subunits ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$) were present in rat olfactory neuroepithelium [3], only $G_{\alpha i2}$-subunits were so far reported in the rat olfactory cilia [22]. Western blot analysis and immunoelectron microscopy confirm the presence of $G_{\alpha i}$-subunits in our olfactory cilia preparations (Fig. 3). Evidence in support of criterion (ii) is provided by a study of muscarinic m4 acetylcholine receptors and type III adenylyl cyclase coexpressed in human embryonic kidney cells [30]. In that study, it was

shown that the adenylyl cyclase III was inhibited due to receptor coupling through G_i. The inhibition of cloned adenylyl cyclase III by $G_{\alpha i2}$-subunits has been also demonstrated in another transfected system [31]. Our results are also consistent with criterion (iii). When $G_{\alpha i}$-subunits were bound by specific antibodies we observed an apparent block to $G_{\alpha i}$ function, which produced an increase in cAMP content, indicating that adenylyl cyclase inhibition was reduced (Fig. 2A).

The dual regulation of the adenylyl cyclase activity we observed in our experiments is different from that with two different effector enzymes, adenylyl cyclase and phospholipase C [7]. We have presented evidence that a single effector enzyme, adenylyl cyclase, coupled with activating and inhibitory G-proteins controls the level of a single second messenger, cAMP. This bi-directional regulation of certain adenylyl cyclases is explained by the presence of independent sites for interaction with stimulatory $G_{\alpha s}$- and inhibitory $G_{\alpha i}$-subunits [32].

We have attempted to limit 'cross talk' between the adenylyl cyclase and some other endogenous enzymes by limiting calcium effects in all experimental procedures. We did this by using EDTA in our solutions, partially purifying the preparation in a sucrose gradient, and by assaying in calcium-free solutions. Membrane preparations in our experiments were also not subjected to calcium shock [33]. Lack of free calcium provides an explanation for the incapacity of the specific inhibitor of Ca^{2+}-calmodulin, KN-62, to produce a noticeable effect on the adenylyl cyclase activity in our experiments.

$G_{\alpha i}$-subunits are present in olfactory cilia. The adenylyl cyclase enhancement in the presence of $G_{\alpha i}$-antibody is controlled by odorants and GTP, and could not be controlled by $\beta\gamma$-subunits [34], Ca^{2+}-calmodulin, or phosphodiesterase. We therefore hypothesize that adenylyl cyclase of olfactory neuron cilia is inhibited functionally through a coupling with a G_i-protein pathway. This mechanism of inhibition may be important for understanding the attenuation of odorant-stimulated cAMP levels in olfactory neurons.

Acknowledgements: We thank Dr. R.R. Anholt and Dr. D.D. Schwartz for comments and critical discussion, Karen Wolfe, Dr. D.W. Young and Randy O. Boddie for technical assistance. Supported by Grants from FAA 93-G-058 (V.V.), NIH DC 01532 (E.E.M.) and Battelle M1-T44-123090 (E.E.M., V.V.).

References

[1] Buck, L. and Axel, R. (1991) Cell 65, 175–187.
[2] Anholt, R.R.H., Mumby, S.M., Stoffers, D.A., Girard, P.R., Kuo, J.F. and Snyder, S.H. (1987) Biochemistry 26, 788–795.
[3] Jones, D.T. and Reed, R.R. (1989) Science 244, 790–795.
[4] Pace, U., Hanski, E., Salomon, Y. and Lancet, D. (1985) Nature 316, 255–258.
[5] Sklar, P.B., Anholt, R.R.H. and Snyder, S.H. (1986) J. Biol. Chem. 261, 15538–15544.
[6] Bakalyar, H.A. and Reed, R.R. (1990) Science 250, 1403–1406.
[7] Fadool, D.A. and Ache, B.W. (1992) Neuron 9, 907–918.
[8] Breer, H. (1994) Biosens. Bioelectron. 9, 625–632.
[9] Boekhoff, I. and Breer, H. (1992) Proc. Natl. Acad. Sci. USA 89, 471–474.
[10] Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802–808.
[11] Borisy, F.F., Ronnett, G.V., Cunningham, A.M., Juilfs, D., Beavo, J. and Snyder, S.H. (1992) J. Neurosci. 12, 915–923.
[12] Liu, M., Chen, T.Y., Ahamed, B., Li, J. and Yau, K.W. (1994) Science 266, 1348–1354.
[13] Brunet, L.J., Gold, G.H. and Ngai, J. (1996) Neuron 17, 681–693.
[14] Kurahashi, T. and Menini, A. (1997) Nature 385, 725–729.
[15] Vodyanoy, V. and Murphy, R.B. (1983) Science 220, 717–719.
[16] Anholt, R.R., Aebi, U. and Snyder, S.H. (1986) J. Neurosci. 6, 1962–1969.
[17] King, S.M., Otter, T. and Witman, G.B. (1986) Methods Enzymol. 134, 291–317.
[18] Abramson, D.R. (1986) J. Histochem. Cytochem. 34, 847–853.
[19] Enslen, H. and Soderling, T.R. (1994) J. Biol. Chem. 269, 20872–20877.
[20] Beavo, J.A. (1995) Physiol. Rev. 75, 725–748.
[21] Mania-Farnell, B. and Farbman, A.I. (1990) Dev. Brain Res. 51, 103–112.
[22] Shinohara, H., Asano, T. and Kato, K.J. (1992) Neuroscience 12, 1275–1279.
[23] Pfeuffer, E., Mollner, S., Lancet, D. and Pfeuffer, T. (1989) J. Biol. Chem. 264, 18803–18807.
[24] Robinson, G.A., Butcher, R.W. and Sutherland, E.W. (1971) Cyclic AMP, Academic Press, New York.
[25] Menevse, A., Dodd, G. and Poynder, T.M. (1977) Biochem. Biophys. Res. Commun. 77, 671–677.
[26] Vodyanoy, V. and Vodyanoy, I. (1987) Neurosci. Lett. 73, 253–258.
[27] McClintock, T.S., Byrnes, A.P. and Lerner, M.R. (1992) Mol. Brain Res. 14, 273–276.
[28] Bruch, R.C. and Kalinoski, D.L. (1987) J. Biol. Chem. 262, 2401–2404.
[29] Jones, D.T. and Reed, R.R. (1987) J. Biol. Chem. 262, 14241–14249.
[30] Dittman, A.H., Weber, J.P., Hinds, T.R., Choi, E.J., Migeon, J.C., Nathanson, N.M. and Storm, D.R. (1994) Biochemistry 33, 943–951.
[31] Chen, J. and Iyengar, R. (1993) J. Biol. Chem. 268, 12253–12256.
[32] Taussing, R., Tang, W.J., Hepler, J.R. and Gilman, A.G. (1994) J. Biol. Chem. 269, 6093–6100.
[33] Chen, Z., Pace, U., Heldman, J., Shapira, A. and Lancet, D. (1986) J. Neurosci. 6, 2146–2154.
[34] Tang, W.-J. and Gilman, A.G. (1991) Science 254, 1500–1503.