Volume 314, number 3, 215–219 FEBS 11866 December 1992

Molecular cloning of G protein α subunits from the central nervous system of the mollusc *Lymnaea stagnalis*

Jaco C. Knol*, Wolfgang Weidemann**, Rudi J. Planta, Erno Vreugdenhil and Harm van Heerikhuizen

Department of Biochemistry and Molecular Biology, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Received 24 September 1992

The central nervous system of the pond snail, *Lymnaea stagnalis*, contains many large, identified neurons which can be easily manipulated making it an advantageous model system to elucidate in vivo the architecture of neuronal signal transduction pathways. We have isolated three cDNA clones encoding G protein α subunits that are expressed in the *Lymnaea* CNS, i.e. $G\alpha_o$, $G\alpha_i$, and $G\alpha_q$***. The deduced proteins exhibit a very high degree of sequence identity to their vertebrate and invertebrate counterparts. The strong conservation of G protein α subunits suggests that functional insights into G protein-mediated signalling routes obtained through the experimental amenability of the *Lymnaea* CNS will have relevance for similar pathways in the mammalian brain.

G protein α subunit; Central nervous system; cDNA cloning; *Lymnaea stagnalis*

1. INTRODUCTION

G protein-mediated signal transduction pathways play an important role in cellular communication (for reviews see [1–4]). Such signalling systems enable neurons to sense physiological and environmental information that is conveyed by a wide variety of messenger molecules dispatched by other cells, and to generate appropriate responses. Specific transmembrane receptors bind the signal molecules and subsequently stimulate the exchange of GDP for GTP on G proteins (guanine nucleotide-binding regulatory proteins) that relay the information to intracellular enzymes or ion channels, thus evoking cellular responses. The signalling process is terminated by the G protein's intrinsic GTPase activity. It is now well-known that G protein-coupled receptors, G proteins and their effectors are all members of large (super)families. We are interested how the multitude of receptor, G protein and effector subtypes that can be co-expressed in a single neuron is exploited so as to transduce the multiple informational inputs that are to be considered in its decision making.

G proteins are heterotrimeric proteins that consist of α, β and γ subunits. The α subunit furnishes the GTPase activity and determines the identity of the G protein since it is thought to dictate receptor and, in many cases, effector specificity. The function of the β and γ subunits, which act as a single βγ subunit, would be receptor coupling and noise suppression. Recently, evidence has accumulated suggesting that βγ subunits can in fact be the signalling moiety for some effectors (see e.g. [5–7]). Molecular biological studies have indicated the presence of at least 16 different G protein α subunit subtypes in mammals. Based on amino acid sequence comparisons these subtypes can be grouped into four families called $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ [4]. The corresponding G proteins have been named accordingly and each modulates a specific (set of) effector(s) such as adenylyl cyclase, phospholipase C, cGMP phosphodiesterase or an ion channel. In the brain a subset of G proteins is known to be expressed: i.e. G_o, G_i1, G_s, G_z, G_q and G_{11} [8–13]. G_o proteins are expressed most abundantly and may comprise as much as 1% of the total membrane protein in brain [14].

Although extensive molecular biological and biochemical studies have provided much information about G proteins, hardly anything is known about the coupling of specific G protein subtypes with receptor and effector subtypes in vivo. This is especially true for the G protein function in neurons, which may be due to the enormous complexity of the vertebrate brain.

The pond snail, *Lymnaea stagnalis*, possesses a simple, surveyable and accessible nervous system which comprises a mere 15,000 neurons clustered in a small number of defined ganglia. The relative ease by which some of its large (50–200 μm) and identifiable neurons can be manipulated [15] makes it an excellent model

Correspondence address: H. van Heerikhuizen, Department of Biochemistry and Molecular Biology, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Fax: (31) (20) 646 1479.

*The first two authors made equal contributions to the studies described in this paper.

**Present address:* Rheinische Friedrich-Wilhelms-Universität, Institut für Zoophysiologie, Endenicher Allee 11-13, D-5300 Bonn 1, Germany.

***The nucleotide sequences of the cDNA clones described in this article have been deposited in the GenBank/EMBL data base under accession numbers Z15094 ($G\alpha_o$), Z15095 ($G\alpha_i$) and Z15096 ($G\alpha_q$).

for the in vivo dissection of neuronal G protein-mediated signalling pathways. Due to their large size, many molluscan neurons are very suitable for micro-injection of a diversity of agents that can perturb (G) protein expression and function (e.g. DNA, RNA, peptides and antibodies), the effects of which can be subsequently monitored by application of various electrophysiological techniques [16,17]. Moreover, a number of identified *Lymnaea* neurons have been well-characterized multidisciplinary [15].

As a first step towards studying the architecture of neuronal G protein-mediated signal transduction pathways and their function in cellular decision making, we have undertaken the isolation of cDNA clones encoding G protein α subunits that are present in the *Lymnaea* CNS. Here we report on the cloning of cDNAs that code for lymnaean $G\alpha_o$, $G\alpha_s$ and $G\alpha_i$ subunits. The primary structures of the deduced proteins have been very conserved (76–90% amino acid identity with vertebrate and invertebrate counterparts), with motifs that are critical for guanine-nucleotide binding and (at least for $G\alpha_s$) effector-coupling being perfectly conserved. This suggests that results of functional studies in our invertebrate model system will have relevance for the understanding of signalling pathways involving G proteins in the mammalian brain.

2. MATERIALS AND METHODS

For the cloning of a *Lymnaea* $G\alpha_o$ homologue, $5 \cdot 10^5$ clones of a λgt10 cDNA library of the adult *Lymnaea* CNS were screened under low stringency conditions with a 1.2 kb *Hha*I DNA fragment containing the entire coding region of a *Xenopus* $G\alpha_o$ cDNA [18]. One clone which still hybridized at high stringency (65°C, 0.2 × SSC), λGAOL23, was plaque-purified and the insert was subcloned in pBluescript KS(+) for sequence analysis.

To pick up α subunit sequences using the PCR technique, two degenerate oligo primers were developed. The sense primer, W1 (5′-GGGAATTCT/CTIT/CTIGGIGCIGGIGAA/GA/TG/CIGG-3′ with I=inosine) is based on the amino acid sequence LLGAGESG and contains an *Eco*RI cloning site. The anti-sense primer, W2 (5′-TAGGGATCCG/TT/CTGNCCNCCNACA/GTC-3′ with N=G/A/T/C), is based on the sequence DVGGQR and contains a *Bam*HI cloning site. Total RNA was isolated from *Lymnaea* CNS tissue [19] and reverse transcribed with oligo-dT as a primer according to the manufacturer's instructions (BRL). An aliquot of the cDNA preparation equivalent to approximately one CNS was used as a template in a PCR reaction with the primers W1 and W2. The resulting 525 bp PCR product was subcloned and clones exhibiting different restriction patterns with respect to the corresponding *Lymnaea* $G\alpha_o$ cDNA fragment were sequenced. To identify full length cDNA clones, clone-specific PCR primers were devised and used in a PCR screening of 52 fractions of a λZAP II *Lymnaea* CNS cDNA library (each fraction containing 100,000 independent clones) according to Gibbons et al. [20]. The corresponding PCR products were used as probes to isolate the pertinent λ clones. The cDNA inserts were excised in vivo as pBluescript II SK(−) phagemids. DNA sequences were determined for both strands using the dideoxy nucleotide chain termination method [21].

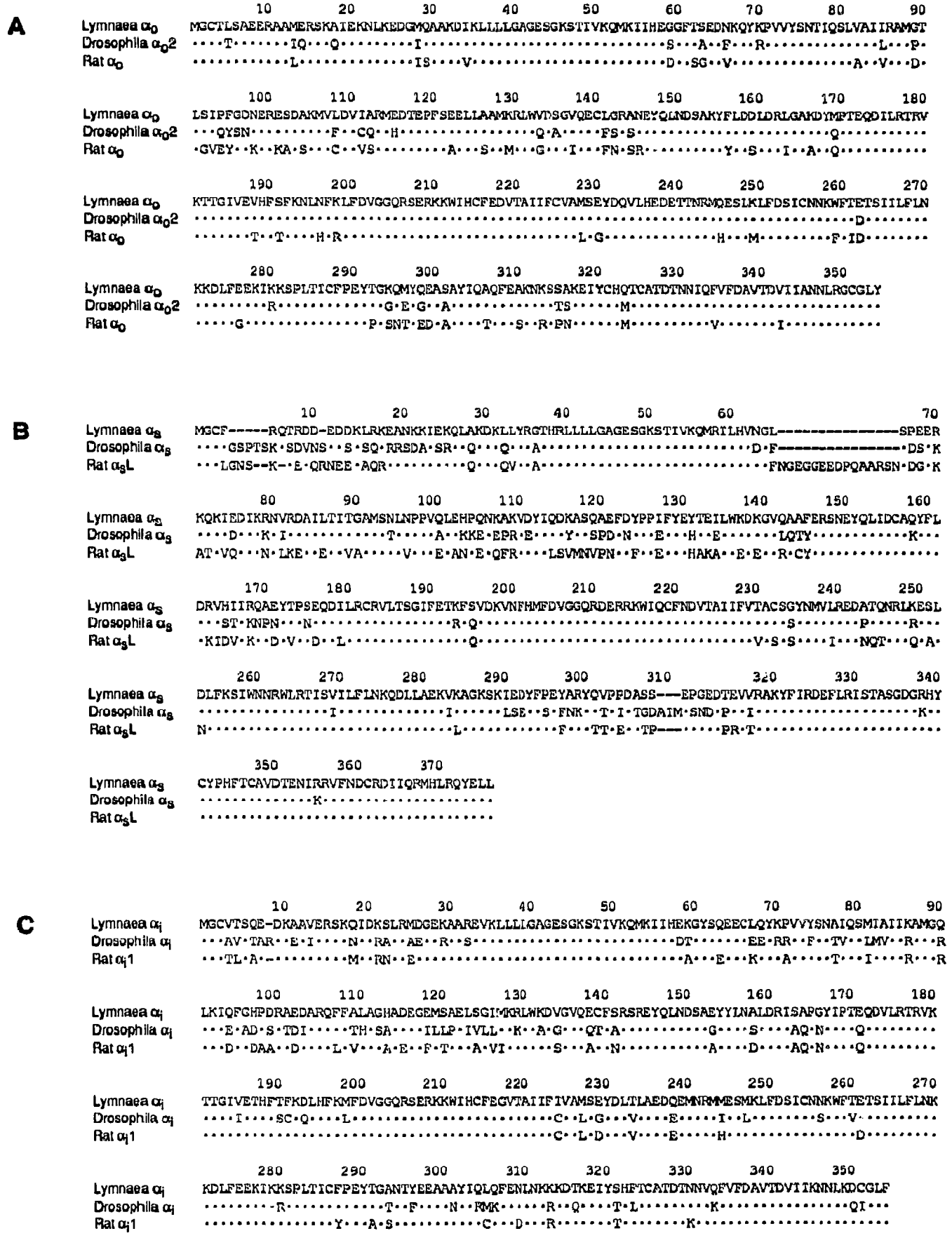
3. RESULTS AND DISCUSSION

Since G_o proteins have been shown to be abundantly expressed in vertebrate neural and neuroendocrine tissues, we first embarked on cloning a $G\alpha_o$ cDNA. A *Lymnaea stagnalis* CNS-specific cDNA library was screened under low stringency conditions with a *Xenopus* $G\alpha_o$ cDNA probe [18]. One clone that still hybridized at high stringency, λGAOL23, was sequenced. The λGAOL23 clone carries an insert of 1300 bp, with a 1062 bp open reading frame being flanked by 111 bp of leader sequences and 127 bp of trailer sequences that do not contain any polyadenylation signal. The predicted protein of 354 amino acids exhibits a high degree of amino acid sequence identity with various $G\alpha_o$ subunits from other organisms (e.g. 80%, 82% and 90% with *Xenopus* [18], rat [8–10] and *Drosophila* [22–24] $G\alpha_o$ forms, respectively; Fig. 1A), whereas the homology with $G\alpha_i$ proteins is less pronounced (68–71%). This strongly suggests that the λGAOL23 cDNA codes for a *Lymnaea* $G\alpha_o$ homologue. The cysteine residue at position −4 relative to the C terminus which serves as a site for ADP-ribosylation of $G\alpha_o$ proteins by pertussis toxin (PTX) has been conserved. Moreover, the central parts of the putative guanine nucleotide-binding domains (regions A, C, G and I in the nomenclature of Halliday [25]) have been perfectly conserved in the *Lymnaea* protein with respect to $G\alpha_o$ subunits of other species.

Subsequently, we decided to use the PCR technique to seek for other G protein α subunits that are expressed in the *Lymnaea* CNS. To be able to pick up multiple α subunit cDNA sequences simultaneously, degenerate oligonucleotide primers W1 and W2 were synthesized on the basis of two of the amino acid sequence motifs that form part of the well-conserved guanine nucleotide binding pocket of G protein α subunits (see section 2). Using these primers, a PCR reaction was carried out with CNS cDNA as a template. The majority of PCR products that were obtained contained sequences derived from the $G\alpha_o$ cDNA which we described above, but two products turned out to contain coding information for *Lymnaea* $G\alpha_s$ and $G\alpha_i$ homologues. cDNA clones containing a complete open reading frame were isolated for both $G\alpha_i$ and $G\alpha_s$ by following a PCR strategy to screen a *Lymnaea* CNS-specific λZAPII cDNA library [20].

→

Fig. 1. Comparison of the amino acid sequence of *Lymnaea* $G\alpha_o$ (A), $G\alpha_s$ (B) and $G\alpha_i$ (C) proteins with *Drosophila* and rat counterparts [8–10,22–24,26,27]. Numbering refers to the *Lymnaea* amino acid sequence. Dots represent identical amino acid residues, dashes represent gaps to allow optimal alignment. The $\alpha_o 2$ and $\alpha_s L$ denotations indicate specific splice forms of the pertinent α subunits: $\alpha_i 1$ is one of three α_i subunits known in mammals.


A
Lymnaea α_o
Drosophila $\alpha_o 2$
Rat α_o
B
Lymnaea α_s
Drosophila α_s
Rat $\alpha_s L$
C
Lymnaea α_i
Drosophila α_i
Rat $\alpha_i 1$

Two cDNA clones encoding a *Lymnaea* $G\alpha_s$ protein were isolated and the coding region of the clone with the longest insert, λGASL11 (3300 bp), was sequenced. Preceded by a 206 bp leader, the open reading frame codes for a protein of 376 amino acids which exhibits a high degree of homology with known $G\alpha_s$ proteins (Fig. 1B). The deduced *Lymnaea* protein product shares 80% and 76% amino acid identity with *Drosophila* [26] and rat [8,9] $G\alpha_s$ subunits, respectively. Both the cores of the guanine nucleotide-binding motifs and the arginine residue that is a site for ADP-ribosylation of $G\alpha_s$ proteins by cholera toxin (CTX) have been conserved (the CTX target site is located at position 183 in the *Lymnaea* protein).

One $G\alpha_i$-specific cDNA clone, λGAIL2, was isolated. It carries a 1200 bp insert with 159 bp of leader sequences being followed by a 1062 bp open reading frame. The predicted 354 amino acid protein highly resembles $G\alpha_i$ subunits from other organisms (Fig. 1C). It shares 85% amino acid identity with the rat $G\alpha_{i1}$ subtype [8,9] and 76% with the *Drosophila* DGα1 protein [27]. Again, the central parts of the GDP/GTP-binding motifs have been perfectly conserved, as has the PTX site of $G\alpha_i$ proteins (position −4 relative to the C terminus).

The strong conservation of all three deduced *Lymnaea* G protein α subunits with respect to their vertebrate and invertebrate homologues indicates their essential role in cell function. The cores of all sequence motifs that are supposed to constitute the domain that is involved in guanine nucleotide-binding and GTP-hydrolysis (regions A, C, G and I [25]) have been perfectly conserved. Conservation continues throughout the α subunits. Substitutions most prominently occur between the A and C regions and near the N-terminus of the proteins, where the $\beta\gamma$ subunit complex is supposed to bind. The C-terminal half is more conserved and it is probably this part of the α subunits that is interacting with receptors and effectors. Indeed, amino acid sequence motifs implicated in the coupling of $G\alpha_s$ with adenylyl cyclase by a study of Berlot and Bourne [28] have been completely conserved. Thus, these communication interfaces of G proteins have been well-conserved in evolution, indicating that G protein-coupled signalling systems do not allow many alterations to occur. This, then, also implicates that results of functional studies performed on *Lymnaea* G protein α subunits should be transferable, in principle, to their mammalian counterparts.

We do not know as yet whether the proteins encoded by the cDNA clones that we have isolated up till now represent all $G\alpha_o$, $G\alpha_i$ and $G\alpha_s$ subunits present in *Lymnaea*. It is known that in *Drosophila* and mammals alternative splicing gives rise to multiple $G\alpha_o$ and $G\alpha_s$ proteins [10,22,29–34], whereas three distinct $G\alpha_i$ genes are known in mammals [9,35,36]. Thus, it is very well possible that multiple forms exist in *Lymnaea* as well.

G_o proteins have been reported to be expressed abundantly in the central nervous system [14]. Therefore, it comes as no surprise that the majority of cDNA sequences isolated from a CNS-specific cDNA preparation by PCR amplification with primers that have been developed on the basis of two amino acid motifs that are highly conserved in α subunits encode a *Lymnaea* $G\alpha_o$ subunit. Although differences in the efficiency of PCR amplification cannot be ruled out, it seems that the $G\alpha_i$ and $G\alpha_s$ subunits are expressed in the CNS at a level that is significantly lower than that of $G\alpha_o$. We have performed preliminary studies on the expression of $G\alpha_o$, $G\alpha_i$ and $G\alpha_s$ mRNAs and proteins in *Lymnaea* (not shown), which seem to confirm the latter notion and indicate that the expression profiles are very reminiscent of the expression patterns of vertebrate G proteins.

One of the PCR primers is based on an amino acid sequence that is derived from the so-called A region of the guanine nucleotide-binding pocket [25]. This sequence is conserved in most members of the $G\alpha_s$ and $G\alpha_i$ subfamilies, but differs slightly from the corresponding sequence in members of the $G\alpha_q$ and $G\alpha_{12}$ families [4]. Hence, the latter will be difficult to pick up with the PCR primers used in this study. However, using other PCR primers we have been able to clone a $G\alpha_q$ cDNA clone which is currently being characterized.

Having shown that in the mollusc *Lymnaea stagnalis* vertebrate-like G proteins are present, we are now aiming to take advantage of the experimental amenability of the *Lymnaea* CNS with its giant neurons, a number of which have already been functionally characterized in a multidisciplinary fashion, to study the coupling of receptors and effectors by G proteins in vivo.

Acknowledgements: The authors wish to express their gratitude to Dr. Loesje van der Voorn for providing the *Xenopus* $G\alpha_o$ cDNA clone, Edwin Roovers for construction of the λZAP II *Lymnaea* CNS cDNA library, Kees Tensen for valuable advice, Maaike le Feber for work done during the cloning of the *Lymnaea* $G\alpha_o$ cDNA clone and Raymond Huismans, Iris van Goethem and Steven Extra for great help during sequence analysis of the cDNA clones described in this paper. This study is financially supported by the Council of Geological and Biological Sciences of the Netherlands Organization for Scientific Research within the research program 'Neuropeptides and Behaviour'.

REFERENCES

[1] Neer, E.J. and Clapham, D.E. (1988) Nature 333, 129–134.
[2] Lochrie, M.A. and Simon, M.I. (1988) Biochemistry 27, 4957–4965.
[3] Birnbaumer, L., Abramowitz, J. and Brown, A.M. (1990) Biochim. Biophys. Acta 1031, 163–224.
[4] Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802–808.
[5] Tang, W.-J. and Gilman, A.G. (1991) Science 254, 1500–1503.
[6] Federman, A.D., Conklin, B.R., Schrader, K.A., Reed, R.R. and Bourne, H.R. (1992) Nature 356, 159–161.

[7] Camps, M., Hou, C., Sidiropoulos, D., Stock, J.B., Jakobs, K.H. and Gierschik, P. (1992) Eur. J. Biochem. 206, 821–831.
[8] Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 3776–3780.
[9] Jones, D.T. and Reed, R.R. (1987) J. Biol. Chem. 262, 14241–14249.
[10] Tsukamoto, T., Toyama, R., Itoh, H., Kozasa, T. and Matsuoka, M. (1991) Proc. Natl. Acad. Sci. USA 88, 2974–2978.
[11] Fong, H.K.W., Yoshimoto, K.K., Eversole-Cire, P. and Simon, M.I. (1988) Proc. Natl. Acad. Sci. USA 85, 3066–3070.
[12] Matsuoka, M., Itoh, H., Kozasa, T. and Kaziro, Y. (1988) Proc. Natl. Acad. Sci. USA 85, 5384–5388.
[13] Strathman, M. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA 87, 9113–9117.
[14] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[15] Geraerts, W.P.M., Ter Maat, A and Vreugdenhil, E., in: Invertebrate Endocrinology (H. Laufer and R.G.H. Downer, Eds.) Vol. 2, Liss, New York, 1988, pp. 141–231.
[16] Harris-Warrick, R.M., Hammond, C., Paupardin-Tritsch, D., Homburger, V., Rouot, B., Bockaert, J. and Gerschenfeld, H.M. (1988) Neuron 1, 27–32.
[17] Kaang, B.-K., Pfaffinger, P.J., Grant, S.G.N., Kandel, E.R. and Furukawa, Y. (1992) Proc. Natl. Acad. Sci. USA 89, 1133–1137.
[18] Olate, J., Jorquera, H., Purcell, P., Codina, J., Birnbaumer, L. and Allende, J.E. (1989) FEBS Lett. 244, 188–192.
[19] Chomzcynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[20] Gibbons, I.R., Asai, D.J., Ching, N.S., Dolecki, G.J., Mocz, G., Phillipson, C.A., Ren, H., Tang, W.Y. and Gibbons, B.H. (1991) Proc. Natl. Acad. Sci. USA 88, 8563–8567.
[21] Sanger, F., Nicklen, S. and Coulson, A.B. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[22] Yoon, J., Shortridge, R.D., Bloomquist, B.T., Schneuwly, S., Perdew, M.H. and Pak, W.L. (1989) J. Biol. Chem. 264, 18536–18543.
[23] De Sousa, S.M., Hoveland, L.L., Yarfitz, S. and Hurley, J.B. (1989) J. Biol. Chem. 264, 18544–18551.
[24] Thambi, N., Quan, F., Wolfgang, W.J., Spiegel, A.M. and Forte, M. (1989) J. Biol. Chem. 264, 18552–18560.
[25] Halliday, K.R. (1984) J. Cyclic Nucleotide Protein Phosphorylation Res. 9, 435–448.
[26] Quan, F., Wolfgang, W.J. and Forte, M.A. (1989) Proc. Natl. Acad. Sci. USA 86, 4321–4325.
[27] Provost, N.M., Somers, D.E. and Hurley, J.B. (1988) J. Biol. Chem. 263, 12070–12076.
[28] Bolet, C.H. and Bourne, H.R. (1992) Cell 68, 911–922.
[29] Hsu, W.H., Rudolph, U., Sanford, J., Bertrand, P., Olate, J., Nelson, C., Moss, L.G., Boyd III, A.E., Codina, J. and Birnbaumer, L. (1990) J. Biol. Chem. 265, 11220–11226.
[30] Strathmann, M., Wilkie, T.M. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA 87, 6477–6481.
[31] Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J. and Spiegel, A.M. (1986) Proc. Natl. Acad. Sci. USA 83, 8893–8897.
[32] Kozasa, T., Itoh, H., Tsukamoto, T. and Kaziro, Y. (1988) Proc. Natl. Acad. Sci. USA 85, 2081–2085.
[33] Ishikawa, Y., Bianchi, C., Nadal-Ginard, B. and Homey, C.J. (1990) J. Biol. Chem. 265, 8458–8462.
[34] Swaroop, A., Agarwal, N., Gruen, J.R., Bick, D. and Weissman, S.M. (1991) Nucleic Acids Res. 19, 4725–4729.
[35] Beals, C.R., Wilson, C.B. and Perlmutter, R.M. (1987) Proc. Natl. Acad. Sci. USA 84, 7886–7890.
[36] Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M. and Kaziro, Y. (1988) J. Biol. Chem. 263, 6656–6664.