Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 303 (2003) 19–23
BBRC
www.elsevier.com/locate/ybbrc

Active transducin α subunit carries PDE6 to detergent-resistant membranes in rod photoreceptor outer segments

Han Liu,[a] Keiji Seno,[a,1] and Fumio Hayashi[b,*]

[a] *Graduate School of Science and Technology, Kobe University, Japan*
[b] *Department of Biology, Faculty of Science, Kobe University, Nada, Kobe 657-5801, Japan*

Received 13 February 2003

Abstract

cGMP-Phosphodiesterase 6 (PDE6) is the central effector enzyme in the phototransduction system of vertebrate photoreceptors. We have recently found that PDE6 accumulates in a detergent-resistant membrane (DRM) fraction in response to excitation of bovine rod phototransduction system. Here, we studied the molecular mechanism of the PDE6 translocation to DRM. Pertussis toxin inhibited the translocation of PDE6. Upon addition of AlF_4^- to dark-adapted ROS, PDE6 translocated to DRM along with a minor fraction of the α subunit of transducin (Tα). The addition of an excess of the inhibitory subunit of PDE6 blocked its accumulation in the DRM, but did not block the translocation of the minor fraction of Tα. These data suggested that the formation of a complex between activated Tα and PDE6 imparted upon Tα a high affinity for the DRM. The translocation of PDE6 to the DRM may be involved in the spatiotemporal regulation of its activity on disk membranes.

Keywords: Phosphodiesterase; PDE6; Transducin; Phototransduction; Photoreceptor; Rod outer segment; DRM; Lipid raft

The phototransduction system in rod outer segments (ROS) of the vertebrate photoreceptor is a G-protein-mediated signaling system. Light-excited rhodopsin interacts with the GDP form of the heterotrimeric G-protein, transducin, and stimulates GDP–GTP exchange on its α-subunit (Tα). GTP-Tα binds the inhibitory subunit (Pγ) of cGMP-phosphodiesterase (PDE6), and in doing so abrogates Pγ's inhibition of the catalytic subunits (P_α and P_β) of PDE6. The resulting decrease in the levels of cytoplasmic cGMP leads to the closure of cGMP-gated channels and hyperpolarization of photoreceptor plasma membranes. A complex between RGS9-1, a regulator of G-protein signaling in ROS, and its cofactor Gβ5L, preferentially deactivates the GTP form of Tα bound to PDE6 by enhancing the GTPase activity of Tα [1–4]. This mechanism enables the system to quench rapidly after PDE6 activation.

Proteins involved in the cGMP-cascade are thought to move freely within the disk membrane of photoreceptors. However, we have recently isolated a detergent-resistant membrane (DRM) fraction from bovine ROS [5]. The presence of DRM in ROS suggested that there were at least two physically distinct membrane domains in ROS. In our experiments, transducin and PDE6 showed a stimulus-dependent translocation between the detergent-resistant and detergent-soluble domains of the membrane [5]. Transducin was recruited to the DRM in a light-dependent manner and was segregated from the DRM when the ROS were exposed to GTPγS. In contrast, PDE6 accumulated within the DRM when light-bleached ROS was exposed to an unhydrolyzable GTP analog, GTPγS.

Here we have studied the molecular mechanism of the PDE6 translocation. It was found that when active Tα is bound to PDE6, it gains a high affinity for unidentified target molecules within the DRM. Furthermore, it was suggested that the RGS9-1/Gβ5L-complex on the DRM is the most plausible candidate for the targeting of active Tα/PDE6. The lateral domain organization on the disk membrane may be essential for

* Corresponding author. Fax: +81-78-803-5717.
E-mail address: fhayashi@kobe-u.ac.jp (F. Hayashi).
[1] Present address: Bio-oriented Technology Research Advancement Institution at Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology.

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00284-5

spatiotemporal regulation of G-protein-mediated PDE6 activity.

Materials and methods

Preparation of Triton X-100-insoluble membrane fraction from bovine ROS. ROS were prepared from frozen dark-adapted bovine retinas in complete darkness by the method of Molday and Molday [6]. Purified ROS were suspended in buffer A (10 mM Mops (pH 7.2), 60 mM KCl, 30 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM BAPTA, 1 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 μM E64), and stored in the dark at −95 °C. Frozen ROS (1 mg protein) were thawed and suspended in 125 μl (final volume) of buffer A. The suspension was incubated under various experimental conditions and then solubilized by the addition of 62.5 μl of 3% Surfact-Amps X-100 (Pierce Biotechnology) in buffer A. The sucrose concentration of the sample was adjusted to 0.9 M by adding 2.4 M sucrose in buffer A and the sample was overlaid with 700 μl of 0.8 M and 500 μl of 0.5 M sucrose in buffer A and then subjected to ultracentrifugation. Following ultracentrifugation (214,000*g* for 16 h at 4 °C), fractions (150 μl) were collected from the tops of the tubes and the pellets were re-suspended with 150 μl buffer A.

SDS–PAGE and Western blotting. SDS–PAGE and Western blotting were performed as described previously [7]. Polyclonal rabbit antibodies were raised against synthetic peptides derived from RGS9-1 (^{2}Thr–^{17}Leu) [7] and Gβ5L (^{17}Cys–^{30}Ser). An antibody against Tα (^{2}Gly–^{15}Cys) was obtained from Calbiochem. Antibody against Tβ (Gβ1; C-16) was obtained from Santa Cruz Biotechnology.

Treatment of ROS with various reagents. ADP-ribosylation of Tα by pertussis toxin was performed as described previously [8]. Pertussis toxin (List Biological Labs) was pre-activated by incubation with 20 mM DTT at 37 °C for 30 min. Dark-adapted ROS were incubated with 10 μg/ml active pertussis toxin, 1 mM NAD, and 100 μM ATP at 30 °C for 1 h in the dark. This treatment by pertussis toxin suppressed the light- and GTPγS-dependent PDE6 activity of the ROS by >95% when it was assessed by the pH-PDE assay (data not shown). In experiments in which aluminum fluoride (AlF_4^-) was used, all buffers contained 10 mM NaF and 30 μM $AlCl_3$, and all experimental procedures were done in the dark. For the study on the effect of exogenous Pγ, recombinant bovine Pγ was obtained as described previously [9]. We added 20 μM Pγ to ROS suspension before adding GTPγS. Reagents described above were added to ROS suspensions 5 min prior to the solubilization, and then, samples were subjected to sucrose-gradient ultracentrifugation.

PDE activity assay. PDE activity was assessed by real-time pH measurement as described previously [10]. To assess the full activity of PDE6 exposed on the surface of the DRM vesicles, the inhibitory subunits of PDE6 were removed by limited digestion by trypsin (Pierce): 25-μl of TPCK trypsin (1 mg/ml) was added to 100-μl of the DRM suspension (880 μg protein/ml) and incubated at 0 °C for 15 min. To assess the PDE activities, aliquots of 5-μl of DRM suspension or 6.25-μl of trypsin-treated DRM suspension were added to the pH-assay medium (180-μl) containing soybean trypsin inhibitor (0.1 mg/ml; Boehringer–Mannheim). Reaction was started by adding 2 mM cGMP.

Results and discussion

Effects of transducin-modulating agents upon the distribution of transducin and PDE6 on floating gradients

As we reported previously, DRMs can be isolated from bovine ROS in the form of a light-scattering buoyant fraction (fractions #3–5 of each panel of

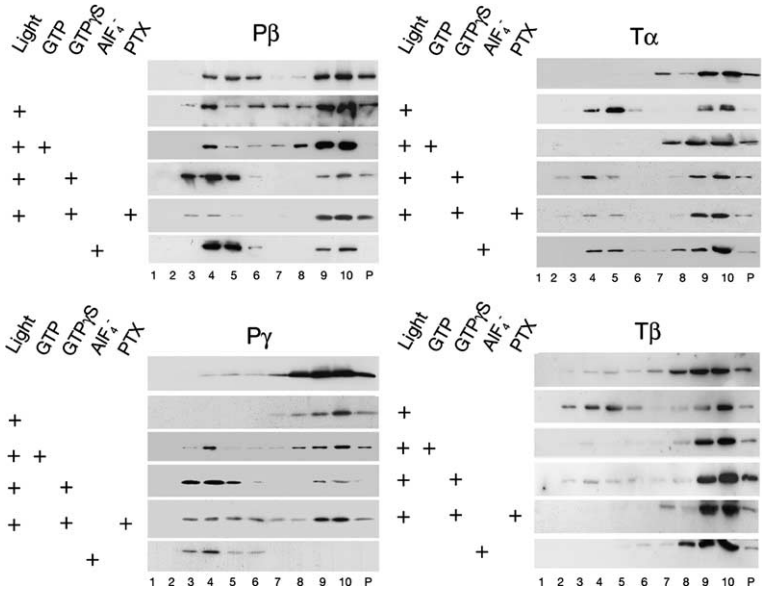

Fig. 1. Effects of various conditions on the distribution of PDE6 and transducin on floating gradient. Dark-adapted bovine ROS (1 mg of protein) were incubated under various conditions, solubilized with 1% Triton X-100 at 0 °C, and subjected to sucrose-density gradient ultracentrifugation. Gradients were separated into 150-μl fractions from top to bottom (fractions #1–10). Pellet fraction (P) was re-suspended in 150-μl of buffer A. Detergent-resistant membranes were distributed in fractions #3–5 and detergent-soluble proteins were in fractions #7–10. Ten μl aliquots of each fraction were analyzed by SDS–PAGE followed by Western blotting with specific antibodies against Pβ, Pγ, Tα, and Tβ. A "+" indicates the addition of following stimuli or reagents: light, ambient room light; GTPγS, 100 μM; GTP, 1 mM; AlF_4^-, 10 mM NaF and 30 μM $AlCl_3$; and PTX, pertussis toxin.

Fig. 1), when ROS are solubilized with 1% Triton X-100 at 4 °C and subjected to sucrose-density gradient ultracentrifugation. Interestingly, we observed stimulus-dependent translocation of transducin and PDE6 between the DRM and the detergent-soluble membrane (DSM) fractions (Fig. 1). When dark-adapted ROS were solubilized in the dark without any additives as shown in the top panels in Fig. 1, both transducin (Tαβγ) and phosphodiesterase (Pαβγγ) were found in the DSM (Fractions #8–10). Light exposure of ROS resulted in the translocation of a large fraction of Tαβγ to the DRM. Upon light excitation of the ROS phototransduction system in the presence of an unhydrolyzable GTP analog, GTPγS, a majority of the PDE6 (Pαβ and Pγ) translocated to the DRM, and a majority of Tα and Tβγ were localized to the DSM as reported previously [5].

To study the molecular mechanism of the accumulation of PDE6 in DRM, we examined the effect of agents that modulate G-protein function on the distribution of the subunits of transducin and PDE6 in detergent-resistant and detergent-soluble membrane fractions of ROS. ADP-ribosylation of Tα by pertussis toxin blocks the interaction of light-bleached rhodopsin and transducin (GDP-Tαβγ), and consequently blocks nucleotide exchange on Tα [8]. We observed that pertussis toxin blocked the light- and GTPγS-dependent translocation of PDE6 to DRM (Fig. 1), suggesting that active Tα is necessary for translocation.

It was expected that sustained activation of transducin is necessary for the accumulation of PDE6 in DRM, since the translocation of PDE6 to DRM required the presence of GTPγS. To test this hypothesis, we examined the effect of GTP on the distribution of transducin and PDE6. We found that GTP did not support PDE6 accumulation in DRM under light conditions (Fig. 1), suggesting that sustained activation of transducin is necessary for the accumulation of PDE6 in the DRM.

In contrast, we found that GTP blocked the light-dependent recruitment of Tαβγ to DRM. This result did not support a hypothesis proposed by Nair et al. [11] that light can induce the translocation of GTP-bound Tα to DRM with RGS9-1. We suppose that the light-mediated accumulation of Tαβγ in DRM reflects a higher stability of the complex of GDP-Tαβγ and metharhodopsin II (MII) on DRM than on DSM. GTP–GDP exchange on Tα would elicit the dissociation of transducin from MII on DRM, leaving Tα and Tβγ in the DSM.

Next, we examined the effect of aluminum fluoride (AlF_4^- on the distribution of PDE6 and transducin (Fig. 1). AlF_4^- can activate transducin even in the dark by mimicking the γ-phosphate of GTP, thereby switching GDP-bound Tα into a conformation that resembles its transition state [12]. Western blot analysis demonstrated that AlF_4^- effected translocation of both the catalytic (Pβ) and inhibitory (Pγ) subunits of PDE6 to the DRM (Fig. 1). It is noteworthy that the addition of AlF_4^- resulted in a translocation of a minor fraction of Tα to the DRM ($5 \pm 3\%$ of the total Tα; $n = 5$), while the majority of Tα and all of the Tβγ subunits remained in the DSM. The translocation of a minority of Tα to the DRM was also observed when light-bleached ROS were treated with GTPγS (Fig. 1). These data suggested that active Tα (Tα*) recruits PDE6 to the DRM and that only PDE6-bound Tα* is retained in the DRM with PDE6.

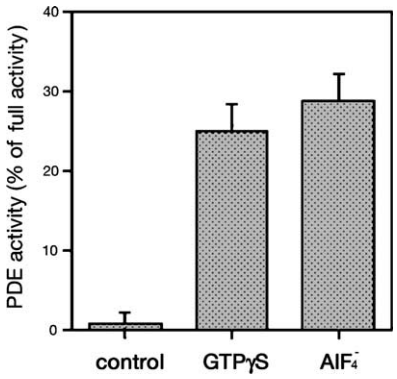

Fig. 2. PDE6 activities in DRMs. DRM was prepared from light-bleached bovine ROS (1 mg of protein) in the presence or absence of GTPγS, or prepared from dark-adapted ROS exposed to AlF_4^- as described previously. PDE6 activity was assayed in terms of the pH-electrode. Activity was indicated by the percentage of full activity induced by trypsin treatment of DRM prepared from light- and GTPγS-treated ROS.

If the PDE6 in the DRM was bound to Tα*, it would be activated. Hence, we measured the PDE activity in the DRM prepared from ROS exposed to light in the presence of GTPγS and from ROS loaded with AlF_4^- in the dark. We found that PDE6 was active in both cases (Fig. 2), with activities that were 25–30% of that observed when the DRM was treated with trypsin. This level of PDE activation was also observed in light-exposed ROS upon addition of GTPγS (data not shown).

Exogenous Pγ blocked the accumulation of PDE6 in the DRM

We next added a 10-fold molar excess of recombinant Pγ to dark-adapted ROS prior to bleaching and the addition of GTPγS. This addition of an excess of exogenous Pγ completely inhibited both PDE activity (data not shown) and PDE6 translocation to the DRM (Fig. 3) induced by light and GTPγS. It is noteworthy that a small fraction of Tα was still observed in the DRM even in the presence of exogenous Pγ. At the same time, the majority of Tα and Pγ were distributed in the DSM (Fig. 3). Therefore, we speculate that there are a limited number of Tα*/Pγ-complex binding sites in the DRM.

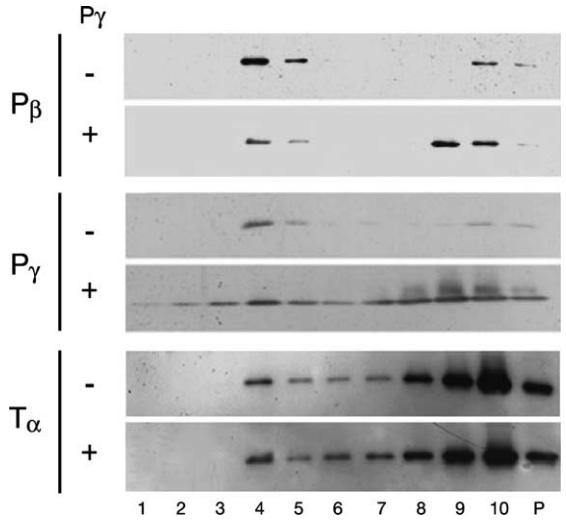

Fig. 3. Effect of exogenous Pγ on the distribution of PDE6 and transducin in floating gradient. ROS (1 mg protein) were exposed to light and GTPγS (100 μM) in the presence or absence of exogenously added recombinant bovine Pγ (20 μM) and DRM was prepared. Proteins in 10 μl aliquots of each fraction and pellet (P) were analyzed on SDS–PAGE and then Pβ, Pγ, Tα, and Tγ were detected by Western blotting.

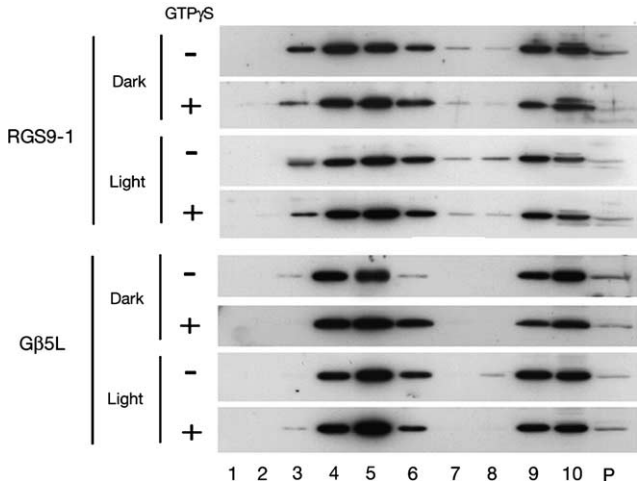

Fig. 4. Distribution of RGS9-1 and Gβ5L on floating gradients. Dark-adapted or light-bleached ROS were incubated in the absence or presence of GTPγS and then DRM was prepared. Ten μl aliquots of each fraction and pellet (P) were analyzed by SDS–PAGE. RGS9-1 and Gβ5L were detected by Western blotting.

The binding sites for active Tα/PDE6 on DRM

The results above suggested that PDE6 gains a high affinity for the DRM upon formation of a complex with active Tα. However, the binding sites for the active Tα/PDE6-complex have not been identified. The complex may have an affinity for a particular lipid environment within the DRM, or it may have an affinity for a protein component located within the DRM, or both. The characteristics of the putative Tα*/Pγ-complex binding sites discussed above are similar to those reported for RGS9-1 complexed with its cofactor Gβ5L. The complex functions as a GTPase activating protein (GAP) in ROS. The RGS9-1/Gβ5L-complex localizes to disk membranes at a low concentration and possesses a much higher affinity for Tα*/Pγ [4,13] than for free Tα* [14]. The RGS9-1/Gβ5L-complex stimulates the hydrolysis of GTP on Tα*/PDE6-complex [13].

Western blot analysis revealed that, under our experimental conditions, approximately 60% of RGS9-1 was consistently detected in the DRM fraction (Fig. 4). A similar fraction of Gβ5L was also constitutively localized to the DRM. Thus, the accumulation of Tα*/PDE6 in the DRM might be due to its association with the RGS9-1/Gβ5L-complex localized in the DRM. However, the reported amount of RGS9-1/Gβ5L seems insufficient to account for the levels of PDE6 detected within the DRM. The concentration of PDE6 in ROS is almost 10-fold greater than that of RGS9-1 [4]. In contrast, almost all of the PDE6 was localized to the DRM upon addition of GTPγS to light-exposed ROS (Fig. 1). Thus, the accumulation of PDE6 in DRM may be dependent not only on the affinity of Tα*/PDE6 for RGS9-1/Gβ5L in the DRM, but also on the characteristic nature of Tα*/PDE6 to form aggregates [15].

It has been suggested that in native disk membranes, there is a lateral domain organization facilitating a high level of rhodopsin activity [16,17]. Even when rhodopsin is incorporated into an artificial, mixed-lipid bilayer, rhodopsin preferentially associates with polyunsaturated lipids and promotes membrane lateral separation onto domains enriched in highly saturated lipids and cholesterol, and highly unsaturated lipids and rhodopsin [18]. It has been shown that a membrane domain enriched in highly saturated lipids and cholesterol is resistant to detergents, while a domain enriched in polyunsaturated lipids is detergent-sensitive [19]. Thus, our experimental results suggest that inactive transducin and inactive PDE6 localize to the fluid, detergent-sensitive membrane domain surrounding rhodopsin, and that PDE6 is recruited to the outer, less fluid membrane domain upon activation by Tα*. If the regulation of PDE6 were mediated through its translocation between these different membrane phases, the shape and size of the DRM and/or DSM would be important for determining the duration of PDE6 activation and the determination of its localization. Elucidation of the structure and function of the DRM within the disk membrane may provide a deeper insight into the mechanism of phototransduction in vertebrate photoreceptors.

Acknowledgments

This work was supported in part by Grant-in-aid 12490023 from the Ministry of Education, Science and Culture of Japan (to F.H.).

References

[1] J.K. Angleson, T.G. Wensel, A GTPase-accelerating factor for transducin, distinct from its effector cGMP phosphodiesterase, in rod outer segment membranes, Neuron 11 (1993) 939–949.
[2] J.K. Angleson, T.G. Wensel, Enhancement of rod outer segment GTPase accelerating protein activity by the inhibitory subunit of cGMP phosphodiesterase, J. Biol. Chem. 269 (1994) 16290–16296.
[3] V.Y. Arshavsky, C.L. Dumke, Y. Zhu, N.O. Artemyev, N.P. Skiba, H.E. Hamm, M.D. Bownds, Regulation of transducin GTPase activity in bovine rod outer segments, J. Biol. Chem. 269 (1994) 19882–19887.
[4] W. He, C.W. Cowan, T.G. Wensel, RGS9, a GTPase accelerator for phototransduction, Neuron 20 (1998) 95–102.
[5] K. Seno, M. Kishimoto, M. Abe, Y. Higuchi, M. Mieda, Y. Owada, W. Yoshiyama, H. Liu, F. Hayashi, Light- and guanosine 5′-3-*O*-(thio)triphosphate-sensitive localization of a G protein and its effector on detergent-resistant membrane rafts in rod photoreceptor outer segments, J. Biol. Chem. 276 (2001) 20813–20816.
[6] R.S. Molday, L.L. Molday, in: P.A. Hargrave (Ed.), Isolation and Characterization of Rod Outer Segment Disk and Plasma Membranes, in Photoreceptor Cells, Academic Press, San Diego, 1993, pp. 131–150.
[7] K. Seno, A. Kishigami, S. Ihara, T. Maeda, V.A. Bondarenko, Y. Nishizawa, J. Usukura, A. Yamazaki, F. Hayashi, A possible role of RGS9 in phototransduction. A bridge between the cGMP-phosphodiesterase system and the guanylyl cyclase system, J. Biol. Chem. 273 (1998) 22169–22172.
[8] F. Bornancin, M. Franco, J. Bigay, M. Chabre, Functional modifications of transducin induced by cholera or pertussis-toxin-catalyzed ADP-ribosylation, Eur. J. Biochem. 210 (1992) 33–44.
[9] A. Yamazaki, V.A. Bondarenko, S. Dua, M. Yamazaki, J. Usukura, F. Hayashi, Possible stimulation of retinal rod recovery to dark state by cGMP release from a cGMP phosphodiesterase noncatalytic site, J. Biol. Chem. 271 (1996) 32495–32498.
[10] F. Hayashi, I. Matsuura, S. Kachi, T. Maeda, M. Yamamoto, Y. Fujii, H. Liu, M. Yamazaki, J. Usukura, A. Yamazaki, Phosphorylation by cyclin-dependent protein kinase 5 of the regulatory subunit of retinal cGMP phosphodiesterase. II. Its role in the turnoff of phosphodiesterase in vivo, J. Biol. Chem. 275 (2000) 32958–32965.
[11] K.S. Nair, N. Balasubramanian, V.Z. Slepak, Signal-dependent translocation of transducin, RGS9-1–Gβ5L complex, and arrestin to detergent-resistant membrane rafts in photoreceptors, Curr. Biol. 12 (2002) 421–425.
[12] J. Bigay, P. Deterre, C. Pfister, M. Chabre, Fluoride complexes of aluminium or beryllium act on G-proteins as reversibly bound analogues of the γ-phosphate of GTP, EMBO J. 6 (1987) 2907–2913.
[13] N.P. Skiba, J.A. Hopp, V.Y. Arshavsky, The effector enzyme regulates the duration of G protein signaling in vertebrate photoreceptors by increasing the affinity between transducin and RGS protein, J. Biol. Chem. 275 (2000) 32716–32720.
[14] W. He, L. Lu, X. Zhang, H.M. El-Hodiri, C.K. Chen, K.C. Slep, M.I. Simon, M. Jamrich, T.G. Wensel, Modules in the photoreceptor RGS9-1.Gβ5L GTPase-accelerating protein complex control effector coupling, GTPase acceleration, protein folding, and stability, J. Biol. Chem. 275 (2000) 37093–37100.
[15] A. Caretta, P.J. Stein, Light- and nucleotide-dependent binding of phosphodiesterase to rod disk membranes: correlation with light-scattering changes and vesicle aggregation, Biochemistry 25 (1986) 2335–2341.
[16] B.J. Litman, D.C. Mitchell, A role for phospholipid polyunsaturation in modulating membrane protein function, Lipids 31 (Suppl.) (1996) S193–S197.
[17] D.C. Mitchell, K. Gawrisch, B.J. Litman, N. Salem Jr., Why is docosahexaenoic acid essential for nervous system function? Biochem. Soc. Trans. 26 (1998) 365–370.
[18] A. Polozova, B.J. Litman, Cholesterol dependent recruitment of di22:6-PC by a G protein-coupled receptor into lateral domains, Biophys. J. 79 (2000) 2632–2643.
[19] E. London, D.A. Brown, Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts), Biochim. Biophys. Acta 1508 (2000) 182–195.